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Reveal the response of enzyme activities to heavy metals through *in situ* zymography

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ABSTRACT

Enzymes in the soil are vital for assessing heavy metal soil pollution. Although the presence of heavy metals is thought to change the soil enzyme system, the distribution of enzyme activities in heavy metal polluted-soil is still unknown. For the first time, using soil zymography, we analyzed the distribution of enzyme activities of alfalfa rhizosphere and soil surface in the metal-contaminated soil. The results showed that the growth of alfalfa was significantly inhibited, and an impact that was most pronounced in seedling biomass and chlorophyll content. Catalase activity (CAT) in alfalfa decreased with increasing heavy metal concentrations, while malondialdehyde (MDA) content continually increased. The distribution of enzyme activities showed that both phosphatase and β-glucosidase activities were associated with the roots and were rarely distributed throughout the soil. In addition, the total hotspot areas of enzyme activities were the highest in extremely heavy pollution soil. The hotspot areas of phosphatase were 3.4%, 1.5% and 7.1% under none, moderate and extremely heavy pollution treatment, respectively, but increased from 0.1% to 0.9% for β-glucosidase with the increasing pollution levels. Compared with the traditional method of enzyme activities, zymography can directly and accurately reflect the distribution and extent of enzyme activity in heavy metals polluted soil. The results provide an efficient research method for exploring the interaction between enzyme activities and plant rhizosphere.

1. Introduction

The rapidly growing population, industrial progress and technical innovations have increased the concentration of heavy metals around the globe (Ali et al., 2017). Heavy metals can be hazardous to soil, plant and human health through the soil-crop-food chain (Shen et al., 2017). Heavy metals usually affect the growth and morphology of plants, as well as microbial metabolism that disrupts the biochemical reactions of the soil (Fang et al., 2017; Hassan et al., 2013). Many metabolic processes require the involvements of soil enzymes and are highly sensitive to the change of enzyme activities. Recently, the soil enzyme activities have been used as a biological indicator to monitor soil quality and environmental health (Hu et al., 2014; Yang et al., 2016). Hence, there is an urgent need to develop a rapid and reliable method to determine soil enzyme activities.

To date, soil enzyme activities have been widely used as an indicator to measure the ecological health of terrestrial ecosystems under heavy metal contamination (Lee et al., 2009; Liang et al., 2014). A previous study indicated that after soil was contaminated by heavy metals, the soil catalase and urease reaction becomes sensitive, reflecting the toxic effects of heavy metals on soil microbial activity (Marzadori et al., 1996). Catalase can break down hydrogen peroxide and prevent organisms from poisoning, and it has been used as a bioindicator for cadmium, chromium, copper, mercury, lead and zinc pollution (Liang et al., 2014; Xian et al., 2015; Yang et al., 2016). Phosphatase plays an important role in the transformation of organophosphorus compounds and has been used as a biological indicator to assess heavy metal pollution (Fang et al. 2017), Lee et al. (2009) and Hu et al. (2014) proposed the dehydrogenase as the catalyst for dehydrogenation of substrates, and suggested it as another indicator of heavy metal pollution. However, the response of enzymes activities to heavy metals was nonuniform, and the selection of enzymes varied between different studies. Additionally, heavy metals enrichment not only affect soil enzymatic activities, but also limit antioxidant enzymatic activities and even the lipid peroxidation in plant cells. With attempts to truly indicate the heavy metal pollution in soil, it is more feasible to determine the activities of enzyme community in both soil and plant *in situ*.

In plants, metal ions can be easily taken up by roots in competition with each other, and then translocated into other organs (Ajm et al.,

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2010). Various symptoms of drug damage are caused by heavy metals, including growth inhibition, chlorosis of leaves, insufficient nutrition and antioxidant enzymatic activities limitation, and even lipid peroxidation in plant cells (Abbas et al., 2017; Liu et al., 2015). To minimize the damaging effects of reactive oxygen species (ROS), aerobic organisms evolved non-enzymatic defense systems and enzymatic protection mechanisms (Kováčik et al., 2012; Liu et al., 2015). The enzyme activities of the roots and rhizosphere are strongly affected by heavy metals stress and root exudates. Therefore, the enzyme activities of rhizosphere are often considered as a crucial indicator for evaluating heavy metal pollution.

The enzyme activity in the rhizosphere reflects the interaction between plant and microorganism, and is a sensitive index for monitoring microbial community composition, activity and function changes (Liu et al., 2017; Razavi et al., 2016). However, the spatial structure of the rhizosphere is characterized by a large, complex, and heterogeneous root-soil interface. Most soil enzymes are extracellular and present either in immobilized or free form (Ge et al., 2017; Rao et al., 2000). Due to complex microbial community structures and diversity, the evaluation of enzyme activities in the rhizosphere needs to consider spatial variability from root to outward and radial (Razavi et al., 2016). The best mean is determining the spatial distribution of rhizosphere enzymes in undisturbed samples. Zymography, a non-destructive *in situ* technique for two-dimensional imaging, now offers an opportunity to visualize enzyme activities-spatial in soil and in the rhizosphere (Razavi et al., 2016; Spohn and Kuzyakov, 2013, 2014). With the application of *in situ* zymography, Razavi et al. (2016) have proved that spatial patterns of enzyme activities vary along the root, and those patterns depend on the plant species. Ge et al. (2017) also employed *in situ* zymography to evaluate the effects of temperature on the dynamics and localization of enzymatic hotspots in the rhizosphere. The direct soil zymography enables the mapping of enzyme activity at the soil surface, in the rhizosphere and the detritusphere. Nonetheless, the application of zymography to enzyme activities in heavy metal contaminated soil is still limited, particularly for the study of spatial distribution of enzyme activities, which are important for the understanding and clarification of the complex interactions between heavy metals and enzymes.

In the present research, we hypothesized that: 1) high heavy pollution will increase enzymatic activity and hotspot area; 2) such an increase in hotspot area is enzyme dependent. For that, we studied the spatial distribution of enzymes is intimately related to the C and P cycles, the results of this study are significant for the understanding of the complex interactions between heavy metals and the distribution of enzyme activities in the rhizosphere. Furthermore, our aims are to improve the soil quality and nutrients and provide a basis for future phytoremediation.

2. Materials and methods

2.1. Sample preparation

The surface soil samples (0–20 cm) were collected in Feng County in Shaanxi Province, China (approximately 106°33' E, 33°48' N). The Feng County, located in the central fold system of Qingling, is one of the largest Zn/Pb production areas. According to the current Chinese system of soil classification, the soil was yellow-brown soil. The soil samples were stored in clean Ziploc plastic bags and immediately transferred to the laboratory. The soil samples were air-dried at room temperature, crushed manually and then passed through a 2-mm sieve. Alfalfa (*Medicago sativa*) seeds were purchased from Beijing Rytway Ecotechnology Co., Ltd. Seeds were surface sterilized with 0.1% H_2O_2 for 5 min, and then rinsed with distilled water for 10–20 times. Properties of the sampling plots are presented in Table 1.

We grew sixteen alfalfa plants (*Medicago sativa*), and each plant grew in a separate rhizobox (18 × 12.5 × 5.2 cm). The rhizoboxes were placed horizontally with one side open, and then slowly injected into the soil. Soil layering was avoided during the loading process. The seeds were germinated on filter paper for 3 days, and then one seedling was planted in each rhizobox at a depth of 5 mm. During 8 weeks of growth, the rhizoboxes remained inclined at 50°, making the root growth in the lower rhizoboxes wall (Ge et al., 2017; Razavi et al., 2016). The samples were kept in a climate-controlled chamber (*i.e.*, temperature = 25 ± 1 °C, daily light period = 16 h, and photosynthetically active radiation intensity = 300 μmol m^{-2} s^{-1}), which was regulated by an automatic temperature control system. In the growth period, the water content of the soil was kept at 60% with the distilled water.

2.2. Soil analysis

The soil moisture was determined gravimetrically in fresh soils at 105 °C overnight. The soil pH of air-dried samples (sieved to 1 mm) was determined using a glass electrode meter (Startorius PB10) in a suspension of 1:5 soil/water ratio (w/v). The soil organic matter (SOM) was determined by a titration method based on the oxidation of organic substances with potassium dichromate (Kalembasa and Jenkinson, 1973). The total N (TN) was measured using the Kjeldahl method (Page et al., 1982). The total phosphorus (TP) was measured by an ultraviolet spectrophotometer (UV3200, Shimadu Corporation, Japan) after wet digestion with H_2SO_4 and $HClO_4$. The soil samples were digested for measuring total heavy metal concentrations. The digestion procedure was based on a modified USEPA Method 3051 A (Element, 2007). Specifically, a 0.200 g soil sample was digested by 15 ml of tri-acidic mixture (HCl, HNO_3, $HClO_4$) with a volume ratio of 1:3:1. The concentrations of Cd, Pb, Zn, and Cu in digested samples were determined using atomic absorption spectrophotometry (Hitachi, FAAS Z-2000, Japan).

For the overall level of soil pollution across the sampling sites, the pollution load index (*PLI*) was determined as Yang et al. (2016):

$$PLI = \sqrt[n]{(Cf1 \times Cf2 \times Cf3 \cdots Cfn)}$$

where *Cf* is the metal contamination factor and *n* is the number of samples analyzed in this study. The pollution can be categorized into four levels: no pollution ($PLI < 1$), moderate pollution ($1 < PLI < 2$), heavy pollution ($2 < PLI < 3$) and extremely heavy pollution ($3 < PLI$) (Liu et al., 2013; Yang et al., 2016). With the calculated *PLI* values, the soil samples in this study were classified into no pollution (N), moderate pollution (M) and extremely heavy pollution (EH).

2.3. Determination of plant index

For the physical plant index, the shoot height and root length were measured with a ruler. The chlorophyll concentration was determined after extraction with 80% (v/v) acetone by measuring the absorbance at 663 and 645 nm as described by Sobrino-Plata et al. (2014). The plant samples were dried at 65 °C for 48 h, and the dry weight was recorded.

For the metal concentrations and nutrients, the plant samples were separated into two portions. One portion was digested with a 10-ml mixture of HNO_3 and $HClO_4$ (*i.e.*, volume ratio = 4:1) for total Cd, Pb, Zn concentration quantified by atomic absorption spectrophotometry (Hitachi, FAAS Z-2000, Japan). The other portion was digested with H_2SO_4 and H_2O_2 for N, P and K concentration measured by flow analyzer. The transport ability of Cd, Pb and Zn from roots to shoots in the plant was evaluated by a transfer coefficient:

$$Transfer\ \ coefficient = H_{shoot}/H_{root}$$

where H_{shoot} and H_{root} are heavy metal concentrations in shoots and roots, respectively.

For the antioxidant enzyme activities, the fresh shoots and roots were firstly homogenized in an ice bath with 1 ml of extraction buffer (*i.e.,* 50 mM phosphate buffer solution containing 1 mM ascorbic acid and 1 mM EDTA) at 4 °C. Then the homogenate was centrifuged at

Table 1
The physicochemical properties of soil samples.

Soil	Pb (mg/kg)	Zn (mg/kg)	Cd (mg/kg)	Cu (mg/kg)	pH	TN (g/kg)	TP (g/kg)	SOM (g/kg)	*PLI*	Grade
1	374.0 ± 0.7	150.3 ± 7.4	2.66 ± 0.12	29.87 ± 0.30	8.82 ± 0.01	0.76 ± 0.01	0.40 ± 0.01	24.05 ± 0.24	0.92	N
2	678.9 ± 2.0	161.1 ± 4.0	5.14 ± 0.16	31.54 ± 1.15	8.74 ± 0.02	1.58 ± 0.01	0.30 ± 0.01	25.81 ± 0.26	1.29	M
3	2490 ± 237	453.2 ± 49.1	26.06 ± 2.47	52.58 ± 1.86	8.78 ± 0.01	1.04 ± 0.01	0.53 ± 0.01	11.76 ± 0.15	3.96	EH

Values are means ± standard (n = 3). Different letters indicate significant differences ($p < 0.05$) among the different samples. TN (total nitrogen), TP (total phosphorus), SOM (soil organic matter), N (none pollution), M (moderate pollution), EH (extremely heavy pollution), *PLI* (pollution load index).

8000*g* for 10 min at 4 °C and the supernatant was used to assay antioxidant enzyme activities (Chen et al., 2013; Liu et al., 2015). The activity of total superoxide dismutase (SOD, EC 1.15.1.1), total peroxidase (POD, EC 1.11.1.7), catalase (CAT, EC 1.11.1.6), and ascorbate peroxidase (APX, EC 1.11.1.11) was correspondingly assessed using the enzyme-specific commercial reagent kit (Suzhou Comin Biotechnology Co., Ltd. Suzhou, China). Based on the manufacturer's instructions, these activity indicators were determined by the absorbance of the supernatant after reactions at different wavelengths (*i.e.*, 560, 470, 240, and 290 nm for SOD, POD, CAT and APX, respectively) (Epoch, US). The SOD determines the ability to inhibit photochemical reduction of nitroblue tetrazolium (NBT). The amount of enzyme required to cause 50% inhibition of the reduction of NBT is defined as one unit of SOD activity. The enzyme activities of POD, CAT and APX were calculated from the initial rate of the reaction using the extinction coefficient of tetraguaiacol, H_2O_2, and ascorbate, respectively.

For the lipid peroxidation, it was evaluated by the malondialdehyde (MDA) content (Yan et al., 2010). The MDA in grounded shoot and root samples was extracted by 10% trichloroacetic acid (2 ml). After centrifugation at 8000*g* for 10 min, the MDA in the supernatant was measured using an MDA reagent kit (Suzhou Comin Biotechnology Co., Ltd. Suzhou, China) based on the absorbance at 532 and 600 nm.

2.4. Soil enzyme activity assays

Various enzyme activities were determined in the soil of the rhizosphere after harvest, including saccharase (EC 3.2.1.26), urease (EC 3.5.1.5), catalase (EC 1.11.1.6), β-glucosidase (EC 3.2.1.21), and alkaline phosphatase (EC 3.1.3.2). According to previous studies, the former two were determined based on substrate applications followed by absorption measurements using a spectrophotometer (UV-2450, SHIMADZU). The required substrate and absorbance wavelengths are sucrose and 508 nm for saccharase (Guan, 1986), urea and 587 nm for urease (Guan, 1986). The catalase activity was expressed as milliliters of 0.02 mol L^{-1} $KMnO_4$ per gram soil per 20-min, and was determined by the potassium permanganate titration method (Guan, 1986). The β-glucosidase and alkaline phosphatase were measured based on the absorption of released *p*-nitrophenol at 400 nm (Eivazi and Tabatabai, 1988) and the released phenol at 578 nm (Guan, 1986), respectively.

2.5. Soil zymography and imaging procedure

After cultivating alfalfa for 8 weeks, direct soil zymography was applied *in situ* to study the spatial distribution of enzyme activity around the roots. We followed the protocol optimized by Razavi et al. (2016). Briefly, specific 4-methylumbelliferone (MUF) substrates were firstly applied to saturate the membranes containing various enzyme species (Spohn and Kuzyakov, 2013). When enzymatically hydrolyzed by enzymes, the MUF substrates become fluorescent and the intensities of enzyme activities become visible and quantified (Dong et al., 2007; Ge et al., 2017). The 4-Methylumbelliferyl-β-D-glucoside (MUF-G) and 4-methylumbelliferyl-phosphate (MUF-P) were the substrates to detect β-glucosidase and phosphatase activity, respectively. Each of these substrates was separately dissolved into a 10 mM MES ($C_6H_{13}NO_4SNa_{.5}$) buffer solution (Sigma-Aldrich, Germany). For each enzyme, a polyamide membrane filter (*i.e.*, diameter = 20 cm and pore size = 0.45 mm) was saturated with the substrates. Then the membranes were cut and adjusted to fit the rhizobox size. Open the rhizoboxes from the low side and apply the saturated membranes directly to the surface of the soil (Dong et al., 2007; Razavi et al., 2016). After incubation for one hour, the membranes were carefully stripped from the soil surface, and the attached soil particles were gently removed with tweezers. After incubation, the membranes were placed in a light-proof room and illuminated by ultraviolet (UV) light. The distance between the UV light resource, the camera (60D, Canon) and the samples was fixed. The substrate is hydrolyzed by the enzyme and the fluorescence intensity is proportional to the activity of the enzyme under the ultraviolet light. To get more accurate information and quantify this, we used Matlab R2016b to analyze the images according to Razavi et al. (2016). In short, the zymograms were first transformed into a 16-bit gray image matrix, and then the noise and camera noise were corrected (Sanaullah et al., 2016). The images were then converted the image data to a double type using the '*Im2double*' function and drew color grid graphics using the '*Meshgrid*' function.

We used the grayvalue obtained from the blank side of the samples as the reference point. After referencing the zymograms, we calculated an average background grayvalue for the zymograms at the zero concentration point on the calibration lines and subtracted this value from all the zymograms. To quantify the zymogram images, a standard calibration that relates the activities of various enzymes to zymogram fluorescence (*i.e.*, fluorescence of the saturated membrane) is required. The calibration was based on zymography of 2.5 × 2.5 cm membranes soaked in a solution of MUF- the fluorescent tag attached to each substrate proxy e with concentrations of 0.01, 0.2, 1, 4, 6, 8, 10 mM. The amount of MUF on an area basis was calculated from the solution volume taken up by the membrane and its size. The membranes used for calibration were imaged under UV light and analyzed in the same way that the samples were. Color scales were given on the right side of the image with the enzyme activities corresponding to the color.

2.6. Statistical analysis of data

All statistical analysis was carried out using SPSS 17.0 for Windows (SPSS Inc, Chicago, USA). A one-way analysis of variation (ANOVA) and a least significant difference (LSD) multiple comparisons ($P < 0.05$) were used to assess the significant difference among different treatments (soil properties, enzyme activities, and so on). The Pearson correlation analysis was performed to measure the pairwise relationship between different variables (*i.e.*, the enzyme activity, heavy metals, and others). All bar graphs were drawn using Origin Pro 9.0. The heat maps of correlation between enzyme activities and properties were performed using HemI software (Heat map Illustration, Version 1.0) (Deng et al., 2014). The analysis of zymograms was processed by Matlab R2016b, and the assay of each zymogram was performed in three analytical replicates.

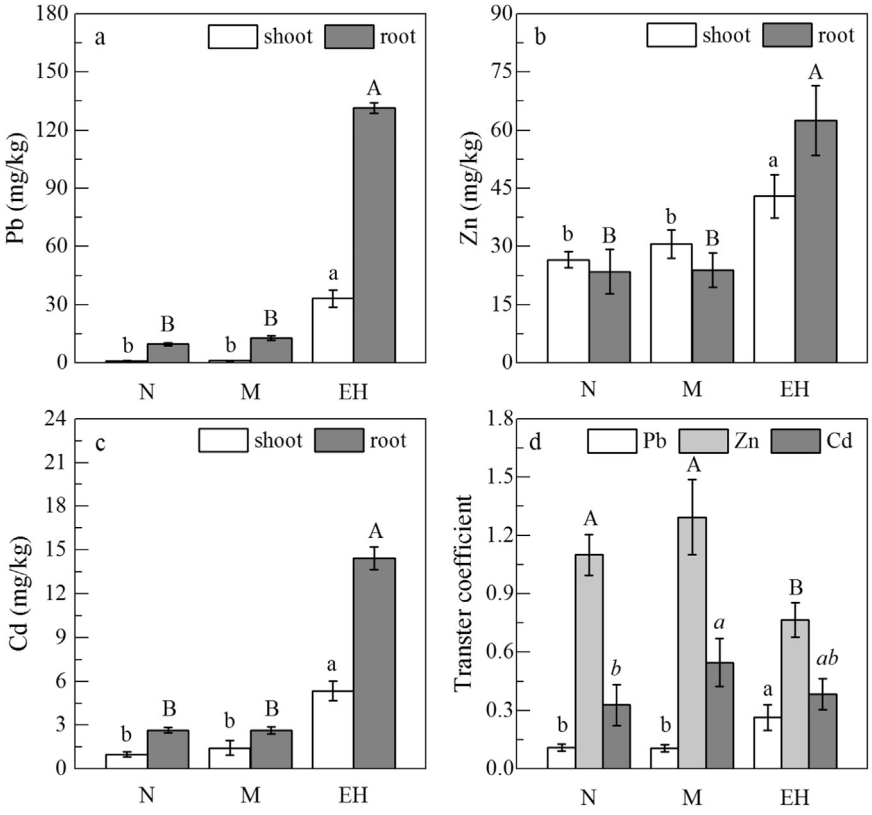

Fig. 1. The metal contents in the alfalfa tissues and transfer coefficient of alfalfa. Values are the means ± standard (n = 4). Different letters indicate significant differences ($p < 0.05$) among the different treatments. N (none pollution), M (moderate pollution), EH (extremely heavy pollution).

3. Results

3.1. Effects of heavy metals on the growth in alfalfa

The increasing pollution level significantly inhibited shoot height and reduced shoot and root biomass ($P < 0.05$), but showed no significant effect on root length (Table S1). Compared with the none pollution and moderate pollution treatment, the extremely heavy pollution treatment substantially reduced the alfalfa shoot biomass by 37% and 30% and root mass by 40% and 19%, respectively. The increasing heavy metal pollution did not significantly reduce the chlorophyll content until M treatment, but significantly reduce in the EH treatment (Table S1).

The Pb concentration in EH treatment was significantly higher than that in the N treatment (*i.e.*, 30.7-fold for shoot; 13.4-fold for root) and M treatment (*i.e.*, 28.3-fold for shoot; 10.3-fold for root), whereas no significant difference was observed between the N and M treatment (Fig. 1a). The shoot and root Zn concentration tended to increase with increasing pollution level, but no significant difference was observed until the M treatment (Fig. 1b). Specifically, the Zn concentration in the EH treatment was 61.5% and ≥ two-fold higher than that in the N treatment for shoot and root, respectively. The pattern of Cd uptake is similar to that of Pb. The shoot and root Cd concentration were significantly higher in the EH treatment than in the N treatment (*i.e.*, 4 and 13 times for shoot and root, respectively), but no significant difference was noticed between the N and M treatment (Fig. 1c).

For both shoot and root tissue, the TN in EH treatment was significantly lower than the other treatments, whereas the TN between the N and M treatment was not significantly different (Table S2). The TP change in response to increasing pollution level showed a similar pattern to the TN change. The TP level in the EH treatment was ≥ two-fold lower than the N treatment. However, compared with the TN change, the TP in root was more sensitive to increasing pollution level, and a significant TP loss already occurred in EH treatment. No significant pollution impact on TK by heavy metals was found among three treatments (Table S2).

3.2. Oxidative damage and quantification of enzymatic antioxidants of alfalfa

To investigate the oxidative damage induced by heavy metals, the lipid peroxidation was estimated by measuring MDA content (Fig. 2a). In alfalfa shoot, heavy metals had no effect on MDA content under heavy metals stress. However, in alfalfa root, the MDA content in EH treatment was 35% higher than that in N treatment.

For the shoot part, the APX activity in EH treatment was significantly lower than that in the N treatment by 18% (Fig. 2b). On the contrary, for the root part, the M treatment enhanced the APX activity by 60%; no further significant APX enhancement, however, was observed in EH treatment. The increasing heavy metals pollution significantly and consistently reduced the CAT activity in both shoot and root part (Fig. 2c). Compared with the CAT activity in N treatment, the reduction was 74% for the shoot and 44% for the root in M treatment. The increasing heavy metal pollution did not significantly reduce the SOD activity in shoot until EH treatment, and the reduction was 16% compared with the N treatment. In the root, the SOD activity in the EH treatment was significantly lower than in the M treatment by 46% (Fig. 2d). The increasing heavy metal pollution showed a significant but inconsistent impact on POD activity ($P < 0.05$) and the impact was tissue-specific (Fig. 2e). Compared with the N treatment for the shoot part, the POD activity was significantly reduced by the M treatment, and then remained unchanged even the pollution further increased in EH treatment. Compared with the N treatment for the root part, the POD activity was significantly reduced by the M treatment, but significantly enhanced by EH treatment.

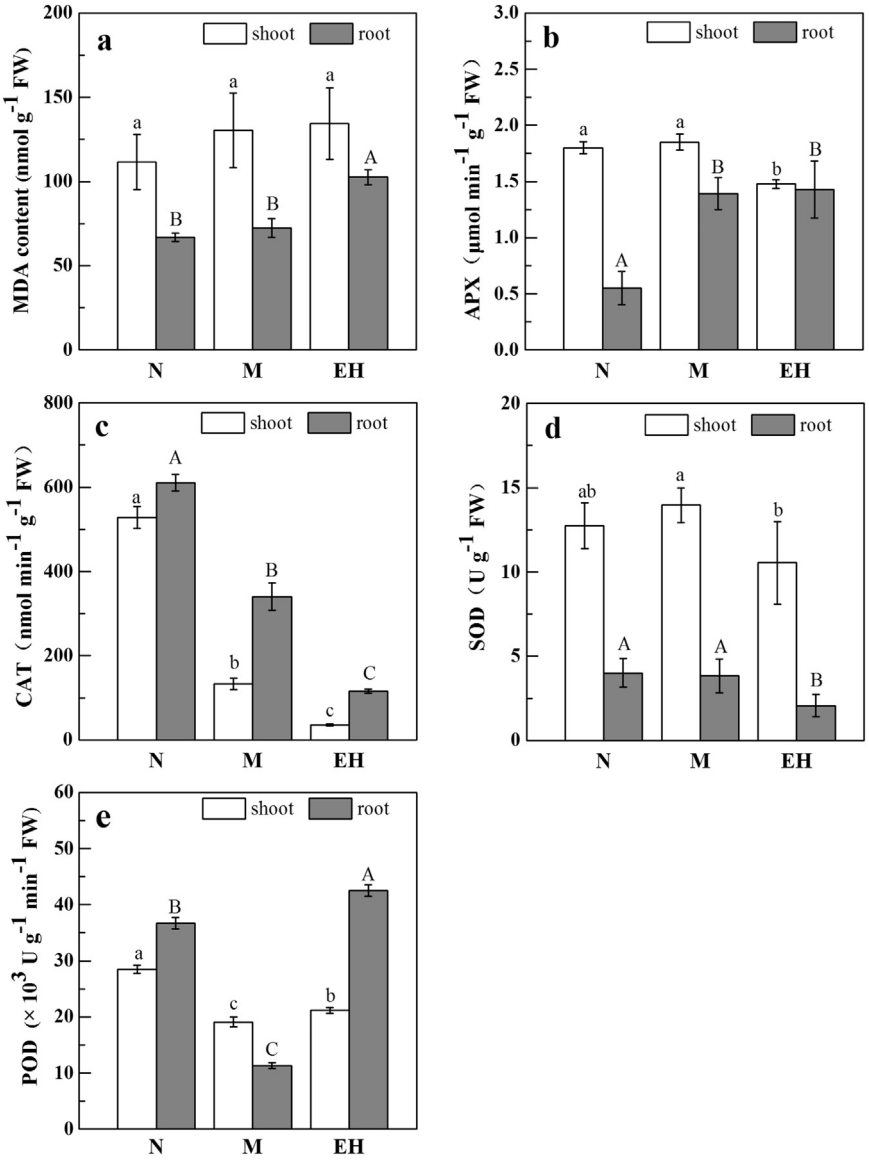

Fig. 2. Effect of different pollution grade on MDA content (a) and enzymatic antioxidants activities of ascorbate peroxidase (APX) (b), catalase (CAT) (c), superoxide dismutase (SOD) (d) and peroxidase (POD) (e) in the alfalfa tissues. Values are the means ± standard (n = 4). Different letters indicate significant differences ($p < 0.05$) among the different treatments. N (none pollution), M (moderate pollution), EH (extremely heavy pollution).

3.3. Soil enzymatic activities

The changes of soil enzymes (*i.e.*, alkaline phosphatase, urease, catalase, saccharase, and β-glucosidase) were shown in Fig. 3. The impacts of increasing heavy metal pollution level on soil enzymes were inconsistent and enzyme-specific.

The catalase activity in EH was significantly higher than other treatments, whereas the catalase activity between the N and M treatment was not significantly different. The saccharase activity change in response to increasing pollution level showed a similar pattern to the catalase activity change. The saccharase in the EH treatment was 31% higher than the M treatment. The urease enzyme activity in the M treatment was exceptionally higher than the other two treatments ($P < 0.05$), and significant difference was found between N and EH treatment. Compared with the N treatment, the β-glucosidase activity in the M treatment and EH treatment was elevated by 34% and 33%, respectively. No noticeable difference, however, was found between the M and EH treatment. No significant difference in alkaline phosphatase activities was found between three treatments. Compatible with the plant enzyme activities, these five types of soil enzyme activities varied significantly between different levels of pollution.

3.4. Distribution of enzyme activities

The zymograms of individual soil and their corresponding zymography images revealed remarkable details about the spatial distribution of enzyme activity along and outward from the roots (Fig. 4). The red, yellow, green and blue color in order on the images represent a decreasing gradient of enzyme activities (*i.e.*, from high to low), and the dark blue represents very low activity.

The greatest phosphatase and β-glucosidase activities were found in the highest level of heavy metal pollution (*i.e.*, the EH treatment) (Fig. 4). The highest average of phosphatase activity and the highest average of β-glucosidase activity was found in the EH treatment and M treatment, respectively (Fig. 5). However, the increase in total β-glucosidase activity was slight and was accompanied with an increase in hotspot area under moderate pollution (Fig. 4). After planting, both phosphatase and β-glucosidase activities were associated with the roots and were rarely distributed throughout the soil (Fig. 4). The hotspots of phosphatase activity were also distributed along roots and accounted for 3.4%, 1.5%, and 7.1%

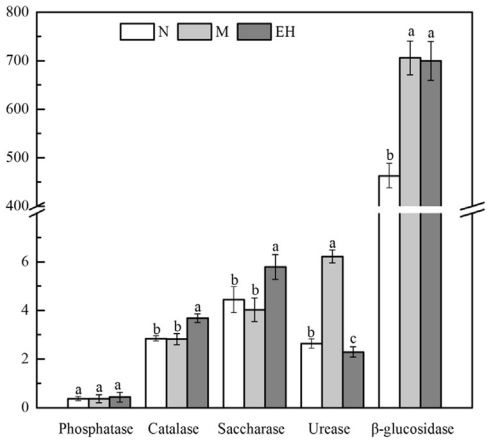

Fig. 3. Influence of different pollution level on soil enzyme activities. Alkaline phosphatase activity (mg P g^{-1} 24 h^{-1}), Catalase activity (ml 0.02 mol L^{-1} $KMnO_4$ g^{-1} (20 min)$^{-1}$), Saccharase activity (mg glucose g^{-1} 24 h^{-1}), Urease activity (mg NH_4^+-N g^{-1} 24 h^{-1}) and β-glucosidase activity (μg PNP g^{-1} h^{-1}). N (none pollution), M (moderate pollution), EH (extremely heavy pollution). Values are the means ± standard (n = 3). Different letters indicate significant differences ($p < 0.05$) among the three treatments.

of total soil surface area, respectively. The localization of β-glucosidase hotspots followed a similar pattern as phosphatase. Thus, heavy metal contents affected the total enzyme activity, but roots impacted the localization of hotspots for both tested enzymes.

3.5. Soil enzyme and antioxidative enzyme activities correlation matrix

Pearson correlation analysis was performed between soil properties, heavy metals and soil enzymes activities (Fig. 6a). Several interesting patterns are found. For the correlations between soil enzymes and soil chemistries, the heavy metals consistently showed strong and positive correlations with the catalase and saccharase. However, the correlations between soil enzymes and TN or TP are more enzyme species-dependent; for example, the TN and TP showed strong and positive correlations with the catalase activity in general, but negative correlations with urease.

For the correlations between enzymatic activities and properties in alfalfa shoot, the APX principally explained the variability of most elements in the alfalfa shoot (Fig. 6b). The APX showed the strongest and negative correlations with all metals, but strongest and positive correlations with TN/TP. For the correlations between enzymatic activities and properties in alfalfa root, the metals showed strongest positive and negative correlations with MDA and CAT/SOD, respectively (Fig. 6c). The TN and TP showed strongest and negative correlations with POD/MDA.

4. Discussion

4.1. The response of plant growth characteristics and antioxidant enzymes to heavy metals stress

The accumulations of heavy metals in plants toxically affect the growth and development of plants. The decline in plant biomass is due to the adverse effects of excessive heavy metals, which inhibits plant growth (*i.e.*, height and length) and reduces the shoot and root biomass (Table S1). Moreover, it is noted that alfalfa exposed to excessive metals is prone to leaf chlorosis, in agreement with the negative impacts of heavy metals on legume (James and Bartlett, 1984).

Our results reflected the discrimination of metal uptake between plant tissues. Although the total uptake amounts of Pb, Zn and Cd significantly increase with increasing metal contaminations in both

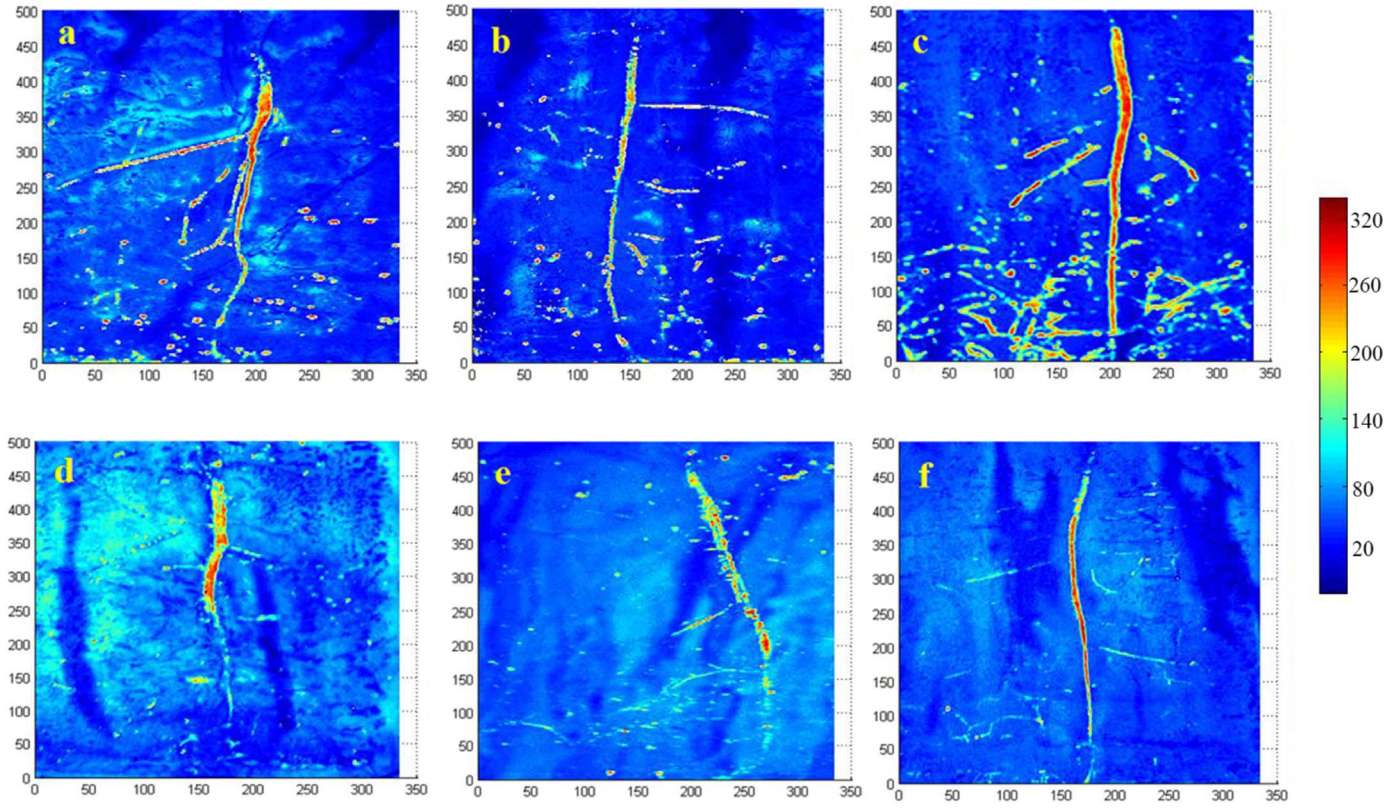

Fig. 4. The zymographs of phosphatase and β-glucosidase activities in three treatments. This showing spatial distribution of enzyme activities along and outward from the root. a. Alkaline phosphatase in N, b. Alkaline phosphatase in M, c. Alkaline phosphatase in EH, d. β-glucosidase in N, β-glucosidase in M and f. β-glucosidase in EH. Side color map indicates enzyme activities (pmol cm^{-2} h^{-1}).

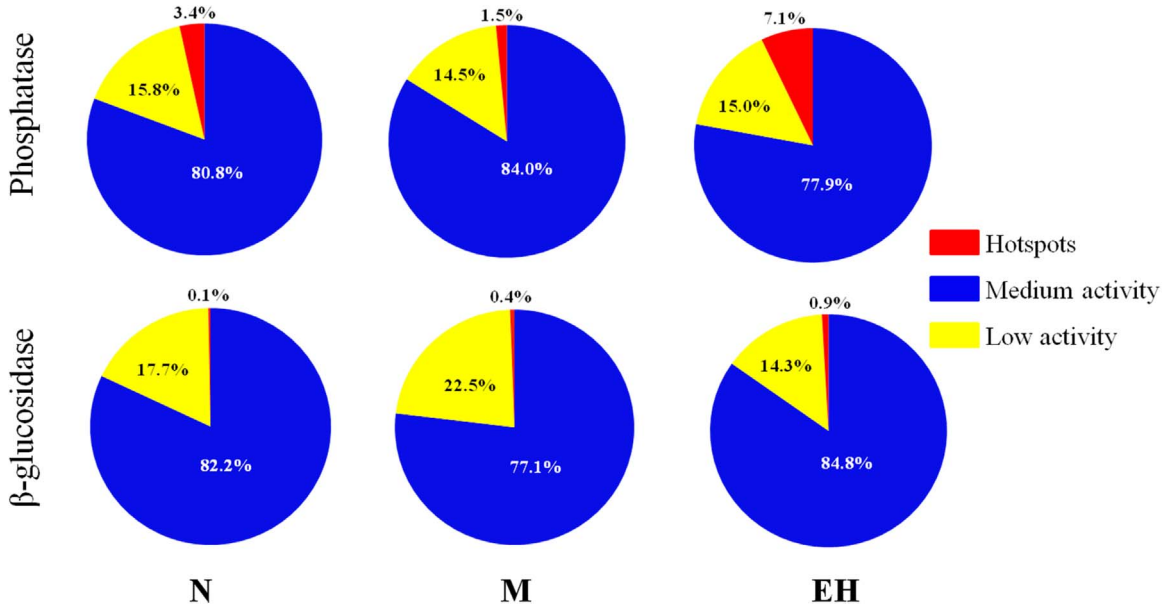

Fig. 5. Contribution of three classes of phosphatase and β-glucosidase activity to total activity on the zymogram areas. Blue: low activity; yellow: medium activity, red: hotspots. N (none pollution), M (moderate pollution), EH (extremely heavy pollution).

shoots and roots, the increase in roots is much higher than that in shoots. Meanwhile, except for the Zn case in the none pollution and moderate pollution treatments, all transfer coefficients were considerably less than 1.0 for plants (Fig. 1d), which indicates the heavy metals are mainly accumulated by roots and only a low level of Pb and Cd is translocated to shoots. Liu et al. (2015) concluded that the uptake amounts of metals are heterogeneous, depending on the plant tissue, plant species, metal species, and metal concentrations, consistent with the observation in this study. The preference or selectivity of metal accumulations by plant roots implies the benefits of phytostabilization of heavy metals.

Our results showed a slight decrease of N content in shoots and roots with increasing metal contaminations, indicating the mechanism of alfalfa to encounter metal stresses by phytostabilization of heavy metals and reducing N content. This conclusion for alfalfa is consistent with the observations for legume plants. The legume plants are sensitive to heavy metals stress (Zhao et al., 2010), and similarly encounter heavy metals stress by reducing N contents. Additionally, intimate inter-relationships among the N content of shoots, chlorophyll content, and photosynthesis in plants have been reported previously (Kong et al., 2015; Możdżeń et al., 2017). Our results found that chlorophyll content was significantly inhibited by excess metals. Therefore, the alfalfa responds to heavy metals stress through decreasing the N content and chlorophyll biosynthesis in plants.

Our results showed the MDA content increased with increasing heavy metal contaminations (*i.e.*, positively correlated with Pb, Zn and Cd in root) (Fig. 2a), implying the membrane damage induced by the peroxidation of polyunsaturated fatty acid as a result of ROS formation and oxidative stress. The cellular membranes in plants are particularly sensitive to oxidative stress from superfluous accumulations of ROS (Chen et al., 2013; Mittler, 2002).

Plants have strong antioxidant enzyme activity to protect cells from the damage of excessive ROS caused by environmental stress (Hojati et al., 2017; Morina et al., 2016). Heavy metals reduced the SOD activity under alfalfa shoot. Compared with the moderate pollution treatment, the alfalfa root held a lower SOD activity in the extremely heavy pollution treatment (Fig. 2d). These results indicate that a mild contamination can induce SOD activity in plant tissue and increase its resistance against stress adversity for survival. However, induced by increasing pollution level, the increase of oxygen free radicals in the leaves exceeds the normal disproportionation ability limit, and the destruction of the leaf membrane and the enzyme system results in the decrease of SOD activity. The SOD activity was related to lead and cadmium, which indicates that lead and cadmium have a great effect on SOD activity of roots. In plant cells, the POD, CAT, and APX play vital roles in regulating the H_2O_2 level for signaling during metabolic changes, but the POD and APX are proposed to be predominantly responsible for modulating H_2O_2 level, especially under stress environments (Chen et al., 2013). In our study, the APX activity was lower in extremely heavy pollution in alfalfa shoot, but higher in alfalfa root (Fig. 2b). The APX activities had varying degrees of changes in shoots and roots of alfalfa under excessive heavy metals stress. The difference between shoots and roots is possibly attributed to the increase of ions concentration, which favors the elimination of more H_2O_2 in the plant. The CAT activity was the lowest in extremely heavy pollution treatment (Fig. 2c). The decline of CAT activity reflects reduced H_2O_2 scavenging capacity (Liu et al., 2015), which results in H_2O_2 accumulation. However, our data showed that POD activity greatly increased under excessive heavy metals stress in shoots and roots of alfalfa (Fig. 2e). Similarly, others previous studies showed POD activity plays an important role in scavenging Al-induced H_2O_2 production in barley seedlings (Chen et al., 2013). The conflicting responses of POD and CAT to heavy metals stress indicate that they may have different mechanisms of action in the process of oxidative stress (Abbas et al., 2017; Liu et al., 2015). The POD augmentation can be also a result of release of those enzymes located in cell wall as response to the stress to which the plants are subjected (Chen et al., 2013; Liu et al., 2015). Under the stress from heavy metals, plants usually clear reactive oxygen radicals by strengthening protection on enzyme activity to maintain stability and integrity of cell membrane. The POD activity was negatively correlated with TN and TP in root. This result indicates that the phosphorus content in plant tissue is closely related to POD activity. Overall, the above results indicate that the alfalfa responds to heavy metals with increasing POD and APX activities, which therefore alleviates heavy metals toxicity in plants.

4.2. *The response of soil enzymes to heavy metals stress*

Previous studies have shown that the soil enzyme activity is affected by the physical and chemical properties of the soil, especially by heavy metals (Yang et al., 2016). In this study, the activities of five enzymes exhibited various changes based on different heavy metal pollution

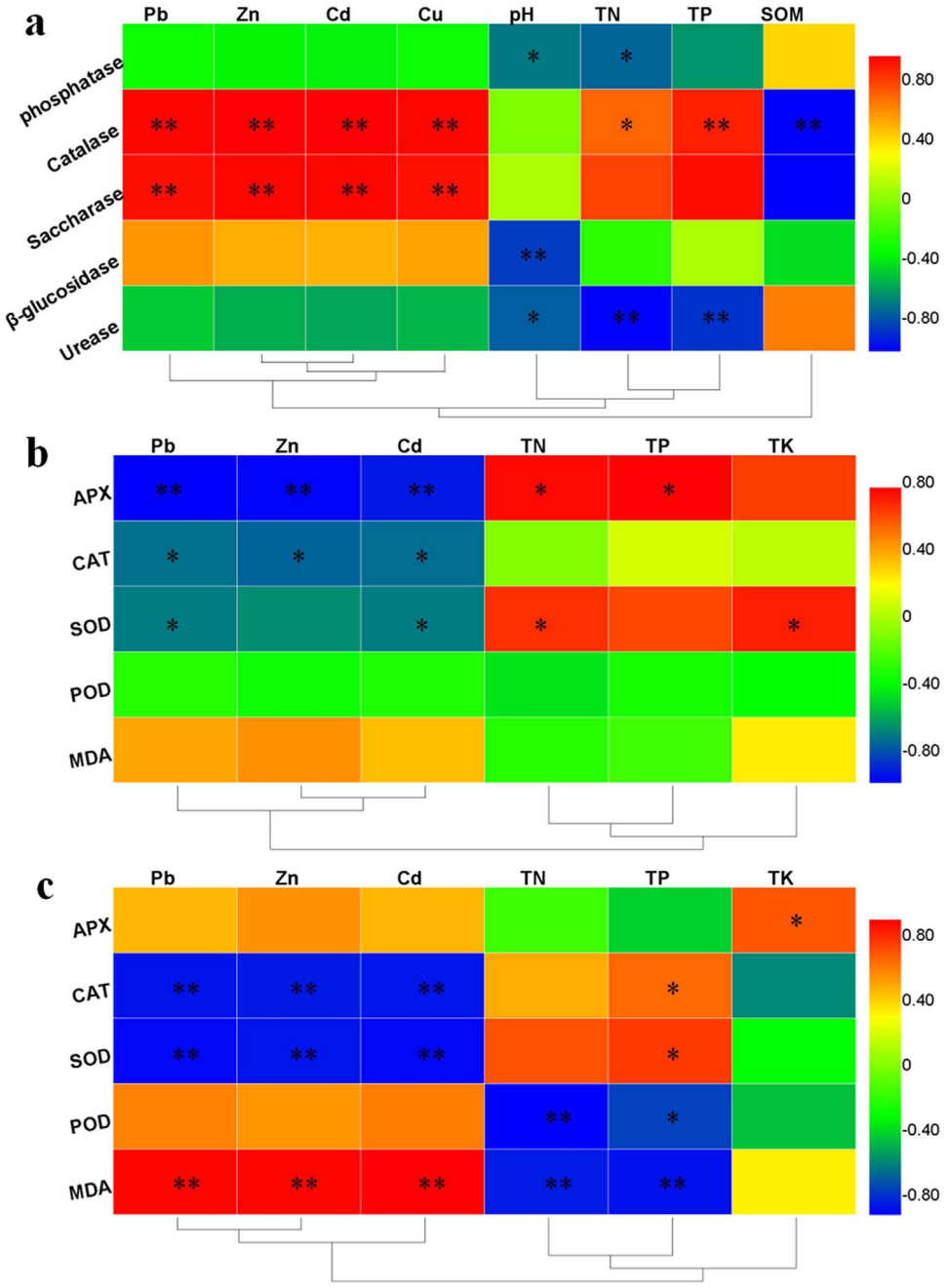

Fig. 6. a: Heat map of correlation between soil enzyme activities and properties in soil samples based on Pearson correlation coefficients; b: Heat map of correlation between enzymatic activities and properties in alfalfa shoot based on Pearson correlation coefficients; c: Heat map of correlation between enzymatic activities and properties in alfalfa root based on Pearson correlation coefficients.

level. The catalase and saccharase activity in the extremely heavy pollution treatment were significantly higher than those in the other treatments (Fig. 3). It may be suspected that metals can cause protein denaturation, lead to complex substrates, interact with residues in enzyme activities, or react with enzyme substrate complexes, resulting in enzyme activity reduction rather than enzyme activity increased (Hemida et al., 1997; Kuperman and Carreiro, 1997). However, Hagmann et al. (2015) conducted the control experiments where metals were added to the enzymatic assays and the ability of the soil itself to act as an inhibitor or activator was assessed. The results showed that the metals or the soil themselves are not simply activating the enzymes, but the metals interacted with the soil for decades resulted in an altered soil microbial community resulting in soil with high extracellular enzyme function.

The metals concentrations (*i.e.*, Pb, Zn, Cd, and Cu) are strongly positive correlated with soil enzyme activities (Fig. 6a), clearly indicating the dominant soil heavy metals to influence soil enzyme activity. Some researchers presented that only oxidoreductases could directly change the valence state of the ions and participate in the detoxification of heavy metals (Hu et al., 2014; Yang et al., 2016). The more active heavy metals confer a higher toxicity, resulting in higher catalase activity to involve in detoxification. Metals interacted with the soil for decades, rather than for a shorter time period of days to months (Hinojosa et al., 2004). The positive correlation between soil saccharase activity and heavy metals in our study may in part depend on the length of time the soil-metal system has been left to “adjust and adapt” (Hagmann et al., 2015; Yang et al., 2016). Yang et al. (2007) noted that phosphatase was positively correlated with Pb, Zn and Cd, but we found

the phosphatase activity showed no correlation with Pb, Zn and Cd. This disagreement might be caused by the different soil physicochemical properties between studies. Fang et al. (2017) discovered that a high phosphorus content presented a positive effect on phosphatase activity. Because the phosphorus is a necessary nutrient for soil microbial metabolism, the existence of different types of phosphorus has a significant effect on soil enzyme activity. In this study, there was no significant difference between three treatments. Plant antioxidant enzymes and soil enzymes reflect the response of enzyme activity to heavy metals from different angles and are related to nutrient elements. However, there was no uniform standard in enzyme to assess heavy metals through accurate method. Therefore, a direct and accurate method suggested by this study can facilitate the assessments of heavy metal pollution level.

4.3. The response of the distribution of enzyme activities in the rhizosphere to heavy metals stress

Our results showed that the spatial pattern of enzyme activity along the roots is affected by heavy metals. The phosphatase and β-glucosidase enzyme activities in plant-soil interface increased with increasing soil pollution. Additionally, the percent hotspot area in the extremely heavy pollution was greater than that in the none pollution (Fig. 4). The increases are likely due to the increasing microbial activity (Bradford et al., 2008; Steinweg et al., 2008) and root exudates by enzymatic activities at high heavy metals (Wan et al., 2016). Plants take up some heavy metals from soil, which may result in the reduction of heavy metal toxicity on enzymes in the rhizosphere (Le et al., 2012). The hotspots are relevant not only in terms of the direct release of enzymes by roots, but also in terms of the low content of heavy metals near the roots and the rhizodeposition (Kuzyakov and Domanski, 2000; Marinari et al., 2014). Furthermore, POD activity was showed the highest in the extremely heavy pollution. Through scavenging H_2O_2 in roots, POD alleviates plant toxicity and increases microbial activity and enzyme activity in rhizosphere (Ali et al., 2017). In addition, some researchers demonstrated that a negative correlation between the phosphatase and β-glucosidase activities and the content of heavy metals and antioxidant enzyme in plants (Ding et al., 2011; Shao et al., 2010).

The distribution of phosphatase activity along the alfalfa root was uniform. A similar uniform spatial distribution pattern is evident in previous studies regarding the acid phosphatase along the roots of the lentil (L. *culinaris*) (Razavi et al., 2016). In contrast, the phosphatase activity decreased in the moderate pollution treatment (Fig. 4). Such a decrease is connected to the phosphorus element. Because of the P is an essential nutrient for plant growth and a component of key molecules such as nucleic acids and phospholipids. The key enzyme reactions require the P involvements (Wardle, 1992), but P content was the lowest in the moderate pollution soil. However, there was no significant difference in soil phosphatase activity between three treatments (Fig. 3). The main reason is that soil enzyme activity is secreted by microorganisms and plants, it is difficult to quantify the reaction of microorganisms and plants under heavy metals stress. The β-glucosidase activities along the alfalfa roots were not uniform and the lowest in none pollution (Fig. 4). The lowest β-glucosidase activity was found in soil enzyme. This is mainly related to the distribution of polymers (*i.e.*, exoenzyme) and heavy metals, and there are oligomeric components (*i.e.*, low molecular weight sugars) and lower heavy metals in rhizosphere (Pathan et al., 2017; Razavi et al., 2016). The values of color intensities of the β-glucosidase activity were the highest in extremely heavy pollution treatment along alfalfa roots. These enzyme activity hotspots result from heavy metals stimulation, plants secretions, and promoting the proliferation of micro-organisms (Wan et al., 2016). In soil, the β-glucosidase activity was also the lowest in none pollution (Fig. 3), this is consistent with the activity of rhizosphere enzyme. Therefore, we conclude that the different soil enzymes react differently to heavy metals, but the level of enzyme activity in rhizosphere could better reflect the effect of heavy metals stress on the soil-plant system. Overall, we visualized the enzyme-specific distribution patterns in soil and in the rhizosphere response to different heavy metals stress through the monitoring of zymography *in situ*.

5. Conclusions

Our results directly reveal the enzyme activities in rhizosphere and their spatial distribution of heavy metals. Over-accumulation of Pb, Zn and Cd in plants shows various toxicity symptoms, including growth inhibition, chlorotic leaves, and a decrease in biomass. This study demonstrates that the catalase and saccharase are the most sensitive to the Pb, Zn and Cd pollutions. The antioxidative activities play a crucial role in protecting against metal-stress. The SOD and CAT activities decreased with increasing heavy metal concentrations. Furthermore, the distribution of enzyme activities shows that both phosphatase and β-glucosidase activities are associated with the roots and were rarely distributed throughout the soil. In addition, the total hotspot areas of enzyme activities were the highest in extremely heavy pollution soil. The results indicated that the spatial distribution of rhizosphere enzyme activities through *in situ* zymography is an intuitive and accurate way to reflect soil heavy metals, which display great advantages over the traditional methods. This study provides important information about the impact of heavy metal pollution on enzymes and may form a basis for future phytoremediation and biomarker studies.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2018.03.015.

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