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RESEARCH PAPER

Maleic acid and EDTA mediated extenuation of Co(II) stress in *Hordeum vulgare* seedlings

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Abstract The present study was performed to assess the effects of maleic acid (MA), ethylenediaminetetraacetic acid (EDTA) amendments on biochemical parameters of 7 days old seedlings of *Hordeum vulgare* L. under Co(II) stress. Application of MA, EDTA, Co(II) alone significantly reduced the root length and shoot length, whereas combination of MA and Co(II), and EDTA and Co(II) enhances the root and shoot length. The antioxidative enzymes such as superoxide dismutase, catalase, guaiacol peroxidase, ascorbate peroxidase and glutathione reductase were significantly enhanced under application of MA, EDTA, Co(II) alone, while their combinations reduced the activities of all the antioxidative enzymes. Multivariate regression analysis and β-regression coefficients showed that β_1(Co) and β_2(MA) have positive regression on the antioxdative enzymes, while β_3(Co × MA) have negative regression with the antioxidative enzymes. Similarly, β_1(Co) and β_2(EDTA), and β_3(Co × EDTA) showed the similar trend as that of Co and MA. Malondialdehyde (MDA) content is increased under Co stress, whereas MA and EDTA alone or in combination with Co reduce the MDA content. Multiple linear regression analysis also showed that MDA content is significantly enhanced under β_1(Co) stress, whereas in combination with MA or EDTA decreases the MDA content.

Introduction

Heavy metals, normally found in diverse ecosystems, immensely affect the organism's health as they bioaccumulate through food chain (Huang, Zhang, Ruan, & Chen, 2017; Zhang, Huang, Chen, Huang, & Ruan, 2016). In recent years, contamination of soil with heavy metals, i.e., Pb, Cd, Cu, Zn, Ni and Cr, has lead to ecological and environmental issues (Kumar et al., 2018). While paying less consideration to other heavy metals, cobalt (Co) toxicity is increasing immensely. Previously limited in serpentine soils and around the Co–Cu deposits ores (Lange et al., 2017), the existence of Co toxicity has spread to other lands, due to its increased utilization in formation of high-grade steels and alloys, and industrial products, i.e., paints, enamels, varnishes and inks (Adriano, 2001).

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Usually the phytotoxicity level imposed by specified heavy metals is associated with their pile up in plant tissues (Davis & Beckett, 1978). The Co content under physiological situations are normally determined that varies from 0.01 to 1.00 μg/g dry matter, with level to some extent exceeds the threshold level resulting in appearance of Co toxicity (Jayakumar & Jaleel, 2009). High content of Co decreases the plant growth. Amongst their cytotoxic properties, metals generate oxidative stress that is reflected in aspects of cellular buildup of reactive oxygen species (ROS) (Gill & Tuteja, 2010), which is accountable for marked injuries to proteins, nucleic acids and lipids. Furthermore, high content of heavy metals may responsible for structural and ultra structural injures in the cell organelles, which is to some degree leads to metal stimulated oxidative stress (Ali et al., 2013).

The plants have adapted protective mechanisms such as antioxidant system that appear as a common response of plant under harmful conditions, being easily mediated by oxidative stress (Hall, 2002). This defense mechanism comprises of various antioxidant enzymes such as SOD, POD, CAT, APX and GR that scavenges the produced ROS (Gill & Tuteja, 2010). A huge collection of such data is existed for various heavy metals (Ali et al., 2014; Gill et al., 2015). But less information is available regarding the biochemical analysis of Co tolerance in *Hordeum vulgare*. It commonly known as barely ranked 4th on production basis in the top five cereals by FAO (Kumar, Chand, & Shah, 2016). It is most important crop grown worldwide for human and animal consumption (Mattiello et al., 2015). Because of phytoremediation potential of barely, it has been extensively used for detoxification of heavy metals such as Al, Cd, Cu, Fe, Cr, Mn, Ni, Pb, Zn etc. (Ciura, Poniedzialek, Jedrszczyk, & Sekara, 2005; Dahbia, Benchaban, & Mohamed, 2013; Mattiello et al., 2015; Ma, Zhu, Shabala, Zhou, & Shabala, 2015). Maleic acid (MA) is a small organic acid which is helpful in remediation of heavy metals (Du et al., 2016). Studies with MA suggested that it forms complexes with Co(II), Ni(II), Mn(II), Cu(II), Zn(II) and Fe(II) (Allan, Baillie, Bonner, Gerrard, & Hoey, 1989; Hossain, Islam, Islam, Salam, & Yousuf, 2012; Rivas, Seguel, & Ancatripai, 2000). MA enhances the metal tolerance in the plants (Hossain et al., 2012). EDTA is also considered as strong chelating agent for phytoremediation of heavy metal ions from the polluted soil (Chen & Cutright, 2001; Li, Li, Fu, Zhuang, & Guo, 2012). It is helps in hindering metal chemical speciation, ion mobility, solubility, and bioavailability in the soil solution phase, uptake by roots, and accumulation in plants (Shahid et al., 2014). Therefore, the present work was accomplished to study the Co(II)-mediated biochemical changes in 7 day old seedlings of *H. vulgare* L. in binary combinations with maleic acid (MA) and EDTA. This paper drafts hypothesis pertinent to ameliorating potential of MA and EDTA against cobalt toxicity in 7 days old *H. vulgare* seedlings.

Materials and methods

Seed material sterilization

The seeds of *H. vulgare* L. var. PL-426 were purchased from Punjab Agriculture University, Ludhiana, Punjab, India. The sterilization of seeds was done by dipping in 0.5% sodium hypochlorite (NaOCl) for 10 min followed by repeated washing with distilled water to get rid of any traces of NaOCl. Commercially available cobalt(II) chloride hexahydrate ($CoCl_2 \cdot 6H_2O$) form of cobalt was used (Figure 1).

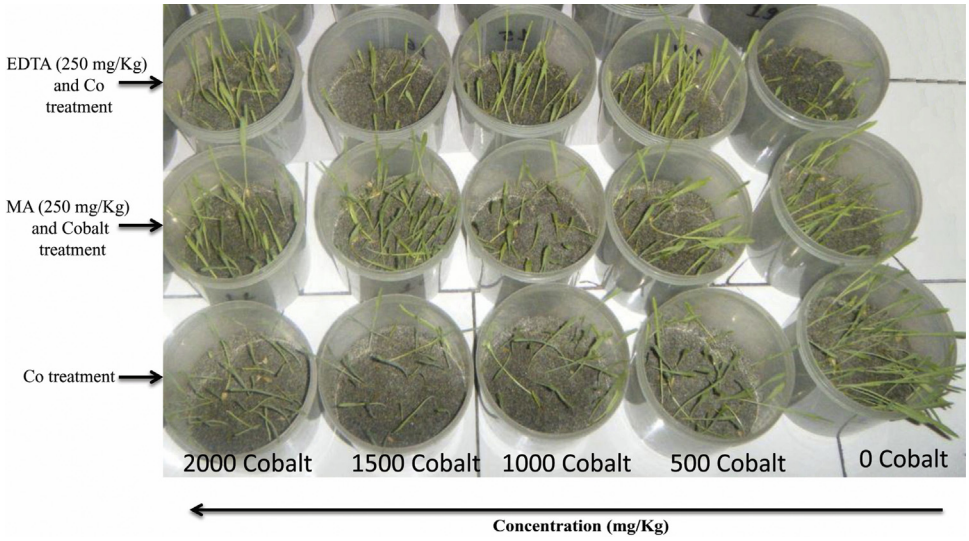

Figure 1 Seedlings of *H. vulgare* growing under Cobalt stress (500, 1000, 1500, 2000 mg/kg) and binary combination of MA (250 mg/kg) with Cobalt stress and EDTA (250 mg/kg) with cobalt stress.
Co: cobalt.
MA: maleic acid.
EDTA: ethylenediaminetetraacetic acid.
mg: milligram.
kg: kilogram.

Table 1 $CoCl_2 \cdot 6H_2O$ (X) treatment in binary combination with MA and EDTA (Y).

Concentration of Y (mg/kg)	Concentration of X(mg/kg)				
	0	500	1000	1500	2000
0	0/0	500/0	1000/0	1500/0	2000/0
250	250/0	500/250	1000/250	1500/250	2000/250

Where, X = $CoCl_2 \cdot 6H_2O$.
Y = EDTA and maleic acid.

Raising of seedlings

Sand was used as growth medium for raising seedlings of *H. vulgare*. The sand was first filtered through a mesh of 300 nm pore size followed by washing with 0.1 N HCl. The latter was washed with distilled water to get rid of any unwanted salts. The seeds of *H. vulgare* were presoaked in distilled water for 2–3 h before sowing in polypropylene containers holding 500 g sterilized sand.

Treatment of cobalt

The treatment dose of cobalt was determined by performing toxicity studies on *H. vulgare* seeds. The IC_{50} of Co was calculated from the data obtained from toxicity studies. Four treatment doses of cobalt viz., 500, 1000, 1500 and 2000 mg/kg of sand were fixed for the present study (Table 1).

Treatment of MA and EDTA

Maleic acid and EDTA were applied in binary combinations with Co to check any alteration in the toxicity of heavy metal. The binary treatment combinations for Co(II) with ameliorating agents (maleic acid and EDTA) as well as treatment of individual chemicals is given in Table 1. Plants were harvested for analysis after 7 days of sowing.

Growth parameters

The plants harvested from various treatments were used immediately for various growth and biochemical parameters. The growth parameters include measurement of root and shoot length.

Preparation of plant extract

Two grams of seven days old *H. vulgare* seedlings were powdered in liquid nitrogen. To this powder was added 6 ml of 50 mM phosphate buffer at pH 7.8, containing EDTA (1 ml), 1 mM of phenyl methane sulfonyl fluoride (PMSF), triton X (0.5%), and polyvinylpyrrolidone 40 (3%). The mixture was centrifuged at 12,000 g at 4 °C for 25 min. The filtrate was stored in −80 °C for further studies.

Protein estimation

The protein content was assessed using the method of Bradford (1976) taking BSA as standard.

Antioxidative enzymes

For enzyme estimation, 1 g of fresh plant material was powdered in liquid nitrogen. To the powdered material was added 50 mM phosphate buffer (pH 7.8) containing 2 mM EDTA, 1 mM DTT, 0.5% (v/v) Triton X-100 and 10% (w/v) PVPP. The whole content was centrifuged at 13,000 g for 20 min. The supernatant was decanted and used for enzyme assays. Enzyme activity (one unit) is referred to as the enzyme required in oxidising of 1 mM of substrate/min/g fw.

Superoxide dismutase (SOD) (EC 1.15.1.1)

Superoxide dismutase was estimated according to the method of Kono (1978). The 3 ml of reaction mixture contained 50 mM sodium carbonate buffer (pH 10.2), 0.03% Triton X-100, 24 μM NBT, EDTA 0.1 mM and enzyme extract (60 μl). Absorbance was measured at 560 nm. The percentage inhibition of NBT reduction was calculated as:

$$\text{where } x \quad x = \frac{\text{Change in absorbance min}^{-1}\text{ (blank)} - \text{change in absorbance min}^{-1}\text{ (test)}}{\text{Change in absorbance min}^{-1}\text{ (blank)}}$$

is the percentage inhibition produced by 60 μl of the sample. Hence 50% inhibition is produced by $(50 \times 60)/x = y$ μl of the sample.

Catalase (CAT) (EC 1.11.1.6)

Catalase activity was determined as per the method of Aebi (1984). The reaction mixture contained 60 μl of enzyme extract and 15 mM H_2O_2 in 50 mM phosphate buffer. Enzyme activity was calculated using the extinction coefficient of 39.4 cm^{-1} mol^{-1}. The decrease in H_2O_2 was followed as decrease in optical density at 240 nm for 30 s at intervals of 3 s at 25 °C.

Table 2 Effect of binary treatments of $CoCl_2 \cdot 6H_2O$, maleic acid (MA) and EDTA on morphological parameters of 7 days old seedlings of *H. vulgare*, two-ANOVA analysis and multiple regression analysis.

Conc. of MA (mg/kg)	Conc. of $CoCl_2 \cdot 6H_2O$ (mg/kg)				
	0	500	1000	1500	2000
Shoot length (cm)					
0	17.92	17.56	11.09	2.88	2.03
250	10.01	11.83	12.60	12.29	12.49
Root length (cm)					
0	10.84	9.14	3.46	1.14	0.48
250	10.34	9.96	9.71	9.24	10.54

Conc. of EDTA (mg/kg)	Conc. of $CoCl_2 \cdot 6H_2O$ (mg/kg)				
Shoot length (cm)					
0	17.92	17.56	11.09	2.88	2.03
250	10.89	13.57	12.77	12.81	13.02
Root length (cm)					
0	10.84	9.14	3.46	1.14	0.48
250	9.37	9.81	9.59	7.69	10.28

Two-way ANOVA F-ratios and Tukey's multiple comparison test (HSD)

Source of variation	Between Co	Between MA	Interaction Co × MA	HSD
Shoot length	177.23***	43.92***	256.18***	3.67
Root length	181.72***	930.83***	160.76***	2.32
Source of variation	Between Co	Between EDTA	Interaction Co × EDTA	HSD
Shoot length	215.69***	106.31***	258.79***	4.42
Root length	202.70***	760.04***	172.18***	2.26

Multiple regression analysis

Shoot length = $19.59 - 0.009\ Co - 0.04\ MA + 5 \times 10^{-5}\ Co \times MA$

Root length = $10.76 - 0.006\ Co - 0.04\ MA + 3 \times 10^{-5}\ Co \times MA$

Shoot length = $19.59 - 0.009\ Co - 0.04\ EDTA + 5 \times 10^{-5}\ Co \times EDTA$

Root length = $10.76 - 0.006\ Co - 0.007\ EDTA + 3 \times 10^{-5}\ Co \times EDTA$

Beta regression coefficients

	β_1 (Co)	β_2 (MA)	β_3 (Co × MA)	r
Shoot length	−1.33	−0.89	1.48	0.957***
Root length	−1.04	−0.09	1.03	0.975***
	β_1 (Co)	β_2 (EDTA)	β_3 (Co × EDTA)	r
Shoot length	−1.31	−0.76	1.41	0.956***
Root length	−1.09	−0.18	1.08	0.962***

Significant at ***P ≤ 0.001, **P ≤ 0.01 and *P ≤ 0.05.
HSD: honestly significant difference.
Co: cobalt.
MA: maleic acid.
EDTA: ethylenediaminetetraacetic acid.
ANOVA: analysis of variance.

Ascorbate peroxidase (APOX) (EC 1.11.1.11)

APOX activity was calculated by the method described by Nakano and Asada (1981) with slight modifications. The reaction was performed at 25 °C in 3 ml reaction mixture containing potassium phosphate buffer (50 mM, pH 7.0), EDTA (0.1 mM), ascorbate (0.5 mM) and H_2O_2 (1 mM). The reaction was started by addition of 60 μl of the enzyme extract in a quartz cuvette.

Guaiacol peroxidase (GPOX) (EC.1.11.7)

Guaiacol peroxidase was estimated according to method given by Pütter (1974). The reaction mixture contained 50 mM phosphate buffer (pH 7), 20 mM guaiacol and 12.3 mM H_2O_2. The rate of oxidation was followed by the increase in absorbance at 436 nm observed spectrophotometrically during 1 min with intervals of 6 s ($\varepsilon = 25.5\ mM^{-1}\ cm^{-1}$).

Table 3 Antioxidative enzymes and MDA content of 7-day old *H. vulgare* seedlings in binary combinations of $CoCl_2 \cdot 6H_2O$ and maleic acid (MA).

Conc. of MA (mg/kg)	Conc. of $CoCl_2 \cdot 6H_2O$ (mg/kg)				
	0	500	1000	1500	2000
Protein content (mg/g FW)					
0	5.58	1.50	0.87	0.24	0.03
250	1.24	2.54	9.59	63.27	67.45
SOD (UA min^{-1} mg^{-1} protein)					
0	9.43	39.77	77.88	356.28	2835.79
250	35.68	25.99	29.22	7.62	9.12
CAT (UA min^{-1} mg^{-1} protein)					
0	0.37	1.71	1.61	1.25	3.38
250	2.31	0.69	0.26	0.09	0.09
APOX(UA min^{-1} mg^{-1} protein)					
0	0.006	0.009	0.009	0.012	0.010
250	0.006	0.007	0.008	0.001	0.001
GPOX (UA min^{-1} mg^{-1} protein)					
0	0.003	0.019	0.047	0.225	1.67
250	0.017	0.013	0.007	0.001	0.001
GR (UA min^{-1} mg^{-1} protein)					
0	0.015	0.045	0.070	0.165	0.293
250	0.034	0.032	0.007	0.001	0.001
MDA (μ mole g^{-1} FW)					
0	1.67	8.62	23.90	26.67	28.46
250	2.31	2.57	12.10	14.01	16.65

MA: maleic acid.
FW: fresh weight.
UA: unit activity.
SOD: superoxide dismutase.
CAT: catalase.
APOX: ascorbate peroxidase.
GPOX: guicol peroxidase.
GR: glutathione reductase.
MDA: malondialdehyde.

Glutathione reductase (GR) (EC 1.6.4.2)

Glutathione reductase activity was determined by using method of Carlberg and Mannervik (1985). Three ml reaction mixture was taken containing 50 mM, pH 7.8 phosphate buffer, 1 mM EDTA, 0.1 mM NADPH, 1 mM GSSG, 75 μl enzyme sample. Decrease in absorbance per minute was recorded at 340 nm. Enzyme activity was calculated using the extinction coefficient 6.22 mM^{-1} cm^{-1}.

Malondialdehyde (MDA) content

Lipid peroxidation is estimated by the method of Heath and Packer (1968). The formation of MDA which is a secondary end product of oxidation of polyunsaturated fatty acid is an indication for the generation of lipid peroxide radicals.

Statistical analysis

All the experiments were performed in triplicates and data was analyzed by using self-coded software in MS-Excel. The results were presented as mean $\pm$ S.D. The data was also analyzed by using two-way analysis of variance (ANOVA) and Honestly significant difference (HSD). The data was also subjected to multiple regression analysis (MLR) to find the effect of Co, MA and EDTA on the biochemical parameters in seedlings of *H. vulgare*.

Results

Effect of Co and MA on shoot and root lengths

The effect of binary treatments of $CoCl_2.6H_2O$ and MA on shoot length and root length are given in Table 2. It was observed that both root and shoot lengths of 7 days old seedlings of *H. vulgare* were decreased in a dose dependent manner with increasing concentration of Co. With addition of MA (250 mg/kg), the effect of cobalt toxicity on seedlings was decreased as was evident from the increase in shoot and root length. There was a significant decrease in shoot length in 1000, 1500 and 2000 mg/kg Co treatment group as compared with control. Minimum root length (0.48 cm) and shoot length (2.03 cm) was observed in 2000 mg/kg per se treatment of Co. F-ratios were found to be significant at $P \leq 0.001$. Multiple regression analysis and β- regression coefficients indicated that Co and MA have negative effect on root length and shoot length, whereas their combined

Table 4 Antioxidative enzymes and MDA content of 7-day old *H. vulgare* seedlings in binary combinations of $CoCl_2 \cdot 6H_2O$ and EDTA.

Conc. of EDTA (mg/kg)	Conc. of $CoCl_2 \cdot 6H_2O$ (mg/kg)				
	0	500	1000	1500	2000
Protein content (mg/g FW)					
0	5.58	1.50	0.87	0.24	0.03
250	3.21	7.72	29.92	66.33	154.41
SOD (UA min^{-1} mg^{-1} protein)					
0	9.43	39.77	77.88	356.28	2835.79
250	21.17	31.49	30.19	19.99	11.71
CAT (UA min^{-1} mg^{-1} protein)					
0	0.37	1.71	1.61	1.25	3.38
250	2.19	0.48	0.21	0.11	0.07
APOX (UA min^{-1} mg^{-1} protein)					
0	0.006	0.009	0.009	0.012	0.010
250	0.006	0.007	0.003	0.002	0.0009
GPOX (UA min^{-1} mg^{-1} protein)					
0	0.003	0.019	0.047	0.225	1.67
250	0.005	0.006	0.002	0.001	0.001
GR (UA min^{-1} mg^{-1} protein)					
0	0.015	0.045	0.070	0.165	0.293
250	0.016	0.009	0.002	0.0009	0.0005
MDA (μ mole g^{-1} FW)					
0	1.67	8.62	23.90	26.67	28.46
250	3.86	4.19	6.80	9.00	13.69

EDTA: ethylenediaminetetraacetic acid.
FW: fresh weight.
UA: unit activity.
SOD: superoxide dismutase.
CAT: catalase.
APOX: ascorbate peroxidase.
GPOX: guicol peroxidase.
GR: glutathione reductase.
MDA: malondialdehyde.

effect has positive effect on the root length and shoot length of 7 days old seedlings of *H. vulgare*.

Effect of Co and EDTA on shoot and root lengths

The outcome of binary treatments of $CoCl_2 \cdot 6H_2O$ and EDTA on shoot lengths and root lengths of 7 days old seedlings of *H. vulgare* are given in Table 2. The root length and shoot length of 7 days old seedlings of *H. vulgare* were reduced under Co and EDTA treatments, and maximum decline occurred at concentration of 2000 mg/kg of $CoCl_2 \cdot 6H_2O$ in the sand as compared to the control. With addition of EDTA, the toxicity of cobalt was significantly decreased. The F-ratios were found to be significant at $P \leq 0.001$. Multiple regression analysis and β-regression coefficients showed that Co and EDTA negatively reduced the shoot and root lengths, whereas their combined effect have positive effect on the shoot and root lengths of 7 days old seedlings of *H. vulgare* L.

Effect of Co and MA on antioxidative enzymes and MDA content

The antioxidative enzymes and MDA content of 7-day old *H. vulgare* seedlings in binary combinations of $CoCl_2 \cdot 6H_2O$and MA are presented in Table 3. The Co(II) treatments resulted in a dose dependent decrease in the protein content in seedlings of *H. vulgare* as compared to the control (5.58 mg/g). Treatment of 2000 mg/kg concentration of $CoCl_2 \cdot 6H_2O$ resulted in maximum decrease (0.03 mg/g; −99%) in the protein content. Maximum increase (67.45 mg/g) in protein content was observed at binary combination of $CoCl_2 \cdot 6H_2O$ and MA (2000 and 150 mg/kg). The results of antioxidative enzymes showed that, *H. vulgare* plants grown in soil containing binary treatment of 2000 mg/kg Co(II) and 250 mg/kg MA showed decline (−99%) in SOD specific activity in comparison to the 2000 mg Co(II) /kg soil treatment. There was lesser (−97%) specific activity of CAT in seedlings of *H. vulgare* grown in soil containing binary combination of Co(II) and MA at concentration ratio of 2000: 250 mg/kg soil as compared to soil containing 2000 mg/kg Co(II) only. The specific activity of APOX was declined in the leaves of *H. vulgare* plants grown in soil given binary treatment of Co(II) and maleic acid at concentration ratio of 2000: 250 mg/kg as compared to Co(II) only treatment at 2000 mg/kg soil. The specific activity of GPOX was declined in the leaves of *H. vulgare* plants grown in soil given binary treatment of Co(II) and maleic acid at concentration ratio of 2000: 250 mg/kg as compared to Co(II) only treat-

Table 5 Two-way ANOVA F-ratios and Tukey's multiple comparison test (HSD) of 7-day old *H. vulgare* seedlings in binary combinations of Co and MA, and Co and EDTA.

Source of variation	Between Co	Between MA	Interaction Co × MA	HSD
Protein content	14.83***	53.15***	17.71***	20.69
SOD	481.24***	673.25***	494.63***	137.29
CAT	1.52**	0.91	2.06**	3.43
APOX	1.61**	0.39	0.40	3.50
GPOX	1.51**	0.0006	0.84	3.49
GR	1.56**	0.22	0.43	3.49
MDA	256.98***	259.86***	24.01***	2.87
Source of variation	Between Co	Between EDTA	Interaction Co × EDTA	HSD
Protein content	29.37***	101.69***	32.17***	27.89
SOD	482.73***	670.78***	493.73***	137.25
CAT	1.50**	1.10**	1.97**	3.46
APOX	1.60**	0.39	0.40	3.50
GPOX	1.52**	0.0003	0.84	3.50
GR	1.57**	0.21	0.43	3.49
MDA	159.70***	354.07***	51.29***	3.05

Significant at ***$P \leq 0.001$, **$P \leq 0.01$ and *$P \leq 0.05$.
Co: cobalt.
MA: maleic acid.
EDTA: ethylenediaminetetraacetic acid.
FW: fresh weight.
UA: unit activity.
SOD: superoxide dismutase.
CAT: catalase.
APOX: ascorbate peroxidase.
GPOX: guicol peroxidase.
GR: glutathione reductase.
MDA: malondialdehyde.
HSD: honestly significant difference.

ment at 2000 mg/kg soil. GR specific activity was maximum in the leaves of *H. vulgare* plants grown in soil amended with 2000 mg Co(II)/kg. Binary combination of Co(II) and MA in the soil at concentrations of 2000; 250 mg/kg showed reduction in GR activity (−100%). Increase (28.46 μmol/g fw; 94%) in the MDA content in seedlings of *H. vulgare* was seen at 2000 mg/kg concentration of $CoCl_2.6H_2O$ in the soil in comparison to the control (1.35 μmol/g fw). There was decrease (2.96 μmol/g fw; −71%) in the MDA content in the leaves of plants raised in binary combination of $CoCl_2{\cdot}6H_2O$ and MA (2000 and 200 mg/kg). The results of two-way ANOVA indicated that different enzymes and MDA content are highly significant with each other (Table 5).

Multiple regression analysis (MLR) showed that Co and MA decreased the protein content, whereas their combined treatments have positive effect on the protein content (Table 6). β-Regression coefficients for all the antioxidative enzymes showed that β_1(Co) and β_2(MA) are positively regressed on the antioxidative enzymes, whereas their combined effect β_3(Co × MA) has negative effect on the antioxidative enzymes in 7 days old seedlings of *H. vulgare*. MLR analysis of MDA showed that Co has positive regression, whereas MA and their combined effect have negative regression with the MDA content.

Effect of Co and EDTA on antioxidative enzymes and MDA content

The antioxidative enzymes and MDA content of 7-day old *H. vulgare* seedlings in binary combinations of $CoCl_2{\cdot}6H_2O$and EDTA are given in Table 4. As compared to the control group (5.58 mg/g) the protein content declined (0.03 mg/g; −99%) in the leaves of plants grown in sand containing $CoCl_2{\cdot}6H_2O$ (2000 mg/kg). Maximum increase (154.41 mg/g) was observed at binary treatment group of 2000 mg/kg $CoCl_2{\cdot}6H_2O$ and 250 mg/kg EDTA respectively. Amendment of soil with 2000 mg/kg Co(II) increased the specific activity of SOD in seedlings of *H. vulgare*, while binary combination of Co(II) 2000: 250 mg/kg EDTA decreased (−100%) the leaf SOD activity. CAT specific activity in the seedlings of *H. vulgare* grown in soil amended with binary combination of Co(II) and EDTA at concentration (2000: 250 mg/kg) was lesser (−98%), while Co(II) treatment at 2000 mg/kg caused increase in CAT activity. Co(II) treatment at 2000 mg/kg increased the specific activity of APOX, while decrease (−91%) in APOX activity was observed in seedlings of *H. vulgare* grown in the soil containing binary combination of Co(II) and EDTA at concentration ratio of 2000: 250 mg/kg soil. There was a high GPOX specific activity 7-days old seedlings of *H. vulgare* grown in soil given binary treatment of Co(II) and EDTA. Maximum decrease in the activity (-100%) was observed at

Table 6 Multiple regression with interaction equations for the antioxidative enzymes and MDA content for the 7-day old *H. vulgare* seedlings.

Multiple regression equations

Protein content = 4.11 − 0.003 Co − 0.07 MA + 0.0002Co × MA
SOD activity = −530.02 + 1.19 Co + 2.83 MA − 0.006Co × MA
CAT activity = 0.552 + 0.001 Co + 0.006 MA − 1×10^{-5}Co × MA
APOX activity = 0.007 + 2×10^{-6}Co + 5×10^{-6}MA − 3×10^{-8}Co × MA
GPOX activity = −0.31 + 0.0007Co + 0.0017MA − 4×10^{-6}Co × MA
GR activity = −0.018 + 0.0001Co + 0.0003 MA − 8×10^{-7}Co × MA
MDA content = 3.54 + 0.01 Co − 0.01 MA − 3×10^{-5} Co × MA

	Beta regression coefficients			r
	β_1(Co)	β_2(MA)	β_3(Co × MA)	
Protein content	−0.07	−0.28	1.15	0.937***
SOD	1.01	0.33	−1.02	0.809***
CAT	0.76	0.55	−1.45	0.865***
APOX	0.46	0.16	−1.17	0.894***
GPOX	1.01	0.34	−1.03	0.816***
GR	1.08	0.34	−1.23	0.964***
MDA	1.05	−0.11	−0.46	0.957***

Multiple regression equations

Protein content = 4.11 − 0.003 Co − 0.12 EDTA + 0.0004Co × EDTA
SOD activity = −530.02 + 1.19 Co + 2.79 EDTA − 0.006Co × EDTA
CAT activity = 0.552 + 0.001 Co + 0.005 EDTA − 1×10^{-5} Co × EDTA
APOX activity = 0.007 + 2×10^{-6} Co + 2×10^{-7} EDTA − 3×10^{-8} Co × EDTA
GPOX activity = −0.31 + 0.0007Co + 0.002EDTA − 4×10^{-6} Co × EDTA
GR activity = −0.018 + 0.0001Co + 0.0002EDTA − 7×10^{-7} Co × EDTA
MDA content = 3.54 + 0.01 Co − 0.005 EDTA − 5×10^{-5} Co × EDTA

	Beta regression coefficients			r
	β_1(Co)	β_1(EDTA)	β_1(Co × EDTA)	
Protein content	−0.04	−0.26	1.13	0.942***
SOD	1.01	0.33	−1.01	0.809***
CAT	0.76	0.47	−1.39	0.861***
APOX	0.45	0.005	−1.11	0.963***
GPOX	1.01	0.33	−1.02	0.816***
GR	1.05	0.17	−1.12	0.967***
MDA	1.06	−0.05	−0.69	0.963***

Significant at ***P ≤ 0.001.
Co: cobalt.
MA: maleic acid.
EDTA: ethylenediaminetetraacetic acid.
SOD: superoxide dismutase.
CAT: catalase.
APOX: ascorbate peroxidase.
GPOX: guicol peroxidase.
GR: glutathione reductase.
MDA: malondialdehyde.

concentration of 1000 Co(II) mg/kg: 500 mg/kg EDTA in the soil as compared 1000 mg Co(II)/kg soil (−85.71%). *H. vulgare* plants when grown in the soil comprising Co(II) at concentration of 2000 mg/kg showed increase in the specific activity of GR. Binary treatment of Co(II) and EDTA in the soil at concentration ratio of 2000 mg/kg and 250 mg/kg led to decrease in GR activity upto −100%. There was an increase (28.46 μmol/g fw; 94.13%) in the MDA content in *H. vulgare* growing in sand containing 2000 mg/kg concentration of $CoCl_2 \cdot 6H_2O$ in comparison to the control (1.67 μmol/g fw). Growing plants in binary combination of $CoCl_2 \cdot 6H_2O$ and EDTA (2000 and 200 mg/kg) was effective in lowering the MDA content (2.96 μmol/g fw; −107.89%) in the leaves. The results of two-way ANOVA indicated that different enzymes and MDA content are highly significant with each other (Table 5).

MLR analysis indicated that Co and EDTA reduced the protein content, whereas combined effect of Co and EDTA have positive effect on the protein content (Table 6). β-Regression coefficients for SOD, CAT, APOX, GPOX and GR indicated that β_1(Co) and β_2(EDTA) have positive regression on the antioxidative enzymes, whereas combined effect

of β_3(Co × EDTA) has negative regression on the antioxidative enzymes in 7 days old seedlings of *H. vulgare* L. MLR analysis of MDA indicated that Co has positive regression, whereas EDTA and their combined effect with Co have negative regression with the MDA content.

Discussion

Cobalt is not an essential element in the plants, however in small concentration it has many beneficial effects. Nevertheless, high content of Co is toxic and leads to morphological and physiological changes in the plants (Chatterjee & Chatterjee, 2003; Gopal, Dube, Sinha, & Chatterjee, 2003; Marschner, 1995). The root length and shoot length of seedlings of *H. vulgare* of present study were reduced under increasing concentration of Co, and this may be attributed to the reduced cell division or cell elongation, or both factors (Hemantaranjan & Trivedi, 2000). The decrement observed in root length and shoot length is also reported under various heavy metals stress in many plant species (Feigl et al., 2003), may be due to reduced mitotic activity of meristem cells and with substantive decline in cell division in the root tips of *Allium cepa* (Dovgaliuk, Kaliniak, & Blium, 2001). Moreover, it is emphasized that heavy metal toxicity also reduced the enlargement of newly generated cells in the elongation zone that leads to reduction in plant growth (Yuan & Huang, 2016).

Plant cells have well organized endogenous machinery of antioxidant molecules that counterbalance the oxidative stress imposed by various stresses like drought, salinity, high/low temperature and pesticides (Gill & Tuteja, 2010). The endogenous antioxidant system consists of various enzymes, viz., SOD, CAT, GPOX, APOX, GR and non-enzymatic metabolites such as ascorbic acid, glutathione, carotenoid, flavonoids etc. (Bowler, Van Montagu, & Inze, 1992). Reactive oxygen species generated as a result of stress create oxidative stress which in turn may increase inbuilt antioxidative response of plant to provide stress resistance (Imlay, 2003).

The enhancement in SOD activity is a periodic modifying mechanism of plants against heavy metal stress, because SOD as first line of protection and changes O^{2-} to H_2O_2, which is further metabolized by diverse enzymes (Gill & Tuteja, 2010). Increase in SOD activity was observed in tomato plants under Co stress by Gopal and co-workers (2003). While mainly regarded as the heavy metal stress enzyme, the enhancement in POD activity provides advantage to the plants, as POD enzyme reduces the H_2O_2 load within cells by exploiting them in other cellular reactions within cytoplasm and peroxisomes (Mittler, 2002). Furthermore, stimulation of POD takes part in decreasing the biological activity of Co, because phenolic compounds that are mainly polymerized by POD enzyme act as Co chelators (Ali et al., 2018). The results of antioxidative enzymes in seedlings of *H. vulgare* in present study were increased under Co and finds supports from Karuppanapandian and Kim (2013).

In seedlings of barley, Co treatment caused significant breakdown of membrane lipids, as indicated by MDA accumulation. Moreover, MDA results in present study are distinct with earlier study in which Co stress reduced the MDA content in green gram cultivated in sand culture for 41 days (Tewari, Kumar, Sharma, & Bisht, 2002). In spinach, MDA content enhanced considerably under application of 500 lM Co (Pandey, Pathak, Pandey, & Pandey, 2009). Similarly, Karuppanapandian and Kim (2013) also reported enhancement in MDA content in Indian mustard which are in confirmation with the results of present study.

Conclusion

The present study shows that MA and EDTA show ameliorative potential in seedlings of *H. vulgare* under Co stress as evident through morphological and antioxidative studies. It is concluded that application of MA/EDTA in fields with high cobalt contamination can mitigate the toxic effects of cobalt.

Conflicts of interest

The authors declare no conflicts of interest.

References

Adriano, D. C. (2001). *Trace elements in terrestrial environments: Biogeochemistry, bioavailability and risks of metals* (2nd edn.). New York: Springer.

Aebi, H. (1984). Catalase *in vitro*. *Methods in Enzymology*, *105*, 121–126.

Ali, B., Qian, P., Jin, R., Ali, S., Khan, M., Aziz, R., ... & Zhou, W. J. (2014). Physiological and ultra-structural changes in *Brassica napus* seedlings induced by cadmium stress. *Biologia Plantarum*, *58*(1), 131–138.

Ali, B., Wang, B., Ali, S., Ghani, M. A., Hayat, M. T., Yang, C., ... & Zhou, W. J. (2013). 5-Aminolevulinic acid ameliorates the growth, photosynthetic gas exchange capacity, and ultrastructural changes under cadmium stress in *Brassica napus* L. *Journal of Plant Growth Regulation*, *32*, 604–614.

Ali, S., Gill, R. A., Mwamba, T. M., Zhang, N., Lv, M. T., ul Hassan, Z., ... & Zhou, W. J. (2018). Differential cobalt-induced effects on plant growth, ultrastructural modifications, and antioxidative response among four *Brassica napus* (L.) cultivars. *International Journal of Environmental Science and Technology*, *15*(12), 2685–2700.

Allan, J. R., Baillie, G. M., Bonner, J. G., Gerrard, D. L., & Hoey, S. (1989). Thermal studies on maleic acid compounds of manganese(II), cobalt(II), nickel(II), copper(II) and zinc(II). *Thermochimica Acta*, *143*, 283–288.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*, *72*(1–2), 248–254.

Bowler, C., Van Montagu, M., & Inze, D. (1992). Superoxide dismutase and stress tolerance. *Annual Review of Plant Physiology and Plant Molecular Biology*, *43*, 83–116.

Carlberg, I., & Mannervik, B. (1985). Glutathione reductase assay. *Methods in Enzymology*, *113*, 484–495.

Chatterjee, J., & Chatterjee, C. (2003). Management of phytotoxicity of cobalt in tomato by chemical measures. *Plant Science*, *164*, 793–801.

Chen, H., & Cutright, T. (2001). EDTA and HEDTA effects on Cd, Cr, and Ni uptake by *Helianthus annuus*. *Chemosphere*, *45*(1), 21–28.

Ciura, J., Poniedzialek, M., Jedrszczyk, E., & Sekara, A. (2005). The possibility of using crops as metal phytoremediants. *Polish Journal of Environmental Studies*, *14*(1), 17–22.

Dahbia, Z., Benchaban, M. M. H., & Mohamed, A. H. (2013). Phytoremediation of contaminated agricultural soil by lead from traffic pollution using a common barley *Hordeum vulgare*. *International Journal of Environment and Resource*, *2*(3), 49–57.

Davis, R. D., & Beckett, P. H. T. (1978). Upper critical levels of toxic elements in plants: II. Critical levels of copper in young barley, wheat, rape, lettuce and rye grass, and of nickel and zinc in young barley and rye grass. *The New Phytologist*, *80*, 23–32.

Dovgaliuk, A., Kaliniak, T. B., & Blium, L. (2001). Assessment of phyto- and cytotoxic effects of heavy metals and aluminum compounds using onion apical root meristem. *TSitologiia I Genetika*, *35*, 3–9.

Du, L., Feng, X., Huang, Z., Liu, B., Jin, Y., Fang, Z., ... & Xia, C. (2016). The effect of U speciation in cultivation solution on the uptake of U by variant *Sedum alfredii*. *Environmental Science and Pollution Research*, *23*(10), 9964–9971.

Feigl, G., Kumar, K., Lehotai, N., Tugyi, N., Molnár, A., Ordög, A., ... & Kolbert, Z. (2003). Physiological and morphological responses of the root system of Indian mustard (*Brassica juncea* L. Czern.) and rapeseed (*Brassica napus* L.) to copper stress. *Ecotoxicology and Environmental Safety*, *94*, 179–189.

Gill, R. A., Zang, L. L., Ali, B., Farooq, M. A., Cui, P., Yang, S., ... & Zhou, W. J. (2015). Chromium-induced physio-chemical and ultrastructural changes in four cultivars of *Brassica napus* L. *Chemosphere*, *120*, 154–164.

Gill, S. S., & Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry*, *48*, 909–930.

Gopal, R., Dube, B. K., Sinha, P., & Chatterjee, C. (2003). Cobalt toxicity effects on growth and metabolism of tomato. *Communications in Soil Science and Plant Analysis*, *34*, 619–628.

Hall, L. J. (2002). Cellular mechanism for heavy metal detoxification and tolerance. *Journal of Experimental Botany*, *53*, 1–11.

Heath, R. L., & Packer, L. (1968). Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics*, *125*(1), 189–198.

Hemantaranjan, A., & Trivedi, A. K. (2000). Effect of foliar applied boron and soil applied iron and sulphur on growth and yield of soybean (*Glycine max* L. Merr). *Indian Journal of Plant Physiology*, *5*(2), 142–144.

Hossain, M. B., Islam, M. S., Islam, M. R., Salam, M. A., & Yousuf, M. A. (2012). Synthesis and characterization of mixed ligand complexes of Co(II) and Fe(III) ions with maleic acid and heterocyclic amines. *Journal of Bangledesh Chemical Society*, *25*(2), 139–145.

Huang, M., Zhang, T., Ruan, J., & Chen, X. (2017). A new efficient hybrid intelligent model for biodegradation process of DMP with fuzzy wavelet neural networks. *Scientific Reports*, *7*(41239).

Imlay, J. A. (2003). Pathways of oxidative damage. *Annual Review of Microbiology*, *57*, 395–418.

Jayakumar, K., & Jaleel, C. A. (2009). Uptake and accumulation of cobalt in plants: A study based on exogenous cobalt in soybean. *Botany Research International*, *2*, 310–314.

Karuppanapandian, T., & Kim, W. (2013). Cobalt-induced oxidative stress causes growth inhibition associated with enhanced lipid peroxidation and activates antioxidant responses in Indian mustard (*Brassica juncea* L.) leaves. *Acta Physiologiae Plantarum*, *35*(8), 2429–2443.

Kono, Y. (1978). Generation of superoxide radical during auto oxidation of hydroxylamine and an assay for superoxide dismutase. *Archives of Biochemistry and Biophysics*, *186*, 189–195.

Kumar, M., Chand, R., & Shah, K. (2016). Evidences for growth-promoting and fungicidal effects of low doses of tricyclazole in barley. *Plant Physiology and Biochemistry*, *103*, 176–182.

Kumar, V., Sharma, A., Kaur, P., Sidhu, G. P. S., Bali, A. S., Bhardwaj, R., ... & Cerda, A. (2018). Pollution assessment of heavy metals in soils of India and ecological risk assessment: A state-of-the-art. *Chemosphere*, http://dx.doi.org/10.1016/j.chemosphere.2018.10.066

Lange, B., van der Ent, A., Baker, A. J., Echevarria, G., Mahy, G., Malaisse, F., ... & Faucon, M. P. (2017). Copper and cobalt accumulation in plants: A critical assessment of the current state of knowledge. *The New Phytologist*, *213*(2), 537–551.

Li, N., Li, Z., Fu, Q., Zhuang, P., & Guo, B. (2012). Effects of chelators and small organic acids on phytoextraction of Cd from soil with *Amaranthus hypochondriacus* L. *Fresenius Environmental Bulletin*, *21*(7), 1879–1884.

Ma, Y., Zhu, M., Shabala, L., Zhou, M., & Shabala, S. (2015). Conditioning of roots with hypoxia increases aluminum and acid stress tolerance by mitigating activation of K+ efflux channels by ROS in barley: Insights into cross-tolerance mechanisms. *Plant and Cell Physiology*, *57*(1), 160–173.

Marschner, H. (1995). *Mineral nutrition of higher plants* (2nd edn.). London: Academic Press.

Mattiello, A., Filippi, A., Pošćić, F., Musetti, R., Salvatici, M. C., Giordano, C., ... & Marchiol, L. (2015). Evidence of phytotoxicity and genotoxicity in *Hordeum vulgare* L. exposed to CeO_2 and TiO_2 nanoparticles. *Frontiers in Plant Science*, *6*(1043), 1–13.

Mittler, R. (2002). Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science*, *7*, 405–410.

Nakano, Y., & Asada, K. (1981). Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant and Cell Physiology*, *22*(5), 867–880.

Pandey, N., Pathak, G. C., Pandey, D. K., & Pandey, R. (2009). Heavy metals, Co, Ni, Cu, Zn and Cd, produced oxidative damage and evoke differential antioxidative responses in spinach. *Brazilian Journal of Plant Physiology*, *21*, 103–111.

Pütter, J. (1974). Peroxidases. In *Methods of enzymatic analysis* (pp. 685–690).

Rivas, B. L., Seguel, G. V., & Ancatripai, C. (2000). Polymer-metal complexes: Synthesis, characterization, and properties of poly (maleic acid) metal complexes with Cu(II), Co(II), Ni(II), and Zn(II). *Polymer Bulletin*, *44*(5–6), 445–452.

Shahid, M., Austruy, A., Echevarria, G., Arshad, M., Sanaullah, M., Aslam, M., ... & Dumat, C. (2014). EDTA-enhanced phytoremediation of heavy metals: A review. *Soil and Sediment Contamination: An International Journal*, *23*(4), 389–416.

Tewari, R. K., Kumar, P., Sharma, P. N., & Bisht, S. S. (2002). Modulation of oxidative stress responsive enzymes by excess cobalt. *Plant Science*, *162*, 381–388.

Yuan, H. M., & Huang, X. (2016). Inhibition of root meristem growth by cadmium involves nitric oxide-mediated repression of auxin accumulation and signalling in *Arabidopsis*. *Plant Cell & Environment*, *39*, 120–135.

Zhang, T., Huang, Z., Chen, X., Huang, M., & Ruan, J. (2016). Degradation behavior of dimethyl phthalate in an anaerobic/anoxic/oxic system. *Journal of Environmental Management*, *184*, 281–288.