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Influence of nickel stress on growth and some important physiological/biochemical attributes in some diverse canola (*Brassica napus* L.) cultivars

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ABSTRACT

To assess the effect of nickel on six canola cultivars a series of experiments were conducted. On the basis of shoot dry weight cvs. Shiralee and Range found to be nickel tolerant, Dunkeld and Ester as nickel sensitive, while the remaining cultivars intermediate. Nickel accumulation in shoots was lower in the nickel sensitive cultivars followed by that in the tolerant ones. Leaf water and osmotic potentials decreased significantly due to high concentration of Ni^{2+}. Decrease in osmotic potential was positively associated with accumulation of total free amino acids. By comparing accumulation of individual amino acids, pattern of accumulation of the amino acids was different in different cultivars. However, only histidine, serine and cysteine increased in appreciable amount in the xylem sap of different canola cultivars. Overall, nickel tolerant cultivars Shiralee and Range showed higher levels of histidine, serine and cysteine under varying levels of nickel than the others. This higher accumulation of histidine, serine and cysteine was positively related to nickel tolerance in all canola cultivars. Thus, differential nickel tolerance in canola cultivars proposed to be associated with relative detoxification of Ni by developing complexes with histidine, serine and cysteine and can be used as potential indicators of nickel tolerance in canola.

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1. Introduction

Of many heavy metals known in nature, nickel (Ni) is essential as trace element for normal plant growth and development, because it is constituent of some important enzymes such as urease. However, high concentration of Ni in growth medium can lead to toxicity symptoms and reduced growth of plants [1]. Toxic effects of high concentrations of Ni in growth medium on plants include alteration in uptake of essential nutrients, chlorosis, reduced CO_2 uptake, disturbances in gas exchange, alterations in water uptake and generation of free radicals and reactive oxygen species that produce oxidative stress [1–4].

There are a variety of mechanisms by which plants can endure high concentration of heavy metals, including restricted uptake and/or translocation of metals, exclusion of toxic heavy metals from cells by ion-selective metal transporters, excretion or compartmentation of heavy metals, production of heavy metal binding factors such as proteins, peptides, amines, amino acids, and formation of complexes with these binding factors and metals to detoxify metals [5–6]. For example, metal tolerant plants accumulated higher proline in response to heavy metal stress as compared to metal sensitive plants, and this accumulation of proline in stressed plants was found to be associated with reduced damage to membranes and proteins [7–8]. In another study, nicotianamine has been shown to chelate Ni and enhanced nickel tolerance in *Thlaspi goesingense* [9]. From these reports it is evident that different amino acids can have an important role in regulating metal toxicity in plants and thus it needs an extensive study. Furthermore, genetic variation and some degree of heritability for Ni stress tolerance have also been reported in Ni hyperaccumulator *Thlaspi spp*. [9–11]. In view of all these reports, the present study was aimed to assess variability for Ni stress tolerance in some elite canola cultivars and to examine up to what extent accumulation of different amino acids has a role in Ni stress tolerance in canola cultivars. In general, the work reported in manuscript is to identify the potential indicators responsible for the tolerance of nickel stress, which could be used in future breeding programs to evolve high yielding canola varieties with improved characters.

2. Materials and methods

Four independent experiments were conducted to assess the response of six selected canola cultivars to varying nickel concentrations. In the first experiment, LD_{50} (Lethal dose at 50% growth)

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concentration of Ni was determined for canola cultivars. In the second experiment, screening of canola cultivars for Ni tolerance was carried out and cultivars were grouped as tolerant, moderately tolerant and sensitive on the basis of shoot dry weight at the adult stage. In the third experiment, physiological and biochemical responses of canola cultivars were assessed under varying concentrations of nickel. In the fourth experiment, accumulation of Ni and amino acids in xylem sap was determined and parallels were drawn between them to assess their role in Ni tolerance.

3. Experiment 1: Optimization of LD_{50} concentration of Ni for canola

In order to determine LD_{50} (nickel concentration where 50% growth was reduced), 20 seeds of each cultivar were allowed to germinate for 15 days under varying concentrations of Ni (0, 10, 20, 30, 40 ... 150 mg L^{-1}). After 15 days, germination percentage, percent viable population size, shoot fresh and dry weights were recorded. The Ni concentration 150 mg L^{-1} was the most toxic concentration, which severely inhibited the growth. At Ni concentration 100 mg L^{-1}, 50% reduction in percent viable population size, and shoot fresh and dry weights was observed and considered as LD_{50}. This Ni concentration was used for further experimentation to screen the canola cultivars.

4. Experiment 2: Assessment of variation in tolerance of canola cultivars to Ni

Seeds of 10 canola cultivars (Shiralee, Range, Torch, Rainbow, Dunkeld, Ester, Con-I, Con-II, Tobin, Frontier) were obtained from Ayub Agricultural Research Institute, Faisalabad, Pakistan. Twenty seeds were sown in earthen of 30 cm diameter containing 6.0 kg homogenously mixed sun-dried sandy clay loam soil with a completely randomized design (CRD) and four replications. After germination the plants were thinned to maintain five seedlings in each pot. After 3 weeks, all plants were subjected to 100 mg L^{-1} Ni as $NiCl_2$ for 4 weeks. On the basis of shoot dry weight in 100 mg L^{-1} Ni, Shiralee and Range, Torch and Rain, and Dunkeld and Ester were categorized as tolerant, moderately tolerant, and sensitive to Ni, respectively, while the other cultivars were found to be intermediate in Ni tolerance. The cultivars so selected were further used to evaluate Ni tolerance on the basis of physiological and biochemical attributes.

5. Experiment 3: Physiological and biochemical responses of selected canola cultivars to Ni

A pot experiment was conducted during the winter 2004–2005 in a net-house at the Botanic Garden of the University of Agriculture, Faisalabad, Pakistan (latitude 31°30 N, longitude 73°10 E and altitude 213 m), with 10/14 light/dark period at 800–1100 μmol m^{-2} s^{-1} PPFD, a day/night temperature cycle of 28/17 °C and 65 ± 5% relative humidity. About 50 seeds of each of six canola cultivar were sown in each earthen pot (30 cm diameter and 20 cm in depth) filled with 6 kg sandy clay loam soil (soil saturation percentage 33%; pH 7.8; EC_e 2.21 mS cm^{-1}). For determining available Ni, soil was extracted in 1 N ammonium acetate solution (1:5) ratio following Allen et al. [16] and for total Ni in the soil samples were digested in a mixture of sulphuric acid and hydrogen peroxide following Wolf [17]. The mean available and total Ni concentration levels in the soil were 0.14 and 29.5 mg kg^{-1}, respectively. After 1 week, the seedlings of comparable size growing equidistantly were thinned to maintain five seedlings per pot. The experiment was arranged in a completely randomized design with four replicates, four nickel treatments (0, 50, 100, 150 mg L^{-1}) and six cultivars. The plants were irrigated with distilled water for 3 weeks before the start of varying Ni treatments as nickel chloride ($NiCl_2 \cdot 6H_2O$) for further 58 d after which time three plants out of five were harvested. Uprooted plants were washed with distilled water and separated into shoots and roots, and then blotted dry before recording their fresh weights. All plants parts were dried at 65 °C until constant dry weight, and their dry weights recorded. Before harvesting the plants for determination of plant biomass, the following physiological parameters were measured:

5.1. Water relations

The 2nd leaf from each plant was excised at 7.00 a.m., and the leaf water potential measurements were made with a Scholander type pressure chamber (Arimad, UK). A proportion of the same leaf used for water potential measurements, was frozen into 2 cm^3 polypropylene tubes at −40 °C in an ultra-low freezer for 2 weeks, after which time plant material was thawed and the frozen sap was extracted by crushing the material with a glass rod. After centrifugation (8000 × *g*) for 4 min, the sap was directly used for osmotic potential determination using a vapor pressure osmometer (Wescor 5500). Leaf turgor pressure was calculated as the difference between leaf water potential and leaf osmotic potential values.

5.2. Total soluble proteins

Total soluble proteins were determined as described by Lowry et al. [12]. Fresh leaf material (0.2 g) was homogenized in 4 mL of sodium phosphate buffer solution (pH 7.0) and centrifuged. The extract was used for the estimation of soluble proteins and free amino acids. The sample extracts were reacted with a Folin phenol reagent and the optical densities read at 620 nm using a spectrophotometer (Hitachi U-2000).

5.3. Total free amino acids

Total free amino acids were determined following the procedure of Hamilton and Van Slyke [13]. For estimation of total free amino acids, 1 mL of each sample as extracted for soluble protein determinations was treated with 1 mL of 10% pyridine and 1 mL of 2% ninhydrin solution. The optical densities of the solutions were read at 570 nm using a spectrophotometer (Hitachi U-2000).

5.4. Total soluble sugars

Total soluble sugars were estimated following the procedures of Malik and Srivastava [14]. Well ground dry leaf material (0.1 g) of each sample was homogenized in 80% ethanol and centrifuged at 3000 × *g*. The residue was retained and repeatedly washed with 80% ethanol to remove all traces of soluble sugars. The resulted filtrate was diluted up to 100 mL with distilled deionized water and reacted with anthrone reagent. The aborbance of the colored solutions was read at 625 nm using a spectrophotometer (Hitachi U-2000).

5.5. Qualitative and quantitative estimation of individual amino acids

Amino acid profile was estimated according to the method of Braithwaite and Smith [15]. One gram fresh leaves were chopped in 2 mL of 6 N HCl in sealed test tubes and incubated at 125 °C in a heating chamber for 24 h. The resulting paste was centrifuged at 15,000 rpm at 15 °C for 10 min. The supernatant was used for separation of amino acid profile through paper chromatography. Individual amino acids were identified by comparing Rf values of

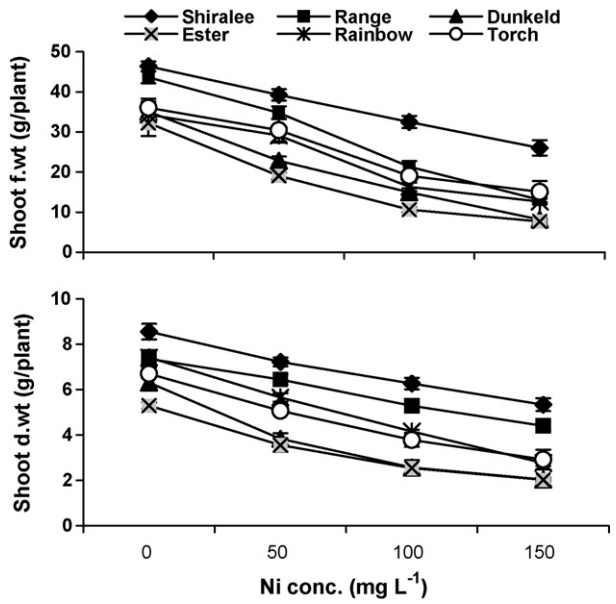

Fig. 1. Fresh and dry weights of shoots and roots of six canola cultivars when 21-day-old plants were subjected to varying concentrations of nickel for 58 days ($n = 4$).

coloured spots and standards in a paper chromatogram. The colored spots of amino acids on paper chromatogram were cut with a scissors and eluted in 3 mL of methanol and the optical densities of the solutions were read at 540 nm using a spectrophotometer (Hitachi U-2000).

5.6. Determination of Ni^{2+} in plant tissues

Ni^{2+} in leaves and roots were determined using the methods described by Allen et al. [16]. Each ground dry plant samples (100 mg) was digested in 2 ml of sulfuric-peroxide digestion mixture until a clear and almost colorless solution was obtained. After digestion, the volume of the sample was made to 10 ml with distilled de-ionized water. Nickel was determined with an atomic absorption spectrometer (PerkinElmer Analyst 100).

6. Experiment 4: Role of amino acids in Ni tolerance

In this experiment, seeds of all selected cultivars were germinated for 1 week, thereafter transferred to perforated polystyrene foam placed in plastic tank ($70 \times 40 \times 25$ cm) containing 30 L full strength Hoagland's nutrient solution (pH 6.5). The hydroponics was continuously aerated. Plants were further grown under varying concentrations of Ni (0, 50, 100, 150 mg L^{-1}) for 6 weeks. Shoots were excised at the base, and the cut surfaces were blotted with absorbent tissue. The root pressure exudates were collected over 8 h period and injected into eppendorf tubes, and then stored at -70 °C. In the xylem sap, amino acid profile and Ni concentration were estimated and quantified following the method of Braithwaite and Smith [15] and Allen et al. [16], respectively.

6.1. Statistical analysis of data

The data were subjected to analysis of variance using a COSTAT computer package (Cohort Software, Berkeley, California). The mean values were compared with the least significance difference test following Snedecor and Cochran [18].

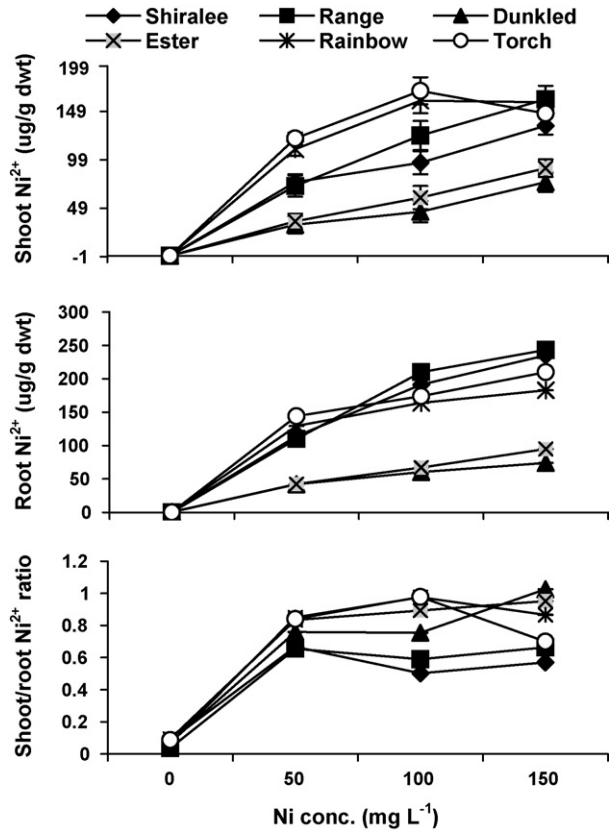

Fig. 2. Nickel (μg/g d wt) in shoots and roots, and shoot/root Ni ratio of six canola cultivars when 21-day-old plants were subjected to varying concentrations of nickel for 58 days ($n = 4$).

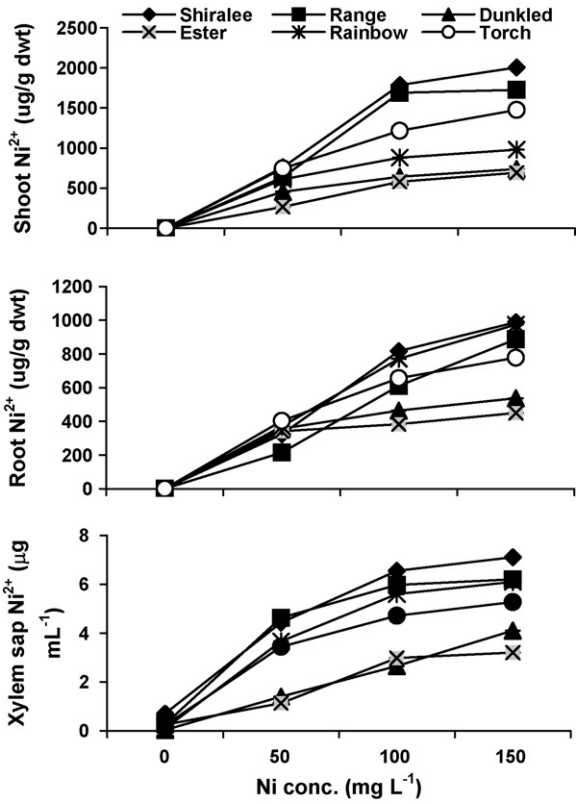

Fig. 3. Nickel (μg/g d wt) in shoots and roots, and xylem sap Ni^{2+} of six canola cultivars when 7-day-old plants were subjected to varying concentrations of nickel for 42 days ($n = 4$).

7. Results

Exogenous application of 100 mg L^{-1} Ni (LD_{50}) proved to be very useful in discriminating canola cultivars as tolerant, moderately tolerant, and sensitive in second experiment. Shoot fresh and dry weight of all selected canola cultivars were consistently ($P < 0.001$) reduced with increase in Ni concentration in rooting medium (Fig. 1). Shiralee and Range were the highest of all the cultivars in shoot dry weight, while Range and Rainbow exhibited intermediate performance. Overall, cv. Shiralee was the most tolerant in terms of shoot fresh and dry weight at all external Ni levels.

Nickel concentration in the xylem sap, shoots and roots of all the canola cultivars was increased with increase in Ni supply in the rooting medium. Furthermore, this accumulation was higher when canola cultivars were grown in hydroponics (Figs. 2 and 3). At 100 mg L^{-1} Ni^{2+}, moderately tolerant cultivars Torch and Rainbow were higher, while sensitive cultivars Dunkled and Ester, lower in shoot Ni^{2+} concentration. However, it was observed that root Ni^{2+} concentration was maximum in cultivars Range and Shiralee and minimum in cultivars Ester and Dunkled, particularly at 100 mg L^{-1} of Ni^{2+} applied through rooting medium. In shoot/root Ni^{2+} ratio Ni tolerant cultivars Shiralee and Range were lower at all levels of Ni^{2+} as compared to the moderately tolerant or sensitive canola cultivars.

Leaf water potential and osmotic potential ($P < 0.001$) in all canola cultivars decreased with increase in Ni concentration in growth medium (Fig. 4). Ester was the highest and shiralee the lowest in leaf water potential of all the canola cultivars at the highest Ni concentration in growth medium, whereas at other Ni concentrations Dunkeld and Ester were intermediate in leaf water potential. Leaf osmotic potential was found to be the lowest in Ni tolerant cultivars (Shiralee and Range), whereas the highest was in Nickel sensitive Dunkeld and Ester as compared with all other cultivars at all concentrations of Ni (Fig. 4). Leaf turgor potential was found to be maintained in Ni tolerant cultivars (Shiralee and Range), whereas it decreased at highest Ni concentrations (Fig. 4).

Leaf total soluble proteins of Ni sensitive or moderately tolerant line decreased when 150 mg L^{-1} Ni was applied through the rooting medium. In contrast, leaf total free amino acids were increased in all canola cultivars. However, maximum increase in total free amino acids was found in Ni tolerant cultivars (Shiralee and Range), whereas Ni sensitive cultivars (Dunkeld and Ester) were the lowest in this biochemical attribute (Fig. 5). Leaf soluble sugars were consistently decreased with increase in Ni supply in rooting medium. However, this reduction was more in Ni sensitive cultivars compared with other cultivars (Fig. 5).

Most of amino acids in leaf were increased in Ni tolerant cultivars with increased in Ni concentration to the growth medium such as histidine, lysine, serine, glutamic acid, cysteine, aspartate, glycine, methionine, arginine etc (Data not shown). However, concentration of histidine, cysteine, lysine and serine in xylem sap were highest in Ni tolerant and the lowest in Ni sensitive canola cultivars at higher concentrations Ni in rooting medium (Fig. 6).

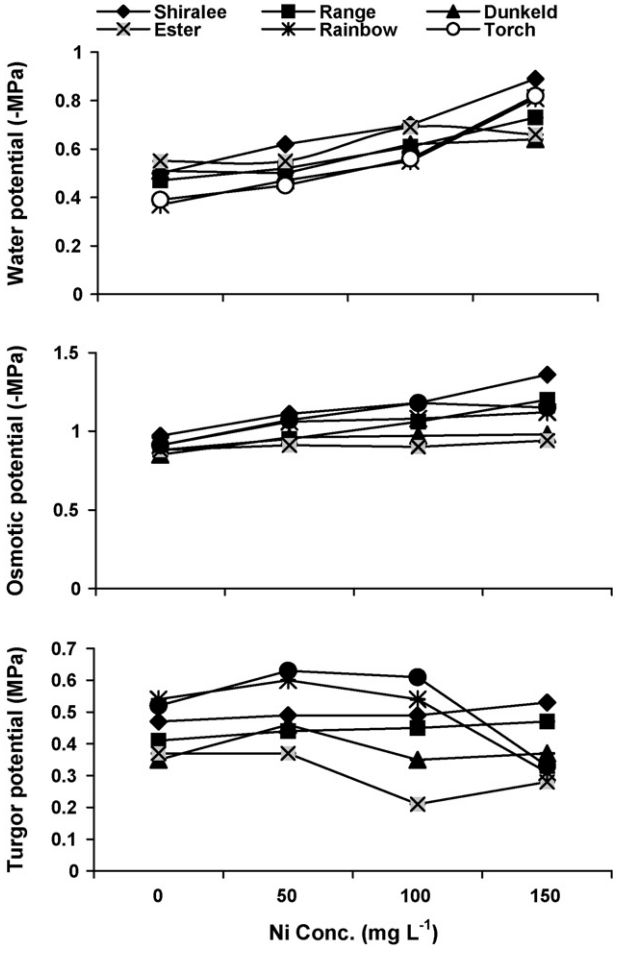

Fig. 4. Leaf water potential, leaf osmotic potential and leaf turgor potential of six canola cultivars when 21-day-old plants were subjected to varying concentrations of nickel for 58 days ($n = 4$).

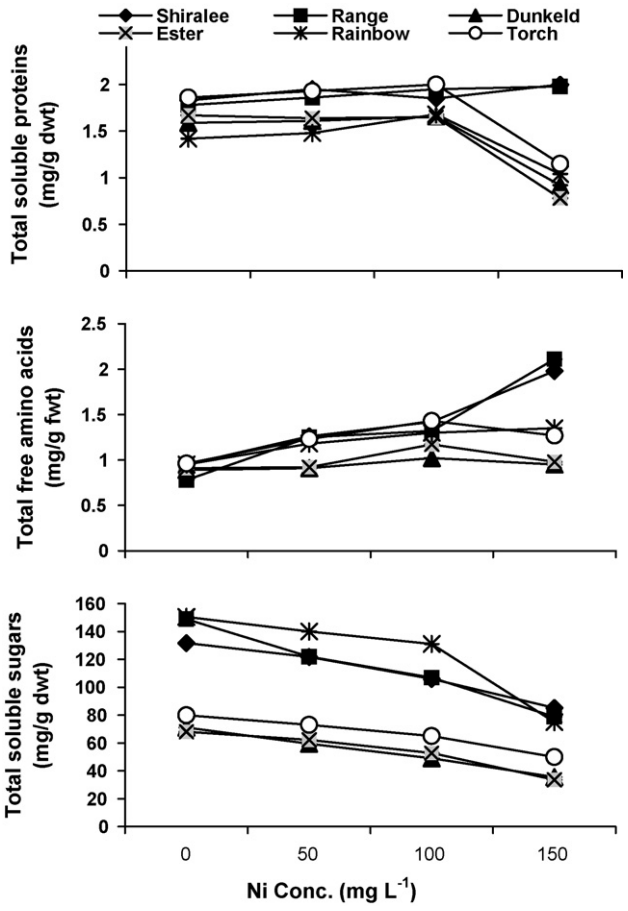

Fig. 5. Total soluble proteins, total free amino acids and total soluble sugars of six canola cultivars when 21-day-old plants were subjected to varying concentrations of nickel for 58 days ($n = 4$).

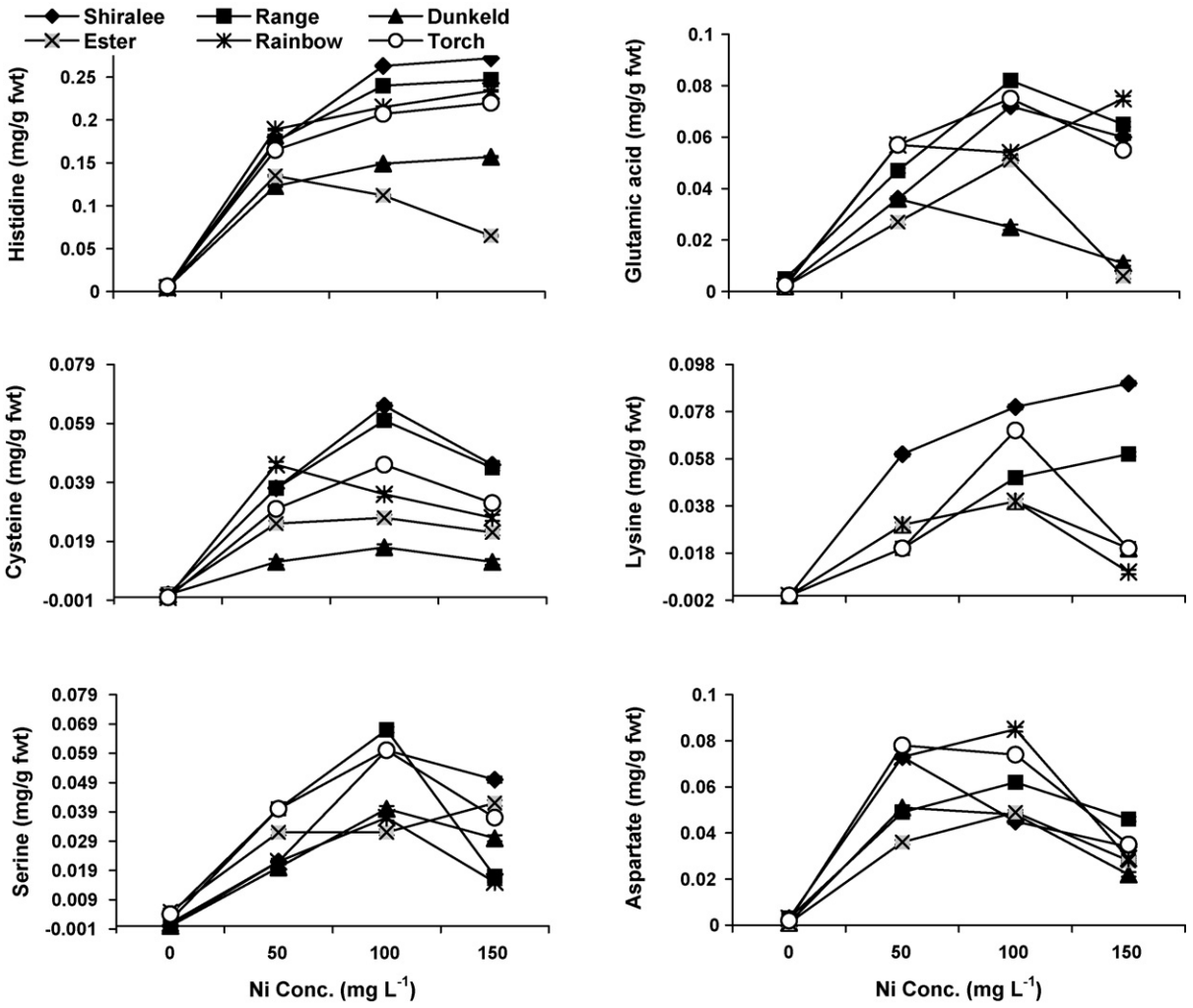

Fig. 6. Accumulation of amino acids in xylem sap of six canola cultivars when 7-day-old plants were subjected to varying concentrations of nickel for 42 days ($n = 4$).

8. Discussions

In the present study, increasing supply of Ni^{2+} in the rooting medium reduced the growth of canola cultivars as has earlier been observed in wheat (*Triticum aestivum* L.) [2] and Matricaria chamomilla [3] at varying concentrations of Ni and Cd. However, a considerable genetic variation in response to Ni stress has been observed in the set of six cultivars/lines of canola examined here. For example, Ni tolerant cv. Shiralee was higher in shoot fresh and dry weight at all levels of Ni^{2+}, while other Ni^{2+} tolerant cv. Range was more sensitive at higher levels of Ni^{2+}. Such variability among canola cultivars to nickel stress may have been due to differences in accumulation or distribution of Ni in shoots and roots [19]. In the present study, moderately Ni tolerant cultivars (Rainbow and Torch) accumulated considerably higher amount of Ni^{2+} in all plant parts i.e. stem, leaves, and roots, compared to the other cultivars. However, all cultivars tended to partition more Ni^{2+} in the roots (Fig. 2). Thus, cultivars having low shoot/root Ni ratio had better ability to retain Ni^{2+} in the roots, possibly by binding and sequestering it in the vacuoles [20], which might have contributed to tolerance to Ni^{2+}. It seems reasonable to propose that variation in sensitivity to Ni stress among six canola cultivars was due to differential accumulation of Ni^{2+} in shoots, contributing to cytosolic detoxification.

Generally, plant water relations of six canola cultivars were adversely affected with increase in Ni^{2+} concentration of the growth medium. If we draw parallels between leaf osmotic potential and leaf nickel concentration, it is obvious that hyper-accumulation of Ni^{2+} caused a decrease in leaf osmotic potential ($r = 0.858^{***}$). In view of Kramer and Boyer [21] hyper-accumulation of heavy metals just like Ni^{2+} and Zn^{2+} is analogous to the process of osmotic adjustment, in which compatible organic solutes and/or inorganic ions (e.g. Na^+, K^+, Cl^-) are accumulated under water or salt stress to lower osmotic potential in the cell to maintain turgor and cellular activity. Likewise, Baker and Walker [22] were of view that metals might be hyper-accumulated to increase osmolarity within the cell. In view of a large number of published reports, it is conceivable that reduction in osmotic potential of plants subjected to any stress (water stress, heat stress, salt stress etc.) may be due to either water loss or an increase in dissolved solutes (organic compatible solutes or inorganic osmotica such as Na^+, K^+, Cl^-, etc.) or a combination of both [23]. Among organic osmotica or compatible osmolytes, soluble sugars and amino acids and their derivatives are more important for maintaining osmoregulation in cells or tissues subjected to stresses. If we draw relationship between soluble sugars or total free amino acids and leaf osmotic potential, it is clear that leaf osmotic potential is positively related to free amino acids (OP vs amino acid $r = 0.646^{***}$) but not to soluble sugars (OP vs soluble sugars $r = 0.00057$ ns). Thus, differential growth responses of six canola cultivars to nickel stress can be related to their differential accumulation of free amino acids. For example, the lowest osmotic potential recorded in nickel tolerant cultivars Shiralee and Range

as compared to other moderately tolerant and sensitive cultivars can be related to their higher accumulation of free amino acids.

Identification of individual amino acids as potential ligands for metal detoxification and tolerance could be of considerable value in identifying potential biochemical indicators for metal tolerance. Thus, despite determining total free amino acids in the nickel tolerant and sensitive cultivars of canola, determination of individual amino acids is more important. In the nickel tolerant and moderately nickel tolerant canola cultivars, accumulation of histidine (His), and cysteine (Cys) increased with increase in external Ni^{2+} supply, whereas there was a small increase in the accumulation of amino acids in nickel sensitive cvs. Dunkeld and Ester. Furthermore, accumulation of these amino acids is positively related to Ni^{2+} accumulation in their leaves (Ni^{2+} vs His $r = 0.90^{***}$; Ni^{2+} vs Cys $r = 0.8007^{***}$) as well as in xylem sap (Ni^{2+} vs His $r = 0.92^{***}$; Ni^{2+} vs Cys $r = 0.711$). These results are similar to the recent findings of Sinha and Pandey [24] who observed that cystein content increased with increase in Ni^{2+} content of *Hydrilla verticillata* when treated with varying concentrations of Ni^{2+}. In addition, Krämer et al. [25] found that Ni^{2+} hyper-accumulator *Alyssum lesbiacum* accumulated more amino acids, particularly L-histidine, whereas non-accumulator *Alyssum montanum* did not accumulate amino acids. In the same study, the authors found that other two hyper-accumulator *Alyssum murale* and *A. bertolonii* also followed the same relationship. Of all the organic acids and proteinacious amino acids, histidine has the highest association constant for complex formation with nickel [26]. The apparent importance of histidine in hyper-accumulator canola cultivars is in striking agreement with a prediction that histidine increases Ni^{2+} tolerance by intracellular detoxification of Ni^{2+} and effective translocation of Ni^{2+} from root to shoot [24]. Recently, Freeman et al. [27] observed that glutathione, cysteine and *o*-acetyle-L-serine are strongly correlated with the ability to hyperaccumulate Ni^{2+} in various *Thlaspi* hyperaccumulators. They concluded that elevated level of cysteine, glutathione and *o*-acetyle-L-serine play a causal role in Ni^{2+} tolerance by enhancing the GSH-dependent antioxidant system. Thus, in view of all these reports and results of the present study, it is suggested that Ni^{2+} is taken up by roots as the free cations under control conditions. However, under nickel stress conditions hyperaccumulator canola cultivars synthesized high amount of histidine and cysteine, which might have enhanced the translocation of nickel from root to shoot and its detoxification, whereas reverse was true for nickel sensitive cultivars of canola. In conclusion, Ni tolerance in canola cultivars appeared to be associated with high accumulation of histidine and cysteine in plants so as to detoxify Ni.

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