International Journal of Science, Environment and Technology, Vol. 5, No 6, 2016, 3651 – 3662

ISSN 2278-3687 (O)
2277-663X (P)

ANTIOXIDATIVE ENZYME ACTIVITIES AND BIOCHEMICAL PARAMETERS IN TWO VARIETIES OF *MACROTYLOMA UNIFLORUM* (LAM) VERDC. UNDER NICKEL STRESS

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Abstract: The present investigation was carried out to examine the toxic effect of nickel on plant growth and antioxidative enzyme activity in *M. uniflorum* (English name – horse gram), and to assess the sensitivity and protective effect of nickel stress in two different varieties of this medicinally important plant. Pot culture experiment was carried out with increasing concentration of nickel (control, 25, 50, 100, 150 and 200 mg of nickel/kg of soil) amended to soil. Seeds of the two different varieties (white and black) were allowed to grow in each of the nickel concentration in triplicate. The plant was gently up rooted on day 21 and 42 corresponding to vegetative and flowering stage of the plant. Root and shoot length, proline and protein content of the leaves were measures and root and shoot tolerance index was calculated. The antioxidative enzyme activity such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.161.1.6) and guaicol peroxidase (POX, EC 1.11.1.7) were estimated in the leaves of both the cultivars of plant on day 21 and 42. Higher concentration of nickel in soil reduced the protein content, root and shoot length following initial increases at lower concentrations of nickel. There was up regulation in de novo synthesis of antioxidative enzymes at lower concentration of nickel which was followed by a state of oxidative damage as evidenced by declining activity of these enzymes. The black variety of horse gram had higher root and shoot tolerance index and sustained to expressed higher activity of antioxidative enzymes at a particular concentration nickel compared to white variety.

Keywords: Nickel, *M. uniflorum*, Antioxidative enzyme, Tolerance index.

INTRODUCTION

Heavy metals pollution has increased over the years due to natural and anthropogenic activities. Bioaccumulation heavy metals such as copper (Cu), cobalt (Co), zinc (Zn), lead (Pb), and nickel (Ni) and their entry to the food chain has resulted in serious implications leading to ecological and health problems. Most agricultural soils contain an average of 25mg/ kg soil dry weight of Nickel and may go up to 7000 mg/ kg in serpentinite and peridotite soil (Brooks, 1987; Holmgren et al., 1993). Nickel is one of the important heavy metal pollutants discharged by mines and smelters. The effluents from the processing plants have been inadvertently used by farmers for plant irrigation leading to increased level of nickel in soil and water. Nickel is considered as an essential nutrient for plant growth, and is

*Received Sep 27, 2016 * Published Dec 2, 2016 * www.ijset.net*

required at low concentration to act as a co-factor for the urease and other enzyme activities (Marschner, 2002). Nickel exposure above the tolerance level in plants has inhibitory effects on plant growth, and the toxicity is manifested by the common symptoms including of retardation of growth, inhibition of photosynthesis, mineral uptake, sugar transport and disturbed water relations (Denkhaus and Salnikow, 2002; Rao and Sresty, 2000). However, effect of stress due to increasing concentrations of nickel in soil on medicinally important plant, *Macrotyloma uniflorum* (Lam) Verdc, especially on antioxidative enzymes activities has not been studied.

Macrotyloma uniflorum (Lam)Verdc. (English name – horse gram), one of the wild pulses of Fabaceae family and has maximum utility as fodder, called as "Horse gram". It has the greatest potential for its utilization as nutraceuticals, forage and food for malnourished people, particularly in drought prone areas of the world. Different parts of the plant (leaf, stem, root and seed) are used in the traditional system of medicine for the treatment of heart disease, asthma, bronchitis, urinary discharges kidney stones, and to relieve phlegm in dry cough (Ghani, 2003). The medicinal properties of different parts of the plant is influenced by several factors including the plants' immediate environment, level of pollutants, content of phytochemicals and associated stress (Sahoo et al., 2015). The medical properties of this plant might be differing based on the variety of the plant species and their stress bearing properties. The cytotoxicity assessment of this medicinal plant in Bangladesh recently revealed presence of kaempferol -3-0- B-D-glucoside, B-sitosterol and stigmasterol (Sarkar et al, 2009). The seeds of the plant have been reported to possess anti-urolithiatic activity (Atodariya *et al*, 2013), and the plant could play a role in antioxidation as it tolerates severe adverse environmental conditions including heavy metal contaminations (Reddy *et al.*, 2005). There are several varieties of horse grams cultivated in India. Black and white varieties are two different commonly cultivated plants in Odisha, and are mainly differentiated on the basis of colour of the seeds, but the antioxidative potential of these two different varieties under nickel stress has not been exploited.

The balance between generation and degradation of ROS is required for metabolic function in stress conditions. The level of ROS in plant tissue is controlled by antioxidative system that consists of antioxidative enzymes and non-enzymatic low molecular mass antioxidants. Metals in excess stimulates the production of free radicals and reactive oxygen species (ROS) such as superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radicals ($OH^{\cdot}$) (Pantola and Shekhawat, 2012). The ROS plays a role in

lipid peroxidation, membrane damage and consequently in plant senescence. Antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT) scavenge ROS. SOD is a metalloprotein that catalyses the dismutation of superoxide to H_2O_2 and molecular oxygen. CAT found predominantly in peroxisomes, dismutates H_2O_2 into H_2O and O_2, whereas POX decomposes H_2O_2 by oxidation of co-substrates like phenolic compounds and /or antioxidants (Pantola and Shekhawat, 2012). The present study documents the antioxidative enzymes activities in leaves and biochemical parameters of root and shoot of two varieties of *M. uniflorum* under nickel stress.

MATERIALS AND METHODS

Certified seeds of two different varieties (white and black) of *Macrotyloma uniflorum* were collected from Orissa University of Agriculture and Technology. The seeds were surface sterilized with 0.1% $HgCl_2$ (mercuric chloride) for one minute and repeatedly washed with distilled water and allowed to germinate in earthen pots. The pots were filled with garden soil, sand and vermin compost in 3:1:1 ratio. The inner surface of pots was lined with a polythene sheet. The soil of each pot was amended with various levels of nickel (control, 25, 50, 100, 150, or 200 mg/kg of soil). Three replicates were maintained for each concentration. Ten seeds were sown in each pot. All pots were watered with regular intervals. Plants were thinned to a maximum of five per pot, after a week of germination. The germination and plant growth was monitored properly and the Plant materials were identified in P.G Department of Botany, Utkal University.

Biochemical parameters

The fresh leaves were collected on day 21 and 42 from each group of treated plants and washed properly with distilled water. One gram of leave were homogenized with 50mM of potassium phosphate buffer at pH 7.8, 50mM EDTA (ethylenediamine tetra-acetic acid), 2mM PMSF (phenylmethylsulphonyl fluoride) and 10%(w/v) PVP (polyvinyl pyrrolidone) using a pre chilled mortar and pestle. The homogenate was centrifuged at 14, 000 RPM for 20 min at 4^oC. The process was repeated two times for each sample and the supernatant was used for estimation of total soluble protein (mg/g fresh weight) and antioxidative enzymes (U/g fresh wt).

Estimation of Protein

The protein concentration in the supernatant from the tissue homogenate was measured as per the method of Lowry *et al* (1951).

Superoxide dismutase

The SOD activity in the homogenate was estimated at 543nm by measuring the inhibition of superoxide driven nitrile formation from hydroxylamine hydrochloride as described by Das *et al*. (1999). The composition of 1.4 ml of cocktail consisted of phosphate buffer 1.11ml (pH 7.4), 75µl of L methionine (20mM), 40 µl Triton X 100 (1% v/v), 75 µl hydroxylamine hydrochloride (10mM), 100µl of 50mM EDTA, 100µl of enzyme extract, and 80µl of riboflavin (50µl). The assay mixture was mixed thoroughly and illuminated for 10 min in 20 watt fluorescent lamp in a wooden box. One ml of Greiss reagent (was prepared by mixing equal volume of 1% sulphanilamide in 5% phosphoric acid and 0.1% N-1- napthyl ethylene diamine) was added to each assay mixture, after 10 min of incubation, and the absorbance was measured at 543nm.The enzyme activity was calculated from the value of V_0/V-1 where V_0 is the absorbance at control (without enzyme) and V-1 is the absorbance of the sample.

Catalase

Catalase activity was measured following the principle of degradation of H_2O_2 by catalase, and the absorbance was recorded at 240nm for 3 min at 15 second interval following method of Aebi (1974). Briefly, the assay mixture (3ml) consisted of 2.0ml of potassium phosphate buffer, 500 µl of supernatant and 500 µl of H_2O_2(hydrogen peroxide). The extinction coefficient of 40$mM^{-1}cm^{-1}$ was used to calculate the catalase activity in the assay mixture.

Guaicol Peroxidase

The activity of GPx was measured by the method of Kar and Feierabend (1984) using extinction coefficient of 26.6 $mM^{-1}cm^{-1}$. The assay mixture (3ml) was composed of potassium phosphate buffer 2.8ml (pH 7.0), 100 µl of supernatant, 50 µl of 10mM H_2O_2, and 50 µl of 18mM guaicol. The increase in absorbance at 470nm due to tetra guaicol formation was recorded at 15 seconds interval for 3 min.

Extraction and estimation of proline

Fresh leave sample of 500mg was homogenized with 10ml of 3% sulphosalicylic acid. The homogenate was centrifuged at 3000rpm for 10min. The assay mixture was composed of 2 ml of extract, 2 ml of acid ninhydrin reagent (1.25g of ninhydrin in 30ml of glacial acetic acid and 20 ml of 6M phosphoric acid) and 2 ml of glacial acetic acid. The assay mixture was heated at 100^oC for one hr followed by rapid cooling in ice bath. 4 ml of toluene was added to the reaction mixture and mixed vigorously in a cyclomixture for 20

seconds. Toluene containing chromophore was aspired and the absorbance was taken at 520nm using Tolune as blank. Proline content was calculated from the standard curve of proline and was expressed as µg/g fresh weight (Bates *et al*, 1973)

Statistical analysis

The data were analyzed using SPSS program for window version 16.0, and were expressed as mean ± S.E. Two way analysis of variance (ANOVA) procedures were used to compare the differences among the treatments and between the two different varieties. The least significant difference (LSD) and Duncan's tests were performed to determine the significance of treatment means at $P<0.05$.

RESULTS

The protein content of leaves at the vegetative stage (day 21 of observation) was significantly lower in black variety compared to its mean level in white variety. However, such difference was not observed in the leaves at flowering stage (Fig-1). Gradual reduction in mean protein content from its respective control level was observed with higher concentration of nickel. Significant reduction was recorded from 100 mg of nickel/ kg of soil in both black and white variety on day 21 whereas such significant reduction was recorded at 50 mg of nickel/ kg of soil amendment on day 42 in both black and white variety.

The mean proline content increased significantly in both the varieties at two different periods of observations with 25 mg of nickel/ kg of soil exposure (Fig 2). The increasing trend continued up to 100 mg of nickel/ kg of soil exposure in white variety at two different observation periods however in black variety the rising trend of proline content was recorded up to 150 mg of nickel/ kg of soil exposure followed by sharp decline by 200 mg of nickel/ kg of soil.

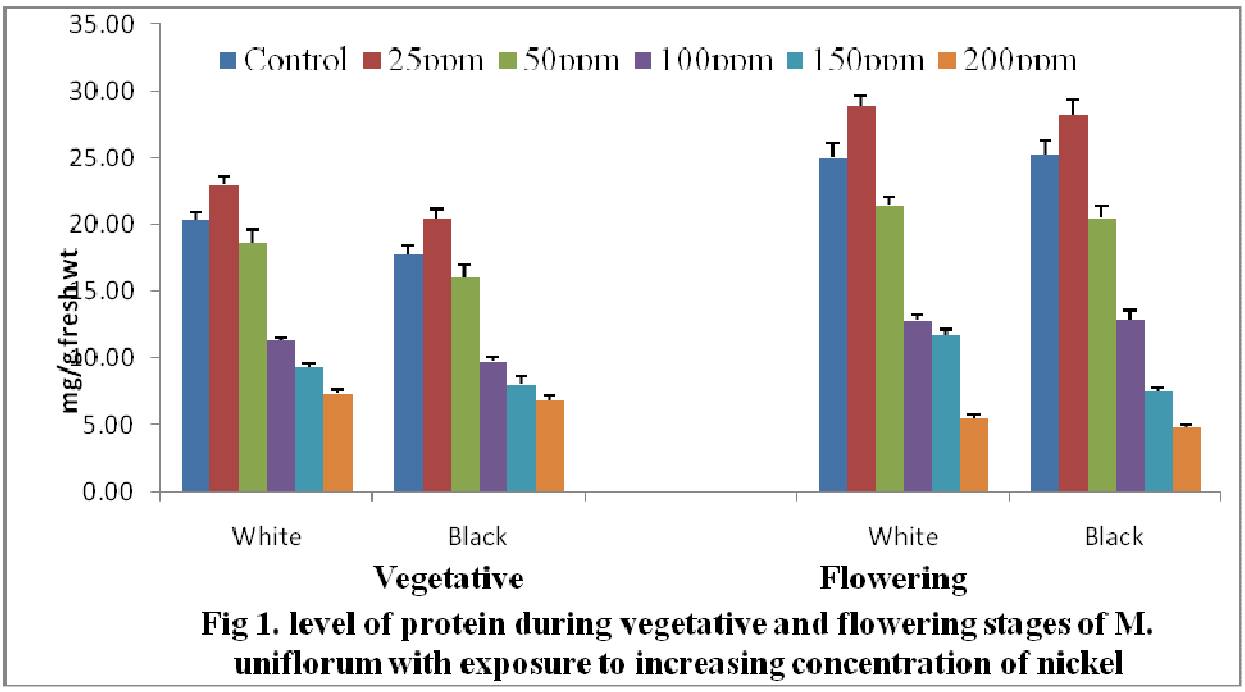

Fig 1. level of protein during vegetative and flowering stages of M. uniflorum with exposure to increasing concentration of nickel

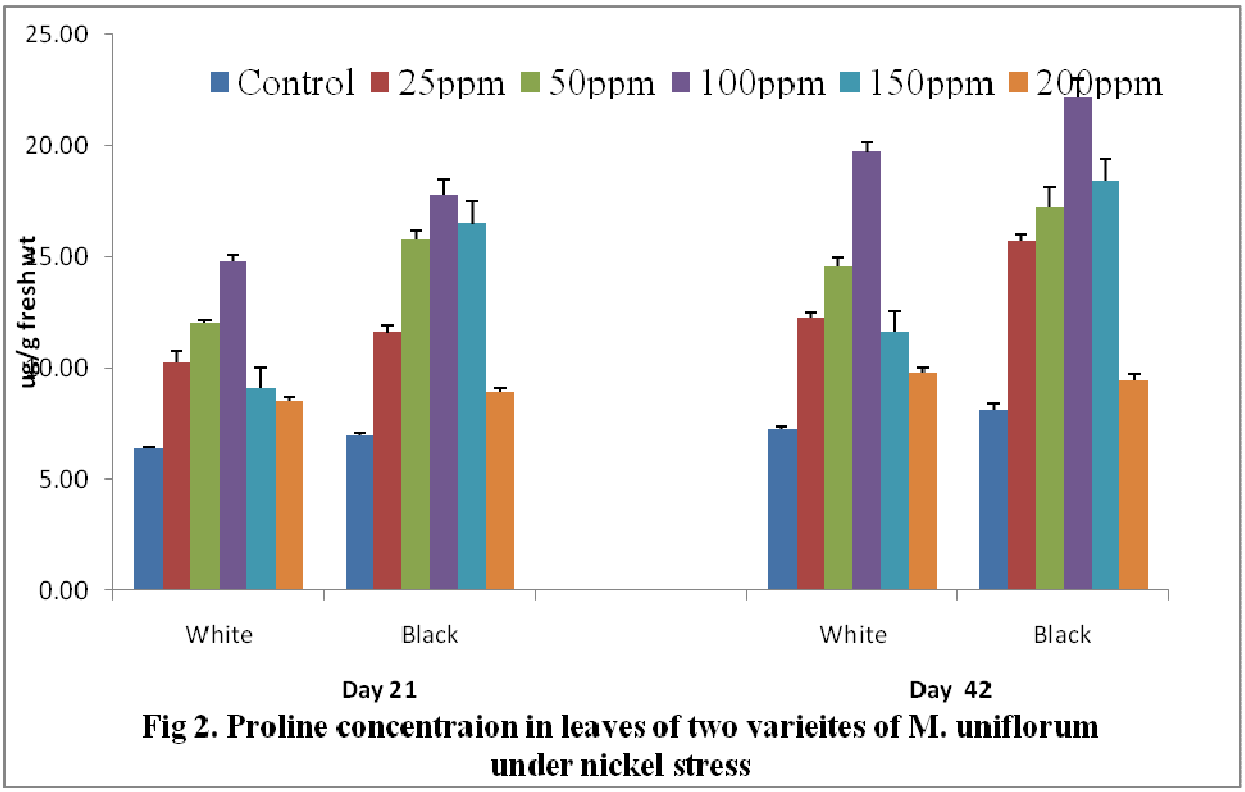

Fig 2. Proline concentraion in leaves of two varieites of M. uniflorum under nickel stress

Fig 3, 4 and 5 shows the mean activity of antioxidative enzymes at two different observation periods in two varieties of *Macrotyloma uniflorum* with increasing concentration of nickel exposure. The black varieties had significantly higher mean activities of SOD, CAT and POX compared to white varieties in control soil. The alteration in mean activity of these three antioxidative enzymes in leaves was quite appreciable with only 25 mg of nickel/ kg of

soil exposure unlike changes in morphological parameters. Initially there was increase activity of the enzymes with lower level of nickel exposure in both the varieties followed by decline in activities with higher concentration of nickel. The up-regulation of antioxidant enzymes with increasing nickel exposure up to 100mg/kg of soil was more pronounced in black variety than the white variety suggesting that black variety had better potential to counter the oxidative changes following nickel exposure. The graph 4 shows that the black variety continued to have increased activity of catalase and peroxidase even up to 100mg of nickel/ kg of soil with respect to catalase activity and statistically comparable activity of peroxidase up to 150 mg of nickel/ kg of soil suggesting that the drop in antioxidative enzymatic activity was more noticeable in white variety than black variety with increasing concentration of nickel.

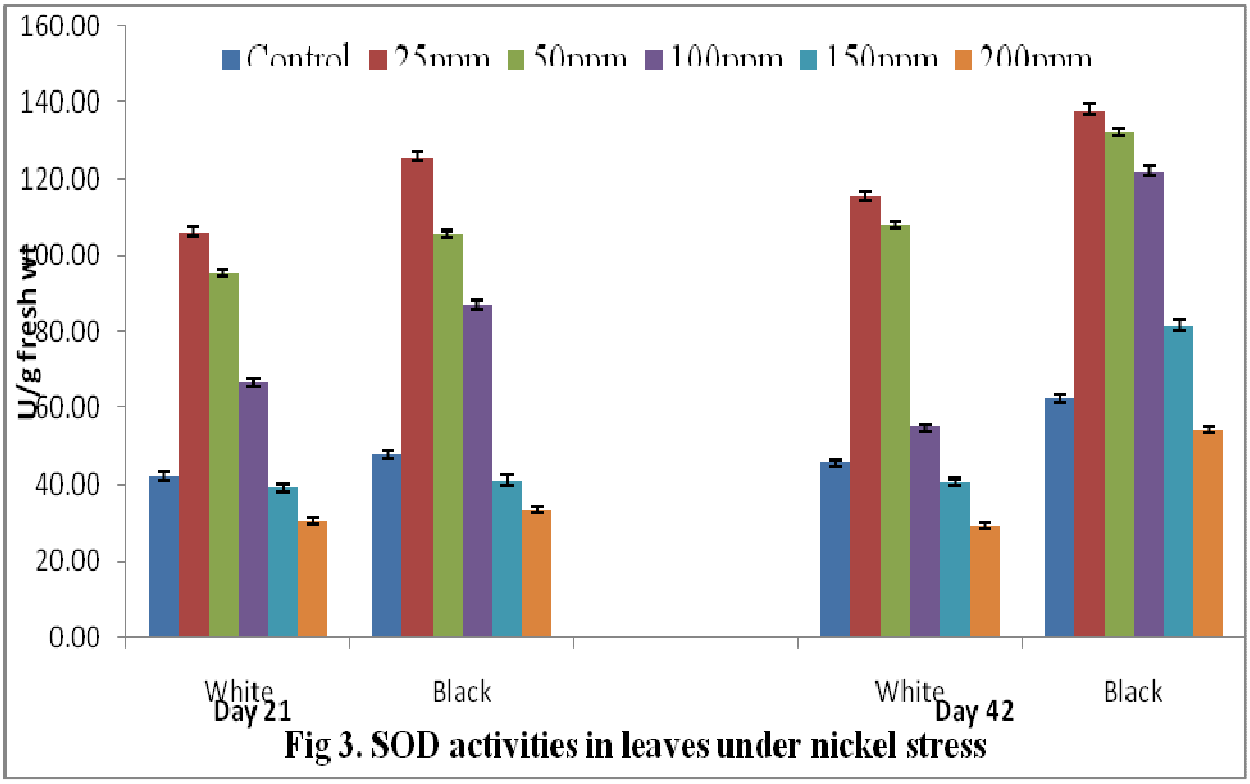

Fig 3. SOD activities in leaves under nickel stress

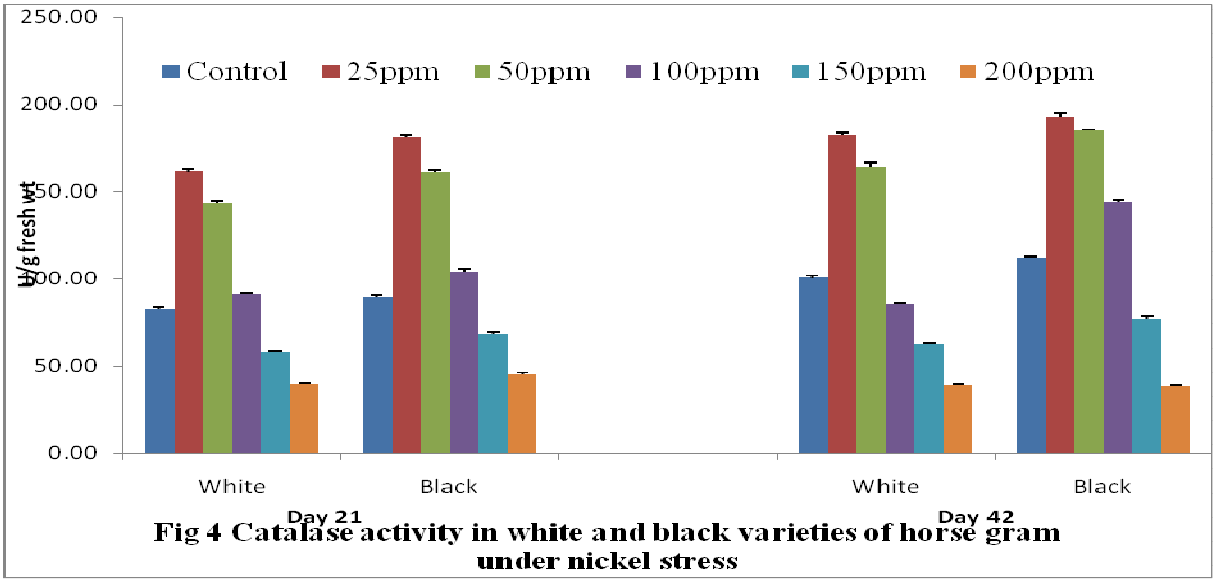

Fig 4 Catalase activity in white and black varieties of horse gram under nickel stress

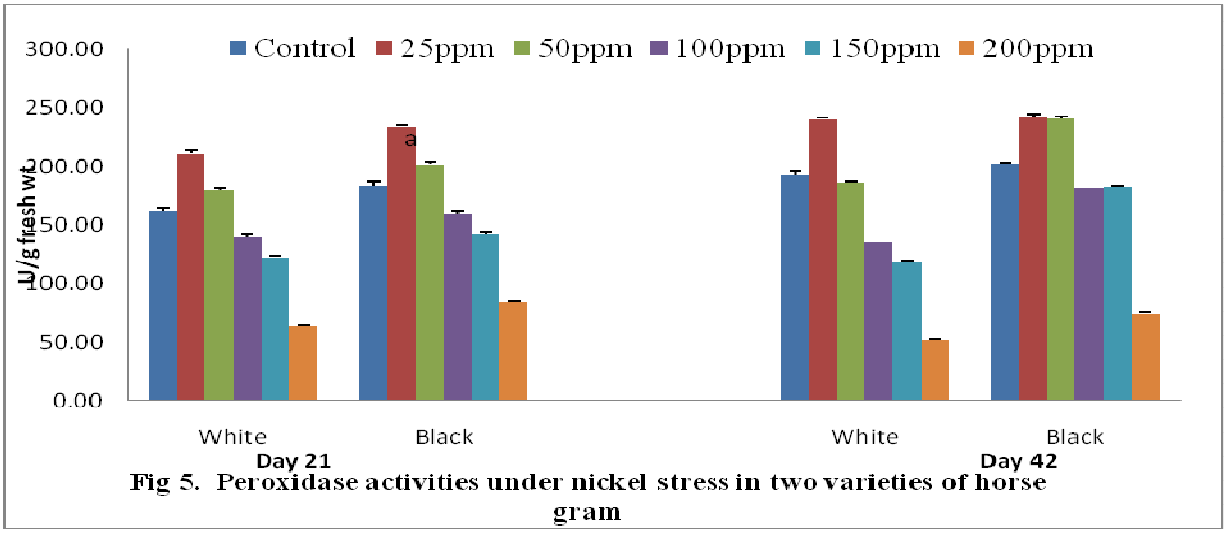

Fig 5. Peroxidase activities under nickel stress in two varieties of horse gram

DISCUSSION

Metal toxicity in plants results from complex interaction of ions with several metabolic processes (Baccouch *et al.,* 2006). Lipid peroxidation of plasma membrane and the resultant disturbance in membrane integrity has been considered as primary effect of heavy metal toxicity. The process starts before appearance of any visible symptoms (Cakmak and Horst, 1991; Baccouch *et al*., 2006). Higher plants protect themselves from accelerated lipid peroxidation as a consequence of oxidative stress through several antioxidant molecules and enzymes such as superoxide dismutase, catalase and peroxidase. The present study analyze the effect of nickel on these antioxidative enzymes and associated changes for the first time on the medicinal plant *M. uniflorum* exposed to increasing nickel stress achieved through amendment of soil.

Nickel contamination of soil and its bioaccumulation in higher plants have several ill consequences on plant growth and metabolism (Seregin and Kozhevnikoca, 2006; Yan *et al.,* 2008). Increasing nickel level to 200mg/kg of soil had visible symptoms of chlorosis, reduced leave size and stunted growth. The strong toxic effects of nickel are reported at 0.5 and 250µm in *Alnus glutinosa* and *Zea mays*, respectively (Baccouch *et al.,* 1998; Wheeler *et al,* 2001). The retardation of plant growth in the present study was noted at nickel concentration of 100 mg/ kg of soil and onwards, and was lower in black variety than white. This might to be due to varied response between cultivars, differing in water potential, and nutrient uptake under nickel stress (Schat *et al.,* 1997).

In the present study, the protein content started declining from 50mg of nickel/ kg of soil, and the proline content continued to rise compared to the control plants with peak level of proline content observed at 100mg of nickel/ kg of soil. This might be due to beneficial role of nickel at lower concentrations and toxicity at higher level. The decrease in protein content might be due to enhanced protease activity under stress condition induced by excessive exposure to nickel and fragmentation of protein, associated with induced lipid peroxidation due to toxic effects of reactive oxygen species (Palma *et al.,* 2002). Increased accumulation of proline in leaves might be related to metal induced water deficit. Higher proline content has earlier been observed in leaves of *Silene vulgaris* treated with copper, cadmium and zinc (Schat *et al,* 1997). Proline contributes to stabilization of subcellular structure and scavenging free radicals, therefore large quantities of proline is synthesized under different environmental stress (Hsu *et al.,* 2003; Kavikishore *et al.,* 2005 and Kumar *et al.,* 2012). The increase in proline content in the present study, and more so in black variety than white, following nickel exposure may be attributed to enhanced protective mechanism for osmoregulation and to counter the ROS generated under nickel induced oxidative stress.

In addition to increase in proline content in leaves, there was increased activity of SOD, CAT and POX with lower level of nickel exposure confirming that there was up regulation of antioxidative enzymes synthesis to counter nickel induced generation of ROS (Pantola and Shekhawat, 2012). The state of oxidative damage of *M. uniflorum* leaves in the present study was observed at higher level of nickel exposure that might have resulted from over accumulation of ROS and failure of antioxidative enzymes to scavenge the generated ROS under nickel stress. SOD is a strong antioxidative enzyme that represents the first line of cell defense against ROS to protect tissue damage. The increase in SOD activity has been reported in several plants under heavy metal stress (Goa *et al.,* 2008). SOD and CAT are

complementary in their action to diminish effects of oxidative stress. POX activity is considered as a potential indicator of sub lethal toxicity of heavy metals in plants (Zhang *et al.,* 2007). Augmentation of SOD, CAT and peroxidase following exposure to lower level of nickel might have resulted from *de novo* enzyme synthesis through induction of antioxidative enzyme gene expression by superoxide mediated signaling transduction, and over production of H_2O_2 due to increased SOD activity (Yan *et al,* 2008). In the present study, the black variety has higher potential to synthesize more of antioxidative enzyme as compared to white variety suggesting that the former had better potential to counter induced-ROS production with increasing nickel exposure. The state of oxidative damage with higher concentration of nickel and degradation of protein was also associated with morphological changes.

CONCLUSION

It is concluded that nickel exposure @25mg/kg of soil has promontory effect in plant growth in both black and white variety of *M. uniflorum*, but exposure above 100mg of nickel/ kg of soil has strong inhibitory effects resulting in oxidative damage. The black variety has better potential for *de novo* synthesis of antioxidative enzymes even with higher concentration of nickel compared to white variety.

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