Exogenous Nitric oxide (NO) induced oxidative stress and increased production of secondary metabolites in *Catharanthus roseus* Callus Cells

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Abstract:

The objective of this study was to investigate the effect of exogenous nitric oxide on cellular response and production of secondary metabolites in *Catharanthus roseus* callus. The cell suspension and callus of *C. roseus* were treated with sodium nitroprusside, and then cell viability, morphology, the amount of H_2O_2, proline, lipid peroxidation, the activity of superoxide dismutase, catalase and peroxidase were evaluated. Also production of total antioxidant, alkaloid, flavonoid and phenolic compounds were estimated. A Significant ($p<0.05$) reduction was observed in viability according to the dose of treatment. Also, the amounts fo H_2O_2, proline, total antioxidant, alkaloid, flavonoid, total phenolic compounds and the activity of the investigated enzymes increased significantly. Malondialdehyde level as a lipid peroxidation indicator was increased significantly in response to treatment. In conclusion, we may announce that the sodium nitroprusside caused cellular membrane damage due to oxidative stress, but an increase in the production of secondary metabolites was observed too. Exogenous NO treatment might be a useful way to increase production of alkaloids at cellular level.

Keywords: Callus, *Catharanthus roseus*, Nitric oxide (NO), Oxidative stress, Secondary metabolites.

Introduction:

Among the chemicals, Nitric oxide (NO) is a small lipophilic gas, relatively unstable with high emission power and a bioactive molecule which is widespread everywhere (Kalantari *et al.*, 2014). During the last decade of the 20th century it was revealed that NO in plants functions as a signal associated molecule involve in a large number of biological phenomena (Luis *et al.,* 2013). Though, it is produced by cytosolic and membrane bound nitrate reductases (Jin *et al.,* 2009), it was reported that the low concentrations of sodium nitroprusside (SNP) as a NO producing agent promotes the growth of shoot, enhances the activities of CAT, POD and SOD and declines the content of MDA (Zhang *et al.*, 2012). On the other hand, the over production or high level of exogenous NO treatment has been correlated with lipid peroxidation and rise in the activity of antioxidant enzymes (Santa-Cruz *et al.*, 2014). Various biotic and abiotic stresses such as heavy metals, temperature extremes, drought, UV radiation and SNP produce free radicals like Reactive Oxygen Species (ROS) and NO in plants which their over production are toxic for membrane system and biological macromolecules (Gill *et al.,* 2010). Free radicals such as H_2O_2, OH^-, O_2 and NO cause oxidation of unsaturated fatty acid double bounds in the membrane phospholipids which breaks the carbon skeleton and finally bring about the oxidative stress. In addition to lipids, oxidative stress induced by free radicals would damage the proteins, carbohydrates and DNA that ultimately lead to plants cell death.

In biological systems, NO rapidly reacts with oxygen and superoxide anion ($^-O_2$) which creates other reactive oxygen species such as NO_x compounds (including NO_2, N_2O_3, and N_2O_4). These compounds can react with cellular amines, thiol or hydroxyl to form the end metabolites of nitrite (NO^-_2) and nitrate (NO^-_3) (Wendehennea *et al.,* 2001). In addition the reaction of NO with $^.O_2^-$ yields peroxynitrite ($ONOO^-$) which is a powerful oxidant that mediates cellular injury (Huief *et al.*, 1993). NO$^.$ also forms complexes with transition metals found in heme- or cluster-containing proteins, that forming iron-nitrosyl complexes. This process alters the structure and function of the target proteins, as exemplified by the activation of soluble guanylate cyclase and the inhibition of aconitases (Hayat *et al.,* 2010). Induction of oxidative stress causes the elevation of secondary metabolite production (Srivastava *et al.,* 2010) as cellular responses to ameliorate the situation which is beneficial to the plants.

Catharanthus roseus is an ornamental and medicinal plant which is native and endemic to Madagascar, which belongs to the Apocynaceae family. *C. roseus* contains many different type of secondary metabolites such as alkaloids and also phenolic compounds (Mujib *et al.*, 2012) with antimicrobial, anti-fungal, anti-diabetic, anti-cancer, and anti-virus activity (Sain and Sharma 2013). The secondary metabolites are natural product of plants which have protective roles against biotic and abiotic stresses. It was previously reported

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that the free radicals induce the production of secondary metabolites in plants. Thus, in this study, we used SNP as a NO producing agent to study the cellular response and production of secondary metabolites in callus of *Catharanthus roseus*.

Materials and methods:

Plant material and Callus culture:

Seeds of *C. roseus* were purchased from Esfahan Seed Pakan Society and soaked in water for 24 hrs. Then grown in green house for 12 days. Explants (0.5-1cm^2) for callus-induction were taken from leaves of 12- days old seedlings. Explants surface was sterilized with sodium hypochlorite 5% (vol/vol) for 5 min and was washed five times with sterile distilled water, then cultured in MS (Murashige and Skoog) medium supplemented with 2,4-D 1.5 mg l^{-1}, kinetin 0.5 mg l^{-1}, sucrose 30 g l^{-1} and agar (Sigma) 7 g l^{-1} at pH=5.8. Cultures were maintained at 22 ± 3^oC under a 16 hrs. photoperiod with a photosynthetic photon flux density of 45 mol m^{-2}s^{-1} provided by cool white fluorescent lamps. After 60 days, the callus was separated from the initial explants and then sub cultured every 18 - 20 days.

Cell suspension culture: Friable calli was on MS medium without agar and were incubated on a shaker at 100 rpm and 25 °C under darkness. Samples were examined with a microscope regularly on a daily basis to ensure the formation of cell suspension. The cells were maintained on a rotary shaker at 25 ± 2 ^{0}C in the dark and sub-cultured weekly by 1:1 dilution with fresh medium. final increased cells were used for viability and morphology tests (Saifullah *et al.,* 2011).

Cell viability Assay: Performing MTT (4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, 80000 cells were cultured in 24 well plates and after treatment with SNP at concentrations of 0, 10, 50, 100, 150, 200, and 250 mmol/L for 1, 3 and 6 days the cells were collected and washed with PBS followed by addition of fresh media to the plates. Then, 10 μl of MTT solution (5 mg ml^{-1} in PBS) was added to each wells and plate was incubated for 4 hrs, in an incubator. After incubation period, to extract crystals of formazan, 100 μl of DMSO was added to each well and the plate was incubated for 30 min. The extracted solutions were transferred to another well and absorbance was taken using an automated microplate reader (SCO diagnostic, Germany) at 505 nm. The viability was reported as percentage of the viable cell (Enikeev *et al.,* 1995).

Fluorescence staining: The cells were suspended in a 24 well plates and treated with 0, 100 and 200 mM of SNP, after 3 days the nuclear morphology of the cells was studied using Hoechst 33342 (1mg ml^{-1} in PBS) following 5 min of incubation in the dark. The diameters of the cells nuclei were also measured in μm with Motic Image software (Micro optical group company, version 1.2). Hoechst is a fluorescent dye which penetrates the cells through the intact plasma membrane and stains the DNA where the changes in nuclear morphology such as chromatin condensation

and fragmentation can be investigated. The morphology of the cell cytoplasm was investigated using acridine orange solution (1mg ml^{-1} in PBS) which stains the nuclei green and the cytoplasm orange. The stained cells were washed twice with PBS, examined, and immediately photographed under an inverted fluorescence microscope (Olympus, IX70) equipped with a camera (DP72) using 40X magnification.

Proline content: The proline content was estimated by the method of Bates et al. (1973). Briefly, the callus was treated with 0, 100 and 200 mM of SNP for 3 days and homogenized in 3% aqueous sulfosalicylic acid and the homogenate was centrifuged at 10000 rpm. The supernatant was used for the estimation of the proline content and added to the reaction medium containing sulfosalicyclic acid, glacial acetic acid and ninhydrin solution. The mixture was kept at 95 °C for 60 min, and then the reaction was stopped quickly by an ice bath. Toluene was added to the mixture and the organic phase was extracted and monitored at 520 nm by spectrophotometer. The concentration of proline was calculated using standard curve.

Lipid peroxidation: Malondialdehyde (MDA) was determined as an indicator of lipid peroxidation according to Metwally et al. (2003). 0.5 g of callus treated with 0, 100 and 200 mM of SNP for 3 days was homogenized in 1 ml 0.1% trichloroacetic acid (TCA) following centrifugation at rpm 4000 for 30 min. The supernatant was treated with 0.5% (w.v) thiobarbituric acid (TBA) at 95^{0}C and cooled quickly. The absorbance was measured at 530 nm wavelength and the non-specific turbidity was corrected by subtracting A$_{600}$ from A$_{530}$ value. The concentration of MDA was calculated using the extinction coefficient (155 μM cm^{-1}) (Perveen *et al.*, 2011).

Measurement of antioxidant enzyme activities: The callus tissue which was treated with 0, 100 and 200 mM of SNP for 3 days was homogenized in 1 ml of potassium phosphate buffer, 50 mM (pH =7) containing 1 mM EDTA and then homogenate was centrifuged at 13000 rpm for 20 min at 4°C. The clear supernatant of the solution containing the enzyme extract was used to estimate the total protein content using lowery method. To determine the antioxidant enzyme activity of superoxide dismutase (SOD), guaiacol peroxidase (POD) and catalase (CAT) equal concentration of protein in the treated samples and control were used.

SOD activity was assayed according to the method of Giannopolitis and Ries (1977). The reaction mixture contained 1 mM riboflavin, 12 mM L-methionine, 0.1 mM EDTA, 50 mM Na$_2$CO$_3$, and 75 mM nitrobluetetrazolium (NBT) and 25 mM sodium phosphate buffer (pH 6.8), with 200 ml crude enzyme extract in a final volume of 3 ml. SOD activity was assayed by measuring the ability of the enzyme extract to inhibit the photochemical reduction of NBT. Glass test tubes containing the mixture were illuminated with a fluorescent lamp (120 W); identical tubes that were not illuminated served as blanks. After illumination for

15 min, the absorbance was measured at 560 nm. One unit of SOD activity was defined as the amount of enzyme activity that was able to inhibit up to 50% the photoreduction of NBT to blue formazan. The SOD activity of the extract was expressed as SOD unit min^{-1} mg^{-1} protein.

CAT activity was assayed by the method of Cakmak and Horst (Cakmak *et al.,* 1991). The reaction mixture contained crude enzyme extract, 10 mM H_2O_2 and 25 mM sodium phosphate buffer. The decrease in the absorbance at 240 nm was recorded for 1 min by spectrophotometer with the extinction coefficient of 39.4 mM^{-1}cm^{-1}. CAT activity of the extract was expressed as unit min^{-1} mg^{-1} protein.

POD activity was assayed by the oxidation of guaiacol in the presence of H_2O_2. The increase in absorbance was recorded at 436 nm (Polle *et al.*, 1994). The reaction mixture contained 50 µL of enzyme extract, 100 µL H_2O_2, 100 µL guaiacol 18 mM and 100 µL potassium phosphate buffer 100 mM (pH=7). POD activity of the extract was calculated using extinction coefficient of 26.6 mM^{-1}cm^{-1} and expressed as unit min^{-1} mg^{-1} protein.

H_2O_2 content: The hydrogen peroxide content was determined according to Sergiev (1997). 0.5 g of Callus was treated with 0, 100 and 200 mM of SNP for 3 days and homogenized in an ice bath with 1ml of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 rpm for 15 min, and 700 µl of the supernatant was added to 700 µl of 10 mM potassium phosphate buffer (pH 7.0) and 700 µl of 1 M potassium iodide (KI). The absorbance of the supernatant was measured at 390 nm in a spectrophotometer. The concentration of H_2O_2 was calculated using standard curve.

Estimation of Total alkaloids: Extraction of alkaloids was according to Ataei et al (2008) with some modification. In brief, 3 g of each treatment was abraded with 10 mL of %96 methanol in the presence of liquid nitrogen and transferred to incubator for 24 hours at temperature 25 °C. The homogenate was centrifuged at 12000 rpm for 10 min and transferred to 80 °C oven for 2 hours to reduce the volume to 1 mL, then, 10 mL of 0.5N sulphuric acid was added to the solution. Using 10N NaOH, the pH of the solution was adjusted to 10, then, 10 mL of chloroform was added, mixed and using separating funnel, the two phases consisting of acidic phase on top and choloroformic phase at bottom was made. Chloroform phase was brought to 10 ml with 96% ethanol and used for further estimation (Ataei-Azimi *et al.,* 2008).

Total alkaloids was estimated according to Srivastava and Srivastava (2010). 5 mL of the cell extract was taken and the pH was maintained at 2-2.5 with dilute HCl. 2 mL of Dragendroff Reagent (DR) was added, and the precipitate formed was centrifuged. The precipitate was further washed with alcohol and then the residue was treated with 2 mL disodium sulfide solution. The brownish black precipitate formed was centrifuged and then it was dissolved in 2 mL concentrated nitric acid and the solution was diluted to 10 mL in a standard flask with distilled water. Afterwards, 1 mL of the solution was taken and 5 mL of thiourea solution (3 % in distilled water) was added to it. The absorbance was measured at 435 nm against the blank containing nitric acid and thiourea. The concentration of total alkaloids was calculated using standard graph.

Preparation of DR.: solution of 0.8g bismuth nitrate pentahydrate in 40 mL distilled water and 10 mL glacial acetic acid was mixed with solution of 8 g potassium iodide in 20 mL distilled water.

Estimation of Total phenolic compounds: Measurements of total phenolic compounds were performed by Folin-Ciocalteu reagent method and gallic acid as standard (Madhavi *et al*., 2013). Briefly, 0.5g of callus treated with 0, 100 and 200 mM of SNP for 3 days was homogenized in 1 ml 80% methanol. After filtration, 30 µl of supernatant solution, 470 µl distilled water and 500 µl of solution 1% (w/v) Na_2CO_3 was added and mixed. After 3 min 500 µl Folin-Ciocalteu reagent was added and then tubes were incubated in dark for 10 min and absorbance was measured at 765 nm. The concentration of total phenolic compounds base on mg of gallic acid was calculated using standard curve.

Estimation of Total Flavonoids: Total flavonoids were estimated according to Marinova (Marinova *et al.*, 2005), briefly, 0.5 g of callus treated with 0, 100 and 200 mM of SNP for 3 days was extracted with 1 mL of 80% (v/v) methanol. Then, the mixtures were ultrasonicated for 20 minutes followed by centrifugation at 12,000 rpm. 50 µL of the supernatant was collected into a test tube, and 1245µl of deionized water was added. After that, 75µl of 5% (w/v) $NaNO_2$ (Sigma, USA) were added and tubes were left to react for 6 min. Then, 150 µl of 10% (w/v) $AlCl_3$ (Sigma, USA) was added and the mixture was incubated for further 5 min. Finally, 500 µL of 1M NaOH (Sigma, USA) was added and a total of 2 mL of the mixtures was transferred to a cuvette, and the absorbance values were measured using spectrophotometer (PG instrument, England) at 510 nm. The concentration of total flavonoids based on mg of catechin was estimated using standard curve.

Estimation of total antioxidant: The total antioxidant was determined using the Benzie and Strain method (Amin *et al.*, 2013). briefly, 30 µL of each enzymatic extract (PBS extract of the callus tissue treated with 0, 100 and 200 mM of SNP for 3 days) and 970 µL of deionized water was mixed, then 1.7 mL of FRAP solution (10 mL of 300 mM acetate buffer pH 3.6, 1mL of TPTZ [Sigma-Aldrich] in 40 mM HCl and 1 mL of 20 mM ferric chloride) was added to the above mixture. After 10 min of incubation in the dark, the absorption was measured using a spectrophotometer (T80^{+}, PG instrument, England) at 593 nm. The concentration of total antioxidant reported as µM mg^{-1} FW.

Statistical analysis: Data were analyzed by SPSS using one-way analysis of variance (ANOVA), Duncan test and taking P < 0.05 as the level of significant. Values are means ± standard deviation (SD) of three different experiments with at least three replications.

Results:

Effect of SNP on cell viability: Treatment of the cells with SNP for 1, 3 and 6 days showed a significant reduction in viability (p<0.05) from 10 mM upwards. The reduction of viability was observed to be time dependent, with the lowest mortality in the 1 day and highest in the 6 days of treatment with respect to each concentration. Results of MTT assay (Table 1) showed that the treatment of the cells with 100 mM of SNP caused approximately 50 percent of the callus cells to die after 3 days. In addition, 200 mM SNP caused 75 percent mortality in 3 days, respectively. Therefore, concentrations of 100 and 200 mM of SNP were chosen to carry on investigation in 3 days.

SNP induced morphological changes: Morphological study of the nuclei of the cells treated with 100 and 200 mM of SNP after 3 days showed chromatin condensation and nuclear breakage (Fig 1).

Also a highly significant reduction (p<0.001) in mean diameter of the nuclei of the cells treated with 100 and 200 mM was observed as compared to the control cells (table 2). It can be also noticed that SNP at these concentrations caused remarkable changes in the morphology of the cytoplasm (Fig 2) such as cytoplasm shrinkage and in some cells complete disappearance of the cytoplasm content compared to the control group of cells.

Effect of SNP on H_2O_2, MDA and proline content: Data analysis showed that the treatment of callus cells with different (100 and 200 mM) concentrations of SNP for 3 days caused significant increase (p<0.05) in H_2O_2 content when compared to the control group (table 3). It was also observed that the increase in H_2O_2 content was dose dependent and the content of the internal hydrogen peroxide was reached to maximum level in the group treated with 200 mM. In addition, treatment of callus cells with the same concentrations of SNP led to highly significant (p<0.001) increase of MDA content in the group treated with 200 mM of SNP when compared with other groups (table 3). Also, a significant dose dependent increase (p<0.05) in proline content of *C. roseus* callus, under SNP treatment was observed when compared to the control group (table 3).

Effect of SNP on the activity of ROS scavenger enzymes: The activity of SOD, POD and CAT increased significantly (p<0.05) in *C. roseus* callus cells after 3 days of treatment with different concentrations of SNP in a dose dependent manner (table 4).

Effect of SNP on Total phenol, flavonoids, alkaloids and antioxidant: The total phenol, flavonoid, alkaloid and antioxidant contents of *C. roseus* callus after treatment with 100 and 200 mM of SNP increased significantly (p<0.05) when compared to the control in a dose dependent manner. However there are exceptions, when total alkaloid content of the *C. roseus*, with respect to 100 mM treatment, did not differ significantly when compared with the control. On the other side, the significant increase of total phenol was not dose dependent (table 5).

Discussion:

Following SNP exposure several biochemical changes take place within the plant cells, which can lead to numerous damages in the structure and functions. The study of such changes may broaden our knowledge about the effect of this chemical on cellular response and secondary metabolite production, which is the basis to understand the cell mechanism to develop new strategies for the improvement of plant tolerance to chemical stress. For several plant species, SNP toxicity was studied using whole plants (Tan *et al.*, 2013; Kumar *et al.*, 2010) and positive correlation was found with respect to the responses of these plants and SNP stress. Cell culture method is a valuable quick and easy screening test for the evaluation of chemical toxicity in several plant species (JinFen *et al.*, 2012; Víteček *et al.*, 2007) and to study the effect of SNP (Li *et al.*, 2011) at the microscopic, physiological, and biochemical levels. Thus, the present study aims to investigate the effects of sodium nitroprusside on *C. roseus* cell biochemistry and morphology, for which limited data are available in literature.

In comparison to the control, treatment with SNP reduced the viability of *C. roseus* cells, dose dependently (analyzed with one way ANOVA) as well as time dependently (the data of two way ANOVA not shown) which demonstrated sensitivity of the species to this chemical. In addition, the morphological changes were observed in the treated cells. The reduction in viability and morphological changes were probably due to the alteration in several metabolic activities and induction of oxidative stress induced by SNP.

Cell death triggered by biotic and abiotic stresses in plants can be classified as programmed cell death (PCD) or necrosis. PCD is an active pattern of cell death which is controlled by genes and its characteristic features are cell shrinkage, chromatin condensation, DNA strand breaks and DNA fragmentation (Ma *et al.,* 2010). The morphological study of SNP treated *C. roseus* cells showed chromatin condensation and cytoplasmic shrinkage, the two main characteristic of PCD. In addition, recently, Vitecek *et al.* (2007) revealed that upon exposure to SNP, tobacco cell suspension cultures undergo PCD which might be due to oxidative stress induced by SNP.

Lipid peroxidation (LPO) has been associated with damages provoked by a variety of environmental stresses. Poly-unsaturated fatty acids (PUFA) are the main membrane lipid components susceptible to peroxidation and degradation (Bidar *et al.*, 2008). The increase in LPO can be correlated with the accumulation

Table 1. The mean percentage of viability of callus cells (*C. roseus*) after 1, 3 and 6 days of treatment with different concentrations of SNP based on MTT assay.

Time(day) / Dose(mM)	1	3	6
0	±7.80 [a] 91.22	8.77±[a] 91.40	±8.44 [a] 90.47
10	2.24 ±[ab] 86.03	3.90±[b] 79.72	±10.71 [b] 66.96
50	±4.01 [b] 77.18	7.59±[c] 65.63	±4.22 [c] 39.87
100	±2.20 [c] 62.57	6.18±[d] 48.44	±6.53 [d] 26.29
150	±9.70 [c] 53.79	6.18 ±35.04 [e]	±6.73 [e] 12.49
200	±3.95 [d] 36.25	6.75±[ef] 23.70	±2.87 [e] 9.22
250	±6.58 [d] 30.11	5.28±[f] 15.80	±1.85 [e] 5.05

Values are means ± SD. Mean different letter codes in a column are significantly different (One way ANOVA, Duncan test, p<0.05).

Fig. 1 Morphology of the cells nuclei stained with Hoechst A) controls, nuclei appeared large and no nuclear breakage was observed (arrows) B) nuclei of the cells treated with 100 mM sodium nitroprusside for 3 days. C) nuclei of the cells treated with 200 mM sodium nitroprusside for 3 days. (Magnification 40X).
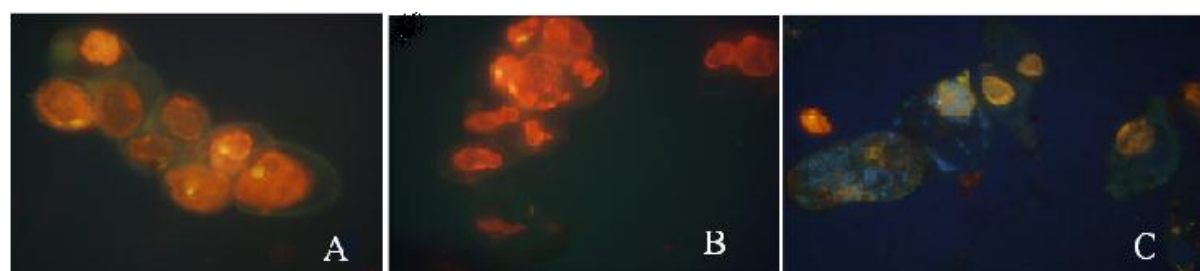
Fig. 2 Morphology of the cells cytoplasm stained with acridine orange A) controls, a distinct cytoplasm was observed and appeared round B) the cells treated with 100 mM sodium nitroprusside for 3 days. C) the cells treated with 200 mM sodium nitroprusside for 3 days. (Magnification 20X).

Table 2. Nuclei diameter of *C. roseus* callus cells after 3 days of treatment with selected concentrations of SNP.

Treatment (SNP)	nuclei diameter (μm)
0 mM	7.74 ±[a] 31.51
100 mM	5.81 ±[b] 27.32
200 mM	6.71 ±[c] 23.50

Values are means ± SD. Mean different letter codes in a column are significantly different (One way ANOVA, Duncan test, p<0.05).

Table 3. Effect of different concentrations of SNP on proline, MDA and H_2O_2 content of *C. roseus* callus cells after treatment for 3 days.

SNP (mM)	MDA ($\mu M\ g^{-1}FW$)	H_2O_2 ($\mu M\ g^{-1}FW$)	Proline ($\mu M\ g^{-1}FW$)
0	9.09 [a] ±0.79	1.05 [a] ±0.06	13.43 [a] ±1.42
100	10.22 [a]±1.26	2.16 [b] ±0.03	16.87[b]±0.93
200	15.75 [b]±2.20	3.36 [c] ±0.10	22.40[c] ±1.34

Values are means ± SD. Means with different letter code in each column differ significantly from each other (ANOVA, Duncan test, p<0.05).

of ions and production of ROS under stresses (Rucińiska-Sobkowiak, 2010). In the present study, it was shown that the treatment of cells with SNP caused significant rise of MDA as the end product of LPO and H_2O_2 which is one of the species of ROS. LPO caused disruptions of the cell membrane integrity which as a result, the internal/external balance of the cells is affected. Thus, in addition to PCD, the induction of LPO via ROS generation due to SNP might be another reason of significant reduction of viability. Also, an

Table 4. Effect of different concentrations of SNP on the activity of ROS scavenger enzymes (SOD, POD and CAT) of *C. roseus* callus cells after 3 days of treatment.

SNP (mM)	SOD ($Umin^{-1}$ mg^{-} protein)	POD (unit min^{-1} mg^{-1}protein)	CAT (unit min^{-1} mg^{-1} protein)
0	$28.75^a \pm 0.74$	$0.14^a \pm 0.11$	$0.81^a \pm 0.18$
100	$48.49^b \pm 0.41$	$0.30^b \pm 0.16$	$1.45^b \pm 0.17$
200	$54.16^c \pm 0.78$	$1.14^c \pm 0.18$	$2.27^c \pm 0.25$

Values are means ± SD. Means with different letter code in each column differ significantly from each other (ANOVA, Duncan test, $p < 0.05$).

Table 5. Effect of different concentration of SNP on total alkaloids, flavonoids, phenolic compounds and antioxidant of *C. roseus* callus cells after 3 days of treatment.

SNP (mM)	Total phenol ($mg\ g^{-1}FW$)	Alkaloids ($mg\ g^{-1}FW$)	Flavonoids ($mg\ g^{-1}$ FW)	Total antioxidant ($mol\ g^{-1}FW$)
0	$6.68^a \pm 0.50$	$6.78^a \pm 0.43$	$34.67^a \pm 0.62$	$4.20^a \pm 1.58$
100	$8.63^b \pm 0.57$	$8.46^a \pm 0.88$	$71.37^b \pm 0.37$	$10.30^b \pm 1.48$
200	$10.18^b \pm 0.34$	$13.57^b \pm 0.77$	$121.62^c \pm 0.02$	$22.29^c \pm 1.37$

Values are means ± SD. Means with different letter code in each column differ significantly from each other (ANOVA, Duncan test, $p < 0.05$).

increase in the activity of scavenging enzymes such as POD, CAT and SOD in *C. roseus* cells was found, which is in accordance with previous works studying SNP stress effects on callus tissue (Yang *et al.*, 2014) and whole plants (Yu *et al.*, 2013). The high activity of these enzymes compared to the control was probably due to an internal increase in the level of hydrogen peroxide and other ROSs. One of the most popular ROSs is $^-O_2^-$, which can be converted to H_2O_2 due to action of SOD (JinFen *et al.* 2012). The H_2O_2 is formed in the cell as a natural product of the metabolic processes (Zhou *et al.,* 2014), and CAT reduces H_2O_2 into water, therefore this enzyme is considered as a main defense with respect to H_2O_2. When the production of hydrogen peroxide is elevated as a response, the activity of CAT is increase too. In some situation, when the level of hydrogen peroxide is beyond the expected CAT activity the other line of defense, such as POD is activated. POD reduces H_2O_2 into water using ascorbate as the electron donor (Moghadam Ali *et al.*, 2013), therefore, POD protects the cell against oxidative damage by detoxifying toxic H_2O_2. An increase in POD activity under SNP stress suggests its role in the detoxification of H_2O_2 in *C. roseus* callus. Therefore, the elevation of ROS scavenging enzymes is expected due to SNP toxicity. Similar response was observed in the maize, wheat and sunflower plants with respect to SNP toxicity (Kumar *et al.,* 2010; Wang *et al.,* 2010; Kalantari *et al.*, 2014). Also, it has been observed that the ROSs interfere with protein polymerization and change in protein structure function relationship (Livanos *et al.*, 2012) which might be the main culprit of morphological changes due to significant reduction in total protein content of *C. roseus* due to SNP toxicity.

The accumulation of proline has been observed in a variety of species grown under abiotic stress and its possible involvement in adaptive mechanisms is postulated (Verbruggen N *et al.*, 2008). Proline plays an important role in osmoregulation, protects against

enzyme denaturation, acts as a reservoir of carbon and nitrogen, regulates protein synthesis as well as cytosolic acidity and/or scavenging of hydroxyl radicals (Alia *et al.*, 1991). A dose dependent proline accumulation was observed in the present study, which might be a cellular response to the SNP toxicity. In addition to aforementioned cellular responses, production of other biochemical factors have been observed, which mainly was in the form of secondary metabolite productions. *C. roseus* produce secondary metabolites such as indole alkaloids and phenolic compounds like: flavonoids, isoflavonoids, terpenoids, tannins and saponins (Ohadoma *et al.,* 2011). This plant is most famous for production of alkaloids such as vinblastine and vincristine which are well known antitumor agents (Gupta *et al.*, 2005). In the present study, we observed a significant increase in the total alkaloids content of *C. roseus* callus cells. Li *et al.,* (2011) showed that the SNP treatment of *C. roseus* increased in the concentration of serpentine, catharanthine, ajmalicine, lochnericine and tabersonine content. In addition, we observed a similar significant increase in the total phenol and flavonoids of the *C. roseus* callus cells in response to SNP treatment. Gupta *et al.,* (2013) reported that the direct addition of the NO donor SNP induced the production of PO, PPO, PAL, β-1, 3 glucanase and phenols gene in the *R. serpentina* callus (Gupta *et al.,* 2013) which are involved in production of phenolic compound and flavonoids. Our result also showed that in response to oxidative effect of SNP total antioxidant in *C. roseus* have increased significantly, which might be considered as a defense response to the toxicity. Phenolic compounds, especially flavonoids are oxidized in presence of ROSs, which results in the inhibition of these species (Michalak, 2006), therefore, accumulation of phenolic compounds in oxidative stress can prevent cellular destruction and increases the cell tolerance, therefore, overproduction of phenolic compounds can be considered as an indicator of oxidative stresses (Keilig *et al.*, 2009).

Conclusion:

We observed a strong cellular response with respect to SNP toxicity, at which the cellular content of proline, secondary metabolites such as total alkaloids, flavonoids and total phenol were increased to compensate the oxidative effect of this chemical. It was also shown that the SNP toxicity can increase the production of total alkaloids, as *C. roseus* is a good source of medicinal alkaloids; therefore, SNP treatment might be a way to increase the production of these therapeutically important alkaloids in cell and callus culture level.

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