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Effects of exogenous nitric oxide and ethylenediaminetetraacetic acid on cadmium toxicity and accumulation in ryegrass

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Abstract

The effects of exogenous nitric oxide (NO) and ethylenediaminetetraacetic acid (EDTA) on cadmium toxicity and accumulation in ryegrass (*Lolium perenne* L.) were studied in a hydroponic experiment. The results show that in plants without Cd application, addition of EDTA and sodium nitroprusside (SNP, an exogenous NO donor) significantly reduced the plant height, root length, and root activity of ryegrass, and significantly increased the $O_2^{\cdot-}$ generation rate and H_2O_2 and malondialdehyde (MDA) content in the aboveground and underground parts of ryegrass. Cadmium stress significantly inhibited ryegrass growth. Addition of SNP or EDTA alleviated Cd toxicity, and addition of both had a better effect. Compared with Cd alone, the shoot height and root length in the Cd+EDTA+SNP treatment increased by 68.8 and 59.6 %, and plant fresh and dry masses by 62.6 and 60.0 %, respectively. Also, the superoxide dismutase activity in the shoots and roots increased by 32.5 and 67.6 %, the peroxidase activity by 49.8 and 67.6 %, the ascorbate peroxidase activity by 134 and 102 %, the MDA content decreased by 30.4 and 21.8 %, and the $O_2^{\cdot-}$ generation rate by 29.0 and 26.1 %, respectively. At the same time, Cd content in the shoots and roots increased significantly by 89.7 and 30.2 %, respectively. Overall, the results suggest that exogenous NO could enhance Cd tolerance of ryegrass, but addition of EDTA could promote plant Cd uptake. Combined application of NO and EDTA increased Cd accumulation in the aboveground parts of ryegrass. In this experiment, the treatment of 100 μ M $CdCl_2$ + 0.25 mM EDTA + 50 μ M SNP showed the best effects in promoting Cd accumulation in ryegrass and enhancing its Cd tolerance.

Additional key words: ascorbate peroxidase, catalase, malondialdehyde, peroxidase, phytoremediation, sodium nitroprusside, superoxide dismutase.

Introduction

With the development of industry and the rapid expansion of cities, soil heavy metal contamination has become increasingly serious. The cadmium (Cd) is listed as a priority pollutant due to its high content in soil and great threat to human health (Chen *et al.* 2015, Moynihan *et al.* 2017). When Cd is absorbed and accumulated by perennial ryegrass, its growth and development could be affected (Bai *et al.* 2015a).

Phytoremediation is a simple, effective, and environmentally friendly method for soil pollution remediation. However, hyperaccumulators generally have disadvantages of small biomass and long growth cycle (Mahar *et al.* 2016, Sarwar *et al.* 2017). *Lolium perenne* L. is a grass ubiquitous in temperate regions. It has a strong adaptability, high growth rate, high yield,

and large absorption capacity for Cd (Yu *et al.* 2005, Hu *et al.* 2013, Bai *et al.* 2015a). By repeatedly pruning and harvesting the ryegrass, soil Cd content could be gradually decreased. Therefore ryegrass can be used to remediate Cd-contaminated soil (Arienzo *et al.* 2004, Lou *et al.* 2013). However, due to the low bioavailability of Cd and low plant biomass, extraction efficiency is rather low (Mahar *et al.* 2016, Sarwar *et al.* 2017). Therefore, various regulatory substances have also been used to stimulate plant growth (Hadi *et al.* 2010, Sharma *et al.* 2019).

Nitric oxide (NO) is a gas signaling molecule ubiquitous in plants. It plays a crucial role in plant resistance to stresses (Sahay and Gupta 2017). It has been reported that NO participates in the regulation of plant response to Cd stress and alleviates Cd toxicity to ryegrass by regulating reactive oxygen species (ROS) accumulation and activity of antioxidants (Wang *et al.* 2013, 2016). Involving Ca^{2+} ,

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Abbreviations: APX - ascorbate peroxidase; CAT - catalase; EDTA - ethylenediaminetetraacetic acid; HO^{\cdot} - hydroxyl radical; MDA - malondialdehyde; $O_2^{\cdot-}$ - superoxide anion radical; POD - peroxidase; ROS - reactive oxygen species; SNP - sodium nitroprusside; SOD - superoxide dismutase.

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cGMP, salicylic acid, and protein kinases, NO signaling can cross-talk with H_2O_2 signaling in plants (Crawford and Guo 2005). Sharma *et al.* (2020) showed that NO can help plants recover from metal toxicity *via* regulating the antioxidant defense system and cell signaling system.

However, there are studies showing that sodium nitroprusside (SNP, an exogenous NO donor) application inhibited the absorption and transport of Cd (Dong *et al.* 2019b). Usman *et al.* (2009) showed that ethylenediaminetetraacetic acid (EDTA), a synthetic chelating agent, can increase heavy metal availability in soil, accelerate heavy metal transport to the aboveground parts of plants, and improve phytoremediation efficiency. Han *et al.* (2018) demonstrated that EDTA application increased Pb and Cd accumulation in both the aboveground and underground parts of *Iris halophila*. In addition, Lambrechts *et al.* (2011) and He *et al.* (2013) showed that EDTA application improved the accumulation of Pb, Cd, and Zn in ryegrass. So far, studies have been conducted on the improvement of phytoremediation efficiency by combined application of plant growth regulators and heavy metal chelators, but few studies have been carried out on combined application of NO and EDTA. Based on the above results, we speculated that adding NO and EDTA could increase Cd accumulation in ryegrass and alleviate Cd stress at the same time.

Therefore, the objectives of this research were to investigate the effects of exogenous NO and EDTA on the physiological characteristics and Cd accumulation of ryegrass under Cd stress and to understand the regulation mechanism of NO and EDTA in ryegrass.

Materials and methods

The experiment was carried out in the plant nutrition laboratory of the College of Resources and Environment, Shandong Agricultural University, China in March 2018. Ryegrass seeds of uniform size were disinfected with 5 % (m/v) NaClO solution, washed with distilled water, and then sprouted on moist filter paper in the dark at 25 °C for 3 d. Seedlings were transferred to plastic seed trays filled with matrix and watered with a half-strength Hoagland nutrient solution for one week and full-strength solution for another two weeks.

Uniform seedlings were chosen and transferred into black glass containers (20 seedlings per container). Eight treatments (Table 1 Suppl.) were set up with three replicates. The containers were arranged in a randomized block design. The experiment was implemented in a controlled environment chamber with a 14-h photoperiod, an irradiance of $400 \mu\text{mol m}^{-2} \text{s}^{-1}$, day/night temperatures of 25/18 °C, and a relative humidity of 65 ± 5 %. Nutrient solution was renewed every two days. The ryegrass plants were harvested after two weeks under the treatments.

Plant fresh masses, plant height, root length, and root activity were determined immediately after harvest, and then plants were dried firstly at 105 °C and then at 70 °C to constant mass to determine shoot and root dry masses. Root activity was assayed by the triphenyltetrazolium

chloride (TTC) method as described by Zhang and Di (2003).

Chlorophyll content was determined according to Knudson *et al.* (1977). Fresh ryegrass leaves (0.5 g) were extracted with 2 cm³ of 95 % (v/v) ethanol in the dark, and absorbance of the extract was determined with a spectrophotometer (Shimadzu AA-6300, Kyoto, Japan) at 665, 649, and 470 nm.

Fresh samples (0.2 g) were ground in 3 cm³ of trichloroacetic acid (0.1 %, m/v). The homogenate was centrifuged at 10 000 g for 10 min and 1 cm³ of the supernatant was mixed with 4 cm³ of 0.5 % (m/v) thiobarbituric acid (TBA). The mixture was heated at 95 °C for 30 min, chilled on ice, and then centrifuged at 10 000 g for 5 min. The absorbance of the supernatant was measured at 532 nm. Non-specific absorption at 600 nm was determined and subtracted. The content of malondialdehyde (MDA) was calculated using the coefficient of absorbance of $155 \text{ mM}^{-1} \text{ cm}^{-1}$.

For determination of electrolyte leaching, fresh samples (0.3 g) were put into a 20-cm³ test tube with 10 cm³ of deionized water, sealed with clean plastic wrap, let stand at room temperature for 30 min, and the initial conductance (S1) was determined using a Leici DDS-307A (Shanghai, China) conductance meter. After that, the tube was heated in water-bath at 100 °C for 20 min, cooled, and the final conductance (S2) was determined. Electrolyte leakage [%] was calculated as $S1/S2 \times 100$.

Fresh samples (1.0 g) were homogenized with 2 cm³ of ice-cold acetone and centrifuged at 10 000 g for 20 min. Titanium reagent (2 % TiCl_2 in conc. HCl) was added to a known volume of the supernatant to give a Ti (IV) concentration of 2 % (m/v). The $\text{Ti-H}_2\text{O}_2$ complex, together with unreacted Ti, was then precipitated by adding 0.2 cm³ of 17 M ammonia solution for each 1 cm³ of the supernatant. The precipitate was washed five times with ice acetone and then dissolved with 1 M H_2SO_4 (3 cm³). The absorbance of the solution was measured at 410 nm against blanks, which had been similarly prepared but without plant tissue (Patterson *et al.* 1984).

Generation rate of O_2^- was measured as described by He *et al.* (2005). Fresh samples (0.3 g) were ground in liquid N_2 and homogenized with 3 cm³ of ice-cold 50 mM sodium phosphate buffer (pH 7.0). After centrifugation at 10 000 g for 20 min, 1 cm³ of the supernatant was added to a mixture of 0.9 cm³ of 65 mM phosphate buffer solution (pH 7.8) and 0.1 cm³ of 10 mM hydroxyl ammonium chloride and incubated at 25 °C for 35 min. The reaction mixture (0.5 cm³) was then added to a mixture of 0.5 cm³ of 17 mM sulfanic acid and 0.5 cm³ of 7.8 mM α -naphthylamine solution. After 20 min of reaction, 2 cm³ of ether was added and mixed thoroughly. The solution was then centrifuged at 1 200 g and 4 °C for 5 min, and absorbance of the supernatant was measured at 530 nm with a spectrophotometer. Absorbance values were calibrated to a standard curve generated with known concentrations of HNO_2 .

For extraction of antioxidative enzymes, leaves and roots were homogenized with 50 mM Na_2HPO_4 - NaH_2PO_4 buffer (pH 7.8) containing 0.2 mM EDTA and 2 % (m/v)

insoluble polyvinylpyrrolidone with chilled pestle and mortar. The homogenate was centrifuged at 10 000 g for 20 min and the supernatant was used for determination of enzyme activities. The whole extraction procedure was carried out at 4 °C.

Superoxide dismutase (SOD) activity was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium following the method of Stewart and Bewley (1980). Catalase (CAT) activity was measured as the decline in absorbance at 240 nm due to the decrease in H₂O₂ concentration according to the method of Patra *et al.* (1978). Peroxidase (POD) activity was measured as the increase in absorbance at 470 nm due to guaiacol oxidation (Nickel and Cunningham 1969). Ascorbate peroxidase (APX) activity was measured as the decrease in absorbance at 290 nm due to the oxidation of ascorbate (Nakano and Asada 1981). The amount of an enzyme that can convert 1 µmole of substrate in 1 min is 1 unit (U).

Proline content was measured using the method of Bates *et al.* (1973). Fresh samples (0.2 g) were homogenized in 5 cm³ of 3 % (m/v) sulphosalicylic acid solution. After extraction at room temperature, proline concentration was determined at 520 nm using a standard curve.

Soluble protein content was estimated using the method of Bradford (1976) using bovine serum albumin as a standard.

Dried samples were ground into powder and digested with HNO₃/HClO₄ (3:1, v/v). Cadmium content was measured by flame atomic absorbance spectrometry (Ali *et al.* 2002).

Statistical analyses were performed using analysis of variance (ANOVA) of SPSS statistics (v. 19.0). Treatment means were separated using the least significant differences (Duncan test) at the 0.05 probability level.

Results

Exposure to Cd significantly inhibited the growth of ryegrass (Table 2 Suppl.). Compared with those in control conditions (CK), the shoot height and root length of ryegrass in the Cd treatment (100 µM CdCl₂) decreased by 28.34 and 27.46 %, respectively, and the fresh and dry masses decreased by 37.21 and 38.05 %, respectively. The addition of 100 µM sodium nitropruside (SNP) alleviated Cd stress on ryegrass and the growth inhibition of ryegrass by Cd was further alleviated by the combined application of 50 µM SNP and 250 µM EDTA. Compared with those in the Cd treatment alone, plant height and root length in the Cd+EDTA+SNP treatment increased by 68.83 and 59.61 %, respectively, and the fresh and dry masses increased by 62.63 and 60.00 %, respectively.

Root activity reflects plant root vitality. Compared with control, the Cd treatment significantly reduced root activity of ryegrass. Root activity of ryegrass in the Cd+SNP and Cd+EDTA treatment was 34.36 and 70.97 %, respectively, higher than that in the Cd treatment and root activity in the Cd+EDTA+SNP treatment was significantly higher (by 106.8 %) than that in the Cd treatment (Table 2 Suppl.). The results showed that both NO and EDTA could

increase the root activity of ryegrass and promote ryegrass growth under Cd stress, and combined application of NO and EDTA showed better effects.

Cadmium stress significantly decreased the content of photosynthetic pigments in the ryegrass (Table 2 Suppl.). Compared with CK, the content of chlorophylls and carotenoids in the Cd treatment were reduced by 29.09 and 22.73 %, respectively. The addition of SNP significantly increased the content of photosynthetic pigments in the ryegrass under Cd stress. Compared with the Cd treatment, the Cd+EDTA+SNP treatment increased the contents of chlorophylls and carotenoids by 70.09 and 82.35 %, respectively. Therefore, combined application of NO and EDTA could alleviate Cd stress and improve Cd tolerance of ryegrass.

Compared with those in CK, MDA content in ryegrass leaves and roots in the Cd treatment increased significantly (by 103.5 and 42.5 %, respectively) indicating membrane lipid peroxidation in ryegrass under Cd stress (Fig. 1A). The addition of SNP or EDTA reduced MDA accumulation, and the combined application of SNP and EDTA was more effective. Compared with those in the Cd treatment, MDA content in the leaves and roots in the Cd+SNP+EDTA treatment decreased by 30.44 and 21.80 %, respectively.

Electrolyte leakage from ryegrass increased significantly under Cd stress (Fig. 1B). Compared with that in CK, the electrolyte leakage in the Cd treated plants increased by 61.71 %. The addition of SNP and/or EDTA reduced electrolyte leakage from ryegrass under Cd stress, and combined application of SNP and EDTA showed better effect. Compared with that in the Cd treatment, the electrolyte leakage in the Cd+SNP+EDTA treatment decreased by 31.26 %.

The O₂⁻ generation rate was significantly higher in the Cd and Cd+EDTA treatments than in the other treatments, and the H₂O₂ content was significantly higher in the Cd treatment than in the other treatments (Fig. 1C,D). Compared with those in CK, the O₂⁻ generation rates in the leaves and roots of ryegrass in the Cd treatment were increased by 48.07 and 63.95 %, respectively, and the H₂O₂ content in the leaves and roots was increased by 78.61 and 103.0 %, respectively. Exogenous SNP or EDTA decreased the O₂⁻ generation rate and H₂O₂ content in ryegrass under Cd stress, and combined application of SNP and EDTA showed better effects. Compared with the Cd treatment, the O₂⁻ generation rates in the leaves and roots in the Cd+SNP+EDTA treatment decreased by 28.99 and 26.11 %, respectively, and the H₂O₂ content by 42.48 and 40.24 %, respectively.

Activity of SOD in ryegrass was significantly decreased under Cd stress (Fig. 2A). Compared with that in CK, SOD activity in the leaves and roots of ryegrass in the Cd treatment was reduced by 26.46 and 40.34 %, respectively. The addition of SNP and/or EDTA helped to recover SOD activity in ryegrass under Cd stress. Compared with that in the Cd treatment, the SOD activity in the shoots and roots of ryegrass in the Cd+SNP+EDTA treatment was increased by 32.53 and 67.61 %, respectively.

The patterns of POD and APX activities were basically similar to that of SOD activity (Fig. 2B,D). Compared with

CK, the Cd treatment significantly reduced POD activity in the leaves and roots by 42.27 and 17.03 % and APX activity by 62.90 and 52.63 %, respectively. The POD and APX activities were also increased in the Cd+SNP, Cd+EDTA, and Cd+EDTA+SNP treatments. Compared with that in the Cd treatment, POD activity in the leaves and roots of ryegrass in the Cd+SNP+EDTA treatment increased by 49.80 and 67.61 % and APX activity increased by 134.1 and 101.6 %, respectively. Compared with that in CK, CAT activity in ryegrass leaves in the Cd treatment increased slightly but not significantly (Fig. 2C), while that in the roots decreased by 42.10 %. Compared with that in the Cd treatment, the CAT activity in ryegrass roots in the Cd+SNP+EDTA treatment increased by 45.45 %.

Proline accumulation can enhance plant resistance to stress. As shown in Fig. 2E, proline content in ryegrass leaves and roots increased significantly in the

Cd treatment. Compared with that in CK, proline content in ryegrass leaves and roots in the Cd treatment increased by 74.95 and 115.1 %, respectively. Addition of EDTA alone reduced proline content in ryegrass under Cd stress, but addition of SNP alone or in combination with EDTA slightly increased proline content. Compared with that in the Cd treatment, proline content in ryegrass leaves and roots in the Cd+SNP+EDTA treatment increased by 8.52 and 7.43 %, respectively.

Soluble protein content decreased significantly in ryegrass under Cd stress (Fig. 2F). Compared with CK, the Cd treatment reduced the soluble protein content of ryegrass leaves and roots by 47.88 and 51.76 %, respectively. Addition of SNP or EDTA alone increased soluble protein content, and combined application of SNP and EDTA had better effect. Compared with that in the Cd treatment, soluble protein content in ryegrass leaves and

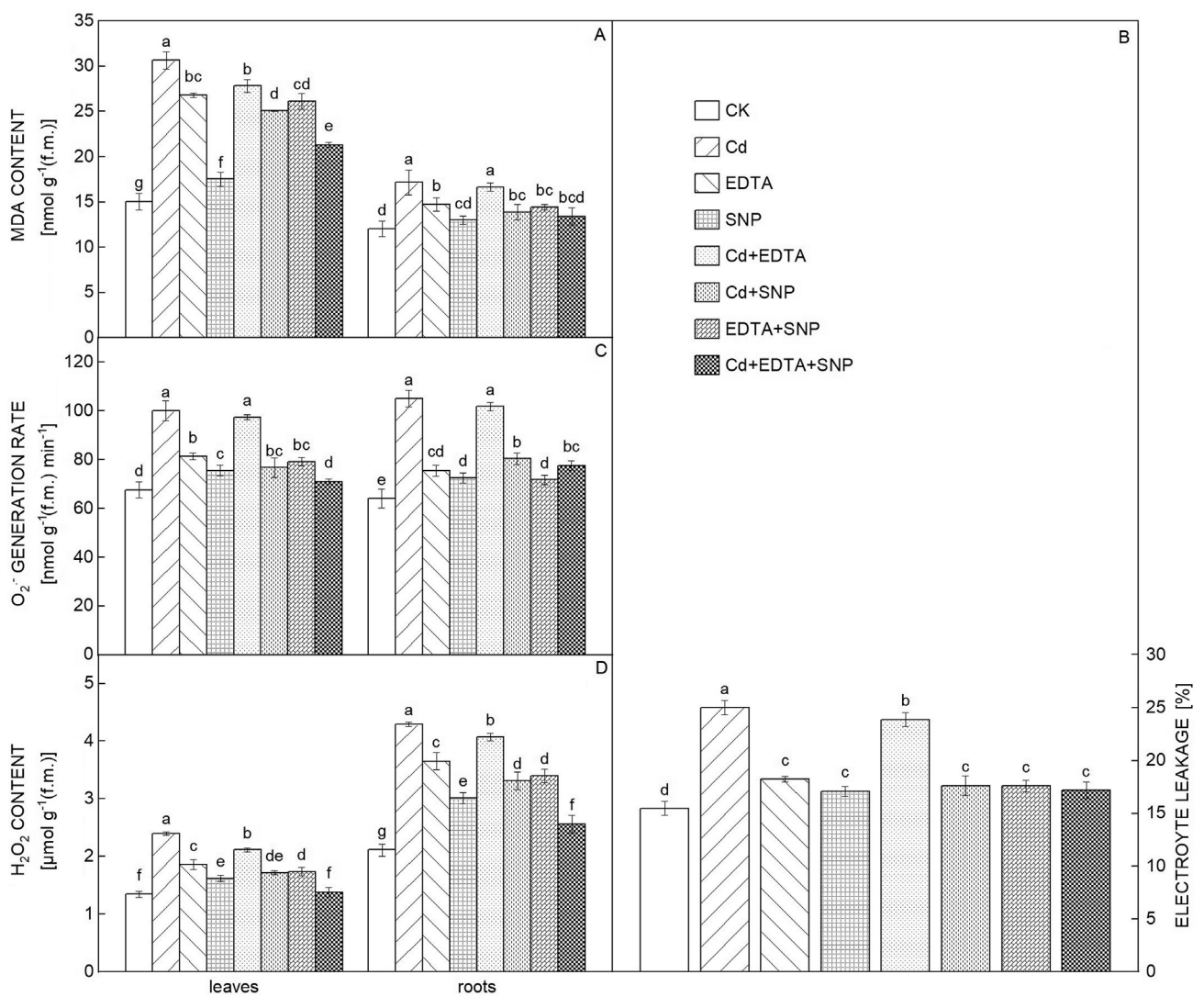


Fig. 1. Malondialdehyde (MDA) content (A), electrolyte leakage (B), O₂⁻ generation rate (C), and H₂O₂ content (D) in ryegrass under different treatments for two weeks. CK - control grown in Hoagland solution, Cd - Hoagland solution with addition of 100 μM CdCl₂, EDTA - 0.5 mM EDTA, SNP - 100 μM sodium nitroprusside (SNP), Cd+EDTA - 100 μM CdCl₂ + 0.5 mM EDTA, Cd+SNP - 100 μM CdCl₂ + 100 μM SNP, EDTA+SNP - 0.25 mM EDTA + 50 μM SNP, Cd+EDTA+SNP - 100 μM CdCl₂ + 0.25 mM EDTA + 50 μM SNP. Means ± SDs, n = 3; different letters in the same column indicate significant differences between treatments at P ≤ 0.05.

roots in the Cd+SNP+EDTA treatment increased by 89.85 and 89.12 %, respectively.

Addition of SNP alone increased Cd content in the roots but decreased Cd content in the leaves of ryegrass treated with Cd (Fig. 3). Addition of EDTA alone significantly increased Cd content in both the leaves and roots of ryegrass. Compared with the Cd treatment, the Cd+SNP+EDTA treatment significantly increased the Cd content in leaves and roots of Cd-treated ryegrass by 89.67 and 30.16 %, respectively, indicating that combined application of SNP and EDTA could significantly improve Cd absorption and accumulation in ryegrass.

Discussion

Cadmium stress can decrease root activity and biomass, cause leaf chlorosis, inhibit plant growth, and even cause plant death in severe cases (Luo *et al.* 2016, Xu *et al.* 2015). In this study, exposure to 100 $\mu\text{mol CdCl}_2$ significantly inhibited the growth of ryegrass (Table 2 Suppl.).

Studies showed that Cd stress increased the activity of chlorophyllase, an enzyme catalyzing chlorophyll hydrolysis (Kaur *et al.* 2017), which explains the low chlorophyll content in the Cd treated plants. When ryegrass was under Cd stress, it seemed that addition of EDTA alone was not able to alleviate Cd stress but even slightly exacerbated plant growth inhibition. The high concentration (0.5 mM) of EDTA itself might exert a stress on plants. It has been reported that low concentrations of SNP might attenuate Pb toxicity in ryegrass exposed to Pb stress (Bai *et al.* 2015b). Addition of SNP effectively alleviated Cd stress on ryegrass, which is consistent with the results of Wang *et al.* (2013) that NO effectively alleviated the Cd-induced suppression of ryegrass growth and chlorophyll synthesis and the findings of Zhuo *et al.* (2017) that NO improved the overall performance of the photo-chemical activities in tall fescue photosystem II under Cd^{2+} stress. Combined application of SNP and EDTA showed an even better effect than SNP alone with improved root activity and increased photosynthetic pigment content, which might be attributed to the chelating ability of EDTA. Studies have

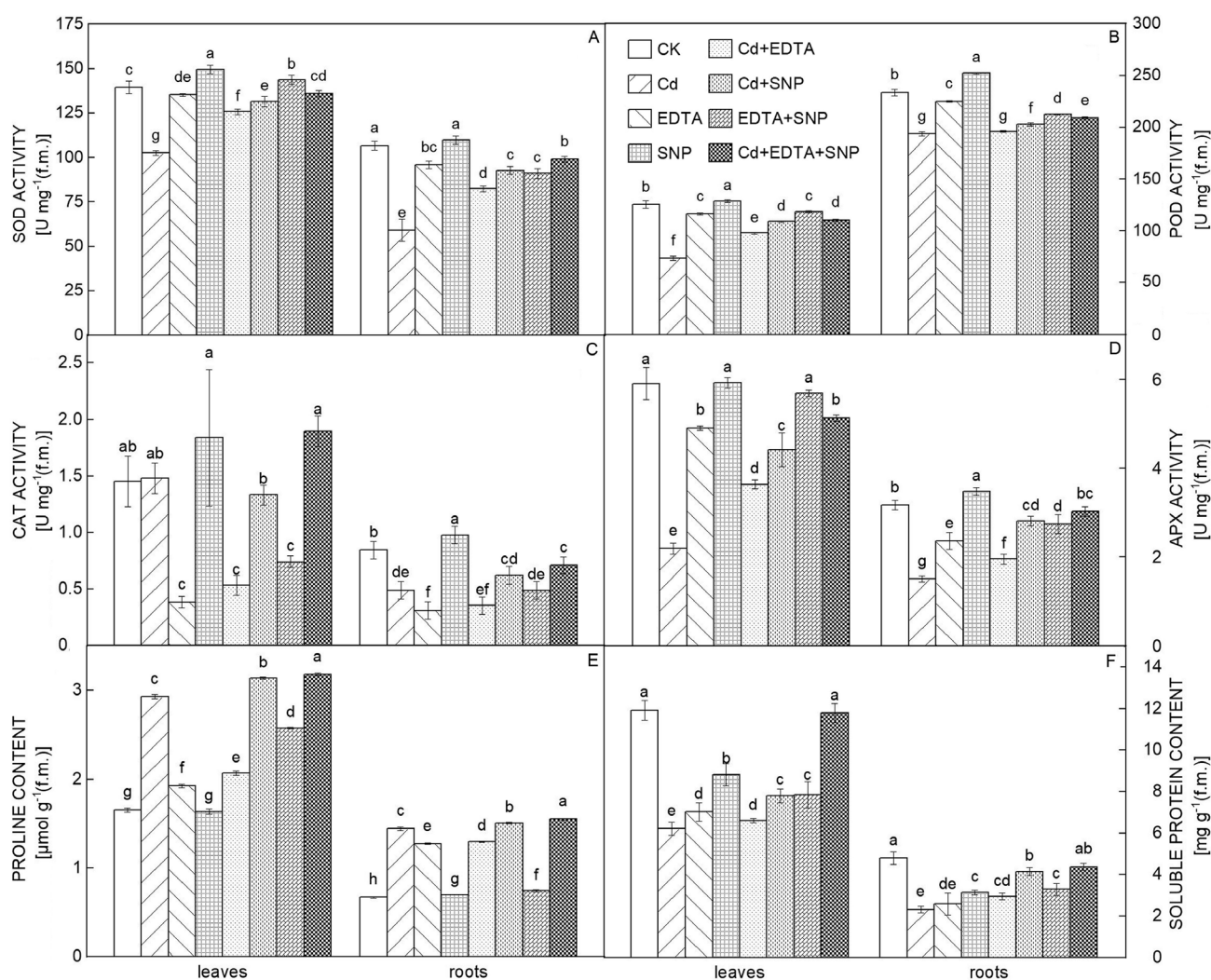


Fig. 2. Superoxide dismutase (SOD, A), peroxidase (POD, B), catalase (CAT, C), and ascorbate peroxidase (APX, D) activities, proline content (E), and soluble protein content (F) in ryegrass leaves and roots under different treatments (for detail see Fig. 1). SNP – sodium nitroprusside. Means \pm SDs, $n = 3$; different letters in the same column indicate significant differences between treatments at $P \leq 0.05$.

shown that metals enter plants in the form of metal-EDTA complexes when EDTA is applied, which is due to the selective absorption of low-potential complexes by plant roots (Bell *et al.* 2003). Schaidler *et al.* (2006) showed that Pb-EDTA, Cd-EDTA, and Fe-EDTA complexes are present in the xylem of *Brassica juncea*. Using high performance liquid chromatography (HPLC), Vassil *et al.* (1998) demonstrated that Pb was transported to the shoots of Indian mustard as Pb-EDTA complex. Therefore, in the Cd+EDTA+SNP treatment, that Cd stress on ryegrass might be alleviated partly due to EDTA's complex with Cd, which reduced Cd toxicity to ryegrass.

Malondialdehyde is the terminal product of lipid peroxidation in plants. It is known that Cd toxicity would increase plant cell membrane permeability, and addition of exogenous NO could reduce membrane lipid peroxidation by reducing $O_2^{\cdot-}$ and H_2O_2 accumulation (Tan *et al.* 2008, Sharma *et al.* 2020). In the Cd treatment, MDA content and electrolyte leakage increased (Fig. 1), indicating that membrane lipid peroxidation occurred, physiological and biochemical functions of ryegrass were impaired, and normal growth of ryegrass was inhibited. However, the addition of SNP alone or the combined application of SNP and EDTA significantly decreased the MDA content and electrolyte leakage in ryegrass under Cd stress. Studies have shown that exogenous NO inhibits the increase in MDA content and electrolyte leakage by reducing lipoxygenase activity (Venkatachalam 2018). Fan *et al.* (2015) also showed that addition of NO significantly lowered MDA content in bermuda grass, which is consistent with the results of this study. In this study, combined application of SNP and EDTA was more effective in alleviating Cd stress on ryegrass than application of SNP or EDTA alone. The reason might be that EDTA and Cd^{2+} formed a relatively stable complex. Adhesion of Cd on cell walls was reduced, more Cd accumulated in mesophyll cell vacuoles and intercellular spaces, and toxic Cd ions were kept from the protoplasm. Therefore, the combined application of SNP and EDTA could reduce membrane lipid peroxidation caused by Cd stress.

The $O_2^{\cdot-}$ and H_2O_2 accumulate in plants under

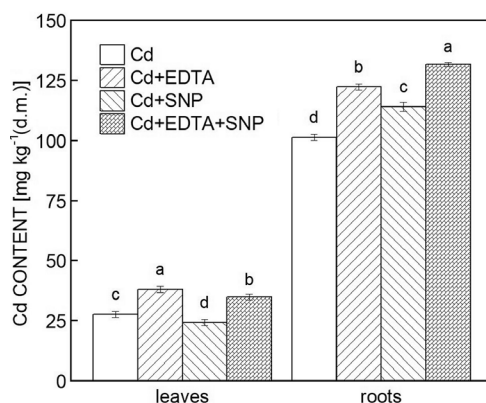


Fig. 3. Cadmium content in ryegrass shoots and roots under Cd stress alone or together with SNP and EDTA (for detail see Fig. 1). SNP – sodium nitroprusside. Means \pm SDs, $n = 3$; different letters in the same column indicate significant differences between treatments at $P \leq 0.05$.

Cd stress, and high Cd content can inhibit the activities of antioxidant enzymes in plants. Accumulation of ROS could inhibit the photosynthesis of plants and damage cell membrane structure (Idrees *et al.* 2012). In this experiment, 100 μM Cd significantly increased the $O_2^{\cdot-}$ generation rate and H_2O_2 content (Fig. 1) and meanwhile inhibited the SOD, POD, and APX activities in ryegrass (Fig. 2), which was consistent with previous results (Wang *et al.* 2013). However, this inhibition was alleviated by SNP or/and EDTA addition, and the combined application of SNP and EDTA was most effective. Kapoor *et al.* (2019) showed that an activated antioxidant system could endow plants with better Cd tolerance. In this study, the better Cd tolerance of ryegrass might be explained especially by the increase in CAT activity. In previous experiments we showed that addition of NO could effectively improve the activities of antioxidant enzymes in peanut under Cd stress (Dong *et al.* 2019a). Exogenous EDTA could form chelates with Cd^{2+} to stop it from replacing the metal ions in the catalytic centers of enzymes in plants, thus improving the activities of antioxidant enzymes under Cd stress. Therefore, addition of SNP or/and EDTA could improve the activities of antioxidant enzymes in ryegrass to remove the excessive ROS and repair damaged cells, and in turn alleviate oxidative stress and improve Cd tolerance of ryegrass.

Soluble protein and proline are osmotic regulators in plants. Besides, proline can remove ROS, improve antioxidant capacity, and stabilize the structure of biological macromolecules (Filippou *et al.* 2013). Proline content increased but soluble protein content decreased in ryegrass under Cd stress (Fig. 2). Combined addition of SNP and EDTA further increased the free proline content and alleviated the inhibitory effect of Cd stress on soluble protein content. Related studies have demonstrated that NO could promote the synthesis and accumulation of proline in plants by increasing Δ^1 -pyrrole-5-carboxylic acid synthetase activity and decreasing proline dehydrogenase activity (Su *et al.* 2018). Therefore, NO and EDTA might improve the osmotic adjustment in ryegrass under Cd stress.

Cadmium absorbed by plants might be transported through the intercellular spaces *via* the apoplastic pathway, or through the protoplast channels *via* the symplastic pathway (White *et al.* 2002). However, studies found that Cd stress could accelerate cell maturation and promote the formation of Casparian strip in the roots, thus making it difficult for Cd to move to the aboveground parts through the extracellular pathway (Krishnamurthy *et al.* 2009). Qiu *et al.* (2012) believe that Cd is mainly transported in plants through the energy-consuming symplastic pathway, and the apoplast pathway only accounts for a small portion. However, the compartmentalization of Cd in vacuoles in the root cells tends to retain the Cd in the roots (Lasat *et al.* 1998), so Cd accumulation in the aboveground parts is generally low. The results showed that Cd content in ryegrass leaves was significantly increased when EDTA was added alone or in combination with SNP (Fig. 3). As Cd and Fe share the transporter IRON-REGULATED TRANSPORTER 1, Cd uptake would be stimulated

when Fe-DEFICIENCY INDUCED TRANSCRIPTION FACTOR, FERRIC REDUCTION OXIDASE 2, and IRON-REGULATED TRANSPORTER 1 trigger iron starvation effects through NO-mediated upregulation (Sharma *et al.* 2020). In addition, studies have suggested that EDTA can chelate heavy metals to increase the concentrations of heavy metals in soil solution and reduce the adsorption of Cd²⁺ on root surface. Therefore, Cd enters plant roots directly (Luo *et al.* 2005). Moreover, metal chelates destroy the Casparian strips in the endodermis of plant roots and enter root tissue, which improves their movement to the aboveground parts.

Conclusions

Combined addition of NO and EDTA alleviated lipid peroxidation and reduction of photosynthetic pigments and improved the osmotic adjustment and antioxidative activity of ryegrass under Cd stress, thus alleviating the inhibition of ryegrass growth. At the same time, the addition of EDTA promoted Cd absorption by ryegrass and significantly increased Cd accumulation in the aboveground parts. Of all the treatments used in this experiment, the Cd+EDTA+SNP treatment was the most effective not only in improving Cd accumulation in ryegrass, but also in enhancing Cd resistance of ryegrass.

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