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Isozyme profiling of Antioxidant Enzyme in *Macrotyloma uniflorum*

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Abstract: The current climate change and pollution scenario has invariably increased the abiotic stress of salinity, heavy metals, and temperature on plants. Abiotic stress impacts the plant's defense system, impacting the crop's growth, yield, and productivity. The present investigation emphasised the antioxidant ability of *Macrotyloma uniflorum* under nickel stress which forms a major part of defense mechanism. It intricately evaluates the antioxidant activities of *M. uniflorum* with respect to different Nickel doses. The antioxidant defense system of plants includes enzymes including super oxide dismutase (SOD), catalase (CAT), peroxidase (POX), glutathione peroxidase (GPX), and dehyrdoascorbate reductase (DHAR). These enzymes act as scavengers to ameliorate the free radicals cellular metabolism produces. Therefore, the present study gives a comprehensive understanding of the antioxidant enzymatic system with special reference to the role of each enzyme in response to the abiotic stress, especially nickel. By studying the isozyme pattern of these enzymes, we can compute the antioxidant activities in a very detailed manner. Different concentrations of Ni in the range of 25 ppm to 125 ppm were taken to find the optimum concentration of Ni for its antioxidant activities. SOD1 isozyme was the most prominent one among all at 25 ppm. The intensity of GPX2 in the black variety is more prominent at 100 ppm as compared to the control. Thus, the results obtained could be easily used to evaluate the minimum concentration of Nickel for antioxidant.

Introduction

Horse gram, also known as *Macrotyloma uniflorum*, is a widely cultivated crop rich in protein, carbohydrate, essential amino acids, and flavonoids with medicinal properties. Legume seeds, including horse gram, have been studied for their antioxidant activities and potential health benefits (Handa et al., 2021; Sahoo et al., 2022a and 2022b). However, environmental stress caused by pollution, including heavy metal contamination, can negatively affect plants' growth and curative abilities. Nickel, in particular, has been recognized as an important micronutrient for plants, but an excess of it can inhibit plant growth and development (Kumari et al., 2023). Nickel toxicity and other heavy metals can also reduce or inhibit photosynthesis in plants (Amanullah and Khan, 2023). Stressful environments cause plant cells to produce more reactive oxygen species (ROS), an inevitable by-product of aerobic metabolism.

Under high levels of nickel stress, the ability of certain cultivars to perform photosynthesis decreased significantly. The plant often responds to stress by producing ROS, which can have negative effects on its metabolism (Kesawat et al., 2023). The plant has a powerful defence against free radicals (Sahoo et al., 2022b; Sarkar et al., 2023). To function properly under stress, plants must balance ROS production and degradation (Swain and Padhy, 2015). Several researchers have noticed increased antioxidants enzyme activities in plants to combat oxidative stress induced by various stresses like as drought, soil salinity, high temperature, chilling, metal stress, anaerobiosis, gaseous pollutants and UV-B radiation (Sharma et al., 2021;

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Sahoo et al., 2022a). Naji and Devraj have observed that HTS elicits a robust enzymatic and non-enzymatic antioxidant system on horse gram (Naji and Rangaiah, 2006). The antioxidative system, which consists of small antioxidants and enzymes, helps regulate levels of ROS in plant tissues (Mansoor et al., 2022). Excessive amounts of metals can lead to the generation of harmful ROS, which can damage plant membranes and cause senescence (Hafeez et al., 2023). A cell is said to be in a state of "oxidative stress" when the amount of ROS surpasses the capacity of its defence mechanisms. Increased ROS amid environmental stress can be harmful to cells because it can lead to lipid peroxidation, protein oxidation, nucleic acid damage, enzyme inhibition, activation of the programmed cell death (PCD) pathway, and finally, cell death. The precise balance between ROS generation and scavenging determines whether ROS will function as a harmful or signalling molecule. Antioxidant enzymes like catalase, peroxidase, and superoxide dismutase help neutralize ROS. Superoxide dismutase specifically helps convert superoxide into hydrogen peroxide, while catalase dismutase hydrogen peroxide into water and oxygen (Zhang et al., 2023).

Antioxidant system of plants

The antioxidant system in plants is made up of antioxidant enzymes and small antioxidant molecules. These components work together to eliminate ROS within plant cells (Biswas et al., 2020). The antioxidant enzyme system constitutes an array of enzymes such as Catalase, Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), gluaiacol peroxidase (GPX), peroxiredoxins (Prxrs) and enzymes of the ascorbate-glutathione (AsAGSH). Which easily varies under stress and are strong indicators of abiotic stresses of plants (Basumatary et al., 2023).

In plant cells, chloroplasts and mitochondria produce superoxide, which is then removed by superoxide dismutase to prevent damage to the cell membrane (Ahmad et al., 2016). Catalase is found in aerobic organisms, primarily in peroxisomes and related organelles. Its main function is to remove hydrogen peroxide produced during certain metabolic processes. Gluaiacol peroxidase is an oxidoreductase enzyme that helps remove hydrogen peroxide by oxidizing specific electron donors (Liu et al., 2023). It is found in plants and is more active under stressful conditions. It is involved in various processes such as cell wall formation and Defence against stress. Soluble peroxidases are found in the apoplast and vacuole, while bound forms are found in the cell wall. Nickel toxicity can induce oxidative stress in plants, increasing hydrogen peroxide production and activating antioxidant enzymes (Basumatary et al., 2023). This study found increased activity of ascorbate peroxidase, superoxide dismutase and gluaiacol peroxidase in response to nickel toxicity in barley roots, indicating their role in scavenging reactive oxygen species. Insufficient activated antioxidant enzymes leave exposed to nickel-induced oxidative stress, resulting in the oxidation of lipids and proteins (Bhat et al., 2023).

Isoenzyme pattern of antioxidant enzymes

Different antioxidant enzymes in plants were expressed in response to exposure to various concentrations of metal stress (Maia et al., 2023). The antioxidant activities can be studied in more detail by exploring the pattern of antioxidant enzymes w.r.t stress. For example, the levels of SOD isoenzymes changed throughout the nickel exposure, with three forms of Mn-SOD showing increased intensity (Subhani et al., 2023). The expression of SOD genes in *Hordeum vulgare* was found to be associated with antioxidant properties and can be under environmental stress. Isozymes profiling shows the activities of antioxidant in an articulative manner. This novel technique leads to exploring the minimum concentration of Ni required for fine-tuned plant defence mechanisms with the least cellular damage. In the present climate change scenario, the work explores the minimum concentration of Ni, a suitable for *M. uniflorum* growth. *M. uniflorum*, a medicinal plant of repute with a wide range of pharmacological activities, has a larger role to play in drug design, which could be explored further.

Scavenging ROS with antioxidant enzymes like catalase (CAT), peroxidases, and SOD, plants can control ROS levels (Bano et al., 2014). The presence of diverse molecular forms of antioxidant enzymes and their potential to alter in response to diverse environmental cues suggest that these isozymes may function in ROS detoxification (Pinhero et al., 1997). Plant tolerance to stress can be studied using isozymes as a biochemical marker (Zhang et al., 2013). Das et al. (2013) proposed a hypothesis that higher activities of antioxidant enzymes, coupled with changes in their isozyme profiles in rice spikelets, provide an important mechanism of tolerance to high-temperature stress.

Materials and Methods

Seeds of black varieties of *M. uniflorum* were obtained from Orissa University of Agriculture and Technology. The seeds were carefully chosen for their uniform size and then sterilized using mercury chloride. The seeds were carefully chosen for their uniform size and then

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sterilized using mercury chloride. They were thoroughly washed before being used for germination studies and pot experiments (Fig. 1).

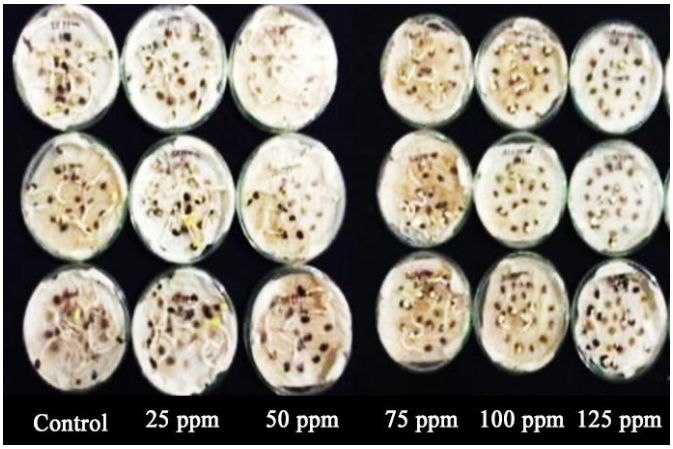

Figure 1. An in vitro germination pattern was observed on day 5 in a black variety of *M. uniflorum* under nickel stress.

Pot Experiment

In a 1:1:1 ratio, garden soil, cow manure, and vermicomposting were placed in the pots. Polythene sheets were used to line the interior surface of the pots. Different amounts of nickel (control, 25, 50, 75, 100, or 125 mg kg-1 soil) were added to the soil in each pot. Each concentration was maintained with six replicates. Each pot held ten seeds that were sowed. Every pot received regular watering. The seeds were allowed to sprout and grow in the pots with various nickel amounts. One week following germination, plants were trimmed to a maximum of five per pot. The Department of Botany, Centurion University, correctly tracked the germination, plant growth, and identification of the plant materials. The plants were harvested from each pot at three different phases of growth such as vegetative (21 days), flowering (42 days) and fruiting stages (63 days). Seeds were collected from the plants after senescence for analysis (Fig. 2).

Figure 2. Black variety of *M. uniflorum* on day 21 grown with different concentrations of nickel.

Analysis of antioxidative enzymes

One gram of leaves was mashed using a mortar and pestle with a mixture of phosphate buffer, EDTA, PMSF, and PVP. The resulting mixture was spun in a centrifuge at a high speed and low temperature. This process was repeated twice for each sample. The liquid collected from the centrifuge was used to measure antioxidative enzymes. Some of the liquid was stored at a very cold temperature for further analysis.

Superoxide dismutase

Both SOD and catalase activities were expressed in units per gram of fresh weight. The activity of SOD in the sample was measured using a method developed by Gao et al., 2012. The absorbance of the mixture was measured and used to calculate the enzyme activity. The activity of catalase was measured using a mixture of sodium phosphate buffer, hydrogen peroxide, and enzyme mixture. The decrease in hydrogen peroxide concentration was measured over a period of 3 minutes using a spectrophotometer. The catalase activity was calculated based on the decrease in hydrogen peroxide concentration.

Catalase

The assay mixture for the activity of catalase consisted of 2 ml of 100 mM sodium phosphate buffer (pH 6.8), 0.5 ml H_2O_2, and 0.5 ml of enzyme mixture. The OD of the reaction mixture was measured spectrophotometrically at 240nm for 3 minutes at 15-second intervals. The catalase activity was estimated by measuring the decrease in H_2O_2 concentration and was calculated by using the extinction coefficient of 40mM/cm. The values were expressed in U/g fresh weight.

Guaicol peroxidase

Using an extinction coefficient of 26.6 mM/cm, the activity of GPx was assessed using the (Kar and Feierabend, 1984) method. The assay mixture (3 ml) contained 100 l of supernatant, 50 l of 10 mM H_2O_2, and 50 l of 18 mM guaicol in addition to 2.8 ml of potassium phosphate buffer (pH 7.0). For three minutes, 15-second intervals were used to measure the increase in absorbance at 470 nm caused by tetra guaicol production. The values were given in fresh weight units per gram (Kar and Feierabend, 1984).

Native PAGE

Native PAGE was performed following the procedure for SDS PAGE with certain changes. The major changes were as follows.

1. SDS was not used in resolving, stacking gel and running and loading buffer preparation.
2. B-mercaptoethanol was not used in loading

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buffer

3. Preheating of sample before loading was not required and
4. Electrophoresis was carried out at 4^0C

Preparation of native polyacrylamide gel

10% resolving gel and 5% stacking gel were prepared for loading the samples. An equal amount of protein (50 µg) was loaded into each lane. A constant current of 15mA was applied to the gel in the electrophoresis apparatus. The voltage was increased to 35 mA when the dye font reached the resolving gel. The power supply was turned off as the dye front reached the bottom of the resolving gel. Gel was removed carefully and subjected to staining to resolve the gel for catalase, SOD and peroxidase isoenzymes.

Superoxide dismutase

The isoenzyme pattern of SOD was observed. The native gel was immersed in a staining buffer containing 50mM potassium phosphate buffer (pH 7.8), 0.11M EDTA, TEMED, 0.003 mM riboflavin and 0.25M NBT for 30 min in the dark. Two 20-watt fluorescent lamps illuminated the gels until the bands became visible as light bands on a violet background.

Catalase

CAT isoenzyme pattern in NATIVE PAGE gel was observed. The gel was incubated in 0.003% H_2O_2 for 10 min and developed in a reaction mixture containing 2% (w/v) potassium ferric chloride and 2% (w/v) potassium ferricyanide. The isoenzymes appeared as colorless band on a deep blue background.

Guaicol peroxidase

GPX isoenzyme pattern was localized in 10% native gel using the method of Rout et al. (2013). The gels were incubated for 30 minutes at room temperature in 0.018M guaicol with deionized water and then immersed in a solution of 0.015% H_2O_2 in 1% acetic acid. A transparent background with radish-brown bands developed in about 10 minutes.

Statistical analysis

The data obtained in the study were expressed in Mean± S.E. The data were analyzed using the SPSS package to compare the mean values using LSD and Duncan test.

Results

Antioxidant activity and isoenzyme pattern

Table 1 displays the SOD, catalase, and peroxidase activities in the black variety of *M. uniflorum* at various growth stages. The SOD activity was highest during the flowering stage in the black variety compared to the vegetative and fruiting stages in control plants. When nickel was added to the soil at a lower concentration, the SOD activity increased. Electrophoretic separation of SOD isozymes showed differences in band intensity between the control and nickel-stressed plants, showing greater changes in intensity. The intensity of SOD isozymes increased up to 100 ppm of nickel and then declined to 125 ppm during the flowering stage. SOD had lower expression levels except at 25 ppm, where the intensity increased compared to the control. Four different forms of SOD were expressed during the fruiting stages. A new form of SOD was found in the stressed plants of this variety, with the highest intensity at 25 ppm of nickel and decreasing afterward. SOD1 was the most prominent form among all the SOD variants in the black variety.

Isozymes of SOD in *M. uniflorum*

The electrophoretic separation for SOD isozymes during the vegetative stage exhibited two isoform bands in control and nickel-stressed plant exposed to increasing concentrations of nickel in the black varieties of *M. uniflorum.* An increase in activity of SOD was observed as a function of external nickel supplementation of 25 and 50 mg kg^{-1} of soil and this is more pronounced in black variety. The black ones had more changes in the band intensity suggesting the ensuing oxidative stress was less in the black variety (Figure 3).

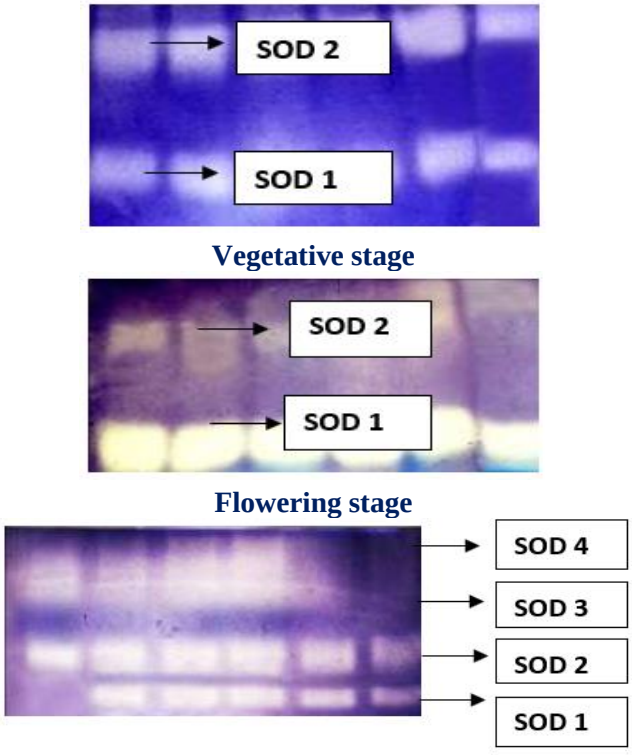
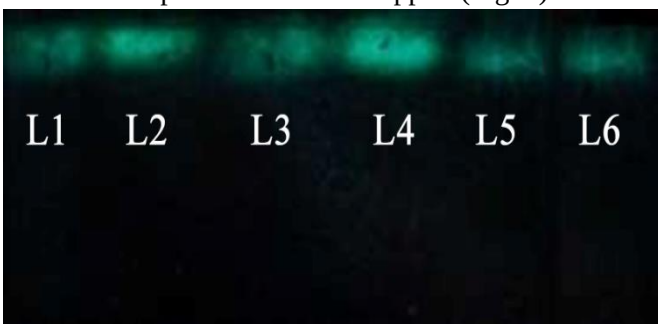


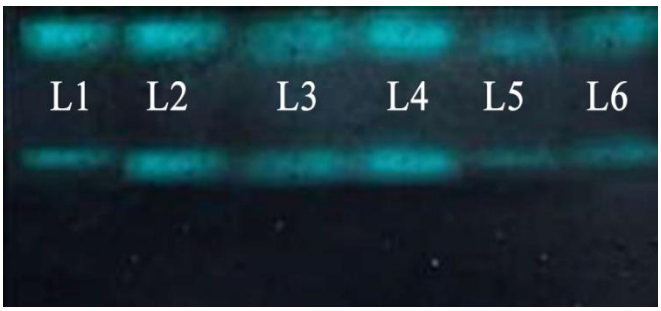
Figure 3. In gel activity of SOD at different stages of growth in black Variety of *M. uniflorum* under nickkel stress. L1-Control, L2-25, L3-50, L4-75, L5-100, L6-125mg of nickel / kg of soil.

Isozymes of CAT in *M. uniflorum* treatment

The activity of CAT enzyme in normal and nickel-stressed plants of *M. uniflorum* was observed. A single form of the CAT enzyme was found in this variety, with its intensity increasing up to 75ppm of nickel and then decreasing. During the flowering stage, two forms of the CAT enzyme appeared, with CAT being more intense. The Guaicol peroxidase enzyme activity in *M. uniflorum* treated with nickel was also assessed at various phases. In the black variety, the activity increased at 25 ppm of nickel during the vegetative stage but declined at higher concentrations. At the flowering and fruiting stages, peroxidase enzyme activity in the black variety was statistically lower only with 75 ppm of nickel and above. The black variety showed better antioxidant enzymatic activity up to a nickel exposure level of 100 ppm. (Fig. 4).



Vegetative stage



Flowering stage

Figure 4. CAT isozymes activity pattern in leaves at different stages of growth in black varieties of *M. uniflorum* under nickel stress. L1-Control, L2-25, L3-50, L4-75, L5-100, L6-125mg of nickel/ kg of soil.

Isozymes of GPX in *M. uniflorum*

Three isozymes of GPX (GPX1, and GPX3**)** were observed after electrophoresis inthe native condition in the stressed plant of the black variety of *M. uniflorum*, whereas two bands were observed in control during the vegetative stage of *M. uniflorum*. The activity of both GPX1 and GPX2 was more prominent in 25- and 50-ppm concentrations of nickel, respectively. Nickel stress induced one more isozyme GPX3 at 100 ppm of nickel in black variety. All the isozymes were more prominent in all the 5 treatments. The band intensity was maximum at 25 ppm with the minimum at 125 ppm (Figure 5). A number of GPX isoforms were visualized after electrophoresis in native gel in the black variety of *M. uniflorum*. In 25 ppm, GPX 1 was more intense and gradually increased up to 50 ppm, followed by a decline at 125 ppm. The band intensity of GPX2 at 25 ppm was more prominent as compared to control. GPX1 in black variety is less prominent. The intensity of GPX2 in the black variety is more prominent in 100 ppm as compared to control.

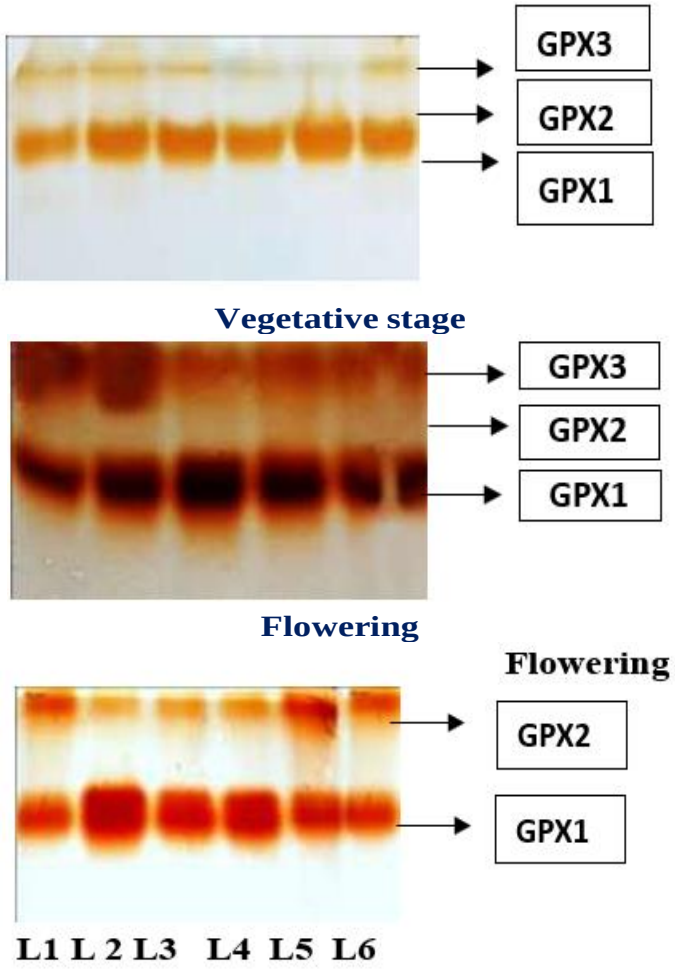


Figure 5. GPX isozymes activity pattern in leaves extracts at different stages of growth in black varieties of *M. uniflorum* under nickel stress. L1-Control, L2-25, L3-50, L4-75, L5-100, L6-125 ppm of nickel.

**Nickel in mg/kg of soil. Values are expressed in Mean ± S.E. Values in parenthesis indicate range. The mean value with different superscripts (small letters) in a column for a particular parameter differs significantly at P≤0.05. * Indicates significantly different mean levels from the other variety within a particular treatment.

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Oxidative stress causes a change in the bio-membrane's selective permeability, which results in membrane leakage and a change in the activity of enzymes that are bound to the membrane (Silva et al., 2018). Therefore, it is crucial to understand how those enzymes behave in order to safeguard against nickel toxicity. It was evidenced that increasing the metal concentration causes an increase in peroxidase and polyphenol oxidase activity in *Arachis hypogea* (Dinakar et al., 2008). When nickel supplementation was present in *M. uniflorum*. At increasing concentrations, the catalase and peroxidase showed an inducible activity. The considerable increase in these enzymes' activity at lower nickel concentrations may significantly impact the breakdown of H_2O_2. This demonstrated that the powerful antioxidant enzymes reported by Parmar et al., 2012 in

Table 1. Antioxidant enzyme activity (U/g.f.w.) in black variety of *M. uniflorum* under nickel stress at three different stages of plant growth.

Parameters		Vegetative	Flowering	Fruiting
SOD	**Ni****	**Black**	**Black**	**Black**
	Control	47.77±4.03^{c*} (44.20-51.21)	62.40 ±4.23^{b*} (58.40 -6.52)	42.43±4.15^{b*} (39.6-47.3)
	25	125.60±4.72^{f*} (120.8-132.5)	138.00±4.85^{f*} (130.8-144.7)	98.32±3.69* (96.2-100.3)
	50	105.64±3.83^{e*} (102.4-108.7)	132.40±3.86^{e*} (130.2-135.7)	82.50±3.96* (80.2-86.5)
	75	87.02±4.22^{d*} (83.5-91.5)	122.22±4.2^{d*} (118.8-126.9)	66.50±4.19^{e*} (62.4-70.3)
	100	40.98 ±4.27^{b} (38.4-45.6)	81.42±4.97^{c*} (76.5-88.3)	40.47±3.85^{b*} (38.8-43.9)
	125	33.38±4.00^{a} (30.2-36.5)	54.43 ±3.97^{a*} (52.4-58.3)	34.43±3.79^{a*} (32.4-37.5)
CAT	Control	89.94±3.8^{c*} (86.8-92.3)	111.77±3.75^{c*} (109.6-114.3)	84.60±3.68^{d*} (82.0-86.3)
	25	181.25±4.13^{f*} (178.6-185.7)	192.53±5.11^{f*} (184.6-198.3)	113.47±4.32^{e*} (109.8-118.3)
	50	161.38±3.92^{e*} (158.4-164.3)	184.48±4.05^{e*} (181.3-188.5)	142.78±3.99^{f*} (140.2-146.4)
	75	104.10±4.91^{d*} (96.4-110.3)	144.57±4.2^{d*} (141.4-148.3)	86.37±4.49^{d*} (82.4-92.5)
	100	68.21±4.09^{b*} (64.3-71.4)	77.48±3.79^{b*} (75.3-80.5)	55.10±3.93^{b*} (52.2-58.3)
	125	45.17±4.01^{a*} (42.1-48.9)	38.47±4.00^{a} (35.2-41.5)	32.53±3.75^{a} (30.8-35.6)
GPX	Control	183.77±6.86d* (170.2-196.3)	202.10±4.36c* (198.4-206.9)	143.70±5.21c (138.2-152.3)
	25	233.70±4.41^{e*} (230.2-238.5)	241.88±5.76d (234.8-251.4)	193.43±4.14e* (189.6-197.6)
	50	201.86±4.98f* (196.8-209.2)	240.78±5.08d* (236.4-249.3)	163.63±5.05d (157.8-170.3)
	75	160.22±4.54c* (154.8-165.3)	182.17±3.87b* (180.4-186.3)	135.03±4.46b* (130.2-139.7)
	100	142.50±4.91b* (136.4-148.9)	182.38±4.22b* (178.9-186.3)	132.90±4.48b* (128.5-137.9)
	125	84.11±4.22a* (80.5-88.8)	74.47±4.10a* (71.9-78.5)	53.23±3.88a (51.1-56.5)

Colocassia esculentum (Parmar et al., 2012) were induced by the moderate concentration of nickel, activating an adequate defensive system against oxidative stress. Antioxidative enzyme activity increased in response to nickel (50 ppm), indicating that oxidative stress was strongly induced in *M. uniflorum.* Nickel stress-induced increase in CAT activity at lower exposure levels in the present experiment was evident in *M. uniflorum.* Nickel stress in the present investigation strongly enhanced GPX activity at lower concentration as a response to metal induced oxidative damage for removal of a major part of H_2O_2. In the present study increase in the SOD activity in *M. uniflorum* suggested an elevated content of superoxide radical in response to nickel stress up to 50 ppm. It might be possible that the inactivation of the enzyme by superoxide anions is the cause of the decreased SOD activity with increased nickel content. Decreased SOD activity with increasing concentrations of nickel might be due to the inactivation of enzyme with superoxide anions. Activity of GPX was more than that of CAT, suggesting that GPX is the major enzyme involved in the metabolism of hydrogen peroxide produced due to the action of SOD on superoxide radicals. The decrease in GPX and CAT at greater levels of nickel may be caused by a shift in protein assembling due to mental stress. In the current investigation, a new type of SOD was found in *M. uniflorum* of this black variety, with the highest intensity at 25 ppm of nickel and decreasing afterward. SOD1 was the most prominent form among all the SOD variants in the black variety. Thus, this work gives a glimpse of the role of antioxidant mechanism under stress conditions and changes in isozymes of antioxidants as a marker for stress tolerance as documented in rice. Before attempting any genetic and environmental change, it is imperative to study the physiological and biochemical pathways (Ashraf and Harris, 2004; Manaa et al., 2013). Numerous research examine how plants react to salt stressors and advance our knowledge of the physiological processes that underlie plant stress response. The majority of these studies on the effects of salinity have focused on species used in agriculture, fodder, and fuel wood. Nevertheless, little is known about how salt affects the development and yield of therapeutic plants.

Future Prospective

In the current era of heavy metal pollution *M. uniflorum,* which has a therapeutic ability for urolithiasis treatment, requires more investigation for its survival and growth. Because they are stationary, plants are powerless against biotic stressors like infections, parasites, grazing and abiotic stresses like drought, flooding, salt, low- to high-temperature swings, UV radiation, nutritional shortages, and heavy metal toxicity. Environmental stressors can affect a plant's growth, development, and output. These conditions frequently cause osmotic stress, increasing the production of reactive oxygen species (ROS) and upsets plant cells' homeostasis and ion distribution. Since membrane peroxidation damages the cell membrane, degrades biological macromolecules, and ultimately leads to cell death, generating and accumulating ROS in plants severely damages cell organelles and functions. The capacity of plants to scavenge the harmful effects of ROS appears to be the most significant. Antioxidants are the front line of defence against the ROS species and are essential to maintaining the plant's health. Antioxidants have wide range of activities in the development and physiology of plants through different variety of mechanism. Before introducing genetic and ecological improvement of abiotic stress, it is essential to study the physiological and biochemical mechanism. Antioxidant enzymes plays a vital role in this process. This work needs further analysis of Ni stress with respect to antioxidant activities.

Conclusions

The present investigation tries to evaluate the Ni concentration required for the development and multiplication of the plant. Nickel forms a major backbone of metalloenzymes such as urease, Ni-Fe hydrogenase and Ni-superoxide dismutase. However, at its high concertation it inhibits plant growth. Excess accumulation of Ni in plants leads to ROS development and oxidative stress. The investigation also showcases the antioxidant activities, a major process for scavenging the ROS. The activity of antioxidant enzymes increased up to 75 ppm of nickel exposure, indicating increased oxidative stress. However, lipid peroxidation levels and nickel uptake increased in a dose-dependent manner. The profiling of isozymes of antioxidant further details their activities in response to Ni stress. The band pattern of isoenzymes changed with different levels of nickel exposure.

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