Protective role of exogenous SNP against heavy metal toxicity in *Brassica oleracea* (var. Capitata)

Narsingh Bahadur Singh*, Himani Singh and Sunaina

Plant Physiology Laboratory, Department of Botany, University of Allahabad, Allahabad

Abstract

The present study is focused on the importance of SNP, a NO donor, as Cd stress modulator on seedlings of cabbage. The exogenously applied Cd induced stress in cabbage seedlings to explore the protective role of SNP. 21-day-old seedlings were grown in Hoagland solution and Cd at 0.5 and 0.7 mM concentration with and without SNP. The biophysical and biochemical parameters were analyzed. Cd exhibited inhibitory effects on growth and metabolism of cabbage seedlings. SNP enhanced growth viz. root and shoot length and fresh and dry weight of the seedlings. The pigment content, sugar, and protein contents decreased in seedlings treated with Cd while increased in SNP with and without Cd. The graded concentration of Cd significantly enhanced the antioxidative enzyme viz. superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) to avoid the oxidative damage caused by heavy metal stress. SNP reduced the oxidative damage and provided protection against heavy metal toxicity.

Keywords: Antioxidants; cabbage; heavy metal; oxidative stress; sodium nitro prusside

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Introduction

A variety of abiotic stress such as drought, salinity, ultraviolet light, and heavy metals cause molecular damage to plants either directly or indirectly through the reactive oxygen species (ROS). Heavy metal toxicity is one of the major environmental problems worldwide. Cd is a highly phototoxic heavy metal released into environment from industry as well as farming practices and rock mineralization (Nriagu and Pacyna, 1988). In soil concentration of Cd is very low. In plants Cd is accumulated through the roots and transported to aerial parts. In plants Cd stunts growth, causes chlorosis and epinasty, alters the ultra-structure of chloroplast, induces photosynthesis inhibition, inactivation of enzymes in CO_2 fixation, and lipid peroxidation, and disturbs nitrogen and sulphur metabolism (Gill et al*.,* 2011). Increased level of malondialdehyde (MDA) and H_2O_2 are major indicators of Cd induced oxidative stress in plant (Dixit et al*.,* 2001). The use of plants as phytoremidiators appeared as key tool in the removal of excess heavy metal from soil and water (Glass, 2000). Protective effects of NO against various stress like metal stress (Kopyra et al*.,* 2006: Hu et al*.,* 2007: Zhang et al*.,* 2008), osmotic stress (Liu *et al.,* 2007), salt stress (Uchida

*Corresponding author
E-mail address: narbahadursingh2012@gmail.com
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et al., 2002), and chilling stress (Neill et al., 2003) have been reported.

SNP alleviates the drastic effect of Cd stress in crops. NO pretreatment alleviates the toxic effects of Cd by preventing the oxidative stress development (Gill et al., 2013: Groppa et al., 2008). Exogenously applied NO minimized the toxic effects of Cd in *Oryza sativa* by increasing pectin and hemicelluloses in root cell walls (Gill et al., 2013) who reported that SNP treatment regulated stress metabolism in Cd stressed seedlings by generating NO. SNP in combined treatment with Cd protects the seedling under metal stress conditions. NO decreased ROS accumulation in roots and leaves in rice under Cd stress (Xiong et al., 2010).

The various experimental results provided evidence on the physiological and molecular mechanism of NO mediated alleviation in Cd induced stress. It promotes seed germination and seedling growth (Kopyra et al., 2003) under unfavorable conditions when plants produce ROS (Singh et al., 2012: *Neill* et al., 2003). The exposure of SNP significantly decreased the negative effects of Cd stress on plant growth (Gill et al., 2013). Nitric oxide is a bioactive signaling molecule (Duner et al., 1999) involved in a various physiological events in plants. NO is a diffusible labile gaseous free radical produced by plants via the oxidation of amino acid arginine to citrulline (Neill et al., 2003).

The nitrification or denitrification cycle provides NO as a byproduct of N_2O oxidation in the atmosphere (Gill et al., 2013). In stress conditions plants produce high level of reactive oxygen species (ROS). NO reacts with ROS and alleviates the oxidative stress in plants (Beligini and Lamattina 1999; Neill et al., 2003). Exogenous NO has been demonstrated to provide resistance against toxicity by heavy metals like copper (Yu et al., 2005) and aluminum (Wang and Yang, 2005). NO provides protection against heavy metal toxicity due to its antioxidant property. SNP mitigates stress induced by Cd in rice leaves (Hsu and Kao, 2004). The purpose of this study is to investigate the effect of NO produced from exogenous SNP on growth and metabolism of cabbage grown hydroponic culture under normal and stress conditions. The aim of study is to elucidate the physiological role of NO as an antioxidant against exposure to Cd Cl_2 at two levels, i.e., 0.5mM and 0.7mM.

Materials and Methods

Seeds of *Brassica oleracea* var. Capitata were purchased from certified seed Agency of Allahabad, Uttar Pradesh, India. Cadmium [Cd $Cl_2.H_2O$] (Molecular weight 201.32) from Lobachemie and sodium nitroprusside (SNP) (Na_2 [Fe $(CN)_5$ NO]. $2H_2O$) [sodium pentacyanonitrosyl ferrate (II)] (molecular weight 297.95g/mol) were purchased from Merck.

Hydroponic culture

The seeds were sown in January, 2013 in the nursery bed (1 m x 1 m) in the Department of Botany, University of Allahabad, Allahabad. The seed bed was irrigated as and when required. 21-day old seedlings of uniform size were transferred as 10 seedlings/box in transparent plastic boxes (height 9 cm, length 23 cm, width 17 cm) filled with 2 L half strength Hoagland solution (Hoagland and Arnon, 1950). After establishment of the seedlings in nutrient culture, the boxes were divided into six sets. In the first set, nutrient solution was replaced by fresh nutrient solution (2 L) and was taken as control. In the second set, Hoagland solution was replaced with fresh Hoagland solution (2 L) containing 200 µM SNP. In the other two sets, the solution was replaced with fresh Hoagland solution (2 L) containing graded concentration of 0.5 mM and 0.7 mM of Cd. In the remaining set, Hoagland solution was replaced with Hoagland solution (2 L) with the graded concentration of Cd with SNP (200µM). Each experimental box was aerated for 12 h a day with aerating tubes. Each box was covered with black sheet to avoid the algal growth in the nutrient medium. The experiment was done in triplicate in glass house condition. After one week of treatment the sampling was done. The first fully expanded leaves of the seedling were sampled for biochemical analyses.

Determination of pigment and protein content

The amount of photosynthetic pigment was determined as per the method of Lichtenthaler (1987). Chlorophyll of experimental plant was extracted with 80% acetone and centrifuged. Supernatant was taken and optical density was measured at 663 nm, 645nm, and 470nm.

Chlorophyll a (µg/ml) = $12.21xA_{663}$-$2.81xA_{645}$
Chlorophyll b (µg/ml) = $20.13xA_{645}$-$5.03xA_{663}$
Carotenoid (µg/ml) = [$1000xA_{470}$-3.27(Chl. a)-104(Chl. b)]/198

Where A is the observed OD

Protein content was determined according to the method by Lowary et al., (1951). Ten mg of first fully expanded fresh leaves of each plant under treatments were homogenized with 1 ml of 1 N NaOH for 5 minutes at 100 ^{o}C. Five ml of alkaline copper reagent were added to it and the mixture was allowed to stand at room temperature for 10 minutes followed by addition of 0.5 ml of Folin-Ciocalteau reagent immediately in the tube. The absorbance of the solution was measured at 650 nm after 30 minutes. The amount of protein was calculated with reference to standard curve of bovine serum albumin.

Sugar content

The quantification of total soluble sugar was done following the method by Hedge and Hofreiter (1962). About 50 mg of the sample was homogenized in 2.5 mL 95% ethanol. After centrifugation 0.1 mL of supernatant was mixed with 4 ml anthrone reagent and heated on boiling water bath. Absorbance was recorded at 620 nm after cooling. The amount of sugar was determined by the standard curve prepared from glucose.

Extraction and assay of antioxidant enzyme

Enzyme extract was prepared by homogenizing (0.25 g) leaves with 0.1 M sodium phosphate buffer (pH 7.0) containing polyvinyl pyrrolidone. The homogenate was centrifuged at 4^{o} C at 15000 g for 30 min in cooling centrifuge (Remi instruments C 24). The supernatant was used for the assay of superoxide dismutase, catalase and peroxidase.

The activity of SOD was estimated by the nitrobluetetrazolium (NBT) photochemical assay following the method of Beyer and Fridovich (1987). The reaction (4 mL) consisted of 20 mM methionine, 0.15 mM ethylene diamine-tetra acetic acid (EDTA), 0.12 mM NBT, riboflavin and 0.5 mL supernatant. Test tubes were exposed to fluorescent lamp for 30 min and identical unilluminated assay mixture served as blank. One unit of enzyme was measured as the amount of enzyme which caused 50% inhibition of NBT reduction. Enzyme activity was calculated as:

SOD units/ml = [(V-v)-1]*200 units/g FW

Where V= absorbance of respective reference and v = absorbance of respective test.

Catalase activity (EC1.11.1.6) was assayed following Cakmak and Marschner (1992). Assay mixture contained 25 mM potassium phosphate buffer (pH 7.0), 2 mL, 10 mM H_2O_2 and 0.5 mL enzyme extract. The rate of H_2O_2 decomposition for 1 min was monitored at 240 nm and calculated using extinction coefficient of 39.4 mM^{-1} cm^{-1} and expressed as enzyme unit g^{-1} FW. One unit of catalase was determined as the amount of enzyme required to oxidize 1 µM H_2O_2 min^{-1}.

Enzyme activity was calculated as:

Activity FW/min. = 250*10X/3

Where X is the observed OD.

POX activities were assayed following McCune and Galston (1989). Reaction mixture contained 2 mL enzyme extract, 2 mL sodium phosphate buffer, 1 Ml 0.1 N pyrogallol and 0.2 mL 0.02% H_2O_2 and determined spectrophotometrically at 430 nm. One unit of enzyme activity was defined as the amount which produced increase of 0.1 OD per minute. Enzyme activity was calculated as:

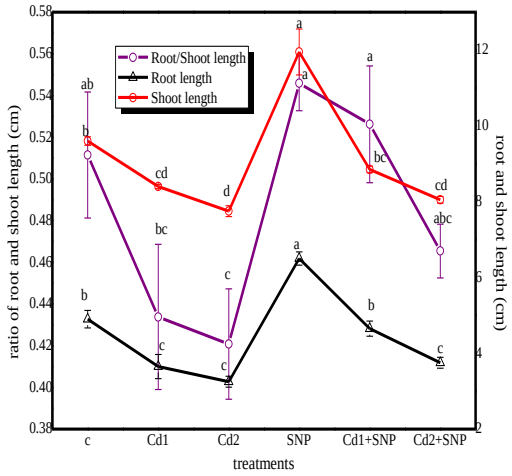
Total activity/g FW /min= 10X*25

Where X is the observed OD.

Statistical analysis

Treatments were arranged in a randomized block design with three replications. Data were statistically analyzed using analysis of variance (ANOVA) by using SPSS software (Version 16 SPSS Inc., Chicago, IL, USA). Appropriate standard error of means was calculated for presentation with tables and graphs. The treatment means were analyzed by Duncan's multiple range test (DMRT) at $P < 0.05$.

Results



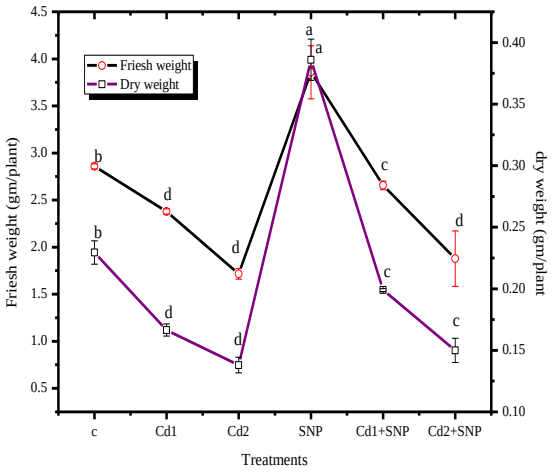
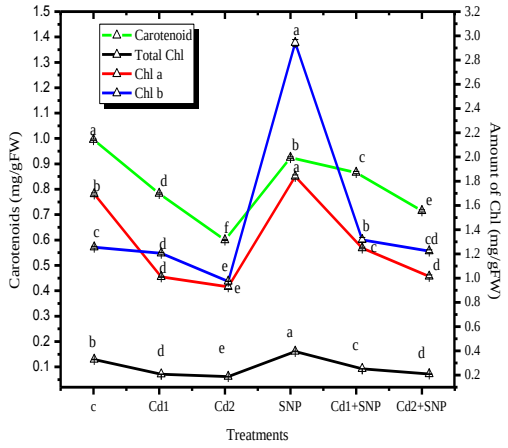


Fig. I: Ameliorating effects of SNP on root, shoot length and root shoot ratio and fresh and dry weight of *Brassica oleracea* var. Capitata against heavy metal stress; Data are mean of three replicates ± SEM. a p<0.001 versus C. C, control; Cd1, 0.5 mM. Cd2, 0.7 mM and SNP, 200 µM concentrations, respectively.



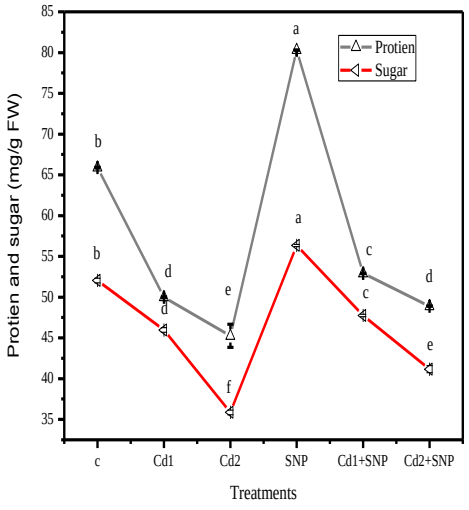


Fig. II: Ameliorating effects of SNP on Photosynthetic pigments, protein, and sugar content of *Brassica oleracea* var. Capitata against heavy metal stress; Data are mean of three replicates ± SEM. a p<0.001 versus C. C, control; Cd1, 0.5 mM. Cd2, 0.7 mM and SNP, 200µM concentrations respectively.

The results pertaining effects of SNP on shoot length (SL) and root length (RL), fresh weight, and dry weight of cabbage seedling were observed under Cd stress. SL and RL decreased in dose dependent manner with maximum decrease of 19 and 27% were recorded in highest concentration of Cd (0.7 mM), respectively. SNP helped in detoxifying Cd to such an extent that SL and RL increased to maximum 3.7 and 1.2 fold as compared with control. Increase in SL and RL corresponded to the both concentrations of Cd (0.5 mM) and (0.7 mM) with SNP caused elevation when compared to Cd treatment alone. The

decrease in root and shoot growth corresponded to increased concentration of Cd. SNP enhanced root and shoot growth. In combined treatments (Cd + SNP) both root and shoot growths were better in comparison to that of Cd treatments. DW decreased significantly ($p<0.05$) under Cd stress in comparison to control with maximum decrease under 0.7 mM concentration, SNP enhanced DW of cabbage (Fig. I)

Cd affected the pigment content of cabbage seedlings. A considerable loss of chlorophyll under the influence of Cd with a maximum 35% decrease was recorded in Cd at concentration 0.7 mM. The amount of chlorophyll increased to 38% in SNP. Carotenoid exhibited greater loss due to Cd with maximum 39% decrease in the highest concentration. Carotenoid followed the trend of chlorophyll. The pigment content decreased due to stress caused by Cd (Fig. II).

Cd treatment significantly declined the protein content in cabbage seedling. The decline in protein content was dose dependent. Higher amount of protein in SNP treatment was recorded as compared with control. Protein content increased in Cd+SNP in comparison to that of Cd. Fig. (II) data showed sugar and protein content decreased in response to Cd. Sugar content of cabbage seedling decreased under the influence of Cd with maximum 34% in 0.7 mM concentration as compared to control. Sugar content increased remarkably in SNP. SNP alleviated the effect of Cd by increasing the sugar content of seedling in combined treatment. Sugar content in Cd (0.7 mM) + SNP treatment recorded an increase over treatment with Cd at 0.7 mM concentration, but always lower than that of control.

. Our results indicated that exposure of cabbage seedlings to Cd with and without SNP significantly affected the antioxidant enzyme system (Fig. III). In presence of Cd the activity of antioxidant enzyme enhanced. The activities of SOD, CAT and POX increased to maximum 12, 63 and 45% respectively in 0.7mM concentration of Cd. However application of exogenous SNP decreased the activity of SOD, CAT and POX. The reductions are 3, 33 and 20% in SOD, CAT and POX enzyme as compared with control.

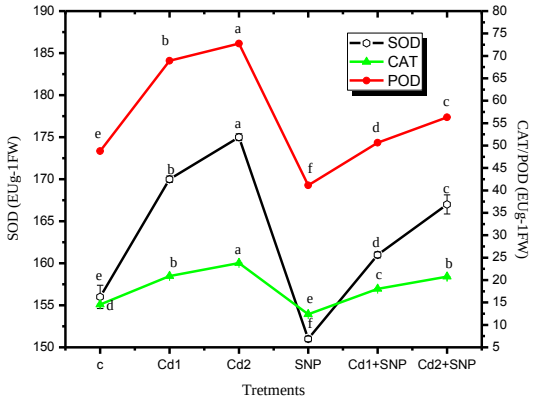


Fig. III: Ameliorating effects of SNP on antioxidant enzyme SOD: superoxide dismutase, CAT: catalase and POD: peroxidase activity of *Brassica oleracea* var. Capitata against heavy metal stress. Data are mean of three replicates ± SEM. a $p<0.001$ versus C. C, control; Cd1, 0.5mM. Cd2, 0.7mM and SNP, 200µM concentrations respectively.

Discussion

Environment stress can adversely affect the growth and metabolism of the plants. Plants have a complex antioxidant defense mechanism to avoid the adverse effect of ROS. The selected doses of Cd have toxic effect on seedlings of cabbage and the application of SNP alleviates the Cd stress. The growth of the seedlings decreased significantly as the concentration of Cd increased. Singh et al. (2003) also found decreasing in growth parameters of *Vignaradiata* under heavy metal stress. Growth was significantly reduced by Cd treatment and partially restored by NO pretreatment (Laspina et al., 2005). Results show inhibition of photosynthesis leading to reduced rate of chlorophyll content which retarded plant growth. According to Caia et al. (2011) pretreatment with SNP reduced loss of chlorophyll in soybean. Increase in chlorophyll content in pea by NO is also reported by Leshemy*et al.,* (1997). Our present study showed that Cd decreased sugar and protein content (Fig II). The inhibition of protein and sugar contents might be due to oxidative damage. Detrimental effect of heavy metals was reported in the carbon metabolism of barley and maize seedling (Stiborova et al., 1987). NO elevated pigments content to restore normal protein and sugar content (Zhang et al, 2008). Total soluble sugar content decreased in stressed leaves with photosynthetic inhibition or

stimulation of respiration (John et al., 2008). To prevent the oxidative damage plant developed a machinery of antioxidants. In the present study antioxidative enzymes like SOD, CAT, and POD increased in Cd stress. The increased activities of SOD and CAT were observed in various plants like tomato (Macias et al., 2002), cucumber (Romero et al., 2005), and mustard (Oracaz et al., 2007). Our result also has similarity with Gao et al., (2010) who found that antioxidant activity increases in *S. nigrum* with elevated Cd concentration. NO exhibits antioxidant properties (Karplus et al., 1991). There are reports that the toxicity of PQ parquet as herbicide diminished by NO in rice leaves (Hung et al., 2002). NO declines in activity of antioxidant enzymes such a SOD, and POX in PQ treated rice and thus decreases the breakdown of protein by ROS. SOD scavenges the highly reactive free radicals (O_2) by converting them into H_2O_2. The toxic H_2O_2 was detoxifying by CAT and POX activities. The antioxidative enzyme system provides the better survival of plants under stressful condition (Mishra et al., 2006).

Conclusion

The present study showed that Cd toxicity induced oxidative stressed condition in cabbage seedlings. NO appears to mitigate the effect of Cd and buttress antioxidant resistance system in the presence of Cd. NO supplementation to stress treatment is reported to quench ROS, reduce oxidative damage, and provide protection against cellular injury.

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