

## EXOGENOUS SODIUM NITROPRUSSIDE INCREASES ANTIOXIDATIVE POTENTIAL AND GRAIN YIELD OF BREAD WHEAT EXPOSED TO CADMIUM

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### Abstract

The cadmium accumulation declines the crop productivity by disturbing the metabolism of plant. Thus, it is very essential to know the process of Cd effects through Cd-induced physio-biochemical determinations as well as its accretion, carrying and the associations with development, mineral nutrients and the antioxidant systems. The experiments were conducted under different Cd (control, 0.3, 0.6, 0.9 and 1.2 mM) regimes without or with an exogenous NO donor, sodium nitroprusside (0.15 and 0.30 mM SNP) on four cultivars of wheat (Punjab-2011, AARI-2011, Millat-2011 and Sehar-2006). The exogenous SNP was effective in improving growth of Cd-stressed wheat plants. Cd stress caused a noticeable repression in flavanoids, total phenolics, soluble proteins, and grain yield components while increased total free ascorbic acid (AsA), proline, glycinebetain (GB), glucose and sucrose, malondialdehyde (MDA), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and the activity of enzymes including catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD) and peroxidase (POD) irrespective of wheat cultivars. More effective of both SNP levels was 0.30 mM. Of wheat cultivars, Punjab-2011 and AARI-2011 performed better than Millat-2011 and Sehar-2006. The exogenous NO was effective in improving total flavanoids, total phenolics, soluble proteins, and grain yield components under Cd stress. Furthermore, NO reversed the effects of Cd on reducing and non-reducing sugars, proline, total free amino acids, H<sub>2</sub>O<sub>2</sub>, MDA and enzyme activity of APX, CAT and POD irrespective of wheat cultivars. Overall, the results elaborated that exogenously applied NO reduced the Cd toxicity in wheat.

**Key words:** Metal toxicity, Antioxidants, Soil remediation, SNP.

### Introduction

The soil pollution is a serious environmental issue due to its unfavourable biological impacts. Due to their widespread occurrence, acute and toxic properties, heavy metals are recognized as the most destructive soil pollutants in agricultural soils (Jamal *et al.*, 2002). Naturally, in the soil, it is found in trace amounts but there are some reports which indicate that high amounts of Cd are present in some soil environments (Arshad *et al.*, 2016). In the soil, Cd has relatively high mobility and even at low quantity exerts toxic effects on biota (Anwer *et al.*, 2017; Sun *et al.*, 2018). Elevated Cd levels in the plants are responsible for reactive oxygen species (ROS) generation (Shekhawat *et al.*, 2008). Chaoui *et al.*, (1997) described that Cd generates ROS through the process of autoxidation. There is an antioxidant system in plants which provide protection from the damage caused by oxidative stress and to hunt these ROS (Noman *et al.*, 2018). Lozano-Rodriguez *et al.*, (1997) described that Cd increases the activities of CAT, APX and SOD enzymes which are involved to hunt the ROS. Enzymes give additional defense under oxidative stress (Ali *et al.*, 2018). The enhancement in the action of these enzymes provides tolerance to plants to face stressful conditions. The decline in the SOD and CAT activity was found under Cd exposure (Sandalio *et al.*, 2001). In shoot, the CAT and SOD activity reduced significantly while in the roots of pea a total failure of these enzymes activity was noticed (Dey *et al.*, 2007). In contrast, increase in the Cd concentration enhanced the activity of SOD in wheat plant (Amirjani, 2012). Various reports have demonstrated strong protective functions of APX, glutathione reductase, glutathione, POD, AsA and carotenoids against Cd toxicity (Tiryakioglu *et al.*, 2006; Noman *et al.*, 2015).

The NO is found involved in promoting germination of seed or breaking dormancy of seed (Ali *et al.*, 2017). It is reportedly found involved in the maturation, inhibition of floral transition, light-mediated greening and mediation of stomatal movement and induces tolerance against different stresses (Mishina *et al.*, 2007; Floryszak-Wieczorek *et al.*, 2007). According to Zhao *et al.*, (2007), NO production is altered (promote or suppress) by both biotic and antibiotic stresses, but it enhances tolerance in plants to specific stresses when applied exogenously.

SNP is the donor of NO and either act as a cytotoxin or a cytoprotectant. According to Beligni & Lamattina (1999), NO acts as an antioxidant or potent oxidant which is dependent on its concentration and severity of the environments. Normally, plant metabolism perturbs due to relatively high dose of NO. Yamasaki (2000) reported that in the plant cell, NO has the capability to damage nucleic acids, proteins and membranes. Hill & Bennett (1970) described that in the leaves of alfalfa and oat, NO decreased photosynthesis. In carrot cell suspensions, the respiration can also be reduced by NO (Zottini *et al.*, 2002). NO may interrelate with other signalling molecules (Wendehenne *et al.*, 2005). For instance, in establishing stress resistance responses NO alleviated the harmful effects of ROS. According to Kopyra & Gwozdz (2003), it not only acts as a signalling molecule but may also function as a regulator for gene expression. It was hypothesized that foliar application of SNP may modulate the Cd toxicity in wheat. Therefore, the aim of current study was to observe the morphological, physiological and biochemical responses of wheat cultivars to exogenously supplied NO under Cd stress. We also determined the optimum level of exogenous NO for reducing the unfavourable effects of Cd on development of wheat plants.

## Materials and Methods

**Experimental design and treatments:** The experiments were laid out in a completely randomized design with four replications under natural environmental conditions (Temperature 23-24°C, Humidity 59%). To fill the plastic pots, 10 kg washed and dried sand was used. With half strength Hoagland's nutrient solution including different CdCl<sub>2</sub> concentrations i.e., 0, 0.3, 0.6, 0.9, 1.2 mM Cd, the sand in each pot was saturated. In each pot ten seeds were sown. After every 15 d, Cd solution of required concentration was applied in excess for maintaining the required level by leaching the solution from the base of pots. The foliar spray of SNP (0, 0.15 and 0.30 mM) was applied at the heading stage. The data for various physio-biochemical parameters was collected after 15 d of foliar treatment.

**Determination of antioxidant activities:** The fresh leaves homogenized in phosphate buffer were used for antioxidant enzymes assay. The APX (Cakmak (1994), CAT (Aebi 1984), SOD (Giannopolitis & Ries (1977), and POD (Chance & Maehly, 1955) activities were determined and expressed as enzymatic units per milli gram protein. The total soluble proteins were determined following the method of Bradford (Bradford, 1976).

**Determination of AsA, total phenolics and flavonoids contents:** Fresh leaves were used for the determination of AsA, total phenolics and total flavonoids. The concentration of AsA was determined by using the method of Mukherjee and Choudhuri (1983). By using Folin-Ciocalteu method (Julkunen-Titto, 1985), the contents of total phenolics were determined. To determine total flavonoids, standard procedure as reported by Zhishen *et al.*, (1999) was followed.

**Determination of tocopherols contents:** Backer *et al.*, (1980) technique was applied to assess the tocopherol contents. A fresh leaf tissue of 0.5 g was homogenized in a mixture of 10 mL of petroleum ether and ethanol. The 1 mL supernatant which was obtained after centrifugation was assorted with 0.2 mL of two percent 2, 2-dipyridyl in ethanol, vortexed and kept the sample in the dark for five minutes. Then it was diluted with four millilitre of distilled H<sub>2</sub>O and assorted well. In the aqueous layer, the resulting colour was measured at 520 nm. A standard graph was made with a known amount of  $\alpha$ -tocopherol and the tocopherol content was calculated.

**Determination of sugars:** For the estimation of sugars (glucose, sucrose and total sugars), the procedure of Riazi *et al.*, (1985) was followed. The 1 g leaves material was ground in 10 mL of 80% ethanol (v/v). For glucose contents, 5 mL of O-tolidine reagent was added in 1 mL of extract and heated at 97°C. After cooling, the resulting colour was read at 630 nm by using a spectrophotometer. For sucrose contents, in 0.1 mL ethanol extract, 0.1 mL of 5.4 N KOH was added. The mixture was heated at 97°C for 10 min, cooled and added 3 mL of anthrone reagent. After heating at 97°C for 5 min and cooling, the absorbance was noted at 620 nm.

**Determination of total free amino acids:** The fresh plant material (0.5 g) was extracted in 0.2 M phosphate buffer

having pH 7.0. A water bath was used to heat the flasks with sample mixture for 30 min. After heating, the volume of each flask was maintained with distilled water up to 50 mL. By using spectrophotometer, the optical density was measured at 570 nm (Hamilton & Van-Slyke, 1943).

**Estimation of H<sub>2</sub>O<sub>2</sub> and MDA concentration:** The method of Velikova *et al.*, (2000) was applied for the estimation of H<sub>2</sub>O<sub>2</sub> concentration. Leaf sample (0.25 g) was grinded in 5 ml TCA. After centrifugation at 12000 g, the absorbance of the supernatant was noted at 390 nm. The concentration of MDA in terms of lipid peroxidation was determined as described earlier (Dhindsa *et al.*, 1981). The calculation was done by using the following formula: MDA contents (nmol/g fresh weight) = (A<sub>532</sub> - A<sub>600</sub>)/1.56 × 10<sup>5</sup> (extinction coefficient)

**Estimation of proline and GB concentration:** The Bates *et al.*, (1973) method was used for the estimation of proline. A standard curve was used for the calculation of proline concentration and the proline was calculated on fresh weight basis. For GB determination, leaf sample having 1.0 g fresh weight was homogenised in 10 mL of distilled water. The 1 mL supernatant was mixed with 1 mL of HCl (2 N). The concentration of GB was estimated at 365 nm by following the process of Grieve & Gratan (1983). A standard curve was used to determine the concentrations of GB.

**Estimation of NO contents:** The method of Ding *et al.*, (1988) and Hu *et al.*, (2003) was applied to estimate the NO content. The fresh leaf sample (0.6 g) was extracted. The pellet was washed with extraction buffer and centrifuged. Then 0.1 g charcoal was added in the two supernatants, vortexed and filtered. The one millilitre filtrate was incubated with one millilitre Greiss reagent for 30 min and absorbance was noted at 540 nm. The NO content was calculated from a standard curve using NaNO<sub>2</sub>.

**Yield parameters:** Yield parameters were estimated at maturity. For this, fertile and non-fertile tillers were counted and after separating the spikes from the plants, its number of spikelets spike<sup>-1</sup>, number of grains spike<sup>-1</sup>, grain yield/plant (g), 100-grains weight (g) and spike length (cm) was estimated.

## Statistical analysis

The data collected was pooled and subjected to analysis of variance technique (ANOVA) by using the GLM module of CoSTAT version 6.2 (CoHort Software, Monterey, CA, USA).

## Results

**Activities of antioxidant enzymes:** In all studied wheat cultivars experiencing root zone Cd stress, a considerable enhancement was observed in antioxidant enzyme activity. Cd-induced increments in APX, CAT, SOD and POD activity that was higher in millat-2011 as compare to other cultivars. Both levels of exogenously applied SNP decreased APX, CAT, SOD and POD activities but more decrease was found at 0.30 mM (Fig. 1A, B, C, D). As compared to other cultivars, Punjab-2011 showed less APX, CAT, SOD and POD activities.

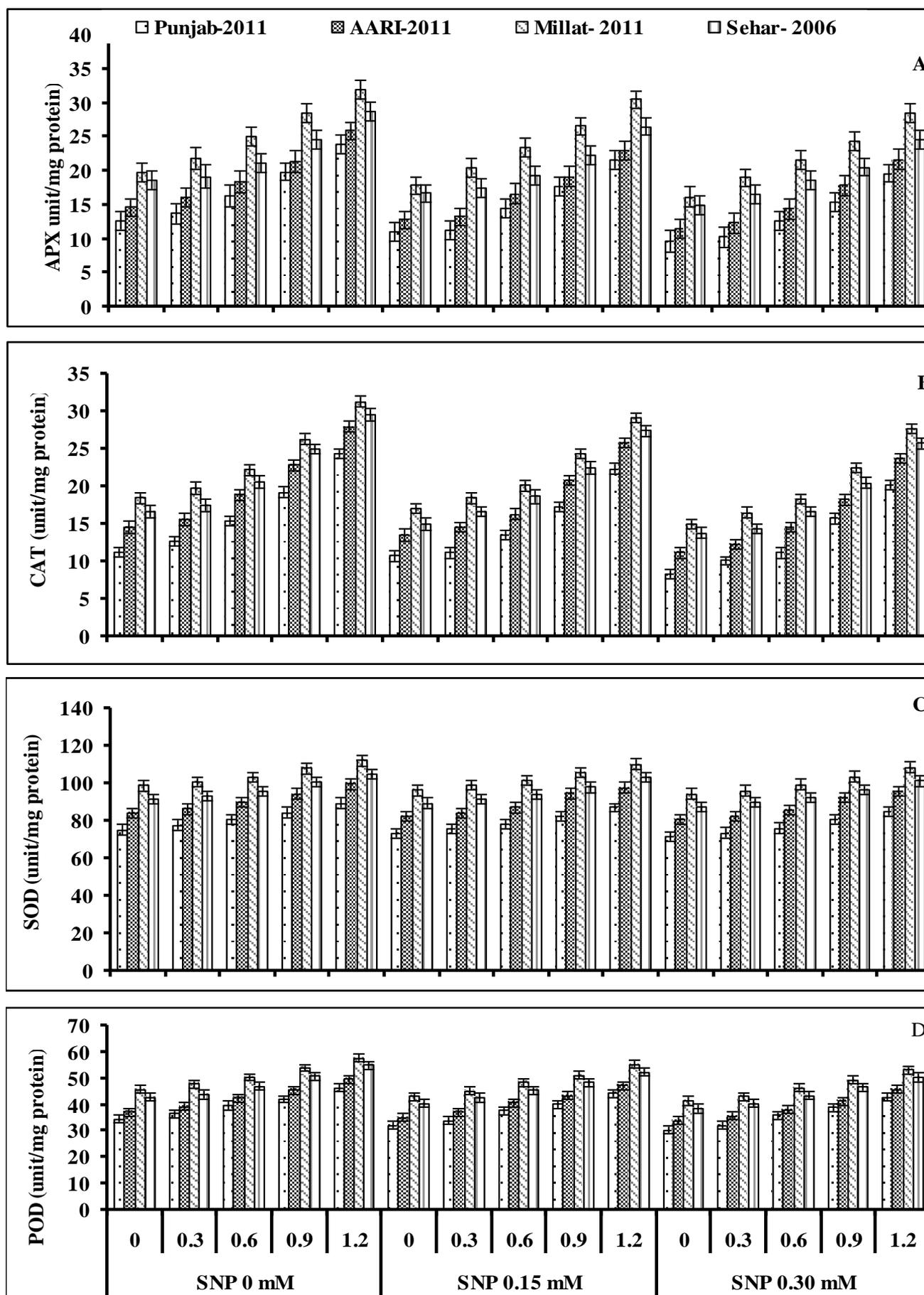


Fig. 1. Effect of exogenous sodium nitroprusside (SNP) on the APX, CAT, SOD and POD contents of bread wheat exposed to cadmium stress (0, 0.3, 0.6, 0.9, 1.2 mM).

Imposition of Cd stress significantly increased ASA and total phenolics contents in all wheat cultivars. But with exogenous application of SNP, AsA and total phenolics were decreased (Fig. 2A, B). In cultivars millat-2011 and Punjab-2011 respectively, more increase in AsA and phenolics was found. Least increase in AsA and phenolics was observed in Punjab-2011 and millat -2011, respectively.

Total flavanoids and tocopherols content declined significantly in all wheat cultivars experiencing root zone Cd levels. Foliarly applied SNP increased total flavanoids and tocopherols content (Fig. 2C, D). As compared to other cultivars, Millat-2011 showed more decrease and Punjab-2011 showed less decrease in total flavanoids and tocopherols content.

Wheat cultivars showed considerable enhancement in leaf MDA and H<sub>2</sub>O<sub>2</sub> contents under root zone Cd levels. The exogenous application of SNP decreased leaf MDA and H<sub>2</sub>O<sub>2</sub> contents (Fig. 3A, B). Punjab 2011 showed more reduction while Millat-2011 showed less reduction in leaf MDA and H<sub>2</sub>O<sub>2</sub> contents.

Cultivars varied significantly as far as total amino acids are concerned. Total free amino acids level significantly incremented while decrease in total soluble protein was observed due to the imposition of Cd stress. The SNP application decreased total free amino acids and increased total soluble protein (Fig. 3D, C). As compared to other cultivars, less increase in total free amino acids was found in Punjab-2011 and more in total soluble proteins.

GB and leaf proline concentration increased in wheat cultivars under root zone Cd regimes. Cultivars also presented significant differences with respect to this attribute. The exogenously applied SNP reduced GB and leaf proline concentration (Fig. 4A, B). As compared to other cultivars, Punjab-2011 showed minimum increase in GB and leaf proline concentration while more increase was found in millat-2011.

Glucose and sucrose increased significantly in wheat cultivars facing root zone Cd. The exogenous application of SNP decreased glucose and sucrose (Fig. 4C, D). As compared to other cultivars, Punjab-2011 showed less increase in glucose and sucrose and millat-2011 showed more increase.

**Yield attributes:** Root zone Cd decreased fertile tillers and non-fertile tillers significantly in wheat cultivars. The exogenous application of SNP increased fertile tillers and non-fertile tillers as compared with untreated control under different Cd levels (Fig. 5A, B). As compared to other cultivars, Millat-2011 showed more decrease in fertile tillers and non-fertile tillers.

Root zone Cd application significantly curtailed the spikelets/spike and grains/spike in wheat cultivars. The SNP exogenous application increased spikelets/spike and grains/spike (Fig. 5C, D). Punjab-2011 showed less decrease in spikelets/spike and grains/spike attributes.

Significant decrease in grain yield/plant and 100-grain weight was observed under root zone Cd regimes in wheat cultivars. The exogenously applied SNP increased grain yield/plant and 100-grain weight (Fig. 6A, B). Millat-2011 and sehar-2006 showed more decrease in grain yield/plant and 100-grain weight as compared to other cultivars.

Spike length of wheat cultivars also decreased significantly under root zone Cd regimes. The exogenous application of SNP increased spike length (Fig. 6C). As compared to other cultivars, Punjab-2011 and ARRI-2011 showed less decrease in spike length, respectively.

Leaf NO increased significantly in wheat cultivars under root zone Cd. Cultivars presented variation in their response with respect to this attribute. The exogenous application of SNP increased leaf NO (Fig. 6D). As compared to other cultivars, Punjab-2011 showed less decrease and millat-2011 showed more decrease in leaf NO content.

## Discussion

In plants, there is present non-enzymatic and enzymatic system to handle with oxidative stress (Noman *et al.*, 2018). In the current work, significant increased was found in APX, POD, SOD and CAT activities. NO application reversed the Cd-induced increases in the activity of these enzymes possibly by mitigating the Cd toxicity. Our observations are aligned with the results of Vanacker *et al.*, (1998) and Asada (1994). The modulated activity of APX and CAT in SNP-treated plants was due to elimination of H<sub>2</sub>O<sub>2</sub>. It is previously reported that the APX enzyme reduces the accretion of toxic levels of H<sub>2</sub>O<sub>2</sub> (Panda *et al.*, 2011). During Cd stress, APX activity increased but application of SNP managed generation of ROS and oxidative stress. This stress management by exogenous supply of SNP proposes its direct correlation with antioxidants. In the current work, the exogenous application of NO reduced the accretion of Cd in the shoots of wheat and enhanced the accretion of Cd in the roots. The results suggested that NO increased Cd uptake in the roots and decreased its transfer to the shoots that enhanced the Cd tolerance in wheat. This mechanism is worthy of further investigations.

Abiotic stress changed synthesis of some proteins (Ericson & Alfinito, 1984; Noman *et al.*, 2015). John *et al.*, (2008) mentioned a decrease in the proteins in *Lemna polyrrhiza* L. due to Cd stress which corresponded to our study. It is estimated that heavy metals are involved in the fragmentation of proteins and lipid peroxidation as a result of toxic effects of ROS, which could cause decline in the protein contents. In contrast, NO enhanced soluble proteins contents that were decreased due to Cd stress. To reduce Cd toxic effects on peanut growth, the exogenous application of NO enhanced the antioxidant ability which was accompanied by the improvements in proteins contents.

Cd stress decreased proline contents while NO application enhanced its endogenous concentrations. In peanut, NO enhanced antioxidant ability and helped in resuming the growth (Chandrashekar & Sandhyarani, 1996), and can also act as an antioxidant. In the current study, proline accumulation is ascribed to the application of NO and the interaction of NO with Cd that improved the Cd resistance in wheat. Therefore, we suggest that NO application can affect proline biosynthesis. Simaei *et al.*, (2011) recommended that proline is capable of scavenge hydroxyl radicals. The protective role of NO possibly is due to its regulatory effects on the endogenous proline contents.

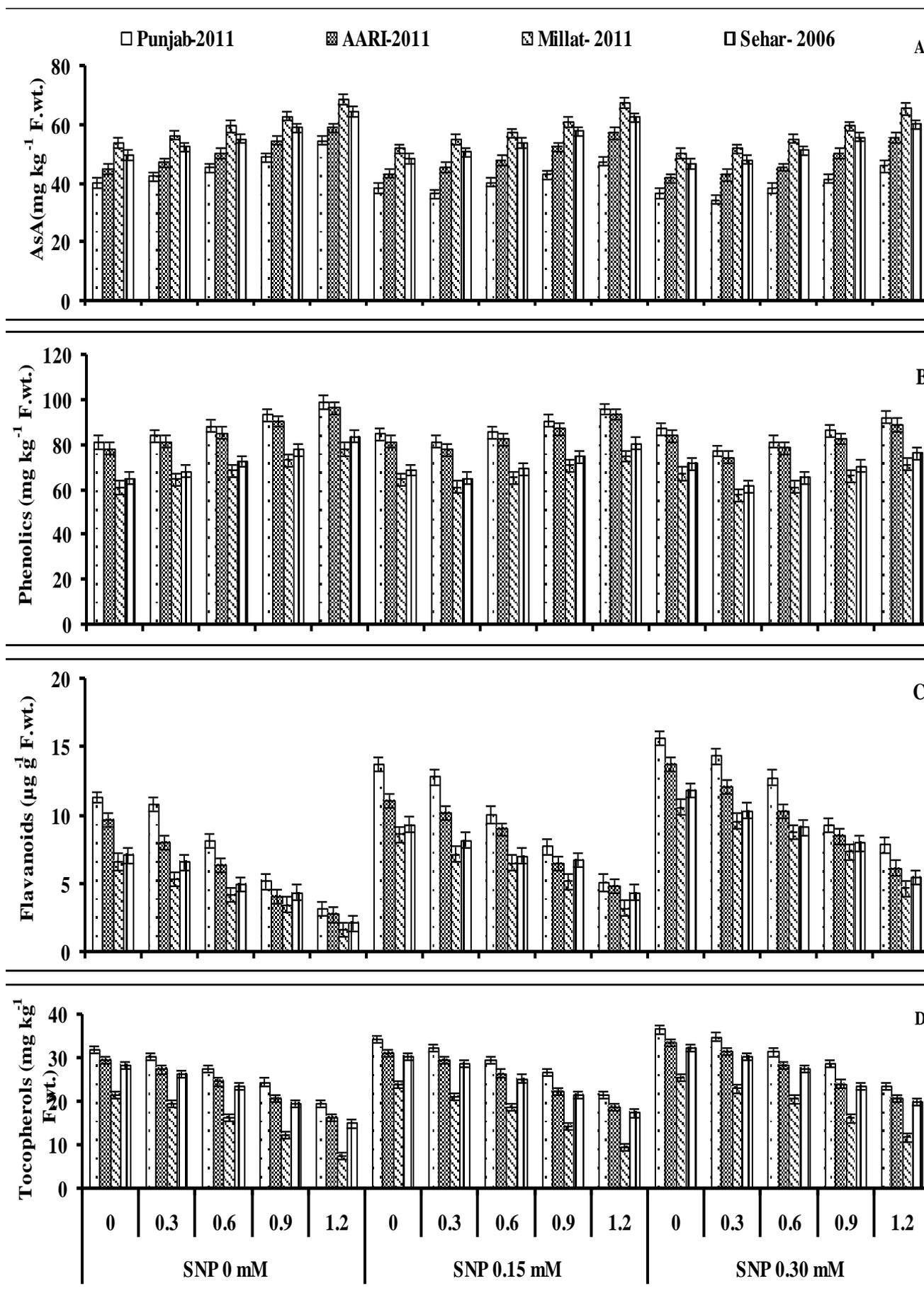


Fig. 2. Effect of exogenous sodium nitroprusside (SNP) on the ASA, phenolics, flavanoids and tocopherols contents of bread wheat exposed to cadmium stress (0, 0.3, 0.6, 0.9, 1.2 mM).

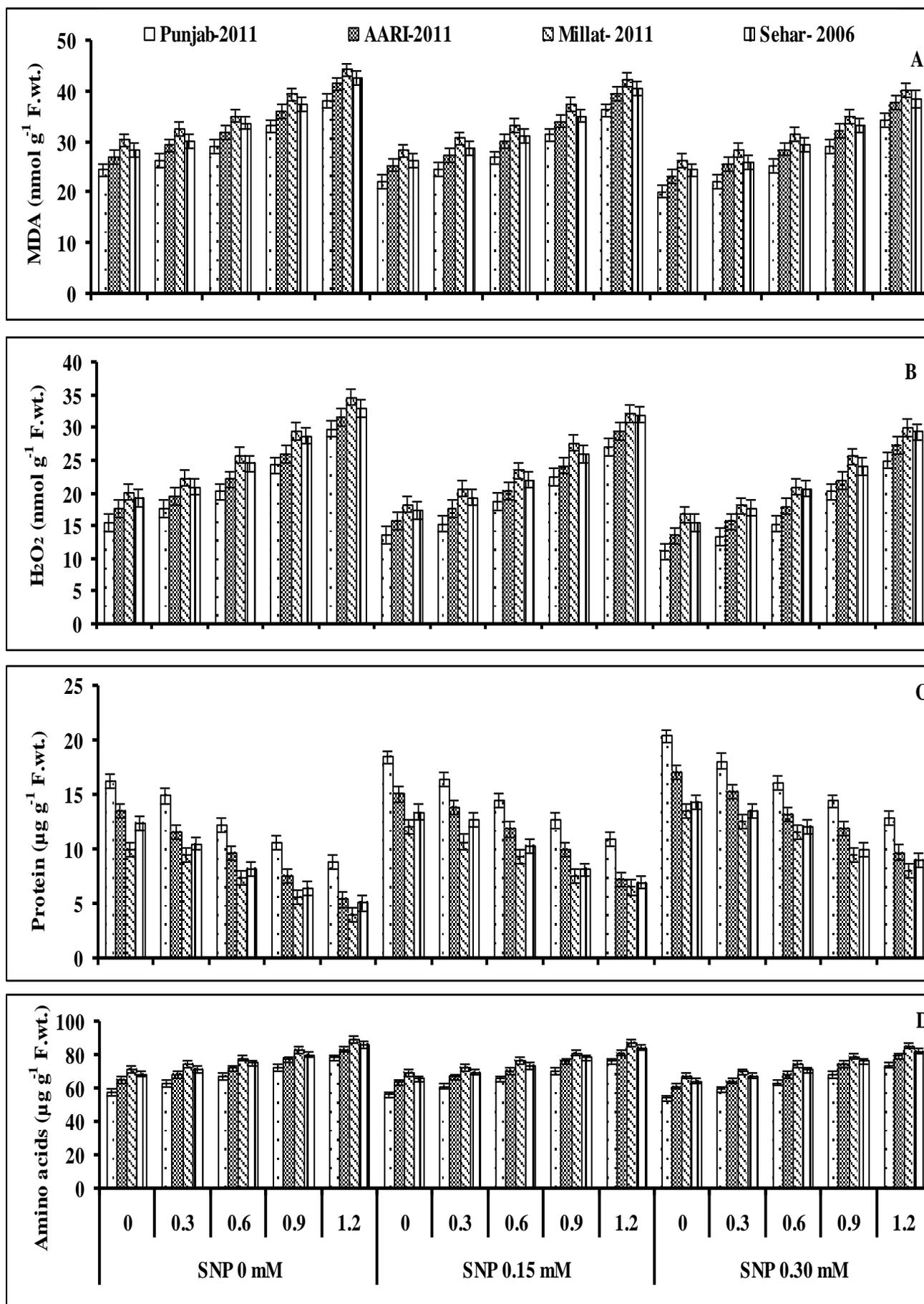


Fig. 3. Effect of exogenous sodium nitroprusside (SNP) on the MDA,  $\text{H}_2\text{O}_2$ , protein and amino acids contents of bread wheat exposed to cadmium stress (0, 0.3, 0.6, 0.9, 1.2 mM).

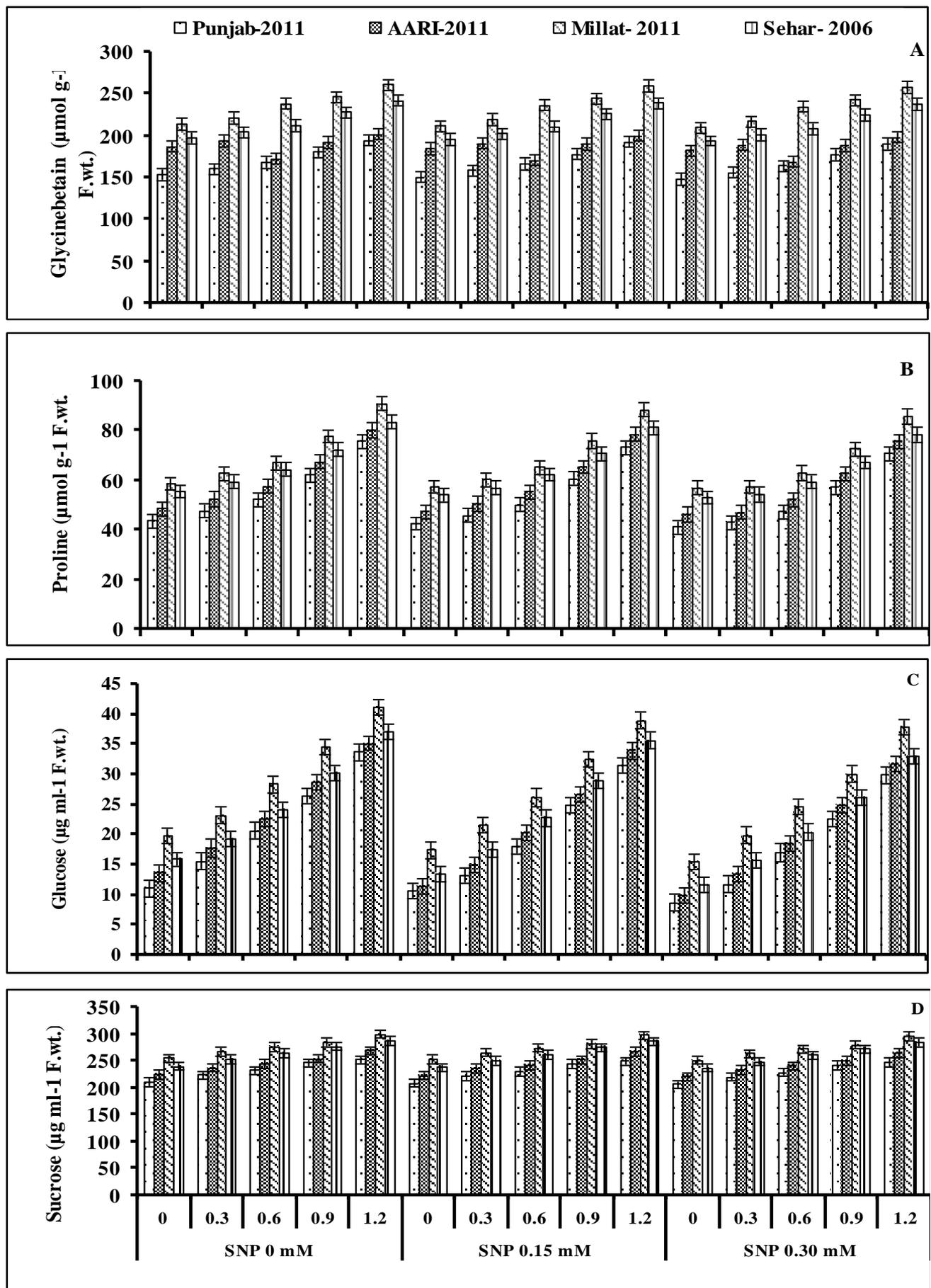


Fig. 4. Effect of exogenous sodium nitroprusside (SNP) on the GB, proline, glucose and sucrose contents of bread wheat exposed to cadmium stress (0, 0.3, 0.6, 0.9, 1.2 mM).

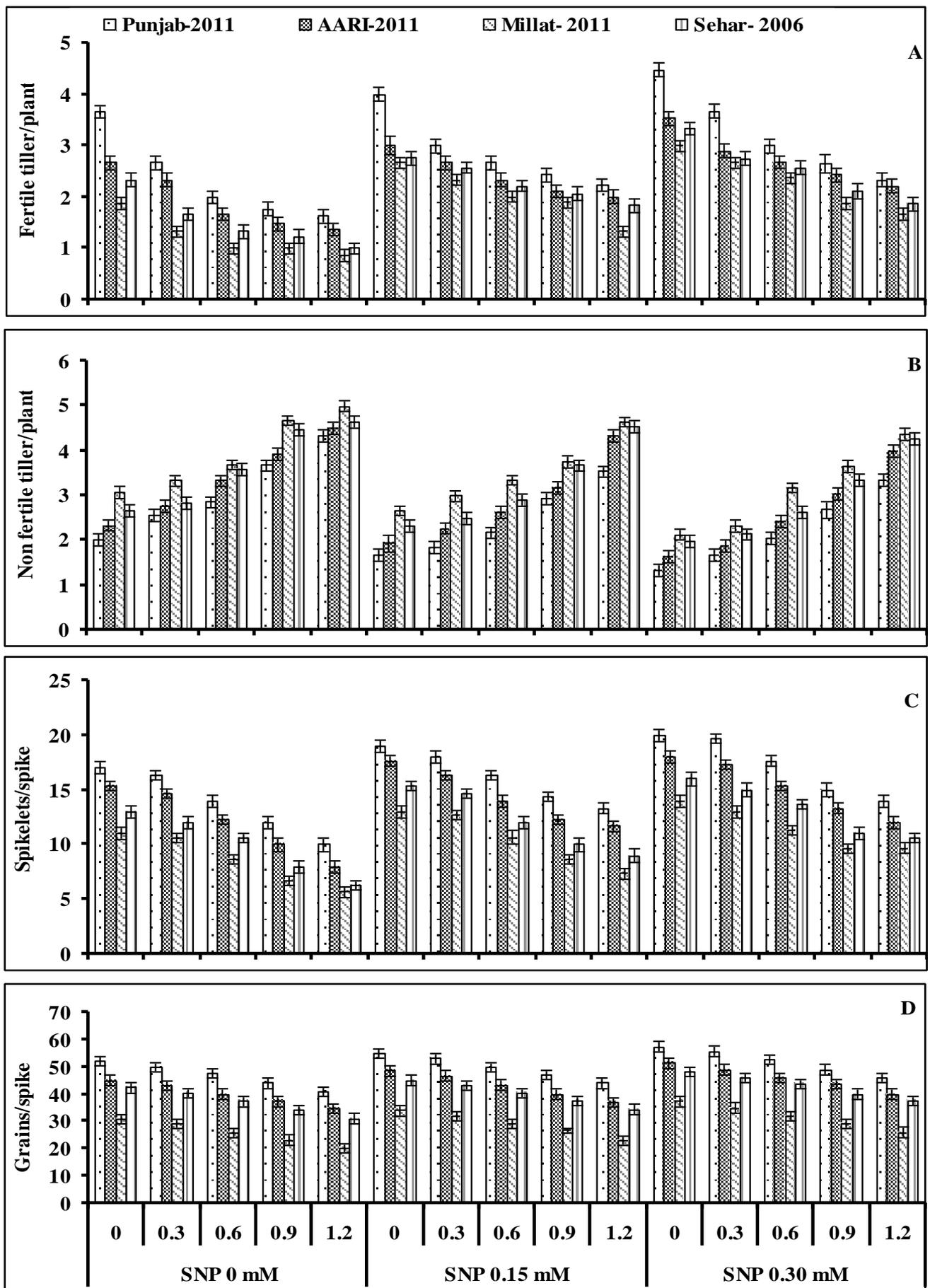


Fig. 5. Effect of exogenous sodium nitroprusside (SNP) on the fertile tiller, non-fertile tiller, spikelets/spike and grain/spike contents of bread wheat exposed to cadmium stress (0, 0.3, 0.6, 0.9, 1.2 mM).

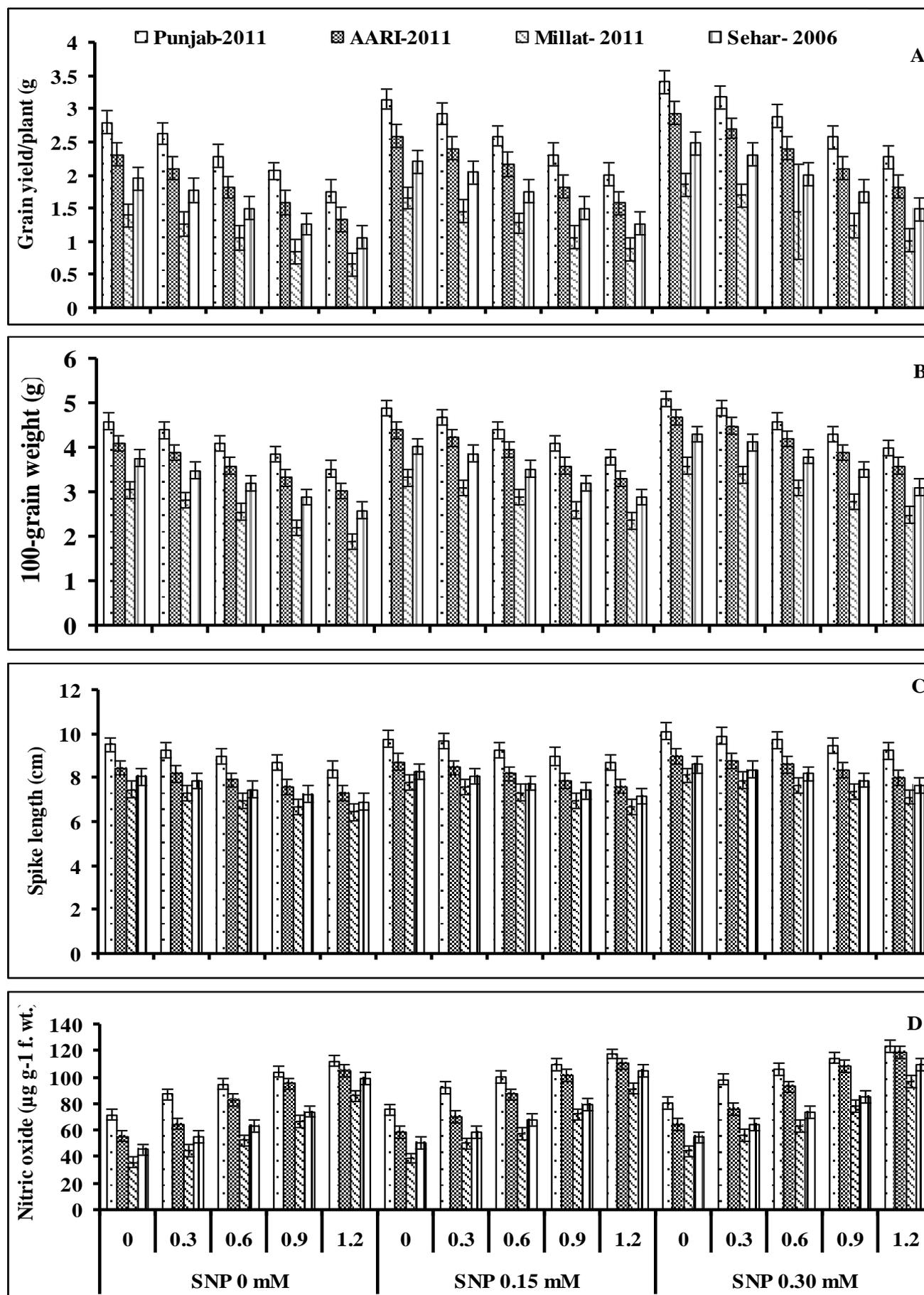


Fig. 6. Effect of exogenous sodium nitroprusside (SNP) on the grain yield, 100-grain wt., spike length and NO contents of bread wheat exposed to cadmium stress (0, 0.3, 0.6, 0.9, 1.2 mM).

Under Cd stress, the enhancement in proline concentration altered glucose, sucrose and the AsA contents. Under Cd stress, the accumulating sugars provided an adaptive system through osmoregulation in the rice plants. Moreover, soluble sugars under stressful environments provided support to the plant for sufficient storage of carbohydrate reserves for the initial metabolism (Hurry & Huner, 1992; Dubey & Singh, 1999). Root or shoot Cd concentration correlated positively with proline, sugars, amino acids, and negatively with leaf water and osmotic potentials suggesting that Cd stress induced water shortage might stimulated proline and sugars synthesis possibly by adjusting both the leaf water and osmotic potential and alleviated the unfavourable effects of Cd. The sugars could play role in enhancing the storage of carbohydrates. The application of SNP under Cd stress provided favourable effects on reducing and non-reducing sugars, amino acids and proline contents that supported earlier results of Pooja *et al.*, (2012). By increasing the contents of AsA, the plants responded to different stresses (Hsu & Kao, 2003; Pant *et al.*, 2011). In Millat-2011, the highest proline contents production was due to antioxidant enzymes that reduced the Cd toxicity. The accumulation of free amino acids was due to Cd stress and the proline accumulation was considered a well-suited cytoplasmic solute (Shah & Dubey, 1998). Cd increased the accumulations of amino acids in accordance with earlier studies (Hsu & Kao, 2003). Under Cd stress, soluble sugars accumulated possibly to regulate the osmotic potential (Chardonnens *et al.*, 1998). The enhancing activities of POD, CAT and AsA, higher proline contents might be helpful in the mitigation of Cd stress. Rosa *et al.*, (2009) suggested that the Cd stress could be reduced with the accumulation of proline.

AsA has involved in scavenging the ROS (Noman *et al.*, 2015). In the control, leaf low AsA contents by NO application showed that the production of AsA contents was improved by Cd stress. The application of SNP in rice lowered the thiobarbituric acid reactive substances, H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup> which is helpful to produce ability in plants against Cd stress (Zhao *et al.*, 2013). The production of AsA under Cd stress was due to H<sub>2</sub>O<sub>2</sub> and MDA. Application of SNP lowered the AsA contents which exposed the protective role of SNP under the elevating Cd stress. Amongst the cultivars, Millat-2011 showed the highest AsA contents indicating its susceptibility to Cd toxicity.

The production of phenolics and  $\alpha$ -tocopherol was suppressed in Cd stress and the application of SNP affected their metabolism. Oxidative stress due to Cd caused reduction in the enzymes of phenolics and  $\alpha$ -tocopherol and the SNP application increased the enzymes activity probably due to decrease of Cd concentration in the shoots. In our work, Cd caused proline accumulation. The application of NO and the interaction of NO with Cd probably improved the contents of proline to increase resistance to Cd stress in wheat. This hypothesis is in a good concurrence with Simaei *et al.*, (2011) who suggested that proline is capable of scavenge hydroxyl radicals. A number of solutes accumulate under Cd stress. Most important solutes are organic acids, GB, proline and amino acids. They enhance

defence against metal stress (Schutzendubel & Polle, 2002), however, the mechanism is largely unknown. Our results showed that the application of NO decreased leaf GB and total free amino acids with respect to control. The increase in Cd concentration at all levels increased leaf total free AsA and GB. The combined effect of SNP and Cd decreased the leaf total free AsA and GB. Among the cultivars, more enhancements in leaf total free AsA and GB were noted in the Millat-2011 as compared with other cultivars under different Cd regimes.

On several plant species, adverse effects of Cd toxicity had been recognized responsible for increases in the overproduction of ROS which smashes the components of cell through oxidative stress via inducing lipid peroxidation (Sharma & Dietz, 2009). MDA is involved in the deterioration of biological systems through reactive free radicals (Hsu & Kao, 2007). Under Cd stress, higher leaf H<sub>2</sub>O<sub>2</sub> and MDA contents were observed that damaged membrane and increased its permeability (Popova *et al.*, 2009). The application of SNP inhibits the increases in the H<sub>2</sub>O<sub>2</sub> and MDA contents which were produced by Cd. SNP caused decreases in H<sub>2</sub>O<sub>2</sub> and MDA contents that provide protection from Cd toxicity which match with the findings of Shah *et al.*, (2001) and Romero-Puertas *et al.*, (2002). Among the cultivars, Millat-2011 suffered much more from Cd stress and had higher amount of H<sub>2</sub>O<sub>2</sub> and MDA compared with other cultivars.

Yield attributes of wheat cultivars such as grain yield per plant, number of grains per spike, 100-grain weight, number of fertile and non-fertile tillers, number of spikelets/spike, and spike length decreased under Cd stress while the application of SNP enhanced these attributes irrespective of cultivars. Exogenously applied SNP with Cd increased the yield of wheat cultivars at all Cd levels. Earlier studies have shown that NO reduces the toxic effect of Cd on the growth and yield of plants. For instance, there are some findings which show that exogenous NO enhanced the biomass and yield of peanut plants under Cd stress (Xu *et al.*, 2014).

## Conclusions

Cd stress caused a marked suppression in total flavanoids, total phenolics and soluble proteins while increased total free AsA, proline, GB, glucose and sucrose, AsA, MDA, H<sub>2</sub>O<sub>2</sub> contents and the activity of APX, CAT, POD and SOD irrespective of wheat cultivars. Punjab-2011 and AARI-2011 were superior to Millat-2011 and Sehar-2006. The 0.30 mM SNP concentration was relatively more efficient. The exogenous SNP was effective in improving total flavanoids, total phenolics, soluble proteins, and grain yield components under Cd stress. Furthermore, SNP reversed the effects of Cd on total free AsA, proline, glucose and sucrose, MDA, H<sub>2</sub>O<sub>2</sub> and the activity of antioxidant enzymes irrespective of wheat cultivars. Overall, the results elaborated that exogenous SNP reduced the Cd toxicity in wheat. It is suggested that large scale, field studies using exogenous SNP should be conducted before any recommendation under Cd polluted environments.

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