Supporting information for

A visual leaf zymography technique for the *in situ* examination of plant enzyme activity under the stress of environmental pollution

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Supporting information M1

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1. Xylanase activity kit instructions

(1) Measurement principle

Xylanase catalyzes the hydrolysis of xylan into oligosaccharides and monosaccharides in a neutral environment. A color reaction, with a characteristic absorption peak at 540 nm, was developed with 3,5-dinitrosalicylic acid in a boiling water bath. The color of the reaction solution was proportional to the amount of reducing sugar produced by the enzymatic hydrolysis. The xylanase activity was calculated by measuring the increased absorption rate of the reaction solution at 540 nm.

(2) Reagent composition

Extraction solution: liquid 65 mL × 1 bottle, stored at 4°C.

Reagent 1: 10 mL × 1 bottle of liquid, stored at 4°C, protected from light.

Reagent 2: 10 mL × 1 bottle of liquid, stored at 4°C, protected from light.

(3) Crude enzyme extraction

Fresh tissue (0.1 g) was mixed with 1 mL of extract solution and homogenized in an ice bath, centrifuged at 4°C (8000 × *g* for 10 min), and the supernatant was then used for testing.

(4) Determination

The spectrophotometer reader was preheated for 30 min and the wavelength was adjusted to 540 nm. The following reagents were added to an Eppendorf (EP) tube:

	Control tube	Sample tube
Sample (μL)	60	60
Extraction solution (μL)	90	90
Reagent 1 (μL)		60
Reagent 2 (μL)	90	

Samples and reagents were mixed and placed in a water bath at 50°C for 30 min. Then, they were immediately inactivated in a boiling water bath for 10 min.

Reagent 1 (μL)	60	
Reagent 2 (μL)		90

Samples and reagents were mixed and color was developed in a boiling water bath for 5 min. Then, 200 μL of the liquid in each EP tube was placed into a micro quartz cuvette to measure the absorbance at 540 nm.

Enzyme activity definition: at 50°C and pH 6.0, the amount of enzyme produced per min per g of sample that was required to decompose xylan into 1 nmol reducing sugar was defined as a unit of neutral xylanase activity.

$$Xylanase\ activity\ \left[nmol/\left(min\cdot g\ fresh\ weight\right)\right]=\frac{\left(\Delta A-0.0058\right)\cdot T\cdot 150}{Dilution\ factor\cdot 1.6904\cdot 10^{6}}=657\cdot\frac{\left(\Delta A-0.0058\right)}{W}$$

where $\Delta A = A_{\text{test tube}} - A_{\text{control tube}}$; 150 is the molecular weight of xylose; T is the reaction time (30 min); dilution factor = 5; and W is sample mass, g.

2. Phosphatase activity (ACP) kit instructions

(1) Measurement principle

In an acidic environment, ACP catalyzes the hydrolysis of disodium p-nitrophenyl phosphate to 4-nitrophenol, with a characteristic absorption peak at 405 nm. The ACP activity was calculated by measuring the increasing absorption rate at 405 nm.

(2) Reagent composition and preparation

Reagent 1: Liquid 60 mL × 2 bottles, stored at 4°C.

Reagent 2: Liquid 50 mL × 1 bottle, stored at 4°C.

Reagent 3: Liquid 50 mL × 1 bottle, stored at 4°C.

(3) Crude enzyme extraction

Fresh tissue (0.1 g) was mixed with 1 mL of reagent 1, homogenized in an ice bath, and centrifuged at 4°C (10,000 × *g* for 10 min). The supernatant was used for testing.

(4) Determination

A microplate reader was preheated for 30 min and the wavelength was adjusted to 405 nm. The following reagents were added to an EP tube:

Reagent name (μL)	Sample tube	Control tube
Sample	10	10
Reagent 1	90	990
Reagent 2	900	

Samples and reagents were mixed and protected from light at 30°C for 30 min.

Reagent 3	400	400

Samples and reagents were mixed, and 200 μL of liquid from each EP tube was placed into a micro quartz cuvette. The absorbance of each tube was then measured at 405 nm. For each sample tube there was a control tube.

Enzyme activity definition: at 30°C, the amount of enzyme produced per min per g of sample that was required to decompose 1 μmol of 4-nitrophenol was defined as a unit of ACP activity.

$$\text{ACP } activity\ \left[\mu mol/\left(min\cdot g\ fresh\ weight\right)\right]=\frac{\left(\Delta A-0.0179\right)\cdot V_1\cdot V_3}{7.3336\cdot T\cdot W\cdot V_2}=0.4545\cdot\frac{\left(\Delta A-0.0179\right)}{W}$$ where

$\Delta A = A_{\text{test tube}} - A_{\text{control tube}}$; V_1 is the total volume of reaction (1 mL); V_2 is the volume of sample (0.01 mL); V_3 is the volume of reagent 1 (1 mL); T is the reaction time (30 min); and W is sample mass, g.

3. β-Glucosidase (β-GC) activity kit instructions

(1) Measurement principle

β-GC catalyzes p-nitrobenzene-β-D-glucopyranoside to p-nitrophenol, which has a maximum absorption peak at 400 nm. The β-GC activity was calculated by measuring the increased absorption rate at 400 nm.

(2) Reagent composition

Extraction solution: liquid 100 mL × 1 bottle, stored at 4°C.

Reagent 1: powder in 1 bottle, stored at −20°C, with 12 mL of distilled water added to the bottle. The powder was fully dissolved just before use. Unused reagents were stored at −20°C.

Reagent 2: 15 mL liquid × 1 bottle, stored at 4°C.

Reagent 3: 15 mL liquid × 1 bottle, stored at 4°C.

(3) Crude enzyme extraction

Fresh tissue (0.1 g) was mixed with 1 mL of reagent 1, homogenized in an ice bath, and centrifuged at 4°C (15,000 × *g* for 10 min). The supernatant was placed on ice for testing.

(4) Determination

A spectrophotometer or microplate reader was preheated for more than 30 min. The wavelength was adjusted to 400 nm. The following reagents were added to an EP tube:

Reagent name (μL)	Sample tube	Control tube
Reagent 1	120	
Distilled water		120
Reagent 2	150	150
Sample	30	30

Samples and reagents were mixed, placed into a 37°C water bath for 30 min, and then immediately placed in a 95°C water bath for 5 min. The EP tubes were mixed thoroughly after being cooled under running water. Each EP tube was centrifuged at 4°C (8000 × *g* for 5 min). The following reagents were added to the supernatant:

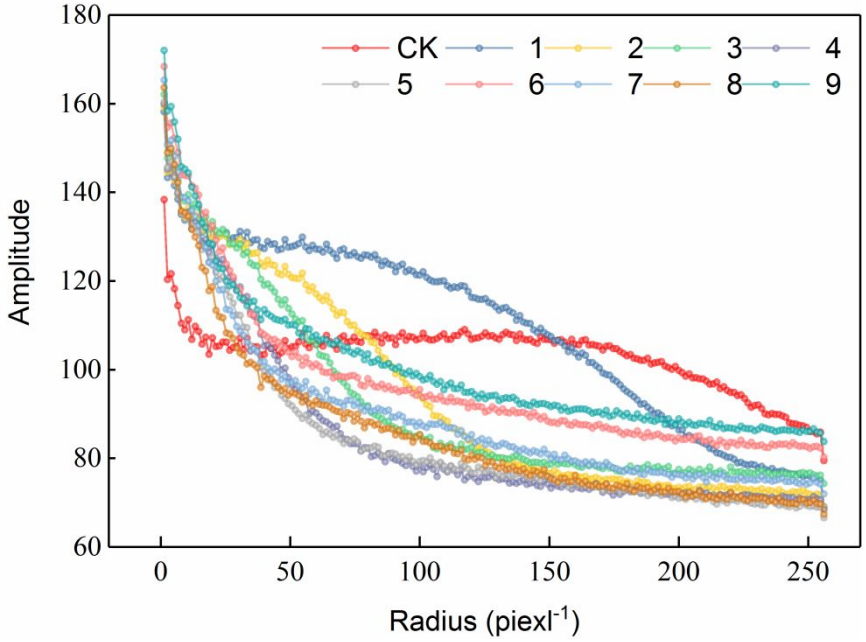
Supernatant	70	70
Reagent 3	130	130

The extract solutions in EP tubes were mixed thoroughly. After standing at room temperature for 2 min, the absorbance of each EP tube was measured at 400 nm. For each sample tube there was a control tube.

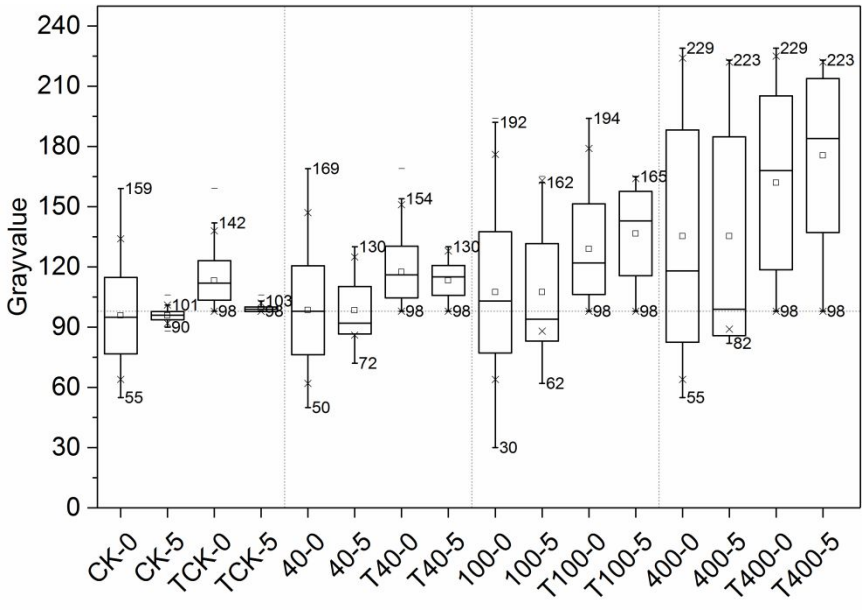
Enzyme activity definition: the amount of p-nitrophenol produced per min per g of tissue was defined as a unit of enzyme activity.

$$\beta - GC\ activity\ \left[nmol/\left(min \cdot g\ fresh\ weight\right)\right] = \frac{\left(\Delta A + 0.0027\right) \cdot V_1 \cdot V_3}{0.00585 \cdot T \cdot W \cdot V_2} = 56.98 \cdot \frac{\left(\Delta A + 0.0027\right)}{W}$$

where $\Delta A = A_{\text{test tube}} - A_{\text{control tube}}$; V_1 is the total volume of reaction (1 mL); V_2 is the volume of sample (0.01 mL); V_3 is the volume of extraction solution (1 mL); T is the reaction time (30 min); and W is sample mass, g.



(a)



(b)

Fig. S1 Estimation of the denoising of images: (a) radial profile plot of FFT images and (b) box chart of calibration images, with or without the reconstruction process at different MUF concentrations.

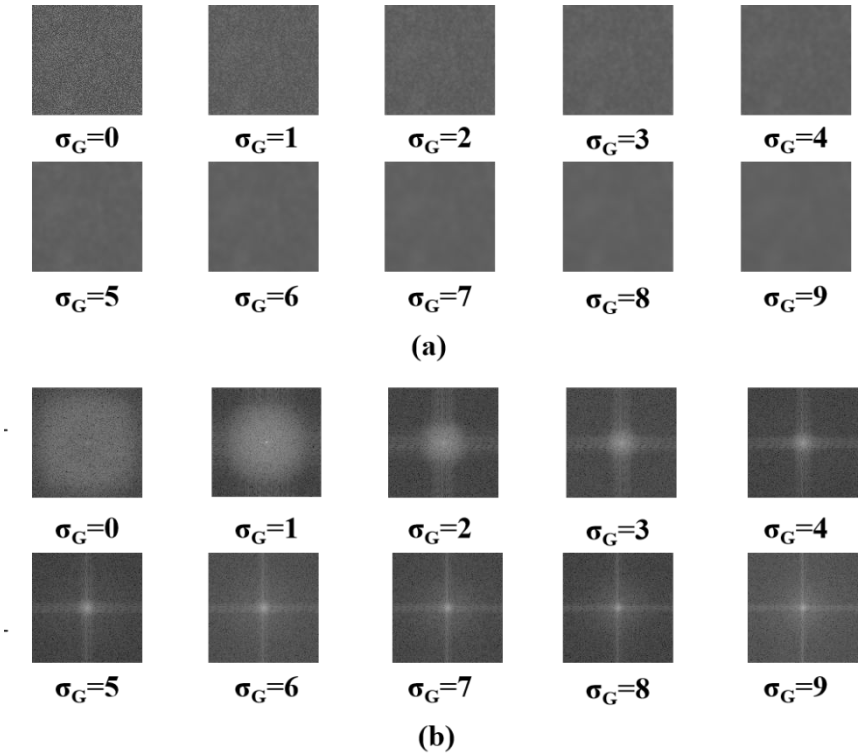


Fig. S2 Spatial domain and frequency domain of the calibration images: (a) spatial domain and (b) frequency domain.

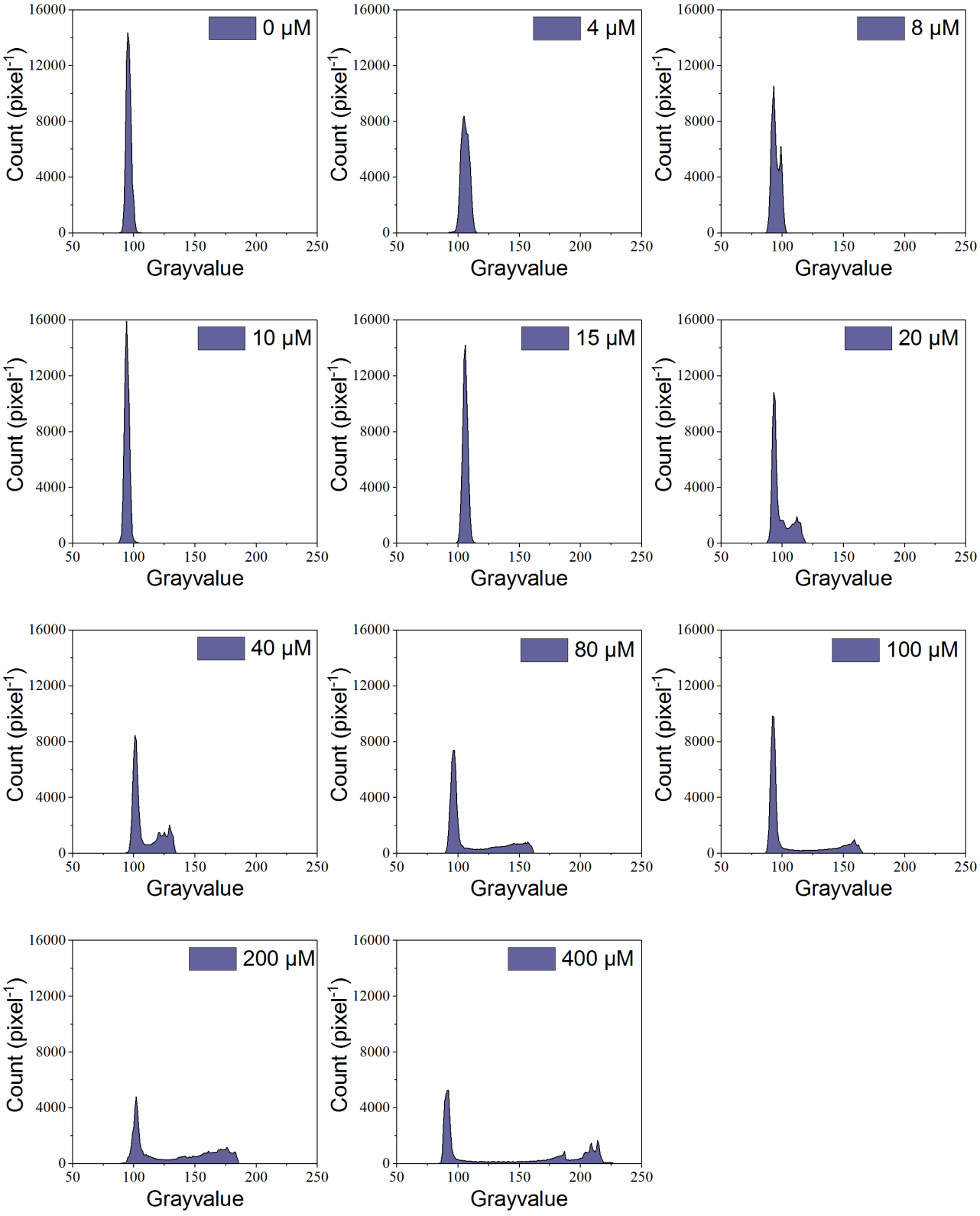


Fig. S3 Histograms of spatial calibration images.

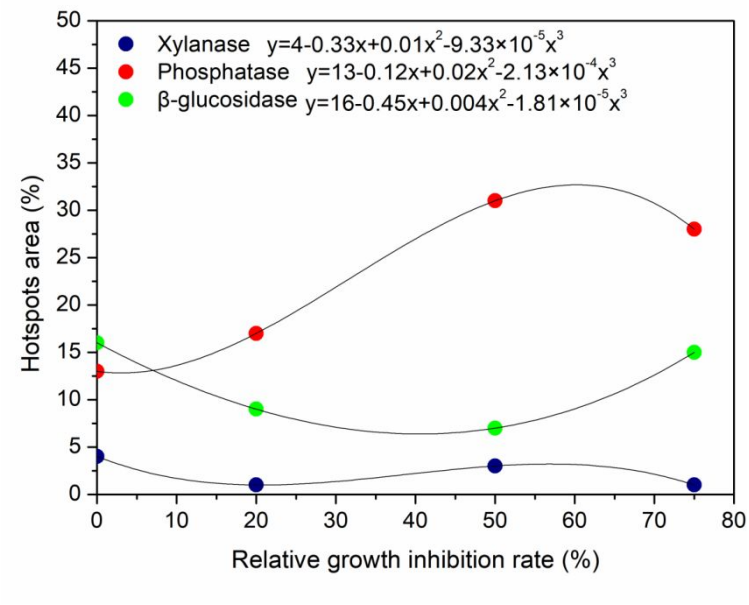
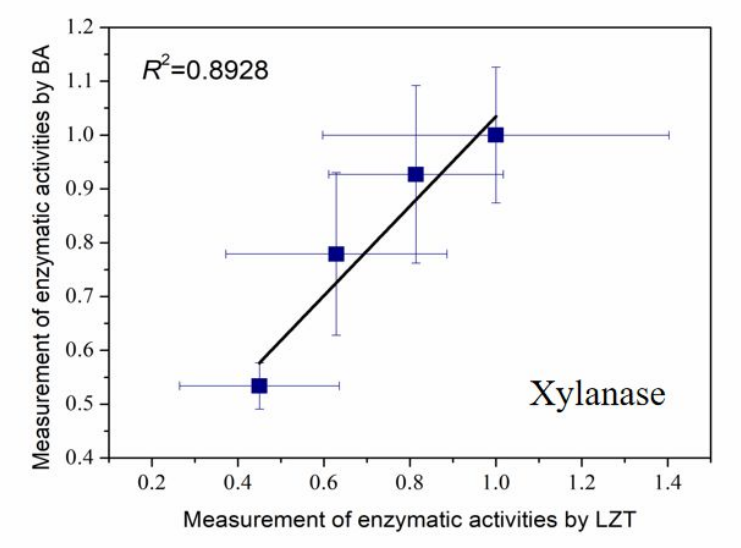
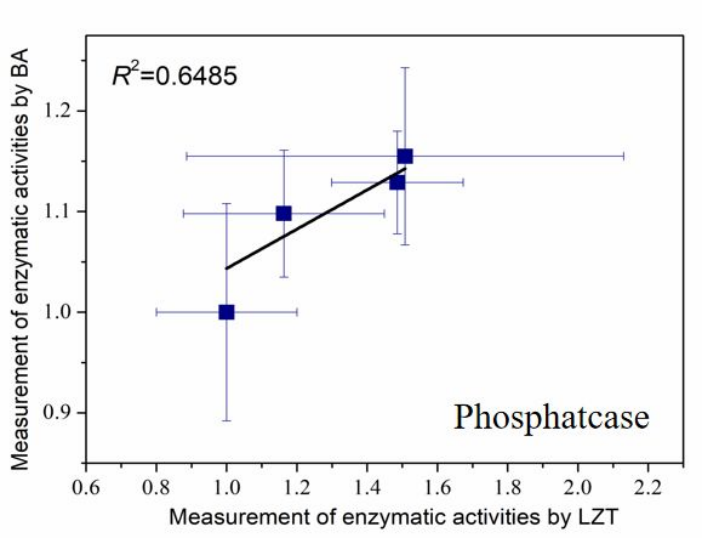


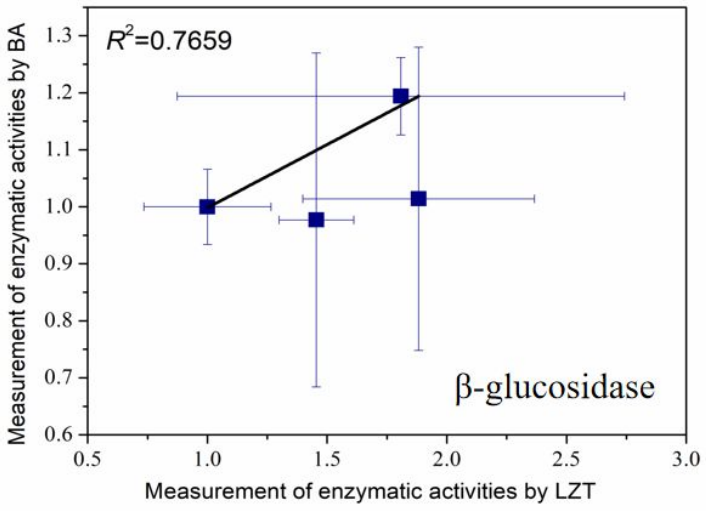
Fig. S4 Relationship between hotspot areas and EC.



（a）



（b）



（c）

Fig. S5 Comparison of the fold changes measured by the leaf zymography technique, with data obtained from a biochemical analysis for (a) xylanase, (b) phosphatase, and (c) β-glucosidase.

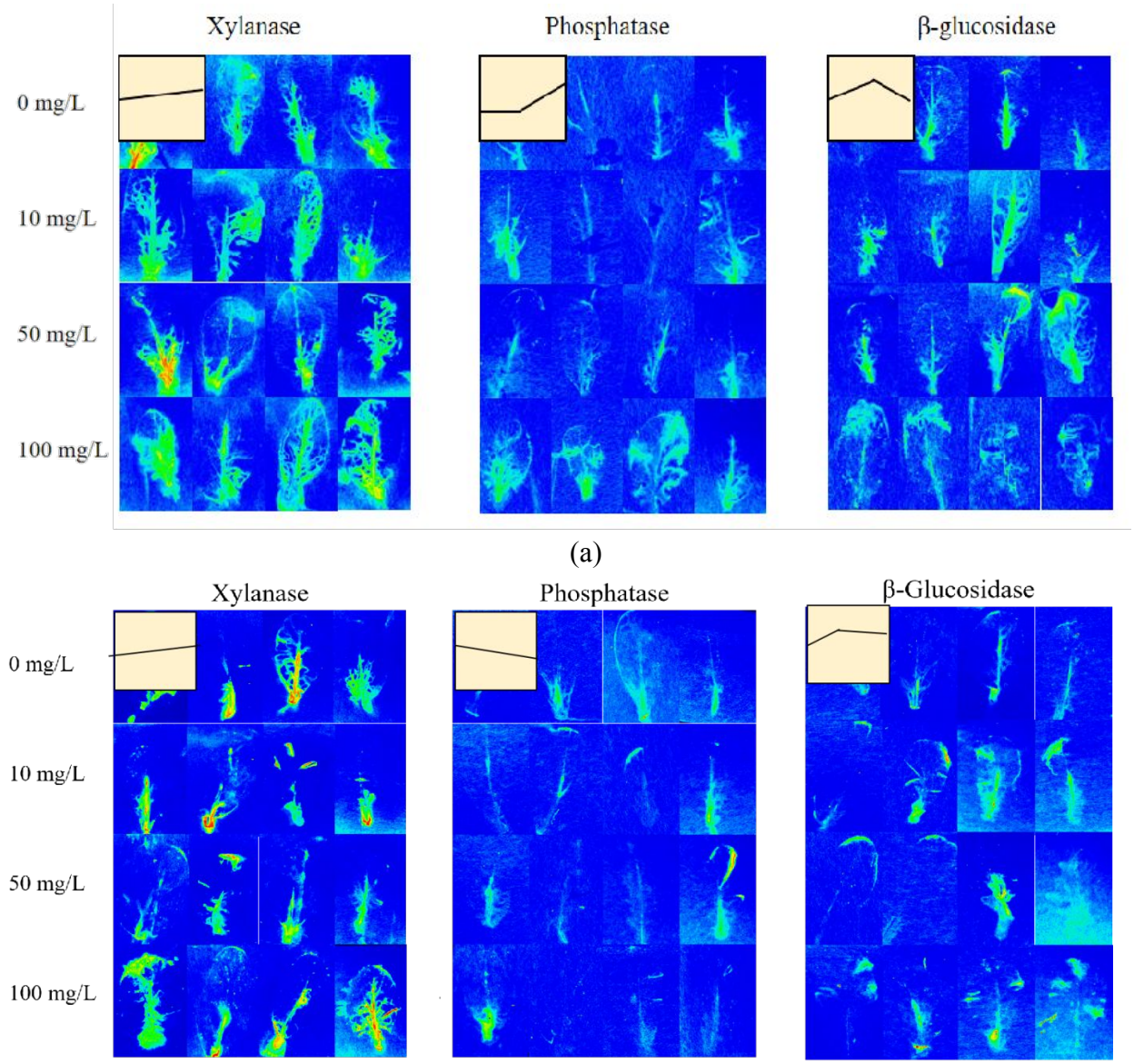


Fig. S6 The spatial distribution of enzyme activities under heavy metal stress: (a) lead stress and (b) copper stress.

Table S1 Sensitive analysis (Equation： $y = A_0 + A_1x + A_2x^2 + A_3x^3 + A_4x^4$)

DBP concentrations (mg/L)	0	10	50	100
			β-glucosidase	
A_0	132.5±1.74	130.8±2.65	147.6±2.08	141.8±2.13
A_1	0.63±0.09	0.91±0.14	0.17±0.09	0.65±0.11
A_2	-0.010±0.001	-0.014±0.002	-0.006±0.001	-0.006±0.001
A_3	8.22E-5±1.00E-5	6.87E-5±1.44E-5	2.87E-5±5.73E-6	1.23E-5±9.74E-6
A_4	-1.55E-7±2.02E-8	-1.00E-7±2.86E-8	-4.32E-8±9.00E-9	6.16E-9±1.82E-8
Reduced Chi-Sqr	126	298	229	203
R-Square(COD)	0.53	0.37	0.52	0.60
Adj. R-Square	0.53	0.37	0.52	0.59
			phosphatase	
A_0	115.2±1.47	130.8±2.65	124.1±1.73	141.8±2.13
A_1	1.49±0.08	0.91±0.14	0.97±0.09	0.64±0.11
A_2	-0.02±0.00	-0.01±0.002	-0.01±0.00	-0.006±0.001
A_3	1.48E-4±7.60E-6	6.87E-5±1.44E-5	6.09E-5±9.77E-6	1.23E-5±9.74E-6
A_4	-2.60E-7±1.481E-8	-1.00E-7±2.86E-8	-7.70E-8±1.96E-8	6.16E-9±1.82E-8
Reduced Chi-Sqr	93	298	125	203
R-Square(COD)	0.74	0.37	0.67	0.60
Adj. R-Square	0.74	0.37	0.67	0.59
			xylanase	
A_0	130.4±1.54	123.3±1.06	130.2±1.68	124.6±1.23
A_1	-0.09±0.078	0.22±0.054	0.019±0.107	-0.006±0.07
A_2	0.002±0.001	-0.007±8.21E-4	-9.59E-4±0.002	-0.002±0.001
A_3	-2.37E-5±6.42E-6	4.14E-5±4.531E-6	-1.16E-5±1.37E-5	1.17E-5±7.37E-6
A_4	5.59E-8±1.15E-8	-7.10E-8±8.18E-9	5.84E-8±3.14E-8	-1.54E-8±1.51E-8
Reduced Chi-Sqr	109	51	105	62
R-Square(COD)	0.46	0.71	0.60	0.61
Adj. R-Square	0.45	0.71	0.60	0.61

Table S2 Hotspots analysis

Enzymes	Activities (%)	DBP concentrations (μmol/L)			
		0	10	50	100
Xylanase	0-25	6758±3488	6440±2660	4966±2273	5275±2452
	25-50	4447±3828	1667±636	1683±1038	1056±346
	50-75	1192±507	528±258	547±187	397±173
	75-100	520±365	152±124	224±107	109±74
β-Glucosidase	0-25	9601±2048	5021±3048	8379±2181	7232±2675
	25-50	3630±1181	3462±539	5182±1741	5023±1944
	50-75	1434±750	2698±1588	1537±792	2205±268
	75-100	707±485	1353±618	605±282	975±296
Phosphatase	0-25	5395±2328	6668±2173	6093±1372	5767±4578
	25-50	1442±583	2787±1412	1995±1597	2363±1205
	50-75	1302±294	1648±963	1582±1164	1885±858
	75-100	1462±555	1403±560	2085±1110	1960±883