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ORIGINAL ARTICLE

Lead and Nickel Accumulation in *Brassica juncea arawali* Growing in Contaminated Soil

Leela Kaur[*1], Kasturi Gadgil[2], Satyawati Sharma[3]

[1] *Department of Environmental Sciences, Maharaja Ganga Singh University, Bikaner (Rajasthan) – 334004, India*

[2]*Centre for Energy Studies, Indian Institute of Technology (I.I.T.) Delhi, New Delhi – 110016, India*

[3] *Centre for Rural Development & Technology, Indian Institute of Technology (I.I.T.) Delhi, New Delhi – 110016, India*

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KEYWORDS

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Lead;
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Phytoextraction;
Salicylic acid

ABSTRACT: *Brassica juncea arawali* plants were exposed to 0, 100, 200, 400 and 800 mg/l concentrations of Lead (Pb) and Nickel (Ni). Plants were treated with control, ethylene diamine tetraacetic acid (EDTA) and salicylic acid (SA) chelant applications at Micromodel experimental site of Indian Institute of Technology, Delhi in 2009. A high level of combined metal concentrations (1600 mg/l) was taken to assess the feasibility of phytoextraction on a high-level metal contaminated soil. Plants were analyzed for growth parameters, biochemical parameters and metal accumulation. EDTA decreased all morphological parameters whereas SA stimulated them. All biochemical parameters showed declination with increasing Pb and Ni concentrations. A higher accumulation of chlorophyll, soluble sugars, soluble proteins and proline occurred in Indian mustard plants treated with SA. Pb and Ni accumulation in plants increased in a dose-response manner with increasing levels of metal treatments and time. EDTA was found to be more efficient chelant than SA for removal of Pb and Ni from contaminated soil.

INTRODUCTION

Heavy metal contamination of soil is one of the prime environmental concerns in the existing era. Heavy metals may indirectly go into the human body via food and cause health issues [1-3]. Our food production depends on soil quality too. Therefore, future generations will face food security problems along with health issues. Hence, scientists are researching the solutions for it. The quality of heavy metal contaminated soil can be improved by removing these heavy metals from soil. Various physical, chemical and biological technologies have been used to remove heavy metals from soil over the last two decades [4, 5]. However, due to high cost and incomplete metal removal, phytoremediation technologies are gaining attention. Phytoremediation technologies use plants to remove contaminants from any medium. One of the mechanisms of phytoremediation for removal of heavy metals from soil medium is phytoextraction which is the uptake of contaminants from soil by plant roots and their translocation and accumulation in shoots [6-8]. The recent phytoextraction researches are on identification of

[*]Corresponding author: leela.kaur@gmail.com (L. Kaur)

hyperaccumulator plants for natural phytoextraction and evaluation of chelating agents for enhancing metal accumulation in plants via induced phytoextraction [9-13].

We aimed to assess metal phytoremediation capacity of *Brassica juncea* with or without chelant application (EDTA and SA). The metals chosen for the study were lead (Pb) and nickel (Ni). Of these metals, Pb is a non-essential element for plants while Ni (in trace concentrations) is essential for plant growth.

MATERIALS AND METHODS

Experimental design

Pot experiment was conducted on *Brassica juncea arawali* with different concentration of combined Pb and Ni at Micromodel experimental site of Indian Institute of Technology, Delhi in 2009. It is situated at 77.09°E longitude and 20.45°N latitude and 28 m altitude above sea level. The mean of maximum and minimum temperature during the study period were 19-42 °C and 4-14 °C respectively. The physicochemical properties of soil used for mustard plant experiments are summarised in Table 1.

Various concentrations of lead and nickel gave to *B. juncea* were 0+0, 100+100, 200+200, 400+400 and 800+800 mg/l of lead and nickel respectively. The treatment was divided into three subgroups viz. (a) no chelant addition, (b) Ethylenediaminetetraacetic acid (EDTA) addition and (c) salicylic acid (SA) addition. EDTA and SA were applied during the 10^{th} week of plant growth (Table 2).

Table 1. Main physicochemical properties of soil

Parameter	Unit	Amount
Texture	-	Sandy Loam
Clay	%	14.2
Silt	%	15.3
Sandy	%	70.5
Electrical Conductivity	mS/cm	0.3
pH	-	7.6
Cation Exchange Capacity	Cmol/kg	18
Organic Carbon	%	0.7
Available N	kg/ha	270
Available P	kg/ha	8.0
Available K	kg/ha	198
Total Pb	mg/kg	0.01
Total Ni	mg/kg	3.0

Table 2. Designed treatments for mustard pot experiment

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

The seeds of *Brassica juncea arawali* were procured from the National Seeds Corporation Ltd. Beej Bhawan, Pusa, and New Delhi. Twenty seeds were sown in 11x11 cm pots containing 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. In chemical treatment, Pb, Ni, EDTA and SA were added as per the designed treatment. The pots were arranged in a complete randomized block design with three replications. Watering was done regularly to maintain optimal moisture level. Seed germination started after the seventh day of sowing and plants were thinned to 3 plants per pot after 30 d of sowing. The first harvesting was done after 30 d of sowing and subsequent harvesting was done on 8^{th} day after chelant application. Third and fourth harvestings were done at flowering stage and maturation stages respectively.

Plant analysis

Morphological parameters of mustard plants 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 tested by percentages of relative seed germination (RSG) and germination index tests. The phytotoxicity of heavy metals was evaluated by the seed germination index (GI). The germination index was calculated according to the following formula [14].

G1(%) = Seed germination (%) × Root length of treatment × 100 / Seed germination (%) × Root length of control

Relative seed germination (%) after exposure to different treatments was calculated as follows [15]:

RSG (%) = Number of seeds germinated in treatment × 100 / Number of seeds germinated in control

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.

Various biochemical parameters like chlorophyll, total soluble sugar, soluble protein and proline content were studied [16]. Arnon method was used to estimate the chlorophyll content in mustard plants leave samples [17]. Total soluble sugars and soluble protein were estimated following the method of anthrone [18] and Bradford [19] respectively. Proline content was measured by ninhydrin method [20].

The dried plant samples were used for the estimation of heavy metals. Metal analysis was done using ICP-OES (Varian Vista-MPX CCD Simultaneous ICP-OES Varian Australia Pty. Ltd). The bioconcentration factor (BCF) which provides an index of the ability of the plant to accumulate the metal with respect to the metal concentration in the substrate was also determined [21].

Data 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.

RESULTS AND DISCUSSION

Effect of treatments on morphological parameters

Effect of chelants EDTA and SA and pollutant treatments on morphological parameters were studied. The germination, length of root and shoot, number of branches and leaves, plant survival (%) and plants fresh and dry weight were determined for judging the growth of plants.

Seed germination and survival

Seed germination and percentage survival of Indian mustard reduced with increasing concentration of Pb and Ni (Table 3). Germination and survival of *B. juncea* enhanced by addition of SA while EDTA decreased it. Germination (%) was significant with time but non-significant with chelant treatments (P=0.33) (Table 4). Addition of EDTA with metals inhibited the seed

germination and these results are corroborated with Ilbas *et al.* [22]. Different dosages of EDTA (30, 60, 90, 120 and 150 mmol/l) were decreased the germination rates of barley seeds. The positive effect of SA for attenuating metal stress in plants can be explained by three reasons: (i) SA may prevent cumulative damage development in response to heavy metals; (ii) SA may alleviate the oxidative damages caused by metals; and (iii) pretreatment with SA may exert a protective effect on the membrane stability [23].

Table 3. Effect of combined Pb+Ni treatments on seed germination and survival of *B. juncea*

Treatment	Seed germination		Relative seed germination (%)		Germination Index (GI)	Survival (%)
	10 d	25 d	10 d	25 d		
T1	$4\pm2.6^{b\,c}$	$17\pm1^{a\,b}$	-	-	-	70^{b}
T2	$3\pm2^{a\,b}$	$16.6\pm3^{a\,b}$	75	98	1.13	$68^{a\,b}$
T3	$2.3\pm1.5^{a\,b}$	$16.6\pm0.5^{a\,b}$	58	98	0.96	$58^{a\,b}$
T4	$2.3\pm0.5^{a\,b}$	15.3 ± 1.5^{a}	58	90	0.8	$56.7^{a\,b}$
T5	1.7 ± 1.5^{a}	13.3 ± 1.5^{a}	41.6	78	0.78	$55^{a\,b}$
T6	3.7 ± 1.5^{b}	16 ± 4^{a}	91.6	94	1.01	$67^{a\,b}$
T7	$4.7\pm2.5^{c\,d}$	16.3 ± 0.57^{a}	116.7	96	1.11	$68^{a\,b}$
T8	$2.7\pm1.5^{a\,b}$	16.3 ± 3.5^{a}	66.7	96	0.92	$65^{a\,b}$
T9	1.7 ± 1.5^{a}	16 ± 3.6^{a}	41.7	94	0.82	$51.7^{a\,b}$
T10	1 ± 1^{a}	12.6 ± 2^{a}	25	74.5	0.60	45^{a}
T11	$4.3\pm1.5^{b\,c}$	18 ± 1^{b}	108	105.8	1.15	82^{c}
T12	6 ± 2.7^{d}	16 ± 2.6^{a}	150	94	1.06	75^{c}
T13	4.6 ± 0.57^{c}	15 ± 2^{a}	116.6	88	0.99	$71.7^{b\,c}$
T14	$4.3\pm0.57^{b\,c}$	15 ± 1^{a}	108	88	0.94	$65^{a\,b}$
T15	$3\pm1^{a\,b}$	14 ± 1^{a}	75	82	0.83	$60^{a\,b}$

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.

Table 4. 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	1.15	0.33
	Time	4.00	889	0.000
	Treatmentsx Time	1.86	1.70	0.07
Survival (%)	Chelant	1.80	1.54	0.11
	Metal	3.09	5.45	0.005
	Chelantx Metal	1.60	0.55	0.96
No. of branches	Treatments	1.77	1.36	0.18
	Time	2.68	18.7	0.000
	Treatmentsx Time	1.48	2.15	0.000

Table 4. Continued

No. of leaves	Treatments	1.77	723	0.000
	Time	2.68	14576	0.000
	TreatmentsxTime	1.48	202	0.000
	Treatments	1.77	415	0.000
Fresh weight	Time	2.68	6605	0.000
	TreatmentsxTime	1.48	53.4	0.000
	Treatments	1.77	107	0.000
Dry weight	Time	2.68	723	0.000
	TreatmentsxTime	1.48	60.1	0.000
	Treatments	1.77	11.6	0.000
Root length	Time	2.68	931	0.000
	TreatmentsxTime	1.48	19.9	0.000
	Treatments	1.77	20.8	0.000
Shoot length	Time	2.68	909	0.000
	TreatmentsxTime	1.48	25.6	0.000

The highest percentage of plant survival (85%) with controls SA and the lowest (45%) with 1600 ppm Pb+Ni+EDTA. In general, the results demonstrated a concentration-dependent inhibition of the plant survival percentage. Chelant did not show significant effect but the difference was significant in metals treatments (P=0.005) (Table 4). Similar results were obtained by another study for white clover treated with 25-100 mg/l of Ni [24]. Heavy metals can induce several abnormalities on ovule development and can indirectly affect the survival of plants [25]. Addition of EDTA with metals declined the plant survival. However, SA enhanced plant survival. SA induces an oxidative burst involving hydrogen peroxide accumulation which acts as the signal transducer for systemic acquired resistance (SAR) [26]. SA has also been seen to induce tolerance to many abiotic stresses [27-29].

Number of branches

The number of branches increased with time in all treatments (Table 5). The differences in number of branches among different treatments were not significant (P=0.18). In all the cases SA influenced growth parameters while EDTA was found to play negative role. ANOVA showed significant differences in the average number of branching both under different metal treatments and between types of metal (except for combined Pb+Ni treatments). The number of branching or tillering gradually increased with time. The number of branches in plant decreased with increase in concentration of metal treatments and these findings are corroborated by another research [30]. Heavy metal tolerance at vegetative stage is crucial for yielding vigorous plants for tolerating metal stress at later stages of growth. The observed reduced degree of branching at higher metal concentration was also observed for Pb in *Solanum melogena* [31] and tomato [30].

Table 5. Effect of combined Pb+Ni treatments on number of branches of *B. juncea*

Treatment	No. of branches with days of treatment			
	30 d	60 d	90 d	120 d
T1	7.0±2.0^{c}	7.7±2.5^{b}	8.3±3.1^{c}	8.7±2.5^{c}
T2	4.3±2.0$^{a\,b}$	6.3±2.5$^{a\,b}$	8.3±2.1^{c}	8.7±2.5^{c}
T3	4.0±2.0$^{a\,b}$	5.7±2.1$^{a\,b}$	7.0±2.1$^{b\,c}$	8.3±1.5$^{b\,c}$
T4	3.7±1.5$^{a\,b}$	5.0±2.1$^{a\,b}$	6.3±1.5$^{b\,c}$	7.3±1.5^{b}
T5	3.3±1.5^{a}	4.3±1.2^{a}	6.0±1.5^{b}	6.3±1.5$^{a\,b}$
T6	5.7±2.1$^{b\,c}$	6.0±1.5$^{a\,b}$	7.0±2.0$^{b\,c}$	7.7±1.2$^{b\,c}$
T7	4.0±2.1$^{a\,b}$	5.7±2.0$^{a\,b}$	7.0±2.0$^{b\,c}$	7.7±1.5$^{b\,c}$
T8	3.7±1.5$^{a\,b}$	5.0±3.0^{a}	6.7±1.5$^{b\,c}$	7.3±2.1^{b}
T9	3.3±1.5^{a}	4.7±2.1^{a}	4.8±1.5$^{a\,b}$	7.0±1.0$^{a\,b}$
T10	3.0±1.5^{a}	4.0±2.0^{a}	4.3±2.0^{a}	5.0±2.0^{a}
T11	8.0±1.0^{d}	8.3±2.1^{b}	8.7±1.5^{c}	12.0±2.5^{d}
T12	7.3±1.5$^{c\,d}$	7.7±2.1^{b}	8.7±1.5^{c}	10.3±2.0$^{c\,d}$
T13	5.0±2.0^{b}	6.0±2.0$^{a\,b}$	7.3±1.0$^{b\,c}$	9.0±2.1$^{c\,d}$
T14	4.3±1.5$^{a\,b}$	5.7±3.1$^{a\,b}$	7.0±1.0$^{b\,c}$	7.3±1.2^{b}
T15	3.7±1.5$^{a\,b}$	5.3±1.5^{a}	6.0±1.0^{b}	7.0±2.0$^{a\,b}$

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

The results of effect of different treatments after 30, 60, 90 and 120 d on number of leaves of *B. juncea* are depicted in Table 6. The differences among treatments were found significant (Table 4). Numbers of leaves increased with time in all treatments and decreased with increased concentrations of Pb and Ni. EDTA did not help in the growth of leaves. Addition of SA enhanced the growth of leaves which are similar to the results of El-Tayeb *et al.* [32] that reported increased growth of leaves in Cu+SA treated sunflower plants.

Table 6. Effect of combined Pb+Ni treatments on number of leaves of *B. juncea*

Treatment	No. of leaves with days of treatment			
	30 d	60 d	90 d	120 d
T1	7.0±2.0$^{a\,b}$	7.7±1.5$^{b\,c}$	9.7±2.6$^{b\,c}$	22.3±2.1^{f}
T2	7.7±2.5$^{a\,b}$	8.7±2.5^{c}	10.3±1.5^{c}	20.0±2.0$^{e\,f}$
T3	7.0±2.0$^{a\,b}$	7.7±2.1$^{b\,c}$	9.0±2.0$^{b\,c}$	17.3±1.5$^{c\,d\,e}$
T4	6.7±2.5^{a}	7.0±2.0^{b}	8.0±1.0^{b}	12.0±1.0$^{a\,b}$
T5	5.8±1.0^{a}	6.0±2.0$^{a\,b}$	6.7±2.1$^{a\,b}$	10.7±2.1^{a}
T6	7.0±2.0$^{a\,b}$	7.7±2.0$^{b\,c}$	9.0±2.0$^{b\,c}$	10.7±2.5^{a}
T7	7.3±1.5$^{a\,b}$	8.0±2.0$^{b\,c}$	9.0±2.5$^{b\,c}$	14.0±2.0^{b}
T8	7.0±2.0$^{a\,b}$	7.3±1.0$^{b\,c}$	8.0±1.0^{b}	12.0±2.0$^{a\,b}$
T9	6.3±1.5^{a}	6.7±2.1$^{a\,b}$	7.0±2.0$^{a\,b}$	11.7±1.5$^{a\,b}$
T10	5.0±2.0^{a}	5.3±2.1^{a}	6.0±1.5^{a}	10.3±2.5^{a}
T11	8.0±2.1^{b}	8.7±1.5^{c}	10.0±3.0^{c}	27.0±2.0^{g}
T12	12.7±1.5^{c}	14±1.0^{d}	15.0±1.0^{d}	20.0±2.0$^{e\,f}$
T13	9.5±2.0$^{b\,c}$	9.7±1.5$^{c\,d}$	11.0±1.0$^{c\,d}$	18.0±2.0$^{d\,e}$
T14	6.3±2.1^{a}	7.3±2.1$^{b\,c}$	8.0±2.0^{b}	14.7±1.5$^{b\,c}$
T15	6.0±2.0^{a}	6.7±1.5$^{a\,b}$	7.0±2.0$^{a\,b}$	11.7±2.5$^{a\,b}$

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.

Root length

Root length was affected by increased concentrations of heavy metals (Figure 1); however, there was direct relationship between root length and time. Overall, 1600 ppm Pb+Ni treatment showed 74% reduction in root length over control. Some treatments were significantly different while others not. The results of reduction in root length due to Pb are in agreement with others [33-35]. The growth inhibition in the presence of heavy metals might be due to some disturbances such as the cellular water status, mitosis, cell cycle and stiffening of cell walls [32]. Similar to our results, reduction of root elongation by EDTA was also observed in other studies [36, 37]. The reason could be the chelating property of EDTA as it can also chelate various essential divalent cations such as Fe, Zn and Cu and therefore, it may disrupt the biochemistry of the leaf cells and ultimately cause cell death [38]. In contrast, negative effect of EDTA was not observed on the rate of root elongation growth of *Typha orientalis Presl* plants [39]. *T. orientalis Presl* plants cultivated with the addition of Pb-EDTA showed high resistance to the phytotoxic effect of metals. Adding 0.1 mmol/l EDTA to 500 mg/l Pb level raised the index of tolerance (IT) value 84% which was close to the control plants whereas after adding a higher concentration of the chelate (0.5 mmol/l EDTA) root elongation growth raised 66%.

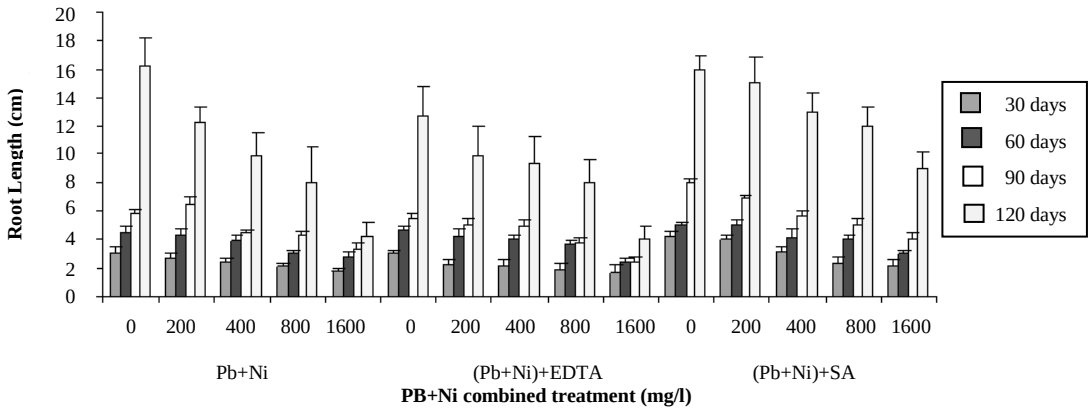

Figure 1. Effect of combined Pb+Ni treatments on root length of *B. juncea*

Shoot length

The decline in shoot length in *B. juncea* was observed with the increasing concentrations of Pb and Ni (Figure 2). All treatments exhibited increase in shoot length with time and it was better in SA treatments. Shoot length was significantly affected by various metal concentrations (Table 4). Our results on positive effect of SA on different plant growth parameters are supported by another study [32]. Similarly the negative effect of EDTA is supported by Sinhal *et al.* [40] who showed that Zn, Cu, Pb and Cd in combination with 30 mg/l concentration of EDTA and citric acid caused significant reduction in growth of marigold in terms of plant height, fresh weight, total chlorophyll, carbohydrate and protein contents.

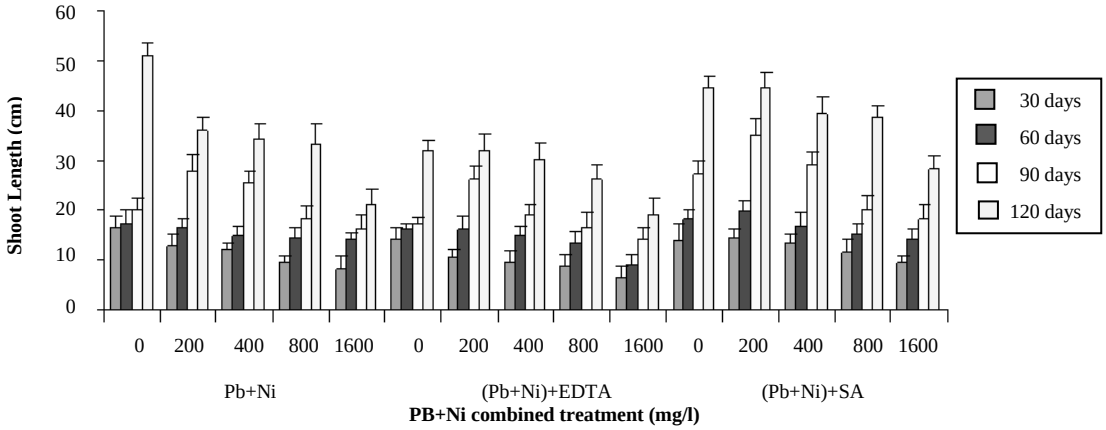

Figure 2. Effect of combined Pb+Ni treatments on shoot length of *B. juncea*

Plant weight

Plant weight significantly increased with the increase in exposure time in all treatments (Figure 3). At 1600 ppm Pb+Ni treatments, fresh weight reduced to about 13% and 56% (as compared to control) with the addition of SA and EDTA respectively for the same time period i.e. 120 d.

Plant showed maximum dry weight in control SA treatment (Figure 4). Dry weight was significantly lower in EDTA treated plants. Fresh weight and dry weight were significantly (P<0.05) different in combined Pb+Ni treatments (Table 4).

EDTA decreased significantly plant dry weight whereas SA stimulated plant dry weight compared to control. Plant dry weight was significantly higher in all SA treatments compared to control; however, the time of application of chelant on plant dry weight is very important.

In general, the sensitivity of given plant species to heavy metal toxicity depends on its concentration, the treatment duration on the plant species, its age and the plant organ examined [23]. In our results, Pb and Ni reduced fresh weight of Indian mustard plants and the reason may be that they were exposed to very high concentration of Pb and Ni and at very early stage of their development.

Similar to our results the adverse effect of EDTA on the growth of plants was also reported by other investigators [35, 41, and 42] which may be due to imbalance 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.* [43] who reported that exogenous levels of SA increased dry yield of maize significantly both in saline and non-saline conditions. Similarly, SA treatments alleviated Cd toxicity in barley seedlings [44], Chinese cabbage [45], maize [46], pea seedlings [23] and rice [47]. Increased dry matter of metal-stressed plants in response to SA may be related to the induction of antioxidant response and protective role of membranes that increase the tolerance of plant to damage.

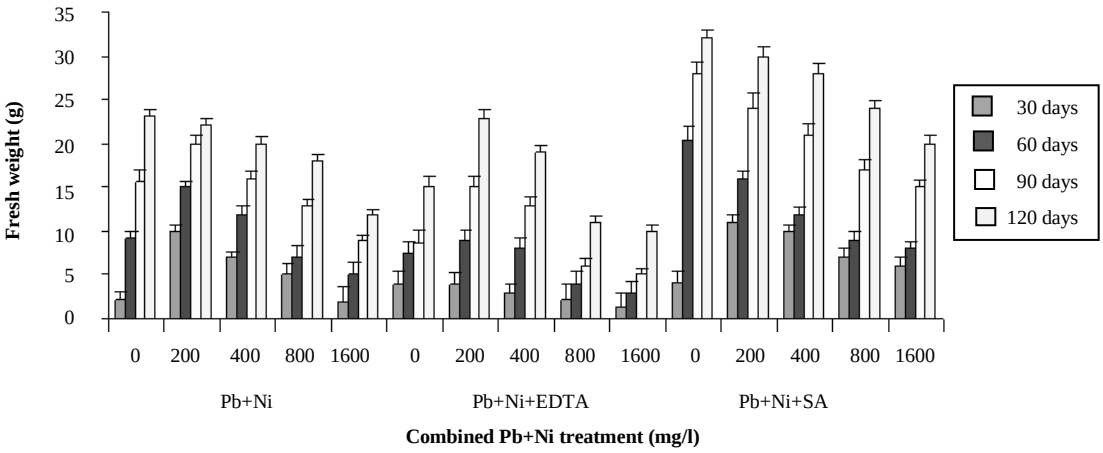

Figure 3. Effect of combined Pb+Ni treatments on fresh weight of *B. juncea*

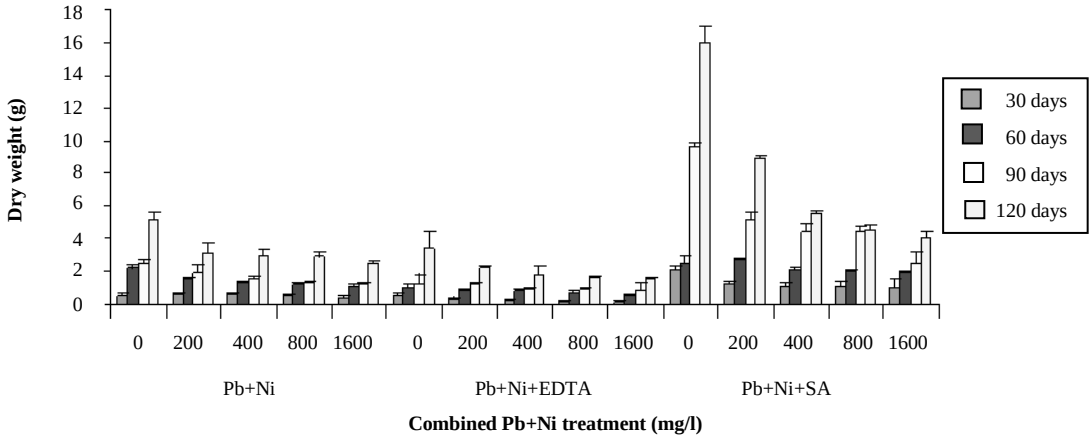

Figure 4. Effect of combined Pb+Ni treatments on dry weight of *B. juncea*

Effect of treatments on biochemical parameters

Biochemical parameters such as chlorophyll, total soluble sugar, soluble protein and proline content were studied. All biochemical parameters showed declination with increasing Pb and Ni concentrations. A higher accumulation of chlorophyll, total soluble sugar, soluble protein 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 changes in chlorophyll a (chl a) and chlorophyll b (chl b) and total chlorophyll (total chl) are represented in Figure 5. With increasing Pb and Ni concentrations, the content of chlorophyll (a, b and total) all decreased. The maximum pigment contents were recorded in control plants. Increased amounts of Chl a, Chl b, and Chl total were observed in plants treated with chelating agents.

The decrease in chlorophyll content at higher metal 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} [48,49]. 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 [50].

SA caused a general increase in chlorophyll content in heavy metal-stressed plants. In agreement with this, SA was also reported to increase the chlorophyll content and stimulate the photosynthetic machinery in maize [44] and barley [46] under Cd stress.

No negative effect of EDTA on chlorophyll content was observed and this is in agreement with [51] who reported similar observation in *Typha orientalis Presl* treated with Pb+EDTA. Increased chlorophyll content was found 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 [52].

Table 7 shows effect of treatments on Chl. a/b ratio of *B. juncea*. The ratio of chlorophyll a/b shows more sensitivity as stress indicator than total chlorophyll content [53]. It is assumed that the conversion of chl b to chl a represents a critical factor for changes in chlorophyll a/b ratio [54]. The ratio of chlorophyll a/b increased slightly with increasing metal treatments which are consistent with the results of Zengin and Munzuroglu [55] and it may be linked to the reduction in light-harvesting chlorophyll proteins (LHCPs) [56]. Adverse conditions are lessened due to decrease in LHCPs content as LHCPs act as an adaptive defense mechanism of chloroplast [57]. Further, greater decreases in chlorophyll b potentially compromise the energy trapping efficiency of photosystem (PS) II and reduce electron transport [58]. Hence, a high chlorophyll a/b ratio also indicates a change in the PS II/PS I ratio in stressed leaves [59]. However the decrease in the chlorophyll a/b ratio may indicate a 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 [60].

Soluble sugar

Carbohydrates that represent one of the main organic constituents of dry matter were found to be affected by excess of Pb and Ni. Effects of treatments on total soluble sugar content of *B. juncea* are depicted in Figure 6. There was a significant reduction in soluble sugars occurred in response to Pb and Ni stress. At higher metal concentration (1600 ppm), soluble sugar got decreased to 55% as compared to control. SA significantly (P<0.05) induced an increase in soluble sugars of metal-stressed plants (Table 8).Our results are consistent with others [32, 45, 52, 61].

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.

However, the accumulation of proteins in plant organs due to heavy metals is well known [62, 63]. 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 [64] 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 [65].

SA induced a considerable increase in the content of protein fractions in Cu stressed sunflower plants [32], as well as Cd and Ni, stressed chamomile plant [66]. Increased protein content was reported in *Typha orientalis Presl* treated with Pb+EDTA [51].

Proline

The content of the stress-indicating amino acid proline increased with the concentration of Pb and Ni (Figure 8). Presence of EDTA reduced proline content in all treatments. Effect of SA was more marked to combat metal stress by increasing proline content than control.

Our findings are corroborated by Saleh [52] 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. Proline content was found to be increased in Indian mustard plants irrigated with distillery effluents [61], distillery and tannery effluents [67] and tannery waste [68]. Moreover, metal toxicity leads to the proline accumulation which might be responsible for the increase in stress tolerance capacity of plants through several functions as osmoregulation, protection of enzymes against denaturation and stabilization of protein synthesis [69, 70]. Binding with metal ions due to the chelating ability of proline can also be a defense mechanism for survival [71].

Salicylic acid is known as a plant antitoxic. Application of SA promoted the proline accumulation and ameliorated the adverse effects of heavy metals on the growth of *B. juncea* which is similar to the findings of others [72, 73].

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

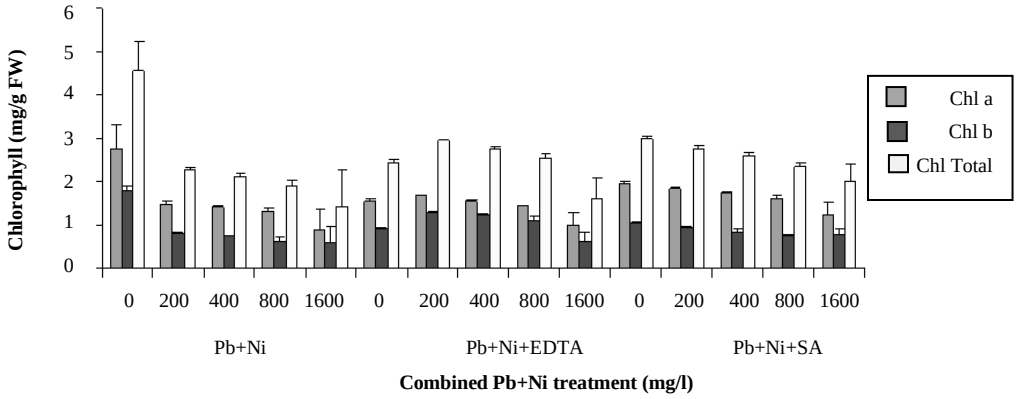

Figure 5. Effect of combined Pb+Ni treatments on chlorophyll content of *B. juncea*

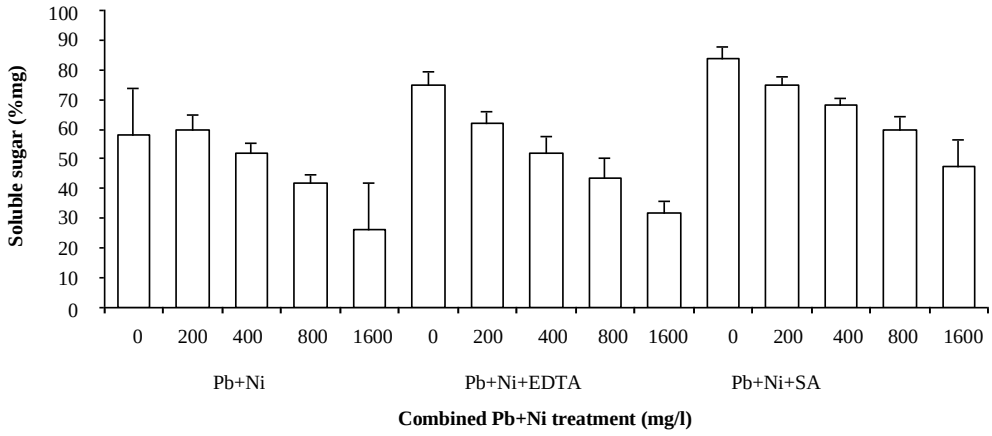

Figure 6. Effect of Pb+Ni treatments on total soluble sugar content of *B. juncea*

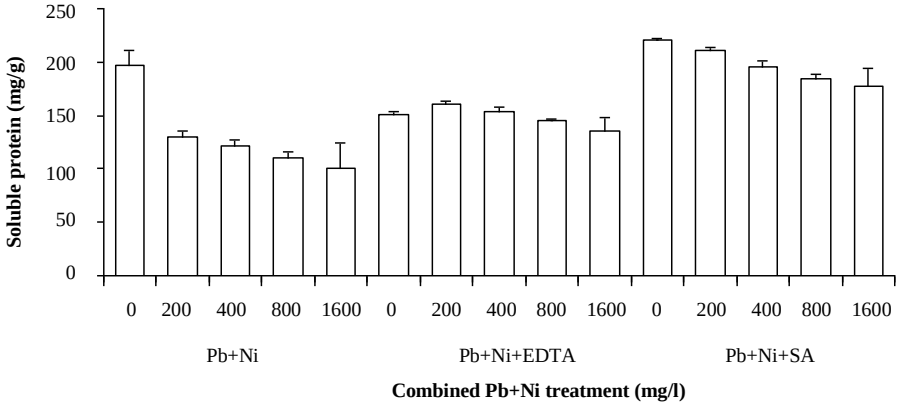

Figure 7. Effect of Pb+Ni treatments on soluble protein content of *B. juncea*

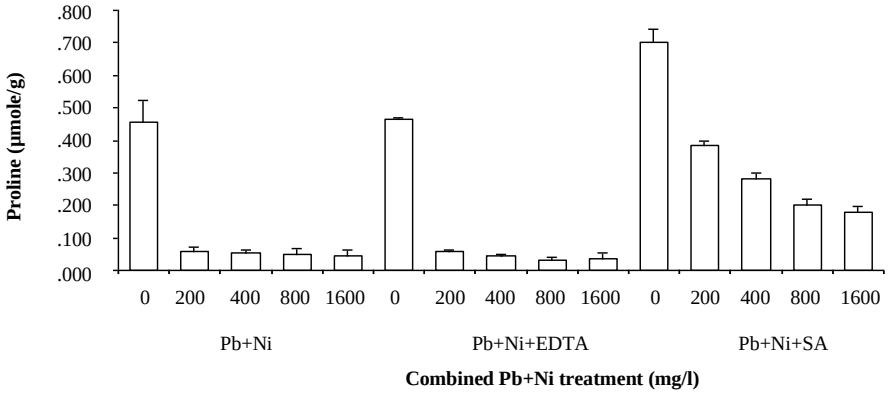

Figure 8. Effect of Pb+Ni treatments on proline content of *B. juncea*

Table 7. Effect of treatments on Chl. a/b ratio of *B. juncea*

Treatment	Chl. a/b
T1	$1.53^{a\,b}$
T2	$1.54^{a\,b}$
T3	$1.82^{b\,c\,d\,e}$
T4	$1.92^{b\,c\,d\,e}$
T5	2.15^{f}
T6	$1.75^{b\,c\,d}$
T7	1.29^{a}
T8	1.26^{a}
T9	1.30^{a}
T10	$1.66^{b\,c}$
T11	$1.92^{b\,c\,d\,e}$
T12	$1.97^{c\,d\,e\,f}$
T13	$2.06^{d\,e\,f}$
T14	$2.20^{e\,f}$
T15	$1.59^{b\,c}$

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.

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

Biochemical parameters	Source of variance	$F_{critical}$	$F_{observed}$	P-value
	Treatments	1.80	37.2	0.33
Chlorophyll	Chl	3.09	479	0.000
	TreatmentsxChl	1.60	3.16	0.07
	Chelant	1.80	27.9	0.000
Soluble sugar	Metal	3.09	3.36	0.03
	ChelantxMetal	1.60	3.15	0.000
	Chelant	1.80	100	0.000
Soluble protein	Metal	3.09	1.94	0.14
	ChelantxMetal	1.60	17.4	0.000
	Chelant	1.80	288	0.000
Proline	Metal	3.09	1769	0.000
	ChelantxMetal	1.60	51.5	0.000

Effect of treatments on metal accumulation

The results pertaining to the effect of combined Pb+Ni treatments on Pb and Ni accumulation by *B. juncea* are presented in Figure 9 and Figure 10 respectively. Pb and Ni accumulation in plants increased in a dose-response manner with increasing levels of Pb+Ni treatments as well as with time. Such metals accumulations were significantly enhanced with EDTA/SA amendment (Table 9). At 1600 ppm Pb+Ni treatment, EDTA improved accumulation of Pb and Ni by 78.4% and 54.7% respectively as compared to control. Accumulation of Pb was greater than Ni in all treatments. Moreover, EDTA was found to be very effective at mobilizing metals through the soil [74].

In the present experiment, levels of Pb and Ni with EDTA and SA were much higher than the control (without chelant). The probable reason may be that EDTA and SA enhanced the capacity of across membrane transport of Pb and Ni or the complexes Pb-EDTA and Ni-EDTA had a higher activity to be transported across membrane. 'Apoplastic pathways have become well-recognized uptake mechanism of chelant and chelant-metal complexes' [38, 75, and 76]. Metal accumulation in plants could occur due to endodermis interruption by providing a way to chelated metals into the stele. High concentrations of chelants may also cause endodermis interruption.

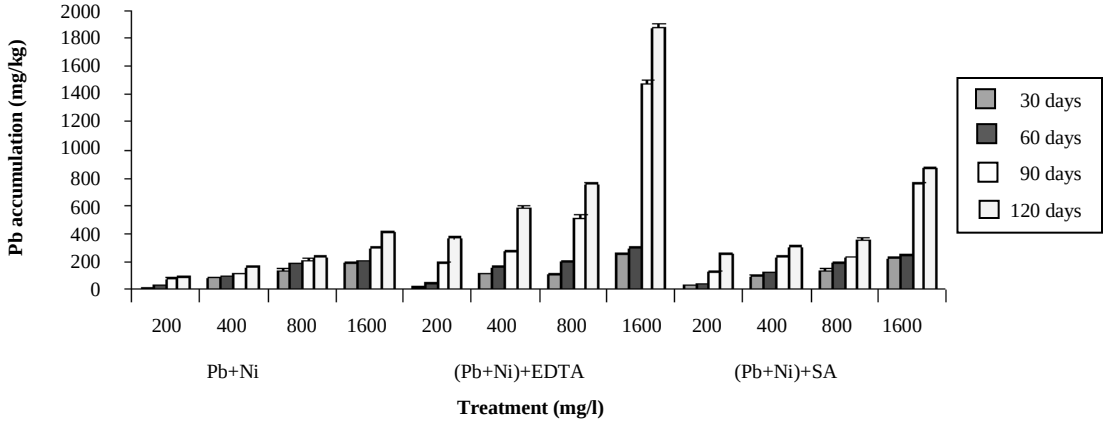

Figure 9. Effect of chelants on Pb accumulation by *B. juncea* in combined Pb+Ni treatments

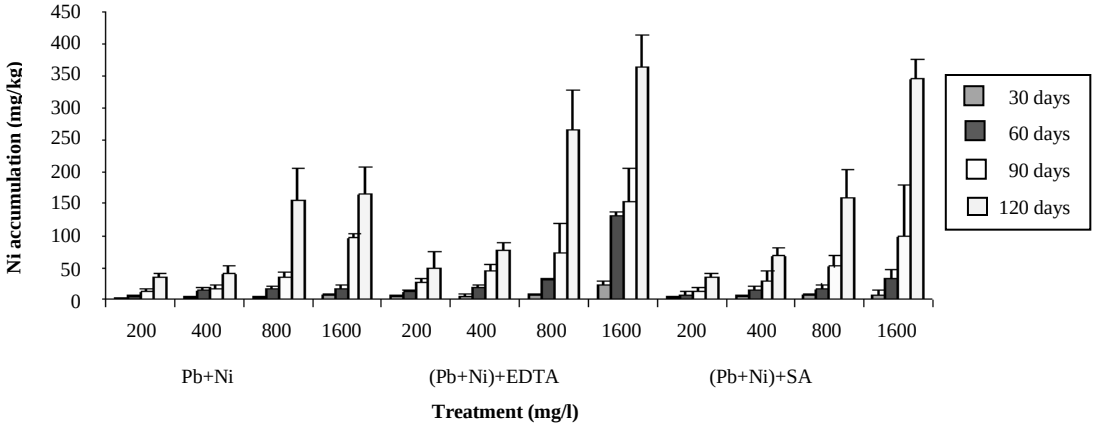

Figure 10. Effect of chelants on Ni accumulation by *B. juncea* in combined Pb+Ni treatments.

Effect of chelants on metal remained in the soil after treatments

The content of heavy metals in the soil of individual pot was analyzed after harvesting the plant biomass. The amounts of Pb and Ni remaining in the pots with different treatments are given in Figures 11 and 12. Statistically significant differences were found among all treatments (Table 9). There was an increase in heavy metal contents in the control treatment, in which soils were polluted with different concentrations of Pb and Ni. Soil contents of Pb and Ni decreased due to enhanced metal uptake by plants in chelants treated soil. 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.

The results of this study indicated that EDTA enhanced the removal of Pb from contaminated soil and also the accumulation of Pb in plants and these results are consistent with those of previous studies [77-84].

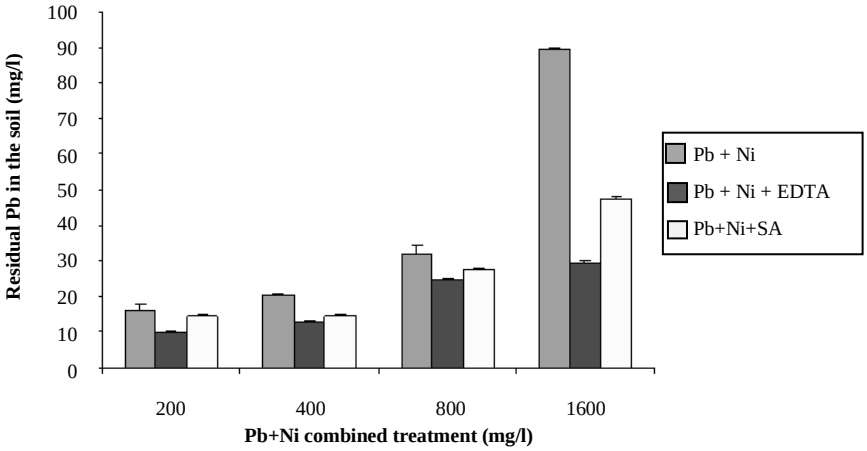

Figure 11. Effect of chelants on Pb remained in combined Pb+Ni treated soils

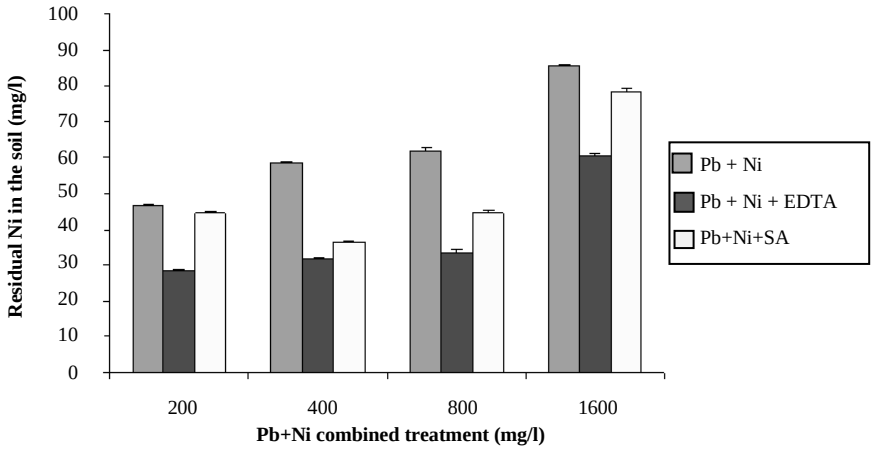

Figure 12. Effect of chelants on Ni remained in combined Pb+Ni treated soils

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

Parameters	Source of variance	$F_{critical}$	$F_{observed}$	*P*-value
Pb accumulation	Treatments	1.88	113	0.000
	Time	2.69	498	0.000
	TreatmentsxTime	1.55	114	0.000
Ni accumulation	Treatments	1.88	43.0	0.000
	Time	2.69	226	0.000
	TreatmentsxTime	1.55	29.9	0.000
Metal remained in soil	Chelant	1.88	11872	0.000
	Metal	2.69	52650	0.000
	ChelantxMetal	1.55	2717	0.000

CONCLUSIONS

Pb and Ni accumulation in plants increased in a dose-response manner with increasing levels of metal treatments and time. Metals accumulations were significantly enhanced with EDTA and SA amendment over control. Accumulation of Pb by *B. juncea* was greater than Ni in all treatments. EDTA was found to be more efficient in metal uptake than SA in *B. juncea*.

Chelate-assisted phytoextraction by *B. juncea arawali* demonstrated better lead extraction as compared to continuous phytoextraction. In chelate-assisted phytoextraction, removal of Pb and Ni by EDTA was found greater than SA from Pb and Ni contaminated soil.

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Conflict of interest

The authors declare that there is no conflict of interest.

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