METHODS PAPER



Development of micro-zymography: Visualization of enzymatic activity at the microscopic scale for aggregates collected from the rhizosphere

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Abstract

Aims Visualization of enzymatic activity links microbial functioning to localization in heterogeneous soil habitats. To assess enzymatic reactions in soil thin layer at the microscopic level, we developed a micro-zymography approach and tested it by visualization of the potential activity of phosphomonoesterase for aggregates collected from the rhizosphere of *Zea mays* L.

Methods We evaluated micro-zymography by applying fluorogenically-labeled substrate i) on individual soil aggregates freshly sampled from the rhizosphere,

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Department of Soil System Sciences, Helmholtz Centre for Environmental Research – UFZ, Halle (Saale), Germany ii) on thin layers of aggregates ($\approx 500 \ \mu m$) saturated with substrate to assess the dynamics of phosphomonoesterase activity, and iii) on maize roots under laser scanning microscope upon the identified hotspots by membrane-based zymography.

Results We found super transparent silicon as the most appropriate fixative to prevent sample drying. We optimized microscope settings to eliminate the soil auto-fluorescence. The fluorescent signal shifted from the free liquid phase towards the aggregate boundaries within 30 min after substrate addition and was finally detectable at the surface of a few aggregates. This was probably due to higher microbial abundance and enzymatic activity on the soil aggregates compared to the liquid phase. The enzymatic activity appeared patchy at the aggregate and root surfaces indicating heterogeneous distribution of hotspots.

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C. Banfield Geo-Biosphere Interactions, University of Tuebingen, Tuebingen, Germany *Conclusions* The methodology including calibration, sample preparation, fixation, and monitoring was developed. The novel membrane-free microzymography approach is a promising tool to identify functional specificity and niche differentiation on roots and soil aggregates. This approach revealed unexplained complexity of competing processes (biochemical, hydrolytic, and physical) due to differently charged reaction products and enzyme-clay complexes.

Keywords Membrane-free micro-zymography · Exo-enzymes · Fixative

Introduction

The majority of soil biochemical processes are mediated by enzymes produced by living macroand microorganisms. (Treseder and Vitousek 2001). Enzymes are secreted by microbial cells into the soil to catalyze the acquisition of carbon or nutrients like nitrogen and phosphorus from soil organic matter (Robinson 2015). Apart from microorganisms and soil fauna (Rao et al. 2017), roots also secrete enzymes to mobilize nutrients from the soil nutrient pool (Badalucco and Nannipieri 2007; Marinari et al. 2014). Thus, living plants and microorganisms are considered the main sources of soil enzymes in agroecosystems. Enzymes are able to move in liquid phase of soil solution either by being attached to the colloidal particles or with microorganisms producing enzymes (Guber et al. 2022). As exo-enzymes are secreted from the cells into the liquid phase, they can end up bound to dead cell envelopes, or to components of the soil matrix such as minerals and particulate organic matter (Rao et al. 2017). It remains a question, how static is the spatial distribution of exo-enzymatic activity, and whether enzymatic activity is higher in free liquid phase or at particle surfaces. Visualization of the enzymatic activity at the corresponding scales could help to solve this question. At the meso-scale (mm-cm), soil zymography is a well-established method that visualizes the spatial distribution of the enzymatic activity in situ in soil (Marinari et al. 2014; Spohn et al. 2013) by application of a substrate-saturated membrane that becomes fluorescent when a MUF-labeled substrate is hydrolyzed (Spohn and Kuzyakov 2013). At the meso-scale, membrane-based zymography has been successfully applied to soil (Sanaullah et al. 2016), different plants (Razavi et al. 2016), root systems (Ma et al. 2018), biopores (Hoang et al. 2016; Razavi et al. 2017), detritusphere (Liu et al. 2017; Ma et al. 2017), and soil aggregates (Guber et al. 2018; Kravchenko et al. 2019a). However, the precision of membrane-based zymography is restricted by the time required to accumulate sufficient signal intensity. Signal spots do not remain sharp due to diffusion of substrates and products of enzymatic reactions through and inside the membrane during the exposure. Therefore, less than 20% of the fluorescent product is detectable on the membrane after one hour of incubation (Guber et al. 2018). In addition, membrane-based zymography requires close contact of the membrane and the soil surface during the entire exposure time to ensure adequate responses only from a thin layer of the porous surface soil (Guber et al. 2021). At finer scales (microaggregates, e.g.), common membrane approaches are unfeasible. Avoiding membranes, e.g. by direct microscopy, could prove to be a way forward. Traditionally, enzymatic activity is determined in soil suspension after destruction of soil aggregates (Marx et al. 2001). Microscopic approaches may not require aggregates disturbance and they enable monitoring the development of enzymatic activity over time and interactions between the soil pores space and aggregates.

Visualization of enzymatic activity at the surfaces of aggregates and in their surroundings requires fixation to prevent aggregate movement and evaporation of the free liquid phase. The bulky shape of aggregates does not allow for the use of cover slips commonly used to fix microbial cells. Generally, the fixation techniques in light microscopy and modern micro-scale approaches are based on chemical or physical fixing agents such as paraformaldehyde, formaldehyde, glutaraldehyde (Fox et al. 1985; Hopwood 1969; Lechene et al. 2007; Peteranderl and Lechene 2004), heat (Barnett et al. 1966), freeze-drying (Abraham et al. 1985), and vapor (Peteranderl and Lechene 2004; Tock et al. 1966) which are not appropriate to trace the development of enzymatic reactions as they inactivate microorganisms and suppress enzymatic activity. Therefore, alternative fixatives are needed for micro-zymography, which are ideally transparent and do not inactivate microorganisms.

Another common limitation of fluorescence-based microscopy is auto-fluorescent plant and soil material (Oburger and Schmidt 2016). Auto-fluorescence depends on the structure or morphology of samples or tissues (Rost 1992) and can be accounted for by mathematical modelling (Steinkamp and Stewart 1986; Van de Lest et al. 1995). However, this needs specific adaptation to adequately distinguish the background in heterogeneous natural matrices like soil (Neumann and Gabel 2002).

The aim of our study was to test whether enzymatic activity can be visualized at micro-scale without membranes directly on soil particles. Therefore, we compared various approaches to fix undisturbed aggregates of the rhizosphere and surrounding free liquid phase without any chemical pre-treatment or washing to avoid reduction of enzymatic activity due to microbial inactivation and protein denaturation. Phosphomonoesterase is a very common enzyme type representing basic microbial metabolism. In comparison to other hydrolytic enzymes, phosphomonoesterase demonstrates consistent (i.e., independent on soil and microbial physiological state) and relatively high extracellular activity for phosphorus acquisition (Ma et al. 2018; Marx et al. 2001; Tischer et al. 2015). Therefore, we tested micro-zymography of phosphomonoesterase. The specific goals of this study were to i) develop a micro-zymography approach to visualize enzymatic activity on the microscopic level combining fluorogenic substrates with fluorescence microscopy, ii) visualize the distribution of enzymatic activity at the surfaces of soil aggregates in the space and time, and iii) test the applicability of micro-zymography at the root surface.

Materials and methods

Soil samples Loam soil was collected from 0–50 cm depth of Haplic Phaeozem near Schladebach in Saxony-Anhalt, Germany. The soil consisted of 32.5% sand, 47.9% silt, and 19.5% clay with an organic carbon content of 8.6 g kg⁻¹ C_{org} , an organic nitrogen content of 0.84 g kg⁻¹ N_{org} , and a pH (CaCl₂) of 6.4 (Rhizosphere Priority Program 2089) (Vetterlein et al. 2021).

Rhizosphere soil of *Zea mays* L. grown in rhizoboxes $(3 \times 8.8 \times 17.8 \text{ cm}, \text{H} \times \text{B} \times \text{L}, \text{Clickbox}(\text{B}))$ with three biological replicates was chosen for

application of micro-zymography approach to ensure high microbial (Cheng 2009; Norton and Firestone 1991) and enzymatic activity (Kuzyakov and Razavi 2019; Nannipieri and Gianfreda 1998; Sanaullah et al. 2016). Sterilized maize seeds were pre-germinated for 48 h. One seedling per rhizobox was planted at one cm depth. The rhizoboxes were covered in aluminum foil to prevent algal growth, and fixed at an angle of 45° to let the roots grow in contact with the transparent front. The rhizoboxes were kept at 22 °C during the day and 18 °C at night, 12 h photoperiod, 350 μ M m⁻² s⁻¹ photosynthetically active radiation, and 65% relative humidity for three weeks. After 21 days, rhizosphere soil was sampled by first shaking the roots to remove bulk soil and then by brushing off the remaining soil adhering to the roots (Vetterlein et al. 2021).

In this study, 4-methylumbelliferyl-phosphate (MUF-P) (Sigma-Aldrich, $\geq 98\%$, CAS: 22919–26-2) was used as the substrate to visualize the potential activity of phosphomonoesterase (E.C. 3.1.3.2). The substrate emits fluorescence when hydrolyzed by the enzyme.

Controls Used to assess auto-fluorescence: 1) A glass slide (to mount the objects for visualization) was considered a control accounting for auto-fluorescence and reflections of the slide surface (without cover slip), 2) Super transparent silicon either alone or with 0.1 M MES buffer (since MES buffer was used for preparation of substrate and to maintain the pH of soil) was applied to assess the auto-fluorescence of super transparent silicon in contact with the liquid phase, 3). To confirm that fluorescence occurs through enzymatic activity, 10 µl of MUF-P was applied to autoclaved and non-autoclaved thin layers of homogenized ($\approx 500 \ \mu m$) soil aggregates inside a plastic ring (with inner diameter of 5 mm) and covered by 10 µl of super transparent silicon. Soil aggregates were autoclaved three times for 30 min at 131 °C and a pressure of 1.5 bar to assess the autofluorescence of minerals and dead cells (Schmidt et al. 2018).

Calibration Different concentrations of 4-Methylumbelliferone (MUF) (Sigma-Aldrich, $\ge 98\%$, CAS: 90–33-5) ranging from 0 to 1 mM were prepared by diluting a 10 mM stock solution with 0.1 M MES buffer and 10 µl were pipetted onto thin layers of soil aggregates and covered by 10 μ l of super transparent silicon to control the difference of light intensity in the background, as well as to obtain a linear regression between the concentrations of MUF and intensity of fluorescence. Intensities were determined 5 min after application of MUF in four technical replicates.

Sample fixation To develop a fixation method which does not inactivate microorganisms, we tested the feasibility of micro-zymography of soil suspensions, of thin layers of soil fixed by agarose gel, and of soil aggregates fixed either by silicon spray or by super transparent silicon. We evaluated the potentials and limitations of each approach (with three technical replicates) based on the original microscopic images and pseudocolor representations with optimized contrast of weak fluorescence signals (refer to the image processing section).

Soil suspensions Were prepared by shaking 50 mg rhizosphere soil with 5 ml deionized water for 2 min by a magnetic stirrer (JENWAY, 1102 Stirrer). Enzymatic assays were performed with 50 μ l of soil suspension and 100 μ l of MUF-P (10 mM) (Koch et al. 2007; Stemmer et al. 1998). Then, 20 μ l of the mixture were pipetted onto a glass slide. The samples were evaluated under the microscope at an amplification of 10×or 20×.

Soil without fixation Fresh intact soil was taken from the rhizoboxes by a sampling ring with inner dimensions of 6×5 mm (height × diameter). Then, 20 µl of MUF-P (10 mM) were added on top of the ring. Each ring was covered by a lid to reduce evaporation of the reagents and soil moisture. After 30 min of incubation at room temperature, a few individual soil aggregates were placed on a glass slide with the help of toothpicks and assessed under the microscope at an amplification of $10 \times \text{or } 20 \times$.

Fixation of soil aggregates with 2% agarose gel To mount individual soil aggregates on glass slides, 50 µl of 2% agarose gel were pipetted onto the slides first. Agarose gel (Sigma-Aldrich, CAS: 9012–36-6) was applied when it was warm (50–60 °C), i.e., before it sets. Then, 20 µl of MUF-P (10 mM) were mixed with the agarose gel. After that, a few rhizosphere aggregates taken from the rhizoboxes were mixed with the agarose gel by a toothpick. The

development of enzymatic activity was visualized after 30 min of incubation at room temperature and at an amplification of $5 \times$.

Soil layers fixed by 2% agarose gel Fresh intact soil was extracted from the rhizoboxes by a ring with the inner dimensions of 6×5 mm (height×diameter). 20 µl of MUF-P (10 mM) were added to the soil. To fix the undisturbed soil layers, 50 µl of warm agarose gel (50–60 °C) were applied to the soil surface. After 30 min incubation at room temperature, a thin layer of soil attached to agarose gel with the approximate thickness of 1 mm was cut with a razor blade. Microscopy of the soil layers was carried out at an amplification of $5 \times$.

Fixation of soil aggregates with silicon spray A thin layer of a colorless silicon spray ("3-In-One PROFESSIONAL", Silikonspray, Art.-Nr.: 49,082/43) was sprayed on a glass slide. Then, a couple of soil aggregates sampled from the rhizosphere were placed at the silicon surface with the help of toothpicks. Thereafter, 20 μ l of MUF-P were pipetted onto the aggregates and assessed under the microscope after 30 min at an amplification of $10 \times \text{or } 20 \times$. Silicon spray was liquid immediately after application, but became solid after about 20 min and formed a protective dry silicone layer which prevented movement of soil aggregates.

Fixation of soil aggregates with super transparent silicon Super transparent silicon is a twocomponent curing rubber (Chen et al. 2010, 2011). Component A (with viscosity of 1000 mPa·s at 23 °C) is a platinum catalyst, while component B (with viscosity of 200 mPa·s at 23 °C) acts as a cross-linker of the siloxane monomer matrix. Super transparent silicon cures by attaching Si-H groups to double bonds at room temperature. Therefore, mixtures of both compounds have a pot life (i.e. the maximum duration when the mixture remains pourable), which is 5000 mPa s within 90 min at 23 °C. Components A and B of super transparent silicon (Wacker, ELASTOSIL®RT 604 A/B, material No: 000009552) were mixed at a ratio of 9:1 before application. It has a low viscosity (800 mPa s at 23 °C), a very low shrinkage rate (< 0.1%), a long curing time (24 h cm⁻¹ at 23 °C), and does not contain any acutely toxic substances. The slow curing prevented shrinkage of silicon, therefore silicon formed a protective layer keeping soil aggregates embedded during the microscopy. The total weight losses of the samples were tested by balance during the microscopy. To ensure good contact between the soil aggregates and the fluorogenic substrate, we applied the substrate first and covered it with the silicon thereafter. Then, microscopy was carried out. Absence of a detectable background fluorescence (before application of MUF-P) and optimal fluorescent signal intensity after application of MUF-P were ensured at 3000 ms exposure time of the camera (Zeiss AxioCam MR5). Therefore, 3000 ms exposure time was fixed for our experiments with super transparent silicon to eliminate the autofluorescence of phosphatase substrate. To identify the optimum concentration of MUF-P, a range of concentrations (0.1-10 mM) were pipetted onto the individual soil aggregates. Then, aggregates were covered by super transparent silicon.

Individual soil aggregates A few rhizosphere soil aggregates (≈ 0.0005 g) were placed on a glass slide. Then, 20 µl of MUF-P were pipetted onto the aggregates. 50–100 µl (based on the number and size of soil aggregates) of super transparent silicon were added as a thin layer on the aggregates and mixed carefully with a toothpick. Then, microscopy was carried out at an amplification of $20 \times$.

Thin layer of soil aggregates A small amount of rhizosphere soil was passed through a 500-µm sieve to homogenize the soil aggregates. Then, a plastic ring with outer and inner diameters of 13 and 5 mm, respectively (Avery Zweckform, No. 3508), was fixed onto the glass slide to prevent the aggregates from moving (Fig. S1). A thin layer of homogenized soil aggregates (≈ 0.01 g) with approximate thickness of 500 µm was put inside a plastic ring. 10 µl of MUF-P were added to the soil aggregates and were immediately covered by 10 µl of super transparent silicon. Microscopy of the soil thin layer was carried out at an amplification of $5 \times$.

Root surface To monitor the distribution of the enzymatic activity directly at the rhizoplane, we applied membrane-based zymography to root segments of *Zea mays* L. Root segments were taken out of the rhizobox and gently brushed to remove most of the soil particles attached to the roots (Vetterlein et al.

2021). Then, they were placed under UV light (45 cm, 15 W black, eurolite[®]) with excitation wavelength of 355 nm. The distance between the UV lamps and root segments was fixed to 6.5 cm. Small pieces $(2 \times 3 \text{ cm})$ of polyamide membrane (Tao Yuan, China) with a pore size of 0.45 µm were saturated with 5 mM MUF-P. The saturated membranes were placed at the root surfaces (Dong et al. 2007; Grierson and Comerford 2000) and were covered by a glass sheet $(0.18 \times 21 \times 30 \text{ cm}, \text{H} \times \text{B} \times \text{L})$ to prevent evaporation and movement. Images were captured by a DSLR camera (D3500, Nikon) after 60 min in the dark. After identifying the hotspots of potential enzymatic activity at the root surface at the mm scale, each root segments was fixed on a glass slide by super transparent silicon. Then, root segments were mounted with ProLongTM Gold Antifade Mountant (ThermoFisher Scientific) and Vectashield (VEKTOR laboratories), respectively and then covered with 0.17 µm 1.5 coverslips. Hotspots were imaged at the µm scale with a confocal laser scanning microscope (CLSM) (Leica Microsystems CMS GmbH).

Microscope settings Imaging was performed on two different fluorescence microscopes; Olympus BX40 microscope (mercury burner) and the Zeiss Axio Imager M1. The images taken with the Zeiss Axio Imager M1 were taken in two different software versions; Axio Vision and ZEN 3.0 (blue edition). The latter is the upgraded form of former and captured higher resolution images. To visualize microscopic objects (e.g., bacterial cells or fungal mycelium), optimal magnification, and exposure times were determined for each field of view to obtain images of highest quality. It is essential to keep the exposure time constant during an experiment, or at least for all images to be compared (Guber et al. 2019; Waters 2009; Webb and Brown 2012). Therefore, to assess the temporal development of an enzymatic reaction, all microscope settings remained unchanged for all images. The most appropriate exposure time depended on the type of object and the fluorogenic substrate. For the dense soil layer, the best exposure time (3000 ms) was longer than common exposure times of quantitative fluorescence microscopy studies on microbial cells stained with fluorogenic dyes (e.g., fluorophores and DAPI). The optimization of the exposure time aimed at strong positive signals without too much interference from other sources (e.g., auto-fluorescence). This simplifies the procedure of auto-fluorescence subtraction from the images. Samples were visualized in bright field (BF) and fluorescence mode (DAPI), using the excitation wavelength of 335–383 nm and the emission wavelength of 420–470 nm.

In addition, applicability of membrane-based micro-zymography at the root surface was tested by a high resolution confocal laser scanning microscope (Leica SP8 confocal system in combination with an inverted DMi8 microscope stand) after mounting with ProLongTM Gold Antifade Mountant (at excitation of 405 nm and detection of 414–482 nm) and Vectashield (at excitation of 488 nm and detection of 515–624 nm), respectively.

Image processing Was carried out in ImageJ version 1.53i (Schneider et al. 2012). The mean background intensity was determined before MUF-P was added (ROI: 1290×967 pixels, corresponding to $36.08-577.3 \text{ mm}^2$ for the individual and the thin layer of aggregates approaches, respectively) from four rectangular regions of interest. To assess the fluorescence emitted from soil surfaces, the background intensity / grayscale value of the control (before application of MUF-P) was subtracted from all pixels' intensities after application of MUF-P. Thereafter, the grayscale values were converted to MUF concentration (µM) based on the aforementioned linear calibration equation. Pseudocolor representations were chosen to improve the visibility of weak fluorescence signals and to support differentiation of activities through color. Pseudocolor images were created by splitting the three channels and applying the jet color map (LUT) to the blue channel. All images were adjusted to the same histogram settings (min: 0, max: 255).

Statistical analyses Were performed in R version 4.0.4 (Team 2019). To test the effect of the fluorescent signal intensity over time, we applied one-way repeated measures ANOVA to contrasting phases (free liquid, aggregate boundaries, and aggregate surface) in three rhizoboxes (biological replicates) with three technical replicates for each contrasting phase. The Tukey's HSD (P < 0.05) test was applied to check for significant time effects. Normality of the values and homogeneity of variances were tested by

Kolmogorov-Sminov test and Fligner-Killeen's test, respectively.

Results

Choice of fixative and substrate concentration

We compared different fixatives and summarized their potentials and limitations for micro-zymography (Table 1; Fig. 1) as follows:

Soil suspensions Fluorescence was emitted from soil aggregates in suspension after 5 min. Due to the low volume on the glass slide, the liquid phase evaporated within 5–10 min, thus reducing the applicability of the soil suspension approach (Table 1a; Fig. 1a).

Soil without fixation After application of the dissolved substrate to the soil in the sampling ring, we observed clear fluorescence from the individual aggregate surfaces, mainly from rod-shaped patches which we assumed to be either bacteria cells or bacterial associations (Fig. 1b). Since no fixative was used, aggregates were easily disturbed and moved by any sudden movement, thus preventing monitoring of enzymatic reaction over time (Table 1b).

Agarose gel Did not only emit auto-fluorescence but also was not solid / rigid enough to provide a flat and smooth surface after cutting. Therefore, in practice, this approach would lead to too much variation, especially in loam soils due to a large number of micro-pores compared to sandy soils (Fig. 1c and d). Since agarose gel needs to be applied while warm (50–60 °C) when viscosity is still low, it is possible that some microorganisms are killed and some enzymes are denaturized (Table 1c and d).

Silicon spray Was transparent, formed flat surfaces and did not emit auto-fluorescence. Immediately after application, it was liquid and soil aggregates could be easily rearranged in it. Within 20 min, the silicon became almost solid and fixed the aggregates very well (Table 1e; Fig. 1e, i and e, ii). The main disadvantage was crystallization of MUF after association with the fast-drying silicon (Fig. 1e, iii).

Comparing the limitations of different fixatives (Table 1; Fig. 1) revealed that agarose gel featured

Table 1 Advantages and limitations of each approach

Approach	Advantages and limitations
a) Soil suspensions pipetted on glass slides (Fig. 1a)	Advantages: Easy application
	Limitations: 1) Aggregates are mobile in the liquid phase, there- fore, imaging in the first minutes after application is not feasible due to mobility, 2) The liquid phase evaporates within 5–10 min due to the low volume on the glass slide, 3) Samples cannot be stored
b) Soil without fixation (Fig. 1b)	Advantages: 1) Substrates easily permeate the soil in the ring,2) Patchy distribution of fluorescence signals on the surface of aggregates is captured
	Limitations: 1) Aggregates are easily disturbed by any sudden movement, 2) Samples cannot be stored
 c) Soil aggregates fixed on glass slides by 2% agarose gel (Fig. 1c) 	Advantages: 1) Aggregates are fixed immediately by agarose gel,2) Possibility of storing the samples for several days
	Limitations: 1) Agarose gel casting does not result in a homoge- neously flat surface, 2) It emits auto-fluorescence, 3) Some air bubbles might be trapped within the gel and cause refraction, 4) It needs to be applied while warm (50–60 °C), thereby killing some microorganisms and possibly altering the substrate
d) Soil layer fixed by 2% agarose gel (Fig. 1d)	Advantages: 1) Aggregates are fixed immediately by the gel, 2) Possibility of storing the samples for several days
	Limitations: 1) Agarose gel casting does not result in a homo- geneously flat surface, 2) It is not rigid enough for cutting with razor blade after 30 min, 3) It emits auto-fluorescence, 4) Some bubbles might be trapped within the gel and cause refraction, 5) It should be applied while still warm (50–60 °C), therefore some microorganisms might be killed, 6) Imaging should be done upside down due to the thick and rough surface of agarose gel. Therefore, distinguishing auto-fluorescence of the gel and fluorescence from of enzymatic activity may not work, 7) Since loamy soil aggregates stick together and clump, this method is not appropriate for loamy soils
e) Soil aggregates fixed on glass slides by silicon spray (Fig. 1e)	Advantages: 1) Silicon is transparent, 2) Silicon gives a perfectly flat surface, 3) It does not emit auto-fluorescence, 4) It becomes solid within 20 min, 5) Aggregates are fixed by silicon
	Limitations: 1) Substrate should be applied before silicon has dried. Otherwise it cannot penetrate inside the aggregates covered by dried silicon, 2) Silicon cannot be applied after the substrate and aggregates because the pressurized gas moves the aggregates on the glass slides, 3) After drying of silicon spray, MUF crystals might be visible around the aggregates, 4) Sam- ples cannot be stored
f) Soil aggregates fixed on glass slides by super transparent silicon (Fig. 1f)	Advantages: 1) Silicon is super transparent, 2) it has flat surface, 3) It does not emit auto-fluorescence, 4) Viscosity is higher than silicon spray, 5) Low shrinkage rate (<0.1%), 6) Possibility of storing the samples for a long time
	Limitations: 1) Long curing time (24 h cm ⁻¹ at 23 °C)

strong auto-fluorescence (Fig. 1c and d). Crystallization of MUF solution was observed around the soil aggregates due to the fast-drying process on silicon spray (Fig. 1e, iii). Therefore, sample fixation with super transparent silicon was selected as the most appropriate fixative for further experiments, because it ensured the most consistent and focused images in comparison to silicon spray and agarose gel. Super transparent silicon almost completely prevented aggregates from drying. This was confirmed by the



Fig. 1 Examples of each approach with both bright field (BF) and fluorescent images under DAPI filter (excitation of 335–383 nm and emission of 420–470 nm); a) loamy soil suspension with 10 mM MUF-P. Scale bar: 50 μ m, b) sandy soil with 10 mM MUF-P. Scale bar: 50 μ m, c) sandy soil with 10 mM MUF-P fixed by agarose gel. Arrows: sand particles. Scale bar: 200 μ m, d) sandy soil with 10 mM MUF-P fixed by agarose gel. Arrows: sand particles. Scale bar: 200 μ m, e, i) loamy soil

with10 mM MUF-P fixed by silicon spray. Scale bar: 50 μ m, e, ii) sandy soil with10 mM MUF-P fixed by silicon spray. Scale bar: 100 μ m, e, iii) loamy soil aggregates with 10 mM MUF-P fixed by silicon spray. Arrows: crystallization of MUF around loamy soil aggregates after 30 min. Scale bar: 100 μ m. f) loamy soil with 5 mM MUF-P fixed by super transparent silicon. Scale bar: 50 μ m

negligible changes in weight (0.1%), especially from the thin layer of homogeneous soil aggregates. Therefore, among all tested fixations, super transparent silicon enabled the best assessment of the development of fluorescence over time and was applied for further experiments.

Appling a range of concentrations of MUF-P (0.1–10 mM) revealed weak enzymatic activity below 5 mM, while a very strong signal of the highest concentration (10 mM) reduced the discernibility of aggregates. Therefore, a concentration of 5 mM was considered as an optimum (Fig. S2).

Controls

Auto-fluorescence of the control samples Glass slides, super transparent silicon and 0.1 M MES buffer (which substituted a substrate in the control treatment) did not exhibit any auto-fluorescence at an exposure time of 3000 ms (Fig. S3). Application of MUF-P to autoclaved soil did not induce auto-fluorescence of the substrate in the liquid phase under UV light (Fig. S4b, middle row). The auto-fluorescence of MUF-P usually observed in the microplate assay (Pritsch et al. 2004) (Fig. S5a) was also visible under

the microscope at exposure time 10,000 ms (Fig. S5b, i). However, shortening the exposure time to 3000 ms considerably diminished the effect of auto-fluorescence (Fig. S5b, ii). Given the average grayscale value was 15.5 ± 0.04 (Fig. S4a) in autoclaved soil, before application of MUF-P, the possible auto-fluorescence of MUF-P did not exceed the background level (Figs. S4b; S5c) and was subtracted with the background from the experimental values. The overall signal intensity in a thin layer of autoclaved soil was almost stable within the time. In the liquid phase however, it slightly decreased in the first 10 min and then remained almost stable over time (Fig. S5c).

Most soil aggregates did not exhibit any detectable auto-fluorescence. The auto-fluorescence of a small number of soil aggregates was differentiated from enzymatic activity by comparison of the signal intensity over time after substrate addition (Fig. 2a and b). Increasing fluorescence intensity on aggregates over time indicated ongoing transformation of the substrate by microorganisms producing MUF through enzymatic activity (Fig. 2, i and i'). Decreasing intensities of the fluorescence indicated sorption of fluorogenic compounds on soil aggregates, while fluorescence constant over time were considered as auto-fluorescence of minerals (Fig. 2, ii and ii').

Fluorogenic substrate Was applied to the autoclaved and non-autoclaved top layer of soil aggregates and then covered by super transparent silicon (Fig. S4). Before application of MUF-P, the autoclaved (Fig. S4a) and non-autoclaved (Fig. S4c) soil aggregates showed little auto-fluorescence with grayscale values of 15.5 and 15.8, respectively. 30 min after application of MUF-P, the mean grayscale value of autoclaved loam soil aggregates was nearly identical 16.3 ± 0.09 (Fig. S4b). Autoclaved quartz particles exhibited auto-fluorescence (Fig. S6a) which increased after wetting. However, it remained stable over time (grayscale values of 27–28) (Fig. S6b) demonstrating absence of enzymatic activity in recently sterilized samples.



Fig. 2 An example of differentiating auto-fluorescence from real fluorescence caused by enzymatic activity on soil aggregates incubated with 0.1 mM MUF-P after 30 min. The background (before application of MUF-P) is subtracted from the fluorescent signals; **a** and **b**) images taken using DAPI filter (excitation of 335–383 nm and emission of 420–470 nm). The

pseudocolor images **a**'and **b**' are based on MUF calibration. Scale bar: 50 μ m. Fluorescence **a** and **a**') immediately after substrate addition, **b** and **b**') 30 min after substrate addition, **i** and **ii**) fluorescence from two aggregates immediately after substrate addition, **i**' and **ii**') fluorescence of the same aggregates after 30 min

Calibration

We observed a linear relationship between the concentrations of MUF in the range from 0 to 1000 μ M and the grayscale values in thin layer of soil aggregates (Fig. 3). The fluorescence of MUF was confirmed to be stable on individual loam aggregates over time (Figs. S7; S8b).

Super transparent silicon

Curing process No weight loss of both individual aggregates and the thin layer of loam soil covered with super transparent silicon was found in the first 30 min of incubation. Thereafter, from 30 to 70 min, the total weight losses of the samples were almost negligible and did not exceed 0.1%.

Visualization of enzymatic activity In the free liquid phase (large pores between the aggregates which became saturated after addition of MUF-P in the first minutes), fluorescence developed within a couple of minutes after substrate addition. However, intensities faded and seemed to concentrate around the aggregates afterwards. The fluorescence at the aggregate boundaries remained rather constant until 50 min of incubation. After 60 min, the fluorescence vanished from the liquid phase and remained highest at the aggregate surfaces (Figs. 4a; S9). Fluorescence was



Fig. 3 Linear regression between MUF concentrations (μ M) and intensities (y = 14.84x-24.673 and $R^2 = 0.998$). The images are shown in pseudocolor for better visibility. The background (before application of MUF) is subtracted from the fluorescent signals. Changes of MUF concentration in the overall image presented as mean values of 4 technical replicates. Given are means \pm standard deviations

determined in three contrasting phases (free liquid, aggregate boundaries, and at aggregate surface) over time after substrate application. The contribution of the liquid phase to the total signal intensity decreased by up to 2.5 times from 6 to 60 min. The relative contribution from aggregate boundaries reached its maximum between 40–50 min. After 60 min, it then sharply dropped, as the activity receded into internal surfaces of aggregates, causing the fluorescence to become spatially more concentrated. Consequently, the relative contribution of aggregates to the total signal intensity almost doubled at the aggregate surfaces at 60 min compared with 6 min (Fig. 4b).

The observed phenomena might be explained by water menisci at the contact points between aggregates and the glass slides. Water menisci form a liquid fringe around the aggregates on the flat glass slide. Hence, the higher signal intensity around the aggregates may either i) reflect a true redistribution of fluorescence towards aggregate boundaries or ii) just a higher signal intensity due to an accumulation of fluorescent products in deeper water menisci around individual particles and soil aggregates or iii) a combination of both. To distinguish both effects, we carried out a test with a thin layer of closely packed, homogenous soil aggregates (sieved to \leq 500 µm), which was thought to lead to a homogenous thickness of water menisci. The distribution of enzymatic activity was monitored over 80 min in liquid (macropores) and solid (at the surface of individual aggregates) phases after subtraction of background intensities of the control samples (before application of MUF-P). Comparing the pseudocolor images of individual aggregates (Figs. 4a; S9) with the corresponding images of the thin soil layer (Figs. 5a; S10a), it was obvious that the development of fluorescence started in the liquid phase, i.e., in large pores between the aggregates, within minutes of substrate addition. Over time, it shifted and became more concentrated in smaller pores at the aggregate boundaries and finally remained only at the surfaces of aggregates. The amount of MUF released from aggregate surfaces increased 10 times from 10 to 70 min after substrate addition. In contrast, in the liquid phase, the amount of MUF decreased by one order of magnitude in the same time frame (Fig. 5). This confirmed the shift of enzymatic activity from the liquid phase (macropores) to the aggregates. Therefore, we conclude that the size of water menisci cannot explain the relatively



Fig. 4 Development of fluorescence over time (shown in pseudocolor) after application of 5 mM MUF-P to individual soil aggregates (%). The background (before application of MUF-P) is subtracted from the fluorescent signals; **a**) an example of the spatial distribution of fluorescence from individual soil aggregates over time shown under both fluorescence (through a DAPI filter, top row) and pseudocolor (bottom row). Each contrasting phase was distinguished by highest color contrast. For example, in the area inside the black rectangular at 6 and

high fluorescent signal developing around the aggregates in 10–50 min after substrate addition (Fig. 6).

Incubation time The total fluorescence, the fluorescence of free liquid phase, and of the aggregate surfaces intersected at 20–40 min (Figs. 5b; S10b). Therefore, 30 min may be considered a threshold value indicating the shift of fluorescence from the

30 min, red, yellow, and cyan colors demonstrate free liquid phase, aggregate boundaries, and aggregate surface, respectively. Scale bar: 50 μ m, **b**) relative contribution of the liquid and solid phases and the liquid–solid boundary to the overall enzymatic activity taken as 100% in individual soil aggregates over time. Letters denote significant difference over time for each contrasting phase tested by one-way repeated measures ANOVA in 3 rhizoboxes (biological replicates). Given are means \pm standard deviations

liquid to solid phase. However, appropriate incubation times always depend on the targets of the studies. To monitor the development of signals around the aggregates, 30–40 min are recommended. In contrast, to demonstrate the fluorescent signals at the surface of individual aggregates, 60–80 min appear appropriate (Figs. 5; S10b).



Fig. 5 Development of fluorescence over time in rhizobox 1 after application of 5 mM MUF-P to a thin layer of homogeneous loamy soil aggregates. The background (before application of MUF-P) is subtracted from the fluorescent signals; a) pseudocolor example images were assumed showing the phos-

Root surface The distribution of fluorescence stemming from potential enzymatic activity was found to be rather homogeneous at root tips as observed via standard zymography (Fig. 7, panels a' and b'). In contrast, imaging root surfaces via micro-zymography

phatase activity in time. Scale bar: 200 μ m, **b**) changes of the enzymatic activity based on MUF concentrations (μ M) in the overall image, aggregate surfaces and liquid phase presented as mean values of 3 technical replicates in each contrasting phase. Given are means \pm standard deviations

and confocal laser scanning microscopy showed a more heterogeneous distribution of fluorescent signals at a micro-scale (Fig. 7, panels i and ii), suggesting that potentially active enzymes may show a patchy distribution along the rhizoplane.



Fig. 6 Schematic illustration of the development of fluorescence over time in soil aggregates after application of 5 mM MUF-P. The transparent silicon covers both solid and liquid

Discussion

Auto-fluorescence Autoclaved loam aggregates (Fig. S4a) emitted weak fluorescence, possibly originating from microbial (Kus 2015) or plant residues (Willemse 1989). In addition, fluorescence might be emitted by some minerals, e.g., Hardystonite and Barylite (Robbins 1983). In our case, autoclaved quartz particles showed higher auto-fluorescence (Fig. S6) than loam aggregates (Fig. S4). The autofluorescence slightly increased after addition of water both at the surfaces of quartz particles and the liquid phase indicating weak reflection of UV light emitted through the microscope objective (Fig. S6b). Some bright non-luminescent minerals like quartz emit bluish fluorescence after being wetted (Ewles 1930). This can explain the bright tiny objects in Fig. S4. Auto-fluorescence may be reduced by CuSO₄ Sudan Black B (Schnell et al. 1999), NaBH₄ (Clancy and Cauller 1998), or Pontamine Sky Blue (Cowen et al. 1985). However, treating soil with such chemicals (e.g., reducing agents) may kill microorganisms and may decrease enzymatic activity. Alternatively, the

phases. The color change in liquid phase and aggregate surface corresponds to fluorescent intensity (μM MUF) shown in pseudocolor images in Fig. 4

background auto-fluorescence can be subtracted by mathematical modelling (Steinkamp and Stewart 1986; Van de Lest et al. 1995) which needs specific parametrization to adequately recognize the background in heterogeneous matrices like soil (Neumann and Gabel 2002). However, according to our results, auto-fluorescence in various sources was stable over time (Fig. 2, ii and ii'), therefore enabling subtraction of these background signals of control samples (before application of MUF-P). In addition, although MUF-P demonstrated auto-fluorescence in autoclaved loam aggregates at very long exposure time (10,000 ms), it was eliminated at 3000 ms (Fig. S5b). The decrease in auto-fluorescence intensity in the first 10 min in autoclaved soil could be due to the quenching effect of soil organic matter as a result of physical sorption by microbially-inactive soil matrix (Tadini et al. 2020). This was confirmed by microplate assay based on sterilized water and on sonicated sterilized soil suspension after application of MUF-P (Fig. S5a). The quenching effect was also detected by micro-zymography as a difference between autoclaved soil and liquid phases (Fig. S5c). Remarkably,

Fig. 7 Examples of maize root segments visualized by confocal laser scanning microscopy; a and b) maize roots (with attached soil particles) fixed on glass slides by super transparent silicon after application of MUF-P-saturated membranes, **a**' and **b**') zymograms of the maize roots, shown in pseudocolor, demonstrating the distribution of phosphomonoesterase activity 6 and 10 min after application a of MUF-P-saturated membrane. Scale bar: 5 mm, i and ii) small parts of maize root monitored by confocal microscopy after being embedded in ProLongTM Gold Antifade Mountant (at excitation of 405 nm and detection of 414-482 nm) and Vectashield (at excitation of 488 nm and detection of 515-624 nm), respectively. Scale bars: 50, and 500 µm, respectively. Image i has been LIGHT-NING processed



the auto-fluorescence remained stable over time as a whole and after a slight decrease in the liquid phase in the first 5 min (Fig. S5c). As the auto-fluorescence intensity was approximately in the same range with the mean background intensity of control sample (before application of MUF-P) (Fig. S4a), it was considered by a subtraction of the background during image processing.

The main fluorescence emission from the soil thin layers Shifted from the liquid phase to small pores within aggregates. It was assumed that higher enzymatic activities in these pores corresponded to higher microbial abundance and activity due to higher C allocation from root exudates into pores with the size of $30-150 \ \mu\text{m}$ compared to larger pores (>180 \ \mmmmm) (Kravchenko et al. 2019b). In contrast to the liquid phase in large pores, steadily increasing fluorescent

signals were detected on a number of aggregates until 70 min, and only leveled off thereafter (Figs. 5; S11). High fluorescence intensities within aggregates and low fluorescence intensities in the liquid phase contained in large pores can be explained by preferential attachment (sorption) of microorganisms to the solid soil fraction. Possible mechanisms of the phenomenon observed in solid and liquid phases are discussed below:

Solid phase Higher abundances of microorganisms at the aggregate surfaces result in higher production of enzymes which, in turn, interact with the organic and organo-mineral soil matrix. After addition of the liquid substrate, a new equilibrium between the solid and free liquid phase has to be established. Therefore, enzymes could be partly desorbed and move in a free- or in a colloid-associated form in solution

(Guber et al. 2022). Enzymes adsorbed onto organic colloids by electrostatic forces and ligand exchange are easily desorbed and re-mobilized. In contrast, they bind firmly to mineral colloids by ion exchange, covalent complexation, hydrogen bonding, van der Waals forces, and hydrophobic forces (Huang et al. 2005; Nannipieri et al. 1996) and they also become immobilized at aggregate surfaces under the influence of adsorption forces mentioned above (Stotzky 1972). Beside enzymes, various chemicals and microorganisms can be transferred by soil mineral colloids (Guber et al. 2022). Given the clay content (20%) of the loam soil used in this study, we assumed formation of enzyme-clay complexes at aggregate surfaces. Coarse and mineral soil colloids $(1 \ \mu m < \emptyset)$ have a higher affinity for enzyme molecules than the fine and organic colloids ($\emptyset < 1 \mu m$) (Guber et al. 2022; Huang et al. 2005). It is also assumed that a fraction of immobilized enzymes maintains activity in the short-term after immobilization (Schimel et al. 2017). Therefore, the concentration of enzymes and consequently the decomposition of MUF-P substrate increased at the surfaces of clay particles forming a substrate concentration gradient from liquid to solid phase which mimics a natural situation after organic P source becomes available in soil (Manzoor et al. 2022; Tietjen and Wetzel 2003). Substrate diffusion and sorption of enzymes on soil aggregates possibly contributed to the shift of fluorescence over time. Larger specific surface areas of small internal pores relative to the large pores, higher amounts of organic matter and of substrate adsorbed to clay would explain higher enzymatic activities inside aggregates (Huang et al. 2005; Tietjen and Wetzel 2003).

Liquid phase Enzyme-clay complexes formed in soil can be transferred to the liquid phase as organomineral colloids (Guber et al. 2022; Tietjen and Wetzel 2003). Since strong fluorescence was first detected in the free liquid phase and it quickly shifted to the interface between aggregate and liquid phases, it was assumed that the part of substrate was decomposed quickly by microorganisms associated with the colloidal fraction and the fluorogenic product was released in the liquid phase. Undecomposed substrate moved towards internal pores of aggregates due to a concentration gradient probably formed as a result of faster substrate consumption caused by locally higher microbial abundance at solid versus liquid phase. As a consequence, at the end of the incubation, the fluorescence was detected at a few aggregates. Remarkably, such a shift from the liquid to the solid phase was not detected when the fluorogenic product (MUF) was added to the soil (Fig. S8). In contrast to substrate addition (MUF-P) (Fig S8a), the distribution of MUF was homogeneous when directly added to the soil (Fig. S8b). This could be due to the charge differences of the MUF (positively charged) produced by cleavage of MUF-P and neutral form of molecular MUF (pKa: 7.79) used for calibration (Nagy et al. 2015; Zhi et al. 2013). Thus, positively charged MUF-products could be bound to negatively charged soil colloids while no binding occurred between colloids and neutral MUF molecules resulting in a homogeneous distribution of the fluorescence in the control samples, where no enzymatic reaction occurred (Figs. S8b; 6). Therefore, the increase in relative contribution of aggregate boundaries (Fig. 4, until 50 min) was likely caused by attraction of fluorescent products around negatively charged aggregate boundaries and not by locally higher enzymatic activity relative to free liquid on glass slides. Such a higher enzymatic activity would have continued in line with internal pores in aggregates (Figs. 4, 5, and 6).

Total fluorescence Demonstrated a decreasing pattern over time, possibly due to product adsorption at aggregate surfaces. In addition, part of fluorescent signal was hidden at the back and inside the aggregates and was not visible in 2D microscopic images (Fig. 5).

The distribution of fluorescence within aggregates after application of fluorogenic substrate was patchy, either reflecting heterogeneous localization of enzymatic activity, or it may be a result of topography (Duman et al. 2010). High-resolution confocal microzymography on the rhizosphere hotspots identified by membrane zymography also revealed an inhomogeneous and patchy distribution of enzymatic activity at the root surface within a hotspot (Fig. 7). This observation, however, requires more solid experimental evidence.

Summary and outlook

We developed a micro-zymography method to visualize the distribution of enzymatic activity on thin layers of rhizosphere soil over time with a spatial resolution of $1-100 \ \mu m$.

By comparing different fixatives, we identified super transparent silicon as the most appropriate fixative for micro-zymography which does not only keep the soil aggregates in place on glass slides but also helps keep them moist and does not destroy microbial cells by an application of interfering chemicals. Micro-zymography enabled monitoring the fluorescence over time, which reflects the development of enzymatic activity in soil. Both for individual aggregates and for a soil thin layer, the development of fluorescence started from the liquid phase, after which the main intensity gradually shifted to the interfaces of aggregates and the free liquid phase, and finally, it became concentrated at the surfaces of aggregates. Fluorescence decreased ten times in the liquid phase while it simultaneously increased ten times at the aggregate surfaces. This reaction could be due to substrate concentration gradients caused by higher microbial abundance and enzymatic activity at the soil surfaces compared to the liquid phase. This microbial response in unsterilized soil was also confirmed by the absence of signal development between 10-30 min in sterilized soil, indicating the absence of microbial and therefore enzymatic activity in the latter. The potential of micro-zymography to assess the development of enzymatic activity in space and time needs to be further tested considering different types of soil and root morphologies.

As an outlook, membrane-free micro-zymography offers promising potential to identify functional specificity and niche differentiation by microorganisms through distribution of their activity at root and aggregate surfaces after robust testing of the method on various soil types and plant species. The next challenge would be to combine zymography with other imaging techniques such as i) imaging the chemical elements by synchrotron-based X-ray techniques, ii) nano-scale secondary ion mass spectroscopy (nanoSIMS), iii) nuclear magnetic resonance (NMR), iv) fluorescence in situ hybridization (FISH). Most of these approaches, however, require resin impregnation or dehydration for preparation of thin layers which affect microbial and enzymatic activity. As visualization of enzymatic activity suggested here is based on the fluorescence of the product of enzymatic reaction, the sample preparation requires gentle treatment maintaining viability of microorganisms and enzymes. Thus, the novel micro-scale approach raises some new questions about the relationship between fluorescence and enzymatic activity which are probably masked by the coarser scale in the "classical" zymography approach.

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Data availability All data and materials support our published claims and comply with field standards.

Code availability No code availability

Declarations

Ethics approval The authors declare that this work is original and has not been published elsewhere, nor is it currently under consideration for publication elsewhere.

Consent to participate Informed consent was obtained from all authors for participation.

Consent for publication The authors have consented to the publication.

Conflicts of interest/Competing interests The authors have no conflicts of interest to declare that are relevant to the content of this article.

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