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Sodium nitroprusside enhances regeneration and alleviates salinity stress in soybean [*Glycine max* (L.) Merrill]

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ABSTRACT

SNP (Sodium nitroprusside) a Nitric oxide (NO) donor, plays varied roles in the growth and development of plants. Hence, in the present study, we have demonstrated that SNP enhances regeneration in half-seed explants of soybean cv. PUSA 9712, when cultured in Shoot Induction Medium (SIM) containing 4.44 μM BA and 30 μM SNP. About (91.0) % of explants formed shoots in the presence of SNP, compared to (76.33) % as observed on SIM with 4.44 μM BA alone. Supplementation of 30 μM SNP in rooting medium (RM) containing 4.93 μM IBA, produced the highest number of rooted plantlets. The plants regenerated in the presence of SNP displayed improved characteristics (mean shoot length = 9.21 cm, mean root length = 14.19 cm) as compared to plants regenerated in control medium devoid of SNP. Salinity stress studies revealed that, application of 50 μM SNP, reduces browning, drying of soybean tissues and increased the plant survivability, further analysis revealed that supplementation of SNP results in stabilization of Na^+ and K^+ ion ratio, activates SOD, CAT enzyme expression and reduce the concentrations of MDA and H_2O_2, thereby reversing the effects of salinity stress. These results signify that SNP enhances regeneration and development rate of soybean plants, and application of SNP alleviates salinity stress manifested by NaCl.

1. Introduction

Global climate change, caused mostly by deleterious human activity, is a major factor which affects the environment and survival of an organism. Among which plants have been remarkably affected by such imbalance in the environment due to their sessile nature compared to animals and humans. Salinity is one of the major environmental threats faced by plant kingdom since it has a multifaceted role affecting plant growth, development, quality, yield and biomass production, due to the changes incurred at structural, biochemical and molecular levels (Zhang, 2015). Salinity affects 42 million hectares of land in Asia (Regional Assessment of soil change in Asia-FAO, 2015) which alarms the necessity for research in physiological, genetic and molecular level, to alleviate salinity stress in plants.

Generally, in plants, resilience towards salinity stress is achieved through ion and osmotic homeostasis, salt-stress signalling pathway, stress damage control and repair signalling molecules, transcription factors, stress-responsive genes, detoxification and growth regulation (Zhu, 2002). Generation of oxidative stress is one of the obligatory outcomes of the salinity challenge, caused due to the accumulation of reactive oxygen species (ROS), which plays a dual role in plant developmental process both, as toxic compounds and signalling molecules during stress conditions. Control of relatively high levels of ROS can be achieved by scavenging these radicals through enzymatic reactions. The stasis between dual roles of ROS can be sustained by a large network of anti-oxidant mechanisms, including various signalling molecules, plant metabolites, enzymes, plant hormones and calcium ions to rectify stress conditions (Miller et al., 2010; Ryu and Cho, 2015). Among them, nitric oxide (NO) plays an important role in alleviating abiotic stress threat in plants reacting quickly with ROS. Typically, NO is involved in cell metabolism and morphogenesis and acts as a signalling molecule in response to various biotic and abiotic stresses (Beligni and Lamattina, 2001).

The positive role of NO in salinity stress tolerance has been extensively studied in various plants (Krasylenko et al., 2010). Involvement of NO in various activities such as leaf water maintenance (Xing et al., 2004), expression of *ATPase* gene (Zhao et al., 2004), activity as a signal molecule in enhancing antioxidant mechanisms (Li et al., 2008), maintenance of ion balance between Na^+ and K^+ to improve plant growth rate (Zheng et al., 2009), promotion of genes expressing

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Abbreviations

NaCl	Sodium chloride
BA	N^6 - benzylaminopurine
GA_3	Gibberellic acid
IBA	Indole-3-butyric acid
RWC	Relative water content
MDA	Malondialdehyde
H_2O_2	Hydrogen peroxide
SOD	Superoxide dismutase
CAT	Catalase

antioxidant enzymes such as SOD, CAT, POX, APX and GPX (Song et al., 2006; Sheokand et al., 2008; Nejadalimoradi et al., 2014) improvement in the accumulation of proline (Vaishnav et al., 2013) and decrease in the production and accumulation of H_2O_2 (Aihong et al., 2011) has already been deciphered.

Soybean [*Glycine max* (L.) Merrill] regeneration in the tissue culture medium is a complicated process, involving multiple growth regulators and their combinations (Paz et al., 2006; Arun et al., 2014, 2016). It was seen in cherry (*Prunus cerasus* L.), that augmentation of regeneration medium with suitable supplements like SNP, a NO donor, reduces the shoot regeneration protocol time and usage of resources (Sarropoulou et al., 2014). In addition to salt stress alleviating character, NO also mimics the action of plant growth regulators (Beligni and Lamattina, 2001). The SNP has been extensively used for biotic, abiotic stress-related and regeneration studies due to the incessant production of NO (Floryszak-Wieczorek et al., 2006) during its supplementation. It has a positive influence on shoot induction in a range of plant species like *Dioscorea opposita* (Xu et al., 2009), *Malus hupehensis* (Han et al., 2009), *Prunus cerasus* L. (Sarropoulou et al., 2014), *Vanilla planifolia* (Tan et al., 2013) and *Chrysanthemum* (Arun et al., 2017).

The experimental crop soybean, is agronomically important, having copious nutritional, health- promoting benefits, hence it has always been a unique target for research. Nevertheless, it is affected by various biotic and abiotic stresses throughout the life cycle which ultimately causes an annual yield loss. Thus more studies are focused on soybean regeneration, genetic transformation, and functional genomic analysis to raise biotic and abiotic resistant soybean plants. Soybean is reported to be severely sensitive to salinity (Greenway and Munns, 1980) as it directly affects metabolism and symbiotic interactions, thereby limiting the plant growth (Dita et al., 2006). Various plant growth regulators, such as abscisic acid, auxin, cytokinins, gibberellins employed in organogenesis, were also determined to be involved in the regulation of plant development and tolerance to diverse stresses including high salinity (Ryu and Cho, 2015). In general, organogenesis protocol required at least three hormones (shoot induction, elongation and rooting) for the regeneration of the whole plant from the explant. As supplemented SNP is believed to elevate these effects along with salt alleviating property, it might be useful to formulate a simple, effective and less complicated regeneration protocol to raise salt tolerant soybean through *in vitro* selection. Till date, the influence of SNP on organogenesis and alleviation of stress conditions has not been studied in soybean. Hence, the present work is carried out to explore the influence of SNP on *in vitro* organogenesis and regeneration in presence and absence of salinity stress, and its ability to bestow tolerance towards salinity stress on soybean in field level.

2. Materials and methods

2.1. Plant material, seed surface sterilization and preparation of explants

Mature and dry soybean seeds of the cultivar (cv.) PUSA 9712 (Fig. 1a) were used in the present study. The seeds were procured from the Indian Agricultural Research Institute (IARI), Pusa Campus, New Delhi, India. The Seeds were germinated and maintained in the greenhouse facility, Department of Biotechnology, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India during the suitable season. The seeds were surface sterilized (Di et al., 1996) and soaked in sterile distilled water (Fig. 1b). Half-seed explants (Fig. 1c) were prepared as previously described (Paz et al., 2006; Arun et al., 2016).

2.2. Experimental design

All sets of experiments were carried out in *in vitro* condition for standardization purpose and later analyzed in *in vivo* condition. The first *in vitro* experiment was performed to analyze the plant hormone mimic the character of SNP. The control plants were grown from half-seed explants on shoot induction medium [SIM:(MS) salts (Murashige and Skoog, 1962), MSIII iron, B5 vitamins (Gamborg et al., 1968), 87.65 mM sucrose, 4.44 μM N^6-benzylaminopurine (BA), and 0.8% agar (pH 5.8)] for 45 days followed by elongation medium ([EM:(MS) salts, MSIII iron, B5 vitamins, 87.65 mM sucrose, 1.45 μM gibberellic acid (GA_3) and 0.8% agar (pH 5.8)]) for 30 days and rooting medium ([RM:(MS) salts, MSIII iron, B5 vitamins, 87.65 mM sucrose, 4.93 μM indole-3-butyric acid (IBA) and 0.8% agar (pH 5.8)]) for 30 days with intermittent sub-culturing every 15 days (Arun et al., 2016). All chemicals were purchased from Sigma, St. Louis, USA.

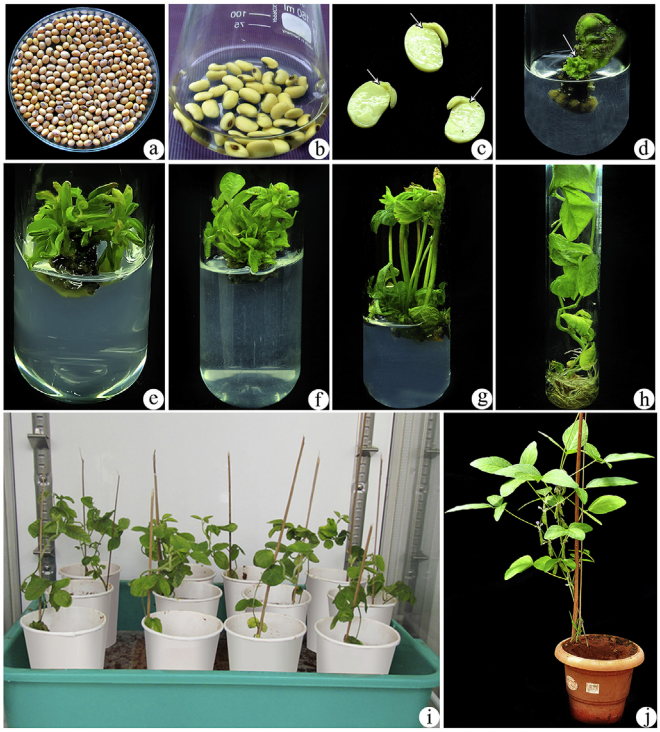

Fig. 1. Effect of SNP on shoot proliferation and rooting from half-seed explants of soybean cv.PUSA 9712. a) mature dry seeds of soybean cv. Pusa 9712 used for preparing the half seed explants, b) one-day old imbibed seeds, c) half seed explants made from imbibed seeds (white arrows direct the embryonic area). d-g) Axillary shoot induction (white arrow indicates the axillary shoot), proliferation and shoot development on SIM containing BA (4.44 μM) and SNP (30 μM) after 45 days of culture, h) Rooted shoot on RM containing IBA (4.93 μM) and SNP (30 μM) after 30 days of culture, i) Hardened plants in plastic cups (sand, soil and vermiculite, 1:1:1 v/v/v), j) Acclimatized soybean plant surviving in greenhouse.

2.3. Effect of SNP on shoot proliferation and rooting from half-seeds

For SNP standardization, different concentrations of SNP (0, 10, 20, 30, 40 and 50 μM) were filter sterilized and added to autoclaved shooting and rooting medium. Explants were subcultured thrice with an interval of 15 days on SIM with SNP to achieve shoot induction and proliferation, followed by 30 days on RIM with different concentrations of SNP with intermittent subculturing after 15 days. The mean number of induced shoots and rooting shoots per explant were recorded respectively. All cultures were incubated at 25 ± 2 °C under a 16h/8h, light/dark, photoperiod at a light intensity of 50 μmol m^{-2} s^{-1} delivered by cool white fluorescent lamps (Philips, Delhi, India). The rooted plantlets were washed with running tap water and then transferred to plastic cups containing soil, sand and compost mixture (1:1:1 v/v/v) and grown in a growth chamber (Sanyo, Osaka, Japan). After two weeks, the plants were transferred into plastic pots containing soil, sand and compost mixture (1:1:1 v/v/v) and grown in the greenhouse under controlled conditions. The Percentage of surviving plants after acclimatization was recorded.

2.4. Effect of SNP on shoot proliferation and rooting in NaCl stress

Half-seed explants with shoot buds were inoculated in different concentration of NaCl (0, 10, 30, 50, 70, 90 and 100 mM) to determine the survival inhibitory concentration of NaCl. In the next set of experiments, the concentration of SNP was standardized at the minimum inhibitory concentration of NaCl, to achieve the highest number of multiple shoots from half-seed explants. Half-seed explants were cultured in fresh SIM supplemented with 70 mM NaCl and different concentration of SNP (0, 25, 50, 75 and 100 μM) for 45 days, with intermittent subculture every 15 days. The surviving explants from each concentration of SIM were further shifted to RIM with respective concentrations of SNP as present in the SIM, supplemented with 70 mM NaCl and were grown for 30 days, followed by transplantation to the greenhouse with optimal conditions. A number of surviving plantlets hardened and acclimatized in the greenhouse conditions was recorded.

2.5. Effect of SNP and NaCl in in vivo conditions

For *in vivo* studies, control plants were germinated, grown in tissue culture medium and acclimatized under greenhouse conditions. After 2 weeks of acclimatization, plants with similar growth pattern were taken and further analyzed for its tolerance against salt stress under *in vivo* conditions. The experimental design consisted of plants treated with three different factors; irrigation with plain water (C-I), irrigation on water supplemented with 70 mM NaCl (C-II) and water supplemented with 70 mM NaCl + 50 μM SNP (C-III) during irrigation for 15 days. Proceeding treatment, plants were analyzed for salt tolerance to investigate the relativity between SNP and salt tolerance.

2.6. Biochemical and physiological analysis

The biochemical and physiological analysis, such as chlorophyll content (Arnon, 1949), RWC (Turner, 1981), proline content (Bates et al., 1973), stomatal aperture (Desikan et al., 2002), Na^+ and K^+ content (Lauchli and Wieneke, 1979), MDA (Heath and Packer, 1968), H_2O_2 (Velikova et al., 2000), SOD (Dhindsa et al., 1981) and CAT (Aebi, 1984) were carried out following respective protocols.

2.7. Statistical analysis

For multiple shoot induction, shoot elongation and rooting, 50 explants were cultured per treatment. All designed experiments were repeated three times with three replicates. Analysis of variance (ANOVA) between treatment means was carried out with the SPSS 16.0 program using Duncan's multiple range test at 5% level.

Table 1
Effect of SNP on shoot proliferation from half-seed explants of Soybean cv. PUSA 9712 on SIM after 45 days of culture.

Treatments		Percentage of explant responding (%)	Mean number of shoots per explant (after 45 days)	Mean shoot length (cm)
BA (μM)	SNP (μM)			
4.44	0.0	76.33 ± 0.29^{f}	15.47 ± 0.25^{f}	5.42 ± 0.18^{e}
4.44	10	85.66 ± 0.25^{d}	26.39 ± 0.25^{e}	6.27 ± 0.25^{d}
4.44	20	89.33 ± 0.26^{b}	31.31 ± 0.22^{c}	8.38 ± 0.18^{b}
4.44	30	91.00 ± 0.25^{a}	38.28 ± 0.22^{a}	9.21 ± 0.22^{a}
4.44	40	88.33 ± 0.22^{c}	33.45 ± 0.25^{b}	7.27 ± 0.21^{c}
4.44	50	82.33 ± 0.29^{e}	29.36 ± 0.30^{d}	4.32 ± 0.23^{f}

Control: Treatment without Sodium nitroprusside.
Results represent the mean (±) standard error of three independent experiments. Mean values followed by the different letters within a column are significantly different according to Duncan's multiple range test (DMRT) at 5% level.

3. Results

3.1. Effect of SNP on shoot proliferation and rooting from half-seeds

Plant hormone similitude role of SNP has been assessed using half-seed explants of soybean. The addendum of SNP along with a BA in SIM evoked a response and increased the mean number of induced shoots (Fig. 1d and e). Lower concentration of SNP (10 μM) had limited impact on shoot induction, whereas hike in concentration developed significant results when compared to BA alone (Table 1). In the present study, the highest number of shoots/explant (38.28) were produced from a single explant at 30 μM SNP along with BA (4.44 μM) after 45 days of starter culture (Fig. 1f and g). Beyond this optimum concentration of SNP, shoot induction reduced (Table 1).

In general, the organogenic protocol of soybean explants requires separate GA_3 phase for shoot elongation (Paz et al., 2006; Arun et al., 2016). However the present protocol did not require separate treatment of GA_3 for shoot elongation, and the SIM with SNP and BA induced the elongation of shoots. The shoot induction and elongation stage have been achieved within 45 days of culture which includes 3 subcultures at 15 days interval. Hence, SNP mediated direct organogenesis did not demand a separate growth regulator for shoot elongation since SNP had the GA_3 like effects on soybean. The SNP also showed a positive influence on rooting of soybean shoots. The number of rooting shoots (12.32) were higher at 30 μM concentration of SNP along with 4.93 μM IBA and were comparatively less in the medium with IBA alone and the medium containing SNP beyond 30 μM concentration (Table 2). The well-established rooting system was observed after 30 days of culture in 30 μM SNP and 4.93 μM IBA supplemented medium (Fig. 1h). Rooted plants were acclimatized in plastic cups (Figs. 1i), and 95% of the plants adapted well and grew into healthy plants (Fig. 1j). Regeneration of soybean from half-seed explants with the aid of SNP requires only 75 days (10–11 weeks).

3.2. Effect of SNP on shoot proliferation and rooting under NaCl stress

Half-seed explants with shoot buds, displayed an inhibition of growth beyond 70 mM NaCl concentration (data not shown). Explants, grown in SIM medium containing NaCl and BA, failed to produce shoots and survive under salt stress. However the explants were cultured on SNP, NaCl and BA containing SIM medium showed tolerance up to some extent, and this tolerance level is dose-dependent (Fig. 2a–f). Experiments with 50 μM SNP revealed better resistance to salt with more number of surviving shoots (32.82) with increased rooting (Table 3). The nature of roots produced under NaCl stress was untypical compared to control roots. Application of 50 μM SNP throughout the regeneration process resulted in (7.48) acclimatized plants.

Table 2
Effect of SNP on rooting of elongated shoots from half-seed explants of soybean cv. PUSA 9712 on RM after 30 days of culture.

Treatments		Percentage of explant responding (%)	Mean number of roots per shoot (after 30 days)	Mean root length (cm)	Mean number of acclimatized plant
IBA (μM)	SNP (μM)				
4.93	0.0	81.33 ± 0.26^{e}	6.49 ± 0.23^{e}	9.35 ± 0.19^{e}	4.46 ± 0.22^{f}
4.93	10	84.00 ± 0.21^{d}	8.28 ± 0.19^{c}	10.43 ± 0.18^{d}	6.05 ± 0.19^{d}
4.93	20	90.66 ± 0.22^{b}	9.53 ± 0.27^{b}	12.41 ± 0.21^{b}	8.25 ± 0.21^{b}
4.93	30	95.33 ± 0.23^{a}	12.32 ± 0.15^{a}	14.19 ± 0.22^{a}	10.09 ± 0.22^{a}
4.93	40	88.66 ± 0.26^{c}	7.75 ± 0.23^{d}	11.17 ± 0.20^{c}	7.65 ± 0.18c^{c}
4.93	50	80.33 ± 0.18^{f}	4.12 ± 0.23^{f}	8.27 ± 0.19^{f}	5.21 ± 0.20^{e}

Control: Treatment without Sodium nitroprusside.
Results represent the mean (±) standard error of three independent experiments. Mean values followed by the different letters within a column are significantly different according to Duncan's multiple range test (DMRT) at 5% level.

Fig. 2. Effect of SNP and NaCl on shoot proliferation from half-seed explants of soybean cv. PUSA 9712. a) 4.4 μM BA, b) 4.4 μM BA and 70 mM NaCl, c) 4.4 μM BA, 70 mM NaCl and 25 μM SNP, d) 4.4 μM BA, 70 mM NaCl and 50 μM SNP, e) 4.4 μM BA, 70 mM NaCl and 75 μM SNP, f) 4.4 μM BA, 70 mM NaCl and 100 μM SNP respectively.

3.3. Effect of SNP and NaCl on growth parameters: In vivo conditions

The irrigation of plants with NaCl (C-II) has affected the various growth parameters. C-II plants displayed reduced growth and their upper trifoliate younger leaves were severely affected by salt stress when compared to C-I plants (Fig. 3a). However, the plants irrigated with SNP (C-III plants) survived under salt stress and displayed better characteristics as compared to C-II plants (Fig. 3a).

3.4. Effect of SNP and NaCl on stomatal aperture

The stomatal aperture of three categories of leaf samples were captured by laser confocal microscope (LSM 510 Meta, CarlZeiss Inc).

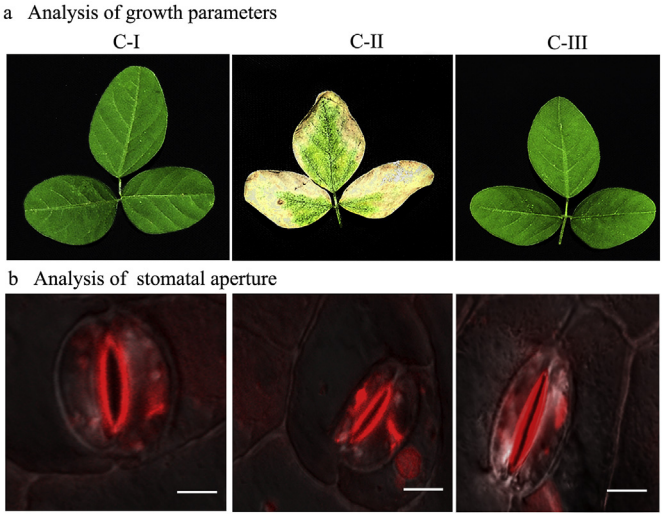

Fig. 3. Effect of SNP and NaCl on *In vivo* conditions of three different categories of plants. C-I plants: irrigation with plain water; C-II plants: irrigation in water supplemented with 70 mM NaCl; C-III plants: water supplemented with 70 mM NaCl + 50 μM SNP during irrigation for 15 days. a) Growth parameters, b) stomata aperture. Bars (C-I, CII and CIII–10 μm).

The lower epidermis layer of the leaves was observed with the detection of propidium iodide (PI) fluorescence with an excitation at 453 nm and emission range between 543 and 620 nm. The closing of the stomatal aperture is one of the adaptive mechanisms to combat the stress and adverse environmental changes. The present study observed that the non-saline conditions did not induce stomatal closing in (C-I) plants, whereas exposure to salinity conditions (C-II) plants resulted in the closure of stomatal aperture, which receded from (60.2–45.4%) (Fig. 3b). Furthermore, application of SNP (C-III) plants, lead to a

Table 3
Effect of SNP on shoot proliferation and rooting from half-seed explants of soybean cv. PUSA 9712 subjected to stress with NaCl.

Treatments		Percentage of explant responding (%)	Mean number of survived shoots[a]	Mean number of rooted plants[b]	Mean number of acclimatized plant
SNP (μM)	NaCl (mM)				
0.0	0.0	78.66 ± 0.22^{d}	15.31 ± 0.25^{e}	6.59 ± 0.23^{d}	5.65 ± 0.22^{c}
0.0	70	56.00 ± 0.23^{f}	4.47 ± 0.25^{f}	0.00 ± 0.00^{f}	0.00 ± 0.00^{f}
25	70	80.33 ± 0.18^{c}	21.48 ± 0.27^{d}	7.27 ± 0.21^{c}	6.39 ± 0.23^{b}
50	70	88.66 ± 0.26^{a}	32.82 ± 0.19^{a}	9.52 ± 0.22^{a}	7.48 ± 0.21^{a}
75	70	82.33 ± 0.29^{b}	27.17 ± 0.22^{b}	8.28 ± 0.25^{b}	4.61 ± 0.25^{d}
100	70	72.66 ± 0.25^{e}	24.25 ± 0.20^{c}	4.22 ± 0.23^{e}	2.63 ± 0.27^{e}

Control: Treatment without Sodium nitroprusside.
Results represent the mean (±) standard error of three independent experiments. Mean values followed by the different letters within a column are significantly different according to Duncan's multiple range test (DMRT) at 5% level.
[a] 4.44 μM BA supplemented along with different concentrations of SNP.
[b] 4.93 μM IBA supplemented along with different concentrations of SNP.

significant reduction in the percentage of stomatal opening (28.6%), where the stomata were forced to close in order to tolerate the salinity condition (Fig. 3b).

3.5. Effect of SNP and NaCl on Na^+ and K^+ content

High uptake of K^+ and reduction in Na^+ content is the practicable survival strategy of plants during salt stress. A higher concentration of Na^+ observed in C-II plants during salinity stress, led them to die in subsequent days (Fig. 4a). However the C-III plants maintained higher concentrations of K^+ as compared to C-II plants (Fig. 4b), and the higher K^+/Na^+ ratio alleviated salt stress, which confirmed the positive role of SNP in development of soybean.

3.6. Effect of SNP and NaCl on chlorophyll content, RWC and proline

Salt stress caused a considerable reduction in chlorophyll content of all NaCl treated plants. There was a significant loss in chlorophyll content in the salt-treated C-II plants, whereas the SNP treated C-III plants were found with less reduction in chlorophyll (Fig. 5a). Salt stress did not affect the appearance of the C-III plants, particularly the leaves appeared with usual green color. However, the leaves of C-II plants were affected by chlorosis, due to salt stress. Salinity stress led to the reduction in relative water content (RWC) of C-II leaves which ultimately affected the growth of the plant. The C-III plants treated with SNP displayed stable RWC, almost similar to that of the plants grown in control conditions (Fig. 5b). An enhancement of proline content in all salt-stressed plants are widely adopted strategy to improve salinity stress tolerance. The C-II plants showed improved accumulation of proline than (C-I) plants. SNP application increased the proline content, as compared to (C-I) and (C-II) plants, which however retained the growth of (C-III) plants (Fig. 5c).

3.7. Effect of SNP and NaCl on MDA, H_2O_2, SOD and CAT

Accumulation of MDA and H_2O_2 under salinity stress conditions increased the toxicity of plant cell which further affects survivability. Main mechanisms of antioxidant enzymes such as CAT, SOD during stress conditions were scavenging H_2O_2 and lowering lipid peroxidation, which ultimately enhanced the growth of the plant by reducing salt stress. NaCl supplement triggered the increased accumulation of MDA and H_2O_2 (Fig. 6a and b) in C-II plants, whereas the level of MDA and H_2O_2 were controlled in C-III plants by increasing SOD and CAT (Fig. 6c and d). Application of SNP caused a reduction in H_2O_2 and MDA content by increasing the activity of SOD and CAT thereby improving the survival of C-III plants under salt condition.

4. Discussion

Soybean is both nutritionally and commercially one of the most valued legumes, hence researchers all around the world are actively involved in various aspects of its improvement. Researchers are trying to update the tissue culture and genetic transformation protocols for soybean in order to improve the traits. Various novel molecules and compounds showing growth enhancing and stress tolerating characters have been excogitated to elucidate their possible role in plant metabolism due to their potential dual application in the dicots. In the present study, we have deciphered the possible role of NO in growth, development and salt stress alleviating activity in soybean.

In the present experiment, the organogenic potential of NO has been studied by supplementing SNP as NO donor. The present experiment observed that SNP functioned similar to cytokinins and improved the multiple shoot induction and proliferation in soybean. Previous studies in *Malus hupehensis* (Han et al., 2009), *Linum uitatissimum* (Kalra and Babbar, 2010), *Vanilla planifolia* (Tan et al., 2013) also agree with our results. As suggested by (Scherer and Holk, 2000), NO donors can mimic and mediate some cytokinin effects in plants and this might be a possible mechanism behind the shoot induction role of SNP. Subculture is the usual method to increase the production of shoots, however, based on the nature of explants, different concentrations of growth regulators need to be supplemented in the culture medium to induce shoot multiplication (Han et al., 2009). In soybean, the continuous subculture in the same growth regulator would result in shoot proliferation (Shan et al., 2005; Arun et al., 2014). Even though BA has

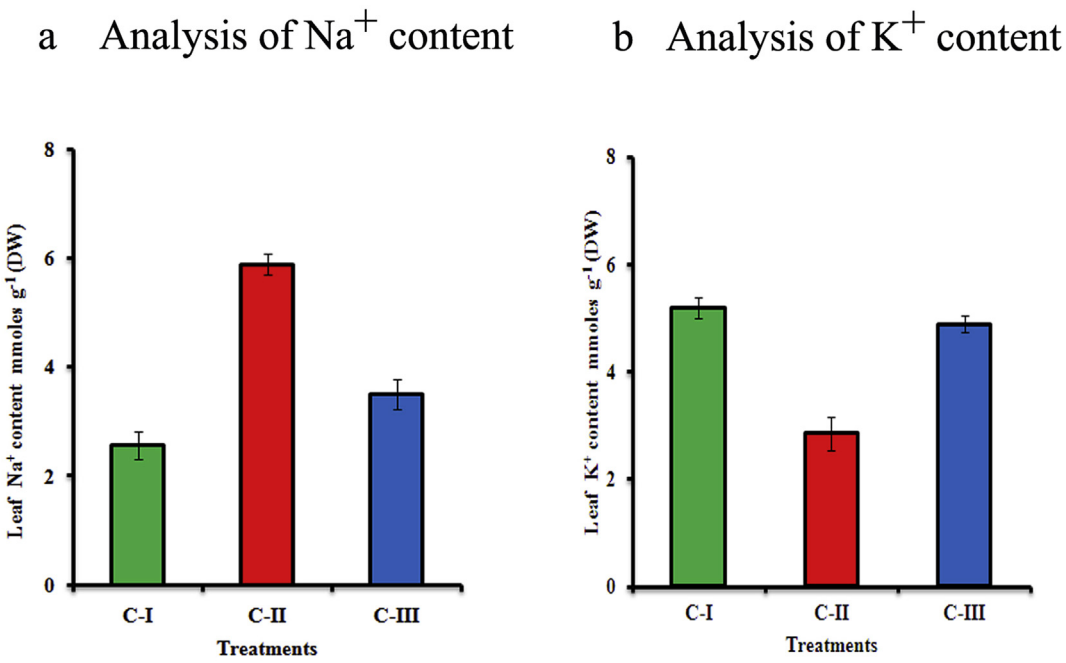

Fig. 4. Effect of SNP and NaCl on *In vivo* conditions of three different categories of plants for 15 days treatments under greenhouse condition. a) Leaf Na^+ content (mmoles g^{-1} DW), b) Leaf K^+ content (mmoles g^{-1} DW). Mean ± standard error is indicated by the bars. Means represented by a similar letter have no significant difference at $p < 0.05$.

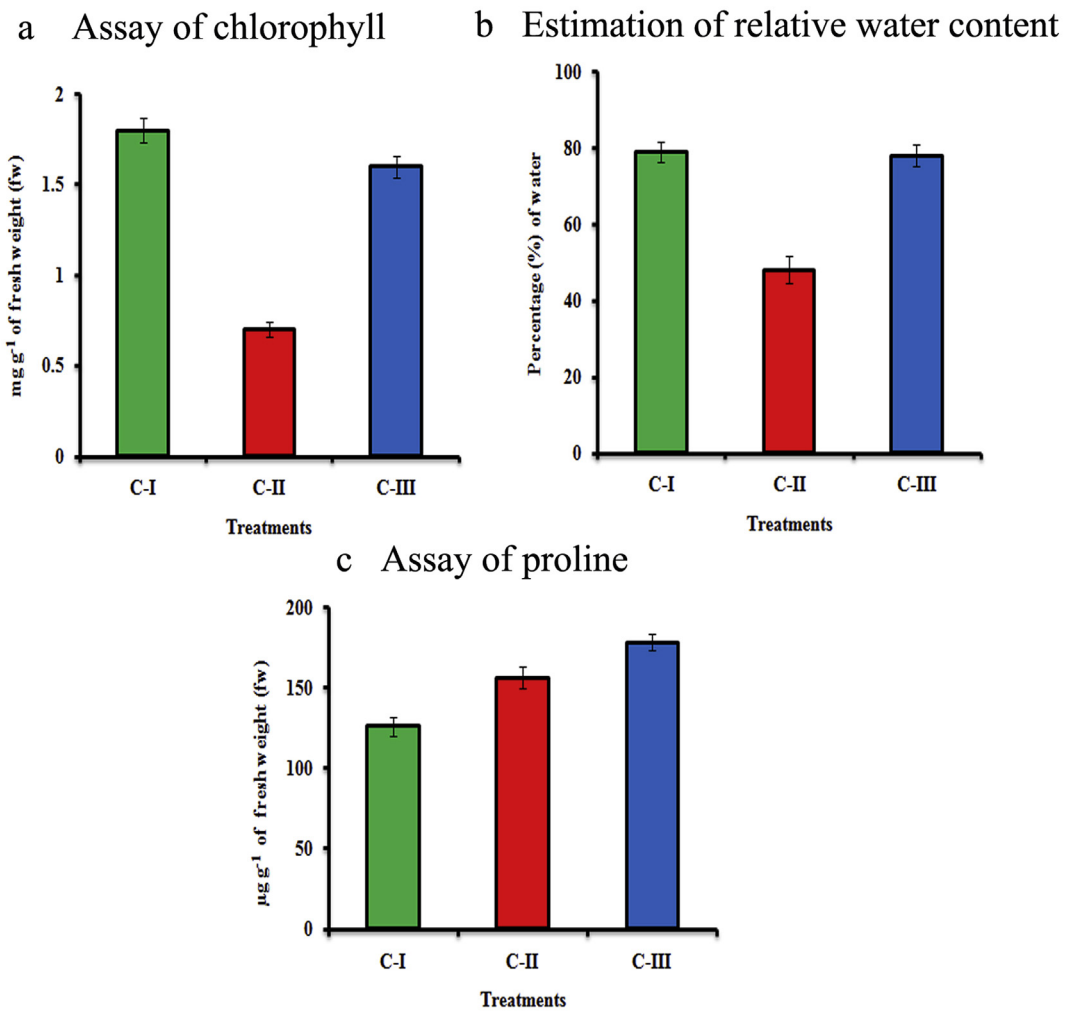

Fig. 5. Effect of SNP and NaCl on *In vivo* conditions of three different categories of plants for 15 days treatments under greenhouse condition. a) Chlorophyll concentration (mg g^{-1} of fw), b) Relative Water Content (%), c) Proline concentration (µg g^{-1} of FW). Mean ± standard error is indicated by the bars. Means represented by a similar letter have no significant difference at $p < 0.05$.

been proved to induce shoot proliferation in many plants, our experiments in soybean with the continuous subculture in BA supplemented medium resulted in rosette shoot clusters which failed to elongate further (data not shown). However, in case of SNP, the second subculture of multiple shoots resulted in shoot proliferation along with shoot elongation with thick shoots since the NO donors may permit the expansion of cell walls by acting upon lipid bilayer of the cell membrane and thus induce cell enlargement and plant growth (Sarropoulou et al., 2014). In the traditional method of half-seed regeneration (Paz et al., 2006), elongation of shoots is achieved with five different growth hormones such as GA_3, IAA, zeatin R, L-pyroglutamic acid and asparagines, which is tedious and time consuming. The present protocol requires none of those growth regulators, and the presence of BA with SNP in the culture medium is adequate for shoot elongation, which shortens the regeneration time. Rooting is one of the well-established character of SNP in various plant species such as maize (Gouvea et al., 1997), cucumber (Pagnussat et al., 2002), mungbean (Huang and She, 2003), and tomato (Correa-Aragunde et al., 2006) which are in line with our results. The root inducing role of SNP can be enhanced by involving auxin signalling pathway which resulted in lateral root emergence and root system architecture modulation (Correa-Aragunde et al., 2006). In the present study, IBA used with SNP produced thick roots with a higher number of lateral roots, when compared to IBA alone (data not shown). The influence of SNP on soybean regeneration is not limited to *in vitro* condition, it played a dynamic role during acclimatization by producing healthy and fast growing plants in *in vivo* conditions too. In the present study, shoot induction, proliferation, elongation and rooting were achieved within 75 days from the day of initiation and this is a crucial outcome amongst soybean regeneration studies, as shoot elongation is a critical bottleneck step in legume regeneration which is a time-consuming process in *in vitro* regeneration (Arun et al., 2014, 2016). The present study overcomes this impediment and established a concise *in vitro* organogenesis protocol which can be applied for genetic transformation studies of soybean. Moreover, shoots induced and elongated were healthy in appearance, suggesting that the application of SNP may help them withstand difficult genetic transformation steps, in particular during the selection of transgenic tissue and plants thereby increasing the transformation efficiency.

We have also investigated the effect of SNP as NO donor on salt stress in both *in vitro* and *in vivo* conditions. From the results of *in vitro* studies, we found that SNP improved the survival and growth of shoots into a rooted plantlet under salt stress. Even though morphological and physiological characters were affected by salinity stress, supplementation of SNP resulted in improvement of plant health. Still, the assessment of their adaptation level under salinity in field conditions is indispensable. Hence we also studied the influence of salinity stress and the stress alleviation potentiality of SNP, on soybean plants *in vivo*. Our results revealed thataugmenting 50 µM SNP with 70 mM NaCl in irrigation solution, negated the salinity stress manifested by NaCl as observed in C-III plants, in contrast to C-II plants which were irrigated

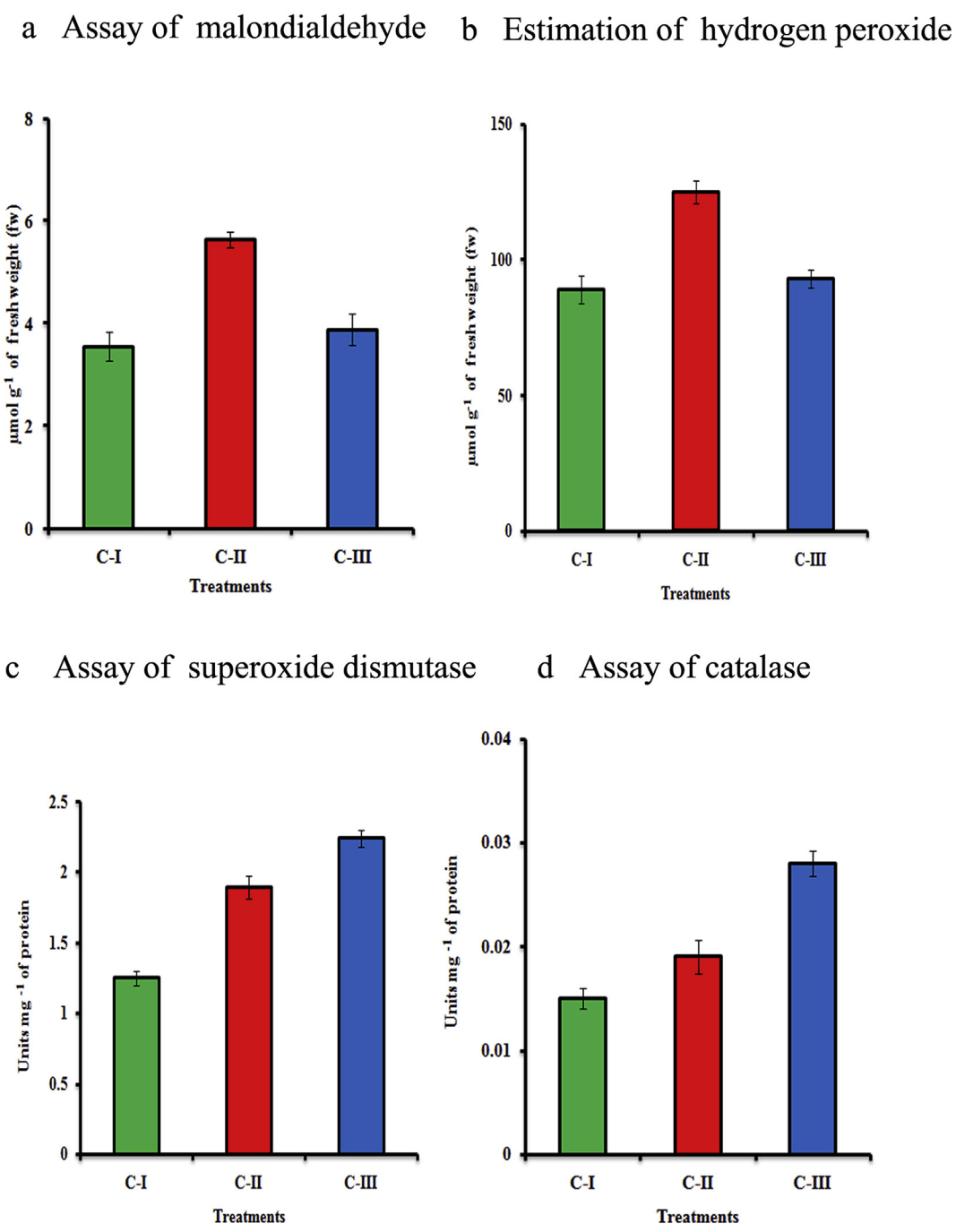

Fig. 6. Effect of SNP and NaCl on *In vivo* conditions of three different categories of plants for 15 days treatments under greenhouse condition. a) MDA content (µmol g^{-1} of FW), b) H_20_2 concentration (µmol g^{-1} of FW). c) SOD (Units mg^{-1} of Protein), d) CAT (Units mg^{-1} of Protein). Mean ± standard error is indicated by the bars. Means represented by a similar letter have no significant difference at $p < 0.05$.

only with 70 mM NaCl. In general, osmotic imbalance, oxidative stress and generation of ROS are the typical outcome of salinity stress, and SNP is believed to alleviate these conditions. Many researchers confirmed that, its mode of action might be direct interaction with H_2O_2, transcription factors, ion channels and enzymes, NO regulated gene expression of ROS scavenging enzymes and redox-based post-translational modification (Beligni and Lamattina, 1999; Qiao et al., 2014; Correa-Aragunde et al., 2015), abscisic acid-mediated stomata closure (Neill et al., 2002), inhibition of lipid peroxidation (Boveris et al., 2000) and ion balance between K^+ and Na^+ (Zheng et al., 2009). Osmotic stress has a negative effect on plant growth and ionic instability, which develop over time and affect the growth rate. The tolerant plants respond with an optimum level of tolerance to these stresses through some natural mechanisms such as ROS, ionic homeostasis and stomatal conductance etc., which facilitate the growth of the plant under salinity stress (Munns and Tester, 2008). We have examined the effect of salt on the growth rate of soybean plants and observed that the new emergence of trifoliate leaves was greatly affected in C-II plants, suggesting that leaf growth is more sensitive to salinity than root growth (Munns and Termaat, 1986). Development of new leaves in C-III plants showed their primary stage of tolerance with the aid of SNP as NO donors, which may contribute to endogenous plant growth regulatory mechanism by acting as a primary signalling molecule, which is incoherence to the observations by (Yaacov et al., 1998; Corpas and Barroso, 2015).

Regulation of stomata has a key role to play in adaptation of plants to stress and changing environmental conditions, with an active response to any stimuli (Damour et al., 2010) and its response towards stress is involved in the expression of inositol 1, 4, 5-triphosphate, cADP-Ribose (cADPR), abscisic acid (ABA), cGMP, MAPK, Ca^{2+}, LEA proteins and the involvement of NO donor in all these pathways has been proved at molecular level and confirmed the role of SNP on stomatal regulation (Garcia-Mata and Lamattina, 2001; Lamattina et al., 2003; Neill et al., 2002, 2008). In our findings, we have noted an increased stomata closure in SNP applied plant as compared to salt stressed. In the current study, the role of SNP in maintaining ion balance has been evaluated and the obtained results confirmed that the SNP stabilized the ratio of K^+ and Na^+ ions during stress condition which are in line with the result of (Zheng et al., 2009) in wheat. Changes in the K^+/Na^+ ratio during salt stress may trigger programmed cell death in organisms since the high accumulation of Na^+ is toxic to cells, therefore it is crucial in sustaining the ratio which is an important aspect of salt tolerance (Yang et al., 2014). This ionic balance has been genetically controlled by PMH^+-ATPase, VH^+-ATPase and NO, acting as second messengers in inducing the expression of these pumps to restore ionic balance. This event has already been proved by (Zhao et al., 2004) and (Wang et al., 2009) in the reed and *Arabidopsis*, respectively. Maintaining the photosynthetic rate without the loss of chlorophyll is a surviving challenge for plants under salinity. SNP upholds chlorophyll content during salt stress in C-III plants. A possible explanation might be the protective effect of NO by preserving the chloroplast membrane against ROS toxicity, NO is also reported to be involved in the chlorophyll metabolic pathway, inhibiting the degradation of chlorophyll and Rubisco (Lazalt et al., 1997; Tu et al., 2003; Lei et al., 2007). Leaf elongation is highly restricted by salt stress as a result of water loss. Thus, high RWC of the plant should be retained throughout the salt stress for the rapid recovery of the stressed plants (Munns, 2002). Application of NO during salt stress improved the RWC by inhibiting water loss and strengthens the growth rate in the present experiments which are in agreement with the results of maize (Zhang et al., 2006) and wheat (Tian and Lei, 2006). Proline, accumulating in large amounts during stress conditions, function as a source of solute for intracellular osmotic adjustments (Stewart and Lee, 1974). Thus, in the present work, improved accumulation of proline was observed in SNP supplemented C-III plants. In contrast to our results, concluded that proline accumulation is not essential for salt tolerance (Lopez-Carrion et al., 2008). In addition, SNP alone improved the tolerance against proline degradation by the improved expression of proline dehydrogenase in *Brassica rapa* plants. Withal, a predominate mechanism in alleviating salt stress is by controlling ROS network to maintain cell homeostasis. The reciprocity between ROS producing and ROS scavenging determines the characteristics of ROS signals and plays a fundamental function in the physiological process of cell during stress conditions (Mittler et al., 2004; Correa-Aragunde et al., 2015). Previous studies reported that NO could act as a signalling molecule in stress conditions and regulate the level and toxicity of ROS either through activating antioxidant enzymes SOD, APX, GPX and CAT and deactivating MDA or, through its antioxidant properties (Neill et al., 2002; Lamattina et al., 2003). The present study proved that the SNP also improves plant growth and increases salt stress tolerance by activating SOD and CAT enzyme expression and reducing the concentrations of MDA and H_2O_2. The activity of SOD is observed to be high when compared to CAT which suggested that the action of SNP pronounced more towards SOD than CAT. These results were in accordance with the results of barley, wheat, (Li et al., 2008; Zheng et al., 2009) and soybean (Vaishnav et al., 2013). The concept behind the action of SNP on ROS has been recently evidenced at the molecular level by Correa-Aragunde et al. (2015) and their study concluded an increased production of ROS due to alterations in intra and extracellular homeostasis, triggering the expression of NO which augments S-nitrosylation of Cys residues of proteins and protect them from subsequent strong and the irreversible oxidation of high ROS concentrations.

5. Conclusion

In conclusion, we found that SNP supplementation along with BA during shoot induction boosts up the regeneration phase of soybean, resulting in rapid *in vitro* regeneration. SNP not only improved the regeneration potential, but also enhanced the salt alleviating characteristics in *in vitro* plants. In addition, plants raised with the help of SNP in *in vivo* with salt stress revealed tolerance to salinity. In this study, we present the clear evidence that the SNP as NO donor could enhance the regeneration potential and salt tolerance by mimicking plant hormone character and signal molecule. Thus the versatile activity of SNP on plants may improve its application in agriculture in order to alleviate salt stress without the tedious process of genetic engineering. Yet revealing of the molecular mechanism behind its action may give hope to identify various genes and transcription factors involved in multiple environmental stresses and the expression of these genes will pave the way to develop salt-tolerant crops without plant species limitations.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101173.

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