INTERNATIONAL JOURNAL OF SCIENCE AND NATURE

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# EFFECT OF NICKEL (Ni) ON CHLOROPHYLL, LIPID PEROXIDATION AND ANTIOXIDANT ENZYMES ACTIVITIES IN BLACK GRAM (*VIGNA MUNGO*) LEAVES

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**ABSTRACT**
Nickel (Ni) is an indispensable micronutrient for plants. At higher concentration Ni becomes toxic for various plant species. In several plants Ni induces change in activity of antioxidant enzymes like superoxide dismutase (SOD), Ascorbate peroxidase (APX) and Catalase (CAT). The objective of this study was to investigate the effect of nickel on photosynthetic pigment, lipid peroxidation and antioxidant enzymes (SOD, CAT and APX) activities in black gram leaves. Seven days old seedlings of black gram were subjected to different concentrations of nickel chloride (20 µM, 40 µM, 60 µM , 80 µM and 100 µM) every alternate day with nutrient solution. Plants were harvested after 10$^{th}$ and 25$^{th}$ days for determined photosynthetic pigment, lipid peroxidation and antioxidant enzymes activities. Photosynthetic pigment decreasd with increase nickel concentration due to oxidative stress. Lipid peroxidation was measured by the production of malondialdehyde in the plants grown on Ni-treated sand and controls. These results indicate that nickel cause oxidative damage in black gram and as an adaptive feature, they increase the lipid peroxidation in leaves. The present results suggested that treatment with different levels of Ni may enhance the antioxidative activities in leaves. We observed that during Ni stress Cu/Zn-SOD was induced (100µM) at 25$^{th}$ day after Ni treatment. Electrophoresis analysis suggested that a significant correlation between concentration of nickel and isozymes pattern of enzymes was observed.
**KEY WORDS**: Nickel, Superoxide dismutase, Catalase, Ascorbate peroxidase, black gram

## INTRODUCTION

Nickel is an essential micronutrients for plant growth and it is also a component of the enzyme urease which is required require for nitrogen metabolism in higher plants (Dixon et al., 2004). Trace elements are essential as micronutrients for normal metabolic function in plants, at higher concentration they are toxic (Seregin and Kozhevenikova, 2006; Chen et al., 2009). Ni is strongly phytotoxic at high concentration (Barcelo et al., 1990; Baccouch et al., 1998). The most common symptoms of Ni toxicity in plants are inhibition of growth, photosynthesis, seed germination, sugar transport (Ali et al., 2009; Leon et al., 2005; Ahmad et al., 2009) and induction of chlorosis, necrosis and wilting (Madhava Rao and Sresty, 2000; Pandey and Sharma, 2002; Nakazawa *et al.*, 2004).

It is known that excessive heavy metal exposure may increase the generation of reactive oxygen species (ROSs) in plants. ROS are efficiently scavenged by antioxidant system in normal condition. ROSs such as the superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($OH^{\cdot-}$) are known to be the main mediators of peroxidative damage. Ni may also induce the lipid peroxidation, cytotoxic protein damage and DNA damage in plant cell (Gajewska et al., 2006; Gajewska and Sklodowska, 2007). Lipid peroxidation produced by extra free radicals is a sign of the presence of toxic substance in the environment: Increased malondialdehyde (MDA) is an indicator of physiological stress. Plants have evolved a battery of antioxidative mechanism to detoxify and eliminate these ROSs. The antioxidant defence system include antioxidant molecule and enzyme like superoxide dismutase (SOD; EC 1.15. 1. 1), ascorbate peroxidase (APX; EC 1. 11. 1. 11) and catalase (CAT; EC 1. 11. 1. 6). SOD is the first enzyme in detoxifying process, converts $O_2^{\cdot-}$ to $H_2O_2$, CAT and APX catalyze the breakdown of $H_2O_2$ (Cakmak and Horst, 1991; Asada, 1992). Previous investigation demonstrated that the effects of Ni on antioxidant enzyme systems have already been studied in some plant species (Gajewska et al., 2006; Gajewska and Sklodowska, 2007; Yan et al., 2008; Song-toa Wang et al., 2010).

*Vigna mungo* L. (black gram) is a bean native to Central Asia. Since mungo beans and sprouts contain high amount of easly digestable proteins, they are good substitute for soya protein in diets (Fery et al., 2002). The objective of the present study was to reveal the mechanism of oxidative stress and defense responses in black gram leaves under Ni stress. We investigated the influence of high Ni concentration on the activities of antioxidative enzymes (SOD, APX and CAT), Photosynthetic pigment and lipid peroxidation in leaves at 10$^{th}$ day and 25$^{th}$ day after Ni application.

## MATERIALS AND METHODS

The chemicals, used in the experimental work, were of AR grade, Molecular grade (Sigma).

**Plant material:**

*Vigna mungo* (black gram) cv. LBG- 645 uniform seeds were surface sterilized for 30 min with 0.1% $HgCl_2$ and germinated in the dark for 7 days over two sheets of *Whatman No.1* filter paper moistened with distilled water. Futher seedlings were transferred in plastic pots which filled with sand and silt. The seedlings were irrigated with nutrient solution for seven days, and then separated into two groups (control and treatment). 7$^{th}$ days after sowing, seedlings of treatment group were irrigated with Nickel chloride ($NiCl_2$) from 20µM to 100µM given every

alternate day with nutrient solution. Each treatment including control was replicated three times. Plants were grown in controlled environment with 16/8 h day/night cycle at 22 ± $5^0$C. Plants were harvested after $10^{th}$ days and $25^{th}$ days of nickel chloride treatment. Fresh plant material was used for enzyme determination, lipid peroxidation and chlorophyll content.

**Estimation of chlorophyll:**

200 mg fresh leaves were homogenized in 5 ml chilled 80% acetone and centrifuged at 10,000 rpm for 10 min and supernatant used for analysis. Chlorophyll was assayed by the method of Arnon (1945). In spectrophotometre the absorbance properties of Chl-a (663nm), Chl-b (645nm) and Carotenoid (480nm) were quantitatively analyzed and the amount of pigment was calculated according to the formula.

**Estimation of lipid peroxidation:** Lipid peroxidation was determined as Malondialehyde (MDA) content after thiobarbutaric acid (TBA) reaction according to Heath and Packer (1968). 200mg fresh plant material was extracted in 5 ml 20% Trichloroacetic acid (TCA) containing 0.5% TBA. The mixture was heated at $95^0$ C for 30 min, quickly cooled, and then centrifuged at 10,000 rpm for 15 min. The absorbance was read at 532nm and 600nm. The concentration of MDA was calculated using an extinction cofficient of $155mM^{-1}cm^1$.

**Estimation of enzymes activity assay:**

SOD activity assay was measured as described by (Beauchamp and Fridovich, 1971). 100 mg fresh leaf material homogenized in 1 ml extraction buffer containing 100 mM phsphate buffer (pH-7.8), 1mM PMSF, 10% PVP, 0.1 mM EDTA and 1.0 mM Triton-X-100. The content was centrifuged at $4^o$C for 20 min at 10,000 rpm. The supernatant was used for enzyme assay. The assay mixture contained 50 mM phosphate buffer (pH-7.8), 0.1 mM EDTA, 13 mM methionine, 75 µM NBT, 2.0 µM riboflavin and 50µl enzyme extract. Riboflavin was added at the end and the reaction mixture were incubated at $25^0$C for 5 min in the dark and later on for 15 min in light. Absorbance was read at 560 nm in UV/Vis. Spectrophotometer (Labomed). The enzyme volume corresponding to 50% inhibition of the reaction (one unit) was calculated. The enzyme was expressed in U/mg protein. CAT activity assay was measured as described by Dhindsa et al. (1981). 100 mg fresh leaf material was homogenized in 1.0 ml extraction buffer containing 100 mM phosphate buffer (pH), 1.0 mM PMSF, 10% PVP, 0.1 mM EDTA and 1.0 mM Triton-X-100. The content was centrifuged at $4^o$C for 20 min at 10,000g. The supernatant was used for enzyme assay. The assay mixture contained 100 mM phosphate buffer (pH-7), 60 mM $H_2O_2$ and 50 µl enyme extract. Decomposition of $H_2O_2$ (extinction cofficient 45.2 $M^{-1}cm^{-1}$) was measured at 240 nm for 4 min. APX activity assay was measured by Nakano and Asada (1981). 100 mg fresh leaf material homogenized in 1.0 ml extraction buffer containing 100 mM phosphate buffer (pH-7.5), 1.0 mM PMSF, 10% PVP, 0.1 mM EDTA, 80 µM ascorbic acid and 1.0 mM Triton-X-100. The content was centrifuged at $4^o$C for 20 min at 10,000 rpm. The supernatant was used for enzyme assay. The assay mixture contained 100 mM phosphate buffer (pH-7.5), 0.5 mM ascorbic acid, 0.2 mM $H_2O_2$ and 50 µl enzyme extract. APX activity was determined by following the decrease in O.D. at 290 nm for 3 min (extinction coefficient 2.8 mM $cm^{-1}$). In all the enzymatic preparations protein was determined by the method of Bradford (1972) using bovine serum albumin (BSA, Sigma) as standard.

**Native PAGE and activity staining:**

100 µg protein from plant exposed to different concentrations of nickel chloride were subjected to discontinous PAGE described by Laemmli (1970). Electrophoretic separation was performed at $4^0$ C for 5 h with constant current of 30 mA per gel. After completion of electrophoresis the gel were stained for the activities of SOD and APX. Gels were stained for SOD isoform by preequilibration in solution of 0.05 mM phosphate buffer (pH- 7.8) and 1.0 mM EDTA for 30 min and then immersed in 2.4 mM NBT, 28 µM riboflavin and 28 mM TEMED for 20 min in dark. The gels were placed in distilled water and exposed in light box for 15 min for identification of individual isoforms, gels were treated with either 2.0 mM KCN or 5.0 mM $H_2O_2$ in the pre-equilibration buffer for 30 min before staining for SOD activity.

Gel was stained for APX isoforms, by equilibration with 50 mM phosphate buffer (pH-7.5) containing 2.0 mM ascorbate for 30 min.The gel was incubated in 2.0 mM $H_2O_2$ for 30 min in dark. Gel was washed with buffer (pH-7.5) for 1 min and submerged in 50 mM phosphate buffer (pH-7.8) containing 28 mM TEMED and 2.5 mM NBT with gentle agitation. The reaction was continued for 10 min and stopped by a brief wash in water.

## RESULTS

Administration of excess Nickel chloride in the nutrient solution was followed by an increase symptoms of toxicity. The results of lipid peroxidation in leaves of black gram in the control and treatment groups are given in table I. In control, plant leaves the level of MDA was 8.63 nmol $g^{-1}$ fresh weight, and significant continuously increase up to 29.33 nmol $g^{-1}$ fresh weight to 100 µM nickel concentration was observed. An immediate and continuous Ni- induced stimulation of lipid peroxidation process was noticed with time. This effect was significant till $25^{th}$ day after Ni application. Enhanced lipid peroxidation could be a consequence of primary effect of Ni stress.

Effect of Nickel on photosynthetic pigment shows in (Table I). Different concentrations of nickel significantly decreased the content of chlorophyll a, chlorophyll b and carotenoids in leaves. Chlorophyll a content was decreased by (22.8% - 52.33%) at $10^{th}$ day and (21.84% -86.21%) at $25^{th}$ day after Ni application, respectively, as compared to the control value. Chlorophyll b content was also decreased by (11.3% - 48.03%) at $10^{th}$ day and (21.14% - 76.11%) at $25^{th}$ day after Ni application, respectively, as compared to the control value. Carotenoid content was decreased by (12.25% - 53.58%) at $10^{th}$ day and (20.18% - 80.6%) at $25^{th}$ day after Ni application, over the respective control.

Superoxide dismutase catalyzes the dismutation of superoxide to $H_2O_2$ and $O_2$. Thus, an increase in SOD activity indicates higher production of endogenous $H_2O_2$, which was seen in the case of nickel treatment, especially under higher concentrations. The SOD activity increased

with nickel concentration up to 40 µM (61% - 806%) than decreased at 60 µM (580%) and further increased up to 100 µM (806%) at 10th day and (53% - 68%) at 25th day after metal treatment, compared to the control value (Table II). At 25th day SOD activity decreased as compared to 10th day plant leaves. APX is a key enzyme in elimination of peroxide. Increase in the APX activity was directly proportional to the nickel metal concentration. The leaves APX activities significantly increased (119%- 141%) and (5% - 86%) under nickel stress at 10th day and 25th day, respectively; compared to the control (Table 2). CAT can eliminate $H_2O_2$ and play a key role in the elimination of $O^-_2$. CAT activity increased by (71% - 480%) and (72% - 385%) compared to control, at 10th day and 25th day after Ni application (Table II).

Electrophoretic analysis suggested that the SOD isozymes profile showed quantitative changes throughout nickel exposure. Three form of Mn-SOD (Mn-SOD 1, Mn-SOD 2 and Mn-SOD 3) were observed with increased intensity with nickel treatment. Highest intensity of Mn-SOD-2 found at 40 µM nickel concentration at 10th day after Ni application (Figure IA). At 25th day after nickel treatment, It was also observed that new isoform of Cu/Zn-SOD expressed at 100 µM (Figure IB). Electrophoretic pattern of APX shown that APX activity continuously increased with increase nickel concentration at 10th (Figure IIA) and 25th day after metal application (Figure IIB).

**Statistical analysis**

All values reported in this work are mean of at least three independent experiments. The mean values ± SE. The significance of differences between control and treatmentmt determined by Student's t-test.

**DISCUSSION**

In this paper, we investigated oxidative damage and capacities of enzymes involved in ROS detoxification in leaves of black gram under Ni stress condition. Typical symptoms Ni toxicity developed 15-17 days after the beginning of treatment. Chlorosis is the common symptoms of Ni toxicity (Pandey and Sharma, 2002). Chlorosis were seen on the leaves of plants treated with 40 µM, 60 µM, 80 µM and 100 µM Ni, No visible symptoms of Ni injury were observed in the case of plants treated with 20 µM. The present results show that the nickel treatment caused a reduction of photosynthetic pigment. This indicates that biosynthesis was inhibited by metals in higher plants (Prasad and Prasad, 1987). Photosynthetic pigment affected by heavy metals due to oxidative stress and subsequent damage through the peroxidation of the chloroplast membranes (Clemens et al., 2002). Chlorophyll content decresed in leaves exposed to Cd stress were also reported in *Vigna mungo* (Singh et al., 2008). Reduced chlorophyll content due to nickel toxicity in different plants species has been well documented (Pandey and Pathak, 2006). Carotenoid content decreased in rice under heavy metal stress (Panda and Khan, 2003).

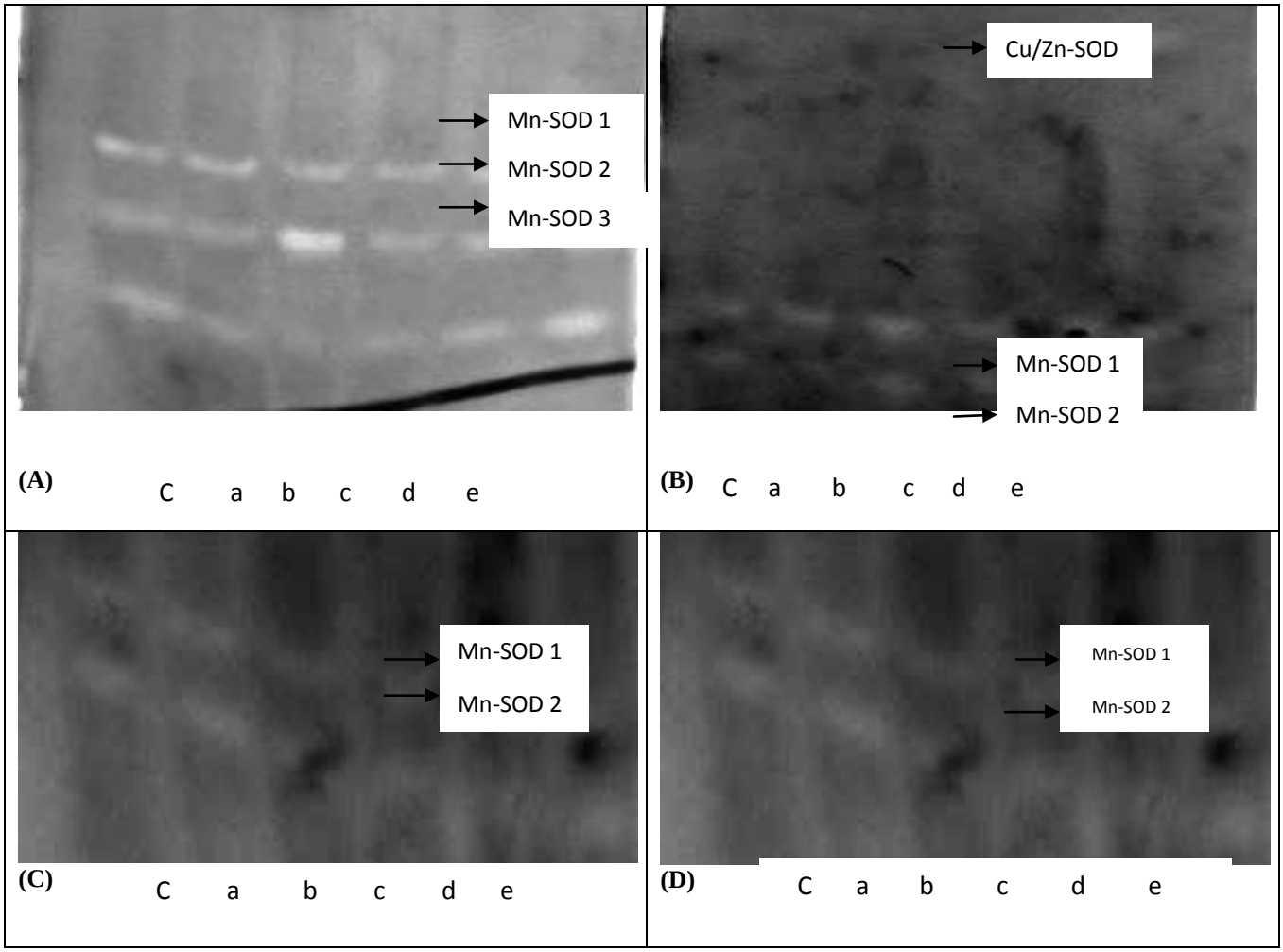

Figure I. Native gels stained for the activity of SOD of black gram leaves exposed to Ni stress, 10th day (A), 25th day (B to D) after Ni treatment, control (B), subjected to $H_2O_2$ (C) and subjected KCN (D) are shown. . Equal amounts (100µg) of protein per lane were loaded on the gel. Lane- C, Control; a, 20µM; b, 40 µM; c, 60 µM; d, 80 µM; e, 100 µM.

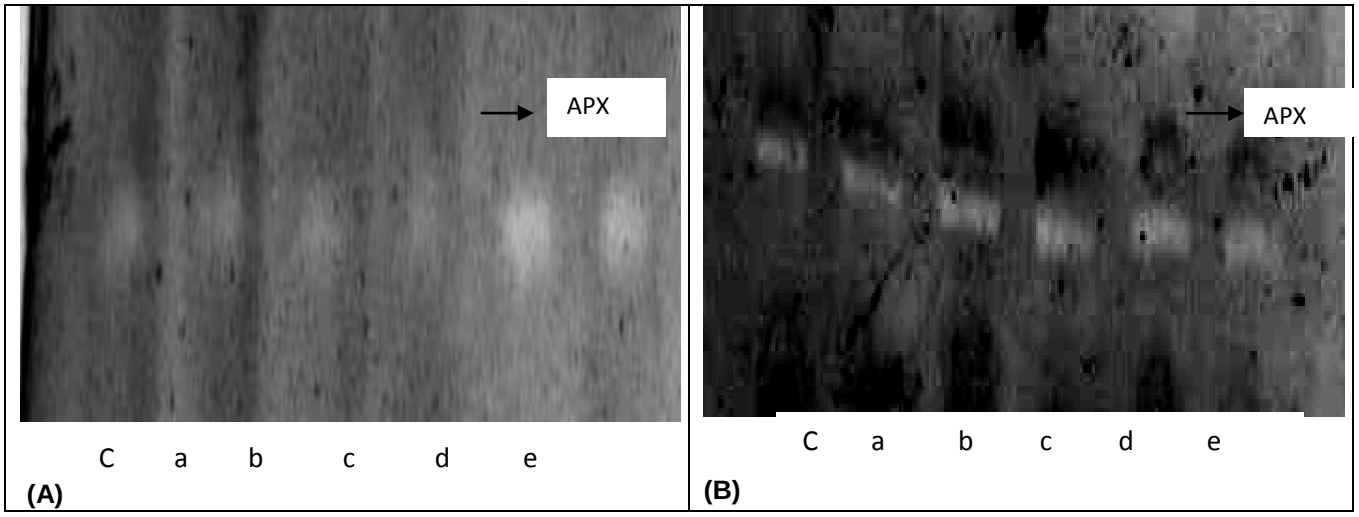


Figure II. Native gel stained for the activity of APX of black gram leaves exposed to Ni stress, 10th day (A) and 25th day (B) after Ni treatment. Equal amounts (100 µg) of protein per lane were loaded on the gel. Lane- C, Control; a, 20 µM; b, 40 µM; c, 60 µM; d, 80 µM; e, 100 µM.

**TABLE I**. Effect of Nickel chloride on Chlorophyll a, Chlorophyll b, Carotenoid and MDA content of black gram leaves at 10th day and 25th after metal application.

| Nickel (µM) | Chlorophyll 'a' [mg $g^{-1}$ (f.m.)] | Chlorophyll 'b' [mg $g^{-1}$ (f.m.)] | Carotenoid [mg $g^{-1}$ (f.m.)] | MDA nmol $g^{1}$(f.m.) |
|---|---|---|---|---|
| 10th Day | | | | |
| Control | 1.96 ± 0.06 | 1.77 ± 0.03 | 1.37 ± 0.14 | 8.63 ± 0.23 |
| 20µm | 1.72 ± 0.03 | 1.57 ± 0.03 | 1.05 ± 0.07 | 10.56 ± 0.34 |
| 40 µm | 1.55 ± 0.02* | 1.46 ± 0.02 | 0.91 ± 0.01 | 15.73 ± 0.23** |
| 60 µm | 1.38 ± 0.03** | 1.36 ± 0.02** | 0.83 ± 0.02 | 17.26 ± 0.34** |
| 80 µm | 1.28 ± 0.05** | 1.23 ± 0.02** | 0.75 ± 0.02 | 24.33 ± 0.40*** |
| 100 µm | 0.93 ± 0.02*** | 0.92 ± 0.03*** | 0.65 ± 0.01 | 29.33 ± 0.37*** |
| 25th Day | | | | |
| Control | 1.14 ± 0.03 | 1.13 ± 0.03 | 0.87 ± 0.03 | 16.06 ± 0.40 |
| 20µm | 0.91 ± 0.02 | 0.89 ± 0.04 | 0.68 ± 0.02* | 17.73 ± 0.25 |
| 40 µm | 0.69 ± 0.03* | 0.66 ± 0.03 | 0.52 ± 0.02* | 1.15 ± 0.33** |
| 60 µm | 0.58 ± 0.05** | 0.54 ± 0.02** | 0.35 ± 0.03** | 25.82 ± 0.25** |
| 80 µm | 0.046 ± 0.03*** | 0.43 ± 0.02*** | 0.23 ± 0.02*** | 33.98 ± 0.36*** |
| 100 µm | 0.31 ± 0.03*** | 0.27± 0.02*** | 0.12 ± 0.02*** | 38.83 ± 0.13*** |

**TABLE 2**. Effect of Nickel treatment on black gram leaves, SOD, APX and CAT activities at 10th day and 25th day after metal application.

| Nickel (µM) | SOD (U $mg^{-1}$ protein) | APX (U $mg^{-1}$ protein) | CAT (U $mg^{-1}$ protein) |
|---|---|---|---|
| 10 day | | | |
| Control | 6.40 ± 0.19 | 90.37 ± 0.58 | 2.86 ± 0.16 |
| 20µM | 10.36 ± 0.14 | 198.77 ± 0.62** | 4.90 ± 0.57 |
| 40 µM | 58.83 ± 0.18*** | 199.17 ± 0.60** | 6.64 ± 0.28** |
| 60 µM | 43.55 ± 0.17** | 199.97 ± 0.54** | 8.48 ± 0.61** |
| 80 µM | 50.78 ± 0.18 ** | 203.56 ± 0.52*** | 12.60 ± 0.69*** |
| 100 µM | 58.04 ± 0.31*** | 217.90 ± 0.49*** | 16.61 ± 0.71*** |
| 25 day | | | |
| Control | 26.48 ± 0.20 | 286.10 ± 0.4 | 5.36 ± 0.41 |
| 20µM | 40.55 ± 0.16** | 300.33 ± 1.4 | 9.26 ± 0.16 |
| 40 µM | 42.46 ± 0.17*** | 428.83 ± 0.4** | 11.66 ± 0.29** |
| 60 µM | 37.41 ± 0.41** | 463.65 ± 3.5*** | 17.36 ± 0.36** |
| 80 µM | 44.59 ± 1.1*** | 498.32 ± 1.3*** | 20.80 ± 0.34*** |
| 100 µM | 49.59 ± 0.17*** | 532.67 ± 1.4*** | 26.03 ± 0.55*** |

In our study, an immediate and continuous Ni-induced stimulation of lipid peroxidation process was noticed. At 10th day of Ni application, lipid peroxidation highly stimulate in leaves. This fast rise of membrane lipid peroxidation could be due to direct contact of membrane roots with the metal (Baccouch *et al*., 2001). These results continue up to 25th day of Ni application with increased lipid peroxidation in leaves. Ni toxicity contributes to enhanced generation of ROS, causing peroxidative damage to membrane lipids, has been reported by (Rao and Sresty, 2000). Many authors also reported, over accumulation of lipid peroxidation product was produced due to heavy toxicity and oxidative damage (Pandolfini et al., 1992; Luna et al., 1994; Chaoui et al, 1997; Mazhoudi et al., 1997).

Many environmental stresses can lead to enhanced production of ROS within plant tissues, and induced SOD enzyme for detoxify these ROS (Alscher et al., 2002). In the present study, there was significantly increase in SOD activity in leaves exposed to different concentration of nickel compared to the control. SOD activity also increased significantly in *Alyssum mantinum* under nickel treatment (Schickler and Caspi, 1999). Similar increase in SOD activity was observed in many plant species under heavy metal stress (Hassan et al., 2005; Qureshi et al., 2007).

Ascorbate peroxidase is a key enzyme of glutathione-ascorbate pathway and eliminate peroxide by converting ascorbic acid to dehydroascorbate (Asada 1992; Foyer and Noctor, 2005). In the present study, the APX activity was found to increase with increase in nickel stress on 10th day and 25th day after Ni treatment. $H_2O_2$, produced in aboundance through rapid dismutation of superoxide by SOD, is efficiently converted in to water and oxygen molecules by APX activity using AsA as a reductant (Qureshi et al., 2007; Qadir et al., 2004). Excess supply of Ni led to increased accumulation of Asc, associated with increased activities of APX.

Our results showed a stimulation of catalase activity after 10th day and 25th day after nickel treatment. the increase in CAT activity suggest elevate level of $H_2O_2$, formed by dismutation of superoxide radicals. Increased CAT activity is generally regarded as a response to heavy metal stress due to the generation of ROS in plant cells (Schützendübel and Polle, 2002).

Our results showed that presence of three SOD isozyme in the leaves at 10th day after Ni treatment. One SOD isozyme was detected markedly, in leaves of 40 µM Ni treated seedling. Isozyme analysis indicated that the increase of SOD activity in leaves was due to Mn-SOD. Howevre, at 25th day after Ni treatment only two isozymes of Mn-SOD were detected. Mn-SOD upregulated under Cd treatment has been reported by (Rodriguez-Serrano et al., 2006) in pea roots. We observed that during Ni stress Cu/Zn-SOD expressed at 100 µM Ni concentration. The Cu/Zn-SOD activity enhanced under heavy metal stress also reported in spinach (Nalini et al., 2009). In addition, the changes in the staining densities of these isozymes were consistant with the changes of the SOD activity as assayed in extract solution. It has been reported that SOD involved in cell defence against oxidative stress (passardi et al., 2005). APX isozyme analysis showed that activity staining induced with increase Ni concentration at 10th and 25th day after Ni application. The increase in the Asc pool along with the increase in activity of APX, indicates that *de novo* synthesis of Asc was enhanced under heavy metal stress (Nalini et al., 2009). The up-regulation of some antioxidant enzyme activities and the isozymes of SOD and APX indicate that excess Ni generate oxidative stress.

## CONCLUSION

In conclusion, our findings showed that there is an imbalance between ROS and ROS scavenging enzymes. Increased SOD, APX and CAT activity might play a role in the defense response of black gram exposed to nickel toxicity. Our results showed that the new Cu/Zn-SOD form expressed by nickel stress on 25th day after treatment. Thus, these findings may contribute to a better understanding of the antioxidant response mechanisms in black gram leaves to Nickel stress.

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