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Deciphering the rhizobium inoculation effect on spatial distribution of phosphatase activity in the rhizosphere of alfalfa under copper stress

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ABSTRACT

Legume–rhizobium symbiosis plays an important role in agriculture and ecological restoration. However, the regulatory mechanisms of rhizobium in alleviating heavy metal stress through the biochemical response of plant-soil system is limited. In this study, alfalfa was inoculated with a copper (Cu)-resistant rhizobium, and its effect on plant growth and the spatial distribution of phosphatase in the rhizosphere under Cu stress was assessed. Our results showed that rhizobium inoculation alleviated Cu-induced growth inhibition, and increased the nitrogen and phosphorus content in alfalfa tissues. Moreover, inoculated plants had a higher Cu uptake than non-inoculated plants, with a much higher increase in the roots than in the shoots; thus, inoculation with rhizobium was shown to decrease the transfer coefficient and promote Cu phytostabilization. The zymograms illustrated that the distribution of phosphatase activities was associated with the presence of roots. Compared with the non-inoculated treatment, the rhizobium inoculation increased the hotspot areas of phosphatase by 26.1% and 39.3% at the Cu 0 and Cu 800 treatments, respectively. In addition, the available phosphorus in the soil showed negative correlations with soil phosphatase activity ($p < 0.05$). The model of partial least squares path modelling (PLS-PM) indicated that soil Cu content directly influenced the hotspot areas of phosphatase activities in the rhizosphere and explained most (86%) of the variation. Thus, the enzymatic hotspots were concluded to mainly be affected by the Cu content of soil, and the phosphatase activities in the rhizosphere were mainly regulated by the ratio of nitrogen to phosphorus. These findings provide a basis for the spatio-temporal dynamics of biogeochemical reactions in the rhizosphere of polluted soils.

1. Introduction

Heavy metal pollution is a potent environmental threat to human health due to its ability to enter the food chain and its high persistence in the environment (Shen et al., 2017; Chou et al., 2019). Over time, copper (Cu) has become one of the main heavy metal contaminants because of its common use in industry and agriculture such as in mining, metal processing, fertilizers, pesticides, and municipal wastes (Li, 2006; Qu et al., 2016). As one of the essential elements for plant growth, Cu participates in multiple biological processes especially for enzymatic reactions (Merlos et al., 2016). However, excessive Cu concentration inhibits plant growth, causes nutrient deficiencies, e.g., nitrogen (N) or phosphorus (P), and damages plant–soil systems (Sun et al., 2015; Wang et al., 2017; Chou et al., 2019).

Legumes have evolved symbiotic associations with soil microorganisms such as rhizobia, leading to the formation of a novel root organ, the nodule, which provides a niche for bacterial N fixation (Chou et al., 2019). In addition, rhizobia can promote plant growth directly (e.g., through increased N and P uptake), and indirectly (through tolerating abiotic stresses like extremes of pH, salinity, and heavy metal pollution). This enhances the defense of plants in stressful environments (Wani et al., 2007; Gopalakrishnan et al., 2015). Therefore, legume–rhizobium symbiosis has been applied to plant heavy metal restoration in recent years (Dary et al., 2010; Ju et al., 2019). There have also been a few studies that have identified the regulatory mechanisms of rhizobium in alleviating Cu stress via the biochemical responses of

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the plant–soil system (Kong et al., 2015; Liu et al., 2015). Some studies, for example, Chen et al. (2018) indicated that rhizobium inoculation reduced reactive oxygen species accumulation, increased glutathione levels, and affected the ascorbate–glutathione cycle to protect alfalfa (*Medicago sativa*) from excess Cu stress. In addition, *Sinorhizobium* inoculation substantially improved antioxidant capacity and alfalfa growth and increased soil N, P, organic matter, and microbial biomass (Kong et al., 2015; Ju et al., 2019). However, the negative effects of heavy metals on plant growth commonly are concentrated the small areas of up to a few millimeters surrounding the living roots (i.e., the rhizosphere), (Hinsinger et al., 2009; Antoniadis et al., 2017). The rhizosphere contains various root exudates and rhizodeposits released by roots, which stimulate microbial activities and enzyme production, and provide the basis for plant–microbe interactions (Bais et al., 2006; Oburger et al., 2014; Kuzyakov and Razavi, 2019). In addition, heavy metal pollution can also decrease metabolic activity and influence bacterial functionality in the rhizosphere. Unfortunately, data on the reaction mechanisms at the root–soil interface in the legume–rhizobium symbiosis under excess Cu conditions are comparatively scarce.

Microorganisms play critical roles in soil nutrient cycling, structural formation, and plant–soil interactions (Wang et al., 2015; Xiao et al., 2017; Zhang et al., 2019a). As components of key molecules such as nucleic acids, phospholipids and amino acids, P and N are two of the main limiting nutrients for microbial growth and activity, and are essential nutrients for plant growth and performance (Xiao et al., 2010; Fang et al., 2017). Phosphatase in soil is produced by microorganisms (i.e., fungi and bacteria) and plants (Zhang et al., 2018; Wei et al., 2019b), and plays an important role in the mobilization of organic forms of P (Madejón et al., 2001). Furthermore, phosphatase is sensitive to heavy metal stress and is widely used as an indicator of heavy metal toxicity in soils (Wang et al., 2007; David et al., 2010). Enzyme activities in the rhizosphere are related to both resource availability and microbial metabolism (Sinsabaugh et al., 2009; Cui et al., 2018), and they adequately reflect the interaction between microorganisms and the local environment (Razavi et al., 2016, 2017; Liu et al., 2017). As energy access limits microbial growth in most soils (Blagodatskaya and Kuzyakov, 2013), high enzyme activity is generally concentrated in small soil volumes with intensive carbon input (Razavi et al., 2016; Wei et al., 2018); these are called enzymatic hotspots (Kuzyakov and Blagodatskaya, 2015). The rhizosphere is the most active microorganism habitat and higher enzyme activities are observed in the rhizosphere, than in bulk soil (Razavi et al., 2016; Kuzyakov and Razavi, 2019), and is thus identified as an important enzymatic hotspot. The hotspot of enzyme activity is a reflection of microbial and root activities, and is heterogeneously distributed in the soil (Wei et al., 2018, 2019a). Many previous studies have used well-mixed bulk soil, which averaged out the estimated effects of the rhizobium inoculation on the phosphatase activities and hotspots in Cu-contaminated soils (Sipahutar et al., 2018; Ju et al., 2019). Therefore, identification of the effects of Cu-contamination on plant–microbial interactions in the rhizosphere can provide unique and important insights into the impact of heavy metals on terrestrial ecosystems.

Soil zymography, a non-destructive in situ technique for two-dimensional imaging, offers an opportunity for visualization of the spatial of enzyme activities in soil and in the rhizosphere (Razavi et al., 2019). We applied in situ soil zymography by placing substrate-saturated membranes in direct contact with roots and soil (Razavi et al., 2016; Sanaullah et al., 2016; Wei et al., 2019a). For the first time, we employed this technique to illuminate the impacts of alfalfa–rhizobium symbiosis in Cu-contaminated soil on the spatial distribution of enzyme activity. The purpose of this study was to: 1) clarify how Cu affects the spatial distribution of phosphatase activity at the root–soil interface, and 2) illuminate the dominant factor that affects soil phosphatase activity under alfalfa–rhizobium symbiosis. We hypothesized that: 1) the enzymatic hotspots would be distributed along the roots, regardless of rhizobium inoculation, and 2) rhizobium inoculation would increase soil phosphatase activity and the hotspot area. We, therefore, visualized phosphatase activity in a Cu-contaminated soil, determined the distribution of Cu concentration in the alfalfa rhizosphere, and measured the N and P contents in the alfalfa plants. This study sheds new light on the role of legume–rhizobium symbiosis and the regulatory mechanisms in counteracting metal toxicity.

2. Materials and methods

2.1. Soil collection

Soil was collected from a pristine area without a history of metal pollution at the Institute of Soil and Water Conservation (ISWC), Chinese Academy of Sciences (CAS), located in Yangling, Shannxi Province, China. The soil was extensively sieved (4 mm mesh) to remove all plant materials and stones, and then divided into three samples. The first sample was used as the control (without Cu addition), while the other two samples were treated with additions of $CuSO_4$ solution, to simulate 400 and 800 mg kg^{-1} Cu-contaminated soil, respectively (hereafter denoted as Cu 0, Cu 400 and Cu 800, respectively). All soil samples were stored in the dark under conditions of 20% humidity and 25 °C for three months to ensure metal stabilization in the contaminated samples. A soil subsample from the control experiment was dried and sieved (2 mm mesh) for physicochemical characterization using standard methods. The selected physical and chemical properties of the soil samples are shown in Table S1.

2.2. Experimental setup

Soil (1.0 kg) was evenly packed into a rhizobox (Fig. S1, inner size: 18.0 cm × 12.5 cm × 5.2 cm) with one removable side, to achieve a uniform soil packing and to avoid soil layering. Immediately before planting, the rhizoboxes were gently shaken to achieve a stable soil packing (Ge et al., 2017). The alfalfa seedlings were germinated on filter paper for three days, and then one seed was planted in each rhizobox at a depth of 5 mm. During alfalfa growth, the rhizoboxes were kept inclined at an angle of 45° so that the roots grew towards the lower wall of the rhizobox due to gravitropism. 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 automated temperature control system. In the growth period, the water content of the soil was maintained at 60% with distilled water.

When the first leaf appeared, the plants were inoculated with a cell suspension of wild-type *Sinorhizobium meliloti* CCNWSX0020. The *S. meliloti* strain is a Cu-resistant bacterium that was isolated from the root nodules of *Medicago lupulina* in lead–zinc mine tailings in China (Fan et al., 2011). *S. meliloti* strains were grown at 28 °C with shaking at 200 rpm in tryptone yeast (TY) extract medium (5 g tryptone, 3 g yeast extract, and 0.7 g $CaCl_2{\cdot}2H_2O$ per liter; pH 7.2), (Robertsen et al., 1981). The bacterial cultures were first standardized (OD 600 nm = 0.8), and then the roots of each seedling were inoculated with bacterial cell suspensions (1 mL). The non-inoculated control received the same amount of sterile distilled water. We tested six treatments: Cu 0, Cu 0 + *S. meliloti*, Cu 400, Cu 400 + *S. meliloti*, Cu 800, and Cu 800 + *S. meliloti*. Sixteen replicates were prepared for each treatment. Plants were harvested at 21 days after inoculation to determine the growth indexes and for other measurements.

2.3. Determination of plant indexes

The harvested shoots and roots were washed four times with sterilized deionized distilled water to remove the Cu^{2+} at the root surface, and then dried at 65 °C for 48 h. The samples (0.3–0.5 g) were placed into digestion vessels and digested with an acid mixture (4:1 HNO_3:$HClO_4$). Then the solution was diluted appropriately with

distilled water before measuring the Cu concentration by an atomic absorption spectrophotometer. The total uptake of Cu was calculated by multiplying the measured Cu concentration by the sample biomass. Plant samples were digested with H_2SO_4 and H_2O_2, and the concentrations of N and P in the digested samples were determined using an injection pump analyzer.

2.4. Measurement of soil physicochemical properties and metals content

Soil moisture was determined by oven-drying 10 g of fresh soil at 105 °C for 48 h. Soil pH was measured in a 1:5 soil/water mixture using a pH meter. Soil organic matter was determined using titrimetric methods, based on the oxidation of organic substances with potassium dichromate (Kalembasa and Jenkinson, 1973). The total N (TN) was measured using the Kjeldahl method (Bremner and Mulvaney, 1982). The total P (TP) was measured using an ultraviolet spectrophotometer after wet digestion with H_2SO_4 and $HClO_4$ (Parkinson and Allen, 1975). The available P (AP) was determined via $NaHCO_3$ extraction. The soil samples were digested to measure the total Cu concentration based on a modified USEPA Method 3051A (Element, 2007). Specifically, a 0.2 g soil sample was digested with 15 mL of tri-acidic mixture (i.e., HCl, HNO_3, and $HClO_4$) at a volume ratio of 1:3:1. The Cu concentration in the digested samples was determined using atomic absorption spectrophotometry. Certified standard reference materials, NIST 2709 San Joaquin soil and NIST 1573a tomato leaves (National Institute of Standards and Technology, USA), were used in the analysis, as per the Quality Assurance and Quality Control protocol.

After plant removal, the spatial distribution of Cu in the soil was measured using field portable X-ray fluorescence. A high definition X-ray fluorescence (HD-XRF) environmental analyzer was used, with a measurement time of 90 s per point. The first scanning point was positioned at a distance of 3 cm from the bottom and 3 cm from the left of the rhizobox. A total of nine scanning points was observed for each rhizobox, i.e., three points with a distance of 6 cm (length) × three points with a distance of 3 cm (width).

2.5. Enzyme activity assays

Whole plants were collected with the soils adhering to the roots. The rhizosphere soil sample was represented by a mixture of the soil attached to the roots from three individual plants, and collected by brushing the soil (with a toothbrush) from the root surface. For each treatment, three rhizosphere soil samples were collected. Then, the rhizosphere soil was used to determine soil enzyme activity after harvest.

The activity of phosphatase was measured following Saiya-Cork et al. (2002). We conducted assays using 96-well plates with eight replicates per sample per assay. The samples of each assay included a blank, a negative control, and a quench standard. Previously defrosted soil samples (1 g) were homogenized with 125 mL of 50 mM buffer for 2 h by a constant temperature shaker. The microplates were incubated in the dark at 25 °C for 4 h. During the incubation, the incubation plates were shaken every hour to ensure that the reaction mixtures were well mixed. To stop the reaction, a 1 mL aliquot of 1 M NaOH was added to each well. The fluorescence was then measured using a microplate fluorometer with 365 nm excitation and 450 nm emission filters (Xu et al., 2017). Following correction of the fluorescence measurements of the assay wells for the negative controls, blanks, and quench standard wells, enzyme activity was expressed as nanomoles of substrate released per hour per gram of 4-methylumbelliferone (MUF) (nmol g^{-1} h^{-1}).

2.6. Soil zymography and imaging procedures

After alfalfa growth, direct soil zymography was applied to study the spatial distribution of enzyme activity around the roots in situ. We followed the protocol optimized by Razavi et al. (2016). The visualization of enzyme activities requires the saturation of membranes with MUF-substrates, which become fluorescent when enzymatically hydrolyzed by a specific enzyme. The 4-methylumbelliferyl-phosphate (MUF-P), buffered by 10 mM MES ($C_6H_{13}NO_4SNa_{0.5}$) was used as the substrate to detect the phosphatase activity. For phosphatase enzymes, polyamide membrane filters (diameter = 20 cm, pore size = 0.45 μm) were saturated with the substrate. The membranes were then cut and adjusted to fit the size of the rhizoboxes. The rhizoboxes were open from the lower side and the saturated membranes were directly applied to the soil surface (Dong et al., 2007; Liu et al., 2017). Zymography for phosphatase enzyme was conducted in different rhizoboxes with three replicates. After incubation for 1 h, the membranes were carefully lifted off the soil surface and any attached soil particles were gently removed using 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, and the samples was fixed. The substrate was hydrolyzed by the enzyme and the fluorescence intensity was proportional to the enzyme activities under the UV light.

The images were processed using MATLAB and ImageJ. Briefly, the zymograms were first transformed into a 16-bit gray image matrix, and the noise and camera noise were then corrected (Sanaullah et al., 2016). 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 performed based on the zymography of 2.5 × 2.5 cm membranes, which were soaked in a MUF-solution with fluorescent tags attached to each substrate proxy at a series of calibration concentrations (i.e., 0.01, 0.2, 1, 4, 6, 8, and 10 mM). The amount of MUF per area was calculated from the solution volume taken up by the membrane and the membrane size. The membranes used for calibration were imaged under UV light and the samples were analyzed in the same way. The relation between the gray values and concentrations was described by a linear fit ($R^2 = 0.98$). Hotspots were distinguished from the surrounding area by the intensity of their color contrast in the digital images. Based on the image references and calibration lines, the color intensity of all pixels exceeding the average value (i.e., > 0.7) were designated as enzyme activity hotspots (represented by a red color in the images), (Ge et al., 2017). Moreover, the hotspot areas were presented as the percentage of total soil surface area.

2.7. Statistical analysis

The data were analyzed with a two-way analysis of variance using SPSS 20.0 (SPSS, Chicago, Illinois USA). Duncan's post-test ($p < 0.05$) was used for multiple comparisons. The final data were expressed as means ± standard deviations. All bar graphs were drawn using Origin Pro 9.0. Pearson correlation was used to measure the pairwise relationship between different variables (i.e., the soil phosphatase activity, hotspots, and others). The heat maps illustrations of correlations between enzyme activities and other properties were performed using HemI software (Version 1.0). Additionally, we determined measures of relative importance of predictor variables using the R package "relaimpo". Partial least squares path modelling (PLS-PM) was used to further identify the possible pathways by which certain categories controlled soil phosphatase activity and occurrence of hotspots. The models were constructed using the "innerplot" function of the R package "plspm".

3. Results

3.1. Plant growth

For both non-inoculated and rhizobium inoculated plants, the increasing Cu concentration strongly inhibited alfalfa growth (i.e., shoot height, root length, shoot and root biomass; Fig. 1A–D, respectively) ($p < 0.05$). Cu concentration and rhizobium inoculation had

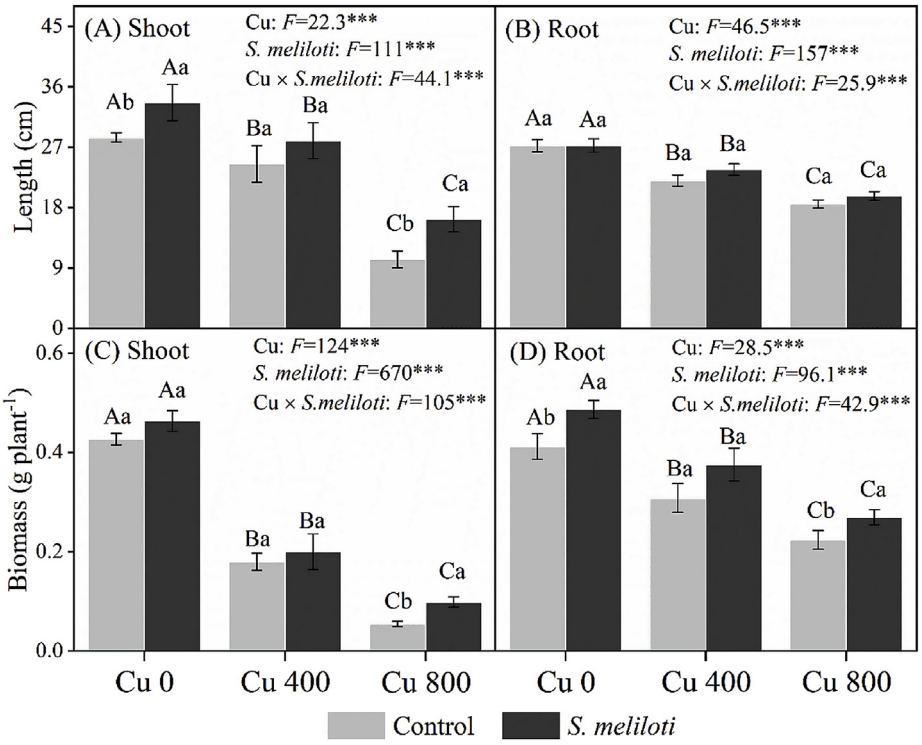

Fig. 1. The effect of inoculation with *S. meliloti* on shoot height (A), root length (B), shoot biomass (C), root biomass (D) in alfalfa with different Cu concentration treatments. The capitalized letters indicate significant differences between different Cu concentrations, whereas the lower-case letters indicate significant differences between non-inoculated and inoculated alfalfa under the same Cu concentration condition ($p < 0.05$). Each value represents the mean ± standard (n = 16). ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

significant main and interactive effects on plant growth ($p < 0.001$). In the Cu 800 treatment, the plants inoculated with rhizobium exhibited better shoot and root growth than the non-inoculated plants. For example, the growth enhancement caused by rhizobium inoculation was 44.7% for shoot biomass, while it was just 16.9% for root biomass (Fig. 1).

Cu concentration and rhizobium inoculation had significant main and interactive effects on the N content in the plant shoots and roots ($p < 0.01$; Fig. 2A and B). The shoots of non-inoculated plants showed consistent N reduction with increasing Cu (i.e., N content decreased by 25.3%–40.1% as Cu increased from Cu 400 to Cu 800), (Fig. 2A). Compared with the non-inoculated plants, the rhizobium-inoculated plants exhibited higher N content in the roots and shoots (Fig. 2B), particularly for the Cu 800 treatment. These findings indicated that rhizobium inoculation enhanced the N content in the plant shoots to counter Cu stress.

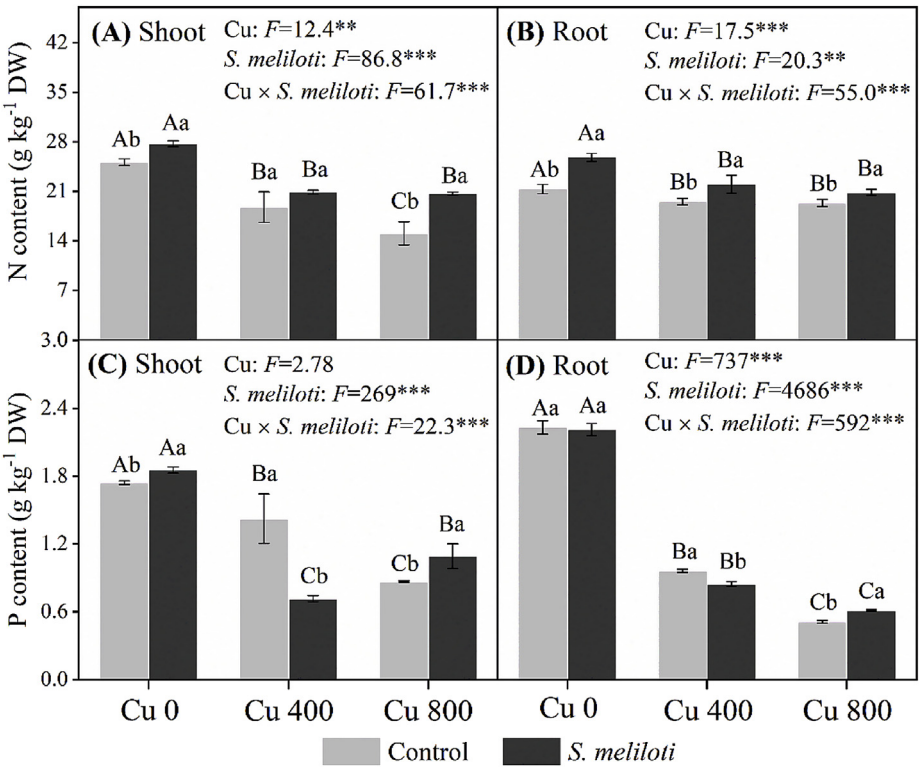

Fig. 2. The effect of inoculation with *S. meliloti* on N content in shoot (A) and root (B), and P content in shoot (C) and root (D) in alfalfa with different Cu concentration treatments. The capitalized letters indicate significant differences between different Cu concentrations, whereas the lower-case letters indicate significant differences between non-inoculated and inoculated alfalfa under the same Cu concentration condition ($p < 0.05$). Each value represents the mean ± standard (n = 3). ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

Table 1
Cu concentrations and the total uptake of Cu in alfalfa tissues.

Treatments		Cu concentrations (mg kg^{-1})		Total uptake (μg plant^{-1})		IF
		Shoot	Root	Shoot	Root	
Cu 0	Control	1.78 ± 0.27 Ba	3.25 ± 0.18 Ca	0.76 ± 0.10 Ba	1.85 ± 0.09 Bb	0.55 ± 0.06 Aa
	S.meliloti	1.51 ± 0.43 Ca	3.30 ± 0.06 Ca	0.71 ± 0.22 Ba	2.22 ± 0.09 Ba	0.46 ± 0.13 Aa
Cu 400	Control	11.1 ± 1.42 Aa	32.6 ± 2.06 Bb	2.03 ± 0.24 Aa	13.9 ± 1.75 Ab	0.34 ± 0.05 Ba
	S.meliloti	8.08 ± 0.86 Bb	56.7 ± 1.36 Ba	1.63 ± 0.10 Ab	29.4 ± 1.95 Aa	0.14 ± 0.02 Bb
Cu 800	Control	12.1 ± 0.66 Aa	49.7 ± 4.86 Ab	0.66 ± 0.07 Bb	15.5 ± 1.52 Ab	0.24 ± 0.03 Ba
	S.meliloti	10.4 ± 0.11 Ab	80.8 ± 1.88 Aa	1.03 ± 0.11 Ba	30.2 ± 2.36 Aa	0.13 ± 0.01 Bb
Factor (Df)		*F* *p*	*F* *p*	*F* *p*	*F* *p*	*F* *p*
Cu (2)		43.8 ***	296 ***	9.97 **	83.8 ***	6.11 *
S. meliloti (1)		108 ***	1970 ***	0.14 NS	519 ***	79.6 ***
Cu × *S. meliloti* (2)		39.9 ***	3.47 NS	12.0 **	27.1 ***	4.32 *

The capitalized letters indicate significant differences between different Cu concentrations, whereas the lower-case letters indicate significant differences between non-inoculated and inoculated alfalfa under the same Cu concentration condition ($p < 0.05$). Each value represents the mean ± standard (n = 3). IF (translocations factor: shoot concentration/root concentration). ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; NS: no significant.

The pattern of P content was similar to that of N in non-inoculated plants. The P content in shoots and roots consistently decreased with increasing Cu concentration ($p < 0.05$), (Fig. 2C and D). Rhizobium inoculation had a significant effect, and Cu concentration and rhizobium inoculation had significant interactive effects, on the P content in plant shoots ($p < 0.01$). The most substantial reduction was observed in the roots as the Cu concentration increased from Cu 0 to Cu 800. Unexpectedly, rhizobium inoculation caused a P reduction by 49.8% in the Cu 400 treatment (Fig. 2C). In roots, the P content in the inoculated plants was also lower than in the non-inoculated at Cu 400 (Fig. 2D).

3.2. Concentrations and total uptake of Cu in plants

Remarkably, the effect of rhizobium on Cu concentration was not uniform between the shoot and root parts of the alfalfa plants (Table 1). Cu concentration and rhizobium inoculation had significant main and interactive effects on the Cu content in the shoots, the Cu uptake in the roots, and the Cu transfer coefficients ($p < 0.05$). Compared with the non-inoculated plants, rhizobium inoculation noticeably decreased the Cu concentration in the shoots but significantly increased the Cu in the roots ($p < 0.05$). Between the non-inoculated and inoculated plants, the difference in Cu uptake in the shoot part was not consistent and depended on specific Cu treatments. However, in the roots, the Cu uptake by the inoculated plants was significantly higher than that by the non-inoculated plants for all Cu treatments ($p < 0.05$). Collectively, the results show that rhizobium inoculation had a more pronounced and consistent effect on the total Cu uptake of roots than the shoots under the same Cu treatments. The Cu transfer coefficients were considerably less than 1.0 for all cases and decreased with increasing Cu concentration. Compared with the non-inoculated plants, rhizobium inoculation significantly and consistently reduced the Cu transfer coefficients ($p < 0.05$).

3.3. Soil phosphatase and distribution of phosphatase activities

The phosphatase activity of rhizosphere soil significantly changed with increasing Cu concentration (Fig. 3). Moreover, Cu concentration and rhizobium inoculation had significant main and interactive effects on phosphatase activity ($p < 0.05$). In the non-inoculated treatments, and compared with the Cu 0 treatment, the phosphatase activity was significantly enhanced in the Cu 400 treatment but was significantly reduced in the Cu 800 treatment. Compared with the non-inoculated plants, rhizobium inoculation increased the phosphatase activity by 13.6% and 38.4% for the Cu 0 and Cu 800 treatments, respectively ($p < 0.05$); while, rhizobium inoculation significantly reduced the phosphatase activity in the Cu 400 treatment ($p < 0.05$).

The two-dimensional distribution of phosphatase activity on the

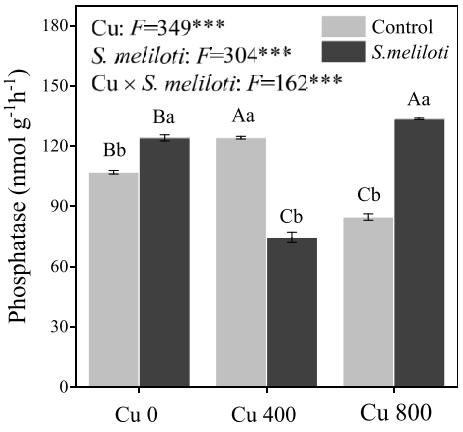

Fig. 3. The effect of inoculation with *S. meliloti* on soil phosphatase activity with different Cu concentration treatments. The capitalized letters indicate significant differences between different Cu concentrations, whereas the lower-case letters indicate significant differences between non-inoculated and inoculated alfalfa under the same Cu concentration condition ($p < 0.05$). Each value represents the mean ± standard (n = 3). ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

zymograms was pronounced for the soil–root surface (Fig. 4). The hotspot areas of phosphatase activity were associated with the roots and were the highest at the root tips, both in the rhizobium-inoculated and non-inoculated treatments. In the non-inoculated treatments, the hotspots contributed to 7.3% of the total area of phosphatase activity and increased considerably to 12.8% and 16.1% in the Cu 800 and Cu 400 treatments, respectively (Fig. 5). Furthermore, Cu concentration had a significant effect, and Cu concentration and rhizobium inoculation had significant interactive effects, on the hotspot areas ($p < 0.01$). Compared with the non-inoculated treatment, the total hotspot areas of enzyme activity were slightly higher in the rhizobium-inoculated Cu 0 treatment. For the Cu 800 treatment, the hotspot areas were 12.8% in the non-inoculated plants, and was accompanied by an increase in hotspot area after rhizobium inoculation (Figs. 4 and 5). However, rhizobium inoculation significantly reduced the hotspot area of phosphatase activity in the Cu 400 treatment ($p < 0.05$).

3.4. Soil phosphatase correlation matrix

Pearson correlation was used to analyze the plant and soil properties and phosphatase activity (Fig. S4). Phosphatase activity showed a negative correlation with the total N content ($r = -0.48$, $p < 0.05$) and AP ($r = -0.55$, $p < 0.05$) in soil, but a positive correlation with the total P content ($r = 0.48$, $p < 0.05$) in soil. The phosphatase activity

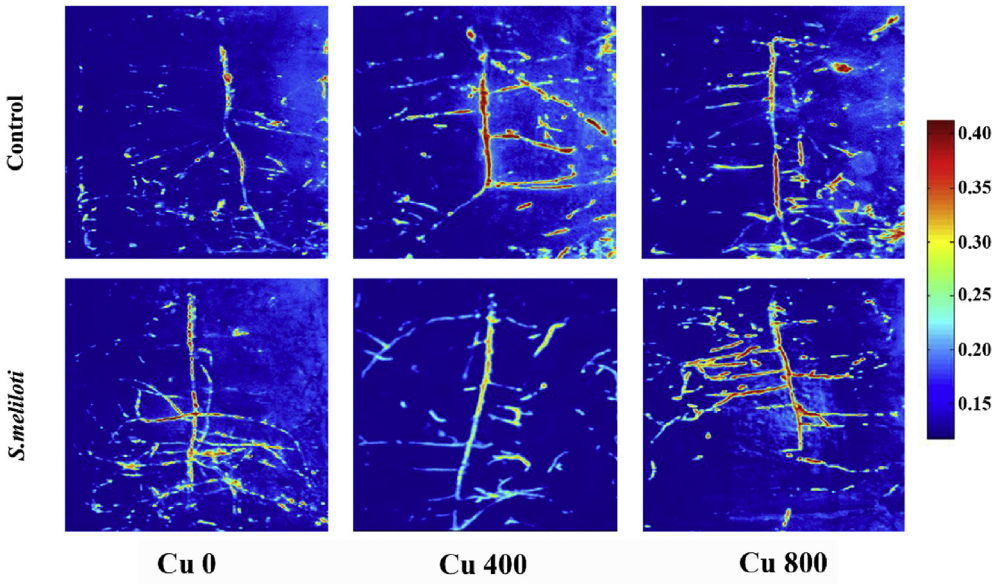

Fig. 4. The zymograms of acid phosphatase activity for the rhizosphere. Side color maps indicate enzyme activities (nmol cm^{-2} h^{-1}). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

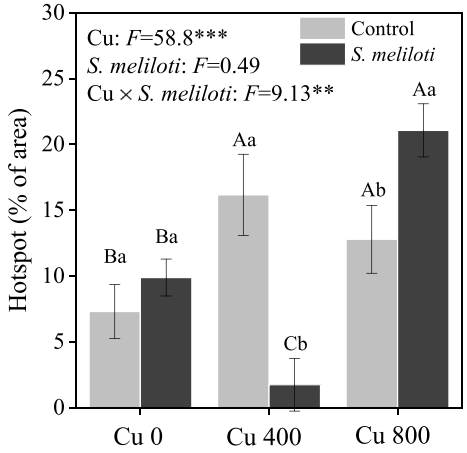

Fig. 5. Enzymatic hotspot areas of phosphatase in non-inoculated and inoculated in the alfalfa rhizosphere. The capitalized letters indicate significant differences between different Cu concentrations, whereas the lower-case letters indicate significant differences between non-inoculated and inoculated alfalfa under the same Cu concentration condition ($p < 0.05$). Each value represents the mean ± standard (n = 3). ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

showed a significantly negative correlation with the shoot N:P ratio ($r = -0.62$, $p < 0.01$) and a positive correlation with the P content in the shoots ($r = 0.61$, $p < 0.01$). In addition, the soil N:P showed the strongest negative correlation with phosphatase activity ($r = -0.60$, $p < 0.01$). The shoot N:P showed a negative correlation with the hotspot areas ($r = -0.50$, $p < 0.05$).

The stepwise linear regression (Fig. 6) identified the P content in the alfalfa shoots as the key variable to the soil phosphatase activity (model strength 98.1%, Fig. 6A). The hotspot area of phosphatase on the other hand was best explained by the Cu content in soil (model strength 98.9%, Fig. 6B). The relative influence of the Cu content in the soil was 13.4% (Fig. 6B). To minimize the confounding interactions among causal factors, PLS-PM was implemented to further reveal the possible pathways through which the soil and plant indexes guide the spatial distribution of phosphatase (Fig. 7). The model indicated a best fit to the data with goodness-of-fit of 0.68. The soil Cu content directly influenced the soil phosphatase activity and the hotspot areas of phosphatase activities in the rhizosphere, which explained 59% and 86% of the variation, respectively (Fig. 7A). The soil N:P (−1.54), plant stoichiometry (−0.80), and AP (−0.62) had negative total effects on the hotspot areas of phosphatase activities, whereas the soil Cu content (0.53) and phosphatase (0.66) induced positive total effects (Fig. 7B).

4. Discussion

Our results showed that the rhizobium inoculation significantly improved the growth of plants in soil contaminated with copper by enhancing nutrition and plant biomass (Figs. 1 and 2). Our results showed a significant decrease of N and P content in shoots and roots with increasing Cu contamination, indicating the mechanism of alfalfa to encounter metal stresses by reducing N and P content (Fig. 2). Remarkably, rhizobium inoculation strongly reduced the P content in the Cu 400 treatment ($p < 0.05$). In addition, rhizobium inoculation increased soil AP in the Cu 400 treatment (Fig. S2). Due to the excessive accumulation of Cu in the roots of alfalfa, the upward transport of P in the underground part may be blocked, so the P content in the shoot is the lowest (Png et al., 2017; Chen et al., 2018). The metal-resistant rhizobia promoted the growth of plants through regulating the biological nitrogen fixation and the consequent effects on metal solubility and bioavailability in soils (Pajuelo et al., 2011). Our results showed that rhizobium inoculation significantly increased the total amount of Cu uptake and Cu concentration in shoots under Cu stress (Table 1). Meanwhile, rhizobium inoculation can reduce the Cu transfer coefficients in the plant. This indicates that the alfalfa–rhizobium symbiosis plays an important role in preventing toxic metal from entering the food chain and ultimately affecting the health of humans.

The hotspots of phosphatase activity were distributed along the living roots, both in the non-inoculated and inoculated treatments (Fig. 4). A continuous distribution of exoenzymes along alfalfa roots is related to the nutrient acquisition strategy along the whole root (Schnepf et al., 2008; Hinsinger et al., 2011). Moreover, our previous studies revealed a homogeneous enzyme distribution along the roots of legumes, probably due to feeding microorganisms (mainly rhizobia) fixing N_2 (Razavi et al., 2016; Duan et al., 2018). Because rhizobia colonization can occur anywhere along the legume root, the roots should maintain an attractive rhizosphere environment for potential symbionts (Koivunen et al., 2003). Furthermore, *S. meliloti* are capable of producing siderophores and can thus relieve plants from heavy metal stress by forming stable complexes with heavy metals, such as Cu

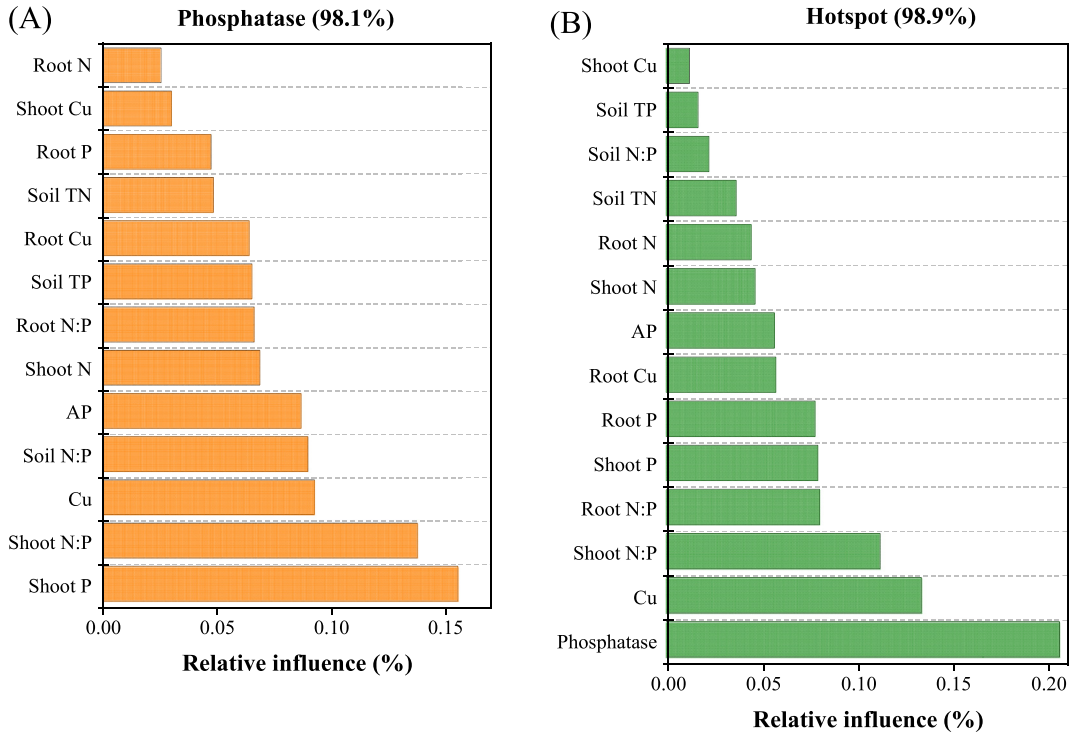

Fig. 6. Relative importance (%) of index drivers for soil phosphatase activity (A) and hotspot (B). Hotspot, the hotspot areas of phosphatase; TN, total N; TP, total P; AP, available phosphorus; Shoot Cu, shoot Cu concentration; Shoot N, shoot N concentration; Shoot P, shoot P concentration; Root Cu, root Cu concentration; Root N, root N concentration; Root P, root P concentration; Shoot N:P, shoot N:shoot P; Root N:P, root N:root P.

(Neubauer et al., 2000), i.e., by reducing the Cu concentration in the soil around the root system, as seen in the present study (Fig. S3). Yang et al. (2019) concluded that *Sedum alfredii* had a lower heavy metal content in the rhizosphere, but that the phosphatase activity was significantly higher than that of bulk soil. The higher enzyme activity of the rhizosphere than of root-free soil depends not only on microbial activity but also on the direct release of enzymes by roots or by lysis of root cells (Jones et al., 2009; Gianfreda, 2015; Zhang et al., 2019b).

The phosphatase activity area along the roots was significantly increased in the treatment of Cu-contamination (Figs. 3–5). Our results showed that the phosphatase activity and the hotspot areas were the highest in the Cu 400 treatment. Cu concentration had significant main and interactive effects on phosphatase activity ($p < 0.05$). Further analysis also indicated that the soil Cu content had the strongest positive effects on the hotspots (Figs. 6 and 7). Compared with the control, the increase in phosphatase was likely due to increased microbial activity (Bradford et al., 2008; Steinweg et al., 2008) and root exudates stimulated by Cu concentration (Wan et al., 2016). Our previous studies

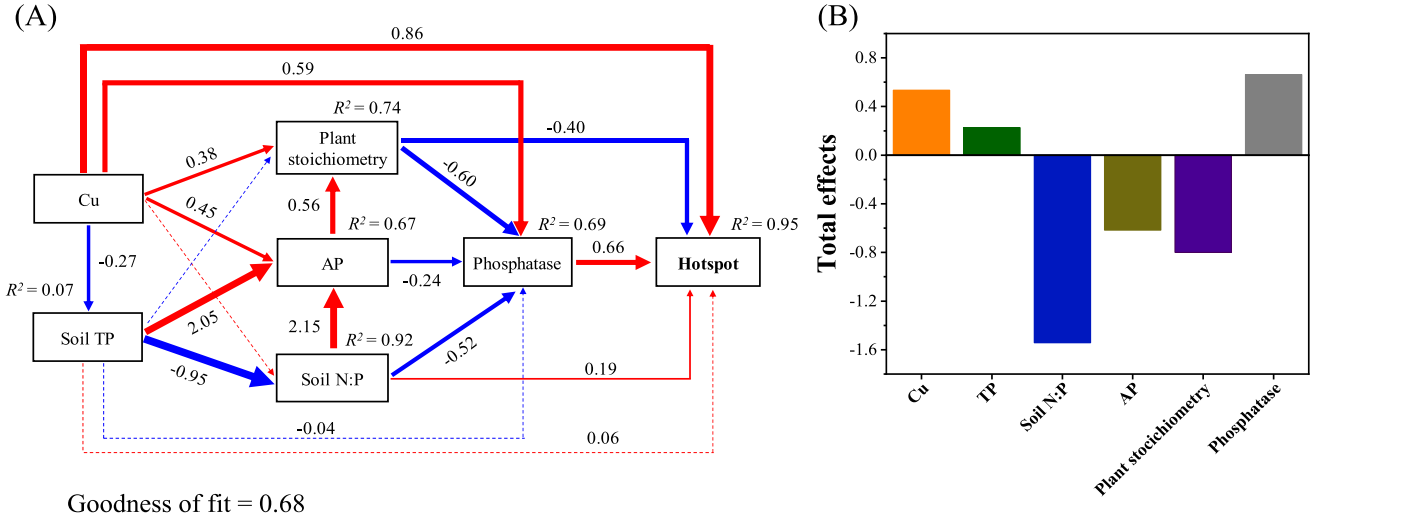

Fig. 7. Partial least squares path modeling (PLS-PM) showed the direct and indirect effects of soil Cu, soil N:P, plant stoichiometry, AP and TP on phosphatase and hotspot. The width of arrows is proportional to the strength of path coefficients. Blue and red arrows indicate positive and negative flows of causality ($p < 0.05$), respectively. Numbers on the arrow indicate significant standardized path coefficients. R^2 indicates the variance of dependent variable explained by the model. Hotspot, the hotspot areas of phosphatase; plant stoichiometry including: shoot (N:P), root (N:P); TP, total P; AP, available phosphorus. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

have consistently indicated that phosphatase activities in the plant–soil interface increased under heavy metal stress (Duan et al., 2018). At the same time, Cu stress can stimulate more total peroxidase in alfalfa roots to alleviate the toxicity, and thus increase microbial activity and enzyme activity in the rhizosphere (Ali et al., 2017). This indicates that exposure to lower concentrations of heavy metal can promote either phosphatase production or secretion as a result of copper-stress conditions. It is possible that enhanced phosphatase production is a metal detoxification response to deposit heavy metal harmlessly away from sensitive cellular sites (Png et al., 2017). However, under high Cu concentrations, the combination of copper ions with the protein-active groups of the enzymes inhibits the activity of phosphatase (Jin et al., 2015). In general, soil enzyme activity varies with the concentration and form of heavy metals (Yang et al., 2016).

The rhizobium inoculation obviously increased the phosphatase activity, especially in the Cu 0 and Cu 800 treatments (Figs. 3–5), mainly because rhizobia can promote root growth and produce a higher abundance of root exudates; in turn, this enhances microbial activity and enzyme activity (Dary et al., 2010; Nannipieri et al., 2012). Our results showed that the rhizobium inoculation can improve the absorption of N and P and provide balanced nutrition to plants (Fig. 2), possibly by stimulating the microbes, which alters the N and P content in the soil, which in turn promotes the production of P-solubilizing bacteria, thereby promoting the phosphatase activity in soil. In addition, such changes may be attributed to the increased soil N content as a result of rhizobia addition, which increases the rhizodeposits and provides more readily available nutrition sources for microbes (Ogunkunle et al., 2018). Previous studies indicated that rhizobium inoculation significantly increased the enzymatic activities which could be due to a shift in the soil chemical properties such as increased N and P and decreased soil toxicity (Sipahutar et al., 2018; Ju et al., 2019). Thus, in the present study, *S. meliloti* not only reduced Cu toxicity, but simultaneously enhanced P and N mobilization and, as a result, promoted plant growth and survival under stressful conditions.

The rhizobium inoculation significantly reduced the phosphatase activity in the Cu 400 treatment (Figs. 3 and 4). The distribution and production of exoenzymes are affected by the demand for nutrients by the plants and microorganisms (Frank and Groffman, 2009). P is an essential nutrient and a component of key molecules, such as nucleic acids and phospholipids, and is involved in controlling key enzyme reactions (Wardle, 1992; Tischer et al., 2015). Previous studies have shown that the elevated soil P content could lead to the reduction of metal availability and the improvement of soil nutrient status, thus promoting root exudation and improving enzyme activities (Benidire et al., 2016). Compared with the non-inoculated treatment, the soil TP content significantly decreased, and the AP significantly increased in the inoculated Cu 400 treatment ($p < 0.05$) (Fig. S2). We further linked the phosphatase activity to the P content. The results indicated that the P content had a positive effect on the phosphatase activity, but that AP had a negative effect on phosphatase activity (Fig. S4). The results of PLS-PM further supported that the AP directly determined the phosphatase activity (Fig. 7). Phosphatase activities reflect the plant and microbial investment for P acquisition (Wei et al., 2018). Generally, microbial production of phosphatases is repressed by inorganic P (Nannipieri et al., 2011; Wei et al., 2019b).

Our results indicated that the soil Cu content had the strongest positive effect on the hotspot areas (Figs. 6 and 7). Soil enzymes are a product of microbial cellular metabolism and can serve as proxies for microbial activity (Spohn et al., 2015). Previous studies have shown that soil enzyme activities decrease exponentially with increasing heavy metal concentrations (Liu et al., 2018). However, in the present study, the extent of soil enzyme activities differed in response to different Cu concentrations. Additionally, the observed decrease in phosphatase was likely due to the highest accumulation of Cu in the alfalfa roots in the Cu 400 treatment (Table 1), and because excessive Cu alters the affinity of enzymes to their substrate (Chou et al., 2019). Therefore, we concluded that the decrease in phosphatase activity was mainly attributed to the increase in AP caused by the inoculation of rhizobium in Cu-polluted soil (Figs. 6 and 7) and that the decrease in the hotspot area was primarily attributed to the addition of Cu to the soil.

5. Conclusions

This study showed that the legume–rhizobium symbiosis functioned in alleviating excess Cu-induced growth inhibition and promoting the phosphatase activity in the alfalfa rhizosphere under high Cu concentration stress. The spatial distribution of phosphatase activities was associated with the presence of roots, which confirmed that the rhizosphere shapes microbial functionality (e.g., nutrient mobilization). More importantly, this study clearly demonstrated that rhizobium inoculation can broaden the hotspot areas of phosphatase, as a result of increased microbial activity and enzyme synthesis by the production of P-solubilizing bacteria, especially under very high Cu stress. In summary, our study provided a direct evidence to illustrate the relationship between Cu, rhizobium and P in the legume–rhizobium symbiosis. The above results will add fundamental knowledge to our understanding of the mitigation mechanism of legume–rhizobium symbiosis in counteracting Cu toxicity and provide a basis for the biogeochemical reactions in the root–soil interfaces of polluted soils and their spatio-temporal dynamics.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2019.107574.

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