Variations of antioxidative responses in two rice cultivars with polyamine treatment under salinity stress

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Abstract The rice varieties *viz.* Nonabokra and Swarna were evaluated on the basis of their responses for oxidative stress induced by sodium chloride (NaCl) and the effects of exogenously applied polyamine thereon. Rice seedlings were treated with 200 mM of NaCl supplemented with two dosages: 1 mM and 2 mM putrescine. Following treatments, plants were evaluated for accumulation of reactive oxygen species (ROS) like O_2^- , H_2O_2 etc. in tissues, lipid peroxidation, protein carbonylation, accumulation of flavonoids and anthocyanin, activities of different oxidative enzymes like guaiacol peroxidase (GPX), catalase (CAT) and glutathione reductase (GR). Preliminary, oxidative stress out of salinity was ensured by plants from significantly higher accumulation of O_2^- and H_2O_2 in the tissues of the NaCl treated varieties. Irrespective of varieties, there recorded a significant variation of the endogenous polyamine profiles under NaCl stress. Interestingly, exogenous application of putrescine had a close relationship on O_2^- and H_2O_2 content for both the varieties. However, Nonabokra was evident as more respondent than Swarna to applied putrescine. The other effects of oxidative stress was impacted on plants as higher values of MDA content, enhanced rate of protein oxidation and putrescine recorded as an

alleviating agent regardless of varieties with dose dependant manner. The generation of ROS and cellular disintegration was accompanied by up regulation of non-enzymatic and enzymatic antioxidation pathways with exogenous application of putrescine. For non-enzymatic antioxidant, it revealed that putrescine was highly effective for sustaining the anthocyanin and flavonoid content in both the varieties under salinity. Whereas, antioxidative enzyme, CAT showed its diminished activity; but activity of GPX and GR were significantly induced under salinity and it was according to the concentration of applied putrescine.

Keywords Antioxidative enzymes · Lipid peroxidation · Polyamine · Rice · Salinity

Abbreviations

CAT	Catalase
DTT	Dithiothreitol
EDTA	Ethylene Diamine Tetra Acetic Acid
GPX	Guaiacol peroxidase
GR	Glutathione reductase
MDA	Malondialdehyde
NN	Nonabokra Normal
NS	Nonabokra Stress (200 mM NaCl; 7 days)
PAs	Polyamines
PMSF	Phenyl Mercuric Sulfonyl Fluoride
Put	Putrescine
PVP	Polyvinylpyrrolidone
PVPP	Polyvinylpolypyrrolidone
ROS	Reactive oxygen species
SN	Swarna Normal
Spd	Spermidine
Spm	Spermine
SS	Swarna Stress (200 mM NaCl; 7 days)

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Introduction

PAs are low molecular weight natural compounds, cationic in nature, are most ubiquitous in plant system implicated in diverse aspect of growth and development. Unlike the other PAs, Spd, Spm which are triamines, putrescine (Put) is typically a diamine. During the recent years, PAs have been under focus by the workers because of its functioning in both perception and modulation of plant's responses to environmental extremities, commonly assigned as abiotic stresses including moisture deficit, excess water causing inundation, excess salt accumulation i.e. salinity, high or intense illumination causing photo oxidation, chilling or freezing etc. (He and Zhu 2008). It is quite established that PA could be acting in ameliorating the salinity or heavy metals in soil for various crops like rice (Yang et al. 2009), maize and tobacco (Liu and Moriguchi 2006), sunflower (Groppa et al. 2003), pulses (Suriyan and Chalermopol 2008), tomato (Santa et al. 2009) etc. Sodidity in the form of sodium chloride (NaCl), the most common salt that induces salinity in soil for different cereals like rice and has been elaborately studied in terms of seed germination, growth and physiological processes, realization of yield, qualitative changes of grain etc. (Quinet et al. 2010). Almost in all the cases, salinity induced oxidative stress has been evident as a potent limiting factor for sustainability and proper growth and development in plants. PAs appear as a broad spectrum of elicitor with divergent effects for interacting the stress perception and its concomitant responses in plants (Mariale et al. 2004). However, a contradiction has also been reported where pre-treatment with PAs, though in higher dosages, might be decreasing the drought tolerance in plants like maize, wheat etc. (Annalisa et al. 2002). Now, rice plants have the moderate concentration of PAs in different plant parts and this expression is variable according to the duration and intensity of salinity exposure. It is reported that in cucumber, tobacco, potato, Arabidopsis, in which endogenous levels of PAs in both free and bound forms are variable and that also becomes the function of both abiotic and biotic stresses as well. However, exogenous application of PAs (Spd, Spm and their obligate precursor Put or its various analogues) to plants activate stress responsive genes, even those are also under regulation of stress hormones like ABA, ethylene etc. (as for eg. Dehydrin, RAB, LEA, ACC synthase, ACC oxidase etc.). The levels of these bio molecules are reported to be increased soon after the exposure of stress condition and also are believed to respond proportionately within a threshold condition of the stress exposure (Tang and Ranald 2005). Salinity effects on rice seedlings could be imparted as feeble and vulnerable for growth reduced tillering, decolonization or yellowing of leaves, non-adequate carbon assimilation, less translocation and subdued growth and consequently yield loss (Wang et al. 2009). More so, salinity otherwise, becomes more detrimental in the forms of oxidative exposure, particularly at early phases of growth or even at the

seedling stages, besides perturbing ionic imbalance in the tissues. Acquisition of salts in excess may perturb the redox of the tissues, causing some sort of over oxidized state in the tissues and produces various free oxygen reactive intermediates viz. $O_2^{\cdot -}$, $^{1/2}O_2$, $OH^{\cdot}$, H_2O_2 , etc. (Zapata et al. 2003). Notwithstanding, the NaCl toxicity and tolerance in plants have been extensively examined, information on plants responses by NaCl induced oxidative exposure are rather less. A general response by different crop species to oxidative stress in relation to PAs is that the later levels are changed with salinity in most cases, as for e.g. Put decreased, while Spd and/or Spm increased. PAs efficiently potentiates the effects of salt and drought induced ROS generation throughout the developmental stages and thus demands as a reliable physiological traits or even markers when selection for tolerance is concerned. Earlier reports demonstrated that long term application of exogenous Put reduces Na^+ and Cl^- accumulation in salt treated rice calli and sustain the grain yield of salt sensitive cultivars exposed to salinity (Quinet et al. 2010). Similar observations have been reported in rice which impacted the efficacy of exogenously applied other PAs like (Spd and Spm) on the endogenous polyamine metabolism. Still, Put, as a di-amine has less been explored on its putative influence of salinity induced oxidative stress in rice. Therefore, understanding the facts and figures it becomes prudent that PAs have been functional elicitors for modulation of stress response in plants under constraint environmental conditions. So, in the present investigation the two rice varieties with their discriminating sensitivity to salinity would be highlighting the impact of PAs on their level of anti-oxidative response. Given that rice with its wider habits and adaptability in various environment is also cultivated in costal saline fallows where salinity is prevalent in different degrees and duration. Thus, it is important to ascertain its responses and reactions to the said condition, which is based on mainly exclusion of salt in excess from the tissues and secondarily, mitigation of oxidative damage by its improved machineries. Since the two varieties viz. Nonabokra and Swarna as employed in the present investigation was assessed for adaptability against ion toxicity caused by NaCl. Thus Nonabokra had been selected as salt tolerant whereas the other variety Swarna was reported to be susceptible (Basu et al. 2010). Therefore, the oxidative exposure and its consideration would be another imperative to justify. With this background the objective of the present study was to clarify the comparative antioxidative responses of two *indica* rice varieties (namely, Nonabokra and Swarna) under simulated condition of salinity and its extent of improvisation with exogenously applied Put.

Materials and methods

The experiment was set in the laboratory of Plant Physiology and Plant Molecular Biology section, Department of Botany,

University of Kalyani, West Bengal, India. The seeds of two traditional semi tall indica rice varieties viz., Nonabokra and Swarna (both are reported as with 110–120 duration, photo-insensitive and nitrogen responsive) were collected from the Regional Rice Research Station, Chinsurah, West Bengal, India. Initially seeds were tested for viability as suggested by Hong and Ellis (2004) under laboratory condition of optimum temperature (27 ± 1 °C) and relative humidity around 80 % in a seed germinator. After successful germination, 1 days old seedlings were transplanted in Hoagland's nutrient media (Hoagland and Arnon 1950) of 2–2.5 lit capacity in a plastic container for 10 days in open air for proper growth and acclimatization under natural condition. The average day and night period during month of June was: average temperature = 30 °C; Relative humidity = 70–80 %, photoperiod = 14/10 h light/dark). The nutrient media was renewed at every 5 days and continued for 15 days. After 15 days, they were divided into 4 treatments in the same medium with three replications of each: 0 mM NaCl, 200 mM NaCl (18 day m^{-1}), 200 mM NaCl + 1.0 mM Put, 200 mM NaCl + 2 mM Put. Admitted well, salinity is offered by a composite effect of other salts of alkali metals like calcium, magnesium etc., however it is the sodium salt which maximally imparts the toxicity to the plants (Munns 2002). The level of salinity was adopted from earlier work on salt tolerance of rice (Roy et al. 2005). All these sets maintained under above said condition for 7 days. Plant samples were harvested by excising the leaves from the stem. Leaves were excised and samples were immediately freezed in liquid nitrogen and preserved in -70 °C (Thermo, USA), for further biochemical analysis. For each analysis, equal amount of freezed leaf samples (from 5 seedlings) with three replications from both control and treatment were taken and following parameters were exercised to evaluate antioxidation strategies with standard protocols

Estimation of sodium ion (Na^+) and potassium ion (K^+) ions in plant tissues

The accumulation of Na^+ and K^+ in the leaf tissues of both control and treatment sets were dried completely at 80 °C and with a mixture of tri acid ($HCl:HNO_3:HClO_4=1:1:1$). After complete digestion of plant materials the solution was made with deionized water and analysis for Na^+ and K^+ was done with atomic absorption spectrophotometer (Schimadzu, Model No. A6800) as described by Liu and Moriguchi (2006).

Determination of lipid peroxidation

Lipid peroxidation of the salinity induced plants and under treatment of Put was measured as content of malondialdehyde (MDA) according to Heath and Packer (1968). 1.0 g of freezed leaf samples was thoroughly homogenized in 5 ml 20 % (w/v) TCA followed by centrifugation at 5,000 rpm

for 10 min under cold condition. The supernatant taken, reacted with thiobarbituric (TBA) acid and MDA content with UV-vis spectrophotometer (Cecil, 210, USA) was measured by reading the absorbance.

Quantification of polyamines

Determination of PAs was done according to Zhao and Yang (2008). 500 mg of freezed leaf tissues was extracted with perchloric acid followed by centrifugation at $26,000 \times g$ for 15 min at 4 °C. The supernatant and reference PAs (Put, Spd and Spm) was dansylated in benzene. The separation of individual PAs were done by TLC on HPTRC silica gel 60 plate (E-Merck) with solvent as chloroform: diethyl amine (100:20). From the fluorescence the on UV-Trans- illuminator the individual PAs were detected and scraped off and eluted with chloroform : acetic acid : water (10:10:1). Finally, the quantification of PAs was done by reading the fluorescence at 337 nm excitation and 495 nm emission by a fluorescence spectrophotometer (Perkin –Elmer, MmPF44B).

Estimation of reactive oxygen species: superoxide (O_2^-) and hydrogen peroxide (H_2O_2)

For determination of O_2^- generation the method was adopted from Elstner and Heupl (1976) with some modification. Freezed leaf sample (1.0 g) was thoroughly ground in 65 mM phosphate buffer (pH 7.8) and supernatant was saved following centrifugation at $5,000 \times g$, for 15 min, at 4 °C. 1.0 ml of assay mixture consisting of 65 mM phosphate buffer (pH 7.8), 10 mM of hydroxylamine hydrochloride and 1.0 ml of supernatant was incubated at 25 °C for 30 min. Following incubation, a mixture of 10 mM sulphanilamide and 7.0 mM alpha-naphthyl amine was added and incubated at 25 °C for 20 min. The changes in absorbance against a reagent blank was recorded spectrophotometrically at 530 nm and generated O_2^- was calculated from a standard of nitrous oxide (NO^-). For H_2O_2 , the leaf sample (1.0 g) was crushed with liquid nitrogen followed by extraction of 10 % TCA. The supernatant was saved following centrifugation at $10,000 \times g$ for 15 min, at 4 °C. The assay mixture of 3.0 ml (consisting of 0.5 ml supernatant, 0.5 mM KI and 10 mM phosphate buffer, pH 7.0) was incubated at 37 °C for 1/2 h. The content of H_2O_2 was determined for recording the absorbance at 390 nm against a reagent blank as suggested by Velikova et al. (2000). For the standards of H_2O_2 , a stock of 30 % (w/v) was used

In order to study the O_2^- and H_2O_2 generation by concerned enzyme, [NAD(P)H-Oxidase], two potent inhibitors namely sodium azide (NaN_3) and imidazole (IMZ) were used (Achary et al. 2008). The leaf extract for both O_2^- and H_2O_2 were incubated with 1 mM NaN_3 and IMZ separately at 30 °C for 15 min. The inhibition for O_2^- and H_2O_2 was detected as described above.

Estimation of anthocyanin, glutathione and flavonoids

The anthocyanin content of the tissues from both control and treatment sets were estimated according to Basu et al. (2009). 1 g of leaf sample (frozen) from both control and treated were thoroughly extracted in 3 ml of methanol-HCl (1 % HCl, v/v), the samples were left at 4 °C in the refrigerator for 2 days. Later on, the extract was filtered and total anthocyanin content was measured by an UV-visible spectrophotometer as the difference between the absorbance at 530 nm and 657 nm. From the data the content was deduced as change of absorbance (A_{530}) per unit of fresh sample.

To determine the contents of glutathione, the frozen plant tissue (1.0 g of leaf) was homogenized in TCA solution under cold condition followed by centrifugation at $15,000\times g$ for 10 min at 4 °C. The reduced form of glutathione (GSH) was estimated according to Hissin and Hilf (1976). The supernatant was taken in 0.1 M phosphate buffer (pH 8.0) followed by adjustment of pH by adding 5 M NaOH and 5 M EDTA, where final pH was recorded at 8.0. The assay mixture containing phosphate buffer and 0.1 % (w/v) O-phthalaldehyde and an aliquot of diluted supernatant was incubated at room temperature for an hour. The fluorescence intensity was monitored at 420 nm (excitation) and 350 nm (emission). For oxidized form of glutathione (GSSG) the diluted supernatant was incubated with 0.4 M N-ethylmaleimide (NEM) for 30 mins. The mixture was diluted by 0.1 N NaOH and adjusted to pH 12.0. An aliquot of the mixture was taken and reacted with same buffer as taken for GSH except 0.1 M NaOH for reading of fluorescence at 420 nm (excitation) and 350 nm (emission). The GSH and GSSG contents were calculated using standards of glutathione.

The flavonoid content of the plant sample was measured according to Basu et al. (2009) with slight modification. The frozen leaf tissue was finely grinded in liquid nitrogen and extracted with n-butanol-water (1:1), the extract was concentrated under vacuum concentrator at 50 °C and residues were collected. By dissolving the residue in 50 % ethanol reacted with 5 % sodium nitrite solution followed by addition of 10 % aluminium nitrite. The reaction was stopped with 1 M sodium hydroxide solution. The absorbance was read at 510 nm with diluent (30 % ethanol) and flavonoid content was determined as described earlier.

Detection of S-adenosyl methionine decarboxylase (SAM-DC) from treated plants

For expression of salinity induced protein for SAM-DC (E.C.4.1.1.50), total protein was extracted from rice plants both under control and treatments. 1.0 g of leaf tissues was homogenized in extraction buffer containing 0.1 M Tris-HCl (pH 6.8), 0.25 mM sucrose, 0.1 mM PMSF, 0.1 mM

TT, 0.5 mM $MgCl_2$, (1:2), 0.1 mM PVPP, 0.01 mM BSA. Following centrifugation at $10,000\times g$, 10 min, 4 °C, the supernatant was concentrated under vacuum (−40 °C) and run on 10 % SDS gel for separation of polypeptides. The protein was electro blotted by transferring on PVDF membrane (Hybond-P, Amersham Pharmacia) with 1X TBS buffer at 40 °C for 2 h in an Electro-Blotter apparatus (Bio RAD Mini trans blot Cell). The membrane was blocked in 5 % BSA in 1X TBS for 2.5 h. It was then probed with purified primary antibody for SAM-DC (a generous gift from Prof. D.N. Sengupta, Department of Botany, Bose Institute, Calcutta, INDIA) in 1: 10,000 dilution with 1X TBS-0.05 % and 0.1 % BSA for over night. The detection of SAM-DC was finally done by incubating the membrane with alkaline phosphatase –conjugated goat anti rabbit IgG (Bangalore Genei) in 1: 1,000 dilution. The detection of SAM-DC was done by developing with substrate solution containing NBT-BCIP (Sambrook and Russell 2001)

Enzyme assays

- i) Guaiacol peroxidase: Steady state measurements for the activity of guaiacol peroxidase (EC 1.11.1.7) were assayed spectrophotometrically using *O*-dianisidine as electron donor and hydrogen peroxide (H_2O_2) as substrate. The enzyme extract was prepared by thoroughly homogenizing the leaf tissues (1.0 g) in liquid nitrogen followed by with 0.1 M potassium phosphate buffer (pH 7.0) under cold condition. The homogenate was centrifuged at $15,000\times g$, at 4 °C for 15 min. The protein content of the supernatant was concentrated using 80 % ammonium sulphate $(NH_4)_2SO_4$ precipitation followed by dialysis and lyophilization (Sambrook and Russell 2001). The protein was measured as per Bradford 3 (1976). The concentrated protein samples was used as source of enzyme and incubated in a assay mixture containing (0.1 M phosphate buffer, pH 6.5; 1.5 mM *O*-dianisidine; 0.2 M H_2O_2 ; 50 µg of protein) at 37 °C. The absorbance was recorded at 430 nm using extinction coefficient of *o*-dianisidine (26.2/mM/cm). One unit of enzyme activity was determined as amount of enzyme required changing the absorbance by 0.1 per unit time (Heu et al. 2009).
- ii) Catalase (EC 1.11.1.6) activity of the samples was assayed according to Nahakpam and Kavita (2011). 1 g of leaf samples was homogenized in 3 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 0.5 mM EDTA, 2 % (w/v) PVP and 0.5 % (v/v) Triton X-100. The homogenate was centrifuged at $22,000\times g$ for 15 min at 4 °C; the supernatant was used for enzyme assay. The total assay mixture of 1.5 ml contained 1,000 µl of 200 mM KH_2PO_4 buffer (pH 7.0), 400 µl of 200 mM H_2O_2 and 100 µl of enzyme extract. The

decomposition of H_2O_2 was followed at 240 nm (extinction coefficient of 0.036/mM/cm) by the decrease in absorbance. Enzyme specific activity is expressed as μmol of H_2O_2 oxidized/min/mg protein.

- iii) Glutathione reductase (EC 1.8.1.7) activity was assayed according to Schaedle and Bassham (1977). Shoot samples of control as well as treated ones of around 1 g were homogenized in chilled mortar and pestle in 30 ml of Tris–HCl buffer (pH 7.6). The homogenate was centrifuged at $22,000\times g$ for 30 min at 4 °C and the supernatant was used for enzyme assay. The reaction mixture in a total volume of 2 ml contained 50 mM Tris–HCl buffer (pH 7.6), 0.15 mM NADPH, 1 mM GSSG, 3 mM MgCl_2 and 200 μl of enzyme extract. The reaction was monitored by the decrease in absorbance of NADPH at 340 nm. The specific activity of enzyme is expressed as μM NADPH oxidized/min/mg protein.

Statistical analysis

All the observations were recorded with three replications ($n=3$) and data were expressed as mean $\pm$ SE. The statistical analysis was performed by one-way (ANOVA) followed by least significance difference (LSD) test taking $p\leq 0.05$ levels of significance (Gomez and Gomez 1984). Windows Microsoft Excel 2003 software was employed for computation, data analysis and graphics.

Results

Concentration of Na^+ and K^+ in tissues under salinity

In general, preliminary, salinity is impacted as an over accumulation of Na^+ and a rapid loss of K^+ from the tissues. Therefore, total Na^+ and K^+ content in both the varieties were considered. Interestingly, a significant ($P\leq 0.05$) accumulation of Na^+ and a loss of K^+ irrespective of varieties under 200 mM NaCl were recorded. However, the accumulation of Na^+ was maximum in Swarna (40.9 %) than Nonabokra (19.2 %) over the control (Fig. 1a). Similarly, for loss of K^+ , Swarna recorded it maximum (42.8 %) and Nonabokra (28.04 %) over their control (Fig. 1b). Application of Put had dose-dependant effects for stabilization of accumulation of Na^+ and K^+ loss significantly ($P\leq 0.05$) in both the varieties. It was recorded that Put could minimized the loss of K^+ by 14.63 % and 18.29 % for Nonabokra at 1 mM and 2 mM concentrations (Fig. 1b) respectively. On the other hand, Put had similar effects for Swarna, where reduction in loss for K^+ was recorded as 11.4 % and 27.14 % by its two doses respectively over those under stress (Fig. 1b).

Effects of NaCl on the content of total free and bound PAs in leaves

The accumulation of total free PAs in the rice varieties (Nonabokra and Swarna) under NaCl and supplemented with varying concentrations (1 mM and 2 mM) of Put recorded some interesting results of the present investigation. It recorded an significant ($P\leq 0.05$) increase in total soluble PAs content irrespective of varieties under salinity over control and it was 19.11 % and 6.25 % in Nonabokra and Swarna respectively (Fig. 2a). However, the response to exogenously applied Put (2 mM) inducing the free PAs recorded significant ($P\leq 0.05$) increase more in case of Swarna (38.23 %) than Nonabokra (27.16 %) (Fig. 2a). This observation indicated that level of polyamine profiles could be induced discriminately in plants and this is modulated as a function of application of exogenous PAs.

The results were also interesting for the bound PAs under salinity irrespective of rice varieties. Both the varieties recorded a significant ($P\leq 0.05$) increase in bound PAs under salinity as compared over control (for Nonabokra it was 31.4 % and for Swarna it was 25.6 %) (Fig. 2b). Such an increase may suggest differential sensitivity for PAs under stress condition for two varieties. Moreover, when plants were treated with exogenous Put, the accumulation of bound PAs was over expressed by higher fold for both the varieties. Still, Nonabokra recorded at both the concentrations of Put (17.64 %) more responsive than Swarna (12.5 %) at 2 mM concentration of applied Put for bound PAs accumulation (Fig. 2b). This may suggest the discrimination in sensation for perception of any inducer molecules (Put in the present case) under stress condition for the two varieties.

Effects of put on O_2^- and H_2O_2 generation under NaCl stress

It is quite expected that NaCl would be effective on development of free radicals according to its potential to induce oxidative stress in the tissues. The salinity at 200 mM concentration had increased the O_2^- and H_2O_2 generation significantly ($P\leq 0.05$) as compared to those under control condition irrespective of varieties. The variety, Swarna, recorded as more sensitive to oxidative damage as it accumulated more O_2^- (52.38 %) than Nonabokra (29.4 %), over the control (Fig. 3a). Similar effects were found for accumulation of H_2O_2 , it was 30.1 % and 20.28 % in Swarna and Nonabokra respectively over control (Fig. 3b).

The exogenous application of Put had registered its effectiveness to down regulate the accumulation of O_2^- and H_2O_2 significantly ($P\leq 0.05$) for both the varieties and it was dose dependant. Likewise, the down regulation of O_2^- by 1 mM Put was recorded 22.7 % and 34.37 % for Nonabokra and Swarna respectively (Fig. 3a). For 2 mM concentration

Fig. 1 Concentration of Na⁺ (a) and K⁺ (b) in the tissues of two rice varieties i.e. Nonabokra and Swarna under salinity (200 mM NaCl) and effects of Put (1 mM & 2 mM) thereon. Data are mean of three replicates $\pm$ SE, compared to the control (0 mM) at $P \leq 0.05$ (*). NN =

Nonabokra under normal condition, NS = Nonabokra under salinity (200 mM), SN = Swarna under normal condition, SS = Swarna under salinity (200 mM)

of Put, the down regulation in O₂⁻ content was much pronounced and it recorded as 56.44 % and 60.12 % for Nonabokra and Swarna respectively (Fig. 3a). So, this showed that Put might have some differential efficacy for antagonism of O₂⁻ generation according to these two rice varieties. Similar trends were also observed by H₂O₂, where Put (1 mM) had reduced the H₂O₂ content by 9.97 % and 11.20 % for Nonabokra and Swarna respectively (Fig. 3b). However, the reductions with 2 mM Put was significant ($P \leq 0.05$) and for Nonabokra and Swarna it was 15.61 % and 20.35 % respectively.

Effects of put on lipid peroxidation under NaCl stress

The assay of MDA content revealed that both the varieties were prone to lipid peroxidation significantly ($P \leq 0.05$) under NaCl stress when compared to that under control condition. The content of MDA increased significantly under NaCl being more in Swarna (56.41 %) than Nonabokra (30.95 %) over control (Fig. 4). It is recorded that exogenous application of Put (1 mM) could reduce the MDA

content by 8.89 % and 3.17 % in Nonabokra and Swarna respectively (Fig. 4). It is noteworthy that application of 2 mM Put significantly ($P \leq 0.05$) reduce the MDA content and Swarna was more responding to Put since it checked lipid peroxidation by 23.63 %. But, Nonabokra recorded 36.05 % at same concentration of Put.

Effects of inhibitors on generation of ROS under NaCl stress

The most common inhibitors for diminishing the generation of ROS are Imidazole (IMZ) and sodium Azide (NaN₃). The two inhibitors down regulating the pathways generating different free radicals for induction of lipid peroxidation. Results presented herein with respect to inhibitor study are based on significant ($P \leq 0.05$) down regulation of the O₂⁻ (Fig. 5a) and H₂O₂ (Fig. 5b) in the tissues under NaCl stress. It is note worthy that the use of IMZ had reduced significantly ($P \leq 0.05$) the activity of O₂⁻ and H₂O₂ by 47.85 and 22.88 % respectively in Swarna. The other variety, Nonabokra also responded significantly ($P \leq 0.05$) well with use

Fig. 2 Changes of bound (a) and free polyamine (b) content in Nonabokra and Swarna under salinity (200 mM NaCl) and effects of Put (1 mM & 2 mM) thereon. Data are mean of three replicates $\pm$ SE,

compared to the control (0 mM) at $P \leq 0.05$ (*). NN = Nonabokra under normal condition, NS = Nonabokra under salinity (200 mM), SN = Swarna under normal condition, SS = Swarna under salinity (200 mM)

Fig. 3 Generation of O_2^- (a) and H_2O_2 (b) in Nonabokra and Swarna under salinity (200 mM) and effects of Put (1 mM & 2 mM) thereon. Data are mean of three replicates $\pm$ SE, compared to the control (0 mM)

of IMZ by reducing O_2^- (40.67 %) and H_2O_2 (21.21 %) content. Another inhibitor i.e. NaN_3 had the same effects on O_2^- and H_2O_2 content for both the varieties. More specifically, Nonabokra had reduced the O_2^- and H_2O_2 content by 23.36 % and 12.85 % respectively; whereas for Swarna it was 30.47 and 11.00 % for O_2^- and H_2O_2 content respectively. This is interesting to note that these two inhibitors have similar effect on down regulation of ROS (O_2^- and H_2O_2) as well as Put with two doses. With this finding it is clear that Put might have inhibited the NAD(P)H-Oxidase activity in similar way as done by the two inhibitors (i.e., IMZ and NaN_3) for the generation of O_2^- and H_2O_2 . Therefore the downregulation of O_2^- and H_2O_2 by application of Put could be inferred upon the involvement of NAD(P)H-Oxidase activity. The results presented with respect to inhibition of ROS by IMZ and NaN_3 might be resembling to that of Put treatment to plants for the same purpose.

Fig. 4 MDA content of Nonabokra and Swarna under salinity (200 mM NaCl) and effects of Put (1 mM & 2 mM) thereon. Data are mean of three replicates $\pm$ SE, compared to the control (0 mM) at $P \leq 0.05$ (*). NN = Nonabokra under normal condition, NS = Nonabokra under salinity (200 mM), SN = Swarna under normal condition, SS = Swarna under salinity (200 mM)

at $P \leq 0.05$ (*). NN = Nonabokra under normal condition, NS = Nonabokra under salinity (200 mM), SN = Swarna under normal condition, SS = Swarna under salinity (200 mM)

Effects of put on anthocyanin, flavonoid and glutathione content under NaCl stress

Anthocyanin and Flavonoids are two predominant phenolics specifically required for down regulation of oxidative stress by non enzymatic pathways. The anthocyanin and flavonoid content were significantly ($P \leq 0.05$) varied when those were exposed to NaCl stress. Interestingly, anthocyanin was more in abundance in both the varieties viz. Swarna and Nonabokra under control and that was 54.92 % and 49.12 % respectively over those under NaCl stress (Fig. 6a). Likewise, flavonoid content was also significantly ($P \leq 0.05$) changed in Nonabokra and Swarna (62.72 % and 61.44 % respectively) under salinity (Fig. 6b). Interestingly, Swarna and Nonabokra had both accumulated more anthocyanin content with Put treatment (at 1 mM) significant ($P \leq 0.05$) and those were 33.33 and 17.14 % respectively when compared only under NaCl stress (Fig. 6a). More over, with higher concentration of Put i.e. at 2 mM, this value though increased (38.29 and 46.67 % for Nonabokra and Swarna respectively), however, not statistically significant ($P \leq 0.05$) (Fig. 6a). As compared to anthocyanin, the changes in flavonoid content was less pronounced with exogenous application of Put (1 mM) and it recorded 14.25 % and 13.22 % in Nonabokra and Swarna respectively to those under only NaCl (Fig. 6b). For 2 mM Put concentration it was significant ($P \leq 0.05$) and the values were 32.75 % and 32.05 % for Nonabokra and Swarna (Fig. 6b). The ratio of oxidized and reduced glutathione is one of the most important criteria in plants maintaining cellular redox, particularly, under exposure of oxidative environment. Thus, when we measure the oxidized and reduced form of Glutathione i.e., GSSG and GSH a significant ($P \leq 0.05$) depletion of reduced form was recorded under stress as compared to that of control for both the varieties namely Nonabokra and Swarna. Interestingly the oxidized form of glutathione (GSSG) was

Fig. 5 Effects of Imidazole (IMZ) and Sodium Azide (NaN₃) on generation of O₂⁻ (a) and H₂O₂ (b) content in Nonabokra and Swarna under salinity (200 mM NaCl) and Put (1 mM & 2 mM). Data are mean of three replicates $\pm$ SE, compared to the control (0 mM) at $P \leq 0.05$ (*).

significantly ($P \leq 0.05$) prone to be sensitized under stress (40.60 % and 57.35 %) as compared over control for Nonabokra and Swarna under salinity (Fig. 6c). Put has also effected for both the cultivars, to recover the GSSG content at 1 mM concentration (41.86 % for Nonabokra and 58.14 % for Swarna) and at 2 mM concentration significant ($P \leq 0.05$) recovery (55.81 % for Nonabokra and 97.34 % for Swarna) compared to salinity respectively (Fig. 6c). However the depletion of GSH was more in Swarna (28.93 %) than Nonabokra (21.33 %) (Fig. 6d). But those varieties could retrieve the level of GSH over salt treated by 19.76 % and 12.5 % for Swarna and Nonabokra respectively under 1 mM Put (Fig. 6d). Whereas, it was 32.61 % and 19.06 % in both the varieties when treated with Put at 2 mM concentration and significant at ($P \leq 0.05$) (Fig. 6d). Alternatively, the ratio of GSSG:GSH (Fig. 6e) content had the similar trend, and statistically significant ($P \leq 0.05$) under salinity and its interaction with Put mainly at 2 mM.

Induction of SAM-DC under salinity stress

SAM-DC enzyme is regarded as a rate-limiting enzyme for PAs biosynthesis with a rapid turn over. This enzyme has a role in synthesis of Spd (a triamine) biosynthesis, with Put as an intermediate moiety and thus confers the stress tolerance. In the present investigation, 25 μ g of total protein was loaded from each treatment for each variety and separated on resolving gel with 10 % sodium dodecyl-sulphate (SDS) by electrophoresis for immunoblotting. From the results of Western blotting, a distinct band of 35 kDa was resolved when hybridized with SAM-DC antisera of rice as a probe, irrespective of varieties as compared to control (Fig. 7). However, in case of Nonabokra Put recorded more inducing for expression of 35 Kd fragment, whereas it was less expressed in case of Swarna under same condition. Therefore, two varieties were variable in response to enzyme expression under Put induction.

NN = Nonabokra under normal condition, NS = Nonabokra under salinity (200 mM), SN = Swarna under normal condition, SS = Swarna under salinity (200 mM)

Effects of put on the activities of guaiacol peroxidase (GPX) catalase (CAT) and glutathione reductase (GR) in rice under NaCl stress

It is found that GPX activity, irrespective of varieties, increased significantly ($P \leq 0.05$) under NaCl stress. Under said condition, Nonabokra recorded 31.76 % over folded activity than control; whereas, in Swarna it was 34.64 % (Fig. 8a). On the other hand, exogenous application of Put had increased the activity maximally in Nonabokra (8.29 %) than Swarna (9.00 %) at 1 mM concentration. But, at 2 mM concentration Swarna had increased the rate (16.94 %), however, for Nonabokra it did not mark any significant ($P \leq 0.05$) change (only 16.09 %) as compared to stress (Fig. 8a). For CAT, on the contrary, it was significant ($P \leq 0.05$) subdued effect that both the varieties recorded under NaCl stress (Fig. 8b). It recorded 54.11 % and 45.91 % less activity in Nonabokra and Swarna under NaCl as compared to those under control condition. Still, the mitigation of impaired activity was not satisfactorily done by Put with its all doses (1 mM and 2 mM) irrespective of varieties. CAT activity, however, was increased marginally by 25.0 % and 14.51 % in Nonabokra and Swarna respectively at 1 mM concentration of Put, when compared to those over only the NaCl treated. Whereas it was (42.64 % and 31.16 %) significant ($P \leq 0.05$) at 2 mM concentration of Put (Fig. 8b). GR, the enzyme is required for the conversion of oxidized glutathione (GSSG) into its reduced form (GSH) with donation of two electrons by NADPH + H⁺. The trend in activity of GR is similar as compared to CAT, it recorded an decreased activity significant at ($P \leq 0.05$) under NaCl stress than that under control for both the varieties (Fig. 8c). Swarna had been maximum in subdued activity (30.62 %) and Nonabokra the minimum (20.28 %) when both were compared to control condition. The exogenously application of Put recorded a distinguishing feature as shown an up-regulation of GR activity irrespective of varieties. Plants were

Fig. 6 Content of Anthocyanin (**a**), Flavonoids (**b**), Glutathione oxidized (GSSG) (**c**), Glutathione reduced (GSH) (**d**) and GSH:GSSG (**e**) in Nonabokra and Swarna under salinity (200 mM) and effects of Put (1 mM & 2 mM) thereon. Data are mean of three replicates $\pm$ SE,

compared to the control (0 mM) at $P \leq 0.05$ (*). NN = Nonabokra under normal condition, NS = Nonabokra under salinity (200 mM), SN = Swarna under normal condition, SS = Swarna under salinity (200 mM)

Fig. 7 Detection of induced SAM-DC (35 Kd) in Nonabokra and Swarna under salinity (200 mM) and effects of Put (2 mM) thereon

relieved from maintaining the down-folded GR activity under NaCl stress when those were treated with Put irrespective of the varieties, Put had maximized the activity by 13.23 % and 12.63 % for 1 mM and it was more significant ($P \leq 0.05$) for 2 mM maximizing the activity by 14.94 and 19.18 % for Nonabokra and Swarna respectively over salt treated (Fig. 8c).

Discussion

The reduced growth and development under salinity stress has well been documented and characterized in different

Fig. 8 Assay of GPX (**a**), CAT (**b**) and GR (**c**) in Nonabokra and Swarna under salinity (200 mM) and effects of Put (1 mM & 2 mM) thereon. Data are mean of three replicates $\pm$ SE, compared to the

control (0 mM) at $P \leq 0.05$ (*). NN = Nonabokra under normal condition, NS = Nonabokra under salinity (200 mM), SN = Swarna under normal condition, SS = Swarna under salinity (200 mM)

crop plants like rice, pepper, potato, tomato, lettuce, melon etc. with proper describing of their physiological and cellular events (Eva et al. 2010). In most of the cases, salinity, besides its specific ion effects on plant systems, also registers an over oxidation state of tissues concerned. When there accumulates a high concentration of salts beyond a threshold values, the inherent genetic plasticity in crop species starts defolding to upregulate and/or down regulate for specific characters. Those characters are concerned with salt induced reactions in plants, highly variable amongst species and thereby demark as salt sensitive or resistant (Roy and Wu 2002). PAs, in general, plays an important role in alteration of plant's cellular metabolism in response to various environmental stresses (Groppa et al. 2003). Therefore, it is imperative to justify the PAs metabolism under oxidative stress. However, preliminarily, plants in the present experiment were mitigated from detrimental effects of NaCl by reduction in sodium absorption and potassium loss under application of exogenous Put. The stabilization of membrane integrity by PAs application under salinity bears the conformity of our findings as reported earlier (Ghosh et al. 2011). From the ongoing experiment, the concentration of accumulated PAs and its profile under salinity and in supplementation with Put became evident in an inconsistent manner. Though the application of PAs is demonstrated to be implicative as one of the molecules for stress response,

however, the findings of the present work suggests otherwise, that PAs sensitivity may vary genotypically. Thus, the changes of free PAs remain significantly varied under salinity irrespective of varieties, still, Put became more affirmative for sensitive variety like Swarna and accumulated more free PAs. On the other hand, for bound PAs it recorded the discriminatory of two varieties for accumulation and was more induced for Nonabokra over Swarna. Therefore, this finding may confirm that underline mechanism for salt sensitivity against salinity is more favored for bound PAs than free. The sensitive or tolerant variety might be discerning not only in their PAs pool, but also the complexes with different cellular moieties (Aronova et al. 2005). Moreover, the content of PAs in plants depends on biosynthesis and its turnover in tissues according to the cellular demands, particularly, under stressful conditions (Zapata et al. 2003.) It is established that SAM-DC is an important enzyme that converts SAM into its decarboxylated form i.e., decarboxylated S-adenosyl methionine (dc-SAM). dc-SAM in turn acts as a precursor of higher polyamines like Spd and Spm. In fact, this enzyme is acted in conjugation with another enzyme spermidine synthase (E.C.2.5.1.16) and spermine synthase (E.C. 2.5.1.22) respectively (Zhao and Yang 2008). At the cellular level, gene for SAM-DC is encoded with a fragment of 35 kDa polypeptide as detected from the present study. In plants SAM-DC is overexpressed with a number of

polypeptide variants to accomplish the biosynthesis of polyamines (Put) under salinity stress (Urano et al. 2004). The expressivity of this enzyme is recorded in a differential manner for both the varieties (Nonabokra and Swarna) and thus could be indicating for PAs sensitivity under salinity stress. Undoubtedly, Spd and Spm are regarded to be essential to protect cellular membrane integrity irrespective of abiotic stress (Zapata et al. 2003). Therefore, SAM-DC, thus, signifies its worth to be a key enzyme for modulation of PAs and that also have been focused in other studies under various stresses (Quinet et al. 2010).

Now, regarding the metals induced oxidative stress the generation of some free radicals are inevitable in plants and those are unstable, still, highly energized in their oxidized states. Those free radicals are required for peroxidation of cellular macromolecules including membrane lipids, preliminary, and thereafter disintegrating the protein and nucleic acids. MDA being one of the products of lipid peroxidation, thus, suggesting oxidative damage of the plants under salinity in this present experiment. Moreover, application of Put on the plants under salinity had significantly decreased the reactive oxygen species (ROS) like super oxide radical (O_2^-) and peroxide (H_2O_2) in the tissues. This might be indicative of the fact that PAs could diminish the lipid peroxidation by down regulating the ROS in the tissues. This could be facilitated either by down regulating the synthesis of ROS or/and lysis of those ROS by enzymatic machineries (Gill and Tuteja 2010). Since, PAs are the characteristic moieties being highly protonated at physiological pH; thus could readily be bound with negatively charged fatty acid tails as well as phospholipid head groups of membrane. Thus, PAs could readily be accessible for membrane stabilization under salinity stress that is evident from this experiment.

In fact, the sources of free radicals like O_2^- is dependent on susceptibility of metal ions (like Fe^{+2} , Al^{+3} , Na^+ etc.) to undergo auto-oxidation from electron transport chain (ETC) on cellular membrane and organelle in plant tissues (Apel and Heribert 2009). PAs may hinder this reaction by accepting the electrons to those mostly with their unsaturated aliphatic chains and thus keep the auto-oxidation of metals in a subdued state (Velikova et al. 2000). In the present experiment, Put was recorded to down regulate the rate of O_2^- and H_2O_2 generation in the salt treated rice varieties. In fact, NAD(P)H-oxidase, either chloroplastic or cytosolic exhibits an oxidation of NADPH and reduction of O_2 into O_2^- . The later in turn is dismutated into H_2O_2 (Sagi and Fluhr 2006). At the cellular level, this could be circumvented by inhibiting one of the enzymes like NAD(P)H-oxidase recruited for generation of O_2^- . There recorded another enzyme called NAD(P)H-peroxidase that also contributes to the generation of ROS. In fact, this enzyme behaves as an oxidase that mediates the oxidation of NADPH and thus reduction of O_2 into O_2^- and H_2O_2

through a complex pathway (Achary et al. 2008). Moreover, NAD(P)H-oxidase may induce an oxidative burst where O_2^- , H_2O_2 and OH^- are generated on the cell surface (Arleta et al. 2009). This was later confirmed based on the substantial observations those recorded the decrease in O_2^- and H_2O_2 generation, when plants were treated with IMZ and NaN_3 , both being typical inhibitors for NAD(P)H-oxidase and NAD(P)H-peroxidase respectively. This fits quite in agreement in down regulation of O_2^- by PAs through down regulation of NAD(P)H-oxidase activity as evident and holds true for the other heavy metals also (Noctor et al. 2006).

Another metabolic pathway that relates the alleviation of salt induced oxidative stress is the accumulation of phenolic compounds with various molecular configurations having intrinsic efficiency to quench excess energy of ROS (Arora et al. 2002). Put, in the present experiment had significantly induced the phenolics accumulation in both the varieties under salinity and more notably, it was dosage dependant of PAs so applied. Hura et al. (2008) reported that over folded accumulation of phenolics may also be substantiated in the tissues through incomplete catabolic procures of higher glycosides as well as pronounced activity of shikimic acid pathway under moderate to severe water stress. The over expression of shikimic acid pathway have been reported in transgenic rice transformed with spermidine synthase (Rodreguez et al. 2009). In other way, PAs exhibit most effective ways for anti oxidation responses, particularly, when those are glycosylated with lactone ring of phenolics. In addition, reports have established the facts that anti-oxidation activity requires more involvement of conjugated phenolics with PAs than free PAs.

The changes in activities of the anti-oxidative enzymes are interesting to note in the varieties of the present experiment, particularly when those are treated with Put. Thus, the activities of GPX, GR and CAT under salinity were significantly different in two varieties when exposed to salinity. However, Nonabokra responded more by several times higher activities than Swarna being induced with Put, as recorded. In actual, PAs can bind to those antioxidative enzymes at their organic or coenzyme moieties or the negatively charged domains of the enzymatic proteins. This is, in fact, required to transport those enzymes to the most vulnerable sites of the cells that undergoes over oxidation to recruit their antioxidation activity (Tang and Ranald 2005). Another possibility may arise from Na^+ or other metal induced denaturation of the enzyme proteins. The roles of PAs are furnished in shielding those proteins on their most vulnerable sites to sustain the native conformity under high ionic environment (Roy and Wu 2002). It is very much well coordinated with the induction of various genes, related to oxidative stress and endogenous accumulation of PAs. These genes also include the organ/tissue specific expression and its regulation under the condition

of oxidative stress or any conditions inducing oxidative stress. Thus, it revealed that high concentration of exogenously applied Spd/Cad had no direct effects on POD/SOD isoforms but might reduce the H_2O_2 concentration of the tissues in *M. crystallinum* (Aronova et al. 2005). On the contrary, there were different organ specific expression of SOD isoforms (Cu/Zn-SOD), those had enhanced activity in root but lesser in leaf tissues (Roy Chaudhury et al. 2007). However, the decreased activity of CAT, irrespective of varieties, in the present experiment could be described as the susceptibility of enzyme protein for denaturation beyond a threshold value of cellular Na^+ concentration as also reported in rice (Morsy et al. 2007) and alfalfa (Wang et al. 2009). GPX, is one of the primary H_2O_2 scavenging enzymes that positions in various cellular organelles like chloroplast, mitochondria, peroxysome, cytosol and even in apoplast. This enzyme being an integral member of ascorbate–glutathione cascade donates an e^- to H_2O_2 either by alone or/and in conjugation with GST catalyzed intermediates (Ashraf 2009). GPX might be accessible to act on relieving the tissue accumulated H_2O_2 , under the condition of over accumulated H_2O_2 even through PAs oxidation. On the other hand, GR is required to replenish the reduced state of glutathione (GSH), which happens to be one of the predominant moieties for non-enzymatic anti-oxidation pathway. The oxidized form of glutathione (GSSG) is readily reduced into GSH by GR. Thus, GR supplements the depletion of GSH under stress or to maintain a threshold value for GSH to GSSG in the tissues. PAs being one of the inducer as suggested by others in the up-regulated activity of GR to replenish the depletion of GSH (Nahakpam and Kavita 2011). Therefore, the possible mechanism underlying the adaptive roles of PAs under salinity was identified from the evocation of antioxidation pathways, at least so revealed from the response of GPX, CAT and GR activity in the present study.

In general, salinity could be detrimental, by inducing an oxidative stress to plants and the tolerance to it depends upon unfolding the genetic plasticity for inducing the antioxidative system. The decline or inadequate expression of anti-oxidative system under this condition is indicative of sensitivity for a variety and to be regarded as susceptible to oxidative damages. It is evident that substantial variations were observed in expression of anti-oxidation pathways of the two varieties i.e. Nonabokra and Swarna under salinity and apparently, Nonabokra seems to be potential candidate for salinity tolerance. The lesser accumulation of ROS and lipid peroxidation, higher accumulation of antioxidant moieties, maintenance of stable anti-oxidative enzyme activities etc., however, might possibly be attributed to Nonabokra for its improved tolerance. Importantly, exogenous application of Put in the present case has rendered its role by induction of both non-enzymatic and enzymatic anti-oxidation

machineries for both the varieties. This supposedly holds the tenability of Put to minimize the accumulation of ROS and repairing the irreversible disintegrations of the plants at cellular levels. Notwithstanding, a short-term exposure to salinity may not necessarily unravel the picture in reality for the plants, particularly, when those were compared to a long-term exposure in rice fallows as well as throughout its life cycle. Moreover, the composite effects out of multi-metal exposure in field must suffice the condition otherwise. Still, the facts and figures, as revealed from the present investigations, may highlight the variability in response of antioxidation pathways between these two rice cultivars those could possibly be useful to understand the underlying mechanism of stress tolerance. In addition, it could be improvising the scope for selection criteria for rice varieties, especially with references to polyamine status in breeding programme under such conditions.

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