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NICKEL-INDUCED CHANGES IN CERTAIN METABOLIC PARAMETERS OF GERMINATING SEEDS OF *BRASSICA JUNCEA* L.

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Despite being essential at certain low concentrations for plant growth and development, nickel turns toxic at elevated concentrations. In the present study, Ni-induced metabolic changes in germinating *Brassica juncea* seeds have been monitored. In response to a 12 h exposure of excised embryo axes from H_2O-imbibed seeds to Ni (100, 200, 400 μM), their ability to reduce TTC (triphenyl tetrazolium chloride), a function of dehydrogenases, was inhibited in a concentration dependent manner. A general metabolic suppression within the embryos due to Ni is suggested. Ni treatment (24 h) of seeds inhibited the α-amylase activity implying a reduction in utilization of starch to sustain germination. The Ni-treated seeds exhibited marginally enhanced lipid peroxidation levels (MDA contents) with concomitantly increased SOD activity suggesting the involvement of altered redox metabolic parameters in Ni-induced inhibitory effects. The findings reveal Ni-dependent metabolic changes in the seeds that might account for reduction in seed germination performance under Ni stress.

Key words: *Brassica juncea*, metabolic changes, nickel, seeds, TTC reduction.

Heavy metal (HM) contamination of soils, mainly due to diverse anthropogenic activities, adversely affects the plant productivity. HMs enter the food chain via accumulation in plants eventually posing a threat to human health (Jarup 2003). Nickel (Ni) is one of the essential micronutrients required for normal plant growth and development (Marshner 1995). It is involved in the structure and therefore in the function of urease (Dixon *et al*. 1980, Eskew *et. al*. 1984) that converts urea into CO_2 and NH_3. Ni deficiency is linked with disrupted urea metabolism causing urea to accumulate in plant tissues that is toxic (Brown *et al*. 1987). On the other hand, higher Ni concentrations interfere with plant processes that lead to growth suppression (Seregin and Kozhevnikova 2006). Plants possess multiple means of cellular HM detoxification that are aimed at reducing the cytosolic HM concentrations (Sharma and Dietz 2006). Role of phytochelatins (PCs), metallotheionins (MTs), certain specific organic- and amino acids in metal complexation is established. Histidine involvement in Ni hyperaccumulation and tolerance of *Alyssum lesbiacum* was demonstrated (Kramer *et al*. 1996).

Phytoremediation offers a plant-based way out for decontamination of soils laden with toxic HM concentrations (Salt *et al*. 1998). This technology essentially depends upon the use of metal hyperaccumulator plants. To date, maximum number of known metal hyperaccumulator plants is that of Ni accumulators many of which belong to the family Brassicaceae. Indian mustard (*Brassica juncea* L.) has been reported to accumulate considerable amounts of heavy metals such as Pb, Cd (Jiang *et al*. 2000, Nouairi *et al*. 2006). For the evaluation of phytoremediation potential of target plants, it is desirable to characterize their responses and the possible mechanism(s) thereof to the elevated HM concentrations. We observed the seed germination and seedling growth of certain common cultivars of *B. juncea* to be differentially inhibited by the surplus Ni concentrations. Of the cultivars considered, cv Varuna and Kranti, with 31 and 49% inhibition of seed germination by 400 μM Ni, were found to be Ni-tolerant and Ni-sensitive, respectively. Furthermore, the cultivar-specific differences in Ni tolerance were found to be correlated with certain metabolic modifications in the seedling (7-d-old) tissue (S. Thakur and S.S. Sharma, unpublished results). However, the elevated Ni concentrations could be expected to influence the early metabolic events within the seeds. Therefore, we have monitored the Ni-induced

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changes in the TTC reduction ability (a measure of dehydrogenases) of excised embryos and certain metabolic changes in the seeds of *B. juncea* cv Kranti.

MATERIALS AND METHODS

Seeds of *Brassica juncea* cv Kranti were procured from a regional centre of G.B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand.

Determination of the Ni effects on TTC reduction ability of excised embryo axes

The effects of Ni on TTC reduction ability, a measure of the activity of dehydrogenases, of the excised embryo axes were determined. The uniform seeds were surface sterilized with 0.1% $HgCl_2$ for 2-3 min. After thorough washing, they were imbibed with distilled water for 24 h. The embryos were excised from the H_2O-imbibed seeds and treated with Ni (100, 200, 400 µM), applied as $NiSO_4.7\,H_2O$, in 50 mm Petri dishes (Tarsons, India) for 12 h. The embryos treated simultaneously with distilled H_2O constituted the control. Following the treatment of desired duration, the embryos were incubated with 0.1% aqueous TTC (triphenyl tetrazolium chloride) solution for 6 h in dark. The stained embryos (due to formazan formation) were homogenized with MetOH (1 ml) and the homogenate was centrifuged at 5000 rpm for 5 min (4ºC). Absorbance of the supernatant was read at 485 nm and the TTC reduction ability was expressed in terms of A_{485} per 5 embryos.

Determination of the Ni effects on certain metabolic parameters of germinating seeds

The surface sterilized seeds were imbibed in solutions containing Ni (100, 200, 400 µM) for 24 h. The seeds treated simultaneously with distilled H_2O constituted the control. The treated seeds were subjected to the following measurements:

α-amylase assay

α-amylase activity was determined following the method described by Filner and Varner (1967). In brief, the seed tissue was homogenized with 0.05 M Tris-HCl buffer (pH 7.2). The homogenate was centrifuged at 10,000 rpm for 10 min and the supernatant served as enzyme extract. The assay mixture containing 1 ml of enzyme extract and 1 ml of substrate (0.15% starch) was incubated for 10 min. Thereafter, 3 ml of IKI reagent was added and absorbance at 620 nm recorded. In the blank (control), enzyme extract was added after the IKI addition. The starch amounts were calculated from a calibration curve and the α-amylase activity was expressed as µg starch degraded min^{-1} mg^{-1} protein.

Lipid peroxidation

The lipid peroxidation was assessed in terms of the malondialdehyde (MDA) contents following the method described by Dhindsa *et al*. (1981). In brief, the seed tissue was homogenized with 0.1% TCA and the homogenate centrifuged at 10,000 rpm for 10 min. The supernatant was reacted with 0.5% thiobarbituric acid (TBA) in 20% TCA. The reaction mixture was heated at 95 ºC for 30 min and cooled rapidly over an ice bath. The mixture was centrifuged at 10,000 rpm for 5 min and the absorbance of supernatant read at 532 nm. The measurements were corrected by subtracting the absorbance at 600 nm. The MDA contents were determined using the extinction coefficient of 155 $mM^{-1}cm^{-1}$ (Heath and Packer 1968).

Superoxide dismutase (SOD) assay

SOD activity was determined following the method described by Beauchamp and Fridovich (1971). The seed tissue was homogenized with K-phosphate buffer (100 mM, pH 7.0). The homogenate was centrifuged at 13,000 rpm for 25 min (4 ºC); the supernatant served as enzyme extract. The reaction mixture contained assay buffer (100 mM K-phosphate, pH 7.8), riboflavin, methionine, NBT and enzyme extract. The reaction mixture was illuminated for 10 min. The absorbance was read at 560 nm using reaction mixture that was not illuminated as blank. A 50% reduction in absorbance was taken as one unit activity.

Determination of protein contents

Protein contents were determined according to the method of Bradford (1976).

RESULTS

Effects of Ni on TTC reduction ability of excised embryo axes

The treatment of embryo axes excised from H_2O-imbibed (24 h) seeds of *B. juncea* with Ni (100-400 μM) for 12 h led to a concentration-dependent suppression of TTC reduction to a corresponding formazan by the embryo tissue (Fig. 1A,B). The TTC reduction was measured after incubation of Ni-treated embryos with TTC for 6 h in dark. A suppression of TTC reduction by 2.4, 23.4 and 48.8% was observed at 100, 200 and 400 μM Ni, respectively (Fig. 1A). In fact, the Ni-induced inhibition of TTC reduction was evident after 3 h treatment with Ni although the magnitude of inhibition was lower than that observed after 12 h treatment (data not shown).

Effect of Ni on α-amylase activity, lipid peroxidation and SOD activity in seeds

Seed treatment with Ni (100-400 μM) for 24 h led to the suppression of α-amylase activity. However, the magnitude of suppression was not concentration dependent as the applied Ni concentrations suppressed the activity similarly. α-amylase activity was observed to be 69.5, 68.5 and 65.4% of control at 100, 200 and 400 μM Ni, respectively (Fig. 2).

The lipid peroxidation, measured in terms of MDA contents, in the seeds of *B. juncea* was increased only marginally due to the Ni treatment. The Ni-induced increase in MDA contents was 4.1, 7.6 and 6.9% at 100, 200 and 400 μM Ni, respectively (Fig. 3A). Simultaneously, the SOD activity in Ni-treated seeds was substantially increased. An increase by 17.5, 19.1 and 18.7% was evident at 100, 200 and 400 μM Ni, respectively (Fig. 3 B).

DISCUSSION

The aim of present study was to monitor the changes in certain metabolic parameters in the seeds of *B. juncea* treated with the elevated concentrations of Ni that might account for the Ni-dependent suppression of seed germination. To begin with, it was examined whether a direct exposure of the excised embryo axes to Ni (100-400 μM) would influence their ability to reduce TTC to a corresponding formazan. The latter is essentially a function of the activity of dehydrogenase enzymes including those from the respiratory metabolism. That Ni suppressed the TTC reduction ability of *B. juncea* embryos in a rather short term treatment indicates a general inhibition of dehydrogenases. This would obviously be expected to contribute to the restricted growth of embryo and in turn the inhibition of seed germination. HMs including Ni have been reported to inhibit the respiration e.g., Llamas and Sanz (2008) reported the inhibition of respiration in shoot and roots of rice plants under Ni stress.

Next, we examined the Ni-induced changes in the activity of α-amylase, as an example of hydrolytic enzymes, in the germinating *B. juncea* seeds. α-amylase hydrolyzes the stored starch into mobilizable sugars to provide energy for growing embryo. Due to Ni treatment, a substantial reduction in α-amylase activity was evident although the magnitude of reduction was generally independent of concentration. The lack of observed dose-dependence might, among other reasons, be a consequence of cellular management of Ni ions allowing their cytosolic concentrations to be maintained within a limited range. HMs influence the enzymes through interaction with functional groups e.g., -SH, that causes the modification of conformation necessary for activity (Van Assche and Clijsters 1990). Besides, Ni ions might have influenced the endogenous GA_3 levels, significant in the regulation of α-amylase synthesis/activity (Chrispeels and Varner 1967). It could be assumed that other hydrolytic enzymes would be similarly affected by Ni leading to the germination inhibition.

Finally, the lipid peroxidation status and the activity of SOD in Ni-stressed seeds were

measured. Indeed, HM stress imposes the cellular redox imbalance via stimulated generation of ROS and a concomitant reduction of antioxidative defence. The resultant oxidative stress strongly accounts for the HM-induced inhibitory effects (Schutzendubel and Polle 2002, Sharma and Dietz 2009). The enhanced tissue lipid peroxidation, measured as MDA contents, is taken as an index of the oxidative stress (Goncalves *et al*. 2009). The induction of oxidative stress by HMs including Ni is well established (Boominathan and Doran 2002, Sharma *et al.* 2004). In the present study, the Ni-treated seeds exhibited only marginally increased MDA levels. Such limited accumulation of MDA might occur due to a simultaneously enhanced antioxidative metabolites and enzymes. Consistent with this, the enhanced SOD activity was observed in the Ni-treated seeds. Indeed, superoxide anion ($O_2^{\cdot-}$) accumulation via membrane localized NADPH oxidase is among the early events towards the development of oxidative stress. HM-induced enhancement of NADPH oxidase is known (Hao *et al*. 2006, Maksymiec 2007). ROS have also been shown to be involved in the regulation of seed metabolism. Taken together, the data suggest a role of oxidative stress in response of seed germination to elevated Ni concentrations. Present findings reveal the interference of elevated Ni concentrations with diverse seed metabolic events ranging from metabolic suppression within the embryos to altered hydrolysis of seed reserves and redox

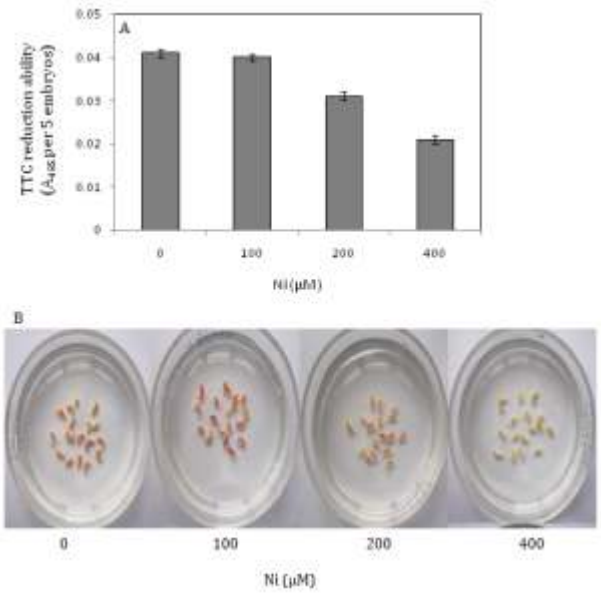

Figure 1. A. Effect of Ni treatment on TTC reduction ability of excised embryo axes from germinating *Brassica juncea* seeds. The embryo axes were treated with stated Ni concentrations for 12 h followed by incubation with TTC for 6 h in dark. Data are arithmetic means ± S.E. (n=6). B. Showing the TTC reduction ability of *B. juncea* embryo axes as affected by Ni treatment.

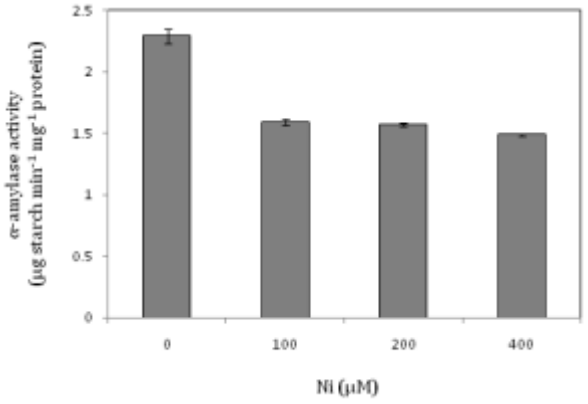

Figure 2. Effect of Ni treatment (24 h) on α-amylase activity in the seeds of *Brassica juncea*. Data are arithmetic means ± S.E. (n=6).

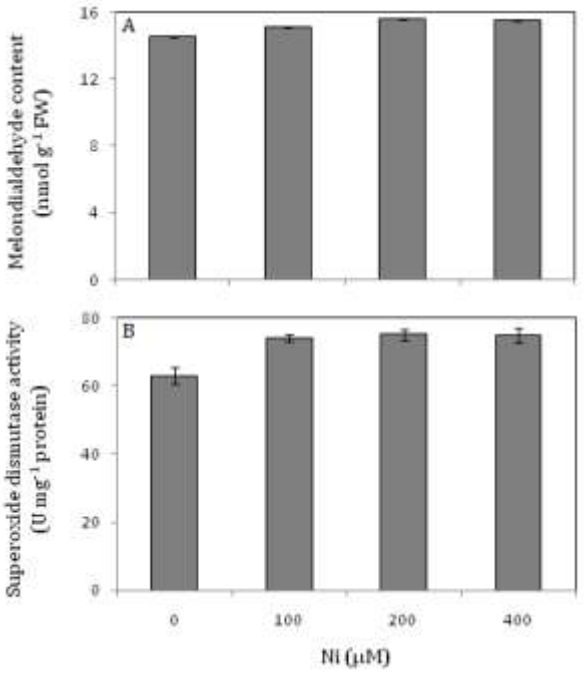

Figure 3. Effect of Ni treatment (24 h) on MDA content (A) and superoxide dismutase activity (B) in the seeds of *Brassica juncea*. Data are arithmetic means ± S.E. (n=6).

parameters. These might account for the inhibition of seed germination.

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