NITRIC OXIDE REGULATED IMPROVEMENT IN GROWTH AND YIELD OF RICE PLANTS GROWN UNDER SALINITY STRESS: ANTIOXIDANT DEFENSE SYSTEM

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Abstract. The current work assessed the effects of exogenously applied sodium nitroprusside (SNP) on rice growth and yield under salt stress. Salt stress induced a significant reduction in biomass and grain yield while increased the plant proline, ascorbic acid, H_2O_2 and MDA contents in all studied cultivars. SOD, POD and CAT activities also significantly increased in salt stressed plants; however, the total phenolics content decreased. Application of SNP as seed priming reduced the adverse effects of salinity on plant biomass production and grain yield, while the accumulation of MDA and H_2O_2 decreased. Of different SNP levels, 0.1 mM regime was more effective in reducing the negative effects of salinity. Among fine rice cultivars, Shaheen basmati performed better, while among coarse rice cultivars the performance of IRRI-6 was better to exogenously applied 0.1 mM SNP. We concluded that exogenous application of SNP up-regulated the antioxidative defense mechanism in salt stressed rice plants which resulted in better yield.
Keywords: *antioxidative potential, biomass, grain yield, nitric oxide, Oryza sativa* L.

Introduction

Reactive oxygen species (ROS) induced lipid peroxidation under salt stressed environment is a major restricting factor for better plant growth and development (Ashraf and Foolad, 2007; Ashraf, 2009; Sian et al., 2015). Important ROS such as $^1O_2$, O_2^-, OH^-, and H_2O_2 being highly reactive in nature, can react with vital metabolites and macro-molecules in cells including photosynthetic pigments, lipids, proteins and DNA (Ashraf, 2009; Sian et al., 2015). Among various cell organelles, the major sites for ROS production are the chloroplast, mitochondria, vacuole and microbodies (Ashraf, 2009). The ROS can initiate a series of destructive processes including the inactivation of antioxidative enzymes (Tanou et al., 2009).

Plants possess an antioxidant defense mechanism to detoxify and scavenge the ROS. Among various antioxidants, flavonoids, tocopherols, phenolics, glutathione, carotenoids and ascorbic acid are non-enzymatic antioxidants; while peroxidase (POD), catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), and ascorbate peroxidase (APX) are enzymatic ones (Ashraf, 2009; Ali and Ashraf, 2011). Of different ROS, production of hydrogen peroxide (H_2O_2) acts as a secondary messenger and helps the plants to rapidly adjust under stressful environmental conditions and mediates defense responses (Miller et al., 2010) but only at low concentrations. Hydrogen peroxide an important ROS also acts as a secondary messenger in low

concentration, activates enzymatic antioxidative defense mechanism (Tanou et al., 2009; Hernandez et al., 2010), facilitates the stability of SOS1 mRNA and Na^+ detoxification under salinity (Chung et al., 2008). Under stressed conditions, such as NaCl salinity, over-production of H_2O_2 directly causes oxidative damages to cellular membranes (Miller et al., 2010).

Among various groups of plant secondary metabolites, phenolics and AsA are important with both biological and antioxidant properties (Posmyk et al., 2009; Ali et al., 2013). The activity of phenolics as an antioxidant metabolite is due to its greater radical stabilization and proton donating ability (Rice-Evans et al., 1996). Plant phenolic content has been reported to be affected under different abiotic stresses including salinity (Ashraf et al., 2010; Jamil et al., 2015). Similarly, AsA also plays its role not only as an important non-enzymatic antioxidant; also contribute efficiently in the completion of enzymatic antioxidation.

The content of MDA can be used as an effective indication to judge the salt tolerance ability of different cultivars (Sairam et al., 2005). Studies reveal that less accumulation of MDA is an indication for improved tolerance against salts stress (Liang et al., 2003; Brankova et al., 2005; Ruiz et al., 2005), because its increased content under salt stress resulted in stunted growth in the plants of sesame (Koca et al., 2007) and tomato (Li, 2009).

Alterations in plant water relations under salt stress are the major obstacle for normal plant growth (Habib et al., 2014). To overcome such problems, plants have adapted a special phenomenon for the accumulation of different osmolytes. It has been reported that among different organic compounds that take part in plant osmotic adjustment, proline is not only an osmoregulator but also an important ROS scavenger (Ashraf and Foolad, 2007). Increased accumulation of proline is a general phenomenon under salt stress, however more accumulation takes places in salt tolerant plants in comparison with the sensitive ones (Ashraf and Foolad, 2007; Habib et al., 2012). Therefore, the amount of proline accumulated could be taken as an important index of salinity tolerance (Ashraf and Foolad, 2007). Different plant species have different potential to tackle the adversative effects of salinity regarding the accumulation and/or synthesis of different organic compounds but several high yielding crop cultivars are not well investigated in this regard (Ali and Ashraf, 2011). So, different techniques are being used for the last two decades for the induction of salt tolerance in these crops and have been found effective to some extent (Ashraf and Foolad, 2007). Use of different chemicals exogenously such as foliar spray or their use as pre-sowing seed treatment is one such technique. Of these exogenously applied compounds, SNP is one such compound (Zhang et al., 2006; Habib et al., 2010). It acts as a free radical which is lipophilic and volatile in nature (Hayat et al., 2010). Apart from its regulatory roles in plants, such as in improving seed germination and seedling growth (Zhang et al., 2006; Habib et al., 2010), it also plays a protective role against different abiotic stresses including water stress (Gracia-Mata and Lamattina, 2001) and salinity (Zhao et al., 2007; Zheng et al., 2009; Habib et al., 2010). Under salinity the different protective role of nitric oxide include the improvement in chlorophyll pigments, gas exchange attributes, and PS-II efficiency (Habib et al., 2013). Its role as protective agent in salinity has been described in wheat (Zheng et al., 2009) and maize (Zhang et al., 2006), where it actively took part in increasing the antioxidant enzymes activity and the activities of proton pumps respectively.

As there are reports available in literature about the protective role of NO_2 against the damages caused by salt stress in crop plants but very few is available about its role in seed yield increments especially in rice in relation with plant oxidative defense mechanism. Therefore the present study was considered with the objective to find out the effects of varying regimes of exogenously applied SNP on the yield of different rice cultivars in relation with its role in enhancing the plant antioxidative capacity with special reference to some enzymatic and non-enzymatic compounds under salinity.

Materials and Methods

The experiment was conducted under natural conditions (day length 13.8 h; relative humidity 45.2%; PPFD, 1275 μmol m^{-2} s^{-1}; the day and night temperatures were 36±3 ^{o}C and 27±2 ^{o}C respectively). The total four cultivars of rice were used in this study, two of these were fine rice (Basmati PB-95 and Bsmati Shaheen), while other two (IRRI-6 and KS-282) were coarse rice cultivars. Before treatment the seeds were surface sterilized with 1% solution of NaOCl for 5 min then washed the seeds thrice with ddH$_2$O. The seeds were subsequently soaked in varying levels (0, 0.1, and 0.2 mM) of SNP for 20 h. Thirty days old seedlings (prepared separately) were transplanted in tubs (each filled with 16 kg of sandy loam soil) arranged in CRD with four replicates of each treatment. The seedlings were established after one week of transplant and salt treatment (0 and 80 mM of NaCl) was applied by increasing the level gradually. The experiment was repeated to validate the results. Data for growth and biochemical attributes was recorded after 30 days of the start of NaCl treatment, while yield attributes were estimated at maturity.

Determination of the activity of different antioxidant enzymes

Enzyme extraction

To determine the activity of various antioxidant enzymes, enzyme extracts were prepared following the method described by Ali and Ashraf (2011).

Estimation of the activities of SOD, POD and CAT

The SOD activity was determined following the method of Giannopolitis and Ries (1977) with some modifications while for estimating the activity of CAT and POD the method of Chance and Maehly (1955) was followed with some modifications.

Leaf ascorbic acid contents

Leaf ascorbic acid content was determined by following a method described by Mukherjee and Choudhuri (1983). Varying levels of pure AsA were used to make the standard curves for measuring the AsA content in the mixture. While the leaf total phenolic contents was measured according to a method described by Julkenen-Titto (1985).

Hydrogen peroxide

Hydrogen peroxide (H$_2$O$_2$) content was determined following the method described by Velikova et al. (2000).

Malondialdehyde (MDA)

The method of Carmak and Horst (1991) was used to determine the MDA content.

Proline content

Leaf proline content was determined by following a method described by Bates et al. (1973).

Yield attributes

Different yield attributes such as number of tillers, weight of hundred grains and grain yield per plant were estimated at maturity after drying properly the spikes under natural sun light.

Statistical analysis

The experiment was arranged in a completely randomized design (CRD) with four replicates and the data so generated for different attributes was analyzed using a software named CoSTAT V 6.3 (developed by, Cohort software, Berkeley, California, USA).

Results

Morphology and growth

Shoot and root fresh and dry biomass significantly decreased in studied rice cultivars grown under salt stress (*Fig. 1*; *Table 1*). Exogenous application of SNP as seed treatment was found effective in increasing the fresh and dry biomass of shoot and root in all rice cultivars under non-stressed and salt stressed conditions. For shoot fresh and dry weights, 0.1 mM SNP was more effective under both non-saline and saline conditions, while in relation with root fresh and dry biomass both SNP levels were found equally effective. Among rice cultivars, IRRI-6 and Shaheen Basmati exhibited higher shoot fresh and dry weights under salt stress, while higher root fresh and dry weights were observed in cvs. IRRI-6 and KS-282.

Imposition of saline stress also significantly reduced the shoot length of all rice cultivars (*Fig. 1*; *Table 1*). Exogenous application of different regimes of SNP as pre-sowing seed treatment significantly reduced the adverse effects of rooting medium salinity on shoot length in all rice cultivars. Of different SNP levels, more increase in shoot length was found in plants raised from seeds treated with 0.1 mM SNP under both non-saline and saline conditions. Maximum increase in shoot length was recorded in cv. Shaheen Basmati under saline conditions, however, under non-saline conditions, maximum increase in shoot length was observed in Basmati PB-95.

Enzymatic antioxidants

The activity of antioxidant enzyme superoxide dismutase (SOD) was increased significantly in all rice cultivars grown under salinity (*Fig. 2*; *Table 1*). Pre-sowing seed treatment with different SNP regimes further increased the SOD activity in all rice cultivars under non-saline and salt stressed conditions and the maximal increase in SOD activity due to SNP seed treatment was found in plants raised from seeds

treated with 0.1 mM level of SNP. However in rice cultivar IRRI-6 both SNP regimes equally increased the SOD activity under non-saline and saline conditions. Comparatively SNP-induced more increase in SOD activity was found in cv. Shaheen Basmati under salinity stress.
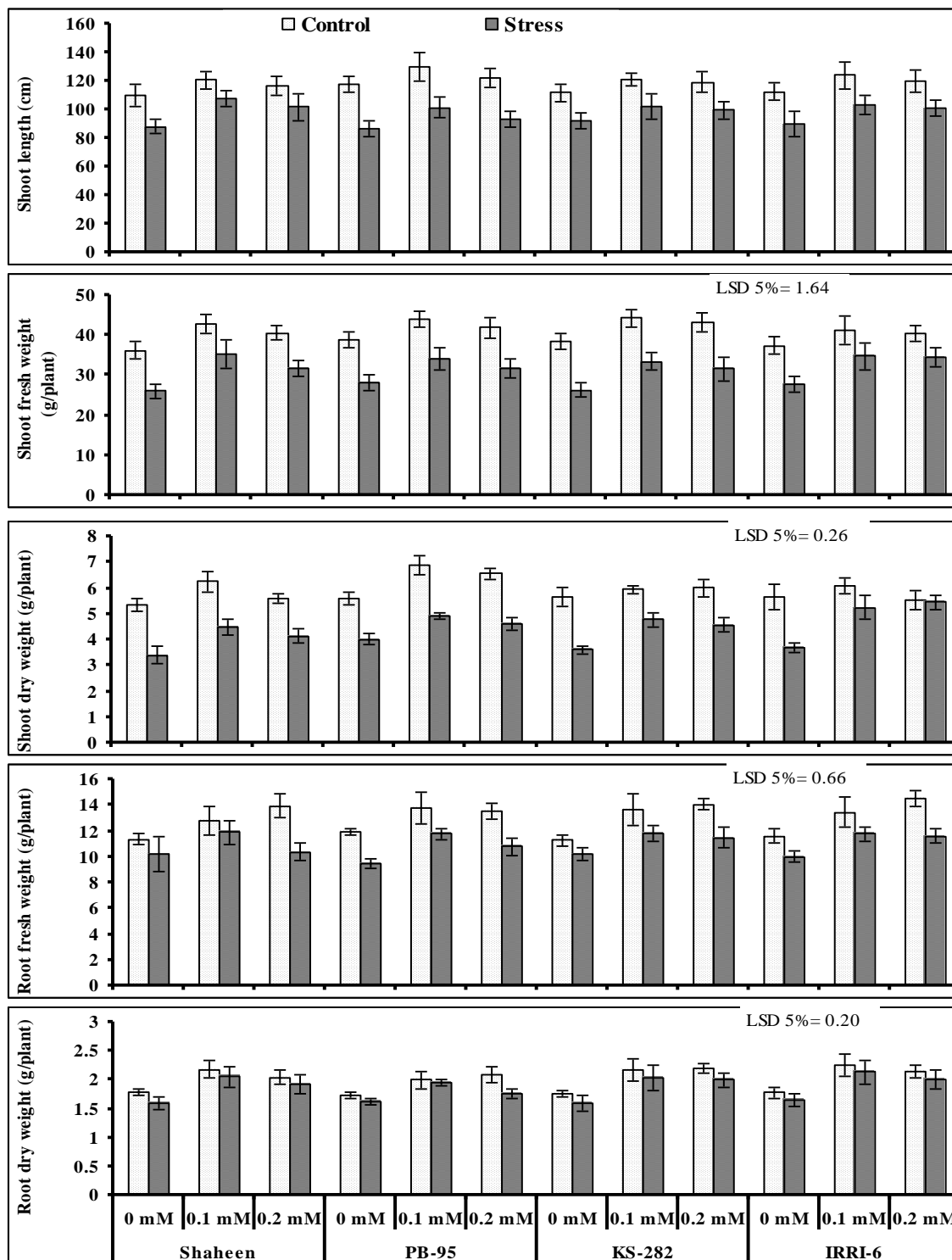

Figure 1. *Growth attributes of four rice cultivars affected by pre-sowing seed treatment with different regimes of SNP grown under salt stress (mean±SE)*

Table 1. *Mean squares from statistical analysis of the data for studied attributes of rice plants of four rice cultivars influenced by exogenously-applied SNP under salt stress*

| SOV	df	Shoot fresh weight	Shoot dry weight	Root fresh weight	Root dry weight	Shoot Length	Number of tillers
Variety (V)	3	15.16 ns	1.74 ***	2.40 ns	0.208 ns	50.04 *	0.177 ns
Salinity (S)	1	2303.40 ***	65.50 ***	85.10 ***	0.432 *	10901.34 ***	29.260 ***
NO (N)	2	26.60 ***	5.64 ***	26.31 ***	2.346 ***	565.36 ***	5.281 **
V x S	3	23.90 ns	1.77 ***	1.10 ns	0.239 ns	31.76 ns	0.454 ns
V x N	6	4.31 ns	0.42 ns	0.09 ns	0.017 ns	7.63 ns	0.072 ns
S x N	2	3.91 ns	0.91 *	7.44 *	0.136 ns	132.01 *	0.947 ns
V x S x N	9	3.24 ns	0.50 ns	1.26 ns	0.036 ns	9.38 ns	0.434 ns
Error	72	10.77	0.26	1.75	0.159	35.72	0.802
SOV	**df**	**SOD**	**POD**	**CAT**	**H_2O_2**	**AsA**	**Proline**
Variety (V)	3	0.54 ns	0.490 ns	0.021 ns	18.84 ns	7.957 ns	1.32 ns
Salinity (S)	1	6850.24 ***	7228.180 ***	1587.620 ***	10157.87 ***	1.227 ***	11961.73 ***
NO (N)	2	105.59 ***	100.730 ***	59.730 ***	274.21 ***	0.107 ***	126.41 ***
V x S	3	0.58 ns	0.013 ns	0.022 ns	17.54 ns	5.044 ns	4.11 ns
V x N	6	0.53 ns	0.033 ns	0.011 ns	1.86 ns	3.721 ns	0.59 ns
S x N	2	28.06 ***	19.576 ***	14.410 ***	22.51 ns	0.003 ns	31.56 *
V x S x N	9	0.28 ns	0.021 ns	0.006 ns	5.62 ns	0.001 ns	1.97 ns
Error	72	1.94	1.661	0.178	18.85	0.002	6.54
SOV	**df**	**MDA**	**Total phenolics**	**100 grain weight**	**Total grain weight/plant**		
Variety (V)	3	3.21 ns	0.056 ns	7.957 ns	0.056 ns		
Salinity (S)	1	25734.95 ***	1229.801 ***	1.227 ***	1229.801 ***		
NO (N)	2	276.74 ***	16.510 ***	0.107 ***	16.510 ***		
V x S	3	19.64 ns	0.128 ns	5.044 ns	0.128 ns		
V x N	6	3.46 ns	0.055 ns	3.721 ns	0.055 ns		
S x N	2	32.43 ns	1.923 ***	0.003 ns	1.923 ***		
V x S x N	9	1.87 ns	0.012 ns	0.001 ns	0.012 ns		
Error	72	12.17	0.210	0.002	0.210		
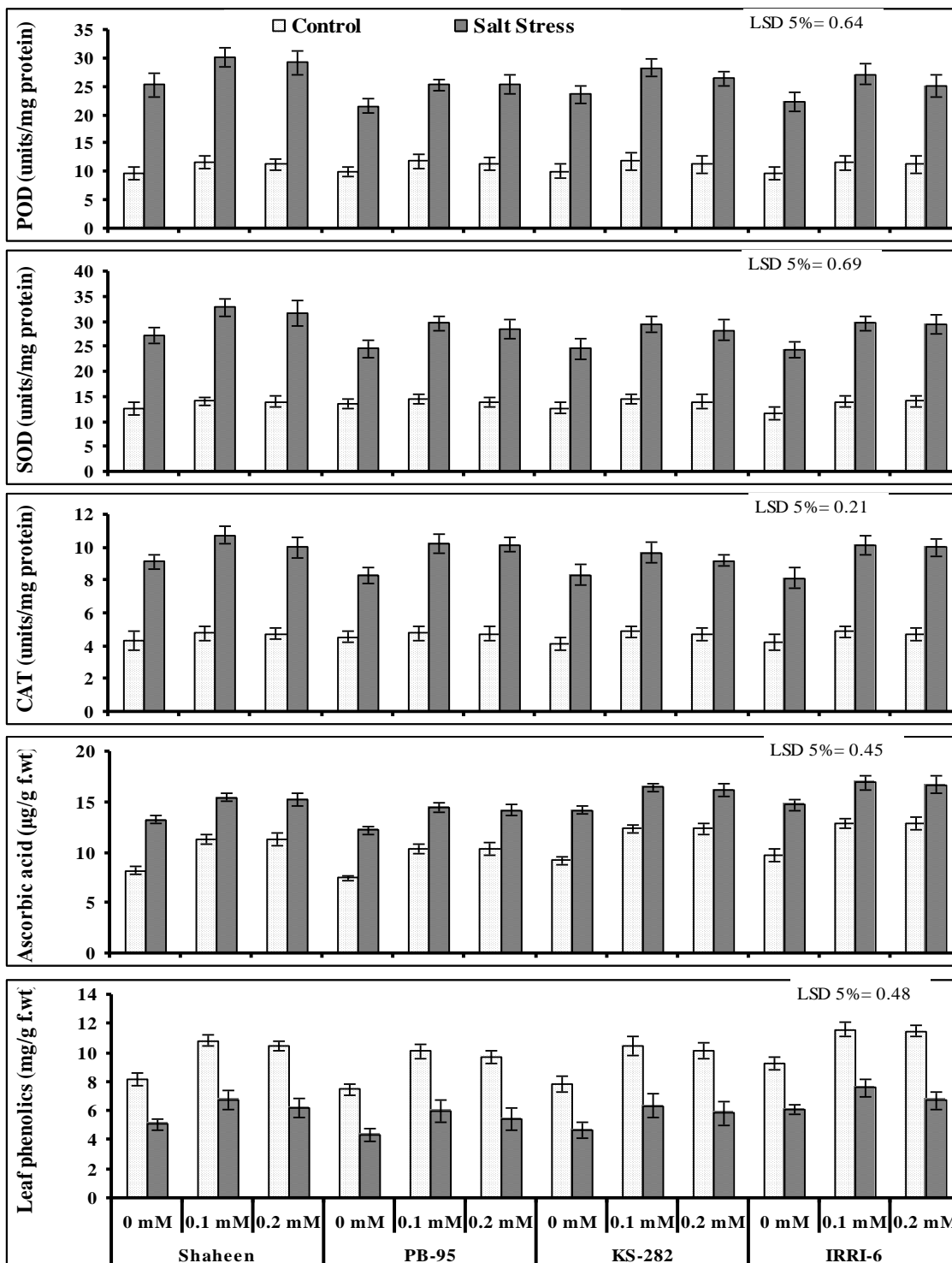
Figure 2. *Enzymatic and non-enzymatic antioxidants of four rice cultivars affected by pre-sowing seed treatment with different regimes of SNP grown under salt stress (mean±SE)*

Rooting medium salinity caused a significant increase in leaf POD activity in plants of all rice cultivars (*Fig. 2*; *Table 1*). Exogenous SNP treatment further increased the POD activity in plants of all rice cultivars grown under both saline and non-saline

conditions and more increase in POD activity was recorded in plants raised from seeds treated with 0.1 mM SNP regime. Among all rice cultivars the maximal increase in POD activity due to SNP treatment was found in cv. Shaheen Basmati and IRRI-6 under salinity stress.

Like SOD and POD, the leaf CAT activity was also enhanced significantly in plants of all rice cultivars when grown under salt stress (*Fig. 2*; *Table 1*). The CAT activity was further increased due to SNP seed treatment under salinity. Both regimes of SNP were found equally effective in improving the activity of CAT. SNP-induced more increase in leaf CAT activity was recorded in cvs. Shaheen Basmati and IRRI-6 as compared with other rice cultivars under salinity.

Non-enzymatic antioxidants

Leaf ascorbic acid content in all rice cultivars was enhanced significantly when grown under salt stress (*Fig. 2*; *Table 1*). Sodium nitroprusside treatment as seed priming further enhanced the leaf ascorbic acid content under salt stress and both SNP levels were found equally effective in this regard. However, under non-saline conditions this increase in leaf ascorbic acid content was more in plants raised from seeds treated with 0.2 mM regime of SNP. More increase in leaf ascorbic acid due to SNP seed treatment was found in cvs. Shaheen Basmati and IRRI-6 under saline and non-saline conditions.

Leaf total phenolics content reduced significantly in all studied rice cultivars due to rooting medium salinity (*Fig. 2*; *Table 1*). Seed priming with SNP was effective in improving the total phenolic content in both saline and non-stressed conditions. Although seed priming with both SNP regimes (0.1 and 0.2 mM) found equally effective in increasing total phenolic content, however, under salinity 0.1 mM SNP regime as seed priming was found more effective Comparatively higher total phenolic content was found in cvs. IRRI-6 and KS-282 under non-saline and saline conditions.

Rooting medium salinity increased the leaf proline content in all studied rice cultivars (*Fig. 3*; *Table 1*). A further increase in leaf proline content was found in plants of all rice cultivars that grown from seeds pre-treated with both SNP regimes. This increase in leaf proline due to SNP priming was found at both SNP regimes in all rice cultivars under salt stress. Comparatively higher increase in proline content was observed in cvs. Shaheen Basmati and IRRI-6 as compared with other rice cultivars.

MDA and H_2O_2 content

A significant increase in leaf MDA was recorded in all rice cultivars due to rooting medium salinity (*Fig. 3*; *Table 1*). Comparatively less increase in MDA due to rooting medium salinity was recorded in cvs. Shaheen basmati and IRRI-6 as compared with other rice cultivars. A significant decrease in leaf MDA content was found in rice plants of all cultivars that were grown from seeds primed with SNP. However, this decrease was found only at 0.1 mM level of SNP.

Imposition of salt stress significantly increased the leaf H_2O_2 content (*Fig. 3*; *Table 1*). Exogenous SNP application was found effective in reducing the H_2O_2 content in all rice cultivars. Seed priming with both SNP levels (0.1 and 0.2 mM) were found equally effective in reducing the leaf H_2O_2 content in all rice cultivars.
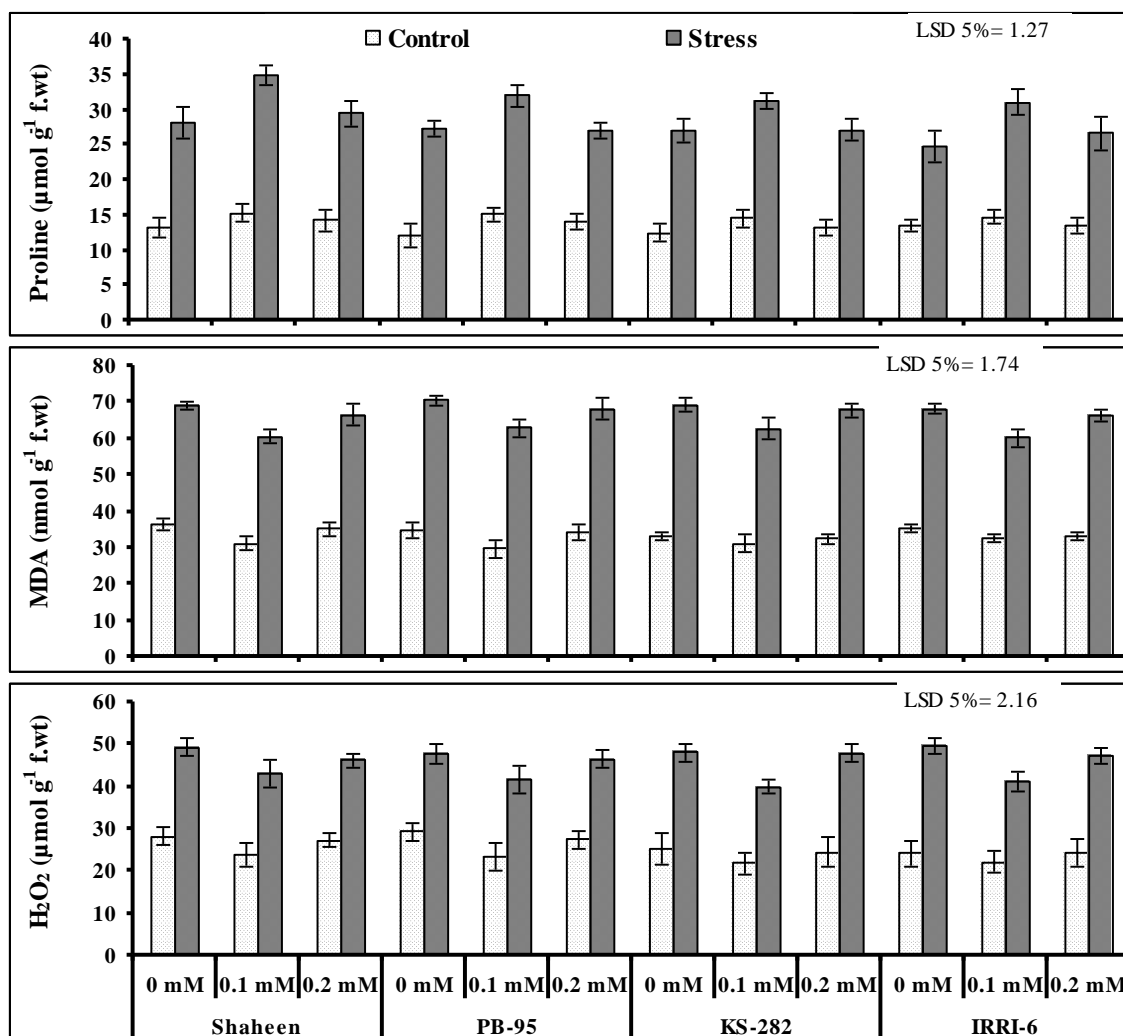
Figure 3. *Leaf proline, MDA and H₂O₂ content of four rice cultivars affected by pre-sowing seed treatment with different regimes of SNP grown under salt stress (mean±SE)*

Yield attributes

Like other growth and physico-chemical parameters, number of tillers was also significantly reduced due to rooting medium salinity in all rice cultivars (*Fig 4*; *Table 1*). A significant ameliorative effect of seed priming with SNP was found on number of tillers under salt stress in all rice cultivars and both SNP levels (0.1 and 0.2 mM) were found equally effective in this regard.

Imposition of salt stress decreased the hundred grain weight in all rice cultivars (*Fig. 4*; *Table 1*). Seed priming with only lower level (0.1 mM) of SNP was found effective in reducing the adverse effects of salt stress on hundred grain weight in all rice cultivars. Comparatively more amelioration on hundred grain weight due to SNP seed priming was recorded in cvs. KS-282 and IRRI-6

Grain yield per plant was also decreased significantly in all rice cultivars due to rooting medium salinity (*Fig. 4*; *Table 1*). Seed priming with SNP was found effective in reducing the adverse effects of salt stress on grain yield per plant in all rice cultivars. Of all SNP levels, 0.1mM was more effective in reducing the adverse effects of salt

stress on grain yield per plant. Comparatively SNP-induced this amelioration on grain yield per plant was more in cvs. IRRI-6 and KS-282.
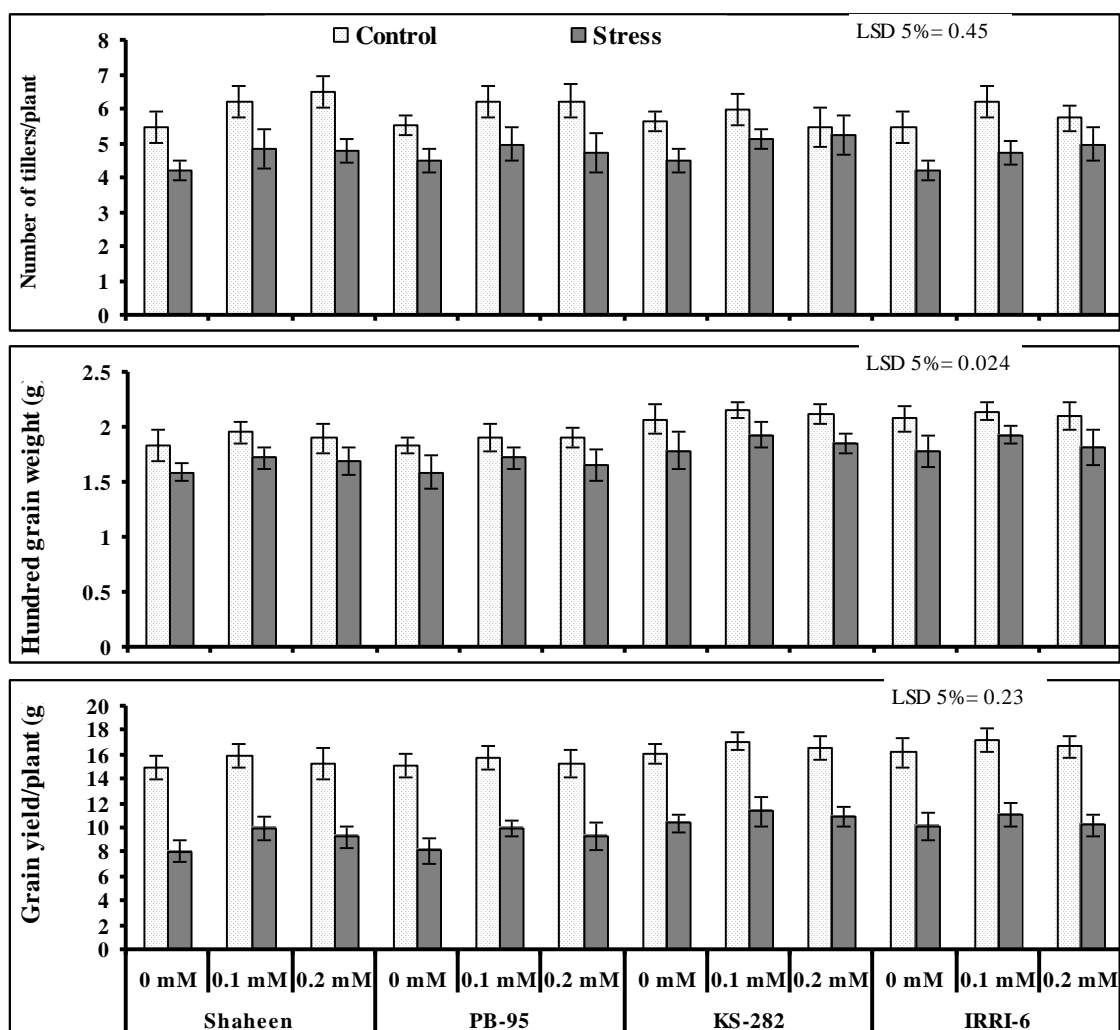

Figure 4. Yield attributes of four rice cultivars affected by pre-sowing seed treatment with different regimes of SNP grown under salt stress (mean±SE)

Discussion

The reduction of yield under adverse environmental conditions including salinity is the result of reduction in plant biomass production and has been the subject of many research studies. This reduction in biomass production under salt stress is the result of metabolic alterations in biochemical and physiological process. These include the disturbances in plant water relations, gas exchange attributes, photosynthetic machinery, as well as the production of ROS in excessive amount (known as oxidative stress). These ROS directly or indirectly damages the cellular components including the membranes, resulting in reduced biomass production (Ali and Ashraf, 2011). Of different ROS, production of H_2O_2 is also an important and common phenomenon which is beneficial as a signaling molecule at lower concentrations (Miller et al., 2010). It has been reported that H_2O_2 increases the activity of plasma membrane H^+-

ATPase, which could in turn maintain the K^+/Na^+ homeostasis (Zhang et al., 2007). Moreover, H_2O_2 has also been reported to facilitate the SOS1 mRNA stability which helped in Na^+ detoxification in *Arabidopsis* (Chung et al., 2008). However, in high concentration H_2O_2 directly damages cellular membranes (Miller et al., 2010). Such damages to cellular membranes due to high production of ROS under salt stress results in accumulation of more MDA due to lipid peroxidation that determines the degree of damage to cellular membranes. Such salt-induced lipid peroxidation results in more leaky membrane (Ashraf and Ali, 2008; Sheokand et al., 2010) that results in lower biomass production and plant yield. Salt-induced increase in MDA content due to oxidative stress has been reported in plants such as canola (Ashraf and Ali, 2008), *Kosteletzkya virginica* (Guo et al., 2009) and wheat (Ashraf et al., 2010). The present findings also depicts that, rooting medium salinity increased the H_2O_2 and MDA contents with a reduction in plant biomass production and grain yield in all rice cultivars. Under such conditions, activity of antioxidative defense mechanism i.e. comprised of enzymatic and non-enzymatic antioxidants is effective to scavenge and detoxify the over produced ROS to reduce their damaging effects. However, the activity of these antioxidants differs among species/cultivars which are important in determining the salt tolerance ability among different cultivars of crop plants (Ashraf, 2009; Ashraf et al., 2012).

Apart from exploring inter-cultivar difference for salt tolerance, exogenous application of different compounds are also being extensively used and found effective in improving plant salt tolerance level (Ashraf and Foolad, 2005) by modifying the various physiological and biochemical processes. Similarly like other compounds, protective effects of exogenously applied SNP against salt induced damages in plants have been well documented (Zhang et al., 2006; Zheng et al., 2009; Habib et al., 2010, 2013). Number of reports has already shown the protective effect of SNP/NO_2 against salt-induced membrane lipid peroxidation in plants (Zhang et al., 2006; Shi et al., 2007). It has been found that NO_2 helps to prevent ROS-induced membrane damages by readily reacting with ROS as well as with lipid peroxyl (LOO^-) and alcoxyl (LO^-) radicals (Sheokand et al., 2010). In present study, exogenous SNP (source of NO_2) application as seed priming was found effective in decreasing the lipid peroxidation in rice plants grown under salt stress.

Normally H_2O_2 production takes places by a reaction of SOD with superoxide, radical while, ascorbate peroxidase (APX) and catalase (CAT) acts as H_2O_2 scavengers (Miller et al., 2010). In the present study, exogenous use of SNP was proved effective in improving the activity of enzymatic antioxidant (SOD, POD and CAT). The increase was also in accordance with previous reports in which increased antioxidant enzymes activity due to SNP treatment have been noted in seedlings of wheat (Zeng et al., 2009), mustard green (Zeng et al., 2011) and *Brassica campestris* (Chang-Li et al., 2011). Moreover, in present study this increase in antioxidative enzyme activities resulted in a decrease in H_2O_2 contents with a reduction in MDA accumulation. This decrease in MDA content with a reduction of H_2O_2 might be due to enhanced scavenging activity of antioxidant enzymes. Such SNP-applied decrease in leaf H_2O_2 under salinity in rice plants is in agreement with the studies has already been reported in *Arabidopsis* (Zhao et al., 2007) and chickpea (Sheokand et al., 2010) grown under salinity.

Leaf ascorbic acid and phenolic contents have significant roles in many metabolic processes in plants (Jamil et al., 2015). These antioxidative compounds play protective roles against salt-induced oxidative damages by scavenging ROS such as singlet

oxygen, hydroxyl radical and superoxide (Ashraf and Ali, 2008). In the present study, exogenous SNP treatment was found effective in increasing the leaf ascorbic acid and phenolic content in rice plants grown under salt stress. Such SNP-induced enhanced synthesis of ascorbic acid and phenolic contents could be due to the involvement of NO2 in the activation of cellular antioxidative defence mechanism (Zeng et al., 2011). SNP-induced such increase in leaf ascorbic acid content has also been found in water stressed rice plants (Shehab et al., 2010). Similarly, it has been found that leaf total phenolic content also increased significantly due to exogenous SNP treatment under adverse environmental conditions (Shehab et al., 2010; Buss et al., 2011).

Leaf proline, a well-known secondary metabolite, plays a significant role as osmoprotectant as well as an antioxidant. As an osmotica, it not only actively takes part in cellular water relations under adverse conditions but also protects the cellular membranes by playing role as an antioxidant (Ali and Ashraf, 2011; Ashraf et al., 2012; Habib et al., 2012). In the present study, leaf proline content significantly increased due to exogenous SNP treatment in rice plants, showing its role as plant stress tolerant compound. SNP-induced accumulation in leaf proline has already been reported in *Kosteletzkya virginica* plants grown under salt stress (Guo et al., 2009).

Conclusions

It was concluded that the SNP seed priming increased the rice plant growth, grain yield and biomass production by the up-regulation of activities of antioxidant enzymes as well as the contents of non-enzymatic antioxidantive compounds which in turn resulted in enhanced ROS scavenging and reduced lipid peroxidation. The exogenous SNP seed treatment also modulated the proline synthesis/accumulation under salt stress there by reducing the salt-induced toxic effects. Regarding various SNP levels, it was concluded that the lower concentration of SNP (0.1 mM) can be used effectively for better yield in rice cultivated in salt rich areas.

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