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Phytotoxicity of nickel and its accumulation in tissues of three *Vigna* species at their early growth stages

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Summary

Seedlings of three *Vigna* species; *V. cylindrica*, *V. mungo* and *V. radiata* were investigated for their nickel (Ni) tolerance. Various growth, photosynthetic attributes and accumulation levels of Ni in the roots and shoots were assessed after exposure to 0 (control), 50, 100, 150 mg kg^{-1} Ni. 100 and 150 mg kg^{-1} Ni induced a significant reduction in germination ($P \leq 0.001$) and fresh biomass ($P \leq 0.05$) of seedlings. The other growth attributes; root and shoot length, dry biomass, leaf number and leaf area were not much influenced by the presence of Ni. A drastic decline was observed for the formation of nodules and chlorophyll a and b contents. A steady increase in metal content of the tissues (root and shoot) was observed with an increase in Ni levels but the pattern of metal accumulation was the same in all species as bioaccumulation of Ni was much profound in the roots (110 mg kg^{-1}) as compared with the shoots (30 mg kg^{-1}). The tolerance indices (TI) of the tissues varied with respect to Ni levels. The roots exhibited higher tolerance indices (TI) than the shoots at their respective levels of Ni. The results indicated a greater sensitivity of chlorophyll molecules and nodulation to Ni. The study depicted inter-specific differences in the degree of Ni tolerance and its accumulation in the plant tissues. Among tested species, *V. mungo* appeared to be sensitive, while, *V. cylindrica* showed successful seed emergence, greater dry biomass along with sustainable chlorophyll biosynthesis and nodule formation. A greater root tolerance index, capacity of roots to accumulate appreciable amount of Ni in addition to restricted transfer of metal to the aerial tissue may signify a better threshold of the species. *V. cylindrica* appeared to cope with Ni toxicity through an integrated mechanism of metal tolerance which may arise from differential accumulation of Ni in the plant parts without damaging the tissue and considerable alteration of important growth parameters. Thus, *V. cylindrica* can be a choice for abandoned soils contaminated with Ni.

Introduction

Nickel (Ni) is among the abundant heavy metals and it constitutes about 0.08% of the earth crust thus it is ubiquitously distributed in soil and water (KUPPER and KRONECK, 2007). Ni toxicity is of serious concerns to agriculture, ecosystem and human health (JARUP, 2003; PANDEY and SINGH, 2011). Rapid industrialization and high anthropogenic pressures in the developing countries have encountered excessive amount of Ni in the environment. Ni originates more frequently from the non-ferrous metal industry, mining, production and disposal of batteries (BOULARBAH et al., 2006). In addition, untreated municipal wastewater, sludge disposal, application of pesticides and phosphate fertilizers are also important contributors of Ni pollution (PANDEY, 2006). Like many other developing countries agriculture soils of Pakistan are contaminated with heavy metals including nickel (ATIQ-UR-REHMAN and IQBAL, 2008).

Ni has been classified among essential micronutrients (BROWN et al., 1987). It is found associated with some metallo-enzymes which are necessary for various plants processes (GIRIDHARA and SIDDARAMAPPA, 2002). However, like many other essential elements its supra-optimal concentrations are strongly phytotoxic (GAUTAM and PANDEY, 2008). Excessive Ni can induce alterations of plant metabolism that leads to the inhibition of germination and growth (KHAN and KHAN, 2010). It is known to produce stunted growth, chlorosis and necrosis of leaf which are visible symptoms associated with Ni toxicity (SEREGIN and KOZHEVNIKOVA, 2006). High concentration of Ni can inhibit dry matter production and chlorophyll biosynthesis (AHMED et al., 2010). Nitrogen fixation which is typical to leguminous plants depends on the ability of *Rhizobium* spp. to form nodules. But excessive Ni has been reported to cause deleterious effects on the genus *Rhizobia* and hence, on nodules formation in a number of leguminous species (VIJAYARENGAN, 2000).

Since, Ni is highly mobile therefore it can easily be translocated from the roots to the aerial parts of the plants (JOZEF et al., 2009). However, its uptake, distribution and bioaccumulation vary considerably in plant species. Evidences (EDEM et al., 2009) show that bioaccumulation of metals in underground and aerial tissues is an indicative of metal tolerance strategy which divides plants into two groups. The first group includes metal excluder species that accumulate and store a significant proportion of heavy metals in their roots while, metal ions are translocated from the roots to the shoots and leaves in the other group called accumulator species (REEVES and BAKER, 2000). Changes in growth responses induced by heavy metals are frequently used to assess tolerance indices of plant tissues which serve to suggest metal tolerance sensitivity (MA et al., 2009).

Both germination and seedling stages are particularly important for successful subsequent development of a crop. The plant species that show heavy metal tolerance at their juvenile stages may produce tolerant adult individuals. Thus, exploration of variation at early growth stages may signify overall potential of a crop for its exploitation on contaminated agriculture lands located in the vicinities of large industries of the country.

Keeping in view the increasing Ni toxicity to crop plants and significant importance of pulses as source of low cost vegetable proteins for low income groups in a developing country like Pakistan, we assessed relative Ni tolerance of three *Vigna* species (*V. cylindrica*, *V. mungo* and *V. radiata*) at their early establishment phases.

We investigated changes in germination, various growth and photosynthetic attributes of the species after their exposure to different levels of Ni in the soil. The Ni content in plant tissues along with tolerance indices for root and shoot growths were also investigated to evaluate tolerance of the species to excessive Ni present in the growth medium.

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Materials and methods

Seeds of three *Vigna* species, *Vigna cylindrica* Skeel var. cp-386, *Vigna mungo* L. var. 6036-7 and *Vigna radiata* var. 97003 were obtained from Pulse Crop Division, Ayub Agriculture Research Institute, Faisalabad, Pakistan.

Air-dried sandy loam soil (pH 7.90) sieved through a 2 mm sieve was thoroughly mixed with $NiCl_2.6H_2O$ (Merck, Germany) at concentrations of 50, 100 and 150 mg kg^{-1} substrate singly at the beginning of experiment, while control plants were without Ni. Since Ni salt was hexa-hydrated therefore actual concentration factor of Ni was used to maintain Ni levels used in the study. Thirty-six earthen pots (height 15 cm and internal diameter 12 cm) were filled with 1.0 kg of soil appropriately. Twenty seeds of each species were sown into these pots. Germination was assessed via seedling emergence and seeds were considered to be germinated when seedlings emerged from the soil bearing at least two young leaves. Data records were made for 10 days until no germination occurred.

For seedling experiment, eight (pre-germinated) 6 days old seedlings were transplanted into each of thirty-six earthen pots (height 30 cm and internal diameter 25 cm) which were filled with 3.5 kg of soil containing Ni at the rate of 0 (control), 50, 100 and 150 mg kg^{-1} substrate. Seedlings were acclimatized for 8 days then thinned out to four in each pot. The experiments were arranged in a complete randomized manner. To simulate field conditions, plants were grown in a wire netting house under natural conditions (temperature $28 \pm 5°C$ and the day-length 12 h). All pots were irrigated with tap water, spraying gently using a spray bottle to avoid leaching. Plants were allowed to grow for 4 weeks, when seedlings were about 15-20 cm tall; bearing at least 10-12 leaves were harvested by taking out whole soil from the pot. Plants were carefully separated from the soil and washed thoroughly with tap and then with distilled water. The data for various growth attributes were recorded. Fresh weights were taken and for dry weight measurements plant material was oven dried at 70°C for 48 h. Leaf samples were scanned using Deskscan and the images produced were analyzed using delta T-scan leaf image analysis software (Delta T Devices, UK).

2 g of leaf material from each treatment was extracted in 80% acetone for the determination of chlorophyll *a* and *b*. The absorbance of filtrates was taken at 645 and 663 nm for chlorophyll *a* and *b* respectively following (ARNON, 1949) using spectrophotometer (2000 Hitachi, Japan). Metal content in plant tissues were determined by the acid digestion method. The well dried root and shoot samples were ground and a wet digestion was carried out with 3:1 HNO_3: $HClO_4$ (v/v) (ALLEN et al., 1986). The concentration of nickel was determined using an atomic absorption spectrophotometer (Analyst 300, Perkin-Elmer, Germany). The standard was AA Ni (Camlab, U.K).

Tolerance indices for plant tissues were calculated following WILKINS, 1978 as:

$$TI\ (\%) = \frac{Root/shoot\ elongation\ (cm)\ at\ Ni\ treatment}{Root/shoot\ elongation\ (cm)\ at\ control} \times 100$$

Thus, three series of tolerance indices for root and shoot for each species were obtained.

Statistical Analysis

Data presented as means ($\pm$ S.E) for each parameter. Data for germination percentage was arcsin transformed following BLISS (1937) then it was subjected to statistical analysis (ANOVA). A two-way analysis of variance was carried out using MS Excel 2000 in order to determine significant effects of different Ni levels as well as to determine inter-specific variability. Least Significant Differences (LSD) between means for species and Ni levels were calculated by employing a multiple range test following DUNCAN (1955).

Results and discussion

The results of the study depicted a differential influence of various Ni levels and variable responses of three *Vigna* species for the attributes studied. The results presented for seedling emergence showed that the maximum number of seedlings emerged at the control for all three species but a gradual decline in seedling emergence was observed with increasing levels of Ni. Seed germination was severely affected by the highest (150 mg kg^{-1}) Ni level. A consistently higher percentage for seedling emergence was observed for *V. radiata* at all levels of Ni except at 100 mg kg^{-1}, where *V. cylindrica* had the maximum (70%) seedling emergence (Fig. 1 A). At low concentration of Ni (50 mg kg^{-1}), germination of seeds of all *Vigna* species was greater. With doubling of the Ni concentration from 50 to 100 mg kg^{-1}, the percentage of seed germination declined by 10%. Germination further declined (more than 50%) when nickel was added at the concentration of 150 mg kg^{-1}. Thus, seed germination was severely ($P \leq 0.001$) affected by the presence of Ni and the species also exhibited significantly ($P \leq 0.05$) variable responses (Tab. 1). Since, germination is the most crucial stage of plant development. Therefore, seed germination is frequently used as an indicator of early response of the plants in a hostile environment (SINGH et al., 2006; TALUKDAR, 2011). HALL (2000) reported that Ni inhibits all energy requiring cellular processes during germination thus, slows down emergence of radicles and plumules. In the present study inhibition of seed germination was also observed and consequently seedling failed to emerge owing to the toxicity of Ni.

Ni has caused no inhibitory effect on root elongation and similarly the species have shown invariable responses for root length (Tab. 1). However, *V. radiata* had greater root length at 100 and 150 mg kg^{-1} Ni (Fig. 1 B). Although various levels of Ni had not influence shoot elongation but there were present significant interspecific differences (Tab. 1). *V. mungo* had consistently the lower shoot length at all Ni levels as compared with *V. radiata* and *V. cylindrica* (Fig. 1 C). 150 mg kg^{-1} Ni had caused a significant reduction ($P \leq 0.05$) of fresh biomass and species had also shown distinct responses (Tab. 1). A consistently greater fresh weight was observed for *V. cylindrica* at all level of Ni (Fig. 1 D). The dry weight also exhibited a similar trend as shown by the fresh biomass (Fig. 1 E). Though, no noticeable influence of Ni level was observed on dry biomass but species had shown significant ($P \leq 0.05$) variable response for dry weight production (Tab. 1).

Impact of different abiotic stresses including heavy metals is frequently perceived through dry weight which serves as one of the realistic predictors because it signifies cumulative effects of various attributes (VIJAYARENGAN, 2000). The presence of Ni did not induce any drastic decline in dry biomass production of these species (*V. cylindrica*, *V. mungo* and *V. radiata*). No suppression of dry biomass can be ascribed for the enhancement of total organic carbon in leguminous plants in the presence of heavy metals as demonstrated by a number of other workers (JIANG et al., 2008; MA et al., 2009).

Fixation of atmospheric nitrogen, which is a base material for protein, is an important function of root nodule bacteria. Moreover, the degree of nodulation is directly related to the nitrogen economy of leguminous plants as it affects their ultimate yield (KHAN and
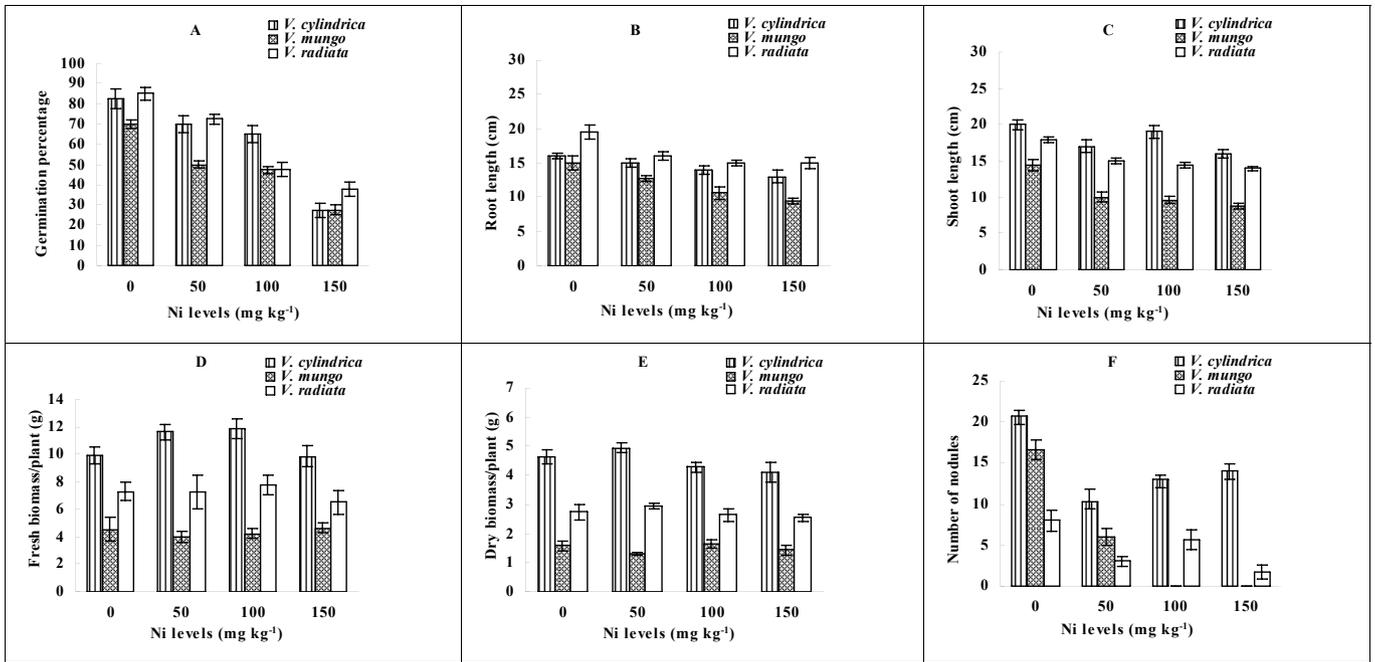
Fig. 1: Effect of varying levels of Ni on various growth attributes: germination percentage (A), root length (B), shoot length (C), fresh biomass/plant (D), dry biomass/plant (E) and number of nodules (F) in three *Vigna* species after 4 weeks growth.
Values presented are means across three replicates. Vertical lines indicate $\pm$ S.E.

Tab. 1: Summary of Analysis of Variance (ANOVA) for various attributes in three *Vigna* species after 4 weeks growth under varying levels of Ni.
Results are from Two Way Analysis of Variance, degree of freedom with df = 3 (levels), df = 2 (species), df = 6 (interaction), LSD by Duncan's multiple range test (P < 0.05), M.S. = mean square, LSD = least significant difference. *, **, ***, at 0.05, 0.01 and 0.001% level of probability respectively, N.S= non-significant. DW = dry weight

Attributes	M.S. Levels	Significant	LSD	M.S. Species	Significant	LSD
Germination percentage	4963.19	***	14.24	793.75	*	11.18
Root length (cm)	184.96	N.S	0.00	217.19	N.S	0.00
Shoot length (cm)	91.85	N.S	0.00	756.75	***	6.88
Fresh biomass/plant (g)	1.94	*	0.77	37	***	0.98
Dry biomass/plant (g)	0.05	N.S	0.00	0.35	*	0.31
Number of leaves	4.93	N.S	0.00	9.36	N.S	0.00
Leaf area (cm^2)	0.06	N.S	0.00	27.38	***	0.59
Number of nodules	190.84	**	4.72	355.08	***	6.02
Chlorophyll *a* (µg g^{-1} DW)	67790.5	***	30.50	1024	N.S	0.00
Chlorophyll *b* (µg g^{-1} DW)	67790.5	***	25.45	1024	N.S	0.00
Root Ni content (mg kg^{-1})	0.00108	***	0.01	0.00001	N.S	0.00
Shoot Ni content (mg kg^{-1})	0.00040	***	0.005	0.0000021	N.S	0.00

KHAN, 2010). A drastic decline (P$\leq$0.01) in the formation of root nodules was observed in the presence of Ni. The responses of the species were distinctly (P$\leq$0.001) variable (Tab. 1). *V. cylindrica* had shown significantly higher number of nodules whereas, the formation of root nodules was severely affected by the presence of 100 and 150 mg kg^{-1} Ni in *V. mungo* and *V. radiata* as the former species had developed no nodules in the presence of higher levels of Ni (Fig. 1 F).

The sensitivity of the genus *Rhizobium* to Ni is well documented (RAJKUMAR and FREITAS, 2008). The poor nodulation in the presence of Ni may have hampered nitrogen fixing system by causing direct toxicity on the *Rhizobia* and/or inhibited legheamoglobin synthesis (JAYAKUMAR et al., 2008). However, better growth of *V. cylindrica* can be correlated with greater number of nodules that carried out more fixation of atmospheric nitrogen leading to an increase in total protein and in turn greater biomass of the species. Thus, our

observations are consistent with the results obtained by CHEN et al., (2003).

Ni levels did not inhibited the leaf number and leaf area (Fig. 2 A and B) in these *Vigna* species. However, data presented in Fig. 2 C and D showed a decrease in chlorophyll *a* and *b*. Several leguminous species growing under Ni contamination have been shown to produce more leaves despite a decrease in chlorophyll content (GOPAI et al., 2002; PANDEY and SINGH, 2011) thus; our results are in close agreement to the finding of these workers. However, decline in photosynthetic green pigments can be attributed to several possible reasons which include oxidative damage of chloroplast membranes, inhibition of chlorophyll biosynthesis and/or replacement of Mg ions in a tetrapyrole rings that have been well documented in different groups of plants in response to heavy metals (NAGAJYOTHI et al., 2009; TALUKDAR, 2011).

Accumulation of Ni in plant tissues was concentration dependent. A steady increase in metal content in both tissues (root and shoot) was observed with an increase in Ni level in the growth medium (Fig. 3 A and B). The metal content of roots was considerably higher in all species as compared to shoots. At the highest Ni level (150 mg kg^{-1}), 73% of the metal uptake was carried out by the roots in *V. cylindrica*. However, metal transfer was considerably lower in the shoots as only 20% of the Ni was transported from soil to the aerial tissue. Thus, metal uptake from soil to roots was higher as compared with its transfer to the shoots. The other two species (*V. mungo* and *V. radiata*) also exhibited a similar trend for Ni transport from soil to plant tissues but no marked contrast was observed between species for metal content at various levels of Ni (Tab. 1). Hence, the capacity of species to accumulate Ni in

their tissues vary markedly, metal seems to be restricted in the roots and a limited transfer to the aerial tissues became evident. Several other studies (REEVES and BAKER, 2000; QURAINY, 2009) also demonstrated differential transport of metals from soil to plant tissues and hyper-accumulators species can accrue far exceeding levels of metals than present in the soil. The hyper-accumulation of Ni in the roots can be a manifestation of detoxification mechanism which may involve binding of metal ions with cell wall, pumping of ions to vacuole or formation of non toxic complexes with specific metal binding protein without changing cellular metabolism and alteration of membrane structure (MAHMOOD et al., 2007; LLAMAS et al., 2008; QURAINY, 2009) thus causing no dramatic decline in growth attributes of the species studied.

Several workers (LI et al., 2003; MADHAIYAN et al., 2007) used root and shoot tolerance indices to predict the sensitivity of the tissues to stress induced by heavy metals. This study depicted that tolerance indices (Tab. 2) were dependant on the concentrations of Ni in both tissues (Tab. 2). Consistently higher tolerance indices were observed for the root tissue in the species at all levels of Ni (Tab. 2). *V. cylindrica* had the maximum root tolerance index (TI=97±0.89) followed by *V. radiata* (TI=89±0.74). However, *V. mungo* had also shown a better shoot tolerance index. These results indicated that in *V. cylindrica* and *V. radiata* the roots demonstrated less sensitive response than the shoots at elevated Ni levels. A higher metal sensitivity of the shoot than the roots have also been reported in other species (SRIVASTAVA et al., 2005). The sensitivity of the shoot can be attributed to higher fraction of cystolic free Ni in the shoots than in the roots thus rendering the aerial tissue more sensitive to metal toxicity despite the fact that the roots accumulated more Ni (GALARDI et al., 2007).
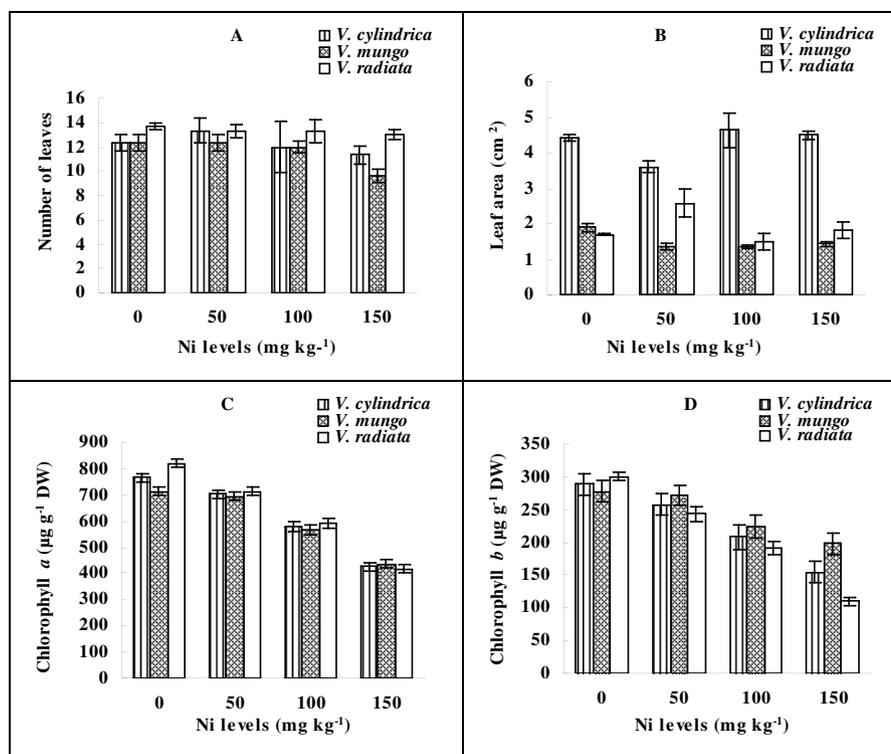
Fig. 2: Effect of varying levels of Ni on various growth attributes: number of leaves (A), leaf area (B), chlorophyll *a* (C) and chlorophyll *b* (D) in three *Vigna* species after 4 weeks growth.
Values presented are means across three replicates. Vertical lines indicate ± S.E. DW = dry weight.
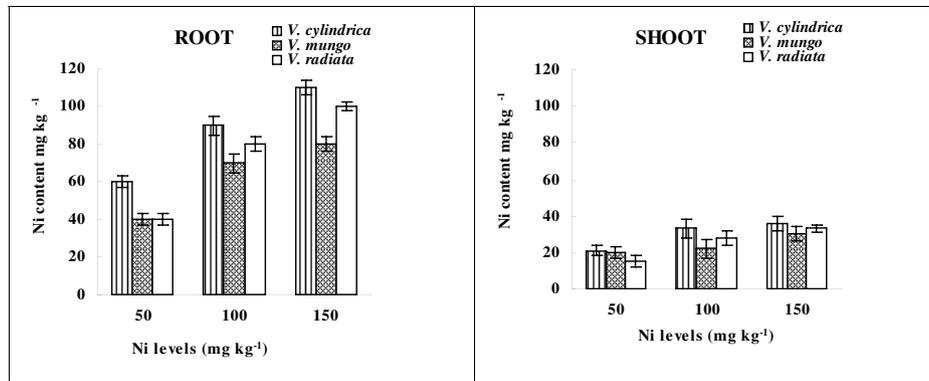
Fig. 3: Bioaccumulation of metal in tissues (root and shoot) of three *Vigna* species.
Values presented are means across three replicates. Vertical lines indicate $\pm$ S.E.

Tab. 2: Tissue tolerance indices (%) of three *Vigna* species after 4 weeks growth under varying levels of Ni.
Values presented are means across three replicates with + S.E.

Species	Root Tolerance Index (%)			Shoot Tolerance Index (%)		
	Ni levels (mg kg^{-1})					
	50	100	150	50	100	150
V. cylindrica	95 $\pm$ 0.95	97 $\pm$ 0.89	89 $\pm$ 0.91	85 $\pm$ 0.82	73 $\pm$ 0.94	61 $\pm$ 0.52
V. mungo	85 $\pm$ 0.45	73 $\pm$ 0.58	76 $\pm$ 0.26	83 $\pm$ 0.46	83 $\pm$ 0.64	77 $\pm$ 0.96
V. radiata	83 $\pm$ 0.45	85 $\pm$ 0.69	89 $\pm$ 0.74	77 $\pm$ 0.69	66 $\pm$ 0.46	53 $\pm$ 0.55

The study demonstrated that vegetative growth parameters in *Vigna* species did not influence by increasing Ni concentrations in the soil. However, chlorophyll content and nodulation appeared to be more susceptible in the studied *Vigna* species in response to Ni contamination. Among the species, *V. cylindrica* had shown better germination and growth responses, less sensitivity of chlorophyll molecules and nodule formation. A greater root tolerance index, better threshold of the roots to accumulate appreciable amounts of Ni along with restricted transfer of metal to the aerial tissue may signify the species as metal excluder. Thus, *V. cylindrica* appeared to possess an integrated mechanism to cope with metal toxicity and can be a potential choice for Ni contaminated soils.

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