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Nitrogen of EDDS enhanced removal of potentially toxic elements and attenuated their oxidative stress in a phytoextraction process☆

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ABSTRACT

(*S,S*)-ethylenediaminedisuccinic acid (EDDS) has a strong capacity to mobilize potentially toxic elements (PTEs) in phytoextraction. It can release NH_4^+-N via biodegradation, which can enhance N supply to soil thereafter promote plant growth and plant resistance to PTEs. However, the advanced feature of released N in the EDDS-enhanced phytoextraction remains unclear. In the current study, the effects of N supply released from EDDS on ryegrass phytoextraction and plant resistance to PTEs were investigated in detail by a comparison with urea. Our results supported that the addition of both EDDS and urea increased N concentration in soil solution, yet EDDS needed more time to release available N for plant uptake and transported more N from root to shoot. Additionally, EDDS significantly increased the concentration of all targeted PTEs, i.e. Cu, Zn, Cd, and Pb, in the soil solution, which results in higher levels of their occurrence in plant biomass compared with urea. By contrast, the supply of N slightly enhanced the ryegrass uptake of micro-nutrients, i.e. Cu and Zn, yet it caused negligible effects on nonessential elements, i.e. Cd and Pb. The mobilized PTEs by EDDS lead to elevated oxidative stress because higher levels of malondialdehyde and $O_2^{\bullet-}$ were observed. The supply of N attenuated oxidative stress caused by $O_2^{\bullet-}$ and H_2O_2, which was associated with enhanced activities of superoxide dismutase and peroxidase. Our results advanced the understanding of the exogenous N supply and metal resistance mechanisms in the EDDS-enhanced phytoextraction. This study also highlighted that EDDS can serve as a N source to ease N-deficient problems in PTEs-contaminated soils.

1. Introduction

Contamination in soil and water caused by potentially toxic elements (PTEs) captured increasing attention in the past decades (El-Naggar et al., 2020; Fang et al., 2020; Palansooriya et al., 2020). Techniques in physical, chemical, and biological aspects have been developed for the contaminated soil to either mobilize the PTEs to another phase, or to immobilize them to more stable forms (Beiyuan et al., 2020; Bolan et al., 2014; El-Naggar et al., 2019b; Liu et al., 2019; Wang et al., 2019a). Phytoremediation is a sustainable and emerging technology, which attracts considerable research interests in the past ten years (Fang et al., 2020; He et al., 2019; Wang et al., 2018). As part of phytoremediation, phytoextraction uses plant to extract contaminants from soils. Its efficacies of removing PTEs from soil is mainly controlled by the transfer efficiencies of PTEs from plant roots to shoots and plant biomass (Antoniadis et al., 2017; Lestan et al., 2008). Hence, besides high tolerance to PTEs, plants with higher biomass and chemical reagents which can promote the mobility of PTEs in soil are preferred in the phytoextraction.

As one of the reagents can enhance mobility of PTEs in soil,

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chelants can facilitate transportation of PTEs into xylem and enhance their transfer efficiencies from roots to shoots of plants, which are commonly used in phytoextraction (Lestan et al., 2008; Luo et al., 2015; Wang et al., 2019b). However, some typical chelants, such as nitrilotriacetic acid (NTA) and ethylenediaminetetraacetic acid (EDTA), were found toxic and/or resistant to soil biota, enzyme activities, and plants (Beiyuan et al., 2018c; Duo et al., 2019; Lestan et al., 2008; Sun et al., 2020). Therefore, biodegradable chelants or low-molecular-weight-organic-acids with high complexation capacities with metals, like (*S,S*)-ethylenediaminedisuccinic acid (EDDS), were recommended to serve as suitable substitutes of the non-biodegradable/toxic chelants in the PTEs-enhanced extraction (Beiyuan et al., 2018b; Ju et al., 2020).

In addition, low soil fertility is severe and worldwide problem (El-Naggar et al., 2019a; Xu et al., 2017). Many PTEs-contaminated soils are also facing this problem. The EDDS is rich in nitrogen and carbon, which might provide available nutrients to microorganisms and/or plants after biodegradation. Studies showed that EDDS can be degraded to N-(2-aminoethyl) aspartic acid ($C_6H_{12}N_2O_4$), then to ethylenediamine ($C_2H_4(NH_2)_2$) (Chen et al., 2010a; Egli, 2001). With the help of microorganisms, the final products of EDDS degradation are carbon dioxide (CO_2) and ammonium (NH_4^+-N). Previous studies showed that supplying exogenous nitrogen in simple forms of ammonium (NH_4^+-N), nitrate (NO_3^--N), and urea ($CO(NH_2)_2$) to the PTEs-contaminated soils can not only enhance plant biomass but also increase phytoextraction efficiencies of the PTEs (Giansoldati et al., 2012). Specifically, NO_3^--N is the form can be easily taken up by plants, yet NH_4^+-N can be transformed to NO_3^--N with the help of enzymes in soils. As a result, nitrogen of both two forms can contribute to higher plant biomass, and indirectly increase the efficacies of phytoextraction.

In detail, the supply of N can promote metal mobility and diffusion rates to root surface, which enhanced metal removal efficiency by phytoextraction (Xie et al., 2009). Elevated content of NO_3^--N caused by applying EDDS and consequently higher N content in the biomass of ryegrass were found in our previous study, compared with adding NTA and citric acid which are biodegradable but have lower N content (Fang et al., 2017). Our previous study suggested that EDDS promoted nitrogen cycling by accelerating nitrogen transformation and improving soil microbial structure and enzyme activities with the extra N supply (Beiyuan et al., 2018a). In addition, Fang et al. (2017) suggested that EDDS leads to a significant increment on the nitrogen transformation (both ammonia oxidation and denitrification) via studying the N cycling gene abundances.

Furthermore, the PTEs can cause oxidation stress by producing of reactive oxygen species (ROS). This is considered as an important aspect to assess phytoextraction nowadays. The ROS are unavoidable by-products in plant aerobic metabolism and harmful to essential cellular components (such as DNA, proteins, and lipids), including superoxide ($O_2^{\bullet -}$) which is the precursor of all oxygen radicals, hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\bullet}$) (Thounaojam et al., 2012). In addition, the promoted oxidative stress leads to lipids peroxidation which is harmful to plant growth. Malondialdehyde (MDA) is the final product of lipids peroxidation, whose level in plants can act as an excellent indicator of plant damage (Sidhu et al., 2018). To alleviate the oxidative stress and tolerate adverse effects caused by PTEs, plants have stimulated antioxidant systems comprise several ROS-removing enzymes, comprising of superoxide dismutase (SOD), peroxidase (POD), catalases (CAT), and ascorbate peroxidase (APX). Previous studies also suggested that extra N supply diminished oxidative stress caused by PTEs (Chen et al., 2018; Shen et al., 2019). Therefore, ROS, MDA, and some ROS-removing enzyme activities were evaluated to further study the effects of EDDS and urea on oxidative stress caused by PTEs in ryegrass.

In this study, we hypothesized that the exogenous N content generated from EDDS degradation can be beneficial for phytoextraction by ryegrass, which might be associated with elevated metal extraction efficiencies, increased biomass, and mitigated oxidative stress. Hence, we evaluated the concentration of PTEs and nutrients in soil solution, total contents of PTEs in both shoot and root biomass of ryegrass, photosynthetic properties, and oxidative stress via ROS and antioxidative enzyme activities. To study the effects of the exogenous N, the above analyses of pot experiments were performed in with no exogenous N, with EDDS, or with urea. Urea was used because it is simple and is one of the most popular N fertilizers without chelating capacity. To the best of our knowledge, there is no research work has elucidated the effects of N supply from EDDS in phytoextraction of contaminated soils by PTEs. The results of this study can strengthen our understanding of the function of N in the phytoextraction, especially for providing a better option for the PTEs-contaminated soils with nutrient-deficiency problems.

2. Materials and methods

2.1. Soil characterization

Topsoil samples (10–20 cm) were collected and preserved in plastic bags in Feng County, China, near a Pb–Zn mine, which were also used in our previous study (Cui et al., 2018). The samples were immediately transported to our laboratory in Yanglin City, Shaan Xi Province. After air dry at room temperature, the samples were sieved (<2 mm) before using. Soil pH (8.08) was determined at a soil-to-water ratio of 1:2.5 (v/v). Total phosphorus and total nitrogen content of the samples are 0.310 and 1.59 g kg^{-1}, respectively. The total phosphorus of the soil samples was determined by an ultraviolet spectrophotometer via the Mo–Sb colorimetric method after a digestion by H_2SO_4 and $HClO_4$ (Duan et al., 2018; Zhu et al., 2018). The total nitrogen was measured by a Nitrogen Analyzer System after the Kjeldahl digestion (Chen et al., 2018). Soil organic matter of the soil sample is 25.8 g kg^{-1} which was determined according to Kalembasa and Jenkinson (1973). Total content of PTEs of the topsoil samples was analyzed by ICP-AES after digestion by a modified USEPA Method 3051 A (0.200 g soil + 15 mL mixture of HCl, HNO_3, and $HClO_4$ at a volumetric ratio of 1:3:1). The ICP-AES results suggested that the soil was contaminated by Cu, Zn, Cd, and Pb (30.1, 160, 4.95, and 652 mg kg^{-1}, respectively).

2.2. Pot experiment

Pot experiments were performed for three different treatments in a greenhouse at 25 °C and natural light condition: SR (soil + ryegrass (*Lolium perenne*)), SRE (soil + ryegrass + EDDS), and SRU (soil + ryegrass + urea). Each treatment contained at least five replicates with a portion of 8 kg of soil sample and covered three different growing time scale (20, 40, and 60 days). Deionized water (DIW) was weekly added to soils of the pot experiment to obtain good soil moisture for ryegrass growth at around 70% of the soil maximum water-holding capacity. A sterilization of ryegrass seeds before sowing was conducted by immersing them in 30% NaOCl solution for 2 min, then washing by tap water at least three times and rising by DIW. One hundred of seeds were cultured for seed germination. After 10 days, the number of pre-germinated seedlings of ryegrass were screened to 40 of similar size in each pot. After 30 days, 5 mmol kg^{-1} EDDS ($C_{10}H_{16}N_2O_8$) and urea ($CO(NH_2)_2$), which have equal N contents, were added respectively into the soil. Theoretically, the EDDS can release an equal amount of

NH_4^+-N as urea when they were applied in the same dosage. After growing for 20, 40, and 60 days, respectively, the plants were harvested and divided into shoot and root samples. The plant samples were cleaned carefully and separately analyzed for nutrients, metal contents, lipid peroxidation, oxidation damage, and antioxidant enzyme activities as shown in Sections 2.3–2.5.

2.3. Metal and NPK content in soil solution, soil, and plant tissue samples

The soil solution samples collected by the Rhizon soil moisture samplers and soil samples of the pot experiment were analyzed for nutrients including total organic carbon (TOC), total phosphorus (TP), and total nitrogen (TN) and potassium (K) and metal concentration for both PTEs (Cu, Cd, Pb, and Zn) and mineral elements (Ca, Mg, Fe, and Al) on Day 20, 40, and 60.

The plant tissues samples were rinsed by tap water and DIW, respectively, for three times, dried at 70 °C in an oven, weighted for recording biomass, and then grounded to around 4 mm for acid digestion (by concentrated H_2SO_4 and H_2O_2 at 1:1 (v/v)). Then the extractant after digestion was analyzed by ICP-AES for metal content, total phosphorus, and total nitrogen as described above.

2.4. MDA, H_2O_2, and $O_2^{\bullet-}$ content and antioxidant enzyme activities in the plant

The level of lipid peroxidation and plant oxidation damage in ryegrass were investigated in detail via determining the contents of malondialdehyde (MDA), superoxide radicals ($O_2^{\bullet-}$), as well as hydrogen peroxide (H_2O_2), respectively on Day 20, Day 40, and Day 60. A dosage of 2 mL of 10% trichloroacetic acid was used to extract the MDA content in plant shoot and root samples. After centrifugation at 8000 *g* for 10 min, the supernatant of the extraction was collected for the below analysis. The amount of MDA, $O_2^{\bullet-}$, and H_2O_2 of the tissue of both shoots and roots were determined by different commercial reagent kits, respectively, which were purchased from Suzhou Comin Biotechnology Co., Ltd. Suzhou, China (Ju et al., 2020).

Fresh plant samples of shoot and root were collected on Day 20, 40, and 60 of the pot experiments to analyzing the antioxidant enzyme activities including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and peroxidase (POD). The clean plant tissue samples were homogenized with 1 mL of extraction buffer in an ice bath at 4 °C. Thereafter, the mixture of tissues and extractant was then centrifuged 15,000 *g* at 4 °C for 15 min, then the supernatant of the mixture was analyzed immediately for SOD, CAT, APX, and POD, respectively, by commercial reagent kits developed by Suzhou Comin Biotechnology Co., Ltd. under different wavelengths (Duan et al., 2018).

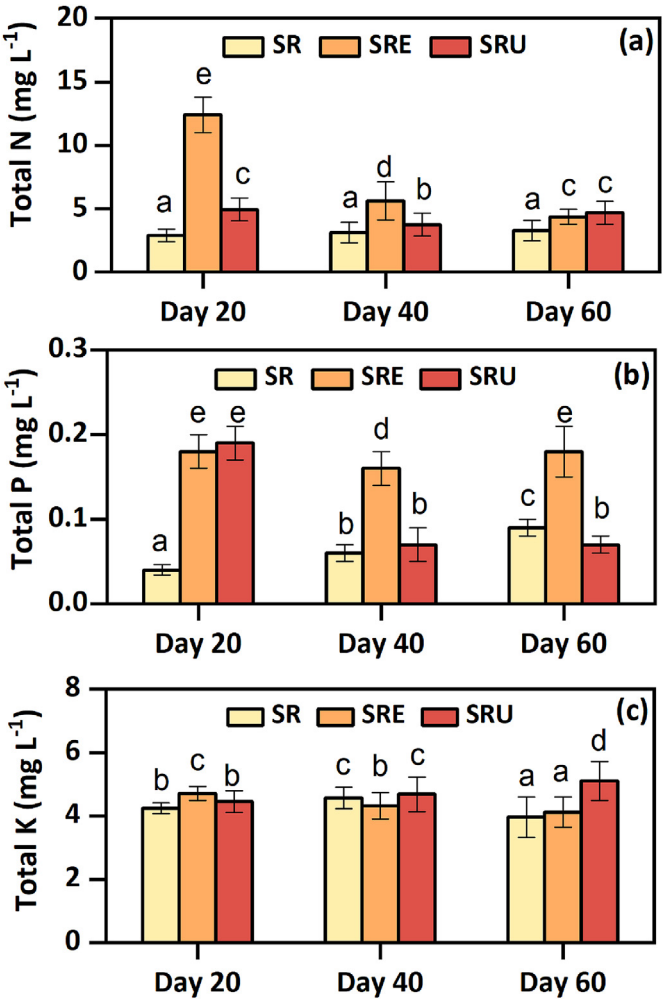

Fig. 1. The total (a) N, (b) P, and (c) K concentration in soil solution under different treatments (SR, SRE, and SRU) on Day 20, Day 40, and Day 60. Each value represents the mean ± SE (n = 4). The small letters stand for statistical significance at the 0.05 level with the LSD tests which was conducted within the same sampling time.

2.5. Photosynthetic rate and chlorophyll content

The plant photosynthetic rate of the pot experiment was studied by a portable photosynthesis system (LI-COR Biosciences, USA). The amount of chlorophyll, as a total of chlorophyll *a* and chlorophyll *b*, was determined according to Duan et al. (2018) and Sobrino-Plata et al. (2014). In detail, after cleaning, the fresh biomass of ryegrass of a 0.20 g was extracted by 25 mL 80% (v/v) acetone and its supernatant after centrifutation was analyzed at 645 nm and

Table 1
Shoot and root biomass of ryegrass under different treatments (SR, SRE, and SRU).

Treatments	Shoot biomass (g Plant^{-1})			Root biomass (g Plant^{-1})		
	Day 20	Day 40	Day 60	Day 20	Day 40	Day 60
SR	1.96 ± 0.12a	4.61 ± 0.53a	8.94 ± 1.12a	0.68 ± 0.03b	1.84 ± 0.16a	3.52 ± 0.48a
SRE	1.74 ± 0.15a	5.86 ± 0.31b	11.2 ± 2.53b	0.52 ± 0.02a	1.72 ± 0.18a	3.64 ± 0.26a
SRU	2.24 ± 0.18b	5.73 ± 0.42b	11.4 ± 1.62b	0.56 ± 0.04a	1.78 ± 0.24a	3.78 ± 0.34a

Notes: SR (soil + ryegrass), SRE (soil + ryegrass + EDDS), SRU (soil + ryegrass + Urea). The values are means ± SD (n = 4). The small letters stand for statistical significance at the 0.05 level with the LSD tests which was conducted within the same sampling time.

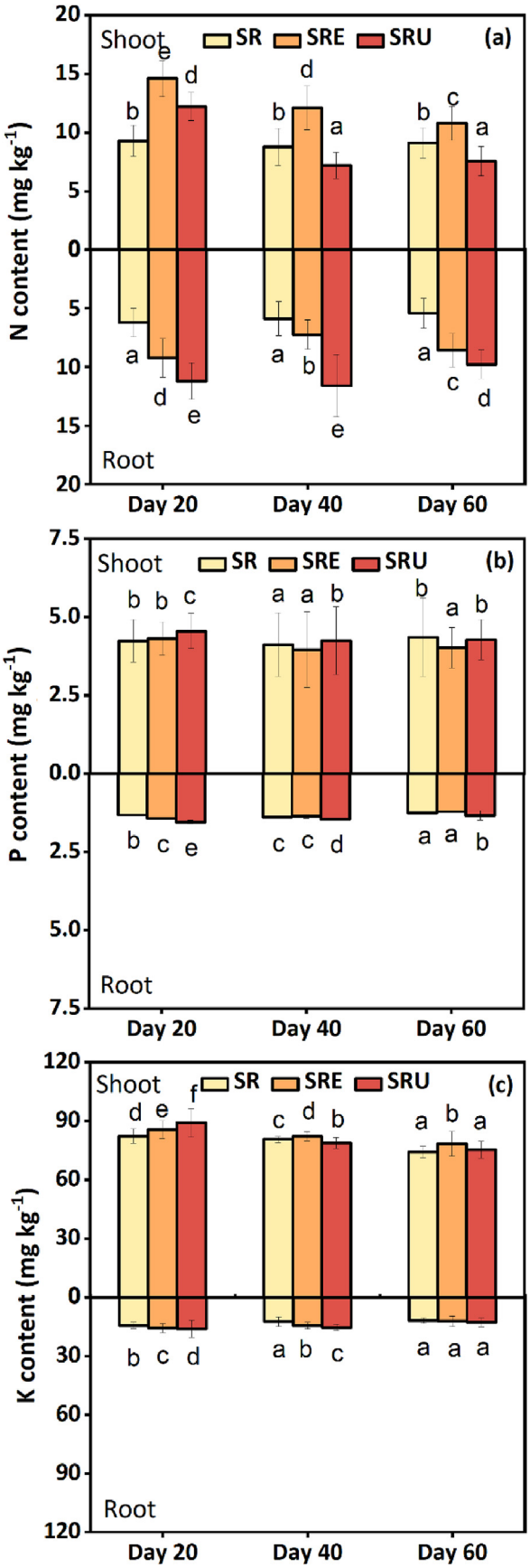

Fig. 2. The total (a) N, (b) P, and (c) K content in plant issues (shoot and root) under different treatments (SR, SRE, and SRU) on Day 20, Day 40, and Day 60. Each value represents the mean ± SE (n = 4). The small letters stand for statistical significance at the 0.05 level with the LSD tests which was conducted within the same sampling time.

663 nm, respectively, for chlorophyll *a* and chlorophyll *b*, by a UV–vis spectrophotometer.

2.6. Statistical analyses

All the data used in this study are average values of replicated experiments (for example, the pot experiment used more than 5 pots for each treatment). Analysis of variance (ANOVA) with the LSD tests was conducted at a significant level of $p < 0.05$ by the SPSS 22 software.

3. Results and discussion

3.1. Effects of EDDS and urea on distribution of nutrients and ryegrass biomass

The total N concentration in soil solution was sharply enhanced by EDDS on Day 20 because its highly N-containing final product after degradation (Fig. 1a), NH_4^+-N, can provide extra nutrients to the plants, compared with the SR. This can be attributed to the delay degradation of EDDS, which was consistent with Meers et al. (2008) that a lag phase of 10–32 days of EDDS degradation is needed in soils. They observed a complete degradation of EDDS after 30–50 days. In the SRU, the N concentration in soil solution on Day 20 is low compared with the SRE, although the urea and EDDS were applied in equal N amounts (Fig. 1a). This can be due to hydrolysis of urea in soils quickly started within 2 days then the urea can be shortly transformed to ammonia then to NH_4^+-N which is easily taken up by plants (Dawar et al., 2011). Both of the N-final product of EDDS degradation and urea hydrolysis is NH_4^+-N which can be quickly converted to NO_3^--N via nitrification. Our previous study suggested that after applying 5 mmol kg^{-1} EDDS, the same dosage as the current study, in a ryegrass phytoremediation study for 2 days, the NH_4^+-N was below its detection limit (<0.01 ppm), while a sharp increase of NO_3^--N amount was observed (Fang et al., 2017). Besides, urea can be quickly converted to NH_4^+-N within 48 h, then to NO_3^--N within 192 h (Francis and Haynes, 1991). The utilization of N can be further interpreted by studying the microorganism activities. In addition to a better microbial community caused by the exogenous N of EDDS and urea, they can cause positive effects on both ammonia oxidation and denitrification processes which can pose different effects on the N utilization (Chen et al., 2010b; Fang et al., 2017; Lu et al., 2012).

The shoot biomass, chlorophyll content, and photosynthetic rate of the SRE decreased firstly on Day 20, then significantly increased (Table 1&2). By contrast, the addition of urea immediately increased the shoot biomass and chlorophyll content which probably due to the quick releasing N from its decomposition (Tables 1 and 2). These further support that EDDS and urea have different N releasing pattern. Additionally, in the hydrolysis process of urea, part of N could be lost because of the generation of ammonia which is gas. The application of both EDDS and urea caused around an improvement by 50% of the total N csontent in total ryegrass biomass (Fig. 2a). However, EDDS induced more N content to the shoot than urea, which is probably due to EDDS can facilitate the N transportation into xylem then increase its transportation efficiency from root to shoot (Luo et al., 2006).

The total P in soil solution was remarkably improved by EDDS (Fig. 1b). Due to its strong dissolved capacity, EDDS can dissolve more P from soils via Fe- and/or Al- bonding phosphates, soil organic matter, and soil colloids (Jalali and Ostovarzadeh, 2009).

Table 2
Total organic carbon (TOC) in soils, Chlorophyll content of ryegrass, and photosynthetic rate of ryegrass under different treatments (SR, SRE, and SRU).

Treatments	TOC (g kg^{-1})			Chlorophyll content (mg g^{-1} DW)			Photosynthetic rate (μmol m^{-2} s^{-1})		
	Day 20	Day 40	Day 60	Day 20	Day 40	Day 60	Day 20	Day 40	Day 60
SR	28.1 ± 2.4a	24.7 ± 2.1a	20.3 ± 1.8a	1.21 ± 0.21b	1.32 ± 0.06a	1.78 ± 0.04a	12.3 ± 1.45c	13.4 ± 1.30b	14.1 ± 1.78a
SRE	204.6 ± 8.9b	62.1 ± 6.2b	22.4 ± 2.6b	1.03 ± 0.08a	1.45 ± 0.07b	2.32 ± 0.18c	9.54 ± 0.09a	12.1 ± 1.25a	15.8 ± 1.39b
SRU	32.4±4a	26.8 ± 3.2a	18.9 ± 2.5a	1.41 ± 0.03b	1.52 ± 0.12b	2.12 ± 0.24b	11.2 ± 1.02b	12.5 ± 1.64a	16.2 ± 1.87b

Notes: SR (soil + ryegrass), SRE (soil + ryegrass + EDDS), SRU (soil + ryegrass + Urea). The values are means ± SD (n = 4). The small letters stand for statistical significance at the 0.05 level with the LSD tests which was conducted within the same sampling time.

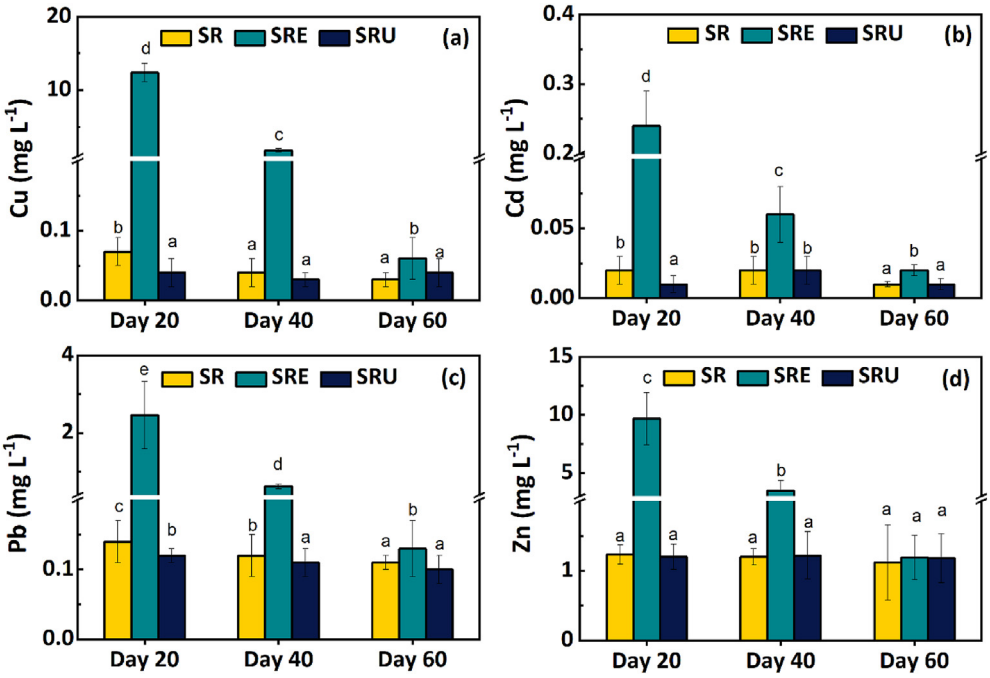

Fig. 3. (a) Cu, (b) Cd, (c) Pb, and (d) Zn contents in soil solutions under different treatments (SR, SRE, and SRU) on Day 20, Day 40, and Day 60. Each value represents the mean ± SE (n = 4). The small letters stand for statistical significance at the 0.05 level with the LSD tests which was conducted within the same sampling time.

Slightly higher amounts of P were mobilized by urea into the soil solution, which stopped after Day 20, compared with EDDS (Fig. 1b), which is probably due to the elevated pH in soil solution caused by its hydrolysis (Hartikainen and Yli-Halla, 1996). However, the remarkably increased total P concentration by EDDS might be not effective for ryegrass uptake, resulting from a marginal increment of total P content in ryegrass biomass (Fig. 2b). The addition of both EDDS and urea caused negligible influence on the total K in soil solution (Fig. 1c) and ryegrass biomass (Fig. 2c). Additionally, the degradation of EDDS released large amount of carbon and significantly enhanced TOC of the soil solution (Table 2), providing large amounts of carbon sources which is beneficial for plant growth.

Both the chlorophyll content and photosynthetic rate were firstly suppressed (on Day 20) then enhanced by the addition of EDDS (on Day 60, Table 2). The latter phenomenon of the increased photosynthesis process could be due to the amount of available nutrients in soil was promoted, especially for N (Duan et al., 2018; Shen et al., 2019), after a considerable time for EDDS degradation.

3.2. Effects of EDDS and urea application on metal mobilization in soil

With the addition of EDDS, more PTEs were mobilized in soil solution. The concentration of PTEs in the soil solution followed an order of Cu > Zn > Pb > Cd on Day 20 (Fig. 3). Some possible reasons are suggested for the order of metal concentration in soil solution: 1) The order of stability constants of metal-EDDS complexes ($logK_{Cu\text{-}EDDS}$ = 18.4 > $logK_{Zn\text{-}EDDS}$ = 13.4 > $logK_{Pb\text{-}EDDS}$ = 12.7 > $logK_{Cd\text{-}EDDS}$ = 10.9 (Begum et al., 2013)) and 2) the total metal content in soil and metal geochemical distribution in soil (Beiyuan et al., 2017). In general, because of the uptake by ryegrass, the concentration of all targeted PTEs in the soil solution was gradually decreased with time. Additionally, it can be affected by metal redistribution with metal-EDDS complexes. For example, the Cu-EDDS complex can react with other metals in soils and form new metal-EDDS complexes (Tsang et al., 2009; Yip et al., 2009). Cations, *i.e.* Ca, Mg, Fe, and Al, were mobilized by EDDS in soil solution (Fig. S1), especially for Ca, which probably results from the higher stability constants which followed an order of $logK_{Fe\text{-}EDDS}$ = 22.0 > $logK_{Al\text{-}EDDS}$ = 12.9> $logK_{Mg\text{-}EDDS}$ = 6.00 > $logK_{Ca\text{-}EDDS}$ = 4.70 (Kolodynska, 2011; Tsang et al., 2009; Yip et al., 2009), as well as their total contents and soil properties. Free EDDS and metal-EDDS complexes in soil can additionally contribute to the dissolution of cation ions with the growing application time (Fabbricino et al., 2013; Komarek et al., 2009; Tsang et al., 2009).

The addition of urea was negligible for metal mobilization in soil (Fig. 3&S1). However, previous studies suggested that urea in soil could be quickly hydrolyzed with soil moisture and release NH_4^+-N and carbon dioxide by the enzyme activities (Dawar et al., 2011).

This reaction can lead to the consumption of hydron and an increment of soil solution pH, thus reduce the soluble and exchangeable fractions of PTEs in soil (Liu et al., 2006). The study also observed an increased proportion of PTEs bond to Fe/Mn oxides in the soil.

3.3. Effects of EDDS and urea on metal distribution in ryegrass

With the assistance of EDDS, the uptake of targeted metals by ryegrass was significantly improved compared with the SR and SRU (Fig. 4). The uptake amount of PTEs of both root and shoot followed an order of Zn > Cu > Pb > Cd on Day 20, slightly different from the order of PTEs in the soil solution, which can be due to the metal uptake preference of ryegrass. The root uptakes of plant nonessential elements, such as Pb (increased by ~4.5-fold) and Cd (increased by ~3.2-fold) were greatly improved with the addition of EDDS (Fig. 4), though the stability constants of Pb and Cd-EDDS complexes are lower than Cu and Zn. This can be attributed to EDDS increased the total soluble concentration of metals in soil solution by forming metal-chelant complexes and it increased the nonselective plant uptake of metals in an apoplastic pathway (Bell et al., 2003; Mahar et al., 2016; Nowack et al., 2006). It mobilized Pb and Cd in soil by metal chelation, which is consistent with the result that more Pb and Cd release to soil solution with the EDDS addition (Fig. 3c&d).

Without the addition of chelant, contaminants like PTEs, are taken up and distributed by the same transporters for essential or micro-nutrients (*e.g.* Fe, Mn, and Zn) (Khan et al., 2014). Divalent micro-nutrients can be quickly taken up and transported from root to other plant tissues (Ondrasek et al., 2019). Therefore, the PTEs, especially for those are divalent, can pass through root and other biomembranes via selective transporters/channels in a similar way. However, the addition of urea did not make significant effects on the root uptake of Pb and Cd (Fig. 4). Increased root uptakes by the addition of urea were only observed for Cu and Zn (~1.6-fold and 1.5-fold, respectively), which are essential micro-nutrients for plants. This might be associated with the enhanced biomass yield of ryegrass which demanded increasing amounts of micro-nutrients. Extra amounts of N supply can contribute to increasing uptake of contaminants by plants which are micro-nutrients, *e.g.* Cu and Zn, besides the effect results from the increment of pH. Therefore, we might make an assumption that the decomposition of N-containing chelants, i.e., EDDS which can finally release NH_4^+-N might also result in higher uptakes of micro-nutrients, leading to an increase of ryegrass biomass. Consistently, a positive correlation between soil or plant N and Cu uptake by *Medicago sativa* was observed (Shen et al., 2019). For nonessential elements for ryegrass, such as Cd and Pb, the addition of N might not help in the same way. However, Cheng et al. (2018) suggested that NH_4^+-N could alter subcellular distribution by increasing the binding of Cd and Cu, therefore increase their uptake by wheat.

The translocation factor (shoot content/root content in biomass) of Cu increased by 3.8-fold on Day 20 was found by EDDS, compared with the SR. The translocation factor of Pb was also enhanced by EDDS at a ~3.2-fold increase on Day 40. This is consistent with previous findings that EDDS is effective for

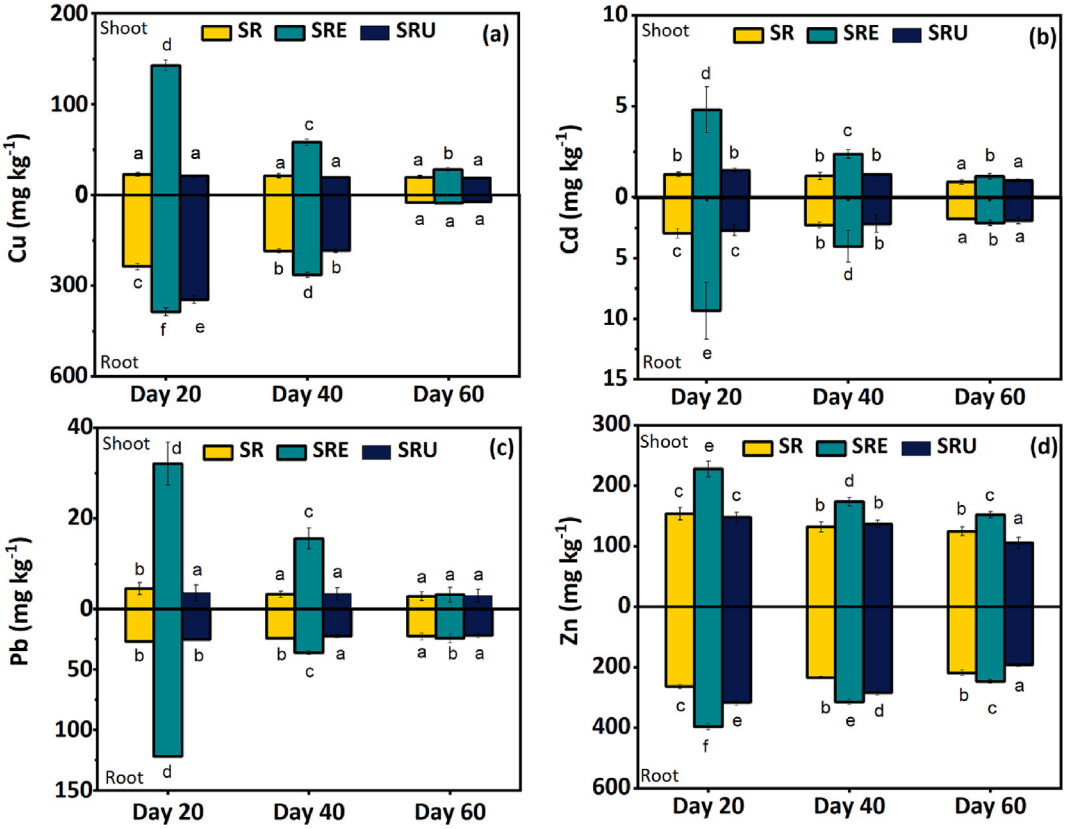

Fig. 4. (a) Cu, (b) Cd, (c) Pb, and (d) Zn contents in plant issues (shoot and root) under different treatments (SR, SRE, and SRU) on Day 20, Day 40, and Day 60. Each value represents the mean ± SE (n = 4). The small letters stand for statistical significance at the 0.05 level with the LSD tests which was conducted within the same sampling time.

translocation Cu and Pb from root to shoot, therefore increased the phytoextraction efficiencies (Luo et al., 2006, 2015). The variation of translocation rate of Cd and Zn by EDDS was negligible compared with Cu and Pb. By contrast, the application of urea only caused limited influence in the translocation rate of the targeted PTEs.

The root uptakes of Fe, Mg, and Al were greatly enhanced by the improved mobilization of metals which results from the application of EDDS, while the shoot uptakes of Mg and Fe were significantly enhanced (Fig. S2). However, the root and shoot uptake of Ca were significantly affected by the addition of EDDS, especially for the shoot. It should be highlighted that the mobilized Al might lead to plant toxicity, which might affect the beginning period of phytoextraction. By contrast, the addition of urea has marginal effects on the cation distribution in ryegrass, because of its low chelating property.

3.4. Effects of EDDS and urea on lipids peroxidation and oxidative stress by PTEs

Enhanced oxidative stress in ryegrass was observed with an enhanced MDA (in both shoots and roots) and $O_2^{\bullet-}$ (in roots) with EDDS (Fig. 5a&b), which is probably due to the enhanced accumulation of PTEs promoted by EDDS (Duan et al., 2018; Ju et al., 2020). The results of enhanced oxidative stress were consistent with the increased content of PTEs in plant tissue and enhanced translocation factors of Cu and Pb as shown in Fig. 4. The enhanced oxidative stress caused by the EDDS-evaluated dissolved PTEs leads to reduction in extraction of PTEs by the plant, which should be avoided or eased. Ju et al. (2020) suggested a co-inoculation of rhizobacteria and rhizobium in EDDS-enhanced phytoextraction to attenuate the oxidative damage, which mostly associated with the enhanced microbial biomass. The MDA accumulation of both root and shoot were significantly increased in the SRE, especially on Day 20 and Day 40. On Day 20, the increment of MDA reached 74.6% and 35.6% for root and shoot compared with the SR, respectively. The higher the MDA levels indicated a higher degree of lipid peroxidation and more severe damage to the plant cell membrane (Cui et al., 2018). EDDS caused notably high MDA levels from Day 20 to Day 60, yet the plant biomass in the SRE showed recovery after Day 40 which might be associated with the newly added N content from the degradation of EDDS.

The $O_2^{\bullet-}$ level was notably enhanced by EDDS in the ryegrass root, which indicates a higher damage level caused by promoted mobility of PTEs can be observed in root in the SRE. However, SOD activities became lower in the SRE (Fig. 6a), which plays a crucial role in quenching $O_2^{\bullet-}$ and convert it to H_2O_2 and O_2 (Gill and Tuteja, 2010; Jia et al., 2019). This might be due to the 10 to 20-fold increase in PTEs concentration in the soil solution probably exceeded the capacity of SOD in ryegrass and caused destruction of the leaf membrane and enzyme systems (Duan et al., 2018). Ehsan et al. (2014) also supported that the activities of SOD, CAT, POD, and APX were enhanced by 10 μM Cd stress yet decreased by 50 μM Cd in nutrient solution.

Theoretically, the reduced $O_2^{\bullet-}$ content leads to elevated H_2O_2 level. However, the variation of H_2O_2 content in both root and shoot after application of EDDS or urea is negligible (Fig. 5c) even with extreme higher levels of PTEs in the soil solution, compared with MDA and $O_2^{\bullet-}$. This could be associated with the ROS-scavenging enzyme activities. A 2-fold increase of APX was found in the SRE,

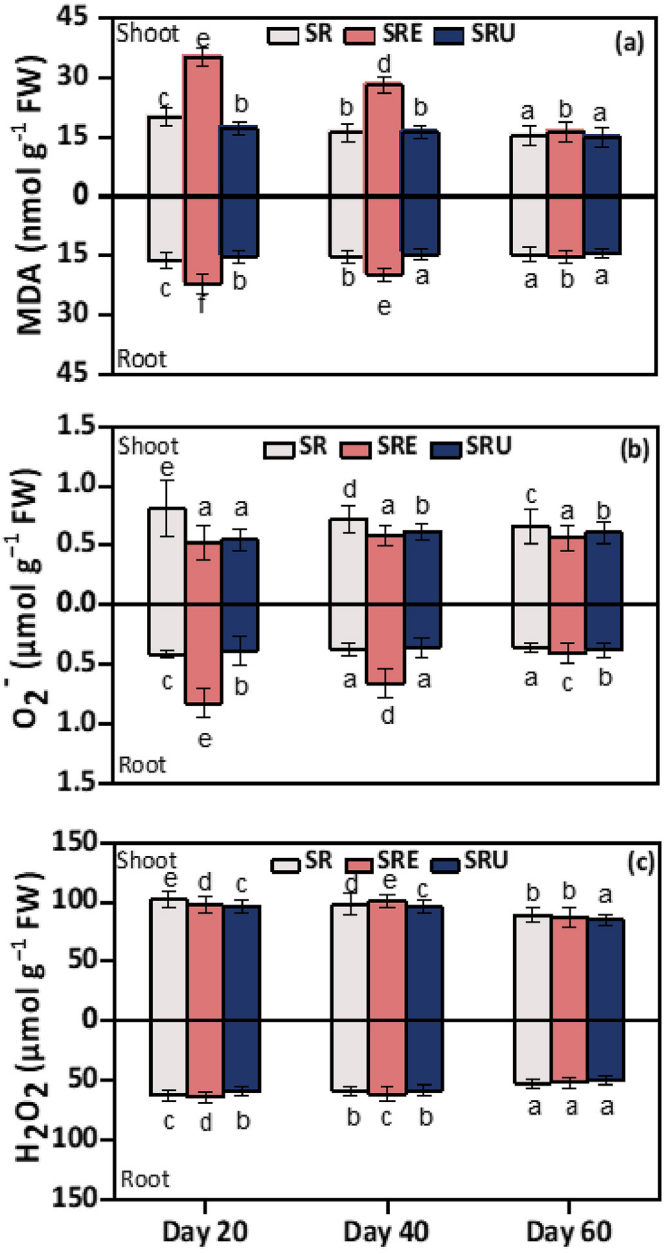

Fig. 5. (a) Malondialdehyde (MDA) content, (b) production of $O_2^{\bullet-}$ and (c) H_2O_2 content in shoots and roots in ryegrass under different treatments (SR, SRE, and SRU) on Day 20, Day 40, and Day 60. Each value represents the mean ± SE (n = 4). The small letters stand for statistical significance at the 0.05 level with the LSD tests which was conducted within the same sampling time.

compared with the SR (Fig. 6c), which probably supported that APX might play a significant role in attenuating H_2O_2 levels in the SRE. The APX is one of the most important enzymes for reducing H_2O_2 levels in plants, especially under strong metal stress (Chen et al., 2018; Mittler, 2002). The POD and CAT activities were slightly reduced in the SRE compared with the SR and SRU, which is probably due to the large amounts of mobilized PTEs by EDDS

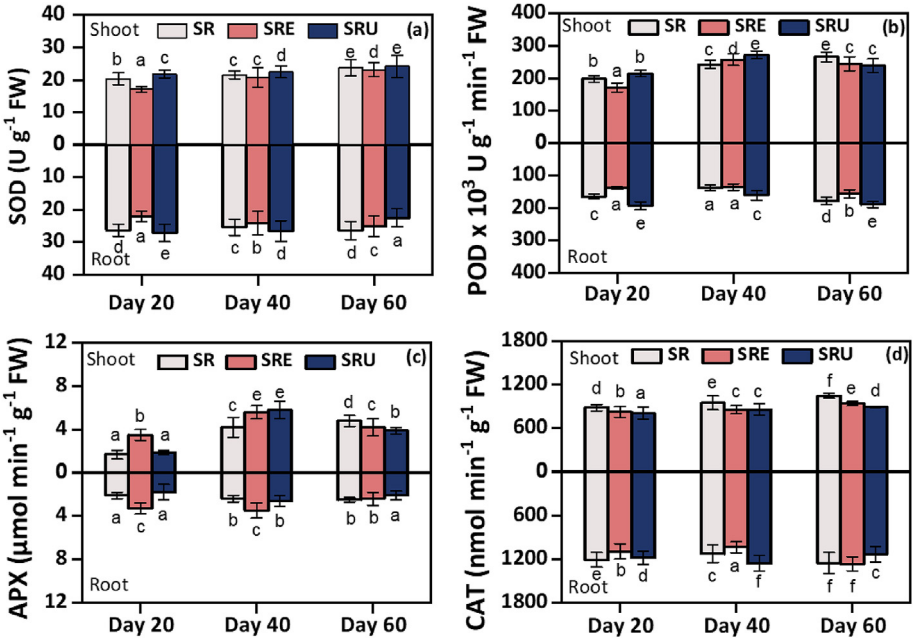

Fig. 6. Activities of ROS-removing enzymes: (a) superoxide dismutase (SOD), (b) Peroxidase (POD), (c) ascorbate peroxidase (APX), and (d) catalases (CAT) in the shoots and roots in ryegrass under different treatments (SR, SRE, and SRU) on Day 20, Day 40, and Day 60. Each value represents the mean ± SE (n = 4). The small letters stand for statistical significance at the 0.05 level with the LSD tests which was conducted within the same sampling time.

contributed to a depression (Fig. 6b&d). The POD is also crucial for decreasing the H_2O_2 level by attenuating H_2O_2 generation; while CAT can access the procedure of turning H_2O_2 to H_2O which cushions detrimental effects from H_2O_2. However, inhibited ROS-scavenging enzyme activities were frequently found in EDDS-enhanced phytoextraction processes.

Though the plant uptake of Cu and Zn were significantly enhanced by urea, the MDA, $O_2^{\bullet-}$, and H_2O_2 content in ryegrass showed a slightly decreasing trend (Fig. 5). This might suggest that micro-nutrients like Cu and Zn, only cause little negative effects on lipids peroxidation and oxidative stress of plants, unlike the nonessential elements. Besides, increment of ROS-scavenging enzyme activities was found in the SRU, especially for SOD and POD which attenuate $O_2^{\bullet-}$ and H_2O_2 content, respectively. The results suggested that the exogenous N supply of urea can alleviate the adverse effects by promoting the plant growth, biomass, and soil microorganic structure and activities, which might indirectly enhance the antioxidant enzyme activities (Shen et al., 2019). This probably indicates that biodegradable chelants, like EDDS, which provide exogenous N supply can also contribute to phytoextraction in the same way. However, the phenomenon could be less obvious due to the block effect by the significant promoted PTEs toxicity.

4. Conclusions

In summary, this study suggests that the N supply of EDDS-enhanced phytoextraction brings benefits from different aspects: 1) EDDS can serve as a slower N source than traditional fertilizers, such as urea, and increase plant uptake of N, because it needs more time to be degraded into available N. 2) EDDS enhances the ryegrass uptake of N especially on its translocation from root to shoot by facilitating the N transportation into xylem. This results in better plant growth and higher biomass of ryegrass, which also caused positive effects on uptakes of some PTEs which are micro-nutrient elements (i.e. Cu and Zn) by ryegrass, but not for nonessential elements (i.e. Cd and Pb). 3) The exogenous N supply can help in alleviating oxidative stress by enhancement of ROS-scavenging enzyme activities in ryegrass associated with increased plant biomass. With the N supply, subtle enhancement on activities of SOD and ROD were found, which are responsible for attenuating detrimental effects from $O_2^{\bullet-}$ and H_2O_2. Therefore, from a novel aspect of exogenous N supply by its degradation, EDDS can not only enhance the removal of PTEs but also improve shoot biomass and ryegrass resistance to PTEs, which are advantageous to phytoextraction in application, especially for contaminated soils with N-deficiency problems. Our study also advances the understanding of the resistance mechanisms of PTEs in the EDDS-enhanced phytoextraction. In addition, future field studies including microorganism activity with a focus on the N supply are urgently needed.

CRediT author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.115719.

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