RESPONSE OF INDIAN MUSTARD (*BRASSICA JUNCEA ARAWALI*) PLANTS UNDER NICKEL STRESS WITH SPECIAL REFERENCE TO NICKEL PHYTOEXTRACTION POTENTIAL

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Abstract

The objective of present paper was to evaluate nickel phytoextraction capacity of Indian mustard plants (*Brassica juncea arawali*) with and without chelant application. The chelants chosen for the study were ethylene diamine tetraacetic acid (EDTA) and salicylic acid (SA). Seeds of Indian mustard were sown in nickel contaminated soils with nickel concentrations from 100 to 800 mg/l as nickel nitrate. Plants were harvested at four stages. Various morphological parameters, biochemical parameters and nickel phytoextraction potential of plants were studied. It was found that seed germination and percentage survival of Indian mustard reduced with increasing concentration of Ni. Addition of SA enhanced germination and survival of *B. juncea*, while EDTA played negative role. Plant growth parameters like numbers of branches and leaves, root length and shoot length decreased with increased concentrations of Ni. In general, EDTA decreased all morphological parameters, whereas SA stimulated them. EDTA treated plants showed 83-90% higher Ni accumulation compared to control for the applied Ni doses of 100-800 mg/l respectively. The Ni metal accumulation order was Ni+EDTA>Ni+SA>Ni. EDTA proved to be more efficient chelant than SA for Ni removal from contaminated soil.

Keywords: *Brassica juncea arawali, Ethelyne diamine tetraacetic acid, Nickel contamination, Phytoextraction, Salicylic acid*

Introduction

Nickel is an essential element in small quantity but its large quantity is detrimental to human health. Ni enters in the human body through consumption of plant foods and soil ingestion (Gall et al., 2015; Yeganesh et al., 2013). The higher concentration of Ni in soils and foodstuff can cause cancer to humans. Nickel mining, electroplating, combustion of fossil fuels and metal plating industries are

DOI: 10.6092/issn.2281-4485/8528

prime sources of nickel contamination in the soil (Harasim and Filipek, 2015; Wuana and Okieimen, 2011). A study by Hu et al. (2017) reported that long-term Ni contamination in agricultural soil increases the occurrence of antibiotic resistance genes when exposed for 4-5 years and at concentrations 0-800 mg/kg Ni. Hence, nickel contaminated soil should be remediated and managed well. In recent times, Ni contaminated soil have been remediated by nickel accumulator plants. Plants of brassicaceae family have potential to accumulate heavy metals from contaminated soil (Syam et al., 2016) especially Indian mustard (*Brassica juncea*) is used in phytoextraction of heavy metal contaminated soil along with biofumigation due to their antimicrobial activity. Hence, these plants are helpful in improving crop yield and soil quality along with remediation of heavy metals (Szczygłowska et al., 2011).

The present paper aims to assess nickel phytoextraction capacity of Indian mustard plants (*Brassica juncea arawali*) with and without chelant application. Ethylene diamine tetraacetic acid (EDTA) and salicylic acid (SA) were applied as chelants.

Materials and methods

Pot Experiment

Pot experiment was set up at Micromodel experimental site of Indian Institute of Technology, Delhi in a complete randomized block design with three replications (Table 1).

Group	Subgroup		
	No chelant	EDTA	SA
Control	T1	T6	T11
100 mg/l Ni	T2	T7	T12
200 mg/l Ni	T3	T8	T13
400 mg/l Ni	T4	T9	T14
800 mg/l Ni	T5	T10	T15

Table 1
Pot experiment treatments.

Twenty seeds of *Brassica juncea arawali* were sown in each pot with the depth of 11 cm and the diameter of 11 cm, filled with unsterilized field soil, farmyard manure (organic carbon 11% total N 0.50, total P 0.65%, total K 2.50% and pH 7.3) and sand in a 2:2:1 ratio for the following treatments. Table 2 shows soil properties and background nickel concentration.

The soil was artificially spiked with nickel nitrate at levels of 100, 200, 400 and 800 mg/l. Chelators including ethylene diamine tetraacetic acid and salicylic acid with the same concentration 2.4 mM were applied during 10^{th} week of plant growth. Plants were thinned to 3 plants per pot after 30 days of sowing.

The first harvesting was done after 30 days of sowing and subsequent harvesting was done on 8th day after chelant application. Third and fourth harvestings were done at flowering stage and maturation stages respectively.

Parameter	Unit	Amount
pH	-	7.2
Electrical Conductivity	mS/cm	0.4
Organic Carbon	%	0.6
Sandy	%	70,0
Silt	%	15.2
Clay	%	14.8
Cation Exchange Capacity	Cmol/kg	16
Available N	kg/ha	265
Available P	kg/ha	7.0
Available K	kg/ha	190
Background Ni	mg/kg	2.0

Table 2
Soil properties and background nickel concentration.

Analysis of Plant and Soil

Plant samples were analysed for morphological parameters, biochemical parameters and nickel accumulation potential. Morphological parameters such as seed germination, plant survival and plant height, number of branches, numbers of leaves, fresh weight and dry weight were determined. Seed germination was examined by percentages of relative seed germination test (RSG) (Hoekstra et al., 2002) and germination index (GI) test (Zucconi et al., 1981) which were calculated as follows:

$$GI\ (\%) = \frac{Seed\ germination\ (\%)\ x\ Root\ length\ of\ treatment\ x\ 100}{Seed\ germination\ (\%)\ x\ Root\ length\ of\ control} \quad [1]$$

$$RSG\ (\%) = \frac{Number\ of\ seeds\ germinated\ in\ treatment\ x\ 100}{Number\ of\ seeds\ germinated\ in\ control} \quad [2]$$

The plant's height was measured. Number of branches per pot and number of leaves per plant per pot were also counted. Root and shoot of the freshly harvested mustard plants were separated manually. Fresh weight was weighed and then samples were dried at 60°C until constant weight to determine total dry weight. The dried plant samples were digested with aqua regia for Ni analysis. Ni concentration of plants was recorded as mg of Ni per kilogram of dry biomass. Biochemical parameters such as chlorophyll, proline, soluble protein and total soluble sugar were estimated by Arnon, Ninhydrin, Bradford and Anthrone methods respectively (Thimmaiah, 1999).
Soil and farm yard manure samples were analyzed to calculate organic carbon, nitrogen, phosphorus, potassium, cation exchange capacity and nickel by the methods of Walkley and Black, Micro-kjeldahl, Olsen, Flame photometer, $BaCl_2$ and aqua regia extraction respectively (Rowell, 1994). Nickel analysis was conducted via ICP-OES (Varian Vista-MPX CCD Simultaneous ICP-OES, Varian Australia Pty. Ltd) with a Ni detection limit of 0.007 mg/l.

DOI: 10.6092/issn.2281-4485/8528

Data and Statistical Analysis

The different data collected for mustard plants were subjected to statistical analyses using SPSS software ver. 17.0 (Chicago, IL, USA). Tools used include descriptive statistics, analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) for statistical significance at 95% confidence level. Analysis of variance was used to assess significant differences among treatments in Microsoft Office EXCEL 2003.

Results and discussion

Effect of treatments on morphological parameters

Effect of nickel and chelants on morphological parameters of mustard plants were determined. Generally, EDTA have adverse effects on morphological parameters of *B. juncea arawali* while SA have positive effects on different plant growth parameters. Table 3 represents variance analyses for morphological parameters of *B. juncea arawali*.

Table 3
F values and significance values of two-way ANOVA for morphological parameters of Indian mustard

Morphological parameters	Source of variance	$F_{critical}$	$F_{observed}$	*P*-value
Germination (%)	Treatments	1.86	1.83	0.05
	Time	4.00	820	0.000
	Treatments x Time	1.86	1.70	0.07
Survival (%)	Chelant	1.80	1.54	0.11
	Ni	3.09	5.45	0.005
	ChelantxNi	1.60	0.55	0.96
No. of branches	Treatments	1.77	2.49	0.003
	Time	2.68	65.9	0.000
	TreatmentsxTime	1.48	3.63	0.000
No. of leaves	Treatments	1.77	8.03	0.000
	Time	2.68	239	0.000
	TreatmentsxTime	1.48	8.80	0.000
Fresh weight	Treatments	1.77	14010	0.000
	Time	2.68	146	0.000
	TreatmentsxTime	1.48	8.80	0.000
Dry weight	Treatments	1.77	107	0.000
	Time	2.68	723	0.000
	TreatmentsxTime	1.48	60.1	0.000
Root length	Treatments	1.77	10.1	0.000
	Time	2.68	539	0.000
	TreatmentsxTime	1.48	14.6	0.000
Shoot length	Treatments	1.77	10.2	0.000
	Time	2.68	1442	0.000
	TreatmentsxTime	1.48	24.9	0.000

Seed germination and survival. The results on the effect of Ni treatments on seed germination and plant survival is given in Table 4. The best results (184.6%) pertaining to RSG, after 10 days, were obtained with 100 ppm Ni+SA treatment and worst (23% RSG) with 800 ppm Ni+EDTA treatment. Here, the highest GI (1.52) was seen with control SA and lowest (0.63) with 800 ppm Ni+EDTA treatment. The difference in germination (%) was significant with chelant treatments and time (Table 3). Seed germination and seedling growth inhibition by nickel has also been reported by many other workers (Ashraf et al., 2011; Jagetiya and Bhatt, 2007; Nasr, 2013; Zhi et al., 2015).

Table 4. *Influence of Ni on seed germination and plant survival in B. Juncea.*

Treatment	Seed germination		Relative seed germination (%)		Germination Index (GI)	Survival (%)
	10 days	25 days	10 days	25 days		
T1	$4.3\pm2.5^{b,c}$	$18.0\pm1.0^{b,c}$	-	-	-	72.0^{a}
T2	$4.7\pm4.0^{b,c}$	$17.0\pm1.0^{a,b}$	108	94.0	0.92	70.0^{a}
T3	$2.7\pm0.5^{a,b}$	$16.6\pm3.0^{a,b}$	61.5	92.6	0.83	68.0^{a}
T4	1.7 ± 0.5^{a}	$16.3\pm1.5^{a,b}$	38.0	90.7	0.78	65.0^{a}
T5	1.0 ± 1.0^{a}	14.0 ± 1.0^{a}	46.0	77.7	0.72	63.0^{a}
T6	$4.3\pm2.0^{b,c}$	$17.0\pm3.0^{a,b}$	100	94.4	1.11	65.0^{a}
T7	$6.3\pm5.5^{b,c}$	$18.3\pm1.5^{b,c}$	146	102	1.27	76.7^{a}
T8	4.0 ± 2.6^{b}	$16.3\pm1.5^{a,b}$	92.0	90.7	1.04	75.0^{a}
T9	$3.0\pm1.0^{a,b}$	$16.0\pm3.0^{a,b}$	69.0	88.8	0.95	65.0^{a}
T10	$2.0\pm1.0^{a,b}$	14.6 ± 2.5^{a}	23.0	81.0	0.63	60.0^{a}
T11	$7.6\pm2.5^{b,c}$	19.0 ± 1.0^{c}	177	106	1.52	84.0^{b}
T12	8.0 ± 1.0^{c}	19.6 ± 0.57^{c}	185	109	1.18	$81.7^{a,b}$
T13	$4.3\pm3.0^{b,c}$	$18.0\pm2.0^{b,c}$	100	100	1.15	71.7^{a}
T14	4.0 ± 2.0^{b}	17.3 ± 0.57^{b}	92.0	96.0	1.50	70.0^{a}
T15	$2.7\pm0.6^{a,b}$	$16.0\pm1.0^{a,b}$	61.5	88.8	0.93	70.0^{a}

Values represent mean ± standard deviation (n=3). Means followed by the same letter within a column do not differ significantly according to DMRT at P=0.05.

Addition of EDTA with metals inhibited the seed germination and these results are corroborated with Ilbas et al. (2006). According to Popova et al. (2009) plants treated with SA can reduce metal stress due to positive effects of SA in preventing cumulative damage development and alleviating the oxidative damages caused by metals. However, plant survival varied from 84% (maximum) in control SA to 60% (minimum) in 800 mg/l Ni+EDTA treatment. In general, the results demonstrated a concentration-dependent inhibition of the plant survival percentage. Addition of EDTA with metals declined the plant survival whereas SA enhanced plant survival.

Number of branches. The number of branches increased with time in all treatments (Table 5). The highest numbers of branches after 120 days were counted in control SA treatment whereas the lowest in 800 mg/l Ni+EDTA treatments. In all the cases SA influenced growth parameters while EDTA was found to play

DOI: 10.6092/issn.2281-4485/8528

negative role. ANOVA showed significant differences in the average number of branching under different Ni treatments. Results also showed that the number of branches in plant decreased with increase in concentration of Ni treatments and these findings are corroborated by Opeolu et al. (2010).

Table 5
Response of number of branches in B. juncea under Ni stress

Treatment	No. of branches with days of treatment			
	30 days	60 days	90 days	120 days
T1	$4.6\pm2.0^{a,b,c}$	$5.3\pm1.5^{a,b}$	$6.6\pm2.0^{b,c}$	$7.3\pm1.5^{a,b}$
T2	$5.3\pm2.5^{a,b,c}$	6.3 ± 2.5^{b}	$6.8\pm1.5^{b,c}$	$9.3\pm2.5^{b,c}$
T3	$4.6\pm1.5^{a,b,c}$	4.6 ± 2.5^{a}	$5.6\pm2.0^{a,b}$	$7.6\pm1.5^{a,b}$
T4	$4.0\pm2.0^{a,b,c}$	4.4 ± 1.7^{a}	4.6 ± 1.5^{a}	5.6 ± 1.5^{a}
T5	$3.0\pm2.0^{a,b,c}$	3.3 ± 1.0^{a}	4.3 ± 1.5^{a}	5.0 ± 1.0^{a}
T6	2.3 ± 1.5^{a}	4.0 ± 1.5^{a}	$5.0\pm2.0^{a,b}$	5.3 ± 2.0^{a}
T7	$4.3\pm1.5^{a,b,c}$	4.6 ± 1.5^{a}	$5.6\pm1.5^{a,b}$	7.7 ± 1.7^{b}
T8	$3.0\pm1.7^{a,b}$	3.6 ± 1.5^{a}	$5.0\pm2.0^{a,b}$	$7.3\pm1.5^{a,b}$
T9	$3.0\pm1.0^{a,b}$	3.4 ± 1.1^{a}	3.8 ± 1.1^{a}	5.0 ± 1.0^{a}
T10	2.3 ± 1.5^{a}	3.1 ± 2.0^{a}	3.3 ± 1.5^{a}	4.3 ± 1.5^{a}
T11	6.6 ± 2.0^{c}	6.8 ± 1.5^{b}	$7.0\pm1.5^{b,c}$	12.0 ± 2.0^{c}
T12	$6.3\pm1.5^{b,c}$	6.6 ± 1.5^{b}	9.6 ± 2.0^{c}	11.0 ± 2.0^{c}
T13	$5.0\pm1.0^{a,b,c}$	$5.0\pm2.0^{a,b}$	$8.3\pm2.0^{b,c}$	$9.3\pm1.5^{b,c}$
T14	$4.0\pm2.0^{a,b,c}$	4.3 ± 2.0^{a}	6.3 ± 1.5^{b}	$6.6\pm2.0^{a,b}$
T15	$3.3\pm1.5^{a,b,c}$	3.6 ± 2.0^{a}	4.6 ± 1.5^{a}	5.3 ± 1.5^{a}

Values represent mean ± standard deviation (n=3). Means followed by the same letter within a column do not differ significantly according to DMRT at P=0.05.

Number of leaves. Numbers of leaves increased with time in all treatments and decreased with increased concentrations of Ni (Table 6).The differences among treatments were found significant (Table 3). EDTA did not help in the growth of leaves. Wu et al. (2004) demonstrated that the leaves of *B. juncea* developed numerous brown dots at 2-4 days after adding 3.0 mmol/kg EDTA to the soil. The whole leaves yellowed and died slowly, indicating phytotoxicity of the EDTA–metal complex. Our results showed that addition of SA enhanced the growth of leaves which are similar to the results of Cu+SA treated sunflower plants (El-Tayeb et al., 2006).

Root length. It is clear from the Figure 1 that root length was affected with increased concentrations of Ni, however, there was direct relationship between root length and time. SA showed better results than EDTA in all treatments. Tripathi and Tripathi (2000) showed that Ni gave promotive effect on root and shoot growth, leaf area, biomass, chlorophyll, protein, carbohydrate and sugar in leaves which were positively and significantly correlated with leaf area, root and shoot length and biomass of *Albizia lebbek* plants. In tomato, the highest significant increase was obtained with 30 mg/kg of Ni soil level for plant height, number of branches, leaf area and root length (Gad et al., 2007).

Table 6
Response of number of leaves of B. juncea under Ni stress.

Treatment	No. of leaves with days of treatment			
	30 days	60 days	90 days	120 days
T1	7.0±1.5^{a,b}	7.7±1.5^{b,c}	9.0±1.5^{b,c}	22.3±1.5^{e}
T2	7.0±1.5^{a,b}	8.7±2.0^{b,c}	9.0±1.5^{b,c}	22.3±2.0^{e}
T3	6.0±2.1^{a,b}	6.7±1.0^{a,b}	7.7±2.1^{a,b}	17.3±1.0^{d}
T4	5.0±2.1^{a,b}	6.0±2.1^{a,b}	7.3±1.5^{a,b}	11.0±1.0^{b}
T5	4.7±2.0^{a}	5.5±2.0^{a,b}	6.7±2.6^{a,b}	9.0±2.0^{a,b}
T6	7.0±2.0^{a,b}	8.3±2.6^{b,c}	8.7±1.5^{b,c}	22.0±1.0^{e}
T7	6.0±2.1^{a,b}	7.7±2.1^{b,c}	8.0±2.0^{b}	12.7±2.0^{c}
T8	5.7±2.0^{a,b}	6.3±2.0^{a,b}	7.0±2.5^{a,b}	11.3±1.5^{b,c}
T9	4.7±1.5^{a}	5.0±2.1^{a,b}	6.3±1.0^{a,b}	11.0±2.5^{b}
T10	4.0±1.7^{a}	4.3±1.5^{a}	5.0±1.0^{a}	7.3±2.5^{a}
T11	9.5±3.0^{b}	9.7±2.5^{c}	11.0±2.0^{c}	26.0±1.5^{f}
T12	8.0±2.0^{a,b}	9.0±1.2^{c}	9.3±1.7^{b,c}	22.7±2.1^{e}
T13	6.3±2.1^{a,b}	7.3±1.5^{b,c}	8.3±1.5^{b,c}	20.0±1.5^{d,e}
T14	5.7±1.5^{a,b}	7.0±1.5^{b}	8.0±1.5^{b}	12.0±1.0^{b,c}
T15	5.3±2.0^{a,b}	6.0±2.5^{a,b}	7.0±2.1^{a,b}	11.3±2.5^{b,c}

Values represent mean ± standard deviation (n=3). Means followed by the same letter within a column do not differ significantly according to DMRT at *P*=0.05.

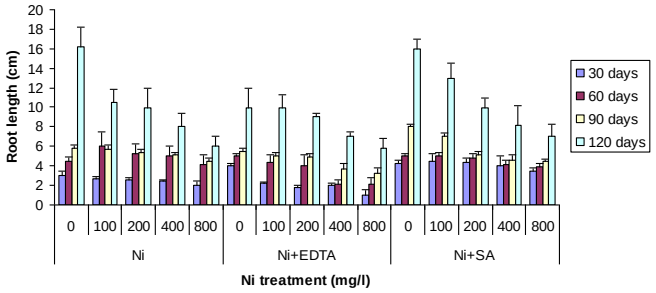

Figure 1
Influence of Ni treatments on root length in B. juncea

Shoot length. 800 mg/l of Ni decreased shoot length to about 37% of the control after 120 days (Figure 2). Shoot length increased with the increase in time in all treatments. Results showed that shoot growth was better in SA treatments compared to EDTA.

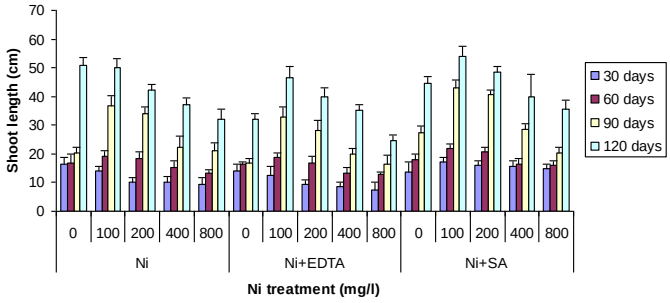

Figure 2
Influence of Ni treatments on shoot length in B. juncea

DOI: 10.6092/issn.2281-4485/8528

Shoot length was significantly affected by different metal concentrations (Table 3). Our results on positive effect of SA on different plant growth parameters are supported by Khodary (2004) and El-Tayeb et al. (2006).

Plant weight. Ni increased fresh weight from 18% (with 800 mg/l Ni) to 54% (with 100 mg/l Ni) of control after 120 days (Figure 3). Plant showed maximum dry weight in control SA treatment (Figure 4). Dry weight was significantly lower in EDTA treated plants as reported by Van Engelen et al. (2007).

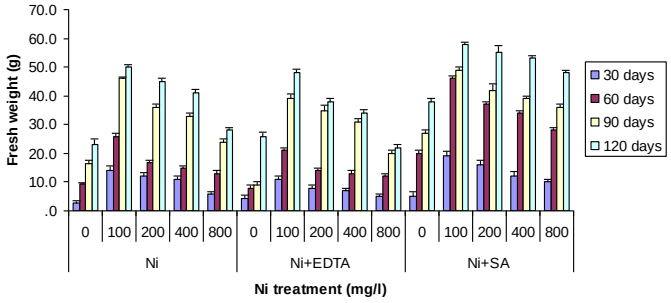

Figure 3
Influence of Ni treatments on fresh weight in B. juncea

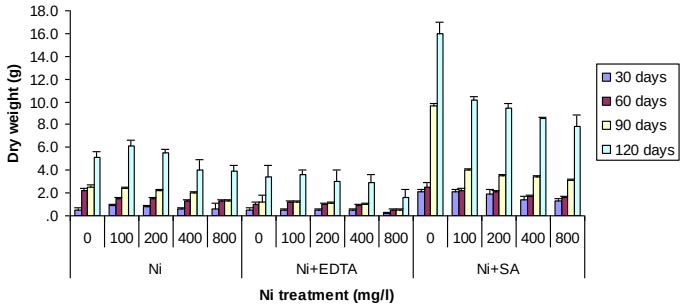

Figure 4
Influence of Ni treatments on dry weight in B. juncea

In general, with the increase in concentration of Ni progressive decrease in plant fresh and dry weight was observed. Growth reduction at higher concentration may be due to the toxic effects of Ni on plants. Similar results were reported by Panwar et al. (2002) and Rajkumar and Freitas (2008). Likewise, other investigators (Chen et al., 2004; Evangelou et al., 2006; Ruley et al., 2006) also reported lower plant yields with the application of EDTA which may be due to disbalance of essential nutrients by EDTA that may lead to cell metabolism disturbance and destabilization of biological membrane. In our results, SA stimulated yields of *B. juncea* and this is in agreement with Gunes et al. (2007) who reported that exogenous levels of SA increased dry yield of maize significantly both in saline and non-saline conditions.

Effect of treatments on biochemical parameters

Various biochemical parameters like chlorophyll, total soluble sugar, soluble protein, and proline content were studied. All biochemical parameters showed declination with increasing Ni concentrations. A higher accumulation of

chlorophyll, soluble sugars, soluble proteins and proline occurred in Indian mustard plants treated with SA. Addition of EDTA enhanced chlorophyll content, soluble sugar and soluble protein but reduced proline content in all treatments.

Chlorophyll. The level of chlorophyll also indicates the toxic nature of heavy metals in the plant system. The changes in chlorophyll a (chl a) and chlorophyll b (chl b) and total chlorophyll (total chl) are represented in Figure 5. With increasing Ni concentrations, the content of Chl a, Chl b, and Chl total decreased. The maximum pigment contents were recorded in control plants.

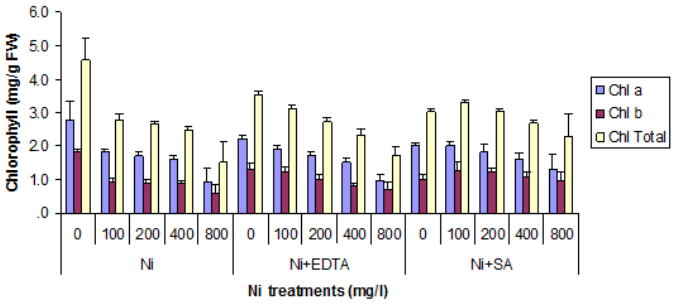

Figure 5
Influence of Ni stress on chlorophyll content in B. juncea

The decrease in chlorophyll content at higher Ni treatment may be due to the degradation of chlorophyll by free radicals generated by metals. The breakdown of photosynthetic pigment may also be due to substitution of Mg^{+2} ion in chlorophyll molecules by metal ions Pb^{+2} and Ni^{+2} (Küpper et al., 1996, 1998).

The metals Pb, Ni, Cd, Mn, Co and their mixture have been reported to enhance the activity of chlorophyllase on chl a decomposition more than that on chl b (Kambhampati et al., 2005).

SA caused a general increase in chlorophyll content in Ni stressed plants. In agreement with this, SA was also reported to increase the chlorophyll content and stimulate the photosynthetic machinery in maize both under control and salinity stress (Khodary, 2004). SA alleviated Cd toxicity in barley seedlings by increasing chlorophyll content (Metwally et al., 2003). Krantev et al. (2008) reported that the chlorophyll content of maize plants reduced approximately 20% and 50% in 15 and 25 µM Cd treatments respectively. However, pretreatment with SA before exposure to Cd led to 17% and 41% restoration of the chlorophyll levels.

No negative effect of EDTA on chlorophyll content was observed. Saleh (2002) found increased chlorophyll content in *Chorcorus olitorius* in response to Ni+EDTA stress due to the formation of heavy metal-EDTA complex and this complex is unable to penetrate the plant membrane.

The ratio of chlorophyll a/b increased slightly with increasing Ni treatments (Table 7) which is consistent with the results of Zengin and Munzuroglu (2005) and it may be linked to the reduction in light harvesting chlorophyll proteins (LHCPs) (Loggini et al., 1999). The decrease in the chlorophyll a/b ratio may indicate a

DOI: 10.6092/issn.2281-4485/8528

proportionately greater effect on photosystem reaction centers compared to light harvesting complexes (LHC), since the reaction centers are relatively rich in chl a, while the LHCs are rich in chl b (Kamel, 2008).
Salicylic acid significantly reduced the Chl a/b ratio in Ni stressed plants as compared to EDTA. It has been reported that SA decreases the Chl a/b ratio in wheat plants (Moharekar et al., 2003). El-Tayeb et al. (2006) also found similar results in sunflower plants for counteraction of Cu toxicity.

Table 7
Response of Chl. a/b ratio in B. juncea under Ni stress

Treatment	Chl. a/b
T1	$1.53^{a, b}$
T2	1.56^{b}
T3	$1.86^{c,d,e}$
T4	$1.88^{d,e}$
T5	$1.94^{d,e,f}$
T6	$1.46^{a,b}$
T7	$1.60^{b,c}$
T8	$1.72^{b,c,d}$
T9	$1.90^{d,e,f}$
T10	1.40^{a}
T11	2.02^{f}
T12	$1.55^{a, b}$
T13	$1.49^{a,b}$
T14	$1.51^{a,b}$
T15	1.37^{a}

Values represent mean ± standard deviation (n=3). Means followed by the same letter within a column do not differ significantly according to DMRT at *P*=0.05.

Soluble sugar. There was a significant reduction in soluble sugar content of *B. juncea* occurred in response to Ni stress (Figure 6). Soluble sugar got decreased to 43% as compared to control in 800 mg/l Ni treatment. SA significantly ($P<0.05$) induced an increase in soluble sugars of Ni-stressed plants. Our results are consistent with others (El-Tayeb et al., 2006; Mba et al., 2007). El-Tayeb et al. (2006) reported that under excess Cu, a higher accumulation of soluble sugars, soluble proteins and free amino acids including proline occurred in sunflower plants treated with 0.5 mM SA. The combined treatment of 5 mg/l Cd+500 mmol/l SA in Chinese cabbage showed that the soluble sugar content increased significantly to about 25.47% in comparison with the control (Mba et al., 2007).

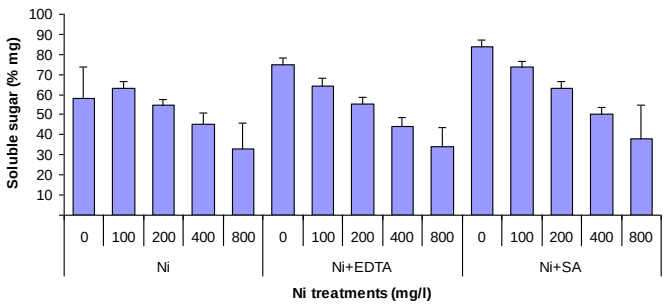

Figure 6
Influence of Ni stress on total soluble sugar content in B. juncea

Soluble protein. From Figure 7, the protein content in Indian mustard plants decreased with an increase in Pb and Ni concentrations. The highest soluble protein content (220±3.5 mg/g) was observed in control SA plants and the lowest (100.5±23 mg/g) in 1600 ppm Pb+Ni treated plants. The protein concentration was enhanced after addition of EDTA/SA.

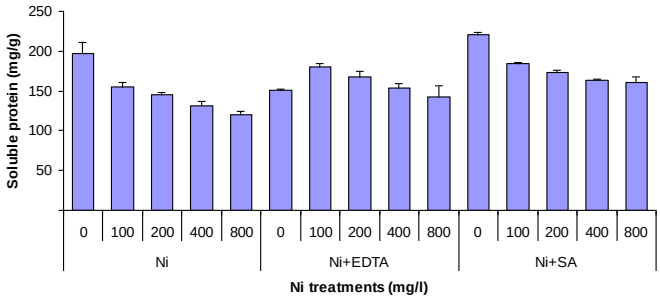

Figure 7
Influence of Ni stress on soluble protein content in B. juncea

However, the accumulation of proteins in plant organs due to heavy metals is well known (Alia et al., 2015; Demirevska-Kepova et al., 2004). The decrease in protein content as observed at higher concentrations of Pb and Ni in *B. juncea* may be because of enhanced protein degradation process as a result of increased protease activity (Palma et al., 2002) found to increase under stress conditions. These heavy metals may have induced lipid peroxidation and fragmentation of proteins due to toxic effects of reactive oxygen species which led to reduced protein content. On the contrary, an increase was observed in protein content in wheat and mustard plants irrigated with effluents which may be attributed to the induction of several stress proteins (Chandra et al., 2009). SA induced a considerable increase in the content of protein fractions in Cu stressed sunflower plants (El-Tayab et al., 2006), as well as Cd and Ni, stressed chamomile plant (Kovacik et al., 2009). Increased protein content was reported in *Typha orientalis Presl* treated with Pb+EDTA (Li et al., 2009).

Proline: The content of proline increased with the concentration of Ni (Figure 8). Presence of EDTA reduced proline content in all treatments. Effect of SA was more marked to combat Ni stress by increasing proline content than control. Our findings are corroborated by Saleh (2002) who reported that proline content was increased significantly with increasing Ni concentration and decreased after EDTA treatment on Chorcorus olitorius treated with Ni (10 and 50 µM) and EDTA (10, 50 and 100 µM) in different combinations. EDTA reduced proline content may be due to formation of heavy metal-EDTA complex. Table 8 illustrates two-way ANOVA summary for biochemical parameters of Indian mustard.

DOI: 10.6092/issn.2281-4485/8528

Biochemical parameters	Source of variance	$F_{critical}$	$F_{observed}$	P-value
Chlorophyll	Treatments	1.80	38.6	0.05
	Chl	3.09	632	0.000
	TreatmentsxChl	1.60	3.19	0.07
Soluble sugar	Chelant	1.80	27.9	0.000
	Ni	3.09	3.36	0.03
	ChelantxNi	1.60	3.15	0.000
Soluble protein	Chelant	1.80	100	0.000
	Ni	3.09	1.94	0.14
	ChelantxNi	1.60	17.4	0.000
Proline	Chelant	1.80	288	0.000
	Ni	3.09	1769	0.000
	ChelantxNi	1.60	51.5	0.000

Table 8
Two-way ANOVA summary for biochemical parameters of Indian mustard.

Ni accumulation in Indian mustard and residual Ni in soil

The Ni concentrations in plants without chelant showed a maximum level of 24±4.6 mg/kg whereas after EDTA application the Ni concentrations increased up to ten fold (241±52.5 mg/kg) with highest Ni treatment after 120 days (Figure 9). EDTA treated plants showed 83-90% higher Ni accumulation rates compared to control for the applied Ni doses of 100-800 mg/l respectively. The metal accumulation order was Ni+EDTA > Ni+SA > Ni.

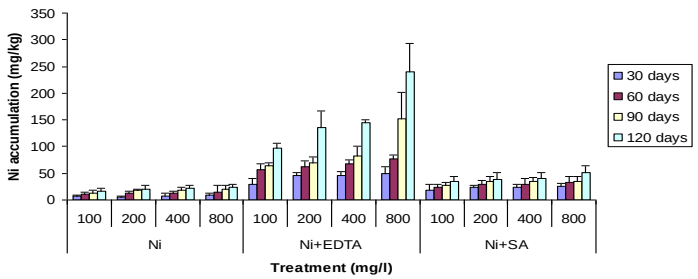

Figure 9
Ni accumulation by B. juncea

Our results are supported by the findings of Meers et al. (2004) who compared the effect of synthetic aminopolycarboxylic acids (EDTA, NTA, and DTPA) with a number of biodegradable, low-molecular weight, organic acids (citric acid, ascorbic acid, oxalic acid, salicylic acid, and ammonium acetate) as potential soil amendments for enhancing phytoextraction of heavy metals (Cu, Zn, Cd, Pb, and Ni) by *Zea mays*. They observed that the translocation efficiency for Pb and Ni was higher in EDTA than SA. Panwar et al. (2002) observed nickel concentration almost double that in control in *B. juncea* exposed to Ni contamination along with EDTA application and found *B. juncea* has the potential to be hyperaccumulator of Ni. Ni accumulation coefficients for *B. juncea* with and without EDTA were 0.37 and 0.21 exposed to 100 mg /kg Ni concentration (Galiulin and Galiulina, 2008). Jean et al. (2008) reported that EDTA was the most effective at increasing the

uptake of Ni in *Datura innoxia*. The translocation factor of Ni was 1.6 fold higher than the control for 1 mmol/kg EDTA.

A Ni-nicotianamine complex was recently identified as the responsible chelator for the translocation of Ni in *Thlaspi caerulescens* (Mari et al., 2006). Other researchers have found that Ni tolerance and transfer through the plant xylem is dependent on free histidine. Although, malic and citric acid were also effective, free histidine translocated the largest amount of Ni (Wycisk et al., 2004). In the absence of EDTA, both histidine and nicotianamine mediated transport may explain the increased Ni translocation. Conversely, once Ni had bound to EDTA it was expected not to be able to complex with histidine, thereby limiting translocation (January et al., 2008).

The specific mechanisms of Ni uptake by plant root systems have not been yet elucidated. The uptake of Ni, like other metals, can be carried out by passive diffusion and active transport (Seregin and Kozhevnikova, 2006). However, the ratio between the active and passive transport depends on the Ni concentration in the nutrient solution. The former is more important at low Ni concentrations (below 34 mmol/l), and at higher concentrations the role of the passive transport mechanism increases due to the Ni toxic effect (Demchenko et al., 2005). Moreover, according to Dan et al. (2002), when the accumulation does not increase in roots with an increase in the applied metal concentration, this indicates a predominantly active uptake of this metal.

Summary of Two-way ANOVA for Ni accumulation in Indian mustard and residual Ni in soil is represented in Table 9.

Parameters	Source of variance	$F_{critical}$	$F_{observed}$	*P*-value
Ni accumulation	Treatments	1.88	43.0	0.000
	Time	1.88	13.3	0.000
	TreatmentsxTime	2.69	203	0.000
Ni remained in soil	Chelant	1.55	21.5	0.000
	Ni	2.69	226	0.000
	ChelantxNi	1.55	29.9	0.000

Table 9 *Summary of Two-way ANOVA for Ni accumulation in Indian mustard and residual Ni in soil*

Soil contents of Ni decreased due to enhanced Ni uptake by plants in chelants treated soil (Figure 10). EDTA was found to be more efficient. This supremacy of EDTA over SA may be due to higher stability constant of metal-EDTA complexes than metal-SA complexes.

DOI: 10.6092/issn.2281-4485/8528

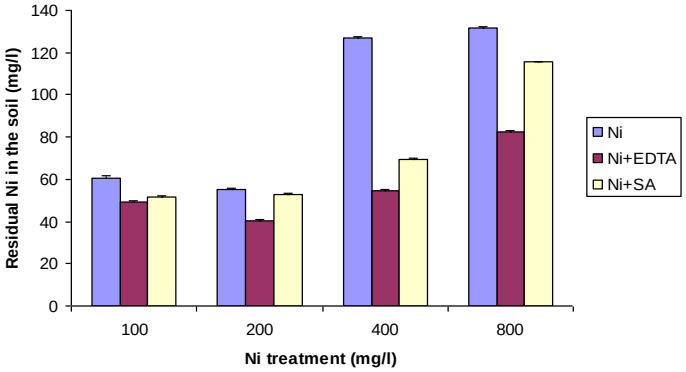

Figure 10
Effect of treatments on Ni remained in soils

Conclusion

EDTA treated *Brassica juncea arawali* plants showed 83-90% higher Ni accumulation compared to control for the applied Ni doses of 100-800 mg/l respectively. EDTA-assisted phytoextraction showed better results than SA-assisted phytoextraction and continuous phytoextraction in Indian mustard plants.

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