



# Nickel Toxicity, Altering ROS Scavenging Mechanism and Impairing Sugar Based Signaling in *Vigna radiata* (L.) Wilczek during Germination and Seedling Establishment

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## Abstract

Nickel is a heavy metal that has a minor requirement in plants and exerts strong toxic effects at low concentrations. Seed germination and pre-greening stages of seedling establishment represent a brief heterotrophic phase in the life of an otherwise autotrophic organism. Germination is also a stage when the seed relies on stored reserves, including minerals rather than carrying out their uptake and has not been studied. The present work is a study of alteration of germination-related biochemical parameters in the presence of Nickel. Manifestations of Nickel induced oxidative stress as well as antioxidant defenses together with sugar sensing and respiratory enzymatic factors were investigated in seeds of *Vigna radiata*. Ni treatment increased electron flow through complex IV in cotyledons. It is proposed that inactivation of  $\alpha$ -amylase adversely affects sugar movement to the growing seedling, leading to poor sugar sensing by hexokinase. These factors, including possible mis-metallation of electron transport complexes, combine to produce the observed symptoms of Nickel toxicity. The altered antioxidants activity can be correlated with Reactive Oxygen Species production and subsequent changes in seed metabolism.

**Keywords** Antioxidants · Electron transport activity · Mis-metallation · Oxidative stress · ROS · Sugar sensing

## Introduction

Heavy metal contamination is a global environmental problem that endangers plant and animal life, with sources ranging from volcanic eruptions and weathering of rocks to industrial activities such as mining, combustion of fossil fuels and industrial sewage which causes Ni and other heavy metals to accumulate in the soil and enter the food chain (Munzuroglu and Geckil 2002; Zeller and Feller 1999; Shah and Nongkynrih 2007; Wuana and Okieimen 2011). Heavy metals

are known to induce changes in redox balance of the cell resulting in oxidative stress (Sreekanth et al. 2013). Secondary effects of oxidative stress such as impairment of membrane function due to lipid peroxidation and oxidation of proteins and nucleic acids compromise cellular function (Gutteridge and Halliwell 1990; Blokhina and Fagerstedt 2010; Dizdaroglu 1993).

Mitochondria are the site for energy production, for providing carbon skeletons for anabolism, while functioning as transient stores for metabolites and  $\text{Ca}^{2+}$  (Turrens 2003 & Duchon 2000). Mitochondria are also known to be the site for generation of reactive oxygen species (ROS) due to electron transfer to oxygen at sites other than the terminal step (Bunik and Sievers 2002; Murphy 2009 and Quinlan et al. 2012). It has been shown that divalent cations like  $\text{Ni}^{2+}$  and others such as  $\text{Pb}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$  and  $\text{Co}^{2+}$  can bind to mitochondrial membranes, disrupt electron transport and possibly lead to uncoupling of phosphorylation (Romanowska et al. 2002). Organisms have developed antioxidative mechanism for ROS scavenging (Noctor and Foyer 1998). These mechanisms are known to suffer damage and operate at a suboptimal level as stress levels rise, leading to a state of greater oxidative stress (Chen et al. 2009; Maheshwari and Dubey 2009).

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Understandably, there has been much more concern about the toxicity of Ni than its deficiency, although there have been very few studies at the stage of seed germination. Exposure to elevated Ni adversely affects biometric parameters (Siddiqui et al. 2011), amylase, protease and ribonuclease enzyme activity causing to retarded seed germination and reduced growth of many crops (Gajewska et al. 2006; Ahmad and Ashraf 2011; Dubey and Anjana 2011). The adverse impact of toxic levels of Ni on the photosynthetic apparatus and performance has been reported (Boominathan and Doran 2002). Metabolic alterations caused by nickel during germination and seedling established have not been probed in detail and form the central idea of the present investigation aims look at alterations to redox balance caused by excess Ni during germination and establishment *Vigna radiata* (Mungbean) seeds/seedlings. Change in ROS levels and the processes used by the developing machinery of the seed in an attempt to combat the oxidative stress generated/induced due to Ni toxicity have been evaluated. The work also examines the effect of Ni toxicity on the activity of respiratory enzymes and evaluates mobilization of starch reserves. The study was focused on germination and seedling establishment as it presents a very useful model, where one part of the plant, the growing axis, witnesses very high metabolic rates to support rapid cell division in this tissue, while the cotyledons serve as stores from which reserves are mobilized just prior to their death.

## Results

### Nickel Accumulation

*Vigna* seeds grown in Nickel chloride solution accumulate a remarkable amount of Nickel during the 48 h period of Ni exposure. Ni accumulation in axis was an order of magnitude higher than that of cotyledons (Table 1). In cotyledons of Ni treated seeds, Ni accumulation was almost twelve fold higher than control cotyledon while in axis of Ni treated seeds, Ni accumulation was almost forty fivefold higher in magnitude when compared to axis of control seeds.

**Table 1** Nickel content

S.No.	Sample	Ni content ( $\mu\text{g g}^{-1}$ DW) Mean $\pm$ SD
1	Control cotyledon	0.673 $\pm$ 0.061
2	0.5 mM Nickel cotyledon	7.929 $\pm$ 0.429
3	Control axis	2.547 $\pm$ 0.322
4	0.5 mM Nickel axis	114.16 $\pm$ 10.61*

\*:\* The mean difference is significant at 0.05 level

### Changes in Biometric Parameters

Treatment of seeds with varying concentrations of  $\text{Ni}^{2+}$  for 48 h did not show any considerable effect on germination since the concentration was chosen this way. However, FW and DW of cotyledons as well as axis and axis length were adversely affected by Ni treatment (Table 2, Fig. 1).

### Effect on Starch Reserve & Soluble Protein Mobilization and $\alpha$ -Amylase Activity

During germination of *Vigna* seeds, remarkable difference in total starch content and  $\alpha$ -amylase activity was observed both in the control and Ni treated seeds. Under Ni stress, axis didn't protrude much, more starch and less  $\alpha$ -amylase activity was observed in cotyledons of Ni treated seeds as compared to control one. A significant increase of 500% starch content was observed in cotyledons of Ni treated seeds when it was compared with control cotyledons whereas axis of both Ni treated and control seeds didn't show much variation in their starch content (Fig. 2 a). In terms of germination affecting enzymes,  $\alpha$ -amylase showed more activity in cotyledons and axis of Ni treated seeds as compared to Ni treated seeds. A significant 19% decrease was observed in cotyledons of Ni treated seeds which can be correlated with more starch accumulation in the same while cotyledons of control seeds showed more  $\alpha$ -amylase activity attributed to less starch accumulation (Fig. 2 b). Protein content shows slight increased mobilization in Ni treated seeds as compared to control one (Fig. 2 c).

### Changes in Enzyme Activities

SOD activity, the first line of defense against the toxic effects of elevated levels of ROS was found to be increased in cotyledons by 45% while in axis it was significantly decreased by 23% of Ni treated seeds than control cotyledons and axis (Fig. 3 a).

Decreased APX activity was observed in cotyledons and axis of Ni treated seeds. A significant decrease of 10% in APX activity in axis and cotyledons (63%) of Ni treated seeds was observed compared to control seeds (Fig. 3 b). GPX activity was decreased in Ni treated seeds compared to control seeds in both cotyledons and axis (Fig. 4 a). The decrease in GPX activity was observed in axis (by 9%) and cotyledons (by 31.2%) of Ni treated seeds was observed as compared to axis and cotyledons of control seeds. GST activity was significantly higher in axis of Ni treated seeds by 82% as compared to axis of control, but cotyledons of Ni treated seeds did not show much change (Fig. 4 b).

An increase in GR activity was observed in cotyledons and axis of Ni treated seeds (Fig. 4 c). GR activity was higher in cotyledons of Ni treated seeds by 160% than control one while

**Table 2** Response of *Vigna* seeds to graded levels of Ni during germination and seedling establishment

S. No.	Ni (mM)	% germination	Axis Length (cm)	Cotyledons FW (mg) Mean $\pm$ SD	Cotyledons DW (mg) Mean $\pm$ SD	Axis FW (mg) Mean $\pm$ SD	Axis DW (mg) Mean $\pm$ SD
1	0.0	100	8.8	520 $\pm$ 9.51	130 $\pm$ 11.67	950 $\pm$ 3.01	82 $\pm$ 2.88
2	0.5	100	2.8	630 $\pm$ 5.16	167 $\pm$ 4.72*	425 $\pm$ 3.24*	39 $\pm$ 1.15*
3	1.0	100	1.87	925 $\pm$ 10.27*	222 $\pm$ 7.21*	125 $\pm$ 5.5*	10 $\pm$ 0.57*
4	1.5	90	1.73	895 $\pm$ 8.81*	214 $\pm$ 9.64*	140 $\pm$ 1.71*	19 $\pm$ 1.15*
5	2.0	100	1.06	930 $\pm$ 6.16*	219 $\pm$ 2.08*	85 $\pm$ 2.33*	11 $\pm$ 1.15*
6	2.5	90	0.91	890 $\pm$ 10.40*	250 $\pm$ 7.63*	115 $\pm$ 2.03*	11 $\pm$ 1.00*
7	3.0	100	0.76	840 $\pm$ 5.05*	221 $\pm$ 6.52*	75 $\pm$ 2.53*	10 $\pm$ 0.57*

Values provided in each column are mean of triplicate observations. \*\* The mean difference is significant at 0.05 level

only 13% increase was observed in case of axis of Ni treated seeds than axis of control seeds.

MDHAR activity was decreased in both cotyledons and axis of Ni treated seeds by 17% and 31% respectively (Fig. 3 c). DHAR activity was increased significantly in cotyledons of Ni treated seeds almost by 136% than control seeds while in axis of Ni treated seeds, DHAR activity was significantly decreased by 39%, compared to control axis (Fig. 3 d).

Among non-enzymatic antioxidants, ascorbate levels were found to increase significantly in both cotyledons and axis of Ni treated seeds by 43% and 33% respectively when compared to control cotyledon and axis (Fig. 5 a). Phenolic content was found to be significantly decreased in cotyledons of Ni treated seeds by 19% when compared with control cotyledon but no significant difference was observed in axis of Ni treated seeds (Fig. 5 b).

### Effect on Oxidative Stress Markers

MDA level showed significant increase of 102% in cotyledon of Ni treated seeds compared to control cotyledons (Fig. 6 a). Protein carbonyl content was increased by 21% in cotyledons

of Ni treated seeds and increased significantly by 106% in axis of Ni treated seeds, compared to control axis (Fig. 6 b).

### Effect of Nickel Toxicity on ROS Levels

Total ROS, H<sub>2</sub>O<sub>2</sub>, O<sub>2</sub><sup>•-</sup> and OH<sup>•</sup> in axis and cotyledon of Ni treated seeds were measured and compared with control seeds. Cotyledons and axis of Ni treated seeds showed less DCFH oxidation to DCF than control seeds (Fig. 7 a), reported in Relative Fluorescence Unit (RFU). Decreased amount of hydrogen peroxide was observed in cotyledons of Ni treated seeds as compared to control cotyledons. Total ROS and H<sub>2</sub>O<sub>2</sub> level in cotyledons of Ni treated seeds showed a significant decrease of 20% and 14% respectively while no significant changes were observed in axis of Ni treated seeds than control axis (Fig. 7 a & b).

A significant decreased hydroxyl by 52% was observed in cotyledons of Ni treated seeds compared to control cotyledon but no significant changes were observed between axis of both sets (Fig. 7 c). Level of superoxide was significantly higher in cotyledons and axis of Ni treated seeds by 34% and 92% respectively than control seeds (Fig. 7 d).

**Fig. 1** *Vigna radiata* seeds grown in distilled water (as control) and in varying concentration of Nickel



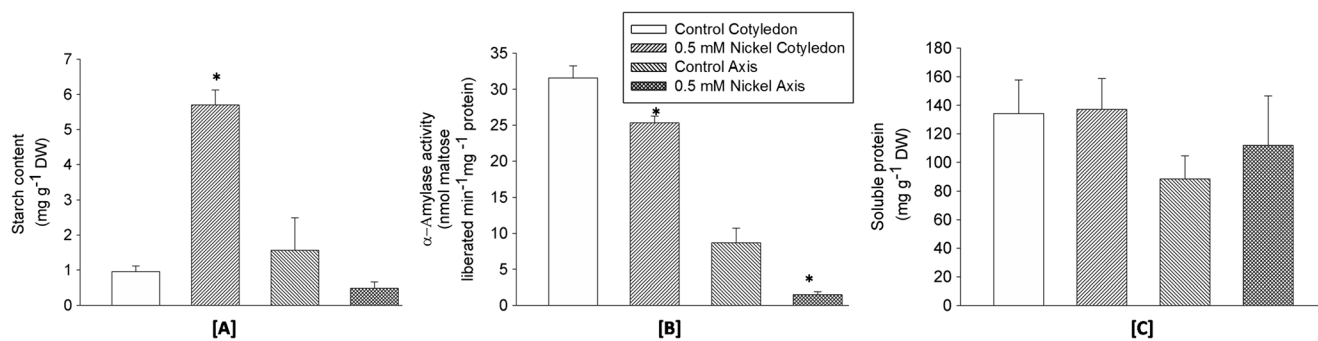


Fig. 2 Effect of 0.5 mM Ni on Starch, soluble protein mobilization and  $\alpha$ - amylase activity

### In Vivo Detection of O<sub>2</sub><sup>•-</sup> and H<sub>2</sub>O<sub>2</sub> Production in Ni Treated Seeds

Figure 8 shows the in vivo staining by NBT in control and Ni treated seeds. Staining of the growing zone demonstrates that O<sub>2</sub><sup>•-</sup> formation is preferentially more in axis of both sets. Similar results were obtained using 3, 5, 3', 5'-tetramethylbenzidine-HCl (TMB) as a probe for H<sub>2</sub>O<sub>2</sub> in the presence of endogenous peroxidase (Fig. 8). H<sub>2</sub>O<sub>2</sub> was more accumulated in growing axis of both sets.

### Effect of Ni Toxicity on Key Enzymes of Electron Transport Complexes Involved in Energy Metabolism

Hexokinase activity showed a significant difference in Ni treated seeds from the control seeds under Ni induced stress. Cotyledons and axis both, respond differently towards Ni stress. While cotyledons of Ni treated seeds show a decrease of 25% compared to control cotyledons, hexokinase activity in axis from Ni treated seeds found to be increased by 37% compared to control axis (Fig. 9 b). SDH activity was

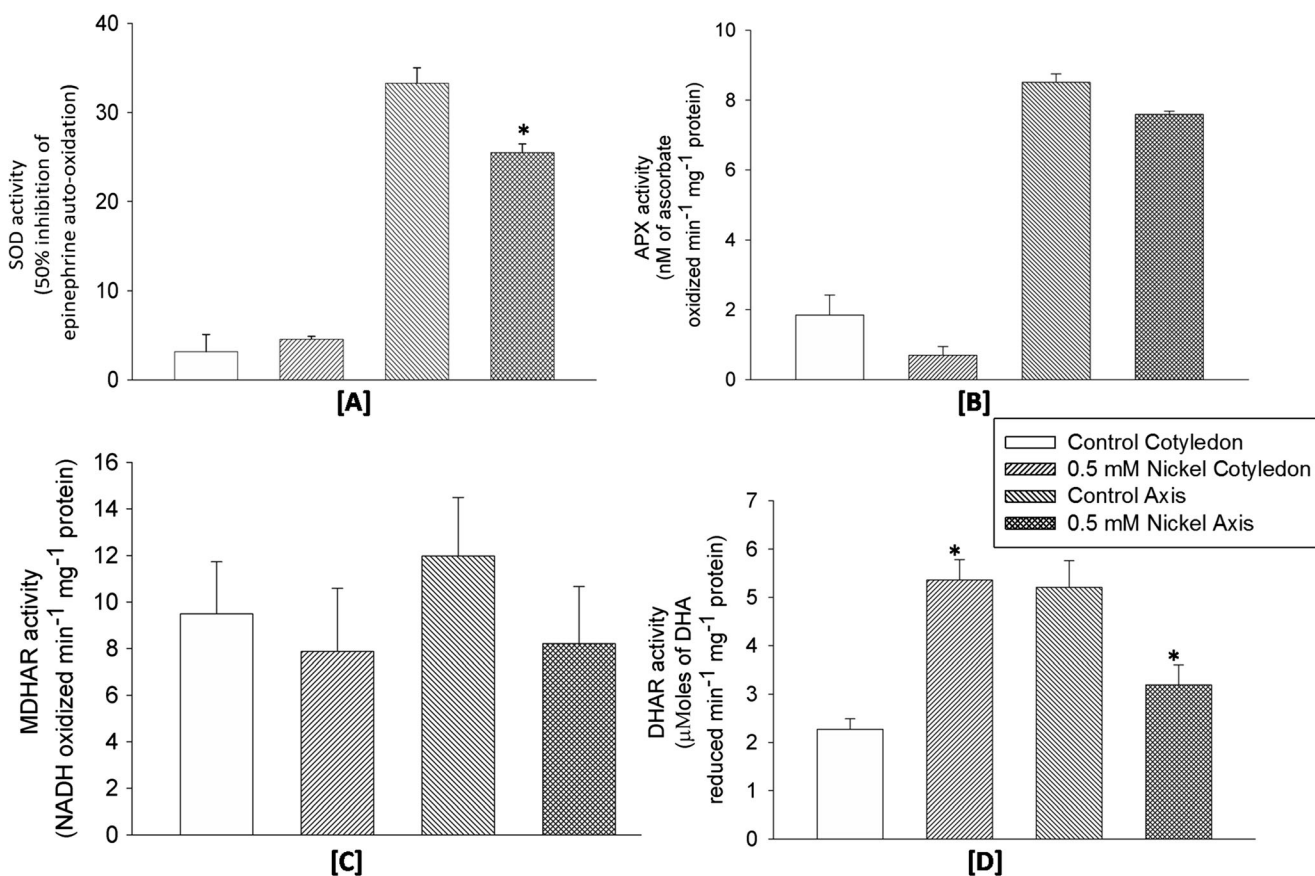
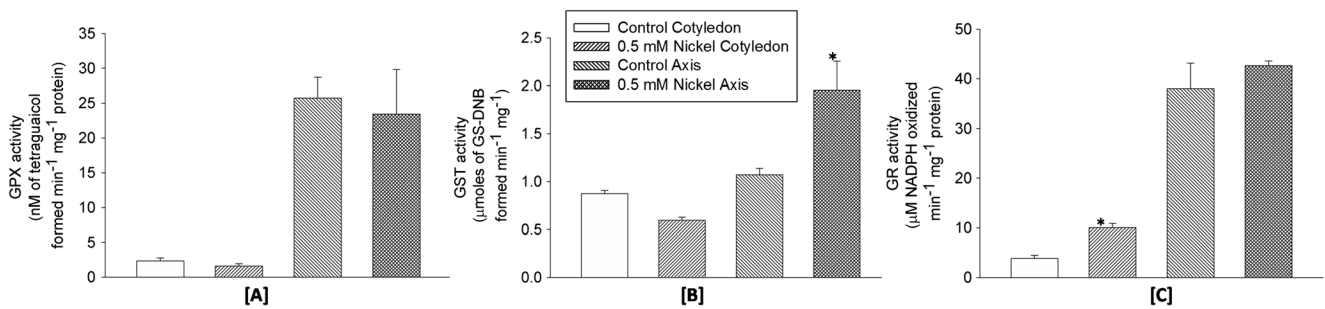


Fig. 3 Effect of 0.5 mM Ni on Superoxide dismutase, Ascorbate peroxidase, Monodehydroascorbate reductase and Dehydroascorbatereductase activity



**Fig. 4** Effect of 0.5 mM Ni on Guaiacol peroxidase, Glutathione-s-transferase and Glutathione Reductase activity

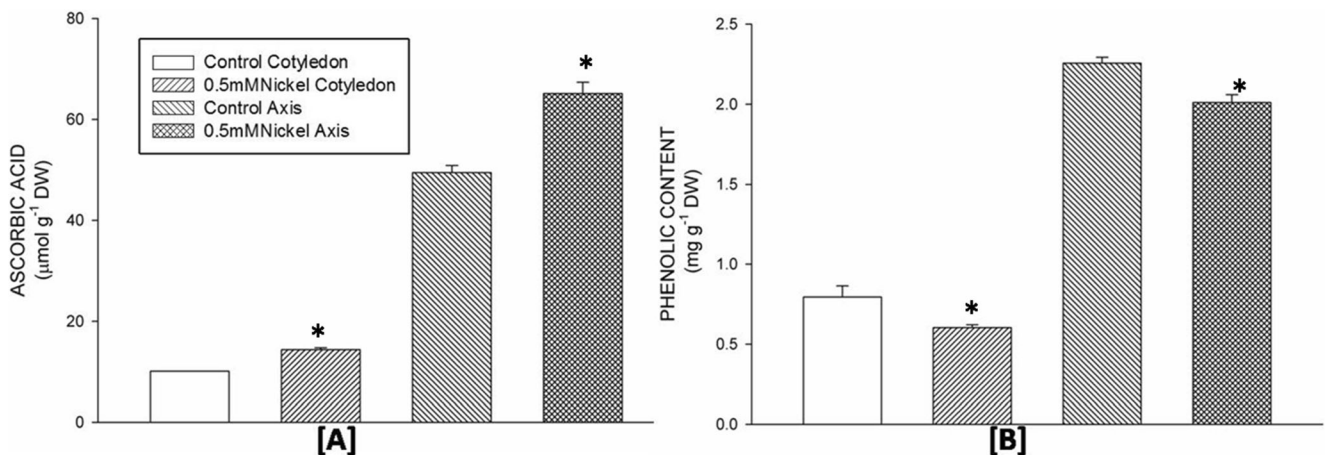
observed to be decreased due to Ni treatment. SDH activity of cotyledons of control and Ni treated seeds didn't show any significant change while axis from Ni treated seeds showed decrease (16%) as compared to axis from control seeds (Fig. 9 A). Cytochrome C oxidase activity was found to decrease due to Ni induced toxicity. Cotyledons of Ni treated seeds showed an increased oxygen consumption of 32% as compared to control one while axis of Ni treated seeds has 13% less Cyt C oxidase activity as compared to control axis (Fig. 9 c).

## Discussion

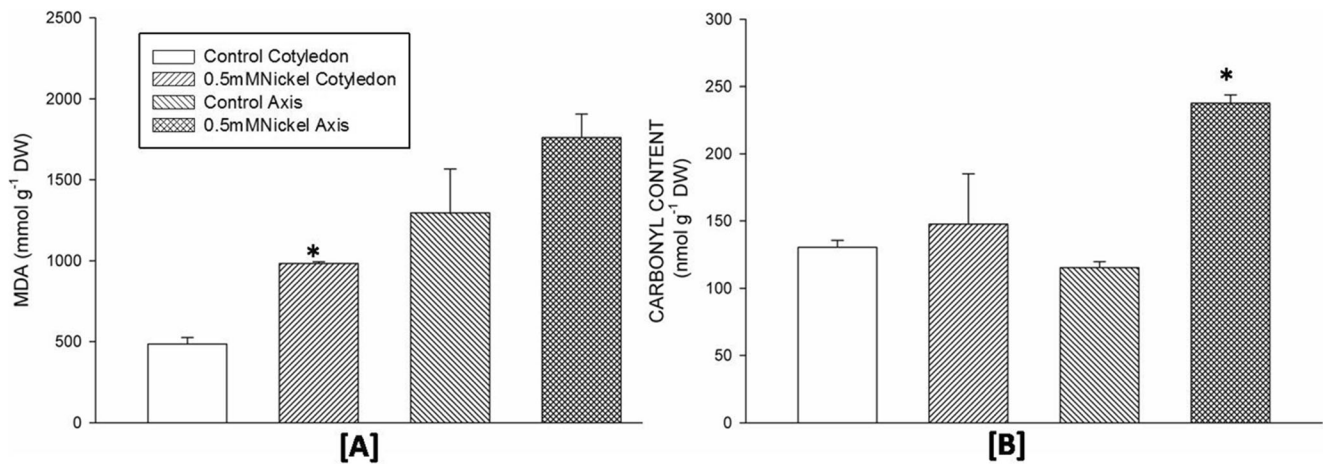
Due to biotic or Abiotic stress in plants, the first observable responses include reduced metabolic activities and decreased rate of growth. It has been suggested that growth reduction due to heavy metal stress generally occurs due to loss of turgor pressure which results in decreased mitotic activity or inhibited cell elongation (Baccouch et al. 2001). In this work, results showed that biometric parameters of *Vigna radiata* seeds are affected by high doses of Ni. Changes were not apparent for the first 24 h of growth under conditions of Ni exposure but by the second day of growth, they manifested as slower growth of axis (Fig. 1). Ni is known to affect growth and water balance of plants (Molas and Baran 2004) and here

it is reflected by the altered fresh weight to dry weight ratio in Ni treated seeds (Table 2).

Desiccation occurring during seed development results in extensive membrane damage. As water is imbibed, reassembly of membranes occurs. In this study, Ni accumulates in *Vigna* seeds to a significant extent when grown in the presence of the metal (Table 1). It can be explained as Ni is present in the imbibing solution; it is likely to be absorbed into the tissues without a specific uptake mechanism. Hence cotyledons show Ni accumulation despite the complete lack of cell division. The rapidly growing embryonic axis represents a completely different picture. Axis tissue exhibits very rapid growth and understandably accumulates Ni along with other mineral ions. During germination, phytase activity releases minerals from cotyledons, mobilizing them towards the growing axis, possibly Ni also begins to accumulate in large amounts, explaining the high Ni content of axis/seedlings. Growth impairment is therefore evident only two days after imbibition, but becomes severe after that stage. Significant difference in MDA content was observed between the control and nickel treated seeds, reflecting more membrane damage under nickel stress (Fig. 6 a). MDA is the decomposed product of polyunsaturated fatty acids (PUFA) of biomembranes and its increased content shows plants under a high level of oxidative stress (Yilmaz and



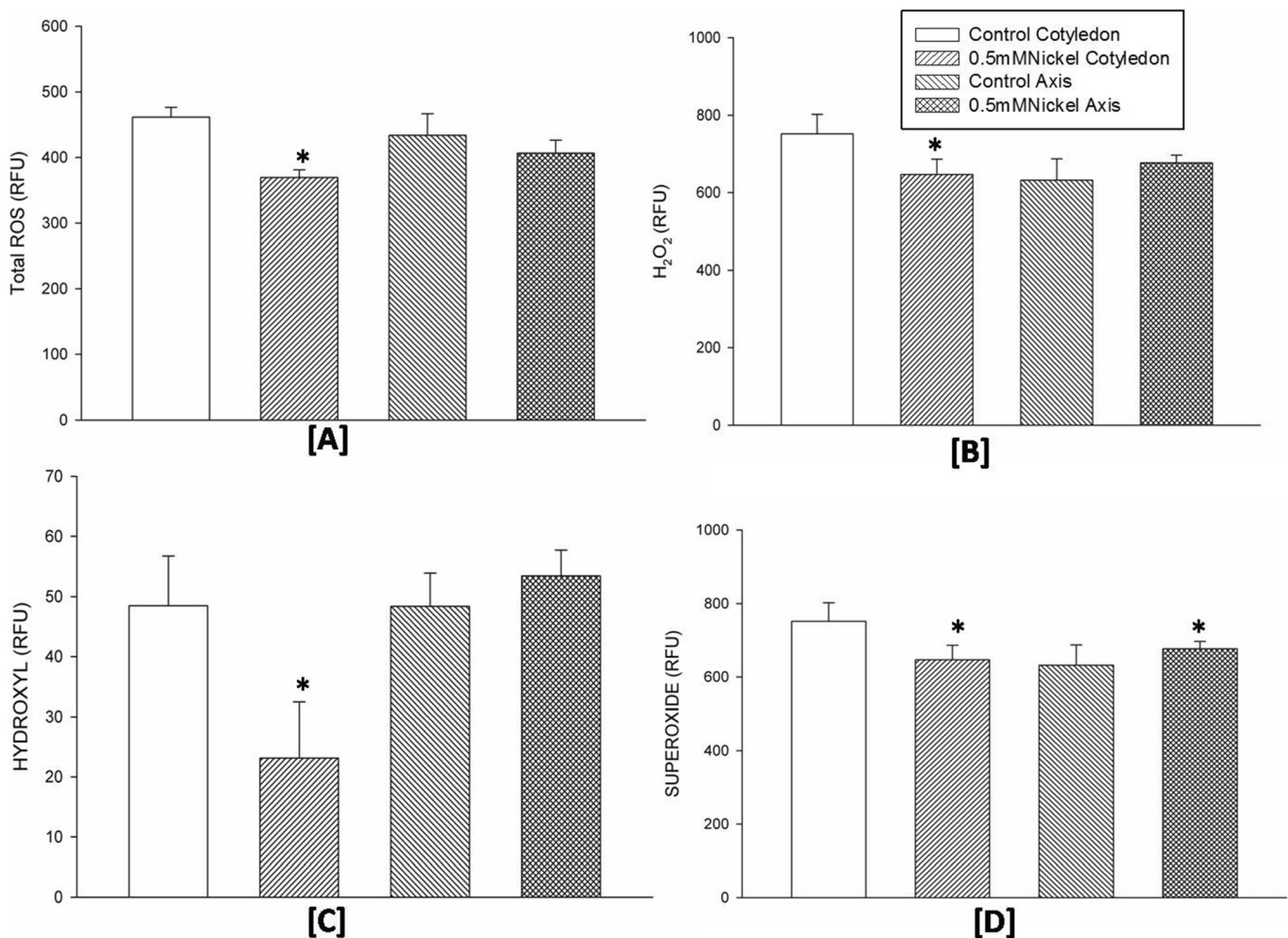
**Fig. 5** Effect of 0.5 mM Ni on Ascorbic acid and Phenolic content



**Fig. 6** Effect of 0.5 mM Ni on Malondialdehyde and Protein Carbonylation content

Parlak 2011). This result is supported by the findings of Baccouch et al. (2001), Yilmaz and Parlak (2011) and Siddiqui et al. (2011) in *Zea mays* roots, *Lemna gibba* and *Triticum aestivum* respectively. Protein oxidation is considered to be a potential marker of oxidative stress together with altered MDA content. Oxidation of proteins can occur

through a number of different mechanisms, such as the formation of disulfide cross-links and glycoxidation adducts nitration of tyrosine residues and carbonylation of specific amino acid residues (Davies 2005). Result demonstrates increased carbonyl content in both cotyledon and axis of Ni treated seeds (Fig. 6 b).



**Fig. 7** Effect of 0.5 mM Ni on total ROS, Hydrogen peroxide, Hydroxyl and Superoxide level

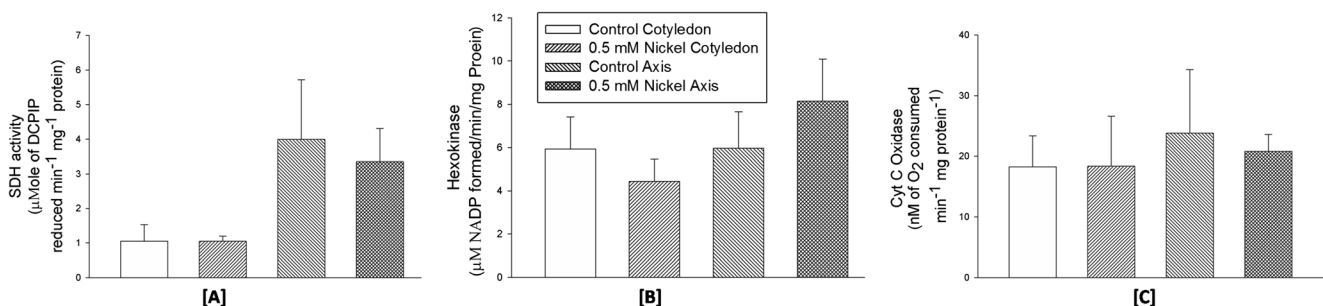
**Fig. 8** in situ staining for superoxide and  $H_2O_2$  by NBT and TMB respectively. (A-1: Control NBT; A-2: 0.5 mM Ni NBT; B-1: Control TMB; B-2: 0.5 mM Ni TMB)



Oxidative stress due to environmental conditions promotes generation and accumulation of ROS, causing oxidation of cellular components and hindering metabolic activities (Suzuki et al. 2012). To overcome oxidative stress, antioxidative mechanism is established by the nature. In this study, activity of antioxidative enzymes has been investigated separately in both cotyledons and axis of control and Ni treated seeds. While SOD activity was found to be high in cotyledons of Ni treated seeds, SOD activity in axis of Ni treated seeds was found to be decreased when compared to control seeds, suggesting the accumulation of Ni in the germinating stage and showed a strategy of defense to lower the oxidative stress (Fig. 3 A). Altered SOD activity can be explained by observing the superoxide level in cotyledons and axis of Ni treated seeds, where significantly low and high levels of superoxide were reported in cotyledons and axis of Ni treated seeds respectively (Fig. 7 d). Further, in vivo staining of the control and treated seeds by NBT explains the altered presence of superoxide free radical in control and Ni treated seeds (Fig. 8). The same pattern of reduced activity in axis of Ni treated seeds was observed in case of APX and GPX (Fig. 3 b & 4 a). GR activity was found to be increased in both

cotyledons and axis of Ni treated seeds while GST activity was higher in axis of Ni treated seeds with decreased activity in cotyledons, compared to control seeds (Fig. 4 b & c). Further, non enzymatic antioxidants such as ascorbic acid and phenolic content were evaluated. Both were found to be increased and decreased respectively in cotyledons and axis of Ni treated seeds (Fig. 5). MDHAR activity was decreased in both cotyledons and axis of Ni treated seeds which can be correlated with ascorbate levels present in the seeds, as later works as substrate for MDHAR (Fig. 3 c). However, DHAR activity was significantly higher in cotyledons of Ni treated seeds (Fig. 3 d) than the control cotyledon, suggesting more non-enzymatic disproportionation of MDHA to DHA within the cell as both are relative participants of Ascorbate-glutathione cycle. High ascorbic acid content was observed in cotyledon and axis of Ni treated seeds which can be attributed to high DHAR activity in them and found to be less in cotyledon and axis of control seeds respectively.

Investigation of ROS levels showed decreased level of total ROS content in axis and cotyledons of Ni treated seeds (Fig. 7 a). This can be attributed to increased levels of SOD, GR,



**Fig. 9** Effect of 0.5 mM Ni on Succinate dehydrogenase, Hexokinase and Cytochrome C oxidase activity

DHAR and GST activities which tried to lower the Ni induced oxidative stress. Levels of superoxide, hydrogen peroxide and hydroxyl were found to be less accumulated as compared to control seeds. Altered  $H_2O_2$  level can be correlated with the level of ascorbic acid, as it acts as a substrate with the greatest reducing activity (Cheruth et al. 2009). In situ staining with TMB showed more accumulation of  $H_2O_2$  in control axis as compared to axis of Ni treated seeds (Fig. 8). It is well known that  $H_2O_2$  plays very important roles in cell signaling in both plants and animals and is important for cellular processes, mainly in plants (Foyer and Noctor 2005). Therefore it can be concluded that due to more  $H_2O_2$  in control axis, their growth was faster than nickel treated seeds where less accumulation of this free radical occurred.

The respiratory enzyme activities may be involved in the response to metal stress conditions. Recently, sugar sensing has been an area that has seen tremendous progress with the proof of concept coming. Hexokinase (HXK) has been involved in sensing sugar levels and coordinating metabolism (Rolland et al. 2006). The enzyme is meant to phosphorylate hexoses prior to their entry into the glycolytic pathway (Granot et al. 2013). In this study, HXK activity (Fig. 9 b) exhibits a very strange behavior as it was increased in the growing axis and decreased in cotyledons of Ni treated seeds. However, decrease of HXK activity does not seem to be an independent factor. The amylase activity also shows significant differences in Ni treated tissues (Fig. 2 b). This is a significant finding as amylase cleaves starch to generate the mobile carbohydrate pool to be transported into the axis, from the cotyledons during germination. Amylase has a recognized  $Ca^{2+}$  binding site, with the ion being necessary for its activity (Bush et al. 1989).  $Ni^{2+}$  has precisely the same valency and hydrated ion radius as  $Ca^{2+}$  (Keilland 1937). As such, exposure to high Ni content may cause amylase to bind significant amounts of Ni to bind with amylase, bringing about its partial inactivation or suboptimal activity (Fig. 2 b). It is well established that metallation of enzymes requires metals at a concentration much higher than the metals availability (Foster et al. 2014) and specific metal delivery systems are required for this process. However, under the conditions of Ni exposure considered in this work,  $Ca^{2+}$  availability is less than  $Ni^{2+}$ . Valency and hydrated ion diameter being very similar, Ni metallation may be favored. Oxidative stress has been observed to cause defects in the metallation of metalloenzymes (Imlay 2014) and the same can be expected in the case of metalloprotein complexes involved in respiratory electron transport. The high concentrations of  $Ni^{2+}$  needed to exert toxicity in comparison to those required for photosynthetically active plants is explained by the fact that at the germination stage and the initial stages of seedling establishment, Nickel toxicity is exerted through a competition between this ion and other elements sequestered in the seed. The cotyledons have comparatively higher electron transport activity and therefore

the major effect appears to be on the axis. However, the possibility of inactivation of amylase being very high in Ni treated seeds, sugar supply to the axis and perhaps sugar sensing through hexokinase is severely hampered. These factors, including mis-metallation may combine to produce the observed symptoms of Ni toxicity.

## Methods

*Vigna radiata* seeds (Var. PDM-139) were obtained from Indian Institute of Pulses Research (IIPR), Kanpur (India). Spectrophotometric analysis for respiratory, enzymatic and non-enzymatic antioxidants was performed with GE Ultrospec 2100 Pro UV Vis Spectrophotometer. ROS were analyzed in Varioskan™ Flash Multimode Reader provided by Thermo scientific. Chemicals were procured from HiMedia Laboratories and Sigma- Aldrich. Seeds were screened for their tolerance range for Ni by imbibing them in the presence of 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM and 0.5 mM, 1 mM, 1.5 mM, 2 mM, 2.5 mM and 3 mM Ni, supplied as hydrated Nickel chloride ( $NiCl_2 \cdot 6H_2O$ ). Germination and seedling growth were monitored. No appreciable difference in seedling growth was observed in initial concentrations up to 0.4 mM (Fig. 1). 0.5 mM Ni was chosen for further studies as seeds showed 100% germination with significantly lesser axis growth in comparison to the control set of seeds germinated in distilled water (Table 2). Seeds were first imbibed for 4 h in the relevant concentration of Ni and transferred to petridishes lined with Whatman1 filter papers soaked in the same  $NiCl_2$  concentration as used for imbibition. Seeds were placed in an incubator adjusted to 32 °C with a 13 h light/11 h dark cycle for 48 h. Seeds imbibed in distilled water were used as control. After 48 h, percentage germination, axis length was measured while FW and DW were recorded separately for cotyledons and axis. Three sets of 10 seeds were randomly selected for each biometric observation.

Cotyledons and axis from control and Ni treated seeds were homogenized separately on ice, using pestle and mortar, in chilled 50 mM Potassium phosphate buffer, pH 8.0 including 100 mM EDTA, 1% (w/v) PVP and 0.5% Triton X-100. The crude extracts were centrifuged at 20,000 g for 20 min at 4 °C and supernatants used for enzyme assays. Protein content was determined according to Bradford (1976) using BSA as a standard.

## Nickel Content Measurement

Ni content was determined as described in Kramer et al. (1997). Control and Ni treated cotyledons and axes were dried at 60 °C for 3 d and repeatedly weighed till constant weight was obtained in three consecutive weighings. 1 g dry tissue was subsequently digested at 180 °C in concentrated nitric



acid. Samples were cooled to RT. 2 ml 30% (w/v) hydrogen peroxide was added to the cooled samples and the mixture heated again at 180 °C till it become clear. Cooled and deionized water was added to a final volume of 5 ml. Ni concentrations were measured using inductively coupled plasma emission spectroscopy (Perkin-Elmer). Certified standards (Sigma) were similarly carried through the digestions and analyzed as part of the quality assurance/quality control protocol. Samples were analyzed in triplicate. Nickel concentration was expressed as  $\mu\text{g Nickel g}^{-1}$  DW of tissue.

### Determination of $\alpha$ -Amylase Activity and Starch Content

The cotyledons and axis were homogenized in chilled 20 mM phosphate buffer (pH 6.9), filtered through four layers of muslin cloth and centrifuged at 10,000 g for 10 min at 4 °C to prepare a 40% w/v crude extract. The supernatants obtained were used for amylase assay. One unit of amylase activity is defined as 1  $\mu\text{mol}$  of reducing groups (maltose) released per minute under the following conditions: pH 6.9, temperature 25 °C and reaction mixture consisting of 1.0 ml starch solution 1.0% (w/v) and 0.25 ml of extracted enzyme solution, incubated for 10 min and then 2 ml Dinitrosalicylic acid reagent was added to each tube. Tubes were incubated in a boiling water bath for 5 min and cooled to RT. 5 ml distilled water was added to each tube and absorbance was measured at 540 nm, using maltose as standard (Bernfeld 1955).

To measure starch, seeds extracted were prepared in 80% methanol containing a pinch of 1 N  $\text{Na}_2\text{CO}_3$ . After 15 min of Centrifugation at 4000 g, Pellets were taken and dried for 4–5 h at RT and then left for drying overnight at 60–70 °C. Dried pellets were hydrated with distilled water for half an hour to get swelled up where starch gains water. Perchloric acid was added and samples were incubated for 10 min. Water was added in the samples and mixed up with a glass rod. After again doing centrifugation at 4000 g for 10 min, supernatant was ready for starch estimation. Supernatant was mixed with Iodine and aqueous NaCl to form a starch-iodine complex and incubated for half an hour. Samples were centrifuged at 4000 rpm for 5 min. Pellet was washed with alcoholic NaCl and again centrifuged at 3000 rpm for 10 min. After washing with alcoholic NaOH, 5–6 ml of distilled water was added to resuspend the pellet. The resultant product was hydrolysed by adding 2 ml of 4 N HCl. The hydrolyzed sample was used for estimating starch by Phenol- $\text{H}_2\text{SO}_4$  method (Sturgeon 1990).

### Antioxidant Enzyme Assays

#### Superoxide Dismutase

SOD was assayed according to Misra and Fridovich (1972). The reaction mixture in a total volume of 1.5 ml contained

100 mM carbonate buffer (pH 10.3), 100 mM EDTA and 40  $\mu\text{l}$  enzyme extract. The reaction was initiated by the addition of 60  $\mu\text{l}$  of 15 mM epinephrine to the reaction mixture. Inhibition of epinephrine auto-oxidation was determined by measuring the increase in absorbance at 480 nm for 3 min at 15 s intervals. One unit of SOD activity was expressed as 50% inhibition of epinephrine auto-oxidation  $\text{min}^{-1} \text{mg}^{-1}$  protein, under assay conditions.

#### Ascorbate Peroxidase

APX was assayed according to Nakano and Asada (1981). 2 ml of the reaction mixture contained 100 mM potassium phosphate buffer, pH 7.0, 5 mM ascorbic acid and 20 mM  $\text{H}_2\text{O}_2$ . Reaction was initiated by the addition of 100  $\mu\text{l}$  enzyme extract.  $\text{H}_2\text{O}_2$  dependent oxidation of ascorbate was followed as decrease in absorbance at 290 nm at intervals of 15 s for 2 min. One unit of enzyme activity was defined as the amount of enzyme oxidizing 1  $\mu\text{mol}$  of ascorbate  $\text{min}^{-1} \text{mg}^{-1}$  protein under the above assay conditions.

#### Glutathione Reductase

GR activity was assayed by following the increase in absorbance at 412 nm due to the reduction of 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) by GSH (Smith et al. 1988). The assay mixture had a total volume of 2 ml and comprised of 250 mM potassium phosphate buffer (pH 7.5), 100 mM EDTA, 1 mM NADPH, 10 mM oxidized glutathione (GSSG), 10 mM DTNB and 200  $\mu\text{l}$  of crude extract. GR activity was followed as the increase in absorbance at 412 nm, at intervals of 15 s for 2 min. One unit of GR activity was expressed as  $\mu\text{mol}$  of NADPH oxidized  $\text{min}^{-1} \text{mg}^{-1}$  protein.

#### Guaiacol Peroxidase

Guaiacol peroxidase activity was assayed as in Vitoria et al. (2001). 2 ml assay mixture contained 1% guaiacol and 0.3%  $\text{H}_2\text{O}_2$  in 50 mM potassium phosphate buffer, pH- 7.0. The reaction was started by the addition of 50  $\mu\text{l}$  of the enzyme extract. The reaction was followed as production of tetraguaiacol by monitoring the change in absorbance at 470 nm for 2 min at intervals of 10 s. One unit activity was defined as the amount of enzyme producing 1  $\mu\text{M}$  tetraguaiacol  $\text{min}^{-1}$ .

#### Glutathione-S-Transferase

GST was estimated following the protocol of Habig et al. (1974). 1 ml assay mixture contained 300  $\mu\text{l}$  of enzyme extract, 1 mM GSH, 1 mM 1-chloro-2, 4-dinitrobenzene (CDNB) in 1 mM potassium phosphate buffer, pH 6.5. Reaction was followed as by monitoring the increase in

absorbance at 340 nm measured at intervals of 15 s for 2 min. One unit of GST activity was defined as formation of GS-DNB adducts  $\text{min}^{-1} \text{mg}^{-1}$  protein.

### Monodehydroascorbatereductase

MDHAR was estimated following the protocol of Hossain et al. (1984). 2 ml assay mixture contained 50 mM HEPES-KOH, pH 7.6, 0.2 mM NADH, 2.5 mM Ascorbate, 0.5 U ascorbate oxidase and 100  $\mu\text{l}$  of enzyme extract. Reaction was followed by monitoring NADH oxidation as a decrease in absorbance at 340 nm measured at intervals of 15 s for 2 min. The activity of MDHAR was calculated using an extinction coefficient of  $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$  for NADH at 340 nm. One unit of MDHAR activity was defined as the amount of enzyme required to oxidize 1  $\mu\text{mol}$  of NADH  $\text{min}^{-1} \text{ mg}^{-1}$  protein.

### Dehydroascorbatereductase Activity

DHAR activity was assayed according to De Tullio et al. (1998) with some modifications. DHAR was measured by following the reduction of DHA (Dehydroascorbate) to ascorbate in a reaction mixture of 2 ml containing 50 mM potassium phosphate, pH 7.0, 0.5 mM DHA, 2 mM GSH and 100  $\mu\text{l}$  of enzyme extract. The reaction was followed by monitoring the increase in absorbance at 265 nm, measured at 15 s intervals for 2 min. DHAR activity was calculated using an extinction coefficient of  $12.59 \text{ mM}^{-1} \text{ cm}^{-1}$  for ascorbate and expressed as  $\mu\text{mol}$  ascorbate produced  $\text{min}^{-1} \text{ mg}^{-1}$  protein.

## Non-enzymatic Antioxidant Assays

### Ascorbic Acid Content

Ascorbic acid content was determined by the method of Omaye et al. (1979). 1 ml of 10% TCA (Trichloro acetic acid) was added to 1 ml of the extract, kept on ice for 10 min and centrifuged at 15000 g for 20 min. Ascorbate was estimated by reacting it with DTC reagent (2, 4-Dinitrophenyl hydrazine–thiourea-  $\text{CuSO}_4$ ) by incubation for 3 h at 37 °C, followed by acidification by the addition of 1.5 ml of 65%  $\text{H}_2\text{SO}_4$  (v/v). Acidified assay mixtures were incubated for 30 min at RT and absorbance measured at 520 nm. Ascorbic acid content was determined using a standard Ascorbic Acid curve. Results were expressed in  $\mu\text{mol g}^{-1}$  DW.

### Phenolic Content

Phenolic content was determined using spectrophotometric method (Folin and Denis 1915). 3 g of Cotyledon and axis were taken separately for extract preparation by grinding in 80% methanol. Prepared extracts were centrifuged at 10000 g

for 10 min and supernatant was further used. The reaction mixture contained 0.25 ml of methanolic extract, 1.75 ml water, 2 ml Folin-Dennis reagent, incubated at RT for 10 min. 2 ml of 1 N  $\text{Na}_2\text{CO}_3$  was added after 10 min to each test tube and again incubated for 1 h. Absorbance was measured at 660 nm. Tannic acid was used as standard. Results were expressed in  $\text{mg g}^{-1}$  DW.

## Determination of Oxidative Stress Markers

### Lipid Peroxidation Estimation

Lipid peroxidation was determined by the Thiobarbituric acid reactive substances (TBARS) assay (Rubin et al. 1976). Sample for determining lipid peroxidation was same as used for antioxidative enzymes activity in above assays. To 0.5 ml of the homogenate, 0.2 ml sodium dodecyl sulphate (SDS), 8.1% (w/v), 1.5 ml 20% acetic acid, pH 3.5 and 1.5 ml thiobarbituric acid (TBA), 0.8% (w/v) and water were added to make up the volume to 4 ml. The contents of the tubes were vortexed, heated to 90 °C in water bath for 1 h and then immediately cooled in ice cold water. To each tube, 1 ml of chilled  $\text{ddH}_2\text{O}$  and 5 ml of a mixture of n-butanol and pyridine (15:1, v/v) was added and the tubes were vortexed and centrifuged at 4000 g for 10 min. The upper layer was aspirated out and used for measurement of colour intensity at 532 nm. 1, 1, 3, 3-tetramethoxypropane (TMP) was used as reference. The amount of TBARS was determined as change in absorbance at 532 nm and was calculated as  $\text{mmol}$  of malondialdehyde (MDA) produced  $\text{g}^{-1}$  DW.

### Determination of Protein Carbonylation

Protein carbonyls were quantified using a derivatization reaction with Dinitrophenyl hydrazine (DNPH), described by Levine et al. (1994). Cotyledons and axis were separately homogenized in ice cold 100 mM K-phosphate buffer, pH 7.4, containing 1 mM EDTA, 2 mM DTT and 1 mM PMSF, in a pre-chilled mortar with a similarly chilled pestle. The homogenate was filtered through four layers of muslin and centrifuged at 27000 g for 20 min at 4 °C. 400  $\mu\text{l}$  of supernatant was mixed with 600  $\mu\text{l}$  of 10 mM DNPH (prepared in 2 M HCl) and incubated at 37 °C for 1 h. The blank was incubated in 2 M HCl only. After incubation, proteins were precipitated with 10% (w/v) trichloroacetic acid (TCA) and the pellets washed thrice with 500  $\mu\text{l}$  of ethanol:ethylacetate (1:1). Washed pellets were finally dissolved in 6 M guanidine hydrochloride and absorption measured at 370 nm. Protein recovery after pellet re-suspension was estimated by Coomassie dye binding assay (Bradford 1976). Carbonyl content was calculated using the molar absorption coefficient of  $22,000 \text{ M}^{-1} \text{ cm}^{-1}$  for aliphatic hydrazone and expressed in  $\text{nmol g}^{-1}$  DW.

## Estimation of Reactive Oxygen Species

Methods involving fluorescent detection of ROS, Hydrogen peroxide and hydroxyl were adopted from Schopfer et al. (2001) while superoxide detection was performed as given in Zhao et al. (2003).

### Total ROS

Seeds were incubated in a 1.5 ml micro centrifuge tube, in darkness, for 1 h in 5  $\mu\text{M}$  2,7-dichlorofluorescin (DCFH) and homogenized in 20 mM of potassium phosphate buffer (pH -6.0) with micro pestle in the same tube. Conversion of DCFH to its oxidized product 2,7 dichlorofluorescein (DCF) was observed as an increase in fluorescence using reagent blank as reference ( $\lambda_{\text{Ex}}$ -488 nm,  $\lambda_{\text{Em}}$ - 525 nm).

### Hydrogen Peroxide ( $\text{H}_2\text{O}_2$ )

Seeds were preincubated for 30 min in 1.5 ml of 20 mM K-phosphate buffer, pH 6.0, to remove pre-formed  $\text{H}_2\text{O}_2$ . The buffer was replaced with 1.5 ml of the same buffer containing 5  $\mu\text{M}$  scopoletin and 3  $\mu\text{g ml}^{-1}$  horseradish peroxidase (HRP) and tubes incubated in darkness at 25 °C on a shaker for 3 h. Fluorescence was observed using reagent blank as reference ( $\lambda_{\text{Ex}}$ -346 nm,  $\lambda_{\text{Em}}$ - 455 nm).

### Hydroxyl Free Radical ( $\cdot\text{OH}$ )

Seeds were incubated for 1 h in 1.5 ml of buffer containing 20 mM 2-deoxy-D-Ribose and centrifuged. 0.35 ml of the post-incubation supernatant was mixed with 0.35 ml of 2-thiobarbituric acid and 0.35 ml of trichloroacetic acid and heated on a boiling water bath for 20 min. The tubes were cooled immediately and centrifuged at 15000 rpm for 15 min. The formation of the breakdown product malondialdehyde was observed as fluorescence, using reagent blank as reference ( $\lambda_{\text{Ex}}$ -532 nm,  $\lambda_{\text{Em}}$ - 553 nm).

### Superoxide ( $\text{O}_2^{\cdot -}$ )

Batches of 5 pairs of cotyledons and 5 detached axes were separately incubated in 1  $\mu\text{g ml}^{-1}$  DHE (Dihydroethidium) in 1.5 ml of 20 mM K-phosphate buffer, pH 6.0, in darkness. Ethidium, the reaction product of superoxide and DHE was measured fluorimetrically using reagent blank as reference ( $\lambda_{\text{Ex}}$ -510 nm,  $\lambda_{\text{Em}}$ - 605 nm).

### In Situ Staining for ROS

In situ staining of superoxide anion and hydrogen peroxide was done according to Han et al. (2013). For superoxide staining, seeds were incubated in 6 mM NBT in 10 mM Tris-HCl

buffer (pH 7.4) at RT, for 30 min. Production of superoxide anion was visualized as production of dark-brown color. For hydrogen peroxide staining, seeds were incubated in 0.42 mM TMB in Tris-acetate (pH 5.0) buffer for 1 h. Production of light blue color indicated presence of  $\text{H}_2\text{O}_2$ .

## Determination of Respiratory Enzymes Activity

### Hexokinase Activity

Hexokinase activity was determined according to Wiese et al. (1999) with some modifications. Seed extracts were prepared in 50 mM HEPES-KOH (pH -7.5), 5 mM  $\text{MgCl}_2$ , 1 mM EDTA, 15 mM KCl, 2.5 mM DTT, 0.1% Triton X-100, 10% glycerol and 2 mM PMSF. The homogenate was filtered and centrifuged at 20000 g for 20 min at 4 °C and the supernatant used for enzyme assays. The 1 ml assay mix contained 50 mM HEPES-KOH (pH -7.5), 5 mM  $\text{MgCl}_2$ , 15 mM KCl, 2.5 mM ATP, 1 mM NAD, 100  $\mu\text{l}$  of sample extract and 2 U of Glucose-6-phosphate dehydrogenase. The reaction was started with the addition of 0.1 M of glucose. Increase in absorbance at 340 nm was followed for 5 min at lapse of 30 s. One unit of activity reduces 1  $\mu\text{mole}$  of  $\text{NAD}^+$   $\text{min}^{-1}$  at RT.

### Succinate Dehydrogenase

The SDH activity was determined by following a decrease in absorption at 600 nm, due to reduction of artificial electron acceptor dichlorophenolindophenol (DCPIP) (Cooper and Beevers 1969). The reaction medium contained 50 mM potassium phosphate (pH 7.5), 20 mM disodium succinate, 10 mM KCN, 3 mg/ml Phenazinemetasulphate (PMS) and 2.5 mM DCPIP. One unit of SDH activity was defined as the amount of enzyme, reducing 1  $\mu\text{mol}$  of DCPIP  $\text{min}^{-1}$   $\text{mg}^{-1}$  protein.

### Cytochrome c Oxidase Activity

Mitochondria were isolated from each set according to Day and Hanson (1977). The cytochrome c oxidase activity was measured with a Clark-type oxygen electrode (Hansatech Ltd., UK) (Neuburger 1985). Isolated mitochondria (0.5 mg protein) were incubated in 75 mM sodium phosphate buffer, 16 mM ascorbate, and 0.1 mM Cytochrome C at 25 °C. The rate of oxygen consumption was measured for 10 min. Subsequently, Sodium cyanide (NaCN) was added to inhibit the enzyme activity. Results were viewed by the 'O<sub>2</sub> view' software provided by Hansatech. Cytochrome C oxidase activity was expressed in nmol oxygen consumed  $\text{min}^{-1}$   $\text{mg}^{-1}$  protein.

## Statistical Analysis

In all experiments, mean values were compared using Tukey, One-way analysis of variance (ANOVA) in SPSS 20.0 software to confirm the variability of data and validity of results. Significance levels of 95% ( $P < 0.05$ ) was considered and has been indicated by ‘\*’.

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