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Soil zymography – A novel *in situ* method for mapping distribution of enzyme activity in soil

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ABSTRACT

Recently, there has been growing interest in the spatial distribution of microbial activity in soil; however, methods for analysis of spatial distribution of microbial activity and for localization of hotspots of enzyme activity in soil are limited. Here were present an *in situ* zymography technique for localization and quantification of enzyme activities in soil by means of thin gels with embedded substrates. After incubation, the substrate remaining in the gel is colored and quantified using calibration curves and digital image analysis. So far, zymography has mostly been used to localize enzymatic activity in electrophoresis gels and in tissue sections. In this study we developed a zymography technique for analysis of the two-dimensional distribution of enzyme activities in soil. The technique was applied to map and quantify protease and amylase activity in the rhizosphere of lupine (*Lupinus polyphyllus*) grown in rhizoboxes. Highest activities, of up to 46 ng mm^{-2} of the soil surface h^{-1} for the protease and of up to 0.90 μg mm^{-2} h^{-1} for the amylase were found in close association with roots. Since zymography is an *in situ* method that does not require destruction of soil structure, it likely pictures enzyme activities more realistically than standard enzyme assays. In conclusion, soil *in situ* zymography offers a promising tool for mapping distributions of enzyme activities in soils in a work- and cost-efficient way.

1. Introduction

Soil microorganisms exist in a highly heterogeneous environment. Therefore, many soil processes that are driven by microbial activity often exhibit high spatial variability. Traditionally, this has not been regarded as an intrinsic property of soils, but rather as a technical problem that had to be overcome by sample homogenization (Nunan et al., 2002). Only recently there has been growing interest in the spatial distributions of microbial activities in soil (Ettema and Wardle, 2002; Nunan et al., 2002). Distribution of microbial activity has been explored by enzyme activities and by abundance of specific functional groups of microbes from the millimeter scale (Grundmann and Debouzie, 2000; Becker et al., 2006) to the plot scale (Enwall et al., 2010; Berner et al., 2011; Wallenius et al., 2011).

Particularly, the spatial patterns of microbial activity in the vicinity of roots (the rhizosphere) have attracted significant scientific interest during the last years (Hinsinger et al., 2009; Sörensen et al., 2009). Due to inputs of easily degradable organic compounds from the roots, microbial biomass and activity are high in the rhizosphere, making this plant–root interface an important hotspot of terrestrial C and nutrient cycling (Kuzyakov, 2002; Hinsinger et al., 2009). The spatial distribution of enzyme activity has been explored in soils at different root zones (Haase et al., 2008), and at different distances from roots (Tarafdar and Jungk, 1987; Kandeler et al., 2002). The common procedure employed in these studies consists of slicing soil in multiple segments, and determining enzyme activities in each of them. Besides being very labor intensive, the approach only provides a one-dimensional insight into enzyme activity with a very rough spatial resolution (Tarafdar and Jungk, 1987; Gahoonia and Nielsen, 1991; Kuzyakov et al., 2003).

Enzymes in soil can be stabilized through interactions with clay minerals and tannins rather than being free in solution (Kandeler, 1990; Nannipieri et al., 2002; Joanisse et al., 2007). Stabilized enzymes can have lower or even no *in situ* activity, since complexation can occlude enzymes, and cause changes in enzyme conformation (Allison and Jastrow, 2006; Nannipieri, 2006). In standard lab assays, enzyme activity is determined in soil slurries that are prepared by sieving, and homogenizing the sample (German et al., 2011). This approach likely releases enzymes that are adsorbed to surfaces or are electrostatically bound, and

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therefore may lead to an overestimation of enzyme activity (Wallenstein and Weintraub, 2008).

In contrast, soil zymography, which we present here, is an *in situ* method that allows the two dimensional quantification of enzyme activities in soil. Zymography uses the activity of enzymes to detect them. A specimen in which a certain enzyme occurs, such as a tissue section, is incubated attached to a gel that contains the enzyme's substrate. Subsequently, the gel is stained in order to visualize the substrate and to identify the areas in which the substrate has been enzymatically degraded during the incubation. Zymography has mostly been used as a qualitative method to identify enzymatically active bands in electrophoresis-gels (Manchenko, 2003). *In situ* zymography techniques have been developed to localize and quantify metalloprotease activity in tissue sections, for example to gain insights into tumor formation (Kleiner and Stetler-Stevenson, 1994; Nemori and Tachikawa, 1999; Wilkesman and Kurz, 2009).

The purpose of this study was to explore the suitability of zymography to map enzyme activities in soil. We developed protocols for quantitative zymography to localize and quantify protease and amylase activity in soil, which were applied to analyze protease and amylase activity in the rhizosphere of *Lupinus polyphyllus* grown in rhizoboxes.

2. Material and methods

2.1. Experimental setup

Lupine (*Lupinus polyphyllus*) was grown for four weeks in rhizoboxes filled with subsoil of a Cambisol. This soil was chosen because it exhibited low TOC and microbial carbon contents (Spohn et al., under review). Since we were interested in the effects of root growth on the spatial distribution of enzyme activities, we assumed that a low background of enzyme activities would be advantageous. The soil had the following characteristics: 24.8% sand, 55.1% silt, 20.1% clay, pH 5.0, 7.9 g kg^{-1} C, 0.6 g kg^{-1} N, 70 μg g^{-1} microbial C. The sampled Cambisol is located in the center of Germany, close to the city of Göttingen and is vegetated by a mixed deciduous forest. Samples were collected from a depth of 21–26 cm, and the soil was passed through a 2 mm sieve.

The rhizoboxes had a trapezoid shape with a height of 13.0 cm, an upper base of 14.0 cm and a lower base of 10.5 cm. Soil was filled into the rhizobox to a final density of 1.2 g cm^{-3}. Two seeds were sown in each rhizobox, although not all germinated. The rhizoboxes were kept inclined by 50° during the cultivation in order to make roots grow along the lower walls of the rhizoboxes. Plants were cultivated at room temperature under natural light for four weeks.

2.2. In situ soil zymography

The protocol for the soil *in situ* zymography was adapted from standard zymography assays for the detection of protease and amylase activity in electrophoresis gels (Manchenko, 2003). The protease zymography is based on the observation that Coomassie Brilliant Blue stains proteins, but not proteolytic products such as small peptides and amino acids. The amylase zymography is based on the characteristic of starch to be stained by iodine, since the iodine ions slide into the coil-shaped starch. These characteristics of proteins and starch are used in standard protocols for the determination of protease and amylase activity (Buroker et al., 1993; Yoo et al., 1987). In these assays, gelantine and starch are incubated with the corresponding enzyme for a defined period of time; then the remaining substrate is stained with Coomassie Brilliant Blue or Lugol's iodine solution and is determined colorimetrically. The method is calibrated with samples of various starch or gelatin concentrations that are allowed to react with the corresponding stain (Buroker et al., 1993; Yoo et al., 1987).

Gels for zymography were prepared in glass-chambers that are commonly used for vertical gel-electrophoresis (Biometra, Göttingen). The chambers consist of two glass slides, which are held together with brackets. Between the two glass slides, 1 mm high glass strips are located. These glass strips, commonly known as spacers, keep the glass slides separated at a fixed distance, so the gel can be cast between them. The chambers are tightened by rubber seals. The gels had a size of 11.0 × 12.0 cm and were 0.1 cm thick. As a matrix, 1% (w/v) agarose (Sigma–Aldrich) was used for all gels. The gels for determining protease activity contained 0.1, 0.05 or 0.01% (w/v) gelantine. The gel for the amylase contained 1.0, 0.5 or 0.1% (w/v) starch (Sigma–Aldrich). For calibrating the protease zymograms, we used gels with 0.00, 0.01, 0.02, 0.03, 0.04, and 0.05% (w/v) gelatin. For calibrating the amylase zymograms, we used gels with 0.00, 0.10, 0.20, 0.30, 0.40, and 0.50% (w/v) starch. The highest concentration of the calibration gels is equal to the concentration of the zymogram gel. Agarose, and gelantine or starch were dissolved in millipore water on a magnetic stirrer at 80 °C. The cast gels were immediately incubated after they had cooled down.

For incubation, the rhizoboxes were opened. To avoid destroying the smooth soil surface when opening the rhizoboxes we did not water the plants on the day we opened the rhizoboxes, since we had observed that this prevents the soil from sticking to the box. The chamber in which the gel was cast was opened by removing one glass slide. The gel was kept on the other glass side for stabilization. The non-covered side of the gel was attached to the soil surface. Soil and gel were wrapped in plastic foil in order to prevent desiccation, and were incubated at 22 °C in the dark. Incubation time was varied between 4 and 18 h. After incubation, gels were removed from the glass slide and were washed in distilled water on a horizontal shaker at 80 rpm and room temperature. The incubated gels were colored and decolored together with the calibration gels on a horizontal shaker at 80 rpm and room temperature. Coloring time was varied between 1 min and 2 h in order to find out which coloring and decoloring period leads to the best contrast.

Fig. 1. Soil rooted by *Lupinus polyphyllus* in the rhizobox for four weeks. The rooted soil was used for the protease zymogram.

Decoloring time was varied between 5 min and 24 h. The purpose of the decoloring was to remove the stain from the areas of the gel where the substrate had been enzymatically decomposed. The gels for the determination of protease activity were colored in Bradford solution (0.15% (w/v) Coomassie Brilliant Blue, 10% (v/v) ethanol, 10% (v/v) acetic acid); and decolored in decoloring solutssion (10% (v/v) ethanol, 10% (v/v) acetic acid). The amylase zymograms were colored in Lugol's iodine solution (5% (w/v) K, 10% (w/v) KI), and decolored in millipore water. Round pieces with a diameter of 5 cm were cut from every calibration gel. All gels were scanned together on an office scanner (Lide 210, Canon).

The digital images of the gels were analyzed using MatLab (The MathWorks). The RGB images were converted to black and white, assigning each pixel a gray value, with black corresponding to a gray value of 0 and white to a gray value of 256. The average gray value in a square of 4.0 cm^2 of each round calibration gel was calculated. Areas that were situated above air bubbles during scanning of gels were excluded. The concentration in the calibration gels was fitted as a function of their gray values. This function was applied to the digital images of the zymograms, resulting in images that display the concentration of the substrate still present in the zymogram after incubation. To illustrate the results, we depicted the values of the grayscale image in color, where red corresponds to the highest gray values (256), and blue to the lowest gray values (0). The colormap is given in Fig. 4. Enzyme activity was calculated based on incubation time and difference between the initial substrate concentrations of the gels and the substrate concentrations after incubation.

3. Results

The lupines grew well in the rhizoboxes and developed a branched root system within four weeks (Fig. 1). The most pronounced contrast for the protease zymograms was achieved with gels containing 0.05% (w/v) gelantine that were incubated with the soil for 6 h, colored for 5 min and decolored for 12 h (Fig. 2A). For the amylase zymogram the highest contrast was achieved with gels containing 0.5% (w/v) starch that were

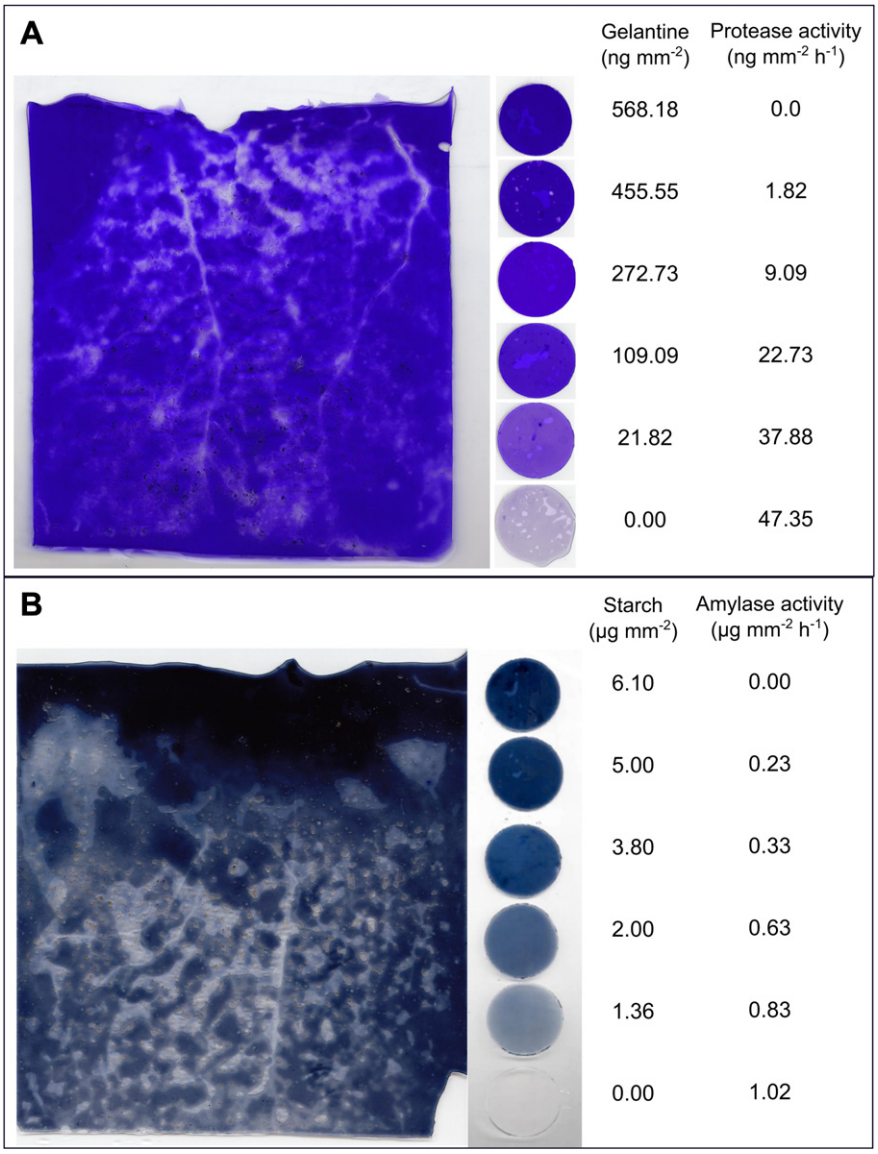

Fig. 2. Protease zymogram and calibration gels (A) and amylase zymogram and calibration gels (B). Next to the calibration gels the substrate concentrations are indicated. In the second line the corresponding enzyme activities are annotated.

incubated for 12 h with the soil, before they were colored for 5 min and decolored for 1 h (Fig. 2B). The enzyme activity was calculated as the difference between the initial substrate concentration of the zymogram and the substrate concentration after incubation divided by the incubation time. We found protease activities between 0.0 and 46.0 ng mm^{-2} h^{-1} (Fig. 2A) and amylase activities between 0.00 and 0.90 μg glucose mm^{-2} h^{-1} (Fig. 2B). An exponential relationship ($R^2 = 0.99$) was observed between the gray values and the gelantine concentration of the calibration gel for the protease zymogram (Fig. 3A). The gray values of the calibration gels for the amylase zymogram were linearly correlated with the starch concentration ($R^2 = 0.94$). The different relationships between gray values and substrate concentrations, exponential and linear, result from different reactivities of the substrate and the stain. The two zymograms presented here were made from different rhizoboxes; however, in previous measurements we found that it is possible to prepare several zymograms from one soil, which allows to analyze the spatial distribution of various groups of enzymes in the same soil. The comparison between the color-transformed zymogram (Fig. 4A) and the photos of the rooted soil (Fig. 1) showed that areas

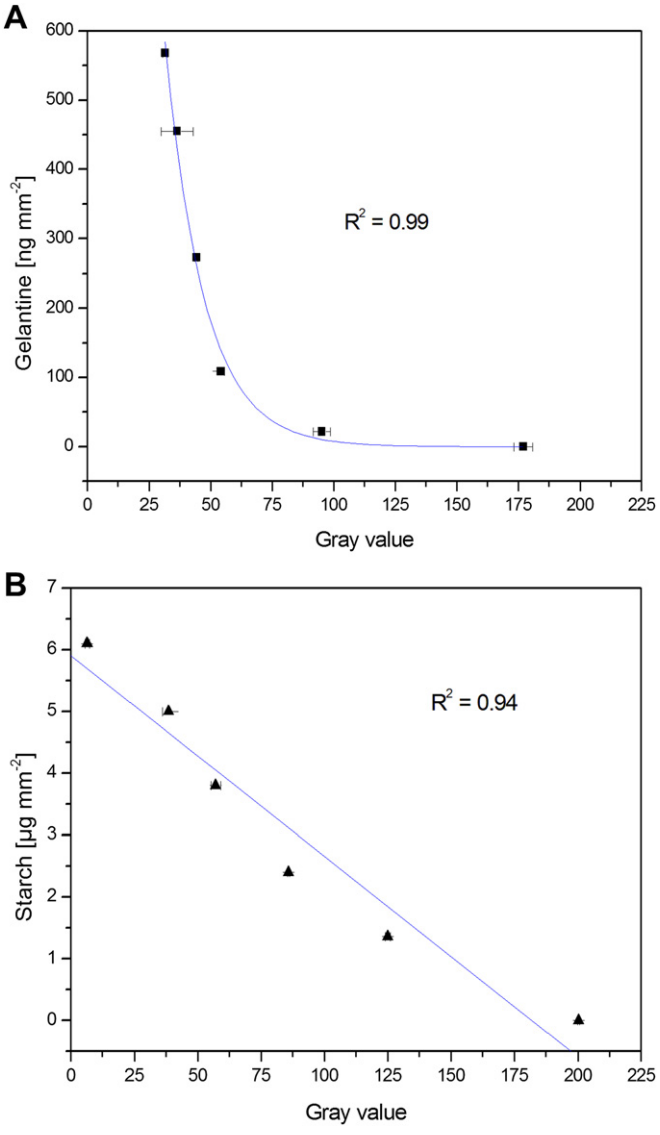

Fig. 3. Relationship between the substrate concentration and the gray value for the calibration gels with gelantine (A) and the gels with starch (B).

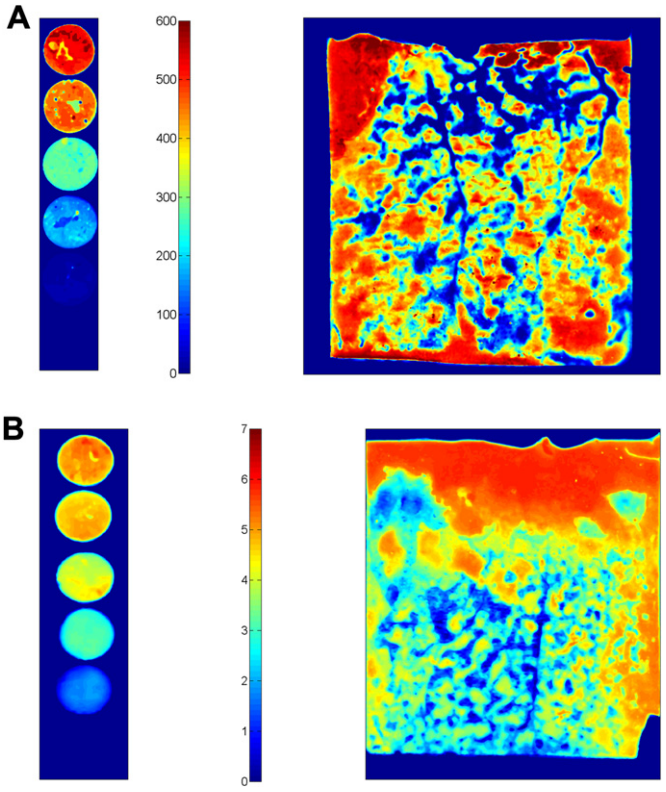

Fig. 4. Calibration gels, color map, and zymograms for protease (A) and amylase (B). For the protease zymogram the substrate concentration is given in ng mm^{-2}; for the amylase zymogram the substrate concentration is given in μg mm^{-2}.

with the highest levels of enzyme activity were associated with the presence of roots.

4. Discussion

Until now distribution of enzyme activity in soil has been determined by cutting soil into pieces and measuring enzyme activity in each of them. For this it has been necessary to destroy the soil structure by preparing a soil slurry. Here we present a novel technique for *in situ* localization and quantification of enzyme activities in soil. The technique allows mapping spatial distributions of enzyme activities at a high spatial resolution.

The correlations (Fig. 3A and B) between the substrate concentrations of the calibration gels and their gray values show that the protease and amylase zymograms can be calibrated with significant confidence. With the zymograms, we measured protease activity of up 46.0 ng mm^{-2} h^{-1} (Fig. 2A) and amylase activities of up to 0.9 μg glucose mm^{-2} h^{-1} (Fig. 2B). These results cannot be compared directly to results of enzyme activities obtained in traditional assays since those are reported on a soil mass basis. However, considering the bulk density of the soil (1.2 g cm^{-3}), and assuming that (a) zymograms detect enzyme activity in the upper 1 mm of the soil, and (b) that diffusion can be neglected, we can calculate maximum protease activities in the order of 38.3 μg g^{-1} and amylase activity of 750.0 μg g^{-1}. Kandeler (2008) reported values of protease activity of 9 μg tyrosine g^{-1} h^{-1} in the subsoil of a Cambisol from the temperate zone. Protease activities of up to 257 μg μg tyrosine g^{-1} soil h^{-1} have been described in topsoil (Dick and Kandeler, 2005). Activities described for amylases differed from 1170 μg glucose g^{-1} h^{-1} in the topsoil of paddy soils (Li et al., 2009) to 109 μg glucose g^{-1} h^{-1} in the topsoil of a tussock grassland (Ross, 1976). This comparison shows that the enzyme activities measured with

the zymograms are in the same range as activities that have been measured in previous studies with common enzyme assays.

What do we measure when we measure enzyme activity with zymography? As with any other enzyme method developed so far, zymograms display potential enzymatic activity, since enzyme activity depends on substrate concentration, pH, temperate and other factors that in the assay might strongly vary from the *in situ* conditions (Wallenstein and Weintraub, 2008). However, zymography differs from other quantitative enzyme methods in that it works *in situ*. This has at least two advantages. First, zymography does not require destruction of soil structure and the preparation of a soil slurry, which solubilizes enzymes. This means that enzyme activities determined by zymography are not overestimated due to the solubilization of complexed proteins as is assumed to happen in common enzyme assays (Allison and Jastrow, 2006; Nannipieri, 2006; Wallenstein and Weintraub, 2008). Second, since the conditions during the zymography assay for the soil microorganisms do not change, it is very likely that they keep producing enzymes at a constant rate. In standard enzyme assays however, the conditions for microbial metabolism change dramatically due to destruction of soil structure, which probably changes enzyme production rates.

The scans of both zymograms clearly exhibited zones of strongly decreased substrate concentrations, i.e. zones that were exposed to higher enzyme activities during incubations than the rest of the gel (Fig. 2). From a comparison with the photos of the rooted soils (Fig. 1) it can be seen that these zones were associated with the presence of roots. For further analyses of the shape of the rhizosphere it would be necessary to develop a technique that allows for projection of the exact position of roots on the zymogram.

Increased enzyme activities in the rhizosphere compared to the bulk soil have been described before. Kandeler et al. (2002) found significantly higher invertase and phosphatase activities in a distance of 0.2–0.8 mm from corn roots grown in a sandy silt loam, while no increases of xylanase activity were observed. Larger zones of increased enzyme activities have been found by Tarafdar and Jungk (1987) who studied phosphatases in the rhizosphere of various plants. The authors reported increased acid phosphatase activity in a distance of up to 3.1 mm from the root, and increased alkaline phosphatase at a distance of up to 1.6 mm from the root. Tarafdar and Jungk (1987) concluded that the increase in phosphatase activity around the root depended upon plant age, plant species and soil type. Similarly, Marschner et al. (2005) reported increased β-glucosidase, phosphatase, and protease activities in the vicinity of the root of a Banksia species compared to the bulk soil.

Previously, spatial distribution of enzyme activity has been determined by taking samples from the area under study in specific intervals and analyzing them separately. In studies on enzyme activities in the rhizosphere usually the soil was cut into slices down to a diameter of 1 mm (Kandeler et al., 2002; Tarafdar and Jungk, 1987). This approach leads to a one-dimensional insight into enzyme activity in the rhizosphere with a rough spatial resolution. Zymography, instead, is a non-destructive *in situ* method for the exploration of two dimensional distributions of enzyme activities with a high spatial resolution. Protocols for the detection of various groups of enzymes in electrophoresis gels have already been described (Manchenko, 2003). Hence, there is a large potential to adopt these protocols to soil zymography and map the spatial distribution of several enzymes in soil. This would allow us to answer questions about the spatial distribution of enzyme diversity. Furthermore, zymography can be combined with other methods for spatial analysis of biogeochemical processes in the rhizosphere. The combination of zymography and autoradiography (Pausch and Kuzyakov, 2010) would allow, for example, to study the relationship between carbon flow from the plant into the soil and enzyme activities in the rhizosphere. Other possibilities are to link zymography to neutron radiography (Carminati et al., 2010) and fluorescence dye imaging (Rudolph et al., 2012) to explore the spatial relation between enzyme activities and movement of water and oxygen in the rhizosphere.

In conclusion, the method developed here offers a promising tool for mapping the two dimensional distribution of enzyme activities in soils. Since the method works *in situ* it likely pictures enzyme activities more realistically than traditional enzyme assays that are based on the destruction of soil structure.

Author contributions

MS designed the research and performed the lab work. Data analysis was conducted by MS and AC. The manuscript was written by MS with contributions from YK.

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