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Effect of nitric oxide (NO) on the induction of callus and antioxidant capacity of *Hyoscyamus niger* under in vitro salt stress

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Summary

Generation of reactive oxygen species (ROS) under salt stress can cause oxidative damage. Nitric oxide (NO) is considered as a functional molecule in alleviating the oxidative damage of salinity to plants through modulating antioxidant metabolism. In the present study, effects of sodium nitroprusside (SNP), a NO donor, on induction of callus and seed germination, and antioxidant capacity of *Hyoscyamus niger* seedlings were studied under 0, 50, 100 and 150 mM NaCl stress. NO stimulated the germination of NaCl-treated seeds. NaCl treatment significantly induced accumulation of H_2O_2 and thiobarbituric acid-reactive substances (TBARS) in *Hyoscyamus niger*, and application of 100 µM SNP stimulated ROS-scavenging enzymes and increased scavenging activity (DPPH), hydroxyl radical (OH•) scavenging activity and chelating activity of ferrous ions, resulting in lower lipid peroxidation induced by NaCl stress. Therefore, it can be concluded that the increased antioxidant capacity by NO might be responsible for its function in alleviating the inhibition of *Hyoscyamus niger* growth and cell damage by salt stress. Also the effect of SNP on the induction of callus of *Hyoscyamus niger* was investigated. Callus fresh weight increased significantly in the combined effect of 50 µM SNP with other plant growth regulators (PGR) compared to control. It is evident that SNP has a direct effect on the induction of callus by interacting with cytokinins and auxins.

Key words: antioxidant capacity, callus, nitric oxide, *Hyoscyamus niger*, salinity.

Introduction

It is a well-known fact that excessive accumulation of salt ions in the soil is increasingly becoming a problem for agriculture production (Zhu, 2003; Baatour et al., 2010). The effects of salt stress on plant physiology have been well documented. Salt stress can cause ion toxicity, osmotic stress and reactive oxygen species (ROS) stress (Mittler, 2002). In plant cells, mitochondria are major sites for the generation of reactive oxygen species (ROS) in nonphotosynthetic cells (Moller, 2001; Fleury et al., 2002). As the results of oxidative stress, protein synthesis is inhibited, lipid is peroxided, enzyme is inactivated, and membrane systems are damaged (Zhu et al., 2004; Tanou et al., 2009). Therefore, the balance between free radical generation and scavenging determines the survival of plants under salt stress. To counteract the oxidative stress, plants have evolved an antioxidant system which is composed of ROS-scavenging enzymes such as catalase (CAT), superoxide dismutase (SOD), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (Jung et al., 2000). Many studies indicated that plant tolerance to salt stress was closely related with the expression and activities of these antioxidant enzymes (Zushi and Matsuzoe, 2009; Seckin et al., 2010) as well as higher antioxidant capacity due to antioxidant metabolites (Zushi and Matsuzoe, 2009). Enhancing the activity of antioxidant enzymes in plants organs is necessary for improving plant tolerance to salt stress. Therefore, increasing free radical scavenging ability by exogenous substance application could be a practical measure in the detoxification of stress-induced excessive free radical production. Recently, rapid and economically viable approaches have been proposed to alleviate the adverse effects of salt stress (Ashraf and Foolad, 2007). In a number of studies, it has been emphasized that exogenous application of elicitors, antioxidants, or plant growth regulators is a meaningful approach in inducing salt tolerance in crops (Ashraf and Foolad, 2007).

Nitric oxide (NO) is a small, highly diffusible gas and a ubiquitous bioactive molecule. Its chemical properties make nitric oxide a versatile signal molecule that functions through interactions with cellular targets via either redox or additive chemistry (Lamattina et al., 2003). NO produced by sodium nitroprusside (SNP) has recently been considered a new phytohormones (Leterrier et al., 2012) and reported to mediate the plants responses to both biotic and abiotic stresses (Crawford and Guo, 2005; Delledonne, 2005); it also plays a crucial role in regulating plant growth and development, including germination, flowering, fruit ripening and organ senescence (Arasimowicz and Floryszak-Wieczorek, 2007).

Moreover, NO is believed to be involved in two respiratory electron transport pathways in mitochondria (Yamasaki et al., 2001; Zottini et al., 2002), where it mediates the modulation of ROS and enhances antioxidant defense system in plants subjected to various abiotic stresses, such as heat (Uchida et al., 2002), iron deficiency (Sun et al., 2007), and salt and heavy metals (Singh et al., 2008). In the past few years, research on function of NO in salt stress tolerance has obtained much interest (Yang et al., 2011; Mostofa et al., 2015). However, the information available is sometimes contradictory, depending on the plant species, severity and duration of the salinity treatments (Begara-Morales et al., 2014; Manai et al., 2014).

In vitro culture of plant in the presence of high concentrations of salt provides a useful tool to study the adaptive mechanisms of plants living in adverse environments. Mechanisms preventing oxidative burst in cells exposed to high salt concentrations are crucial for cell survival. Although the antioxidant system is undoubtedly involved in the strategies used by the tissues to survive under high salt concentrations, the variation in the degree of responses has demonstrated that multiple mechanisms rather than a single mechanism which may be responsible for the adaptation of the tissues to resist salt stress.

Black henbane, *Hyoscyamus niger* L., known as a medicinal herb belongs to the family Solanaceae and is widely distributed in Europe and Asia (Sawant, 2004). The plant contains tropane alkaloids (hyoscyamine, hyoscine and scopolamine) as well as flavonol glycosides (quercitin, rutin, kaempferol), which are amongst the oldest drugs used in medicine (El Jaber-Vazdekis, 2009). Tropane alkaloids, are widely used in medicine for their mydriatic, anticholinergic, antispasmodic, analgesic and sedative properties. The seeds are used in traditional therapy for the treatment of a variety of health problems such as asthma, cough, colic, diarrhea and genitourinary complaints such as irritable bladder (Gilani, 2008). In traditional Chinese medicine the seeds of this plant are used as an antispasmodic, sedative, and analgesic agent, as well as for the treatment

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of stomach cramps, heavy coughs, neuralgia, and manic psychosis (MACY, 2002). *Hyoscyamus* species is one of the four plants used in Ayurveda for the treatment of Parkinson's disease.

In previous research, sodium nitroprusside (SNP) as a NO donor was used to counteract the effect of salt stress. However not any data are available on the effect of NO as a precursor in antioxidative responses of *Hyoscyamus niger* against *in vitro* salt stress. The aim of this work is designed to study the effects of NO on alleviation of oxidative damages induced by salt stress. Comparing these responses can be useful for understanding the physiological and biochemical mechanisms of NO in this plant that has to cope with salt stress.

Materials and methods

Experiment 1: Seed germination and salt stress

Seeds of *Hyoscyamus niger* were obtained from Pakan Bazr Company, Isfahan, Iran. Seeds surface were sterilized for 1 min with 70% ethanol and for 10 min with 10% sodium hypochlorite, then rinsed several times with sterile distilled water. Sterile seeds then were placed on half-strength MS medium (MURASHIGE and SKOOG, 1962) containing treatment of 0, 50, 100 and 150 Mm NaCl and 0, 100 and 200 µm SNP under aseptic condition. SNP ($[Na_2Fe(CN)_5]\cdot NO$) was used as NO donor. The medium was supplemented with 3% sucrose and solidified with 0.8% agar. The pH of the medium was adjusted to 5.8 before autoclaving. After four weeks some biochemical and physiological parameters were measured.

Experiment 2: Induction of Callus

Callus induction was obtained from hypocotyl explants of *Hyoscyamus niger* obtained from seedling germinated on MS medium with a combination of SNP (0 and 50 µm) and 2 concentrations (0.5 and 1 mg L^{-1}) of α 6-benzyl aminopurine (BAP), kinetin (kin), and 2,4-dichlorophenoxy acetic acid (2,4-D). Before autoclaving at 121 °C for 15 min, pH of the media was adjusted to 5.8.

Seed germination percentage

Since all swollen seeds germinated, seed germination percentage was calculated by the following formula (thus differentiating swollen from unswollen seeds):

Seed germination percentage**=** Number of seeds showing swelling of the embryo/Total number of seeds

Determination of antioxidant enzyme activities

Three hundred mg fresh tissue were ground with 3 ml ice-cold 50 mM phosphate buffer (pH 7.8) containing 0.2 mM EDTA, 2 mM ascorbate and 2% PVP. The homogenates were centrifuged at 4 °C for 20 min at 12,000×g and the resulting supernatants were used for determination of antioxidant enzyme activities.

SOD activity was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium following the method of STEWART and BEWLEY (1980). CAT activity was measured as the decline in absorbance at 240 nm due to the decrease of extinction of hydrogen peroxide (H_2O_2) according to the method of PATRA et al. (1978). POD activity was measured by the increase in absorbance at 470 nm due to guaiacol oxidation (NICKEL and CUNNINGHAM, 1969). GR activity was measured according to FOYER and HALLIWEL (1976), which depends on the rate of decrease in the absorbance of NADPH at 340 nm.

Determination of antioxidant activity

For determination of antioxidant activities, 0.3 g sample was suspended in 3 ml of serine borate buffer (100 mM Tris-HCl, 10 mM borate, 5 mM serine, and 1 mM diethylenetriaminepentacetic acid, pH 7.0). The slurry was centrifuged at 5,000×g for 10 min at 4 °C and the supernatants were used for the in vitro antioxidant assays. All samples were placed on ice during the experiments.

Radical scavenging power (RSP) of tissue extracts were assessed by the method of MANDA et al. (2010). 100 µl of the extract was added to 2.9 ml of a DPPH (1×10^{-4} M) solution, and kept for 30 min at room temperature under the darkness. The resulting color was measured at 520 nm against blanks. A decreasing intensity of purple color was related to a higher radical scavenging activity, which was calculated using the following formula:

$$\mathrm{RSP}(\%) = \left[1 - \left(\frac{A30}{B30}\right)\right] \times 100$$

Where A30 is absorbance of sample and B30 is absorbance of blank at 30 min reaction time.

Hydroxyl radical (•OH) scavenging activity was measured according to the method of MANDA et al. (2010). The reaction mixture (2.1 ml) contained 1 ml $FeSO_4$ (1.5 mM), 0.7 ml H_2O_2 (6 mM), 0.3 ml sodium salicylate (20 mM) and 100 µl of extracts of *Hyoscyamus niger* tissue. This mixture was incubated at 37 °C for 1 h, after which the absorbance of the solution was measured at 562 nm. The percentage scavenging effect was calculated as:

$$\text{Scavenging effect}(\%) = \left[1 - \left(\frac{A1 - A2}{A0}\right)\right] \times 100$$

Where A0 was the absorbance of the control (without extract) and A1 was the absorbance in the presence of the extract, A2 was the absorbance without sodium salicylate.

Fe^{2+}-chelating activity was measured according to the method of MANDA et al. (2010). The reaction mixture (2.0 ml) contained 100 µl of *Hyoscyamus niger* tissue, 100 µl $FeCl_2$ (0.6 mM), and 1.7 ml deionised water. The mixture was shaken vigorously and left at room temperature for 5 min; 100 µl of ferrozine (5 mM in methanol) were then added, mixed, and left for another 5 min to complex was measured at 562 nm against a blank. Disodium ethylenediaminetetraacetic acid (EDTA-Na_2) was used as the control.

The chelating activity of the extract for Fe2+ was calculated as:

$$\text{Chelating effect}(\%) = \left[1 - \left(\frac{A1 - A2}{A0}\right)\right] \times 100$$

Where A0 was the absorbance of the control (without extract) and A1 was the absorbance in the presence of the extract, A2 was the absorbance without ferrozine.

Determination of H_2O_2 concentration

The H_2O_2 concentration was determined according to PATTERSON et al. (1984). The assay was based on the absorbance change of the titanium peroxide complex at 415 nm. Absorbance values were quantified using standard curve generated from known concentrations of H_2O_2.

Determination of lipid peroxidation

Lipid peroxidation was determined in terms of thiobarbituric acid-reactive substances (TBARS) concentration according to the method of CAVALCANTI et al. (2004). Three hundred mg of fresh sample was homogenized in 3 ml 1.0% (w/v) TCA at 4 °C. The homogenate was centrifuged at 12,000×g for 20 min and 1 ml of the supernatant was

added to 3 ml 20% TCA containing 0.5% (w/v) thiobarbituric acid (TBA). The mixture was incubated at 95 °C for 30 min and the reaction was stopped by quickly placing in an ice bath. The cooled mixture was centrifuged at 10,000×g for 10 min, and the absorbance of the supernatant at 532 and 600 nm was read. After substracting the non-specific absorbance at 600 nm, the TBARS concentration was determined by its extinction coefficient of 155 mM^{-1} cm^{-1}.

Glycine betaine content

The amount of glycine betaine was estimated according to the method of GRIEVE and GRATTAN (1983). The plants tissues were grounded with 20 ml of deionised water for 24 h at 25 ºC. The samples were then filtered and filtrates were diluted to 1:1 with 2 N H_2SO_4. Aliquots were kept in centrifuge tubes and cooled in ice water for 1 h. Cold KI-I_2 reagent was added, and the reactants were gently stirred with a vortex mixture. The tubes were stored at 4 ºC for 16 h and then centrifuged at 10,000×g for 15 min at 4 ºC. The supernatant was carefully aspirated with a new glass tube. The periodide crystals were dissolved in 9 ml of 1,2-dichloroethane. After 2 h, the absorbance was measured at 365 nm using glycine betaine as a standard and expressed in mg g^{-1} DW.

Total soluble protein content

Total soluble protein content was measured according to BRADFORD (1976) using bovine serum albumin (BSA) as a protein standard. Fresh leaf samples (1 g) were homogenized with 4 mL Na-phosphate buffer (pH 7.2) and then centrifuged at 4 °C. Supernatants and dye were pipetting in spectrophotometer cuvettes and absorbance was measured using a spectrophotometer at 595 nm.

Statistical analysis:

The experiment was carried out as bi-factorial in a completely randomized design (4 salinity level × 3 SNP level) with 3 replications. Analysis of variance (ANOVA) for different responses parameters were evaluated by SAS 9.4 software package. Least significance difference (LSD) test was used to determine the significant difference among the mean values at the 0.05 level.

Results

In vitro seed germination: Control of dormancy release and germination of plant seeds

It was observed that the seeds started germination in the five days after placing on the medium. Complete seedlings with two leaves appeared in the 2rd week. When salinity was applied singly, seed germination percentage was low compared to control. In all levels of salinity, Medium supplemented with 100 µM SNP significantly increased seed germination (Fig. 1). Medium supplemented with SNP 100 µM in control resulted in 100% seed germination. Interaction effect of salinity* SNP showed that SNP different levels in each level of salinity was significant (Tab. 1).

H_2O_2 concentrations and lipid peroxidation

Compared to control, salt stress significantly induced accumulation of H_2O_2 in *Hyoscyamus niger* tissue ($P < 0.05$), and application of exogenous NO dramatically reduced concentration of H_2O_2 ($P < 0.05$) (Fig. 2), and effect of salt stress was blocked by addition NO in medium. Under normal conditions, exogenous NO did not significantly affect H_2O_2 in *Hyoscyamus niger* tissue.

TBARS was used to indicate lipid peroxidation, similar with the change of H_2O_2, salt stress significantly increased TBARS concentration in *Hyoscyamus niger* tissue ($P < 0.05$) (Fig. 2). In 50, 100 and 150 mM NaCl when seedlings were treated with 100 µM SNP, TBARS concentration was significantly lower than non-treated plant. In our experiment, it appears that NO has protective effect on membrane damage *Hyoscyamus niger* tissue.

Comparison of SNP different levels in each level of NaCl showed that SNP effect was statistically significant for H_2O_2 and TBARS concentration except in control (Tab. 2).

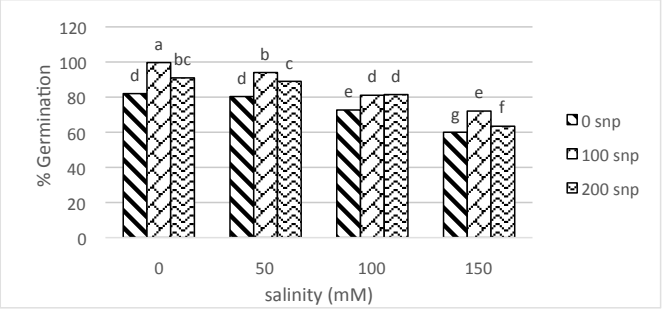

Fig. 1: Effects of SNP on seed germination of *Hyoscyamus niger* under NaCl stress. Means with different letters are significantly different ($P<0.05$).

Tab. 1: Salt*SNP effect separate by salt for germination. *, **, ***, significant at 0.05, 0.01, and 0.001 levels, respectively.

Salt (mM)	df	Mean Square
0	2	234.10***
50	2	143.44***
100	2	74.08***
150	2	115.96***

Antioxidant enzyme activities

As shown in Fig. 3, salt stress significantly increased the activities of SOD, CAT, POD and GR in *Hyoscyamus niger* than those of the control groups, which may be a reflection of the oxidative stress induced by salt stress. With increasing NaCl in the medium, POD activities increased until 100 mM and then decreased (Fig. 3). Application of SNP increased activities of all antioxidant enzymes under salt stress. Under normal conditions, activities of POD and GR antioxidant enzymes were not significantly influenced by application of exogenous SNP. Treatment of plants with SNP increased the activity of CAT and APX enzymes in control, and stress conditions.

Interaction effect of salinity* SNP showed that SNP different levels in each level of salinity was significant except for POD and GR enzymes in the control (Tab. 3).

Antioxidant capacity

DPPH scavenging capacity, •OH scavenging capacity and metal chelating capacity were usually used to express antioxidant capacity of plant tissues. Compared to the control, salt stress dramatically decreased antioxidant capacity of *Hyoscyamus niger* tissue, and reduced DPPH scavenging capacity, •OH scavenging capacity and metal chelating capacity (Fig. 4).

While application of exogenous SNP significantly alleviated the inhibition of antioxidant capacity by salt stress, and increased DPPH scavenging capacity, •OH scavenging capacity and metal chelating capacity compared to the salt stress. Under normal conditions, ap-

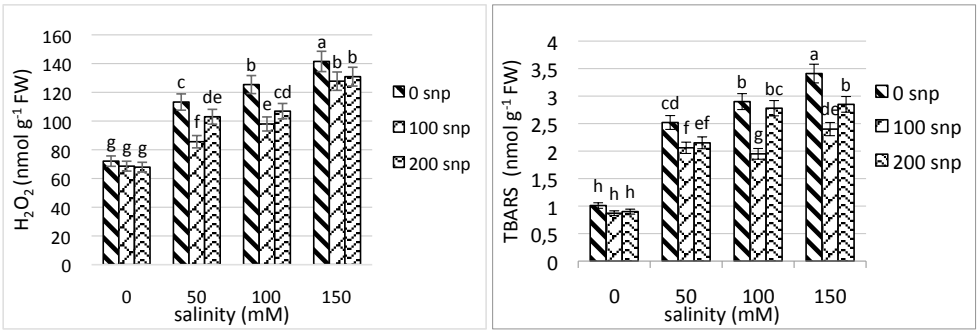

Fig. 2: Effects of SNP on the concentrations of H_2O_2 (A) and TBARS (B) of *Hyoscyamus niger* under NaCl stress. Means with different letters are significantly different (P<0.05).

Tab. 2: Salt*SNP effect by salt for H_2O_2 and TBARS concentration. *, **, ***, significant at 0.05, 0.01, and 0.001 levels, respectively. ns, non-significant.

	Mean Square		
Salt (mM)	**df**	**H_2O_2**	**TBARS**
0	2	15.19^{ns}	0.016^{ns}
50	2	584.91^{***}	0.178^{*}
100	2	587.32^{***}	1.50^{***}
150	2	155.39^{**}	0.75^{***}

Tab. 3: Salt*SNP effect by salt for POD, CAT, SOD and GR. *, **, ***, significant at 0.05, 0.01, and 0.001 levels, respectively. ns, non-significant.

Salt (mM)	**df**	**POD**	**CAT**	**SOD**	**GR**
0	2	8.38^{ns}	73.50^{**}	2918.11^{**}	0.0032^{ns}
50	2	129.53^{***}	273.79^{***}	661.44^{***}	0.036^{***}
100	2	245.29^{***}	346.77^{***}	2550.33^{***}	0.240^{***}
150	2	85.20^{**}	43.11^{***}	868.11^{**}	0.163^{***}

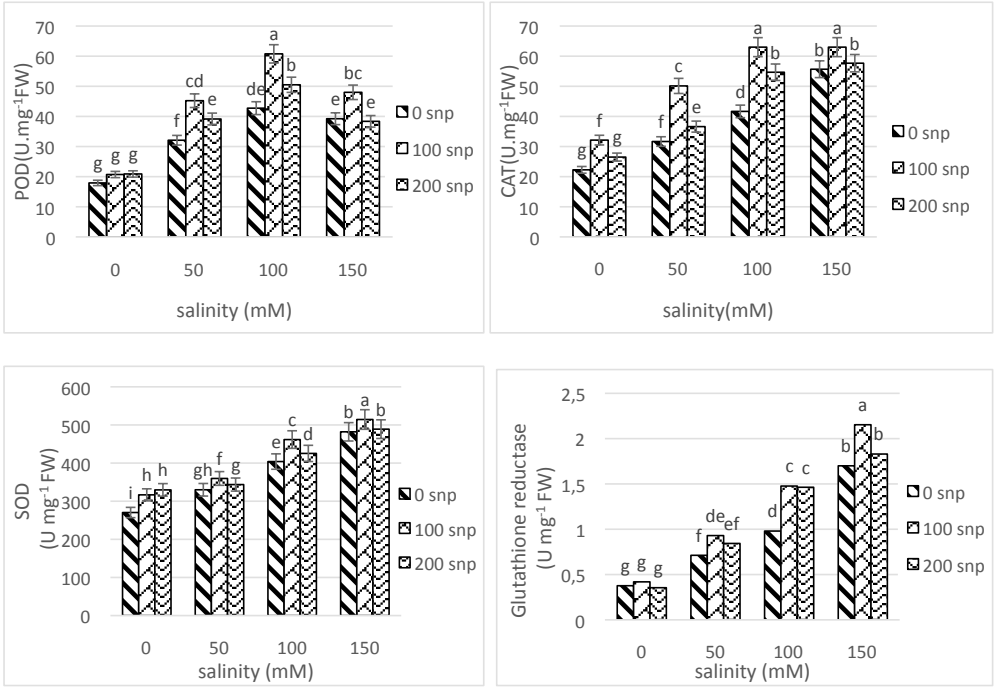

Fig. 3: Effects of SNP on the activities of SOD, CAT, POD and GR of *Hyoscyamus niger* under NaCl stress. Means with different letters are significantly different (P<0.05).

plication of exogenous. NO did not greatly change the antioxidant capacity of *Hyoscyamus niger* tissue (Fig. 4).

Comparison of SNP different levels in each level of NaCl showed that SNP effect was not statistically significant in control for DPPH scavenging and metal chelating and 150 mM NaCl for DPPH scavenging (Tab. 4).

Glycine betaine concentration

As shown in Fig. 5, salt stress significantly induced accumulation of glycine betaine in *Hyoscyamus niger* (P < 0.5), and application of SNP significantly increased glycine betaine concentration under salt stress, Like the change of other parameters, under normal conditions, glycine betaine concentrations in *Hyoscyamus niger* tissue were not

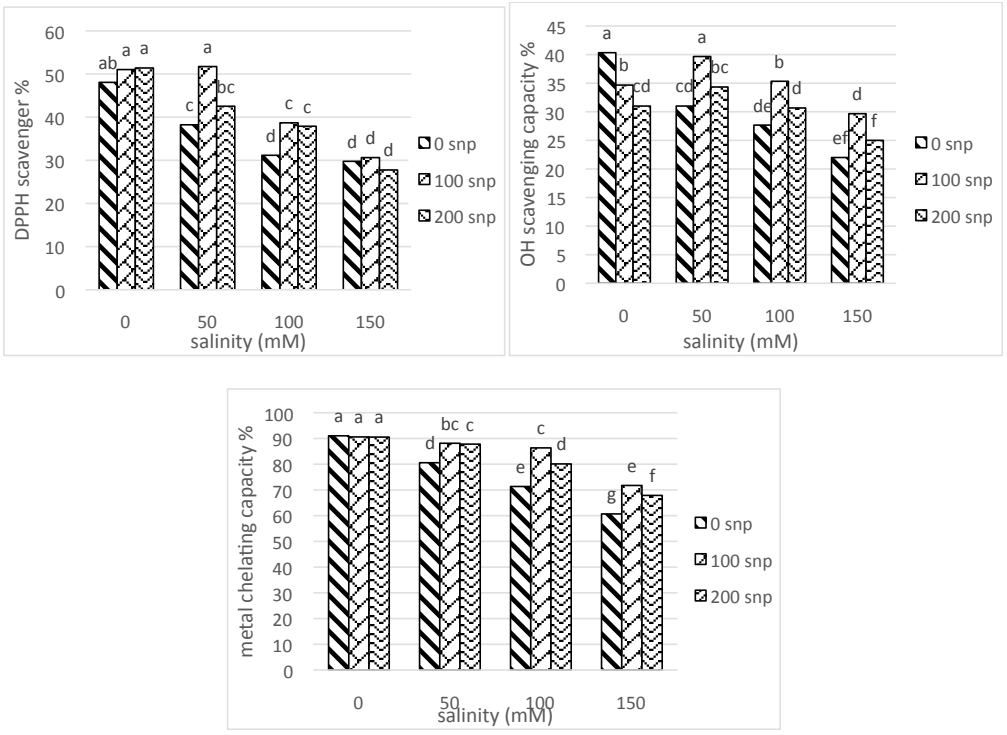

Fig. 4: Effects of SNP on DPPH scavenging activity, hydroxyl radical (•OH) scavenging activity and Fe^{2+} chelating activity of *Hyoscyamus niger* under NaCl stress. Means with different letters are significantly different (P<0.05).

Tab. 4: Salt*SNP effect by salt for DPPH scavenging, OH scavenging and OH scavenging. *, **, ***, significant at 0.05, 0.01, and 0.001 levels, respectively. ns, non-significant.

Salt (mM)	df	DPPH scavenging	OH scavenging	Metal chelating
0	2	10.023^{ns}	66.33***	0.22^{ns}
50	2	142.69***	57.33**	54.84***
100	2	51.30*	44.77**	170.35***
150	2	6.48^{ns}	44.77**	94.63***

significantly changed by application of exogenous SNP. Interaction effect of salinity* SNP showed that SNP different levels in each level of salinity was significant except in control (Tab. 5).

Total soluble proteins

Addition of NaCl caused a significant reduction in soluble proteins content. This reduction was more pronounced in 150 mM NaCl. In the lower salt concentrations (control, 50 mM), when plants were treated with 100 µM SNP, they showed higher amount of protein in comparison with their controls. 100 µM SNP was found to be most effective (Fig. 6). Interaction effect of salinity*SNP showed that SNP different levels in each level of salinity was significant (Tab. 6).

Callus induction:

The results varied with respect to plant growth regulators (PGR) and SNP (Tab. 7). Among eight callus induction media, media supplemented with 0.5 mg^{-1} 2,4-D, 0.5 mg^{-1} BAP and 50 µM SNP was the most average of callus fresh weight (Tab. 7). It was significantly higher than all other media combinations. The concentration of higher BAP and 2,4-D had a decrease in callus fresh weight. The minimum day until callus induction was recorded with media supplemented with 0.5 mg^{-1} 2,4-D, 0.5 mg^{-1} kin and 50 µM SNP (Fig. 7).

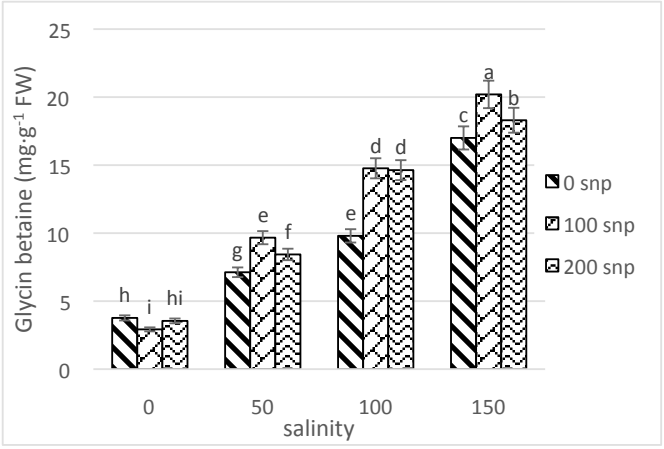

Fig. 5: Effects of SNP on Glycine betaine of *Hyoscyamus niger* under NaCl stress. Means with different letters are significantly different (P<0.05).

Tab. 5: Salt*SNP effect by salt for glycine betaine. *, **, ***; significant at 0.05, 0.01, and 0.001 levels, respectively. ns, non-significant.

Salt (mM)	df	Mean Square
0	2	0.57ns
50	2	4.85***
100	2	24.023***
150	2	7.77***

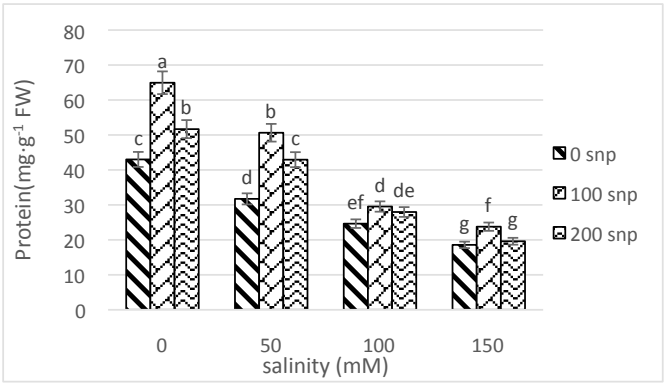

Fig. 6: Effects of SNP on Total soluble proteins of *Hyoscyamus niger* under NaCl stress. Means with different letters are significantly different (P<0.05).

Tab. 6: Salt*SNP effect by salt for total protein. *, **, ***, significant at 0.05, 0.01, and 0.001 levels, respectively. ns, non-significant.

Salt (mM)	df	Mean Square
0	2	366.093***
50	2	271.37***
100	2	18.78*
150	2	22.73*

Tab. 7: Effects of SNP and plant growth regulators on callus induction of *Hyoscyamus niger* hypocotyl. Means with different letters are significantly different (P<0.05).

PGR	SNP (µM)	Callus FW (g)	Day until induction of callus
0.5 2,4.D + 0.5 BAP	0	0.25± 0.03 d	10± 0.57 b
	50	0.54± 0.04 a	8± 0.57 c
1 2,4-D + 1 BAP	0	0.19± 0.015 ef	11.66 ± 0.57 a
	50	0.2± 0.01 ef	8 cd
0.5 2,4-D + 0.5 kin	0	0.35± 0.035 c	10.3± 0.57 b
	50	0.43± 0.04 b	7.33± 0.57 d
1 2,4-D + 1 kin	0	0.16± 0.03 f	11.66 ± 0.57 a
	50	0.23± 0.01 de	11.33± 0.57 a

Discussion

Exogenous NO has a strong stimulating effect on seed germination under stress or no-stress conditions (Beligni and Lamattina, 2000). Some studies provided evidence that NO could counteract the inhibitory effect of salinity in the process of germination (Zheng et al., 2009). In agreement with these, we observed that exogenous NO treatment significantly stimulated seed germination under severe salt stress in *Hyoscyamus niger* (Fig. 1).

In this work, SNP, as an exogenous donor, significantly reduced

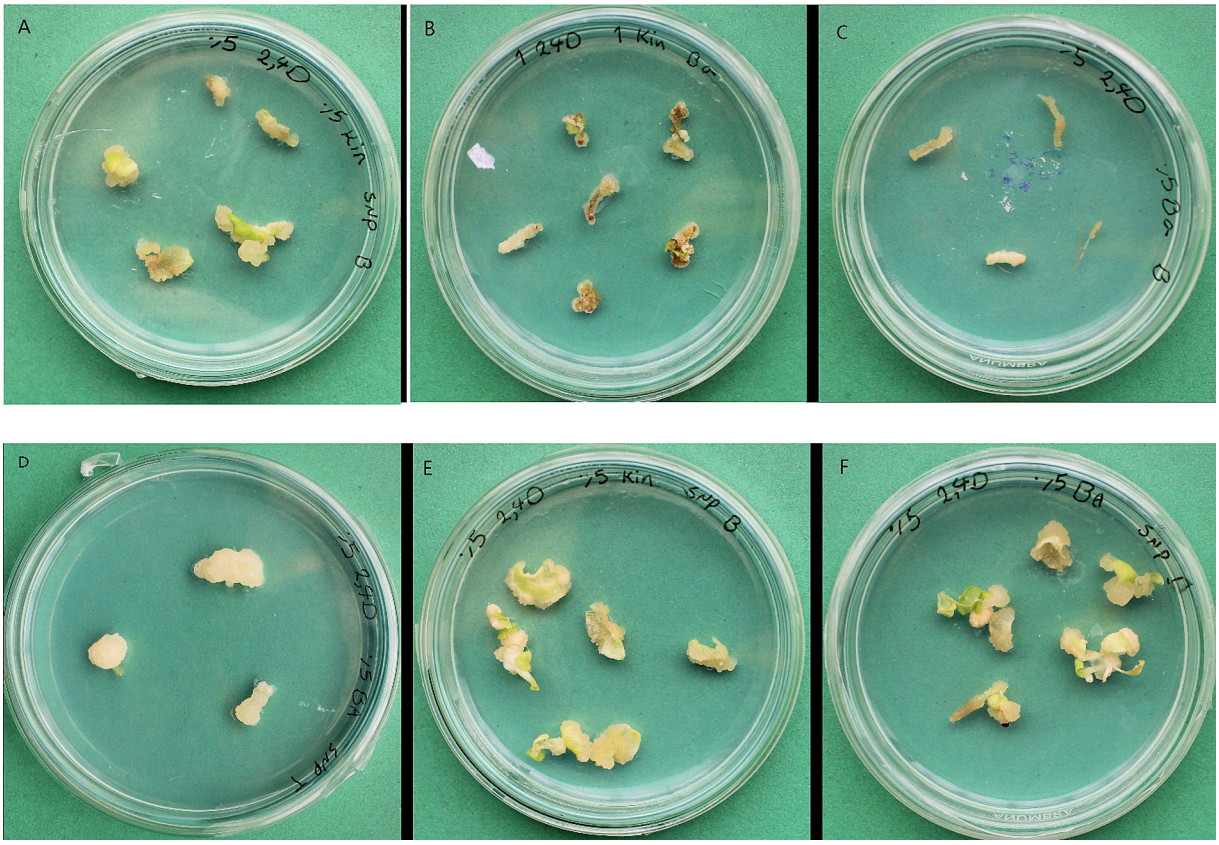

Fig. 7: Effects of SNP and plant growth regulators on callus induction of *Hyoscyamus niger* hypocotyl. **A**: 0.5 mg/l 2,4-D+ 0.5mg/l kinetin and 50 µM SNP; **B**: 1 mg/l 2,4-D + 1 mg/l kinetin; **C**: 0.5 mg/l 2,4-D+ 0.5 mg/l BAP; **D**: 0.5 mg/l 2,4-D+ 0.5 mg/l BAP and 50 µM SNP; **E**: 0.5 mg/l 2,4-D+ 0.5 mg/l kinetin and 50 µM SNP; **F**: 0.5 mg/l 2,4-D+ 0.5 mg/l BAP and 50 µM SNP.

oxidative stress in *Hyoscyamus niger* imposed by salt stress, and reduced the accumulation of H_2O_2 and TBARS accumulation. Salt-stressed *Hyoscyamus niger* plant accumulated higher levels of H_2O_2 and TBARS contents (Fig. 2), which might be due to membrane destruction caused by ROS-induced oxidative damage. Exogenous application of NO reduced levels of TBARS and H_2O_2 in NaCl-treated *Hyoscyamus niger* plants (Fig. 2), which is in agreement with the observations of ZHENG et al. (2009) and KHAN et al. (2012). Therefore, application of NO could be an effective practice to protect plants against oxidative injury caused by salt stress. NO alleviates abiotic stress through different metabolism, and antioxidant capacity modulation was reported to be one of important pathways in many investigations (HAO et al., 2009; QIAO and FAN, 2008).

The activities of SOD, CAT, POD and GR in the presence of SNP under salt stress were also higher than those under salt stress alone (Fig. 3). Exogenous application of NO increased activity of CAT, SOD, POD and APX in seashore mallow (GUO et al., 2009), mustard (ZENG et al., 2011), wheat (RUAN et al., 2002) and protected plants from oxidative damage under salt stress. In tomato, exogenous application of NO increased the activity of antioxidant enzymes SOD, POD, CAT, APX, non-enzymatic antioxidant ascorbate and reduced glutathione under salinity stress thus helping to alleviate salt-induced oxidative damage (WU et al., 2011). NO can act (i) as a direct scavenger of ROS, and (ii) antioxidant system inducer to enhance the expression of antioxidant enzyme-encoding genes (GROSS et al., 2013). NO applied exogenously may also induce the synthesis of endogenous NO (HAO et al., 2008; ZHAO et al., 2009; FAN and LIU, 2012), which can function as signaling molecule or ROS scavenger under prolonged stress conditions by regulating/enhancing the activities of antioxidant enzymes (HAO et al., 2008; FAN and LIU, 2012).

Antioxidant capacity including DPPH-radical scavenging activity, hydroxyl radical scavenging activity and the chelating activity of ferrous ions were measured for further investigation of NO function in antioxidant capacity induction of *Hyoscyamus niger* under salt stress. In the present experiment, exogenous NO reversed the inhibition of DPPH-radical scavenging activity, hydroxyl radical scavenging activity and the chelating activity of ferrous ions by salt stress in *Hyoscyamus niger* (Fig. 4), furthermore, compared to the change of antioxidant enzymes, their changing degrees were much higher related with lower accumulation of H_2O_2 and TBARS, this was accordance with the observed results in *Cakile maritima* (KSOURI et al., 2007). H_2O_2 can also lead to the production of OH• and is very reactive on important biomolecules or membranes in plants (KARUPPANAPANDIAN et al., 2011). In this study, as suggested by results in lipid peroxidation in membranes of *Hyoscyamus niger* plants, the OH• scavenging activity decreased at high-salt concentration. However, both SNP concentrations under stress conditions improved the capacity of OH• scavenging. Therefore, the enhanced scavenging ability of OH• inhibited ROS accumulation in plant, and thus plants were protected from lipid peroxidation of membrane systems and oxidative damages. Also JASID et al. (2008) reported that NO acts as an antioxidant and ROS scavenger

Increase of DPPH radical scavenging activity, hydroxyl radical scavenging activity and the chelating activity of ferrous ions in plants usually depends on antioxidant metabolites (KSOURI et al., 2007), there were reports indicated that exogenous NO could induce synthesis of these antioxidant metabolites (KSOURI et al., 2007; WU et al., 2007; FERREIRA et al., 2010), therefore, the stimulation of antioxidant capacity by NO might be attributed to induction of antioxidant metabolites synthesis and they might be greatly responsible for lower lipid peroxidation in *Hyoscyamus niger* under salt stress. To make sure the hypothesis of metabolites function in increasing antioxidant capacity induced by NO.

Osmolyte adjustment via glycine betaine accumulation has been reported to inhibit accumulation of ROS, protect photosynthetic machinery and activate stress-related genes (CHEN and MURATA, 2008). In this study, NaCl stress induced glycine betaine accumulation in *Hyoscyamus niger*, and exogenous NO increased glycine betaine level under salt stress (Fig. 5). Recently, many studies have indicated that NO appears to be involved in the metabolism of glycine betaine in stress tolerance (LÓPEZ-CARRIÓN et al., 2008).

In the present study, a similar accumulation trend of total soluble proteins was recorded in *Hyoscyamus niger* under NaCl stress (Fig. 6). Soluble proteins play a main role in osmotic adjustment under NaCl stress and can provide storage form of nitrogen (SINGH et al., 1987). Increase in soluble protein content under stress may be the result of enhanced synthesis of specific stress-related proteins (DOGANLAR et al., 2010). Thus, application of NO to salt-stressed *Hyoscyamus niger* provoked a remarkable increase in levels of total soluble proteins and glycine betaine perhaps to provide a better protection to plants exposed to stress. The protective nature of the osmolytes under NO treatment corroborates with the findings of HAYAT et al. (2012), KHAN et al. (2012), KAUSAR et al. (2013), and DONG et al. (2014) in various plants. Noticeable accumulation of glycine betaine, total soluble proteins due to NO application might enhance salt tolerance of cells through osmotic regulation.

Also, the results indicated that addition of 50 μM SNP led to increase in Callus induction compared with the control (Tab. 7). Elicitations are considered to be an important strategy towards improved *in vitro* production of secondary metabolites. Various biotic and abiotic factors added to the medium of callus production influence their production by activating genes for de novo synthesis or by stimulation the physiological processes leading to enhanced accumulation of such products. In a previous study conducted in CAB-6P, Gisela 6, and M.M 14 cherry rootstocks, the callus formation percentage was 100% in all SNP (0-50 μM) + 17.6 μM BAP + 2.68 mM NAA treatments (SARROPOULOU et al., 2014). According to XU et al. (2009), 87% callus induction frequency was obtained when *Dioscorea opposite* Thunb tuber explants were treated with 40 μM SNP. Thus, the effect of SNP on callus formation is genotype-dependent.

In conclusion, exogenous NO could significantly alleviate the growth inhibition of *Hyoscyamus niger*, and protect mitochondria and cell wall from damage under salt stress, which might depend on the higher antioxidant capacity modulated by NO.

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