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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

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