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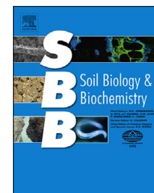

Root development impacts on the distribution of phosphatase activity: Improvements in quantification using soil zymography

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ABSTRACT

Zymographic methods for the 2D distribution of phosphatase activity in soils have markedly advanced our understanding of root-soil-microbiota interactions. Robust quantitative approaches for 2D assays, which use 4-methylubelliferyl phosphate (4-MUP), are needed to advance a mechanistic understanding of enzyme behaviour and distribution in soils. We present improvements to the method for phosphatase zymography in rhizobox studies, involving (1) a systematic evaluation of 4-methylumbelliferone (4-MU)-based calibration functions in relation to image exposure time and (2) the development of advanced image analysis tools for lateral and longitudinal distributions of phosphatase activity along barley roots (*Hordeum vulgare* L., cv Optic). Exposure time (<1–32 s) affected the slope and intercept of 4-MU calibration equations by 4.4- and 5.8-fold, respectively. In lateral root profiles, a linear relationship was found between phosphatase activity and root hair length at 0 cm (7 nKat mm^{-2}), 0.2 cm (48 nKat mm^{-2}), and 2 cm (234 nKat mm^{-2}) distance from the root tips ($r = 0.9795$, $p < 0.0001$); an algorithm designed to optimise estimates of phosphatase activity longitudinally confirmed this relationship ($r = 0.9462$, $p < 0.0001$). To improve the precision and accuracy of fluorescence-based soil zymography, careful control of calibration and imaging conditions and further development of advanced image analysis techniques are recommended.

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1. Introduction

Plant and microbial phosphatase enzymes catalyse the mineralization of organic phosphorus (P) in soil (Eivazi and Tabatabai, 1977). Standard methods for the measurement of soil phosphatase activity originally relied on the incubation of soil with organic P substrates and, later, artificial chemical substrates such as 4-methylumbelliferyl phosphate (4-MUP) or *p*-nitrophenyl phosphate (Nannipieri et al., 2011). In 'batch' incubations such as these, the product of hydrolysis is quantified spectrophotometrically or fluorometrically and is normalized by incubation time and soil mass (e.g., mol s^{-1} g^{-1}, Kat g^{-1}) (Dick, 2011). Methods for the 2D visualization of phosphatase activity in soil (soil zymography) have developed in the last 30 years (Oburger and Schmidt, 2016). These methods provide a means of evaluating the spatial heterogeneity of phosphatases and have advanced our understanding of how plant genotypes, living and dead roots, fertilizer treatments, and microorganisms affect the cycling of P in the rhizosphere (Spohn et al., 2013; Spohn and Kuzyakov, 2013, 2014; Spohn et al., 2015).

The first described non-destructive method for the 2D analysis of chemical changes in the rhizosphere was a compendium of protocols for visualizing redox-sensitive metals, soluble phosphate, pH, and phosphatase activity (Dinkelaker et al., 1993a, 1993b). The 'Hohoneim Box' (rhizobox) consisted of a narrow layer of soil placed at an angle, such that roots grow into a quasi 2D pattern against a removable transparent plate. At the end of the growth period, roots are exposed by removing the plate so that biochemical and 2D image analyses can be conducted. These 2D assays involve pressing a gel and/or filter paper against the surface of the exposed

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roots and soil. The filter paper is impregnated with a substrate, which when hydrolysed, will indicate the presence of enzyme activity or a specific analyte by colour change or fluorescence. Whilst the original 2D method for phosphatase zymography was based on 1-naphthyl phosphate, resulting in red zones of hydrolysis on filter papers (Dinkelaker & Marschner, 1992), later studies such as Dong et al. (2007) have used MUP followed by fluorescence imaging.

The *in situ* measurement of phosphatase distribution developed by Spohn et al. (2013) is used to visualize root and soil-associated phosphatase activity on a 2D plane. An agarose gel (1% w/v) is placed on the soil surface and acts as the mobile phase for soil enzymes. A nylon filter paper, impregnated with the phosphatase substrate (4-MUP), is placed on the gel surface and allowed to incubate at ambient temperature. Root and soil phosphatases that diffuse through the gel to the filter paper (and which are active at the designated pH of the assay) will hydrolyse MUP to its fluorescent product (4-methylumbelliferone, 4-MU). Acid or alkaline phosphatase activity is defined by the pH of the assay, which is controlled using modified universal buffer (MUB) at pH 6.5 or pH 11, respectively (Spohn and Kuzyakov, 2013).

The use of fluorescence imaging to measure phosphatase activity is powerful due to its sensitivity, relative ease of image collection, and the large amount of fine scale information gained on the spatial heterogeneity of the phosphatase activity in soil. The filter deployment procedure in soils is relatively straightforward and fluorescent gel images can be collected with equipment standard to molecular laboratories (gel casting box, gel imaging system). A key challenge however is to establish a suitable methodological pipeline for the collection and quantitative analysis of such images. To date, most studies that have utilized the 2D zymography method have been qualitative with few exceptions, including the first short communication on MUP-based zymography (Dong et al., 2007). Dong et al. (2007) quantified the size of phosphatase-active areas based on binned pixel counts (i.e., 50–100, 100–150 … >250 pixels) and found differences between Douglas fir and birch tree roots of varied ages. Recently, gridding approaches have been used to quantify the average activity across a single image (Hofmann et al., 2016; Liu et al., 2017), whereby a grid is overlaid on an image and the mean gray-value intensity is recorded wherever a root crosses a gridline. Mean gray-values at root-grid intersections are then averaged to obtain a single intensity value for each replicate. Replicate filters can then be used to carry out statistical comparisons across treatments. Using this approach, Hofmann et al. (2016) found statistically significant effects of P availability on acid phosphatase activity in the rhizosphere of young beech trees. Whilst both qualitative and quantitative pixel-based and gridded approaches are useful, only a fragment of the information embedded in the image is captured.

All current methods for quantification of phosphatase activity, including batch and 2D methods, rely on the same chemistry and are therefore vulnerable to similar limitations, such as the unknown specificity of soil enzymes to the assay substrate and that only active, non-degraded and mobile enzymes are targeted (Nannipieri et al., 2011). Additionally, an artefact of the 2D method is that incomplete filter drying can lead to blurred images, and poor contact between the filter-gel and soil may create an erroneous perception of heterogeneity in a sample (Oburger and Schmidt, 2016). Regarding the collection of images, fluorescence intensity is captured in a digital photograph of the filter under UV light. As for any digital image, image quality depends on the type of light sensor used (e.g., charge coupled device [CCD], complementary metal-oxide semiconductors [CMOS]) and the resulting specifications of the imaging system (e.g., dynamic optical range, image resolution), which in turn determines the accuracy of the digital information obtained. A lack of understanding of the imaging system may lead to a loss of information or inaccurate fluorescence measurements, for example due to over-exposure, under-exposure or photo bleaching. The processing of 2D experimental data is also time-consuming in the absence of suitable image analysis tools (Downie et al., 2015).

Current mechanistic studies of plant-microbiota-soil dynamics seek to quantify the impact of edaphic and biological factors on the distribution of phosphatase activity in the rhizosphere. For example, studies of 2D phosphatase activity distribution have revealed several important biogeochemical mechanisms in root-soil systems, particularly regarding the role of microorganisms and soil amendments in the turnover of P and C (Spohn et al., 2013; Spohn and Kuzyakov, 2013; Kuzyakov and Blagodatskaya, 2015; Spohn et al., 2015; Hofmann et al., 2016; Liu et al., 2017). The ability to quantify the relative extent and decay of phosphatase activity across and along the root axis is also of interest for estimating the total soil volume affected by various phosphatases and to model the various physical and biochemical mechanisms of nutrient turnover in the rhizosphere (e.g., 'hot spots'; Kuzyakov and Blagodatskaya, 2015).

Improvements are therefore needed to expand the quantitative capacity of soil zymography methods. In this paper, we present amendments to the 4-MUP based soil zymography method for studying the 2D distribution of phosphatase activity in root-soil systems. The study focuses on two critical factors for improving the accuracy and precision of measurements: the calibration of phosphatase activity using 4-MU and downstream digital image analysis. Specifically, we (1) investigate factors affecting the quality of calibration relationships and propose improvements to the calibration protocol and imaging system to optimise the precision and accuracy of the technique and (2) develop an image processing pipeline to obtained standardised measurements of phosphatase activity using a root reference coordinate system. Finally, (3) we apply these analytical approaches in root-soil systems of barley (*Hordeum vulgare* L.) and analyse the patterns of phosphatase activity across perpendicular and longitudinal root axes.

2. Materials and methods

2.1. Plant growth

Barley (*Hordeum vulgare* L., cultivar 'Optic') plants were grown in rhizoboxes in a cultivated humus-iron podzol sampled from a permanent grassland site at Glensaugh, near Laurencekirk in Aberdeenshire, Scotland (56°53′42.29″N -2°32′00.42″W). The soil is derived from acid igneous and metamorphic rocks and Old Red Sandstone sandstones and under the Scottish system belongs to the Strathfinella association and series (Macaulay, 1981). The 'Glensaugh' soil has a very low available P content (6.7 mg kg^{-1} Olsen extractable P), is acidic (pH 4.5 in $CaCl_2$ 1:2 w:v) and contains a relatively large proportion of citrate-extractable P in organic forms (73%) (Stutter et al., 2015; Giles et al., 2017). The soil was sieved (<2 mm), dried, and then re-wet prior to filling the rhizoboxes. The rhizoboxes (15 cm × 10 cm x 1 cm) were constructed of two plexiglass and opaque plastic panels, which were separated by 1-cm plastic spacers and clamped together. The bulk density of soil in the rhizoboxes was ~1.1 g cm^{-3}. Soil moisture (~20%) was maintained by weight during the growth period. Barley seeds were pre-germinated on distilled water agar (0.1% w/v) and transplanted into rhizoboxes, which were placed at a 45° angle for ten days of growth under controlled growth conditions (22 °C day/14 °C night, 16 h light at a minimum 200 W m^{-2}).

2.2. Soil zymography

Gels and nylon filter papers were prepared with 40 mM MUB at

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