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ORIGINAL PAPER

Spatial distribution and catalytic mechanisms of β-glucosidase activity at the root-soil interface

Muhammad Sanaullah[1,2] · **Bahar S. Razavi**[1] · **Evgenia Blagodatskaya**[1,3] · **Yakov Kuzyakov**[1,4]

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Abstract We compared modifications of soil zymography, a new in situ technique to visualize enzyme activities, based on contact of fluorgenic substrate-saturated membranes with soil either through the gel layer (*gel zymography*) or without gel application (*direct zymography*). We coupled zymography with quantitative measurements of enzyme kinetics to characterize catalytic mechanisms of β-glucosidase activity at the plant-soil interface including root surface (rhizoplane), rhizosphere, and bulk soil. Direct zymography refined and focused image resolution. The area of hotspots (i.e., spots with most intensive enzyme activity) as well as color intensity ratios estimated using direct zymography exceeded by a factor of 2 the corresponding values obtained with gel zymography. As determined by direct zymography, the percentage of hotspots associated to root surfaces was 58–68 % of total hotspot area. Hotspot area comprised only 6.8 ± 0.1 % of the total area of an image and 9.0 ± 3 % of the root surface area. The intensity of β-glucosidase activity, however, was up to 20 times higher in the hotspots versus bulk soil. The contribution of rhizosphere to β-glucosidase activity of the whole image (77–82 %) was four times higher than the contribution of the root surface. Enzyme kinetic parameters indicated different enzyme systems in bulk and rhizosphere soil. Higher substrate affinity and catalytic efficiency in bulk than in rhizosphere soil suggested relative domination of microorganisms with more efficient enzyme systems in the former. Coupling direct zymography and kinetic assays enabled mapping the two-dimensional (2D) distribution of enzyme activity at the root-soil interface and estimating the catalytic properties of root-associated and soil-associated enzymes.

Keywords Zymography · Rhizosphere hotspots · Enzyme mapping · Root exudates · β-glucosidase · Enzyme kinetics · Root-soil interface

✉ Muhammad Sanaullah
sanasial@gmail.com

✉ Evgenia Blagodatskaya
janeblag@mail.ru

[1] Department of Agricultural Soil Science, Georg August University of Göttingen, Büsgenweg 2, 37077 Göttingen, Germany

[2] Institute of Soil and Environmental Sciences, University of Agriculture, Faisalabad, Pakistan

[3] Institute of Physicochemical and Biological Problems in Soil Science, 142290 Pushchino, Russia

[4] Institute of Environmental Sciences, Kazan Federal University, Kazan, Russia

Introduction

The rhizosphere is the soil volume affected by the presence of growing plant roots and is a key hotspot, characterized by very tight biotic and abiotic interactions (Hinsinger et al. 2009). Many active substances such as secondary metabolites and enzymes excreted by living roots participate in the interactions between the roots and their environment, forming hotspots of root-soil microenvironment (Bais et al. 2004; Palacios et al. 2014; Pii et al. 2015; Valentinuzzi et al. 2015). The spatial structure of the rhizosphere defines a large, complex, and heterogeneous root-soil interface. All processes and functions ongoing in the rhizosphere are dominated by the activities of roots, rhizosphere microorganisms, and root-microbial interactions. Complex compounds released into the rhizosphere through rhizodeposition can strongly impact the neighboring soil, causing a rhizosphere effect (Whipps 2001). Due to rhizodeposits and especially their soluble part—exudates—

microbial biomass and enzyme activities are high in the rhizosphere. This makes this root-soil interface an important hotspot of nutrient cycling (Marinari et al. 2014). Nonetheless, the distribution of enzyme activity along the roots and the relative abundance of the hotspots at the root-soil interface remain unclear.

Enzyme activities are the primary biological drivers of organic matter decomposition and nutrient cycling (Treseder and Vitousek 2001). Most soil enzymes are extracellular and are present either in immobilized or free form (Gianfreda and Bollag 1994; Rao et al. 2000). In addition to being released by microorganisms, these enzymes can also be secreted by roots to mobilize nutrient pools for soil (Badalucco and Nannipieri 2007; Marinari et al. 2014). Despite higher activity in the rhizosphere, it remains unclear whether the microbial mechanisms of organic matter decomposition differ in these hotspots and in bulk soil. This may be revealed by enzyme kinetics parameters (Nannipieri and Gianfreda 1998; Pathan et al. 2015). They describe the catalytic activity and enzyme-substrate affinity (Nannipieri et al. 1988, 1996; Zhang et al. 2009) and the probable distribution of enzyme activity in the rhizosphere (Nannipieri et al. 2012; Uksa et al. 2015; Wallenstein and Weintraub 2008).

The spatial distribution of the root-soil interface can be separated into (1) the rhizoplane, i.e., root surface with root-associated microorganisms (hereafter root-associated activity) and (2) the rhizosphere per se, i.e., soil affected by root exudation (Marinari et al. 2014) (Fig. 1). These parts are completely different from the bulk soil that is not affected by roots. In such rhizosphere-affected microhabitats, the in situ enzyme activities are very dynamic and are responsive to changes in microbial biomass and community composition (Ling et al. 2014; Waldrop et al. 2000). Therefore, not only potential activity but also the catalytic properties of the enzymes can vary significantly in the rhizosphere compared with bulk soil (Nannipieri and Gianfreda 1998). The strong variability of enzyme distribution and biochemical nutrient cycling at the soil microscale requires methods for their accurate assessment.

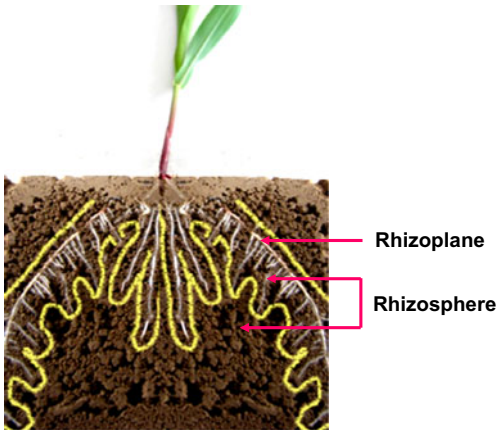


Fig. 1 Visual description of root-soil interface, illustrating rhizoplane (root surface with root-associated microorganisms); the rhizosphere (soil directly affected by root exudation, in between *yellow lines*). Bulk soil without roots was also used for comparison

One of the most realistic methods to detect enzyme activities is their direct visualization in undisturbed soils. In situ measurements include histochemical techniques (Joner et al. 2000), electron microscopy of soil sections (Ladd et al. 1996), and zymography (Marinari et al. 2014; Spohn et al. 2013). Zymography is a recent technique used to analyze the two-dimensional distribution of enzyme activities in soil by integrating fluorescent substrates (Spohn and Kuzyakov 2013). This technique is useful for a broad range of enzymes at microscales and is a powerful tool providing information on potential hydrolytic activities, enzyme forms, and their localization (Vandooren et al. 2013). Soil zymography enables measuring the distribution of exoenzymes using membranes coated with substrate molecules that become fluorescent when hydrolyzed. These membranes are incubated on the soil surface protected by a 1-mm-thick gel and are subsequently photographed under ultraviolet (UV) light that excites the fluorescent molecules (Spohn and Kuzyakov 2013). One of the limitations of the recent zymography technique is the difficulty to separate root-associated enzyme activities from soil enzyme activities. This is because the gel application to the root-soil interface used in zymography can cause artifacts because enzymes or substrates diffuse into the gel and potentially blur the hotspot focus. One of mitigating possibilities is to reduce gel thickness. This, however, can reduce but not eliminate the diffusion problem.

In this paper, we present an improved soil zymography by enabling direct contact of substrate-saturated membranes with plant roots and associated microorganisms without gel application. We used this approach to study the spatial distribution of β-glucosidase activity (responsible for decomposition of cellulose, the most abundant plant polymer) in the rhizosphere of maize (*Zea mays* L.). We coupled zymography with the determination of enzyme kinetics in rhizosphere hotspots and in root exudates in order to confirm visual observations by quantitative data. Our study aimed (1) to determine the spatial distribution of β-glucosidase activity at the root-soil interface, (2) to estimate the relative contribution of the rhizosphere and rhizoplane hotspots to total enzyme activity, and (3) to compare the catalytic properties of root-associated and soil enzymes.

Materials and methods

Site description

Soil samples were taken from the top 25 cm of an arable loamy Haplic Luvisol located on a terrace plain of the Leine

River north-west of the city of Göttingen (Holtensen, Germany). The area where the soil was collected has a temperate climate with a mean annual temperature of 8.7 °C and a mean annual precipitation of 645 mm. The soil had the following physicochemical properties: 7 % sand, 87 % silt, 6 % clay, pH 6.0, 12.6 g kg^{-1} total organic C, 1.3 g kg^{-1} total N, 1.4 g cm^{-3} bulk density, and 30 % field capacity (Kramer et al. 2012; Pausch and Kuzyakov 2012). After sampling, the soil was air dried, mixed, and sieved (<2 mm).

Growth conditions

Pre-germinated maize (*Zea mays* L.) seedlings were grown at 70 % field capacity for 4 weeks in rhizoboxes filled with soil to a final density of 1.4 g cm^{-3}. The rhizoboxes had an inner size of 12.3 × 12.5 × 2.3 cm. One seedling was sown in each rhizobox. Three rhizoboxes without plants were also maintained as an additional control for determining enzyme kinetics in bulk soil without plants. Three replicates were carried out in the experiment. The rhizoboxes were kept inclined at 50° during the cultivation in order to make roots grow along the lower panels of the rhizoboxes. Incubation temperature was kept at 20 ± 2 °C during whole growth period. Day length was 14 h, and light intensity was approximately 400 μmol m^{-2} s^{-1} at the top of the canopy.

Modified zymography approach

After 4 weeks of maize growth, β-glucosidase activity was determined by zymography by integrating substrate that becomes fluorescent when hydrolyzed (Spohn and Kuzyakov 2013). Briefly, 2 mg of 4-methylumbelliferyl-β-D-glucopyranoside (EC 3.2.1.21, Sigma-Aldrich, Germany) were dissolved in 2 ml of MES buffer to obtain a saturated solution at pH 6.5. This amount of substrate was chosen based on preliminary tests. Nylon membrane filters (Tao Yuan, China) with a diameter of 14.2 cm, and a pore size of 0.45 μm were soaked in the solutions of β-glucosidase substrate. These polyamide membrane filters are hydrophilic and chemically resistant to alkaline solutions and organic solvents. For the incubation of these substrate-saturated membranes, the rhizoboxes were opened from the lower, rooted side. We compared three different modifications: (1) substrates-saturated membranes were incubated on the surface of agarose gel protecting the soil; 1-mm-thick agarose gels were prepared by dissolving 1 % agarose in water at 80 °C. After 1 h incubation at 20 °C, the membranes were removed and photographed under UV light (360 nm wavelength) in the dark to excite fluorescent molecules. This method was termed *gel zymography*. (2) To avoid a possible diffusion effect of substrate/enzyme on the zymography image, we directly applied the membrane to the same soil surface used for gel zymography. After incubation for 60 min, the few soil particles attached to membranes were gently removed and photo images were prepared as in the original method. This methodological modification was termed *direct zymography*. A quenching effect of soil particles was also tested by a 60-min application of membranes saturated with the series of 4-methylumbelliferone (MUF) concentrations to the soil surface. However, in contrast to polyamide membrane filters obtained from Sartorius-Stedim, Biotech (Spohn and Kuzyakov 2013), no significant quenching effect was detected on fluorescence intensity after using nylon membrane filters (Tao Yuan, China). (3) The third modification was used to determine the distribution of enzyme activity associated with the root surface, i.e., rhizoplane, after direct zymography; the same plants were separated gently from the soil and put on a glass plate. The roots were covered by membranes saturated with buffer to check for possible remaining fluorescence. Buffer-saturated membranes showed no remaining fluorescent signals. Thereafter, the freshly substrate-saturated membranes were directly applied to the roots without any gel protection. This modification was termed *root zymography*. Here, we assumed that disturbance due to root extraction from soil may affect the overall enzyme activity but that the spatial distribution of rhizoplane enzyme activities remains similar over the short-term. The membranes were incubated on plant roots for 20 min and then the photo images were obtained under UV light, as mentioned above. Twenty minutes of incubation time for root zymography was found sufficient in preliminary tests because longer incubations showed no change in image resolution. This reduced incubation time was also important in order to avoid root mortality because we used the same plants for determining enzyme kinetics in root exudates thereafter.

A calibration curve was prepared from membranes that were soaked in solutions of MUF at different concentrations (0, 0.5, 1.0, 2.0, 6.0, and 10 mM). These calibration membranes were also photographed under UV light in the same way as the zymogram membranes.

Image analysis

Images were processed and analyzed using the open source software Octave (GNU, version 3.6.4). Firstly, digital images were transformed to 16-bit grayscale images. The images were then refined with "jet color map," which ranges from blue to red and passes through the colors cyan, yellow and orange. Color scales were given on the right side of the image with color intensity values. Consistency in pixel quality was maintained by pasting 4 cm^2 frames of black and white color on each image, ensuring the same color scale and pixel quality for all zymograms. All the zymograms were referenced based on the gray value received from the blank side of zymograms as the referencing point. After referencing the zymograms, we calculated an average

background gray value through the zymograms of calibration lines at concentration of zero and subtracted this value from all the zymograms. Note that the same filters were applied to all of the images, including the calibration base line. Based on referencing of images and of calibration line, color intensity <0.5 was considered as background, whereas an intensity >0.5–1.0 represented enzyme activity. In order to transform zymogram images to graphical representation, digital image histograms were developed as bar charts, which showed the distribution of pixel values according to the color map. These histogram graphs show the number of pixels of the zymogram images at each 0.01 color intensity value occurring in that image. Numbers of pixels as well as area of whole image were calculated based on these histograms. All pixels with the color intensity exceeding average value (i.e., >0.75, see "Results") were assigned to the hotspots for β-glucosidase activities. The contribution of roots with associated microorganisms to total area of β-glucosidase activity was calculated by subtracting the number of pixels corresponding to the area of roots from the total area of β-glucosidase activity. In order to compare the intensity of β-glucosidase activity in the hotspots and in the whole gel, direct, and root zymogram images, the color intensity ratio was calculated as the ratio of mode values of color intensity in the hotspots and in the whole zymogram of β-glucosidase activity (Ortiz Jaramillo et al. 2015). Prior to calculating color intensity ratio, the background color intensity (0.5) was subtracted from all color intensity values.

Enzyme kinetics

For determination of soil enzyme kinetics, soil particles (0.5 g) attached to the plant roots were carefully sampled directly from the hotspots recognized by zymography; this fraction was considered as rhizosphere hotspot soil (Fig. 1). Enzyme kinetics in bulk soil (i.e., from rhizoboxes without plants) was determined for the comparison of catalytic properties of soil with or without plants. We used soil suspension (1:100) as a common approach of soil enzymology to determine enzyme kinetic parameters (Makboul and Ottow 1979; Nannipieri et al. 1982, 2012). We tested whether the soil suspension-based approach is sensitive enough to distinguish the differences in catalytic properties of enzymes between rhizosphere hotspots and bulk soil.

To determine the enzyme kinetics in root exudates, the roots were gently washed and all soil particles attached to the root surface were removed. These washed roots were incubated in 50 ml Hoagland nutrient solution for 1 h to obtain root exudates and to determine kinetics of enzymes secreted by roots and by root-associated microorganisms. Nutrient solution was used to get vigorous root functioning and to avoid osmotic stress and reuptake of secreted enzymes. The nutrient solution volume was similar to the 50 ml soil suspension based on 0.5 g of soil, which we used for the soil enzyme kinetic assay.

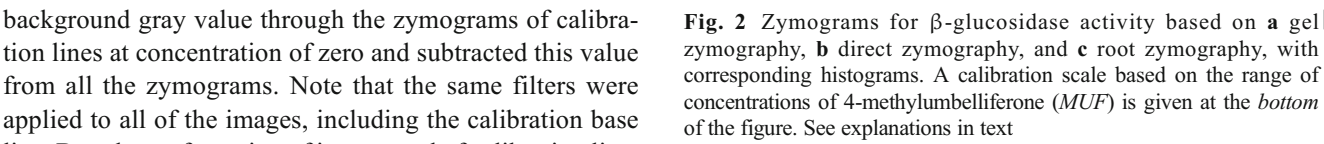


Fig. 2 Zymograms for β-glucosidase activity based on **a** gel zymography, **b** direct zymography, and **c** root zymography, with corresponding histograms. A calibration scale based on the range of concentrations of 4-methylumbelliferone (*MUF*) is given at the *bottom* of the figure. See explanations in text

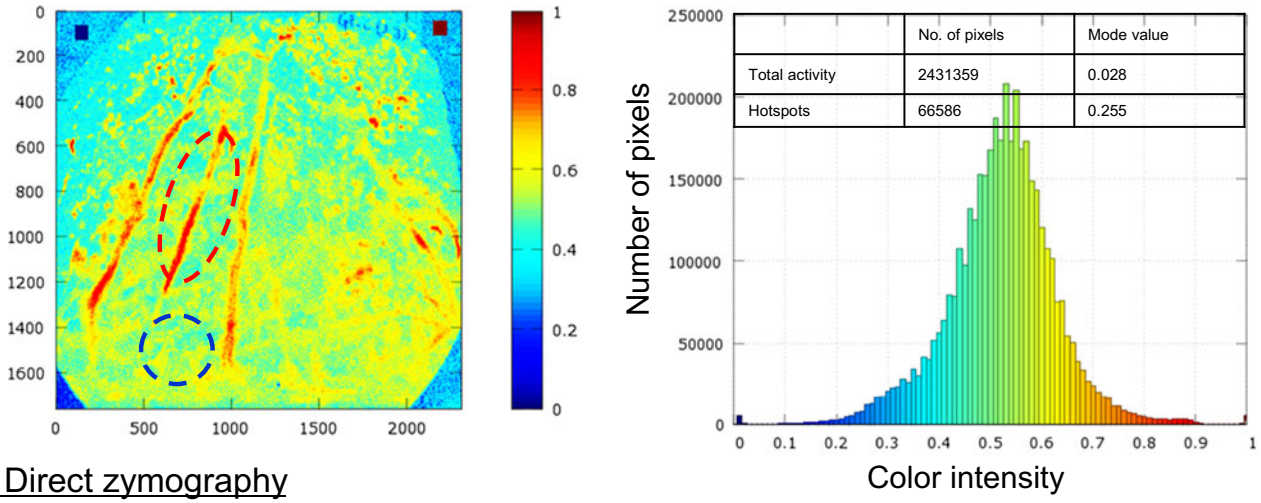
The enzyme kinetics was assayed using fluorogenically labeled substrates based on MUF (Pritsch et al. 2004; Sanaullah et al. 2011). For the detection of β-glucosidase activity, MUF-β-D-glucopyranoside was pre-dissolved in 2-methoxyethanol (Hoppe 1983), and sterile MES buffer was used to prepare stock and working solutions. We determined enzyme activities in a broad range of substrate concentrations (0–200 μmol g^{-1}). Saturation concentrations of fluorogenic substrates were determined in preliminary experiments.

Half a gram soil (dry weight equivalent) was suspended with 50 ml water using low-energy sonication (40 J s^{-1} output energy) for 2 min. Then, 50 μl of soil suspension or solution with root exudates were added to 150 μl of each substrate solution (containing 50 μl MES buffer) and were incubated for 2 h in a 96-well microplate (Puregrade, Germany). The calibration solutions were prepared using soil suspension (50 μl) and MUF to obtain series of concentrations 0–120 μmol. For enzyme activity determinations in root exudates, pure Hoagland nutrient solutions were used rather than soil suspension for the calibration curve. Fluorescence was measured in microplates at an excitation wavelength of 355 nm and an emission wavelength of 460 nm, slit width of 25 nm, with a Victor^3 1420–050 Multi-label Counter (PerkinElmer, USA). Enzyme activity was expressed either as MUF release in soil as nanomoles per gram of dry soil per hour, or, for root exudates, as nanomoles per gram of dry root biomass per hour. The assay of each enzyme at each substrate concentration was replicated three times. The kinetic parameters V_{max} and K_m along with the catalytic efficiency constant (K_a), i.e., ratio of V_{max}/K_m, were calculated using the Michaelis-Menten equation.

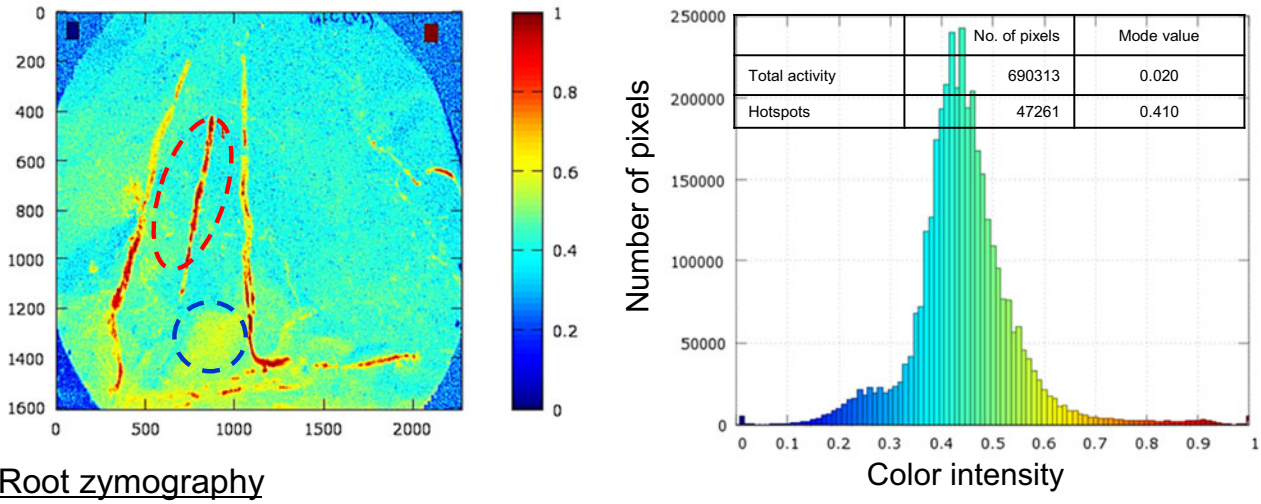
$$V = (V_{max}[S]) \Big/ (K_m + [S]) \quad (1)$$

Where V_{max} is the maximal rate of enzymatic activity; K_m is the half saturation constant or the substrate concentration at ½V_{max}; and S is the concentration of the substrate. The parameters of Eq. (1) were fitted by minimizing the least-square sum using ModelMaker software (version 3.1, Cherwell Scientific Publishing, Inc., 1999). We used the V_{max}-to-K_m ratio (K_a) as a proxy for catalytic efficiency (Tischer et al. 2015).

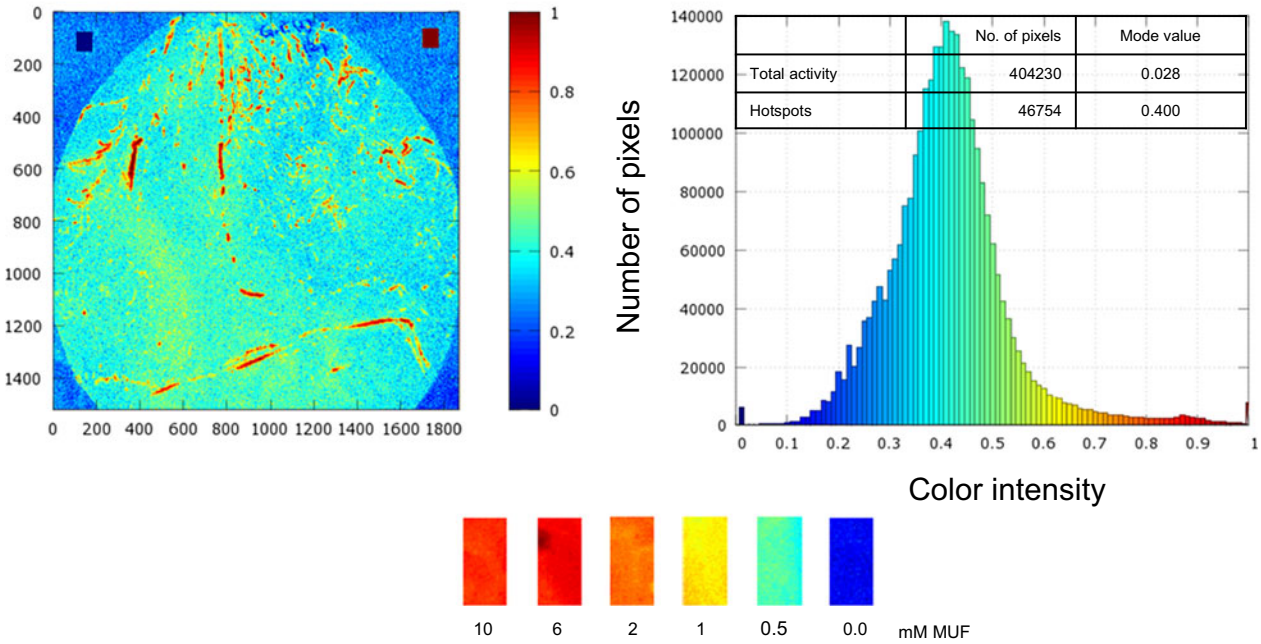
(a) Gel zymography



(b) Direct zymography



(c) Root zymography



Statistical analysis

Significance of differences between variables was tested using one-way ANOVA, where $p < 0.05$ was considered the threshold value for significance. The R software (version 3.1.1) was used for these statistical analyses.

Results

Enzyme zymography

The two-dimensional distribution of β-glucosidase activity on gel zymograms was pronounced for the roots and for the rhizosphere (Fig. 2a). It was, however, difficult to distinguish root- and soil-associated activities at the root-soil interface (e.g., at the areas emphasized by dotted red lines). The blurring left open whether an activity gradient around the roots was due to (i) the diffusion of root-associated enzymes, (ii) diffusion of MUF substrates through the gel, or (iii) an activity of the rhizosphere soil. Furthermore, it was equivocal to interpret color intensity between two roots as a rhizosphere soil activity (e.g., the area emphasized by the dotted blue line, Fig. 2a). Even when the enzymes originated microbially (in the rhizosphere), the precise estimation of the hotspot area was hampered by fluorescence scattering through the gel. Direct zymography for β-glucosidase eliminated this diffusion effect and focused on both the soil-associated and root-associated enzyme activities (Fig. 2b).

The next step of root zymography was designed to improve the precision of estimation of hotspots distributed at the root surface (rhizoplane) in the absence of soil enzymes. This step strongly focused on the distribution of β-glucosidase activity on the root surface and improved the monitoring of hotspot distribution along the root (Fig. 2c). Digital image histograms for gel zymograms of β-glucosidase activity mostly had a broader-range intensity, having more colors with a higher number of pixels compared with direct membrane contact (Fig. 2). Mid-range histograms with high color contrast in direct and root zymograms confirmed the visual brightness of the images and the stronger focus on both soil-associated and root-associated enzymes as compared with gel application.

The contribution of β-glucosidase activity of the rhizoplane to the total activity area was 23 % in the gel approach but was 5 % lower in the direct membrane application (Fig. 3). Total β-glucosidase activity was mainly attributed to rhizosphere soil activity (77–82 %). When considering solely hotspots, the contribution of rhizoplane to the hotspot area was three times higher than the contribution of rhizosphere (Fig. 3). The contribution of root-associated activity to hotspot area was 68 and 58 % based on gel and direct zymographies, respectively. Thus, direct membrane application revealed an up to 10 % overestimation of the hotspot area by the gel technique.

The contribution of hotspots to the total area of enzyme activity in the two-dimensional (2D) images was 3.3 ± 0.8 % in gel zymograms and it doubled (6.8 ± 0.1 %) when estimated by direct application of membrane to the soil surface (Fig. 4). Root zymography revealed that the contribution of hotspots at the rhizoplane was 9.0 ± 3 % of β-glucosidase activity at the root surface. The color intensity ratio calculated by the mode values of color intensities of hotspots and of total activity was doubled by direct zymography compared with gel zymography (Fig. 4). The intensity of enzymatic processes revealed by the color was 10–20 times higher in the hotspots than the color intensity of the whole image (Fig. 4).

Enzyme kinetics

β-glucosidase activity differed significantly between the rhizosphere hotspots and bulk soil, indicating different enzyme systems (Fig. 5a).

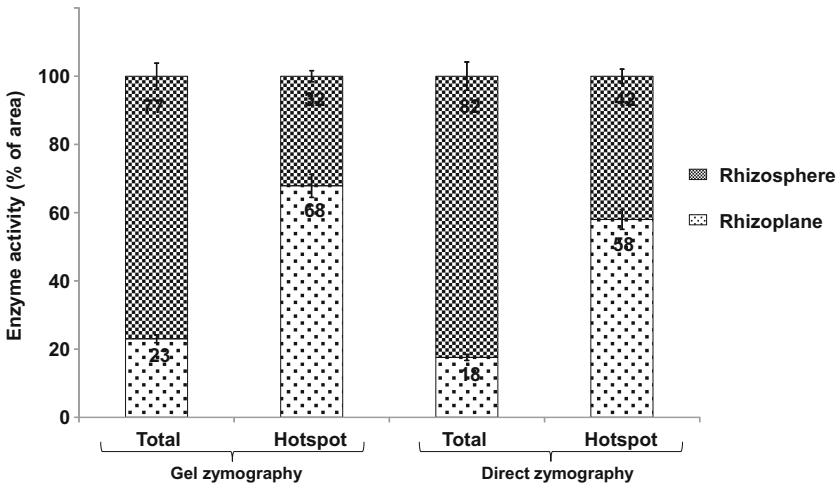


Fig. 3 Relative contribution of rhizoplane and rhizosphere soil to total and hotspot enzyme activity (% of area) for β-glucosidase calculated from zymograms of gel, direct, and root zymographies. Data represent mean ± SE ($n = 3$)

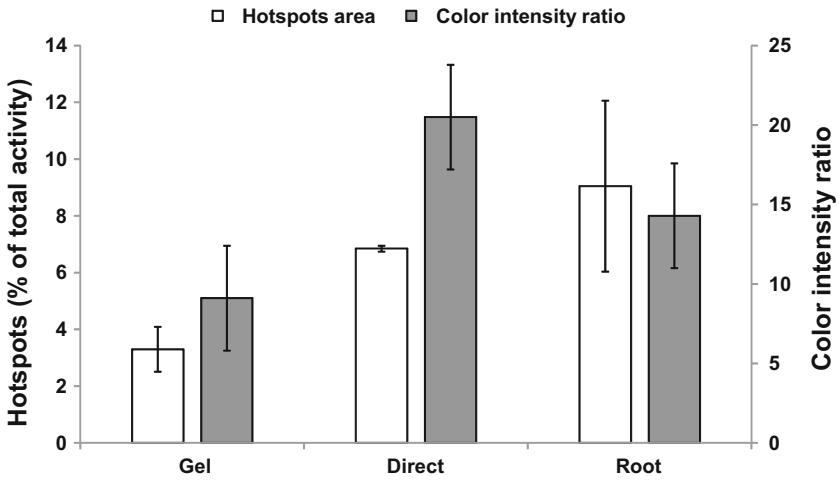


Fig. 4 Contribution of hotspots to total β-glucosidase activity and color intensity ratios of β-glucosidase activity in the hotspots to total area of activity calculated from zymograms of gel, direct, and root zymographies. Data represent mean ± SE ($n = 3$)

The V_{max} decreased 1.5 times and K_m 2.5 times in bulk soil versus rhizosphere (Table 1). At the same time, the catalytic efficiency constant (K_a) for bulk soil was almost 1.5 times higher than in rhizosphere soil.

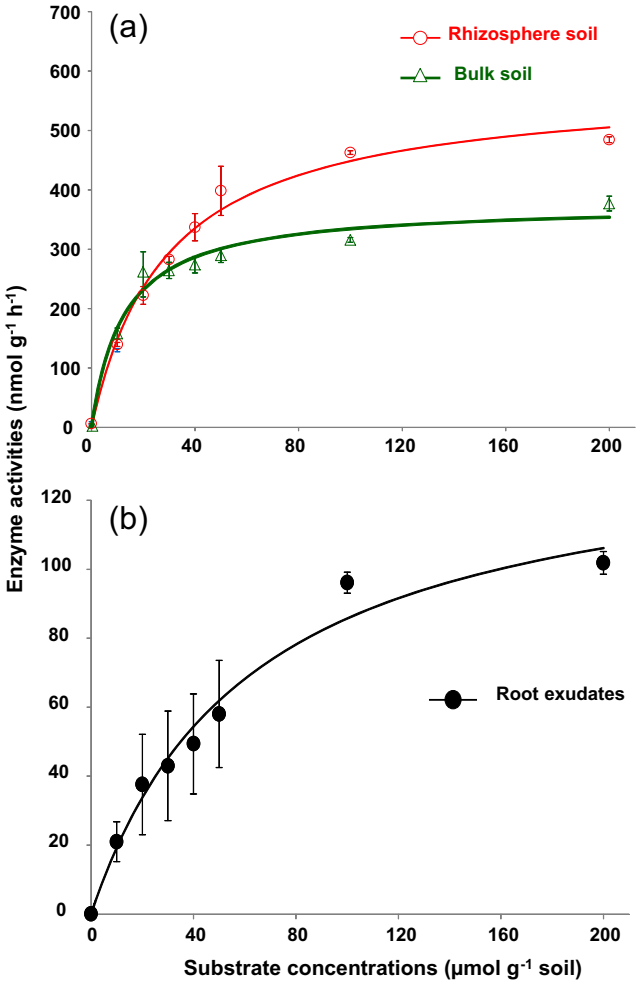


Fig. 5 kinetics of β-glucosidase activity in **a** the rhizosphere and bulk soil and **b** in root exudates sampled from living maize roots incubated in nutritive solution. Data represent mean ± SE ($n = 3$)

The V_{max} of β-glucosidase activity of rhizoplane microorganisms in root exudates was 139 ± 12 nmol g^{-1} of root h^{-1} and K_m was 63 ± 12 μmol g^{-1} soil, resulting in a catalytic efficiency constant (K_a) of 2 ± 0 nmol MUF μmol substrate^{-1} h^{-1} (Fig. 5b; Table 1). In order to compare β-glucosidase activity in root exudates with the rhizosphere activity, we roughly calculated activity in root exudates as nanomoles per gram of soil per hour. This was possible because, after 4 weeks of plant growth, the roots homogeneously penetrated the whole soil volume of the rhizobox. We assumed that the whole soil volume in the rhizobox was directly affected by roots and root exudation. We therefore divided total dry root biomass by total soil weight in the rhizobox to convert β-glucosidase activity in root exudates to nanomoles per gram of soil per hour. This comparison revealed that β-glucosidase activity in root exudates was almost negligible, i.e., 4000 times lower than that of the rhizosphere soil.

Discussion

Advantages of gel vs. direct zymography

Direct membrane application coupled with root zymography refined image resolution and improved hotspot area estimations. Larger hotspot areas and contrast ratios using direct zymography confirmed higher visual brightness of the images and more focused activity of both soil-associated and root-associated enzymes as compared with gel application (Fig. 6). The overestimation of root-associated (for 5 %) and hotspot activities (for 10 %) was revealed by gel zymography in comparison with direct zymography. Such an overestimation of the area of enzyme activity associated with roots by gel zymography can be a consequence of enzymes/substrate diffusion through the gel. Direct zymography, therefore, improved the precision of semi-quantitative 2D mapping of enzyme activities.

Table 1 Michaelis-Menten kinetic parameters, V_{max} (nmol g^{-1} h^{-1}), K_m (μmol g^{-1} soil), and catalytic efficiency (K_a), i.e., ratio of V_{max}/K_m (nmol-MUF μmol substrate^{-1} h^{-1}), for β-glucosidase in the rhizosphere and bulk soil (calculations based on dry soil weight) and in root exudates (calculations based on dry root weight)

	V_{max}	K_m	K_a
Rhizosphere soil	578 ± 25	29 ± 4	20 ± 3
Bulk soil	376 ± 18	12 ± 2	30 ± 6
Root exudates	139 ± 12	63 ± 12	2 ± 0

Values represent mean ± SE ($n = 3$)

Thus, the direct membrane application is a step forward in the development of soil zymography approach, avoiding possible shortcomings and artifacts due to substrate diffusion and fluorescence scattering through the gel. Such artifacts can be greater at high soil moisture. Artificially reduced soil moisture for the sake of better image resolution for gel zymography can cause drought stress for soil microorganisms and thus affect enzyme activities. That is why a direct application of membrane to the soil is preferable over the gel-based zymography approach. Although we demonstrated this based on one enzyme, we assume that the peculiarities of hotspot distribution will work for the range of soil enzymes.

Contribution of soil- and root-associated enzyme activities

The 2D images distribution revealed that the contribution of the rhizosphere soil to total β-glucosidase activity was significantly higher (77–82 %) than that of the rhizoplane. Nonetheless, hotspots of β-glucosidase activity were mainly associated with the rhizoplane. These hotspots largely reflect the inputs of easily degradable organic compounds from the roots (Hinsinger et al. 2009; Högberg and Read 2006; Kuzyakov et al. 2002; Marinari et al. 2014), which stimulate microbial abundance on the rhizoplane (Brimecombe et al. 2000; Kang and Freeman 2007; Pathan et al. 2015). Even though the contribution of hotspots to the total enzyme activity area (3–7 %) was relatively low, a large portion of soil processes occurs in these microsites (Spohn and Kuzyakov 2014), and the processes intensity was 20 times higher in the hotspots versus soil. As β-glucosidase is mainly released by microorganisms (Blagodatskaya and Kuzyakov 2008; Kang and Freeman 2007), the higher activity (hotspots) on the maize rhizoplane indicated higher microbial abundance near root exudation (Kang and Freeman 2007).

Enzyme kinetics

Zymography approach enabled soil sampling directly from rhizosphere hotspots for determination of enzyme kinetic parameters. Common in soil enzymology determination of enzyme kinetics in soil suspension (Nannipieri and Gianfreda 1998; Nannipieri et al. 1982) was sensitive enough to distinguish differences between rhizosphere hotspots and bulk soil. The higher potential reaction rate (V_{max}) of β-glucosidase in the rhizosphere hotspots confirmed a higher activity than that operating in bulk soil because the rhizosphere soil is richer in organic substrates (Hinsinger et al. 2009). The higher K_m for rhizosphere soil compared with bulk soil can be interpreted as a dominance of copiotrophic microorganisms with a lower enzyme affinity to the substrate and a lower substrate use efficiency (Panikov 1995). An increase in K_m indicated a decrease in overall enzyme functioning in the rhizosphere at low substrate amounts. This was confirmed by a lower catalytic efficiency of β-glucosidase in the rhizosphere as compared with bulk soil (Gianfreda and Bollag 1994; Makboul and Ottow 1979). Lower K_m values and the higher catalytic efficiency (K_a) of β-glucosidase in bulk soil suggested efficient enzyme systems of organics

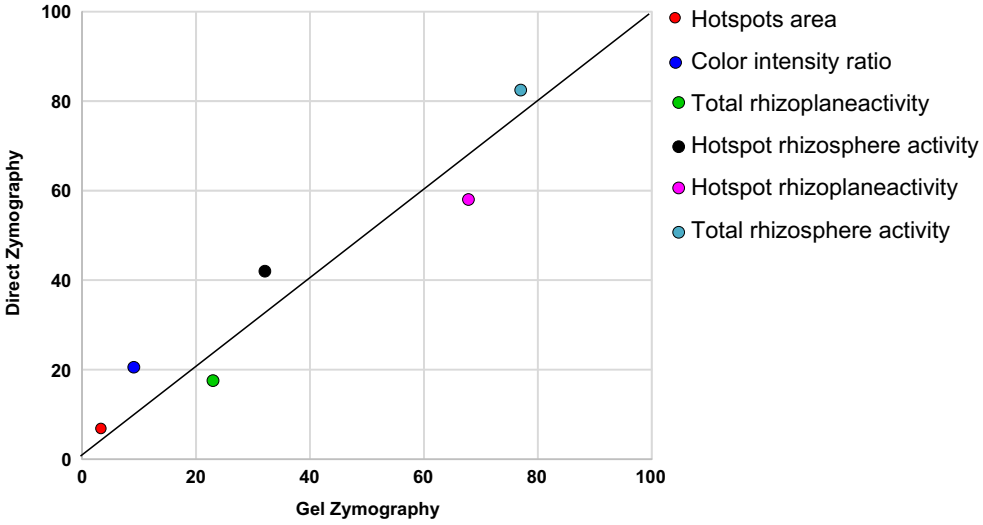


Fig. 6 Correlation analysis of direct vs. gel zymography considering total hotspots area, color intensity ratio, and total and hotspots activities of rhizoplane and rhizosphere

decomposition versus the rhizosphere. Thus, compared with the rhizosphere hotspots, bulk soil is dominated by oligotrophic microorganisms because oligotrophs have a high catalytic efficiency and a high rate of dispersion of enzyme-substrate complexes (Button 1991).

The very low contribution of root exudates to soil β-glucosidase activity demonstrated that the main source of β-glucosidase production is soil microorganisms (Blagodatskaya and Kuzyakov 2008). Coupling the dual zymography approach (i.e., direct and root zymography) with quantitative determination of enzyme kinetics enabled to differentiate both the relative contribution and catalytic properties of root-associated (including rhizoplane microorganisms) and soil-associated enzyme systems in the rhizosphere.

Conclusions

Direct zymography coupled with root zymography refined the image resolution and demonstrated that direct membrane application is a step forward in the development of the soil zymography approach. Direct zymography avoids possible shortcomings and artifacts due to MUF diffusion and fluorescence scattering through the gel. The 2D images of β-glucosidase activity distribution revealed that the rhizosphere soil contribution to total β-glucosidase activity was four times higher than that of the rhizoplane, and that hotspots were present mainly at rhizoplane surfaces. For the first time, we used the spatial distribution of enzyme activity obtained by direct zymography to determine enzyme kinetic parameters in soil sampled directly from rhizosphere hotspot. The distinctly different affinity of enzyme systems to substrate indicated possible domination of different microbial species in the rhizosphere hotspots and in bulk soil (Nannipieri and Gianfreda 1998) that needs to be proven by soil proteomic and genomic approaches (Pathan et al. 2015; Pii et al. 2015). Enzyme kinetic parameters demonstrated a higher catalytic efficiency of β-glucosidase in bulk soil compared with the rhizosphere soils. In conclusion, we demonstrated a two-dimensional distribution of enzyme activity at the root-soil interface. Coupling direct zymography and kinetic assays enabled accurately estimating both the relative contribution and catalytic properties of root-associated and soil-associated activities in the rhizosphere.

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