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Deciphering the nitric oxide, cyanide and iron-mediated actions of sodium nitroprusside in cotyledons of salt stressed sunflower seedlings

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ABSTRACT

Nitric oxide (NO) is an endogenous signaling molecule in plants. Sodium nitroprusside (SNP), an established NO donor used in plant science research, simultaneously releases NO, cyanide (CN^-) and iron (Fe) in solution. Since cyanide and iron mask NO effect of SNP, its use in NO research is debatable. Deciphering the action of SNP through NO, CN^- or Fe has been undertaken in the present work. Cotyledons from salt stressed sunflower seedlings grown in the presence of NO donors were subjected to spectrofluorometric analysis of NO, CN^- and Fe contents, and proteome and biochemical analyses. Diethylenetriamine NONOate (DETA) proved to be a better NO source since SNP enhanced ROS accumulation in the tissue. Abundance of 127 proteins is modulated by salt stress. SNP and exhausted SNP (exSNP) alter the abundance of 117 and 129 proteins, respectively. These proteins belong to primary metabolism, stress-response, transport, translation, proteolysis, chaperone, regulatory, and storage. Salt-responsive proteins, such as, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK) and isocitrate dehydrogenase are negatively modulated. DETA and SNP lower the activities of GAPDH and S-adenosylmethionine synthase (SAMS). Abundance of heat shock 70 kDa protein and actin are sensitive to both NaCl and NO. SNP affects plant growth by modulating proteome though iron, cyanide and NO. Its use only as an NO donor is thus debatable. exSNP control also releases substantial amount of cyanide and iron, thus questioning its use as control in NO research.

1. Introduction

Nitric oxide (NO) is an important endogenous signaling molecule in various physiological processes in plants. It has a protective role against abiotic stress conditions such as, salinity, drought, UV-radiation or heavy metals [1,2]. NO modulates the activities of enzymes such as superoxide dismutase (SOD), glutathione reductase (GR) and heme oxygenase (HO) in sunflower seedlings as an early and long distance signaling response to salt stress [3–5]. Exogenous application of SNP during salt stress significantly alleviates salt stress-induced oxidative damage and enhances growth and dry weight of seedlings of many plant species [6–9]. SNP is the most extensively used source of NO in life science research. As an iron-nitrosyl derivative, it is an inorganic complex containing Fe (II) and NO^+. Free or membrane-bound thiolate anions attack SNP and decompose it into disulfide (RSSR), NO and CN^-, which limits its usefulness as an NO donor [10–12]. NONOates, are, on the other hand, one of the purest NO donors. Diethylenetriamine/nitric oxide adduct (DETA NONOates) is a dimeric covalent complex of NO and amine. The decomposition reaction follows first-order kinetics and releases up to 2 mol of NO per mole of NONOate. The first-order decomposition, predictable NO release rate and non-reactive property with biological reactants, makes NONOates valuable compounds in NO research [10].

The β-cyanoalanine synthase (β-CAS) pathway principally metabolizes cyanide produced as a by-product of ethylene biosynthesis, and β-CAS activity increases with the concomitant increase in ethylene

Abbreviations: β-CAS, β-cyanoalanine synthase; DETA, diethylenetriamine NONOate; EDTA, ethylenediaminetetraacetic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GR, glutathione reductase; HO, heme oxygenase; HSP70, heat shock 70 kDa protein; LC-MS/MS, liquid chomatography–tandem mass spectrometry; NO, nitric oxide; OD, optical density; PBS, phosphate buffer saline; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; RT, room temperature; SAMS, *S*-adenosyl methionine synthase; SDS, sodium dodecyl sulfate; SNP, sodium nitroprusside; SOD, superoxide dismutase

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biosynthesis so as to maintain cyanide homeostasis. β-CAS catalyzes the formation of β-cyano-alanine and H_2S from cysteine and cyanide [13]. An octaploid amphiploid genotype generated by crossing hexaploid bread wheat and a diploid wheatgrass growing in salt marshes, is salt-tolerant and exhibits higher abundance of β-CAS, MnSOD, aconitase, serinehydroxymethyl transferase and malate dehydrogenase [14]. Salt stress disturbs the normal metabolism and increases the tissue content of Mg, Ca, Na and Fe while K, N, Zn and Cu contents decrease in french beans [15]. In *Andrographis paniculata*, high salinity levels significantly increase the contents of Na, Fe, Zn and Cu while N, P, K, Ca, Mg and Mn contents decrease [16]. Osmotic and salt stresses enhance ROS production and cause oxidative damage in plants [17,18]. Significant increase in $O_2^{\cdot-}$ content under salt stress has been reported in maize [19,20], wheat [21,22], pea [23], rice [24], and sunflower [25]. Moreover, salt stress increases H_2O_2 content in maize [20], wheat [22,26,27].

Investigations at the proteome level are important to examine the underlying physiological mechanisms of salt stress tolerance in plants [28]. Proteomic analysis of 34 plant species has been utilized to create a database of 2171 salt stress-responsive proteins [29]. Although the roles of NO in salt tolerance have been addressed in plants such as Arabidopsis [30] and *Populus euphatica* [31], contradictory results have been reported and its roles are dependent on the plant species and severity of salt stress [32,33]. Various categories of proteins are modulated by salt stress in plants [34–37]. Analysis of total citrus leaf proteome demonstrated overlapping roles of NO and H_2O_2 in acclimation to salt stress. Pre-treatments with either SNP (100 μM) or H_2O_2 (10 mM) before subjecting to salt stress (150 mM NaCl) lead to protein carbonylation and reduction of the *S*-nitrosylation level of proteins [38]. Maize seedlings show co-regulation of more than 58% of proteins by NaCl or NaCl + SNAP [39]. Twenty thee percent of these proteins belong solely to carbohydrate metabolism. Exogenous NO application as 100 or 300 μM of SNP to tomato seedlings subjected to salt stress (120 mM NaCl) ameliorates salt stress-induced oxidative stress by significantly increasing the activities of antioxidant enzymes such as SOD, GR, peroxidases and ascorbate peroxidases [40]. Recent investigations from the author's laboratory have also demonstrated the important roles of various ROS scavenging enzymes, such as GR [4], Cu/Zn SOD [25], phospholipid hydroperoxide glutathione peroxidase (PHGPX) [41], and that of non-enzymatic components such as reduced glutathione in modulating sunflower seedling growth under salt stress [4]. Exogenous NO application using SNP (100 μM) in *Avicennia marina* improved salt tolerance accompanying the regulation of the abundance of proteins involved in primary metabolism, energy metabolism, photosynthesis and stress response [42].

With this background information in view, present investigations aim at a thorough analysis of SNP-modulated growth responses in sunflower seedlings vis-a-vis other established NO donor (DETA) though pharmacological studies, proteomic and biochemical approaches. Using sensitive colorimetric and fluorescent probes, iron, CN^- and NO released from SNP and its control (exSNP) have been analyzed both in aqueous solutions and in tissue homogenates of seedling cotyledons subjected to SNP and DETA treatments. Physiological role of SNP has been clearly segregated into those caused by NO, CN^- or iron though proteomic approach and subsequent biochemical analyses.

2. Materials and methods

2.1. Plant growth and treatments

Sunflower seeds (*Helianthus annuus* L., cv. KBSH 53) were obtained from the University of Agricultural Sciences, Bangalore. Prior to sowing, seeds were thoroughly washed with a liquid detergent (Teepol) and imbibed in tap water for 1 h followed by surface sterilization with mercuric chloride (0.005% of $HgCl_2$). The seeds were then washed in running tap water for 1 h, followed by imbibation in distilled water for 2 h. Seeds were sown on moist germination sheets placed in plastic trays. Pharmacological treatments such as those of: SNP, exhausted SNP and DETA were given at equimolar concentration of 250 μM and supplemented in half strength Hoagland solution in the absence or presence of 120 mM NaCl. These treatments were provided once the radicle emerged from the seeds. Seedlings were raised up to 2 d in dark at 25 °C. Cotyledons were harvested from seedlings with uniform growth pattern and stored at −80 °C until further use after freezing in liquid nitrogen.

2.2. Estimation of nitric oxide (NO) content in aqueous solutions and biological samples

For estimation of NO in the aqueous solutions of NO donors, the reaction mixture contained 15 μl (250 μM) of NO donors and 1.5 μl (2.5 μM) of MNIP-Cu in a total reaction volume of 600 μl made up by 10 mM potassium phosphate buffer (pH 7.4). Fluorescence was measured spectrofluorometrically using MNIP-Cu (ex. 330 nm, em. 420 nm). For biological samples, cotyledons (500 mg fw) harvested from dark grown sunflower seedlings were finely ground in liquid nitrogen using chilled pestle and mortar and homogenized in Tris buffer (100 mM, 1 mM PMSF, pH 7.0). The homogenates were vortexed for 2 min and centrifuged at 10,000 g for 20 min at 4 °C. 20 μl of the total soluble protein (TSP) fraction from each sample was mixed with 1.5 μl of MNIP-Cu in 10 mM potassium phosphate buffer (pH 7.4) in a total reaction volume of 600 μL. The reaction mixture was incubated in dark for 30 min at 25 °C. As a control, reaction mixture containing all the constituents except protein sample was used. NO content in each sample was estimated spectrofluorometrically using MNIP-Cu (ex. 330 nm, em. 420 nm) according to Yadav et al. [43] with modifications.

2.3. Estimation of cyanide content in aqueous solutions and biological samples

For estimation of cyanide in the aqueous solutions of NO donors, the reaction mixture contained 36 μl (250 μM) of NO donors and 1 μl of probe in a total reaction volume of 450 μl made up by 10 mM potassium phosphate buffer (pH 7.4). Fluorescence was measured spectrofluorometrically using Cyanide orange (Ursa BioScience, USA; ex. 470 nm, em. 575 nm). For biological samples, cotyledons (500 mg fw) harvested from dark-grown sunflower seedlings were finely ground in liquid nitrogen using chilled pestle and mortar and homogenized in Tris buffer (100 mM, 1 mM PMSF, pH 7.0). The homogenates were vortexed for 2 min and centrifuged at 10,000 g for 20 min at 4 °C. 30 μl of the total soluble protein (TSP) fraction from each sample was mixed with 0.5 μl Cyanide Orange (Ursa BioScience, USA) in 10 mM potassium phosphate buffer (pH 7.4) in a total reaction volume of 450 μL. The reaction mixture was incubated in dark for 30 min at 25 °C. As a control, reaction mixture containing all the constituents except protein sample was used. Cyanide content in each sample was estimated spectrofluorometrically using Cyanide Orange (ex. 420 nm, em. 575 nm).

2.4. Estimation of Fe (II), Fe (III) and total Fe (II + III) content in the aqueous solutions and biological samples

Concentrations of Fe (II), Fe (III) and total Fe (II + III) in 250 μM aqueous solutions each of the NO donors (DETA and SNP) and exSNP were estimated at 593 nm using iron colorimetric assay kit (BioVision, Inc., USA). For estimation of Fe (II), the reaction mixture contained 154 μl (250 μM) of NO donors and 300 μl of iron probe in a total reaction volume of 615 μl made up by iron assay buffer. For estimation of total Fe (II + III), the reaction mixture contained 154 μl (250 μM) of NO donors, 15 μl of iron reducer and 300 μl of iron probe in a total reaction volume of 615 μl made up by iron assay buffer. All reaction mixtures were incubated in dark for 30 min at 25 °C and the O.D was

measured at 593 nm. Control reaction was set without NO donors. Iron standard curve was prepared using the Iron Standard provided in the kit. Fe (III) content was calculated by subtracting Fe (II) content from total Fe (II + III) content.

For biological samples, cotyledons (500 mg fw) harvested from dark grown sunflower seedlings were finely ground in liquid nitrogen using chilled pestle and mortar and homogenized in Tris buffer (100 mM, 1 mM PMSF, pH 7.0). The homogenates were vortexed for 2 min and centrifuged at 10,000 g for 20 min at 4 °C. For estimation of Fe (II), 10 µl sample was mixed with 300 µl of iron probe in a total reaction volume of 615 µl made up by iron assay buffer and incubated in dark for 30 min at 25 °C. After incubation, O.D was measured at 593 nm. For estimation of total Fe (II + III), 10 µl sample was mixed with 15 µl of iron reducer and 300 µl of iron probe in a total reaction volume of 615 µl made up by iron assay buffer and incubated for 30 min at 25 °C. After incubation, O.D was measured at 593 nm. Control reaction was set without proteins. Fe (III) content was calculated by subtracting Fe (II) content from total Fe (II + III) content.

2.5. Proteomic analysis of total soluble proteins

Sample preparation and mass spectrometric analysis of total soluble proteins was done according to Jain et al. [44]. Cotyledons (500 mg fw) from 2 d old, dark-grown sunflower seedlings raised in the absence or presence of 120 mM NaCl and NO donors (SNP and exhausted SNP) were ground to fine powder in liquid nitrogen and dispersed in the extraction buffer (100 mM Tris-HCl, 5% sucrose, 1 mM EDTA, 1% SDS, 1% protease inhibitor cocktail, pH 7.5). Homogenates were centrifuged at 10,000 g for 20 min at 4 °C. Purification of total soluble proteins in the supernatant was carried out by using 1D Gel clean-up kit. Total protein content was quantified by Bicinchoninic acid (BCA) assay. Aliquots containing 30 µg of protein of respective samples were loaded on 12% gel for SDS-PAGE to separate the proteins. After electrophoresis, the gel was fixed and proteins were stained with Coomassie Blue G-250. Whole lanes were subjected to in-gel digestion of the proteins. For destaining, the gel was excised into cubes and gel pieces were washed for 10 min in 60% acetonitrile (CAN) followed by 10 min in water. For dehydration, the gel pieces were incubated with 100% CAN for 10 min. For reduction of proteins, gel pieces were incubated with 5 mM DTT for 10 min at 60 °C. The proteins were further incubated with 25 mM iodoacetamide solution in dark for 15 min at RT. For removal of excess DTT and iodoacetamide, the samples were incubated with 100% CAN for 10 min and then washed three times with 50 mM ammonium bicarbonate (ABC), 100% CAN and 60% CAN. The cleaned samples were air dried for 15 min at 37 °C and later incubated for 10 min with 50 mM ABC containing 0.01 µg µl^{-1} trypsin solution (Sigma-Aldrich Pvt Ltd, USA). Following 10 min incubation, the samples were further incubated overnight at 37 °C and 25 mM ABC was added to ensure that all the gel pieces were immersed completely. 60% ACN/0.1% TFA were added to the supernatant containing the gel pieces and incubated by gently shaking for 10 min. Afterwards, the supernatant was transferred to a fresh tube and the gel pieces were incubated for 30 min with 99.9%/0.1% TFA solution. In a speedvac, supernatants containing the eluted peptides were then dried and stored until further analysis at −20 °C. The pre-fractionated dried samples were dissolved in 2% ACN/0.5% TFA and incubated with agitation at RT for 30 min. LC-MS/MS analysis was performed on a LTQ-Orbitrap XL (Thermo Scientific). Samples were injected automatically and loaded onto the trap column. After 5 min, peptides were eluted and separated on an analytical column by reversed phase chromatography operated on nano-HPLC (Ultimate 3000, Dionex) with a non-linear 170 min gradient using 5% ACN in 0.1% formic acid in water (A) and 0.1% formic acid in 98% ACN (B) at a flow rate of 300 nl min^{-1}. The gradient settings were: 5–140 min: 14.5–90% A, 140–145 min: 90% A - 95% B, 145–150 min: 95% B and followed by equilibration for 15 min to starting conditions. From the MS prescan, the 10 most abundant peptide ions were selected for fragmentation in the linear ion trap if they exceeded an intensity of at least 200 counts and were at least doubly charged. During fragment analysis a high-resolution (60,000 full-width half maximum) MS spectrum was acquired in the Orbitrap with a mass range from 200 to 1500 Da. For peptide identification, Swiss-Prot database was used and results were displayed using Scaffold software.

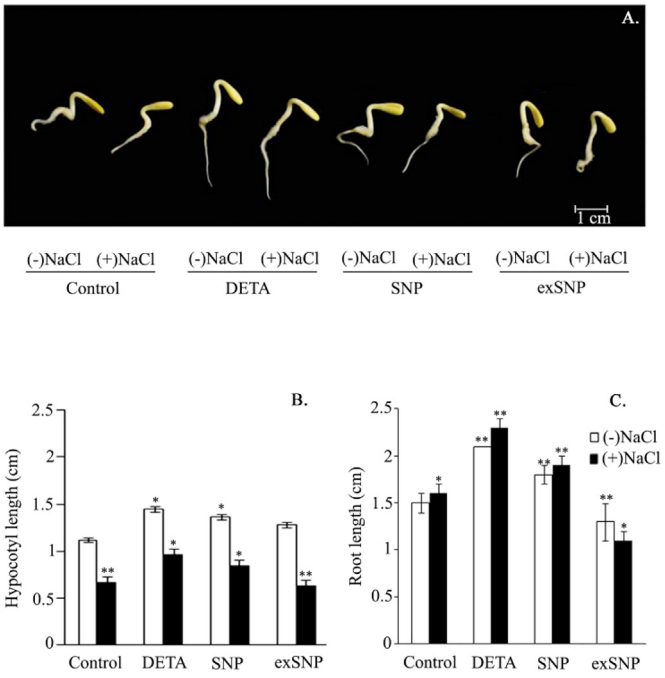

Fig. 1. A. Sunflower seedling growth in response to salt stress and NO. Seedlings were raised in dark in the absence or presence of 120 mM NaCl in Hoagland medium supplemented with NO donors (DETA and SNP, 250 µM each) and exSNP for 2 d. B. Hypocotyl length of sunflower seedlings in response to salt stress and NO. Note enhanced hypocotyl extension in response to DETA and SNP. C. Root length of sunflower seedlings in response to salt stress and NO. Note enhanced root length in response to DETA as NO donor. Statistical analysis was done using SPSS 22 *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared with control (without NaCl and NO donors) according to one-way ANOVA. Bars indicate SE ($n = 3$). Abbreviations: DETA-diethylaminetriamine; SNP-sodium nitroprusside; exSNP-exhausted SNP.

3. Biochemical analyses

3.1. Western blot analysis of actin (ACT8) and heat shock 70 kDa protein (HSP70)

Tissue homogenization and sample preparation: Cotyledons (500 mg fw) harvested from dark grown sunflower seedlings were finely ground in liquid nitrogen using chilled pestle and mortar and homogenized in Tris buffer (100 mM, 1 mM PMSF, pH 7.0). The homogenates were vortexed for 2 min and centrifuged at 10,000 g for 20 min at 4 °C. Protein content was quantified by Bradford assay for protein estimation [45] and mixed with reducing Laemmli buffer. Laemmli buffer consists of 60 mM Tris (pH 6.8), 10% glycerol, 2% (w/v) SDS and 0.008% bromophenol blue.

SDS-PAGE for separation of proteins: Aliquots of cotyledon samples containing 30 and 50 µg of protein for actin and HSP70, respectively, were loaded on a pre-cast 4–15% linear gradient resolving gel (Biorad, USA) for vertical electrophoresis using Miniprotean tetra Cell (Biorad, USA). Electrophoresis was performed at 25, 35, 55, 75 V for 10 min each; 100 V for 20 min and 150 V for 30 min.

Transfer of separated proteins onto nitrocellulose (NC) membrane: Subsequent to resolution of polypeptides, gel was taken out from the cassette and washed in transfer buffer [0.025 M Tris, 0.192 M

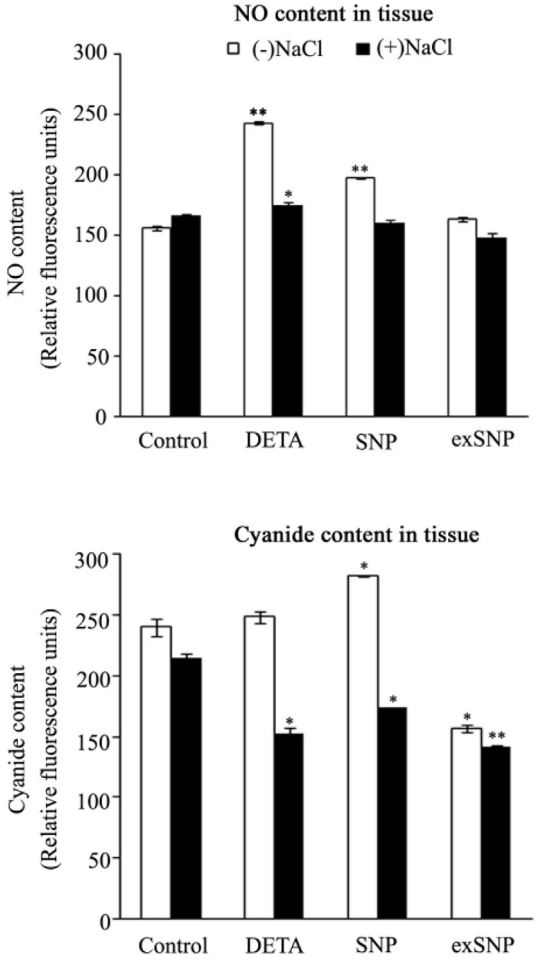

Fig. 2. NO and cyanide contents in sunflower seedling cotyledons. Estimation of NO content was done spectrofluorometrically using MNIP-Cu (ex. 365 nm, em. 420 nm) as NO probe. Cotyledons raised in the presence of DETA alone (in control growth conditions) exhibit highest NO content. Spectrofluorometric estimation of cyanide content was undertaken in 10,000 g supernatant of 2 d old seedling cotyledon homogenates raised in the absence or presence of 120 mM NaCl and supplemented with 250 μM of NO donors (DETA or SNP) or exSNP, using the fluorescent probe- Cyanide Orange (Ursa BioScience, USA; ex. 420 nm, em. 575 nm). Cotyledons raised in the absence of NaCl exhibit highest cyanide content. exSNP brought about least elevation in cyanide content irrespective of salt stress. Statistical analysis was done using SPSS 22 *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared with control (without NaCl and NO donors) according to one-way ANOVA. Bars indicate SE ($n = 3$). Abbreviations: DETA-diethylaminetriamine; SNP-sodium nitroprusside; exSNP-exhausted SNP.

glycine, 0.1% (w/v) SDS and 20% methanol] for 15 min at 4 °C. A complete transfer of proteins was obtained on NC membrane at 400 mA current for 1 h at 4 °C. Prior to Western blot analysis, NC membrane was activated in deionized water for 5 min followed by incubation in transfer buffer (10–15 min). Filter paper (GE Healthcare, UK) was also cut to the size of NC membrane and pre-soaked in transfer buffer. Filter papers (4 pieces), activated NC membrane, gel and filter paper (4 pieces) were packed together to prepare the transfer sandwich, which was then placed inside the transfer unit. To check the complete transfer of proteins, the membrane was stained with Ponceau S stain (prepared by dissolving 500 mg Ponceau S in 1% acetic acid).

Detection of actin abundance: The blot with transferred proteins was first rinsed with MilliQ water to completely remove Ponceau S stain and then incubated in the blocking buffer [5% BSA, 0.2% Tween 20 in phosphate buffer saline (PBS; 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4; final volume = 1 L), pH 7.4] for 1 h at RT to prevent non-specific binding of primary and secondary antibodies to the membrane. Subsequently, membrane was incubated overnight at 4 °C in an orbital shaker with anti-actin antibody (ACT-8, diluted 1:800 in blocking buffer) obtained from Sigma-Aldrich Pvt Ltd, USA. The membrane was then washed thrice in wash buffer (0.2% Tween 20 in PBS, pH 7.4) for 5 min each and incubated for 1 h at RT on an orbital shaker in secondary antibody (anti-rabbit IgG conjugated to alkaline phosphatase obtained from Sigma-Aldrich Pvt Ltd, USA) in a dilution of 1:500 in wash buffer. Finally, the membrane was washed thrice in wash buffer for 5 min each and was developed using freshly prepared BCIP/NBT (1 Sigma Fast tablet dissolved in 10 mL deionized water) for 5–10 min till the desirable color intensity was obtained. Once the desirable color intensity was obtained, the membrane was placed in deionized water.

Detection of heat shock protein 70 kDa abundance: The blot with transferred proteins was first rinsed with MilliQ water to completely remove Ponceau S stain and then incubated in the blocking buffer [5% BSA, 0.2% Tween 20 in phosphate buffer saline (PBS; 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4; final volume = 1 L), pH 7.4] for 1 h at RT to prevent non-specific binding of primary and secondary antibodies to the membrane. Subsequently, membrane was incubated overnight at 4 °C in an orbital shaker with anti-HSP70 antibody (1:3000 diluted in blocking buffer) obtained from Agrisera, Sweden. The membrane was then washed thrice in wash buffer (0.2% Tween 20 in PBS, pH 7.4) for 5 min each and incubated for 1 h at RT on an orbital shaker in secondary antibody (anti-rabbit IgG conjugated to alkaline phosphatase obtained from Sigma-Aldrich Pvt Ltd, USA) in a dilution of 1:2000 in wash buffer. Finally, the membrane was washed thrice in wash buffer for 5 min each and was developed using freshly prepared BCIP/NBT (1 Sigma Fast tablet dissolved in 10 mL deionized water) for 5–10 min till the desirable color intensity was obtained. Once the desirable color intensity was obtained, the membrane was placed in deionized water.

3.2. Estimation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity

GAPDH activity was measured according to Lindermayr et al. [46] by estimating the increase in absorbance due to reduction of NAD^+ to NADH. Cotyledons (500 mg) harvested from 2 d old, dark-grown seedlings raised in the absence or presence of 120 mM NaCl were ground to fine powder with liquid nitrogen and homogenized in grinding medium (100 mM Tris-HCl, pH 7.5). The homogenates were reduced with 10 mM DTT for 20 min at RT and were then centrifuged at 10,000 g for 20 min at 4 °C to obtain total soluble proteins (TSPs). Residual DTT was removed with PD-10 desalting columns. Protein was quantified according to Bradford [45]. Each aliquot containing 150 μg protein was added to a reaction mixture of 1 mL containing 100 mM Tris-HCl, 50 μM arsenate, 100 μg mL^{-1} glyceraldehyde-3-phosphate and the reaction volume was adjusted to 950 μL. The reaction was initiated by addition of 50 μl of 10 mM of NAD^+ and the change in absorbance was monitored at 340 nm for 1 min. Enzyme activity was calculated using extinction coefficient of 6.22 $mM^{-1}cm^{-1}$ for NADH. One unit of enzyme activity was defined as the amount of enzyme required to reduce 1 mM NAD^+ min^{-1} at 25 °C.

3.3. Estimation of S-adenosyl methionine synthase (SAMS) activity

S-adenosyl methionine synthase (SAMS) activity was measured according to Wang et al. [47]. Cotyledons (500 mg) harvested from 2 d old, dark-grown seedlings raised in the absence or presence of 120 mM NaCl were ground to fine powder with liquid nitrogen and homogenized in grinding medium [50 mM Tris-HCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 2% polyvinylpyrrolidone, pH 7.6]. The homogenate was centrifuged twice at 10,000 g for 15 min at 4 °C to obtain TSPs. Protein content was quantified by Bradford assay for protein estimation [45]. The reaction mixture contained 100 mM Tris-

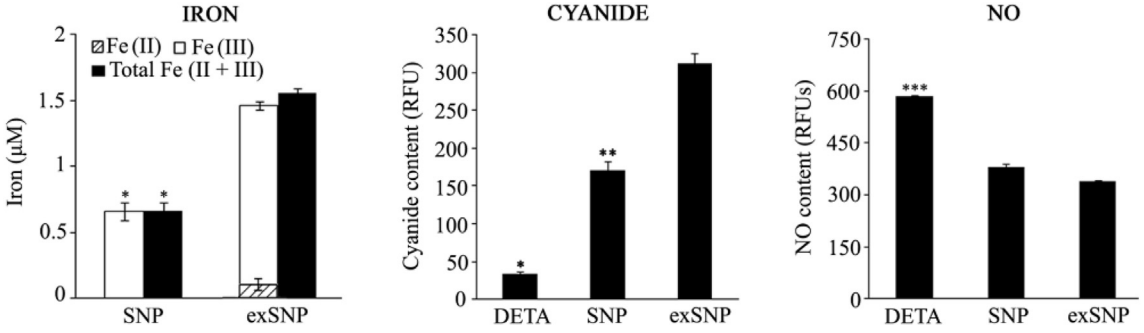

Fig. 3. Estimation of Fe (II), Fe (III) and total Fe (II + III) content, cyanide and NO content in the aqueous solutions of NO donors. Concentrations of Fe (II), Fe (III) and total Fe (II + III) in 250 μM each of the NO donors (DETA or SNP) and exSNP were estimated using iron colorimetric assay kit (BioVision, Inc., USA). exSNP solution has much higher iron content as compared to that in freshly prepared SNP solution. Negligible amount of free iron was present in DETA. Spectrofluorometric estimation of cyanide content was undertaken in 250 μM solutions of NO donors and exSNP using Cyanide Orange (Ursa BioScience, USA; ex. 420 nm, em. 575 nm). Cyanide content was maximum in exSNP solution and negligible in DETA. Spectrofluorometric estimation of NO was undertaken in the 250 μM solutions of NO donors (DETA and SNP) and exSNP using MNIP-Cu (ex. 365 nm, em. 420 nm). DETA proved to be the source for NO in solution. Statistical analysis was done using SPSS 22 *p < 0.05, **p < 0.01 and ***p < 0.001 compared with control (exSNP) according to one-way ANOVA. Bars indicate SE (n = 3). Abbreviations: SNP-sodium nitroprusside; exSNP-exhausted SNP; RFUs-relative fluorescent units.

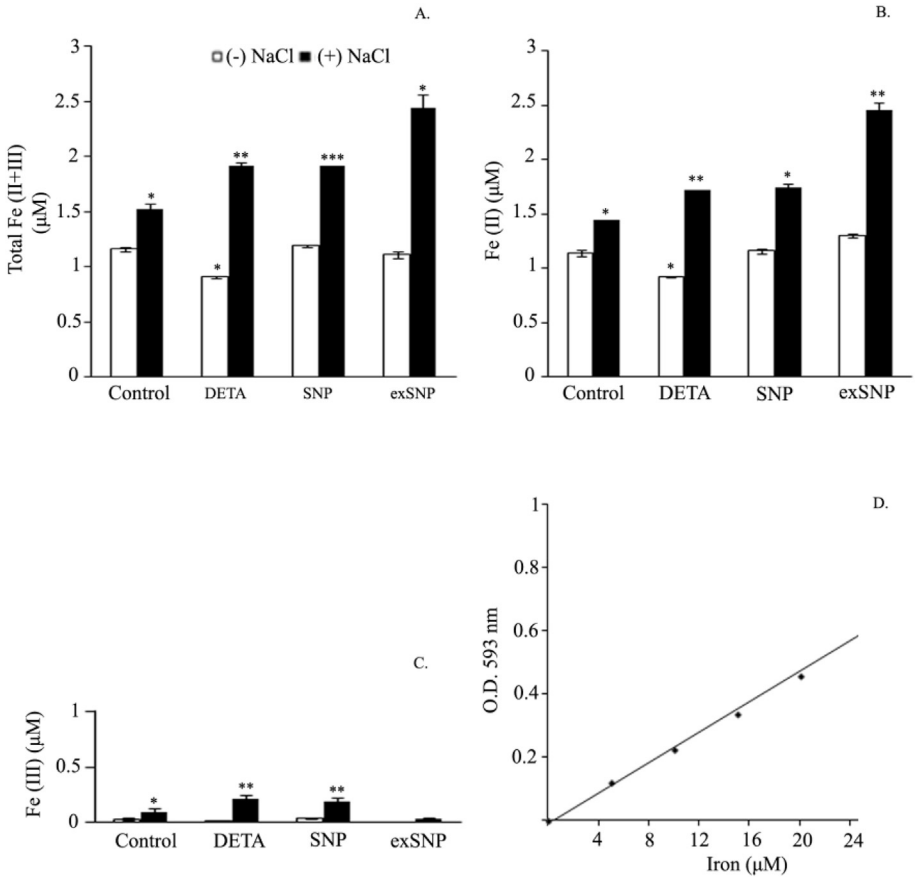

Fig. 4. A. Total Fe (II + III), B. Fe (II) and C. Fe (III) contents in sunflower seedling cotyledons. Concentrations of Fe (II), Fe (III) and total Fe (II + III) in 10,000 g supernatant of cotyledons derived from 2 d old dark grown sunflower seedlings raised in the absence or presence of 120 mM NaCl and supplemented with 250 μM of NO donors (DETA or SNP) or exSNP were estimated using iron colorimetric assay kit (BioVision, Inc., USA). Seedling cotyledons grown in the presence of NaCl and exSNP exhibited maximum Fe (II) and total Fe (II + III) content. D. Iron standard curve. Statistical analysis was done using SPSS 22 *p < 0.05, **p < 0.01 and ***p < 0.001 compared with control (without NaCl and NO donors) according to one-way ANOVA. Bars indicate SE (n = 3). Abbreviations: DETA-diethylaminetriamine; SNP-sodium nitroprusside; exSNP-exhausted SNP.

HCl, 1 mM methionine, 2 mM ATP, 5 mM dithiotheitol, 20 mM $MgCl_2$, 150 mM KCl, pH 8.0. To start the reaction, 40 μg of TSP was added to the reaction mixture with final volume of 1 mL and SAMS activity was measured immediately at 340 nm using a UV spectrophotometer.

3.4. Statistical analysis

For statistical analysis, SPSS 22.0 statistical program (SPSS Inc, Chicago, IL, USA) was used. Effect of various treatments was analyzed statistically using one-way ANOVA and categorized accordingly to various levels of significance (*p < 0.05, **p < 0.01 and ***p < 0.001).

All experiments were performed at least thrice.

4. Results

Earlier recently published work from the author's laboratory forms the basis for using NO donors at 250 μM [48]. These investigations also demonstrated DETA as one of the cleanest (not releasing any other ions detrimental to plant growth) NO source and SNP as one of the least preferred NO source based on ROS production as a result of its application. Likewise, use of 120 mM NaCl as a salt stress provider is also based on our established observations whereby a concentration of NaCl beyond 120 mM proves toxic and leads to visible poor seedling growth

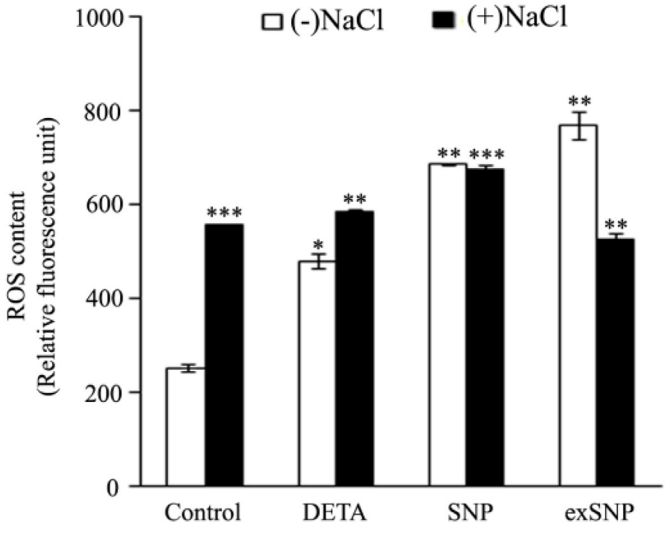

Fig. 5. ROS content in sunflower seedling cotyledons. Spectrofluorometric estimation of ROS in 10,000 g supernatant of cotyledons derived from 2 d old dark grown sunflower seedlings raised in the absence or presence of 120 mM NaCl and supplemented with 250 μM of NO donors (DETA and SNP) and exSNP, using 2′, 7′- dichlorofluorescin diacetate (Sigma, USA; ex. 495 nm, em. 525 nm). All NO donors lead to ROS elevation in control tissue, it being two, thee and four fold of control value in response to DETA, SNP and exSNP, respectively. Relative to salt stressed seedlings, ROS accumulation to such an extent from salt stressed seedlings treated with DETA, SNP or exSNP was not evident. It remained much or less in similar range. Statistical analysis was done using SPSS 22 *p < 0.05, **p < 0.01 and ***p < 0.001 compared with control (without NaCl and NO donors) according to one-way ANOVA. Bars indicate SE (n = 3). Abbreviations: DETA-diethylaminetriamine; SNP-sodium nitroprusside; exSNP-exhausted SNP.

and death [43].

In the present work, exogenous application of NO using NO donors (DETA and SNP, 250 μM each) ameliorates salt stress in sunflower seedlings, as is evident from enhanced hypocotyl extension and root growth (Fig. 1). In general, NO exerts protective effects in response to abiotic stress in plants. However, our results indicate nearly equal NO content in both control and salt stressed seedling cotyledons. In solution, NO production from DETA is about 35 and 42% higher than that from SNP and exSNP, respectively. Interestingly, after exhausting NO in SNP stock solution, no significant difference in NO generation is evident between SNP and exSNP aqueous solutions (Fig. 2). Nonetheless, NO content in seedling cotyledons raised in control conditions in the presence of SNP is 21% higher than in seedling cotyledons raised in the presence exSNP. Furthermore, NO content in seedling cotyledons raised in the presence of DETA is 19% and 33% higher than in seedling cotyledons raised in the presence of SNP and exSNP, respectively. In the seedlings raised in the presence of 120 mM NaCl, NO content is maximal in the cotyledon homogenates of seedlings raised in presence of DETA. However, in presence of salt stress, similar NO content is evident both in SNP and exSNP treated seedling cotyledon homogenates. DETA in solution releases 16% more NO than SNP. Sunflower seedling cotyledon grown in presence of DETA exhibits 95% increase in endogenous NO content, while those grown in presence of SNP exhibits only 45% increase.

Experiments carried out in solution using 250 μM each of DETA, SNP and exSNP brought forward a very interesting finding. Both SNP and exSNP produced significantly high levels of cyanide in solution (Fig. 3). DETA produced negligible amount of cyanide in solution. Furthermore, exSNP releases 51% more cyanide in solution compared to SNP. Cyanide levels were comparatively less in seedling cotyledons obtained from salt-stressed condition alone or salt stress in combination with NO donors–DETA and SNP (Fig. 2). Similar low cyanide levels were observed in seedling cotyledons from control condition (raised only in Hoagland solution) and those raised in presence of DETA (−NaCl). Higher levels of cyanide were evident in the seedling cotyledons raised in the presence of SNP (−NaCl). Noteworthy decline was observed in cyanide levels in seedling cotyledons obtained from both exSNP with or without NaCl.

Upon dissolution in iron assay buffer, 250 μM of SNP and exSNP release iron in the form of Fe (III). However, exSNP releases significantly higher concentration of Fe (III) (51%) compared with SNP (Fig. 3). DETA in solution produces negligible amount of either Fe (III) or Fe (II). Fe (III), Fe (II) and total Fe (II + III) estimation in seedling cotyledons raised in the control conditions (with or without NaCl) or in the presence of 250 μM each of DETA, SNP and exSNP (with or without NaCl) showed that Fe (II) as a major component of total Fe (II + III) present in biological systems (Fig. 4). Interestingly, cotyledons obtained from sunflower seedlings raised in the presence of exSNP + NaCl exhibit significantly enhanced accumulation of Fe (II).

Sunflower seedling cotyledons grown in presence of NO donors, DETA, SNP and exSNP in control conditions (absence of salt) have ROS content higher than the control seedlings by two, three and three fold, respectively (Fig. 5). In general, ROS content in sunflower seedling cotyledons is more than two fold in response to salt stress as compared to control conditions. NO donors, DETA and SNP bring about only a marginal increase in ROS content in sunflower seedling cotyledons grown in salt stress conditions. exSNP leads to maximum ROS production in control seedling cotyledons but its impact remains similar as in salt stress (without exSNP) condition. Thus, salt stress and application of NO donors (DETA and SNP) enhanced accumulation of ROS in sunflower seedling cotyledons.

In order to further understand the role of NO in combating salt stress tolerance, liquid chromatography–tandem mass spectrometry (LC-MS/MS) technique has been used to detect cytosolic proteins which are differentially modulated in cotyledons derived from 2 d old sunflower seedlings subjected to 120 mM NaCl with 250 μM SNP as NO donor. Detailed proteome analysis has also been undertaken using exSNP in the absence or presence of salt stress since SNP also releases cyanide and free iron. A wide range of proteins from various cellular components and biological processes have been detected from tissue homogenates (10, 000 g supernatant). They are classified into 10 functional categories on the basis of their biological roles, using UniProt as: primary metabolism, stress-response, transport, translation, proteolysis, chaperone, regulatory, storage, other metabolic proteins and miscellaneous category.

A total of 138 proteins were detected by LC-MS/MS. A list of all cytosolic proteins detected by proteomic analysis (LC-MS/MS) from cotyledons of 2 d old seedlings subjected to salt stress (120 mM) and NO donor (250 μM) and exhausted SNP is provided [Supplementary data]. In response to salt stress, the abundance of 127 proteins is altered.

The highest number of proteins being modulated by NaCl belong to the category of primary metabolism (37%) followed by translation proteins (13%); chaperone proteins, stress-response proteins, proteolysis (9% each); transport proteins (7%); other metabolic proteins and miscellaneous (6% each); and regulatory and storage proteins (2% each) (Fig. 9). In response to SNP, both in absence or presence of salt, abundance of 117 proteins is altered. However, in response to exSNP, abundance of 129 is altered both in the absence or presence of salt stress. Highest number of proteins modulated by SNP and exSNP belong to the category of primary metabolism irrespective of salt stress. Four proteins namely, actin, HSP70, GAPDH and SAMS have been selected for further physiological investigations from among the cytosolic proteins (10,000 g supernatant) obtained from 2 d old sunflower seedling cotyledons which show noteworthy regulation in response to SNP and exSNP as identified by proteomic analysis (Table 1). Western Blot analysis of actin (ACT8) and HSP70 expression in cotyledons derived from 2 d old seedlings shows significant change in the abundance of ACT8 and HSP70 proteins in response to NaCl stress (Fig. 6). This

Table 1
Cytosolic proteins (10,000 g supernatant) from the cotyledons of 2 d old seedlings showing noteworthy regulation in response to sodium nitroprusside (250 μM SNP) and salt stress (120 mM NaCl).

Proteins	Treatment and fold change	Functions
Aconitase	**exSNP + NaCl = -1.8** NaCl = 0.9 exSNP = 0.2 SNP = 0.1 SNP + NaCl = −0.8	Catalyzes the stereo-specific isomerization of citrate to isocitrate via cis-aconitate in the TCA cycle.
Actin	**exSNP = 3.4** **NaCl = 3.3** **SNP + NaCl = -2.5** exSNP + NaCl = −1.4 SNP = 0.4	Functions in maintaining and controlling cell shape and architecture.
ATP synthase gamma chain	**SNP + NaCl = -1.7** exSNP = 1.0 NaCl = 0.4 exSNP + NaCl = −0.6 SNP = −0.4	Produces ATP from ADP. The gamma chain is important in regulating ATPase activity and the flow of protons though the CF_0 complex.
ATP synthase subunit beta	**exSNP + NaCl = -1.9** SNP + NaCl = −1.5 exSNP = 0.8 NaCl = 0.3	Produces ATP from ADP using a proton gradient across the membrane generated by electron transport complexes of the respiratory chain.
Glyceraldehyde 3-phosphate dehydrogenase	**NaCl = 1.6** SNP = 0.1 SNP + NaCl = −0.8 exSNP + NaCl = −0.2 exSNP = −0.1	Catalyzes the conversion of glyceraldehydes 3-phosphate to D-glycerate 1,3-bisphosphate as a tetramer in the sixth step of glycolysis for gluconeogenesis.
Heat shock 70 kDa protein	**exSNP + NaCl = -2.7** **NaCl = -2.4** SNP + NaCl = 1.0 exSNP = 0.9	Assists a large variety of protein folding processes in the cell by transient association of their substrate binding domain with short hydrophobic peptide segments within their substrate proteins.
Ribulose-phosphate 3-epimerase	**SNP + NaCl = 2.2** exSNP = 1.0 NaCl = 0.5 exSNP + NaCl = 0.3 SNP = −0.2	Converts D-ribulose 5-phosphate into D-xylulose 5-phosphate in the pentose phosphate pathway.
S-adenosylmethionine synthase 2	**NaCl = -1.4** SNP + NaCl = 0.5 exSNP + NaCl = −0.1 exSNP = −0.6	Catalyzes the formation of S-adenosylmethionine from methionine and ATP.
Sucrose synthase 2	**SNP = 1.9** NaCl = 1.0 exSNP = 1.0 SNP + NaCl = −0.6 exSNP + NaCl = −0.8	Key enzyme in plant sucrose catabolism.
Tubulin beta-1 chain	**exSNP + NaCl = -2.7** **SNP + NaCl = -1.7** exSNP = 0.5 SNP = −0.4 NaCl = −0.1	Major constituent of microtubules. It binds with 2 mol of GTP, one at an exchangeable site on the beta chain and other at a no-exchangeable site on the alpha chain.

coincides observations from proteome analysis where there is a three and one fold increase in the abundance of actin and HSP70, respectively, in response to NaCl stress. Exogenous NO application, however, leads to considerable increase in the abundance of actin in both, salt-stressed and control seedling cotyledons. DETA (−NaCl) enhances the abundance of HSP70. Salt stress enhances GAPDH activity while it leads to reduction in SAMS activity. NO inhibits the activities of both enzymes in control conditions as well as salt-stressed conditions. These observations from biochemical analyses are in conformity with proteome analysis, whereby both SNP and exSNP lead to decrease in the abundance of GAPDH in salt-stressed conditions while in the control conditions its abundance is not significantly affected. Interestingly, the abundance of SAMS is reduced by exSNP in both control and salt-stressed conditions, however SNP does not bring any significant changes in its abundance in these conditions.

5. Discussion

5.1. Nitric oxide confers protection against salt stress

Salt stress induces NO accumulation in sunflower seedling root tips [43]. Earlier pharmacological studies have shown that exogenous application of NO donors, such as SNP, confers protection to plants against salt stress by regulation of ion homeostasis, protection from oxidative damage and promotion of plant growth [6,49]. On similar lines, NO donors such as, DETA and SNP, ameliorate the detrimental effects of salt stress by promoting hypocotyl and root growth of sunflower seedlings. However, depending on the plant species and the severity and duration of salt stress, the role of NO in salt stress tolerance is sometimes contradictory [33,40].

5.2. Cyanide and iron released from SNP mask its NO effect

SNP simultaneously releases NO, CN^- and iron in solution (Fig. 7). In plants, the biosynthetic pathway of ethylene is the ubiquitous source of cyanide (Fig. 8). 1-aminocyclopropane-1-carboxylic acid (ACC), the direct precursor of ethylene, is converted to ethylene along with the release of cyanoformic acid by ACC oxidase [50,51]. Under high salinity, leaves and roots of the halophytes *Thellungiella salsuginea* and *Cakile maritime* accumulate high levels of ACC than in the glycophyte *Arabidopsis thaliana* [52]. Various components of ethylene biosynthesis are expressed in abundance in the salt-tolerant genotype of soybean [53]. In tobacco leaves, high concentrations of NaCl stimulate β-CAS and cysteine synthase activities, and leads to H_2S accumulation [54]. Lower levels of cyanide in the salt-stressed sunflower seedling cotyledons (present work) can be explained on the basis of earlier literature which suggests that the activity of enzyme involved in cyanide catabolism i.e. β-cyanoalanine synthase is enhanced during salinity stress [14,54]. â-CAS activity increases in tobacco [55], wheat [56] and rice [57] upon exposure to exogenous KCN. These results show that endogenous â-CAS activity helps in avoiding cyanide toxicity imposed by moderate external CN^- exposure. On similar lines, treatment with exSNP (present work), implicating small doses of cyanide in the plant tissue system, is likely to activate enzyme machinery involved in the catabolism of cyanide. Thus, plants are able to tolerate cyanide toxicity.

Iron is taken up by plants as Fe (II) as it is more soluble than Fe (III).

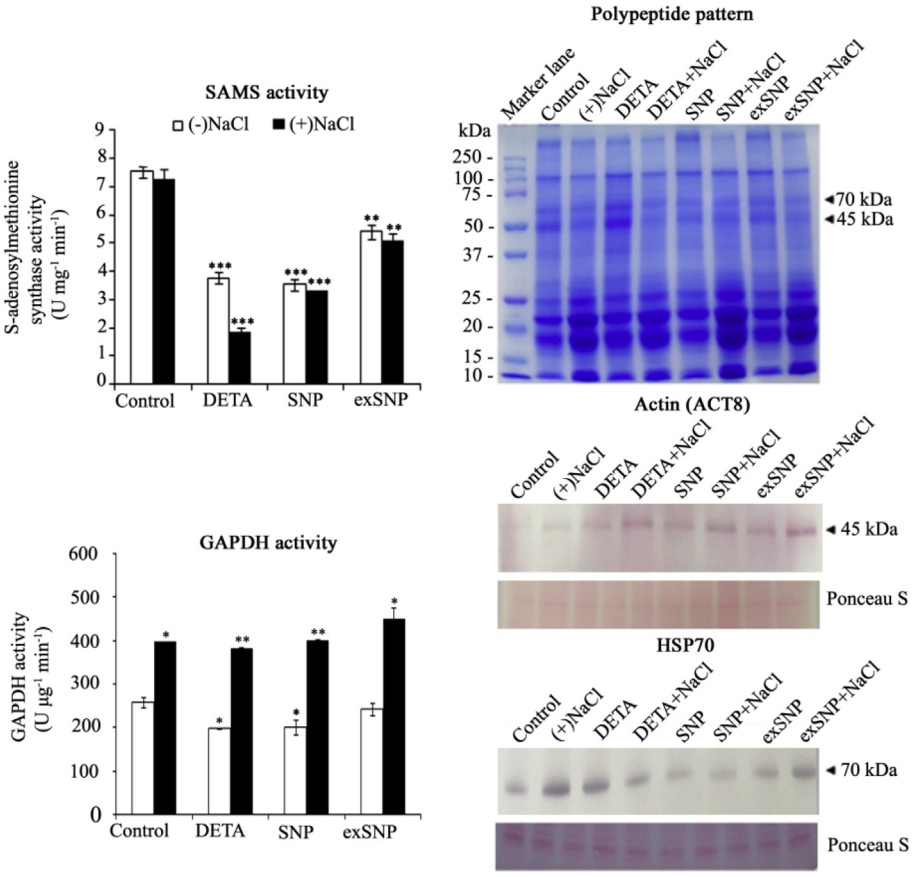

Fig. 6. Effect of NO donors on *S*-adenosylmethionine synthase (SAMS) activity, GAPDH activity, polypeptide pattern, actin (ACT8) abundance and HSP70 abundance in seedling cotyledons. All assays were performed on 10,000 g supernatant of cotyledons derived from 2 d old dark grown seedlings raised in the absence or presence of 120 mM NaCl and supplemented with NO donors (250 µM each of DETA or SNP). An inverse relationship between SAMS activity and salt stress was evident. Note the enhanced GAPDH activity in salt stressed seedlings. Bands corresponding to ACT8 and HSP70 have been marked in the whole tissue polypeptide profile. The abundance of ACT8 was enhanced in response to NO donors and salt stress. The abundance of HSP70 was greatly enhanced in salt stress condition in the absence of NO donors. Statistical analysis was done using SPSS 22 *p < 0.05, **p < 0.01 and ***p < 0.001 compared with control (without NaCl and NO donors) according to one-way ANOVA. Bars indicate SE (n = 3).Abbreviations: DETA-diethylaminetriamine; SNP-sodium nitroprusside; exSNP-exhausted SNP.

Plants have evolved two mechanisms for iron acquisition- Strategy I and Strategy II. The preferential iron uptake among dicotyledonous by strategy I occurs as Fe (II) [58]. In the present study, unlike DETA which releases negligible amount of free ion, both SNP and exSNP release iron as Fe (III). Interestingly, iron accumulates in the cotyledons as Fe (II), since sunflower is a strategy I plant (dicot). Accumulation of Na^+ and Cl^- at toxic levels in the rhizosphere of salt-affected soils causes severe nutritional imbalance in maize owing to the strong interference of these ions with the uptake of essential mineral elements [59–61]. Present study demonstrates enhanced accumulation of iron in salt-stressed seedlings. Salt stress-induced iron accumulation is significantly enhanced in exSNP treated seedlings as well. SNP releases CN^- (five equivalents of CN^- per mole of SNP) and free iron along with NO [11,12] (Fig. 7). The present study has also given evidence that irrespective of irradiation and heat treatments, SNP (exSNP) releases NO as well as deleterious by-products such as free iron and cyanide, with exSNP releasing these deleterious ions at even higher concentrations than SNP. Hence, cyanide and iron mask the NO effect of SNP which limits its use as an NO donor, and also exSNP should not be ideally used as NO controls/sham controls in life science research.

5.3. SNP also enhances tissue ROS levels

In many plant species, ROS production and activity of ROS scavenging enzymes increase during abiotic stress to potentially scavenge toxic levels of ROS and mitigate the oxidative stress damage caused by salt stress [4,25,41,62–65]. Present work has demonstrated that SNP significantly enhance tissue ROS levels in the absence or presence of salt stress thereby all the more highlighting the limitations of the use of SNP as an NO donor in life science research.

5.4. Proteome modulation in relation with salt stress

In the present work, a total of 138 proteins display differential abundance with respect to salt stress (120 mM NaCl) and NO donors (Supplementary table 1). Abundance of 127 proteins is modulated by salt stress alone (Fig. 9). Meanwhile, SNP and exSNP, both in the absence or presence of salt, modulated the abundance of 117 and 129 proteins, respectively. These proteins belong to 10 different functional categories namely, primary metabolism, stress-response, transport, translation, proteolysis, chaperone, regulatory, storage, other metabolic proteins and miscellaneous category (Fig. 9; Table 2). Interestingly, SNP or exSNP treatments lead to increase in abundance of many proteins which are also modulated by salt stress and these proteins belong to the categories of stress-response, transport, translation, proteolysis, chaperone, regulatory, storage and miscellaneous proteins (Table 2).

NaCl and NO significantly alter the abundance of proteins involved in primary metabolism indicating their ability to regulate energy balance. In response to salt stress, carbohydrate, amino acids and fatty acid metabolisms are critically modulated. It affects activities of enzymes participating in photosynthetic electron transport and carbon fixation [29]. In the present work as well, salt-responsive nature of proteins, such as RuBisCo small, fructose-bisphosphate aldolase, aconitate hydratase, GAPDH, PGK, isocitrate dehydrogenase etc, is evident. These proteins are, however, negatively modulated by NO donor treatment in salt-stressed sunflower seedling cotyledons. GAPDH is a highly conserved enzyme in the glycolytic pathway and plays a critical role in carbon economy. It is down-regulated in shoots of alfalfa subjected to salt stress [66]. Other earlier works have reported NO-induced inhibition in GAPDH activity in Arabidopsis [46] and *Nicotiana tabacum* [67], as a result of reversibility of its *S*-nitrosylation [46]. In the present work, physiological investigations undertaken on the estimation of the activity of GAPDH demonstrated that, in general, the activity of GAPDH

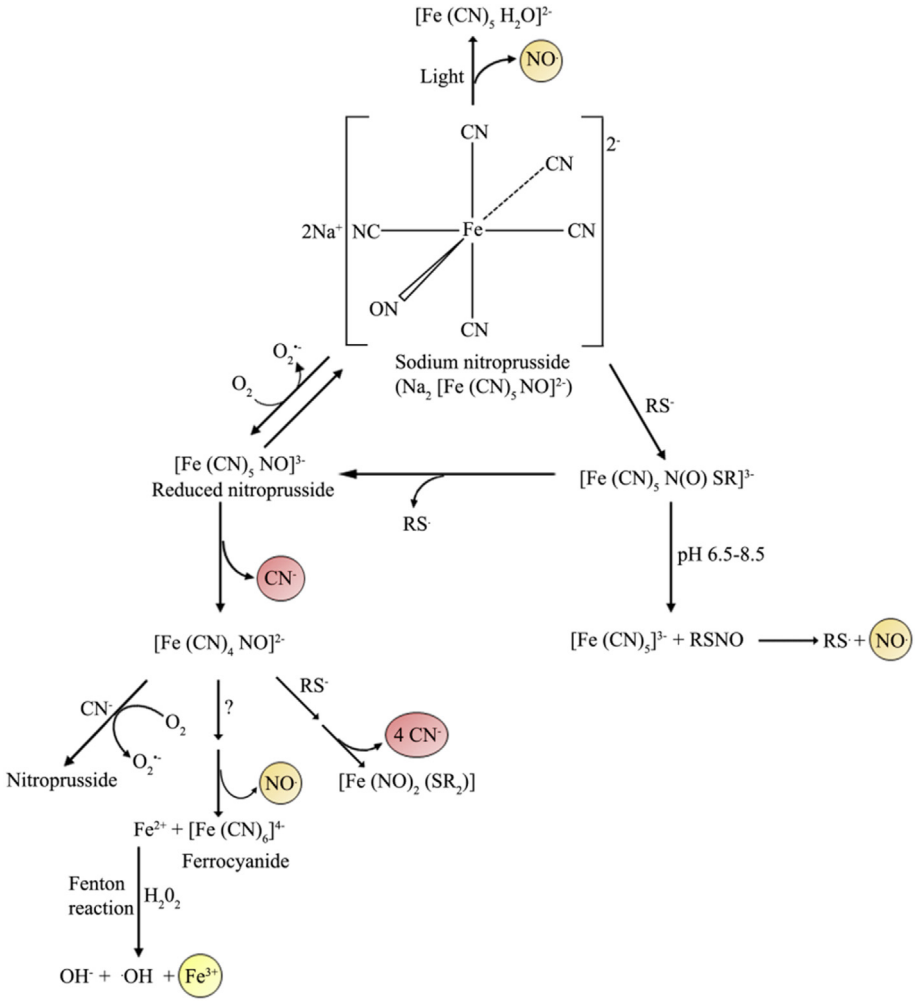

Fig. 7. Proposed reductive metabolism of sodium nitroprusside (SNP) in tissue. The metabolism of SNP produces various metabolites including cyanide anions, ferric ions and nitric oxide molecules. Abbreviations: CN^--cyanide anion, RSNO- S-nitrosothiol, RS^-- thiyl radical, NO·-nitric oxide, Fe (II)-ferrous ion, Fe (III)-ferric ion.

in 2 d old sunflower seedling cotyledons is higher in response to salt stress as compared to control conditions (absence of salt) (Fig. 3). In presence of NO donors, DETA and SNP (in control conditions) enzyme activity is lowered. NO significantly inhibits enzyme activity, thus indicating the possible role of NO in regulating glycolytic pathway.

Due to the involvement of some amino acids in osmotic adjustment, an understanding of their metabolism is important in salt stress tolerance. SAMS catalyzes the biosynthesis of *S*-adenosyl methionine (SAM) from methionine. It is also the precursor molecule for ethylene, polyamines, phytosiderophores and biotin biosynthesis [68]. Expression level of *SAMS* has been reported to increase under salt stress [69–71]. Salt stress and NO treatments (−NaCl) lead to decrease in abundance of SAMS in sunflower seedling cotyledons (present work). Interestingly, SAMS activity in soybean roots has also been reported to decrease under flooding conditions although it is not significantly changed by low temperature, gibberellic acid, calcium and NaCl [47]. In congruence with earlier reports, the activity of SAMS is similar in 2 d old sunflower seedling cotyledons grown in the absence or presence of 120 mM NaCl. In Arabidopsis, following exogenous NO application, SAMS gets *S*-nitrosylated and is consequently inhibited [46]. On similar lines, NO inhibits the activity of SAMS in sunflower seedling cotyledons in the absence or presence of salt stress (present work). DETA, a better NO donor than SNP, inhibits SAMS activity significantly as compared to SNP or exSNP (Fig. 3).

Exposure of plants to nearly all stress conditions also leads to gene expression and synthesis of heat-shock proteins (HSPs) [72,73]. HSPs are important molecular chaperones involved in re-folding the misfolded proteins. Transcription of these proteins is induced in all living cells exposed to stress conditions. In *Pohlia nutans*, *PnHsp70* encodes for a mitochondrial chaperone which is induced by salinity, drought, cold or hot temperature and UV radiation [74]. A study of the impact of salt and genotype on the physiology and root proteome variations in *Solanum lycopersicum* lead to the identification of four HSPs among many other proteins. Only the cytosolic HSP70 varied in its expression with both salt factors and genotype. This protein gets up-regulated in all genotypes by salt stress. A similar proteomic analysis to explore salt responsive proteins in *Sesuvium portulacastrum* revealed the induction in expression of *Hsp70* [75]. HSP70 has thus been proposed as a biomarker of salt stress which highlights its role in protection of plants against stress conditions. In congruence with these earlier reports, proteomic analysis of sunflower seedling cotyledons demonstrated the salt responsive nature of HSP70 and its abundance in response to salt stress. NO also leads to accumulation of HSP70 (Fig. 6).

Salt stress is also known to induce dynamic changes in the cytoskeleton. Actin is an important structural component of the plant cytoskeleton. It is classified into vegetative and reproductive classes which differ not only in their spatial and temporal expressions but also regulate different functions in diverse cell types. The vegetative class is further divided into two subclasses. Vegetative subclass I consists of ACT2 and ACT8 which are expressed constitutively. Subclass II consists of ACT7 whose expression is highly regulated. The most abundant vegetative isoform is ACT2 (50%), followed by ACT7 (40–50%) and ACT8

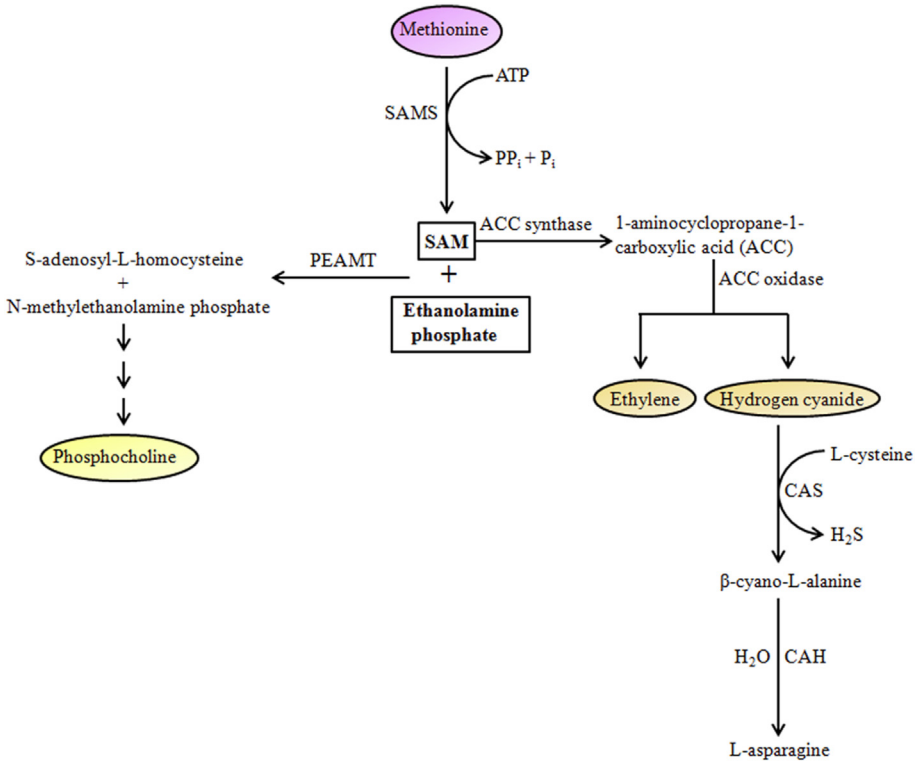

Fig. 8. Interrelationship among the routes of biosynthesis of ethylene, cyanide and phospholipids biosynthesis. Note that all these biomolecules emerge from a common precursor i.e. methionine. Abbreviations: SAMS- S-adenosyl methionine synthase; SAM- S-adenosyl methionine; PEAMT- Phosphoethanolamine N-methyltransferase; CAS- Cyanoalanine synthase; CAH- Cyanoalanine hydratase.

(10–15%) [76,77]. In Arabidopsis, salt stress (150 mM NaCl) induces actin filament assembly and bundle formation. Long-term exposure to high concentration of salt stress treatment (200 mM NaCl) causes polymerized microfilaments to depolymerize [78]. Microtubules (MTs) initially depolymerize and get re-polymerized in response to salt stress in Arabidopsis seedlings. The depolymerization of MTs happens both with low (50 mM) and high (100 mM) NaCl concentrations, suggesting that depolymerization of MTs is necessary to withstand salt stress [79].

In plants, the cytoskeleton is increasingly recognized as one of the downstream targets of nitric oxide (NO), as it is involved in many processes controlled by NO, including those modulated by abiotic and biotic stress [80]. In the root apices of maize, NO modulates the organization of actin cytoskeleton, and degree of its modulation is specific to a cell-type and stage of development [81]. Similarly, in Arabidopsis, during cold treatment the organization of actin filaments is modulated by NO. Actin is one of the candidate proteins which have been reported to undergo *S*-nitrosylation upon GSNO treatment in cotyledons of sunflower seedlings subjected to salt stress [44], and potato leaf extracts [82]. In plants, like Arabidopsis and *Nicotiana tabacum*, tubulin has also been recently reported to undergo tyrosine nitration [83,84]. Tyrosine nitration of tubulin is potentially involved in microtubule organization in plant cells [85]. Proteomic analysis in sunflower seedling cotyledons demonstrated the salt responsive nature of actin and its increase in abundance in response to salt stress (Table 1). NO also leads to accumulation of actin (Fig. 3). Thus, salt stress induces dynamic changes in the plant cytoskeleton by regulating the abundance of actin and it is also a downstream target in NO-mediated signaling in response to stress conditions.

In the present study, the abundance of different subunits of ATP synthase and V-type proton ATPase has been found to be modulated by salt stress. This is in congruence with earlier reports on chloroplastic ATP synthases [29]. In Arabidopsis and *Citrus aurantium*, ATP synthase subunit alpha and beta have been reported to undergo *S*-nitrosylation and *in vivo* tyrosine nitration upon exogenous NO treatment [84,86,87]. Present study also showed the abundance of these proteins to be differentially modulated by NO (Supplementary table 1).

Protein synthesis is an important component in adaptation to salt stress. In the present work, several components of translation machinery exhibit altered abundance, with most proteins being positively modulated in response to salt stress, including ribosomal proteins (30S, 40S, and 60S), elongation factors and initiation factors. This is in congruence with earlier reports on ribosomal proteins, tRNA synthases, translation initiation and elongation factors [37,88–92]. The observed enhanced abundance of translation machinery may assist in maintenance of normal cellular activities under adverse saline conditions. Proteins involved in proteolysis, such as components of ubiquitin proteasome pathway, various peptidases and proteases, are known to exhibit change in abundance in response to salt stress [92,93]. These proteins can regulate turnover rates of proteins and can also modulate other biochemical processes like transcription and signaling [29]. Similarly, the enhanced abundance of 26S protease regulatory subunits, proteasome subunits and tripeptidyl peptidase-2 in sunflower seedling cotyledons under salt stress can facilitate degradation of irreversibly damaged proteins (present work). Proteins of regulatory nature, such as oxygen evolving enhancer protein (OEE), are also salt-responsive. OEE modulates the activity of photosystem II to combat salt stress [92,93]. In *Solanum tuberosum*, NO donor treatment enhanced the accumulation of OEE2 [94] while OEE1 has been reported to undergo *S*-nitrosylation [82]. In the present work, salt stress reportedly leads to an increase in the abundance of OEE1 and OEE2. Thus, NO may modulate regulatory proteins like OEE to combat salt stress in plants. In the present study, some proteins involved in other metabolic processes like cell wall biogenesis or degradation and purine nucleotide metabolism, have also been observed to be salt-responsive in sunflower seedlings. Adenylate kinase 4 and dTDP-4-dehydrorhamnose reductase are negatively modulated by salt stress. Furthermore, storage proteins, such as 11S globulin, are salt-responsive in sunflower seedlings. NO increases its abundance along with that of oleosin, which is a crucial structural component of oil body membrane. Earlier work from the author's laboratory has showed that NO plays a protective role during proteolytic degradation of oleosins prior to or accompanying lipolysis [43].

Based on earlier literature, Table 3 segregates the molecular

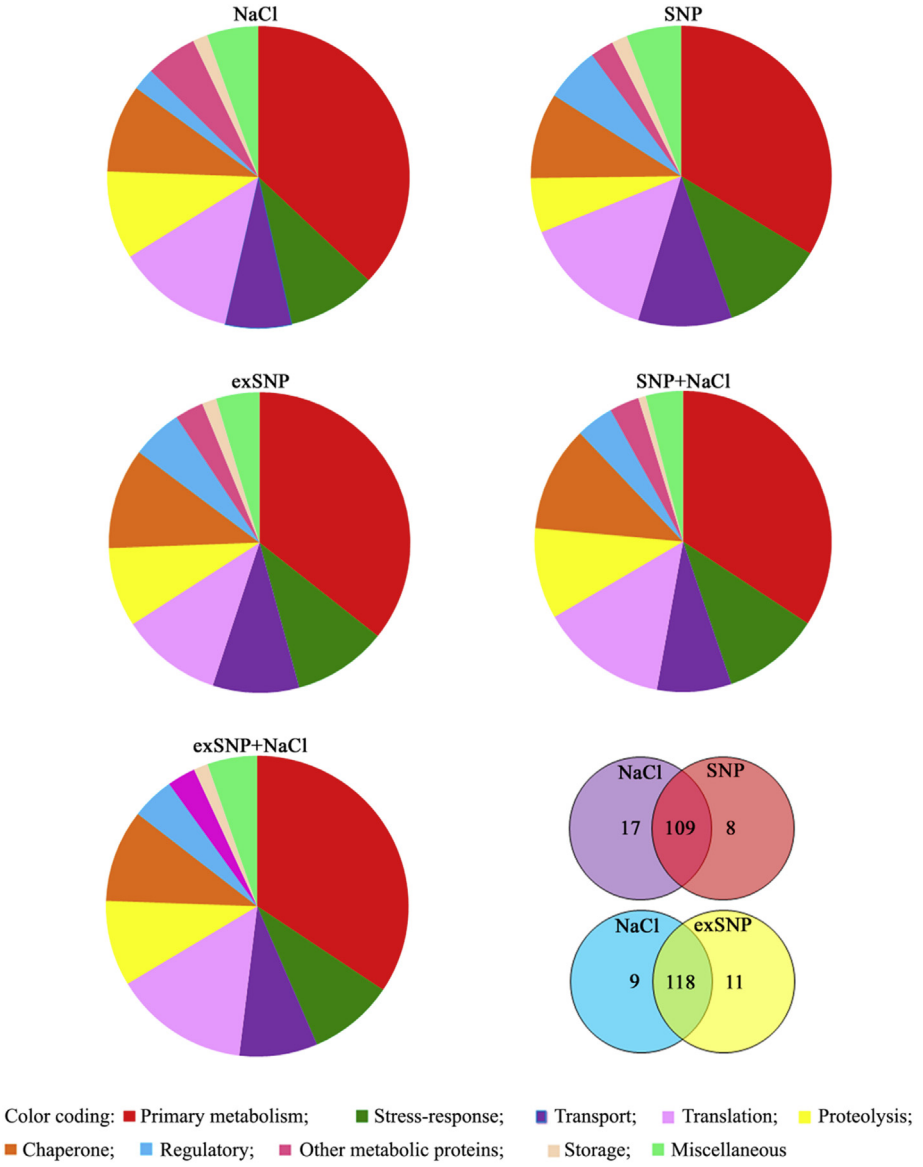

Fig. 9. Different categories of major cytosolic proteins in the 10,000 g supernatant of cotyledon homogenates in response to the indicated treatments provided to sunflower seedlings. Abbreviations: NaCl-sodium chloride; SNP-sodium nitroprusside; exSNP-exhausted SNP.

Table 2
Differential regulation of cytosolic proteins from 10,000 g supernatant of 2 d old seedling cotyledons.

Protein categories	NaCl (%)	SNP (%)	exSNP (%)	SNP + NaCl (%)	exSNP + NaCl (%)
Primary metabolism	37	34	36	34	34
Stress-response	9	11	10	11	9
Transport	7	10	9	8	8
Translation	13	14	11	14	15
Proteolysis	9	6	9	10	9
Chaperone	9	9	11	11	10
Regulatory	2	6	5	4	5
Other metabolic	6	3	3	3	3
Storage	2	3	3	1	2
Miscellaneous	6	6	5	4	5

mechanism of SNP action on some of the proteins of different metabolic categories. Thus, SNP action is evidently being mediated by NO, cyanide and iron. Enhanced production of NO under stress conditions causes a number of biochemical aberrations by post-translational modifications (PTMs). *S*-nitrosylation and protein tyrosine nitration are major NO-mediated PTMs. From the available literature, most of the NO-modulated proteins like GAPDH, ribulose bisphosphate carboxylase small chain, serine hydroxymethyltransferase 1, oxygen-evolving enhancer protein 1 and actin have been reported to undergo *S*-nitrosylation (Table 3). Abiotic stress conditions, such as low temperature, salt stress and high light bring about significant modulation of *S*-nitrosylation of proteins [38,95–97]. In *Citrus aurantium*, *Nicotiana*

Table 3
Segregation of SNP modulated cytosolic (10,000g supernatant) proteome analysis from the cotyledons of 2 d old dark grown sunflower seedlings, into probable effects of NO, CN and iron, based on available literature.

Protein	Nitric oxide				Cyanide				Iron			
	NO donor	System	Effect	Reference	Donor	Plant species	Effect	Reference	Treatment	Plant species	Effect	Reference
PRIMARY METABOLISM												
Aspartate aminotransferase (AST) 1				–				–	Iron deficiency	*Cucumis sativus* L.	AST activity increased in roots.	[101]
Aspartate aminotransferase			NO may be involved in the alteration of AST release induced by abamectin in rats.	[102]*				–				-
Glyceraldehyde-3-phosphate dehydrogenase	SIN-1	Human platelet	Immediate minor inhibition	[103]*				–				–
	SNP	Adult rat brain	Enhances ADP-ribosylation	[104]*				–				–
	GSNO (1000 μM), SNP (1000 μM)	*Arabidopsis thaliana*	*in vitro* GAPDH activity reduced upto 90% and 50% by GSNO and SNP respectively due to *S*-nitosylation.	[46]				–	Iron deficiency	*Beta vulgaris* L	Up-accumulation of the protein	[105]
	GSNO (500 μM)	*Solanum tuberosum*	S-nitrosylated	[82]				–				–
	DEA/NO	*Nicotiana tabacum*	*In vitro* GAPDH activity reduced by 25% (50 μM DEA/NO) and 85% (500 μM DEA/NO).	[67]				–				–
Glyceraldehyde-3-phosphate dehydrogenase A		*Arabidopsis thaliana*	*In vivo* tyrosine nitration	[84]				–				–
Aconitate hydratase 1 (Aconitase)	NOC-9 (5 mM)	*Nicotiana tabacum*	Decrease in activity by 50% (30 min) and 90% (1 h)	[106]				–	Iron deficiency	*Lycopersicon esculentum*	Protein expression increased	[109]
		Arabidopsis thaliana	NO inhibits aconitase activity as indicated by increase in citrate levels (70%).	[107]				–				–
	NO bolus (50 μM), NOC-12, and NOC–18	Recombinant pig heart mitochondrial aconitase	55% inactivation (30 min NO bolus); Aconitase activity diminished to 60% (NOC-12) and 25% (NOC-18) of initial values after 30 min.	[108]*				–				–
Succinyl-CoA ligase [ADP-forming] subunit beta				–				–	Iron deficiency	*Lycopersicon esculentum*	Protein expression increased	[109]
Ribulose bisphosphate carboxylase small chain	GSNO (25–500 μM)	*Kalanchoe pinnata*	Reduction of initial and total carboxylase activity in a dose-dependent manner	[110]				–				–
	SNP (100 μM)	*Citrus aurantium*	S-nitrosylation	[87]				–				–
Phosphoglucomutase				–				–	Iron deficiency	*Lycopersicon esculentum*	Protein expression increased	[109]
Serine hydroxymethyltransferase 1	GSNO or GSH (1 mM)	*Arabidopsis thaliana*	S-nitrosylated	[86]				–				–

(*continued on next page*)

Table 3 (*continued*)

Protein	Nitric oxide				Cyanide				Iron			
Triosephosphate isomerase	GSNO or GSH (250 μM)	*Arabidopsis*	S-nitrosylated	[46]				–				–
	GSNO, GSH, or GSSG (200 μM)	*Arabidopsis* undergoing H (hypersensitive disease resistance response)	S-nitrosylated	[111]				–	Iron deficiency	*Beta vulgaris* L.	Up-accumulation of the protein	[105]
Isocitrate dehydrogenase [NADP]	500 μM SNP	*Citrus grandis*	Decrease in activity	[112]				–	Iron deficiency	*Lycopersicon esculentum* root tips and leaf extracts	Fe-depletion leads to 10.5 fold increase in ICDH activity	[113]
Phosphoglycerate kinase		*Arabidopsis thaliana*	*In vivo* tyrosine nitration	[84]				–	Iron deficiency	*Beta vulgaris* L.	Protein increasing in Fe-deficient condition	[115]
	GSNO (500 μM)	*Solanum tuberosum*	S-nitrosylated	[82]				–	Iron deficiency	*Beta vulgaris* L.	Up-accumulation of the protein	[105]
Adenosylhomocysteinase		*Arabidopsis thaliana*	*In vivo* tyrosine nitration	[84]				–				–
Malate dehydrogenase	SNP (100 μM)	*Actinidia deliciosa*	Downregulated protein	[114]				–	Iron deficiency	*Beta vulgaris* L.	Protein increasing in Fe-deficient condition	[115]
				–				–	500 μM Fe (III)EDTA	*Zea mays*	Increase in abundance	[116]
S-adenosylmethionine synthase	SNP(100 μM)	*Medicago sativa*	Enhanced accumulation of the enzyme	[117]				–	Iron deficiency	*Cucumis sativus* L.	Protein concentration is increased	[118]
Aspartate aminotransferase			NO may be involved in the alteration of AST release induced by abamectin in rats.	[102]*				–				-
STRESS- RESPONSE PROTEINS												
Catalase	SNAP, GSNO,NOC-9	*Nicotiana tabacum* cv. Xanthi-nc	Inhibition	[119]	KCN	*Spinacia okracea*	Inhibition	[120]	Iron deficiency	*Pisum sativum*	Activity declined by 51% in young Fe-deficient leaves	[100]
	GSNO or GSH (1 mM)	*Arabidopsis*	S-nitrosylated	[86]	NaCN	*Carthamus tinctorius*	50% inhibition of enzyme activity of catalase isoforms (pH optimum −6.5, 41.8 μM NaCN; pH optimum- 8.5, 46.6 μM)	[121]				–
Catalase	GSNO (500 μM)	*Solanum tuberosum*	S-nitrosylated	[82]	Cyanide (5 mM)	*Phaseolus vulgaris, Medicago sativa* (nodules)	Inhibition	[122]				–
				–	KCN	*Beta vulgaris*	50% inhibition was observed at 6.5 X 10^{-4}M KCN.	[123]				–
TRANSPORT PROTEINS												

(*continued on next page*)

Table 3 (*continued*)

Protein	Nitric oxide				Cyanide		Iron			
ATP synthase subunit alpha	GSNO or GSH (1 mM)	*Arabidopsis thaliana*	S-nitrosylated	[86]	–					–
		Arabidopsis thaliana	*In vivo* tyrosine nitration	[84]	–		Iron deficiency	*Beta vulgaris* L.	Protein increasing in Fe-deficient condition	[115]
ATP synthase subunit beta	SNP (100 μM)	*Citrus aurantium*	S-nitrosylation	[87]	–		Iron deficiency	*Beta vulgaris* L.	Protein increasing in Fe-deficient condition	[115]
		Arabidopsis thaliana	*In vivo* tyrosine nitration	[84]	–					–
REGULATORY PROTEINS										
Oxygen-evolving enhancer protein 2	GSNO (200 μM)	*Solanum tuberosum*	Accumulation following NO-donor treatment	[94]	–					–
14-3-3-like protein				–	–		500 μM Fe (III)EDTA	*Zea mays*	Increase in abundance	[116]
Oxygen-evolving enhancer protein 1	GSNO (500 μM)	*Solanum tuberosum*	S-nitrosylated	[82]	–					–
STORAGE PROTEIN										
Oleosin			NO plays a protective role during proteolytic degradation of oleosins prior to/accompanying lipolysis.	[43]	–					–
MISCELLANEOUS										
Actin	GSNO (500 μM)	*Solanum tuberosum*	S-nitrosylated	[82]	–					–

(*)- from animal literature.

tabacum and Arabidopsis, a number of proteins have been reported to undergo differential *S*-nitrosylation under salt stress [38,67,98]. Salt-stress induced protein tyrosine nitration has been reported in cytosolic and cell wall-associated proteins [99]. In addition, release of cyanide and free iron upon SNP dissociation in solution limits its usefulness as an NO donor in biological research and the results obtained there from are to be analyzed with due caution [11,12]. Catalase, a ROS-scavenging enzyme, is also likely to be inhibited by NO and cyanide. Iron (Fe) deficiency also leads to a decline in catalase activity [100]. Fe deficiency also enhances aspartate aminotransferase 1 activity and phosphoglucomutase expression, and increases the abundance of 14-3-3-like protein (Table 3). Thus, the response to Fe deficiency includes proteins involved in primary metabolism and regulation.

6. Conclusions

To sum up, NO donors, such as SNP, ameliorate the detrimental effects of salt stress by promoting hypocotyl and root growth of sunflower seedlings. Irrespective of irradiation and heat treatments, SNP (exSNP) releases NO as well as deleterious by-products such as free iron and cyanide, with exSNP releasing these deleterious ions at even higher concentrations than SNP. Hence, cyanide and iron mask the NO effect of SNP which limits its use as an NO donor. Furthermore, exSNP does not seem to be a reliable control for NO-related pharmacological treatments to plants. SNP also significantly enhances tissue ROS levels irrespective of salt stress, thereby further highlighting its limitations as an NO donor. SNP or exSNP treatments enhance the abundance of many proteins positively modulated by salt stress as well. These proteins belong to the categories of stress-response, regulatory, transport, proteolysis, and storage proteins. NO significantly inhibits the activities of GAPDH and SAMS. Proteomic analysis of sunflower seedling cotyledons has further demonstrated the salt responsive nature of HSP70 and its abundance in response to salt stress. NO also leads to accumulation of HSP70 and actin. Salt stress induces dynamic changes in the plant cytoskeleton by regulating the abundance of actin, and it is also a downstream target in NO-mediated signaling in response to stress conditions. SNP action in plants is thus evidently being mediated by NO, cyanide and iron. Present findings hold promise for future investigations on post-translational modifications of cytosolic proteins in response to the three molecular species (NO, CN^- and iron) released from SNP.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2019.03.008.

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Disclosures

The authors have no conflicts of interest to declare.

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