Nitric oxide mitigates salt-induced oxidative stress in *Brassica juncea* seedlings by regulating ROS metabolism and antioxidant defense system

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Abstract

The present investigation was designed to determine the interaction of nitric oxide with other antioxidants in relieving oxidative stress induced by NaCl at morphological, physiological and molecular level. 15 days old seedlings of *B. juncea* were subjected to 50 mM NaCl alone, 100 μ M SNP alone and in combination (SNP + NaCl) in hoagland growth medium for 96 h and to analyze the cellular homeostasis and salt tolerance mechanism via examining growth, stress parameters, enzymatic and non enzymatic antioxidants and expression level of NR. Exposure of 100 μ M sodium nitroprusside to mustard seedling enhanced photosynthetic pigment content and prevented plant growth inhibition. Accumulation of MDA and H₂O₂ was more pronounced in individual NaCl treated seedling than in the combination of NaCl and SNP. Applying SNP enhanced NR activity by 1.70 folds and increased NO production by 2.26 folds than individual salt treated roots. Furthermore, the activities of CAT, GPX and NR act synergistically with endogenous NO level whereas APX work antagonistically. In addition, the study also demonstrates that NO regulated NaCl induced transcriptional expression of NR. Induction of BjNR in Indian mustard roots lead to enhanced the plant resistance against salinity stress. The present finding revealed that NO confers increased *B. juncea* tolerance to salt stress by stimulation of antioxidants and reestablishment of cellular redox status.

Keywords Salt stress · Nitric oxide · Nitrate reductase · *Brassica juncea* · Antioxidant defense system

Introduction

Increasing salinization of soil and water is a global concern which affects the agricultural production worldwide. This problem becomes greater in the region with dry climate, infrequent rainfall and extreme temperature leading to high evapotranspiration rate (Hasanuzzaman et al. 2018; Khator et al. 2019; Mahawar and Shekhawat 2019). All of these characteristics are common in arid and semiarid regions of India. In fact, the United Nation Environment Program estimated that approx 20% of irrigated agriculture land in the world is affected by soil salinity (Hasanuzzaman et al. 2018). Salinity stress influences various physiological and metabolic pathways of the plant which mainly depends on concentration and duration of stress that eventually leads to

a diminution in crop production (Verma et al. 2015; Khator and Shekhawat 2019; Khator et al. 2020). In plant soil, NaCl is the most prevalent and soluble salt and its elevated concentration incite two principal effects on plant; osmotic stress and ion toxicity. During the preliminary stage of salinity stress, plant root system reduces water absorption capacity and enhances water reduction capacity from the leaves tissue due to osmotic stress stimulated by high salt accumulation in the plant (Munns 2005; Khan et al. 2012). Consequently, salinity stress is regarded as hyperosmotic stress. Moreover, increased concentration of Na⁺ and Cl⁻ in the cytosol causes a severe ionic imbalance which leads to electrolyte leakage and also affects the substantial physiological disorders including membrane disruption, nutrients imbalance; impairment in the ability to neutralize the reactive oxygen species and variation in antioxidant enzyme activity (Mahawar and Shekhawat 2016; Khator and Shekhawat 2019). Photosynthetic inhibition and fractional closure of stomata are other negative consequences of salt stress which constitutes to restrict plant development and productivity (Ahmad et al. 2016).

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It has been well documented that salinity stress can induce over-accumulation of ROS in different cellular compartments viz. chloroplast, mitochondria and peroxisome, thus causing the oxidative burst in terms of peroxidation of lipid, oxidation of protein and nucleic acids (Hasanuzzaman et al. 2018) which ultimately reduces the plant growth and production (Fatma et al. 2016; Ahmad et al. 2016). To minimize the oxidative stress, plants have developed potential antioxidant defense system to counteract ROS mediated toxicity (Verma et al. 2008; Shekhawat et al. 2008, 2009, 2010, 2019; Khator and Shekhawat 2020). The antioxidant defense system comprises non-enzymatic antioxidants which include; ascorbic acid (AsA), glutathione (GSH), proline, phenolic compounds, alkaloids, non-protein amino acids, and α -tocopherols and enzymatic antioxidants include; superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), guaiacol peroxidase (GPX) and glutathione S-transferase (GST) (Hasanuzzaman et al. 2012; Verma and Shekhawat 2012; Khator and Shekhawat 2018; Mahawar and Shekhawat 2018; Mahawar et al. 2018a). Hence, cellular detoxification is the most important tolerance mechanism to overcome salt-induced oxidative stress.

Nitric oxide is an endogenous bioactive signalling molecule in a plant that contribute in various physiological alterations including germination of seeds, root formation, closure of stomata, programmed cell death and environmental stress responses (Mostofa et al. 2015; Ahmad et al. 2016; Su et al. 2018; Wang et al. 2018; Khator and Shekhawat 2019). It has been well reported that NO acts as an antioxidant by improving the plant tolerance against salt-induced oxidative stress (Hasanuzzaman et al. 2018). Recently, NO has been recognized as small gaseous redox radical which interacts with a broad range of signalling molecules, thereby altering the activities of the protein, decreased glutathione content, accumulation of proline and peroxynitrite which facilitates to combating salinity induced stress. In addition, NO mitigate NaCl stress by regulating the ionic homeostasis and improving the photosynthetic potential and plant growth (Ahmad et al. 2016; Hasanuzzaman et al. 2018; Sehar et al. 2019). Notably, Nitric oxide has been established as a chain breaker or potential inhibitor of reactive oxygen species that cause oxidation of biomolecules (Fancy et al. 2017). Interestingly, numerous reports revealed that the application of SNP (NO donor) exerts positive effects on antioxidant defense machinery and reactive oxygen species metabolism in different plant species such as tomato (Wu et al. 2011), mangrove (Chen et al. 2014), Indian mustard (Gupta et al. 2017) and pea (Yadu et al. 2017) under salt stress which can be inhibited by using the scavenger of nitric oxide i.e. cPTIO [2-(4-Carboxyphenyl)—4, 4, 5, 5 tetramethylimidazole-1-oxyl-3-oxide]. Strikingly, 2,2'(hydroxyl nitroso hydrazono)

bis-ethanimine (DETA) and S-nitroso-*N*-acetylpenicillamine (SNAP) are another NO producing compounds to counteract the NaCl induced stress by upregulating the antioxidant enzyme activity and reducing MDA and ROS level (Egbichi et al. 2014; Ahmad et al. 2016).

Brassica juncea is an imperative oil-seed crop, which frequently occurs under saline conditions and it is cultivated predominantly in the arid and semi-arid regions (Khator and Shekhawat 2019). It is regarded as an important salt-tolerant crop because it is more suitable to assess the source of salt stress tolerance in plants. Major region of Thar Desert experiences the scarcity of water and extreme saline conditions which eventually lead to drought and water stress. *B. juncea* adapted to these adverse desert climatic conditions due to substantial potential to cultivate in salt-affected areas (Khator and Shekhawat 2019). Concerning the vital function of NO in plant response to salinity stress, the present investigation was designed to illustrate the cytoprotective role of NO in alleviating NaCl induced oxidative stress by regulating the antioxidant defense system and ROS metabolism in *B. juncea* seedlings. In addition, exogenous NO triggers increased accumulation of proline, antioxidant enzyme activity and expression of NO biosynthetic enzyme i.e. nitrate reductase in salt-affected *B. juncea*.

Material and methods

Plant material, growth conditions and treatments

Seeds of *Brassica juncea* var. DPY 561 were collected from the National Bureau of Plant Genetic Resources, Jodhpur. Surface sterilization of seeds was done with 0.1% Mercuric chloride for 1–2 min to remove fungal contamination and rinsed thoroughly with autoclaved distilled water 4–5 times. Seeds were germinated in a glass petriplate containing autoclaved whatman filter paper no. 1 soaked with sterile distilled water (10–20 ml) and kept at 25 °C in BOD incubator (vaioetra) under dark condition. The uniformly sprouted seedlings were randomly selected and transferred to plastic pots (12 × 12 cm²) containing 1 L of Hoagland nutrient solution (pH 6.8–6.9, using Elico LI 120 pH meter). Each plastic pot contained ten plants. These pots were maintained in thermostatically controlled culture room at 25 ± 2 °C and 50% relative humidity. The Hoagland medium was replaced from the fresh Hoagland medium on alternate days so as to evade nutrient scarcities to seedlings. After 15 days of growth, plants were subjected to the following treatments for 96 h.

1. Hoagland nutrient solution (control) without treatment (0 mM NaCl + 0 μ M SNP)
2. NaCl alone (50 mM)
3. SNP alone (100 μ M)

4. NaCl (50 mM) + SNP (100 μ M)

The concentrations of NaCl and NO (SNP) was selected on the basis of preliminary experiment where the NaCl concentrations ranged from 10 to 100 mM (10, 30, 50, 70, 100 mM) NaCl and SNP concentrations ranged from 10 to 200 μ M (10, 25, 50, 100, 150, 200 μ M) SNP were subjected to the plants for 96 h. In the case of NaCl and SNP treatment, 50 mM NaCl and 100 μ M SNP concentrations were more appropriate for the plant because at this concentration higher NO content was recorded. Thus, 50 mM NaCl and 100 μ M SNP concentrations were selected for further experimental work. After 96 h of the above treatments, plant tissues were harvested to determine various growth, stress and antioxidant parameters. The experiments were carried out in three replicates and arranged in a completely randomized block design.

Measurement of plant growth parameters

Growth parameters were determined in terms of root length (RL), shoot length (SL), fresh weight (FW) and dry weight (DW) of seedlings. For this, seedlings were harvested and rinsed with deionised water. Fresh weight (FW) and dry weight (DW) were calculated from control as well as treated plant tissues (Adair Dutt 125 A SCS). For dry weight measurement, the fresh plant tissue was placed overnight in a hot air oven (Nsico Hicon Oven) for dehydration at 65 °C and weight of dehydrated tissue was calculated. The percent tolerance was calculated by fresh weight treated/fresh weight control $\times$ 100 according to Wilkins (1978). The leaf water content was calculated by the formula (Fresh weight – Dry weight/Fresh weight) $\times$ 100.

Determination of photosynthetic pigments and protein content

Photosynthetic pigments (chlorophyll) were estimated by the procedure of Arnon method (1949) and its content was expressed in milligram per gram fresh weight (FW) of tissue. Amount of chlorophyll in treated plant/Amount of chlorophyll in control $\times$ 100 the given formula was used for calculating the stability index of chlorophyll. Protein content was determined according to the method described by Lowry et al. (1951).

Estimation of oxidative stress and ROS accumulation

Lipid peroxidation was estimated by De Vos et al. (1989) method to determine the production of malondialdehyde (MDA) content with 2-thiobarbituric acid (TBA). Plant tissue (0.5 g) was ground in 10 ml of 0.25% thiobarbituric acid in 10% trichloroacetic acid. The plant extract was incubated

at 95 °C for 30 min and then immediately terminated the reaction on an ice bath. The resultant reaction mixture was centrifuged at 10,000 $\times$ g for 15 min at room temperature. The optical density of the supernatant was recorded at 532 and 600 nm using Elico double beam SL 210 UV VIS spectrophotometer. The MDA content was estimated from non-specific absorbance at 600 nm deducted from the absorbance at 532 nm. The MDA content was expressed in nmol g⁻¹ fresh weight of tissue by using extinction coefficient 155 mM⁻¹ cm⁻¹.

H₂O₂ level in plant seedlings was determined spectrophotometrically by Alexieva et al. (2001) method. For H₂O₂ estimation in *B. juncea* seedlings, 0.5 g of fresh plant tissue was homogenized in 5 ml of 0.1% (w/v) trichloroacetic acid (TCA) in an ice bath. Homogenate was centrifuged at 12000 $\times$ g for 15 min. The reaction mixture contained 0.5 ml of the plant extract, 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M potassium iodide. Reaction was carried out for 1 h in dark conditions and optical density was recorded at 390 nm using UV–Visible spectrophotometer. The H₂O₂ accumulation was calculated using an extinction coefficient of 0.28 mM⁻¹ cm⁻¹.

Proline estimation

Proline estimation was done according to Bates et al. method (1973). Plant tissue (0.5 g) was crushed in 5 ml of freshly prepared 3% sulphosalicylic acid and centrifuge at 9500 $\times$ g for 20 min. Reaction mixture contained 2 ml of supernatant, 2 ml of acid ninhydrin (1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid) solution and 2 ml of glacial acetic acid. The mixture was incubated at 100 °C for 1 h in a hot water bath and immediately cooled on an ice bath to stop the reaction. 4 ml of toluene was added to the above reaction mixture. It was vortexed for 1 min and the absorbance of separated chromophore recorded at 520 nm. Toluene was served as a blank. The proline content was estimated from a standard curve prepared using L-proline. Amount of proline was represented in microgram per gram fresh weight of tissue.

Extraction and assay of antioxidant enzymes

For the extraction of antioxidant enzymes 0.5 g plant tissue was homogenized in 5 ml of 50 mM sodium phosphate buffer (pH 7.0) Homogenized mixture was centrifuged at 9500 $\times$ g, 4 °C for 20 min, the resultant supernatant was utilized as enzyme extract to assay the antioxidant enzymes activity.

Catalase (EC 1.11.1.6) activity was calculated by Aebi (1974) method. The reaction mixture consisted of 50 mM phosphate buffer (pH-7.0), 9 mM H₂O₂ and an appropriate quantity of enzyme in the total volume (3 ml). Decline in the

absorbance was recorded at 240 nm. The H_2O_2 molar extinction coefficient at 240 nm was taken as $0.039 \text{ mM}^{-1} \text{ cm}^{-1}$.

Ascorbate peroxidase (EC 1.11.1.11) activity was determined according to the procedure given by Chen and Asada (1989). The reaction mixture consisted of 1.9 ml of 50 mM phosphate buffer (pH 7.0) containing 0.6 mM ascorbic acid, 0.1 ml of enzyme extract. The reaction was started by adding 1 ml of 0.1% H_2O_2 . Rate of Ascorbic acid oxidation was assayed by the decline in absorbance at 290 nm for 3 min (extinction coefficient $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$).

Guaiacol peroxidase (EC 1.11.1.7) activity was assayed by the method of Putter et al. (1974). Guaiacol peroxidase (GPX) activity was estimated from the content of tetraguaiacol (final product of the reaction). The 3 ml reaction mixture contained 50 mM phosphate buffer (pH-7.0), 20 mM guaiacol solution, 3.7 mM H_2O_2 and 0.1 ml enzyme extract. Increase in the absorbance was taken at 436 nm (extinction coefficient $26.6 \mu\text{M}^{-1} \text{ cm}^{-1}$).

Assay of nitrate reductase activity

The Nitrate reductase (EC 1.6.6.1) activity was determined by the procedure of Datta and Sharma (1999) with some modifications. 0.5 g of plant tissue was homogenized in 1 ml of 50 mM potassium phosphate (pH 8.0) buffer containing 1 mM EDTA, 25 mM cysteine and 3% (w/v) bovine serum albumin (BSA). The homogenate was centrifuged at $12000\times g$, 4°C for 20 min. The supernatant was used as an enzyme source to assay NR activity. The assay mixture consisted of 200 μl of 50 mM KNO_3 , 200 μl 0.5 mM NADH, 400 μl enzyme extract, 1200 μl 50 mM potassium phosphate buffer (pH 7.0). The total 2 ml reaction mixture was incubated for 30 min at 25°C . Subsequently, 0.5 ml of 14 mM 1-naphthylamine and 0.5 ml of 58 mM sulphanilamide was added in the following reaction mixture to estimate the amount of nitrite released. The resultant mixture was incubated for 15 min at 25°C and the absorbance of the pink colour complex was recorded at 540 nm. Assay mixture without NADH served as blank.

Semi-quantitative RT-PCR

The extraction of total RNA was carried out from the roots of *B. juncea* using RiboZol™ (Biotechnology Grade, AMRESCO) according to the manufacturer's instruction. After the isolation of RNA, cDNA was amplified by PCR using following primers for BjNR (GenBank accession No.: XM 013793229.1) amplifying 896 bp region, forward 5'-AGGAGATGAGGTTTGTGGCG -3' and Reverse 5' CCACTCCCCTGCAACTTTGT 3' and for actin amplifying 196 bp region, forward 5' GTTGGGATGAACCAGAAGGA 3' (Accession number: 1458691/2), Reverse 5' GAGGAGCCTCGGTAAGAAGA 3' (Accession number: 1458692/4).

Amplification of actin cDNA from the *B. juncea* was utilized as the internal standard. cDNA was amplified by polymerase chain reaction as follows: 25 μl of reaction mixture contained $10\times$ PCR buffer, dNTPs (10 mM), MgCl_2 , forward primer (10 μM), reverse primer (10 μM), Taq polymerase (3U/ μl) and cDNA template. Cycling conditions for polymerase chain reaction were as follows— 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing 64.3°C for 1 min, extension 72°C for 2 min and final extension of 72°C for 5 min. The amplified transcripts were detected after electrophoresis on 1.2% agarose gel stained with ethidium bromide and transcript bands were visualized using Gel Doc Imaging system (Bio-Rad Laboratories Inc., Germany).

Nitric oxide assay

Nitric oxide content in *B. juncea* seedling was determined according to Amooaghaie et al. (2015) method using Greiss reagent. Fresh plant tissue (0.5 g) was homogenized in pre-chilled mortar and pestle with 3 ml of 50 mM pre-cooled acetic acid buffer (pH 3.6) containing 4% zinc acetate. Supernatant was obtained after the centrifugation of homogenate at $10,000\times g$ for fifteen minutes at 4°C . The obtained pellet was washed with one millilitre of the extraction buffer and centrifuged it again. Supernatants were combined after two times centrifugation and subsequently added 0.1 g of charcoal for neutralization. Further the filtrate was collected after vortexing and filtration. An equal ratio of filtrate (1 ml) and Greiss reagent (1 ml) (1% sulphanilamide and 0.1% N-1naphthylethylenediaminedihydrochloride in 5% H_2PO_4 solution) was incubated for half an hour at room temperature. The absorbance of the resultant mixture was recorded at 540 nm and the content of NO was calculated using the standard curve of sodium nitrite.

Statistical analysis

SPSS 16 version software was used for the statistical analysis. Three biological replicates of both the treatments as well as control were used for each independent experiment. The mean values of different parameters were analyzed and difference between the control and treated plants (mean $\pm$ standard error) were interpreted by one-way ANOVA at $P \leq 0.05$ as a level of significance using Duncan Multiple Range Test (DMRT).

Results

Effect of SNP (NO donor) and NaCl stress on the growth parameters

B. juncea seedlings treated with NaCl stress (50 mM) alone resulted in progressive declined growth parameters.

As shown in Table 1, salinity stress alone reduced fresh weight, dry weight, root length, shoot lengths by 18.51%, 14.28%, 60.66%, 75.63%, respectively, with respect to control whereas no significant decrease was observed in case of leaf water content and tolerance index. Application of SNP (100 μ M), NO donor increased the fresh weight, dry weight, root length, shoot length, leaf water content and tolerance index by 36.36%, 16.66%, 85.68%, 63.46%, 5.41% and 36.36% respectively relative to salinity alone treated plant (Table 1). However, in plant treated with the combination of NaCl + SNP, the percentage increases in fresh weight, root length, shoot lengths and tolerance index was 35.29, 107.49, 73.07 and 25.92, respectively, while no significant change was observed in case of leaf water content with respect to NaCl alone treated plants (Table 1).

Effect of SNP and NaCl on photosynthetic pigments

Photosynthetic pigments including chlorophyll a, chlorophyll b and total chlorophyll were drastically declined in salt-treated *B. juncea* seedlings than that of control seedlings. As presented in Table 2, salinity stress decreased the amount of chlorophyll a by 17.77%, chlorophyll b by 30.08% and total chlorophyll by 33.82% in leaves while no notable decrease was recorded in chlorophyll a, chlorophyll b and total chlorophyll of shoots tissue as compared to control. The inhibitory effects of salinity stress on the content of Chl

a, Chl b and total Chl were reduced by the application of SNP. Treatment of SNP enhanced content of Chl a by 32%, Chl b by 80.60% and total Chl by 47.16% in leaves and no significant change were noticed in shoots (Table 2). Compared with NaCl treated plant, the application of SNP with NaCl treated plant was more effective in improving Chl a, Chl b and total chl content in *B. juncea* seedlings. Addition of SNP to NaCl treated plant increased the Chl a, Chl b and total Chl content by 45.94%, 131.25% and 33.82% in leaves as compared to NaCl alone treated plant (Table 2). In case of shoots, the combined treatment of NaCl + SNP enhanced the content of Chl a, Chl b and total Chl by 14.28%, 12.5% and 13.59% respectively, in contrast to NaCl stress alone. Moreover, Chlorophyll stability index of leaves and shoots was reduced in NaCl treated seedling than that of control. Exogenous application of SNP (SNP + NaCl) was found to mitigate the salinity induced oxidative stress by increasing the CSI of leaves and shoots i.e. 1.45 times and 1.21 times, respectively, higher as compared to NaCl stress alone (Table 2).

Effect of SNP and NaCl on protein and proline content

In response to NaCl stress, the total soluble protein was slightly increased by 12.65% and 16.66% in leaves and shoots, respectively, than those of control whereas in roots

Table 1 Effect of nitric oxide (100 μ M sodium nitroprusside) on fresh weight, dry weight, root length, shoot length, leaf water content and tolerance index in seedlings of *Brassica juncea* subjected to 50 mM NaCl for 96 h

Treatments	Fresh weight (g)	Dry weight (g)	Root length (cm)	Shoot length (cm)	Leaf water content (%)	Tolerance Index (%)
Control	0.27 $\pm$ 0.009 ^c	0.07 $\pm$ 0.006 ^b	17.16 $\pm$ 0.520 ^c	5.50 $\pm$ 0.693 ^c	74.07 $\pm$ 3.25 ^b	100.00 $\pm$ 3.725 ^c
NaCl	0.22 $\pm$ 0.020 ^d	0.06 $\pm$ 0.004 ^c	10.41 $\pm$ 0.751 ^d	4.16 $\pm$ 0.576 ^c	72.72 $\pm$ 4.56 ^c	81.48 $\pm$ 7.456 ^d
SNP	0.30 $\pm$ 0.012 ^b	0.07 $\pm$ 0.005 ^b	19.33 $\pm$ 0.770 ^b	6.80 $\pm$ 0.635 ^b	76.66 $\pm$ 5.11 ^{ab}	111.11 $\pm$ 12.62 ^b
NaCl + SNP	0.34 $\pm$ 0.017 ^a	0.078 $\pm$ 0.006 ^a	21.60 $\pm$ 0.664 ^a	7.20 $\pm$ 0.722 ^a	77.05 $\pm$ 4.8 ^a	125.92 $\pm$ 10.43 ^a

Values are Mean $\pm$ SE of three replicates and are statistically significant according to Duncan multiple range test ($P \leq 0.05$), within different treatments. Data points marked with the same letters show insignificant differences ($P \leq 0.05$) within different treatment

Table 2 Effect of nitric oxide (100 μ M SNP) on Chlorophyll a, Chlorophyll b, Total chlorophyll and Chlorophyll stability Index in seedlings of *Brassica juncea* exposed to 50 mM NaCl for 96 h

Treatments	Leaves				Shoots			
	Chl a (mg g ⁻¹ FW)	Chl b (mg g ⁻¹ FW)	Total Chl (mg g ⁻¹ FW)	Leaves CSI (%)	Chl a (mg g ⁻¹ FW)	Chl b (mg g ⁻¹ FW)	Total Chl (mg g ⁻¹ FW)	Shoots CSI (%)
Control	0.43 $\pm$ 0.01 ^{bc}	0.21 $\pm$ 0.01 ^{bc}	0.65 $\pm$ 0.05 ^{bc}	100.00 $\pm$ 6.05 ^b	0.07 $\pm$ 0.004 ^b	0.032 $\pm$ 0.003 ^b	0.103 $\pm$ 0.007 ^b	100.00 $\pm$ 6.35 ^b
NaCl	0.41 $\pm$ 0.02 ^c	0.21 $\pm$ 0.01 ^c	0.62 $\pm$ 0.04 ^c	96.52 $\pm$ 5.31 ^c	0.06 $\pm$ 0.008 ^c	0.030 $\pm$ 0.005 ^b	0.096 $\pm$ 0.005 ^c	93.59 $\pm$ 2.88 ^c
SNP	0.45 $\pm$ 0.03 ^b	0.24 $\pm$ 0.02 ^b	0.70 $\pm$ 0.03 ^b	108.02 $\pm$ 7.41 ^b	0.07 $\pm$ 0.006 ^b	0.034 $\pm$ 0.003 ^b	0.106 $\pm$ 0.010 ^{bc}	102.91 $\pm$ 4.69 ^b
NaCl + SNP	0.54 $\pm$ 0.01 ^a	0.37 $\pm$ 0.02 ^a	0.91 $\pm$ 0.05 ^a	140.66 $\pm$ 6.92 ^a	0.08 $\pm$ 0.008 ^a	0.036 $\pm$ 0.004 ^a	0.117 $\pm$ 0.003 ^a	113.88 $\pm$ 8.94 ^a

Values are Mean $\pm$ SE of three replicates and are statistically significant according to Duncan multiple range test ($P \leq 0.05$), within different treatments. Data points marked with the same letters show insignificant differences ($P \leq 0.05$) within different treatment

the protein content was almost equal to the untreated plant. In addition, the application of SNP further elevated the soluble protein content by 15.82% and 34.09% in leaves and shoots while a gradual decrease was noticed in protein content of roots over the control plant (Fig. 1a). Subsequently, *B. juncea* seedlings treated with the combination of NaCl and SNP shown a marked increase in protein content by 21.34%, 43.59% and 33.33% in leaves, shoots and roots, respectively, in comparison to NaCl alone treatment (Fig. 1a).

Salinity stress triggered the induction of proline accumulation by 1.58 -folds, 2.31 -folds, 1.89- folds higher in leaves, shoots and roots than that of control in *B. juncea*. The application of SNP increased the level of proline by 77.86%, 166.66% and 116.94% in leaves, shoots and roots with respect to control (Fig. 1b). Further, the supplementation of SNP to NaCl treated plant enhanced the content of proline by 1.39 times, 1.31 times, 1.26 times greater in leaves, shoots and roots respectively in contrast to NaCl treated plant (Fig. 1b). However, the higher proline accumulation

was noticed in leaves than in shoots and roots tissue in all treatments i.e. NaCl, SNP, NaCl + SNP (Fig. 1b).

Effects of SNP and NaCl on the oxidative stress parameters

As illustrated in Fig. 2, the content of both MDA and H_2O_2 was significantly increased in NaCl treated *B. juncea*. Individual NaCl stress enhanced the MDA content by 1.56 folds, and 1.39 folds in leaves and roots compared to control plants. H_2O_2 level was elevated by 1.59 folds in leaves over the control whereas insignificant change was recorded in individual NaCl treated shoots and roots respectively (Fig. 2). The individual application of SNP decreased the accumulation of MDA by 21.99% and 46.48% in leaves and roots and H_2O_2 by 33.51% in leaves while no notable decrease was found in shoots and roots, respectively (Fig. 2). In the combined treatment of NaCl + SNP, the higher reduction in the accumulation of MDA and H_2O_2 was found in leaves and roots

Fig. 1 **a** Protein content and **b** Proline content in leaves, shoots and roots of *B. juncea* treated with 50 mM NaCl alone and 100 µM SNP (NO donor) in presence or absence of 50 mM NaCl for 96 h. Values are Mean $\pm$ SE ($n=3$) and are statistically significant according to Duncan multiple range test ($P<0.05$), within the individual salt treatment. Data points marked with the same letters show insignificant differences ($P\leq0.05$) within different treatments

Fig. 2 **a** MDA content and **b** H_2O_2 content in leaves, shoots and roots of *B. juncea* treated with 50 mM NaCl alone and 100 µM SNP in presence or absence of 50 mM NaCl for 96 h. Values are Mean $\pm$ SE ($n=3$) and are statistically significant according to Duncan multiple range test ($P<0.05$), within the individual salt treatment. Data points marked with the same letters show insignificant differences ($P\leq0.05$) within different treatments

but not progressive decline was noticed in shoots compared to individual application of NaCl and SNP (Fig. 2).

Effect of SNP and NaCl on enzymatic antioxidants

The activity of antioxidant enzymes was determined to investigate the role of SNP in the tolerance of NaCl stress by *B. juncea* seedlings. The activity of catalase was found to be higher in leaves tissue while the activity of other enzymes including ascorbate peroxidase, guaiacol peroxidase and nitrate reductase were found to be higher in roots tissue than leaves and shoots tissues in all the treatments (Fig. 3). The activity of APX and GPX were elevated by 15.47% and 24.55% in NaCl exposed roots compared to control (Fig. 3b, c) whereas NR activity decreased by 14.92% in roots under salt stress (Fig. 3d). In addition, the activity of catalase was reduced by 36.38% in NaCl alone treated leaves. However, NaCl had no considerable effect on catalase activity in shoots and roots with respect to control (Fig. 3a). In contrast, the application of SNP enhanced the activity of CAT by 14.13% in leaves, NR activity by 22.98% in roots while

the activity of APX and GPX was decreased in leaves, shoots and roots (Fig. 3a–d). Interestingly, the maximum increase in the activity of the antioxidant enzyme (CAT, GPX and NR) was noted in the combined treatment of NaCl and SNP in compared to individual NaCl treated plant (Fig. 3a, c, d). On the other hand, the activity of APX was declined by the individual supplementation of SNP and in the combination with NaCl stress than the control plant (Fig. 3b).

Effect of SNP and NaCl on NR transcript level

The expression of nitrate reductase (NR) gene in the roots of *B. juncea* under different treatments including NaCl alone, SNP alone and combination of NaCl + SNP was presented in Fig. 4. The individual NaCl stress resulted in low intensity of band represents the reduced level of NR expression. On the other hand, the application of SNP enhanced band intensity confers the induced expression of NR but maximum increase in NR expression was observed in the combined treatment of NaCl and SNP. However, the effect of combined treatment (NaCl + SNP) was found more pronounced than individual

Fig. 3 a Catalase, b Ascorbate peroxidase c Guaiacol peroxidase, d Nitrate reductase activity under different treatments (50 mM NaCl alone, 100 μM SNP and combination of 50 mM NaCl+100 μM SNP) in leaves, shoots and roots of *B. juncea* for 96 h. Values are

Mean $\pm$ SE ($n=3$) and are statistically significant according to Duncan multiple range test ($P<0.05$) within the different treatment. Data points marked with same letters show insignificant differences ($P\leq 0.05$) within different treatments

SNP. In comparison to control roots, the NR expression was induced by the addition of SNP and in the combination with NaCl stress (Fig. 4).

Effect of SNP and NaCl on NO accumulation

The content of NO was found to be increased with increase in NaCl concentration. The accumulation of NO was enhanced by 39.10% in roots at 50 mM NaCl compared to the control plant. After 50 mM NaCl, the NO content was drastically declined up to the 100 mM NaCl while no significant results were obtained in case of leaves and shoots (Fig. 5a). *B. juncea* seedlings treated with individual SNP treatment, the elevated level of NO recorded in roots which was 3.03 folds higher at 100 μ M SNP than control and thereafter NO content almost remains constant up to 200 μ M SNP (Fig. 5b). Moreover, the addition of 100 μ M SNP with 50 mM NaCl stress improved the endogenous level of NO by 2.26 folds in roots as compared to individual NaCl stress. However, the insignificant results were obtained in leaves and shoots of *B. juncea* (Fig. 5c).

Discussion

Nitric oxide is a bioactive signalling molecule which facilitates the improvement in plant growth and development under different environmental conditions (Verma et al. 2013; Fancy et al. 2017; Amooaghaie et al. 2018). In the present investigation, NaCl stress significantly declined the growth and yield of *B. juncea* (Table 1), which is in agreement with previous reports on different plant species such as *Oryza sativa* (Mostofa et al. 2015) and *Cicer arietinum* (Ahmad et al. 2016). Combined application of SNP with NaCl improved the plant growth by increasing the fresh weight, dry weight, root length and shoot length. The study is consistent with the finding of the earlier reports on different crops such as *Brassica nigra* (Amooaghaie et al. 2018), *Eruca sativa* (Mahawar et al. 2018a, b) and *Solanum lycopersicum* (Ahmad et al. 2018). The study conducted on the *B. juncea* and *C. arietinum* by Ahmad et al. (2016) and Fatma

et al. (2016) reveals that the application of SNP promoted the plant growth under a saline environment which might be due to the fact that NO acts as a relaxant for plant cell wall, protect the lipid membrane, enhanced the fluidity of membrane and also triggers the enlargement of cell and plant growth. In addition, NO also improved the osmotic potential of cell and enhanced the viscosity of cytoplasm (Dong et al. 2014).

The photosynthetic pigments was found to decrease in salt-treated *B. juncea* (Table 2) which might be due to chlorophyll destruction, reduction in chlorophyll synthesis and susceptibility of pigment-protein complexes (Rasool et al. 2013). The study was confirmed by Ahmad et al. (2016) in *Cicer arietinum*. The fall in Chl content might be associated with diminution in plant growth and biomass (Ahmad et al. 2016). Salt stress-induced decline in photosynthesis has been accredited to a number of factors including disruption of Na⁺ and Cl⁻ ions homeostasis, essential mineral nutrients (Khan et al. 2010), fractional stomatal closure and enhanced ROS accumulation in chloroplasts (Meneguzzo et al. 1999). The addition of SNP was found to be enhanced the content of photosynthetic pigments in *B. juncea* seedlings by protecting the membrane of chlorophyll-containing cell organelle against NaCl induced oxidative stress (Kausar et al. 2013). The present study is corroborated with the previous results in different crop plants under salinity stress including rice (Habib et al. 2013) and chickpea (Ahmad et al. 2016).

In response to NaCl stress, total soluble proteins play a vital role in maintaining osmotic homeostasis and it can act as a reservoir of nitrogen. Interestingly, the soluble protein content was amplified under stress condition which is responsible for the stimulation of stress-related proteins (Doganlar et al. 2010). The increased production of total soluble protein in salt-induced oxidative stress was reported by Mahawar et al. (2018b) in *Eruca sativa*. To reduce the negative effect of NaCl stress, plant triggers the increased production of osmolyte i.e. proline in cytosol of the cell (Abdel Latef and Miransari 2014). In the current investigation, a similar trend of the proline accumulation was noticed in *B. juncea* seedlings exposed to NaCl stress (Fig. 1). The elevated level of proline towards the salt stress was documented in *Nitraria tangutorum* by Liu et al. (2016). In fact, proline has been well established as osmoregulator in plant cell under salinity stress (Ahmad et al. 2016). Additionally, proline also acts as a stabilizer of the plasma membrane, energy reservoir, free radical scavenger and can provide cell protection against salt-induced oxidative stress. Beside this, the accumulation of proline increased the nitrogen fixation in *Medicago truncatula* plants under salt stress (Verdoy et al. 2006).

However, *B. juncea* seedlings exposed to salinity stress caused the greater accumulation of reactive oxygen species (H₂O₂) and malondialdehyde content (MDA) (Fig. 2),

Fig. 4 The effect of 100 μ M SNP (NO donor) on the expression of NR gene in roots of *B. juncea* under salt stress. NR and Actin gene transcripts were analyzed by semi quantitative RT PCR

Fig. 5 **a** The concentration-dependent effect of NaCl (10 mM, 30 mM, 50 mM, 70 mM and 100 mM) and **b** SNP (10 μM, 25 μM, 50 μM, 100 μM, 150 μM and 200 μM) on Nitric oxide content in leaves, shoots and roots of *B. juncea* for 96 h. **c** Nitric oxide content in *B. juncea* seedlings treated with 50 mM NaCl alone and 100 μM

SNP in presence or absence of 50 mM NaCl for 96 h. Values are Mean $\pm$ SE ($n=3$) and are statistically significant according to Duncan multiple range test, ($P<0.05$). Data points marked with same letters show insignificant differences ($P\leq 0.05$) within different treatments

there by inducing electrolyte leakage which is due to ROS mediated disintegration of the cell membrane and increased activity of lipoxygenase. The study is consistent with a previous report of Fatma et al. (2016) in *B. juncea*. Strikingly, the over-accumulation of ROS and the peroxidation of the lipid membrane is an illustrious marker for determining the extent of oxidative stress. Nevertheless, the supplementation of SNP inhibited the peroxidation of the lipid membrane and provides cell protection against NaCl induced oxidative stress. The study conducted by Ahmad et al. (2018) in *Solanum lycopersicum* revealed that NO decreased the production of free radical by reducing the MDA level. In the present investigation, the reduction in H_2O_2 level and lipid peroxidation in SNP treated *B. juncea* seedling may have accredited to the induction of antioxidant defense system that potentially scavenges ROS (H_2O_2). Ahmad et al. (2016)

have been documented that NO functions as a potent antioxidant, free radical scavenger and reducing the electrolyte leakage in chickpea plant (Ahmad et al. 2016).

To counteract the NaCl induced oxidative injury, plant cell has ability to stimulate antioxidant defense machinery that strongly inhibit ROS accumulation and re-established the cellular redox status (Hasanuzzaman et al. 2018). The upregulation of antioxidant defense system is an important tolerance mechanism to minimize the ROS induced toxicity in salt-affected plants. The present study reported that the activity of antioxidants including APX and GPX was upregulated whereas the activity of CAT and NR (nitric oxide biosynthetic enzyme) were downregulated in *B. juncea* under saline stress (Fig. 3). The decreased activity of CAT and NR under salinity stress have been reported by Hayat et al. (2012) in *Solanum lycopersicum* and Chen et al. (2014) in

Fig. 6 Schematic representation of the cytoprotective mechanism underlying the NO-mediated alleviation of NaCl induced oxidative stress in *B. juncea*. Salinity stress exhibit the negative effect on the growth of *B. juncea* through the over-accumulation of ROS which leads to oxidative damage to biomolecules. However, sodium nitroprusside (NO donor) exert the positive effect on plant growth by upregulation of antioxidant activity and also responsible for maintaining the cellular redox homeostasis. Thus, the NO-mediated stimulation of antioxidants increases *B. juncea* tolerance towards salinity stress

Aegiceras corniculatum and. In contrast, increased activity of APX and GPX under salinity stress has been reported in *A. corniculatum* (Chen et al. 2014) and *C. arietinum* (Ahmad et al. 2016). The application of SNP improved the activity of antioxidant enzymes in NaCl treated *B. juncea* which is consistent with the observation of Fatma et al. (2016) in mustard and Ahmad et al. (2016) in chickpea plant. Further, SNP also induced the expression of nitrate reductase gene, which is responsible for increased activity of nitrate reductase under NaCl stress. Applying 100 μ M SNP enhanced the expression of NR in *B. juncea* roots (Fig. 4) which are consistent with the observation of Kuruthukulangarakoola et al. (2017) in *A. thaliana*. Thus the observed results suggested that NO can mitigate the salt-induced oxidative stress by reducing the production of free radical, peroxidation of lipid and enhancing the activity of antioxidants, thereby improving *B. juncea* tolerance towards the NaCl stress.

The effect of SNP in response to NaCl stress was evaluated by determining the endogenous level of NO and also measured the change in concentration of NO by the supplementation of sodium nitroprusside (Fig. 5). The NO content decreased under salinity stress which was further enhanced by applying SNP in salt-treated *B. juncea* roots. Consequently, SNP (NO donor) has triggered the accumulation of NO in *B. juncea* roots. The present study is in accordance with the findings of Ahmad et al. (2016) and Fatma et al. (2016) in *C. arietinum* and *B. juncea*. The present investigation indicated that NO acts as a downstream regulator of ROS producing enzyme and upstream regulator of antioxidant enzymes. The preceding equivocal

and contradictory observations suggested that the direct link between up regulation of antioxidant enzymes and increased NO accumulation.

Conclusion

The outcome of research provides evidence that cytoprotective role of NO in the mitigation of salt-induced oxidative stress in *B. juncea* seedlings. The application of 100 μ M SNP (NO donor) improved the photosynthetic pigments and proline accumulation which leads to enhanced growth of *B. juncea*. Moreover, NO has been responsible for maintaining the cellular redox homeostasis which was directly associated with *B. juncea* tolerance against salinity stress (Fig. 6). Exogenous supplementation of SNP (100 μ M) allayed salinity induced oxidative damage by stimulation of antioxidant defense and inhibition of ROS and MDA accumulation. The induced expression of NR gene in combined treatment of 100 μ M SNP + 50 mM NaCl confers that the potential antioxidant properties of NO in *B. juncea* under NaCl induced oxidative stress. The study also demonstrates that NO acts as a strong antioxidant and provides cellular protection against NaCl induced oxidative stress by direct or indirect detoxification of ROS. Consequently, the application of SNP or alteration in its endogenous level might be new possibilities for salinity stress tolerance in the era of climate change.

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Author contributions GSS designed and conceptualized the study, KK executed the experiments, and all the authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest Authors declared that there is no conflict of interest.

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