Soil Biology and Biochemistry 127 (2018) 156–167

Contents lists available at ScienceDirect

Soil Biology and Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Quantitative soil zymography: Mechanisms, processes of substrate and enzyme diffusion in porous media

Andrey Guber[a,*], Alexandra Kravchenko[a,b], Bahar S. Razavi[b,c], Daniel Uteau[d], Stephan Peth[d], Evgenia Blagodatskaya[b,e], Yakov Kuzyakov[f]

[a] Department of Soil, Plant and Microbial Sciences, Michigan State University, East Lansing, MI, USA
[b] Department of Agricultural Soil Science, University of Göttingen, Göttingen, Germany
[c] Department of Soil Science and Plant Nutrition, University of Kiel, Kiel, Germany
[d] Department of Soil Science, University of Kassel, Witzenhausen, Germany
[e] Institute of Physicochemical and Biological Problems in Soil Science, 142290, Pushchino, Russia
[f] RUDN University, Moscow, Russia

ARTICLE INFO

Keywords:
In situ 2D zymography
Soil enzyme activity
Microbial hotspot's localization
Diffusion processes

ABSTRACT

Soil membrane zymography enables 2D mapping of enzyme activities on the surface of soil samples. The method is based on diffusion of components of enzymatically-mediated reactions to/from membrane, and, thus, reflects the distribution of enzyme activities at the intact soil surface. Zymography has been already successfully implemented in numerous soil ecology applications. Here we identify two methodological aspects for further improvement and expansion of the method at micro and macro scales: first, accounting for the area of contact between the soil surface and the zymography membranes and, second, accounting for diffusion effects during the zymography procedure. We tested three methods, namely, laser-scanning, staining with a fluorescent product (e.g. MUF: 4-methylumbelliferone), and X-ray computed micro-tomography, for assessing the area of the soil surface in contact with the membranes. We quantified diffusion of MUF, enzymes and substrate between the substrate-saturated membrane and soil as well as diffusion processes during membrane zymography via HP2 software. Diffusion of the substrate from the membrane and of the MUF-product to the membrane was detected, while there were no clear evidence of enzyme diffusion to/in the membrane. According to the model simulations, the enzyme activities detected via 2D zymography probably represent only a small portion, about 20%, of the actual reactions within the soil volume that is in both direct contact and in hydrological contact with zymography membranes. This is a result of omnidirectional diffusion of reaction products. The membrane contact with the soil surface estimated by three methods ranged from 3.4 to 36.5% further signifying that only a fraction of enzymes activity is detectable in a course of 2D soil zymography.

1. Introduction

Soil zymography is a new technique for *in situ* visualization and quantification of two-dimensional distribution of enzyme activities in soil and rhizosphere studies (Hoang et al., 2016; Ge et al., 2017; Liu et al., 2017; Razavi et al., 2016, 2017; Sanaullah et al., 2016; Spohn et al., 2013; Spohn and Kuzyakov, 2014). During zymography a membrane saturated with an enzyme-specific fluorogenic substrate is placed on the surface of a soil sample. Upon a contact of the substrate with soil enzymes, a fluorescent product (e.g. MUF: 4-methylumbelliferone, or AMC: 7-amido-4-methylcoumarin) is released and its presence on the membrane is then detected under UV light. The fluorescing pattern on the membrane reflects spatial distribution of active enzymes on the soil surface.

The key difference between the membrane zymography and classical measurements of enzyme activities in soil slurries is the enzyme-substrate accessibility. Classical enzyme assays maximize access of substrate to all potentially reactive enzyme sites by ensuring sample destruction and detachment followed by the release of enzymes from soil matrix to the suspension (Schimel et al., 2017). However, in intact soil, an occurrence of a contact between a substrate and an enzyme depends on the presence of transport avenues and on diffusion processes taking place within them. Membrane zymography to a certain extent emulates the diffusion processes taking place in the intact soil.

Abbreviations: MUF, 4-methylumbelliferone; WM, Water-saturated Membrane; SM, Substrate-saturated Membrane; CT, computed tomography

* Corresponding author.
E-mail address: akguber@msu.edu (A. Guber).

https://doi.org/10.1016/j.soilbio.2018.09.030
Received 8 June 2018; Received in revised form 26 September 2018; Accepted 27 September 2018
Available online 28 September 2018
0038-0717/

Just as in soil, the fluorescent patterns on the membrane depend on diffusion. This feature is both a blessing and a curse of the method, as it enables clear visualization of the real processes occurring in soil, but substantially complicates interpretation of its results.

Interpretation of the fluorescent pattern on soil zymograms is based on the assumption that spots with high fluorescent levels correspond to localities with high enzyme activity, while spots with no fluorescence correspond to soil surface localities without enzymes. However, the observed fluorescent pattern is likely a complex outcome of diffusion of the involved chemical compounds, i.e., enzymes, substrates, and fluorescent products. Below we describe the hypothesized concept of physical processes taking place during zymography measurements.

The activity detected in a course of soil zymography is generally attributed to extracellular enzymes excreted by roots or microorganisms, which exist either immobilized on surfaces of the soil matrix and organic materials or in a free form in the soil solution (Gianfreda and Bollag, 1994; Rao et al., 2000). The immobilized enzymes are assumed to be strongly attached, thus immobile, while the free enzymes are assumed to be mobile (Nannipieri and Gianfreda, 1998). While it is presumed that both forms exist (Stotzky, 1986; Nannipieri et al., 1996), their relative sizes are generally unknown and likely dynamic, due to fluctuations in enzyme production by roots and microorganisms, and continuous biochemical enzyme degradation and their immobilization by soil particles and organic material. The soil surface in contact with zymography membrane is, typically, very uneven. Thus, as the distances between enzymes located on/near the soil surface and the membrane increase, the potential contributions of enzymes to the fluorescent signal on the membrane decrease (Fig. 1). Free enzymes in the soil solution can potentially reach the membrane by diffusion or react with dissolved substrate within the water film boundary between the membrane and soil surface. Immobilized enzymes can be broadly divided into three groups in terms of their position with respect to the membrane: 1) enzymes in direct physical contact with the membrane, 2) enzymes in hydrological contact with the membrane through water films, and 3) enzymes without any contact with the membrane. When the membrane with a substrate is placed on a soil surface, the enzymes in direct contact with the membrane (group 1) are the first to be involved in catalytic activities. The substrate readily available for this enzyme group is decomposed quickly and the released MUF immediately contributes to appearance of a fluorescent signal on the membrane. Involvement in catalyzes of the enzymes connected with the membrane through water films (group 2) depends on the time needed, first, for the substrate to diffuse from the membrane and reach the enzymes, and, second, for the released MUF to diffuse back to the membrane. The immobilized enzymes with no contact with the membrane (group 3) are unlikely to contribute to the fluorescent signal, since there are no means for the substrate and MUF transport to/from them (Fig. 1). Thus, the fluorescent signal detected on the membrane under UV-light may originate from multiple sources including immobilized and free enzymes. Upon reaching the membrane, the MUF products can also laterally diffuse within it during the incubation and thus decrease the spatial resolution of the zymogram. Higher catalytic activity of free enzymes as compared to immobilized ones (Rao et al., 2000) further complicates interpretation of soil zymograms. The same complications exist in standard fluid-based enzyme assays.

It should be noted that diffusion, in contrast to convective flow, is omnidirectional, and rates of either vertical or lateral diffusion of substances, enzymes, and products, e.g. MUF, in soils and membranes are generally unknown. Therefore, quantification of *in situ* enzyme activity in the soil and the rhizosphere, based on membrane zymography, requires accurate assessments of diffusion pathways and rates for all involved chemicals. We assessed following diffusion pathways involved in zymography analysis: 1) diffusion of enzymes from soil into zymography membranes; 2) diffusion of MUF and substrate within the membranes; 3) diffusion of substrate from the membrane to soil; and 4) diffusion of MUF from soil to the membrane.

Quantifying diffusion is greatly complicated by the ubiquitous unevenness of soil surfaces and related water films, which introduce a large uncertainty into size (area) and quality of contact between the soil surface and the membrane, the problem being particularly substantial in well aggregated and coarse textured soils. Due to differences in sizes and shapes of soil particles and aggregates, presence of soil pores and incompletely decomposed plant residues, a soil surface is never perfectly flat, and thus the actual area in direct physical contact between the membrane and the soil may be relatively small (Fig. 1). Its size can be difficult to measure, though theoretically, on an ideally sliced dry soil surface, the contact area should be equivalent to the volumetric fraction of solid substances in soil, i.e. = [1.0 - soil porosity]. However, in moist soil the indirect contact areas, e.g. via water films, can be much larger in size than the direct contact areas (Fig. 1), but the quality of this type of contact is inferior to direct contact. By quality of the contact here we refer to spatial accuracy with which the fluorescent signal on the membrane can reflect activity of the enzyme. Due to tortuosity of diffusion pathways, such spatial accuracy resolution is expected to be much lower for indirect contact areas. The size of indirect contact depends on the soil water content and the soil water retention properties. Knowing the positions of the direct and indirect contacts on the membrane is an important prerequisite for soil zymogram's

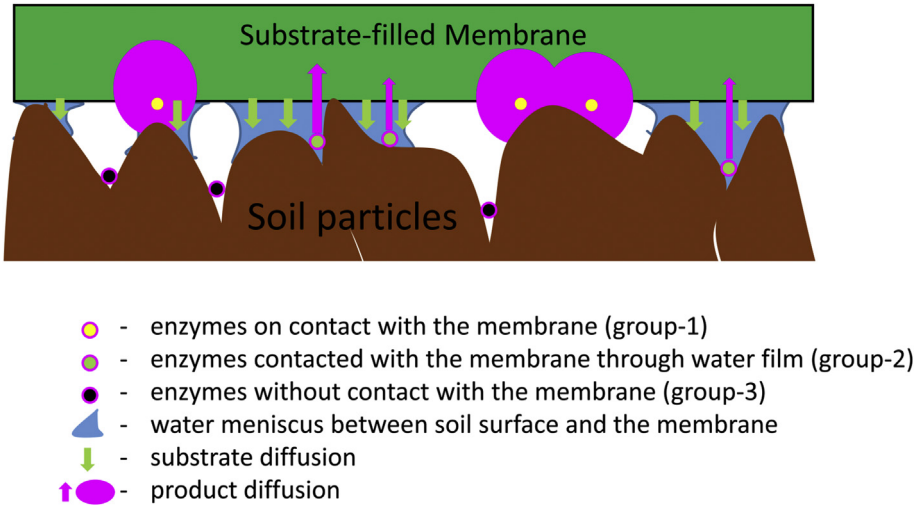

Fig. 1. Hypothetical pathways of the substrate and product diffusion between the membrane and soil surface, when a membrane with substrate is incubated on the soil surface during zymography analysis.