Soil Biology & Biochemistry 67 (2013) 106–113

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

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

Distribution of microbial- and root-derived phosphatase activities in the rhizosphere depending on P availability and C allocation – Coupling soil zymography with ^{14}C imaging

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ARTICLE INFO

Article history:
Received 3 April 2013
Received in revised form
11 August 2013
Accepted 12 August 2013
Available online 30 August 2013

Keywords:
Rhizosphere processes
Phosphorus mobilization
Phosphatase activity
Belowground carbon allocation
Microscales imaging
Rhizodeposition
Exudation
Autoradiography

ABSTRACT

Despite its importance for terrestrial nutrient and carbon cycling, the spatial organization of microbial activity in soil and in the rhizosphere is poorly understood. We related carbon allocation by roots to distribution of acid and alkaline phosphatase activity in the rhizosphere of *Lupinus albus* L. To do so, we further developed soil zymography – an *in situ* method for the analysis of the two-dimensional distribution of enzyme activity in soil – integrating fluorescent substrates. Soil zymography was combined with ^{14}C imaging, a technique that gives insights into the distribution of photosynthates after labeling plants with ^{14}C. Both acid and alkaline phosphatase activity were up to 5.4-times larger in the rhizosphere than in the bulk soil. While acid phosphatase activity (produced by roots and microorganisms) was closely associated with roots, alkaline phosphatase activity (produced only by microorganisms) was more widely distributed, leading to a 2.5-times larger area of activity of alkaline than of acid phosphatase. These results indicate a spatial differentiation of different ecophysiological groups of organic P mineralizing organisms. The spatial differentiation could be either between microorganisms and *L. albus* or between microorganisms that produce exclusively alkaline phosphatases on the one hand, and *L. albus* and root associated microorganisms that produce acid phosphatases on the other hand. The spatial separation of different organic P mineralizing organisms might alleviate a potential competition between them. While alkaline phosphatase activity strongly decreased with P fertilization, acid phosphatase activity was not affected by fertilization, suggesting that alkaline phosphatase-producing microorganisms react more strongly to it than other organic P mineralizing organisms. Alkaline phosphatase activity was high in parts of the rhizosphere where relatively little recent photosynthates were allocated, indicating that rhizodeposition and the activity of alkaline phosphatase-producing microorganisms are not directly related. Our study indicates, first, a spatial differentiation of organic P mineralization by various ecophysiological groups that react differently to inorganic P fertilization and second, that rhizodeposition and alkaline phosphatase-producing microorganisms were not directly related. Finally, we conclude that soil zymography with fluorescent substrates is a very promising approach for studying the distribution of a broad range of extracellular enzymes at microscales.

1. Introduction

The rhizosphere is a hotspot of nutrient and carbon (C) cycling (Hinsinger et al., 2009) that strongly shapes nutrient and C cycling in terrestrial ecosystems (Högberg and Read, 2006). Since nutrient and C cycling in the rhizosphere strongly vary at microscales, their study requires spatially explicit methods (Schimel and Bennet, 2004; Watt et al., 2006; Marschner et al., 2011). At present, spatially explicit methods for the study of rhizosphere processes are limited, which is one reason why the spatial organization of the rhizosphere is poorly understood. Especially, a lack of spatially explicit methods for the determination of the distribution of enzyme activity in the rhizosphere has been emphasized several times (Wallenstein and Weintraub, 2008; Burns et al., 2012). Here we enhanced an existing method for *in situ* analysis of the distribution of enzyme activity (Spohn et al., 2013a) and combined it with ^{14}C imaging. This allowed us to study the spatial distribution of alkaline and acid phosphatase activity in relation to the belowground allocation of recent photosynthates at a high resolution.

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http://dx.doi.org/10.1016/j.soilbio.2013.08.015

Phosphorus (P) is among the most important plant-growth limiting nutrients in soils. Due to its rapid precipitation, only a small portion of inorganic P present in soil is soluble (Hinsinger, 2001). The chemical forms of P in soil differ not only with parent material, soil pH and vegetation cover, but also with time and the extent of pedogenesis (Walker and Syers, 1976). Calcium phosphates represent the main primary mineral source of inorganic P in little or only moderately weathered soils with neutral to alkaline pH, whereas in acidic and more progressively weathered soils phosphates are bound or occluded by iron, and aluminum (hydr)oxides predominate. The organic P pool increases during initial soil development and declines again with further weathering (Walker and Syers, 1976).

Plants and microorganisms have developed several mechanisms to mobilize P, i.e. to mineralize organic P and to solubilize bound inorganic P. They can release protons and organic ligands such as oxalate and citrate that solubilize bound inorganic P (Illmer et al., 1995; Hinsinger, 2001). In order to mineralize organic P, plants and microorganisms produce extracellular phosphatases. While microbes are capable of producing both acid and alkaline phosphatases, plants only can produce acid phosphatases (Dick et al., 1983; Juma and Tabatabai, 1988; Nannipieri et al., 2011). The activity of extracellular phosphatase in soil has been reported many times to be negatively correlated with availability of inorganic P (Olander and Vitousek, 2000; Sinsabaugh et al., 2008).

With respect to P acquisition, the plant–microbial relationship can be competitive as well as mutualistic. Microorganisms can increase P availability for plants by P solubilization and mineralization (Richardson et al., 2009; Spohn and Kuzyakov, 2013; Spohn et al., 2013b). However, they may also decrease the availability of P to plants by P immobilization in the microbial biomass, decomposition of P-mobilizing organic compounds released by roots, and counteracting root-induced pH decrease by proton consumption during ammonification (Marschner et al., 2011). According to Marschner et al. (2011) microbial and plant P foraging occur in different regions of the rhizosphere, which might alleviate a potential competition between roots and microbes. While plants mostly take up P at the root tip and in the proximal elongation zone, microbial P uptake is highest in the root hair zone, i.e. in the zone of maximal rhizodeposition (Marschner et al., 2011). However, this concept of spatial differentiation of microbial and plant P acquisition has not, to our knowledge, been tested yet.

Due to C limitation, many microbial populations in soil show features of a dormant state (Joergensen et al., 1990; Vance and Chapin, 2001). Yet, even trace amounts of easily degradable organic C can strongly stimulate their activity (De Nobili et al., 2001; Joergensen et al., 1990). A significant source of easily degradable organic C (OC) are rhizodeposits. Rhizodeposits include root cap and border cell loss, death and lysis of root cells, gaseous losses, passive and active release of solutes (root exudates) and insoluble polymer secretion (mucilage) from living cells (Hinsinger et al., 2009; Jones et al., 2009). Estimates of the total allocation of photosynthates to roots range between 20% and 50% for herbaceous plants, of which approximately one half is released into the soil (Kuzyakov and Domanski, 2000). Due to inputs of easily degradable rhizodeposits, a large population of soil biota resides in the rhizosphere (Kuzyakov, 2002). Their abundance in the rhizosphere ranges from two-times (for Protozoa) up to more than 1000-times (for denitrifiers) higher compared to root-free soil (Westover et al., 1997). Root exudates can strongly stimulate microbial organic P mineralization (Spohn et al., 2013b). However, the spatial relation between rhizodeposits and enzyme activity has not, to our knowledge, been studied.

We, first, hypothesize that alkaline phosphatases – that are exclusively produced by microorganisms – are differently distributed in soil than acid phosphatases that are also produced by plants. Second, we hypothesize that alkaline phosphatases are higher in areas of high rhizodeposition than in areas of low rhizodeposition, since microorganisms can be strongly stimulated by rhizodeposits. Third, we hypothesize that P fertilization leads to a strong decrease in phosphatase activity, since plants and microorganisms can draw on the easily available P source and do not have to mineralize P.

Recently, a new method was developed to map the distribution of enzyme activity in soil *in situ* at high resolution and was applied for analysis of protease and amylase activity in the rhizosphere (Spohn et al., 2013a). Here, we developed the method further using fluorescent enzyme substrate, and we combined soil zymography with ^{14}C imaging, to gain insights into the distribution of photosynthates in roots and in soil at microscales. The use of fluorescent substrates in soil zymography bears the advantage that the distribution of various enzymes can be measured based on the same calibration line.

2. Material and methods

2.1. Experimental setup

Lupinus albus L. (Saat-Union GmbH, Isernhagen) was grown in rhizoboxes in sandy soil. The rhizoboxes had an inner size of 12.3 × 12.5 × 2.3 cm and were inclined by 50° in order to make the roots grow along the lower wall of the rhizobox. Soil from the Ahe horizon of a Podzol from central Germany (51°31′01 N, 9°39′15 E) was mixed with quartz sand (in a ratio of 3:1) in order to lower the P concentration. The properties of the mixed soil were 93.6% sand, 5.2% silt, 1.2% clay, 0.5 g kg^{-1} C, and 10 (±1) mg kg^{-1} $NaHCO_3$-extractable P. Total N and microbial C were below the detection limit. The soil was filled into the rhizoboxes to a density of 1.4 g cm^{-3}. In half of the boxes, the soil was amended with 0.1 mg P g^{-1} as KH_2PO_4 dissolved in water according to Aldén et al. (2001), and Demoling et al. (2007). In total, there were four replicates of P amended soil and four controls. The water content in the rhizoboxes was adjusted to 60% water holding capacity, and was kept stable throughout the experiment. The plants were grown in a climate chamber (Binder) at 60% air humidity with 14 h photoperiod at 24 °C and 10 h darkness at 19 °C.

2.2. Soil zymography

After cultivating the lupines for 28 days, the spatial distribution of extracellular acid and alkaline phosphatases was analyzed by soil zymography. Soil zymography is a novel method that allows for *in situ* mapping of the distribution of enzyme activity. It is based on a gel screen containing the enzyme's substrate that is incubated attached to undisturbed soil (Spohn et al., 2013a). In this study the zymography technique was further developed by integrating substrates that become fluorescent when they get hydrolyzed. Methylumbelliferyl-phosphate (Sigma–Aldrich) was dissolved in modified universal buffer (Skujins et al., 1962) to a concentration of 12 mM. The buffer had been adjusted to either pH 6.5 for acid phosphatase activity or pH 11.0 for determining alkaline phosphatase activity. Membrane filters of polyamide (Sartorius) with a diameter of 14.2 cm and a pore size of 0.45 μm were soaked in the solution and subsequently oven dried at 30 °C for 10 min. For preparation of gels, 1% agarose was dissolve at 80 °C in universal buffer with a pH of 6.5 for acid phosphatase or pH of 11.0 for alkaline phosphatase. Gels were cast in systems usually used for vertical gel-electrophoresis (Biometra). The gels had a size of 0.1 × 12.0 × 11.0 cm. Membranes and gels were prepared directly before analysis of enzyme activity. For the incubation, the lower side of the rhizoboxes was opened, exposing the lupine's roots. The agarose gel was attached to the soil, and the membrane was placed on top of it. After 40 min of incubation at 20 °C, the membrane was removed, and oven dried for 4 min at 30 °C, while the gel was discarded. The dried membrane was placed on an epi-UV-desk (Desaga) in the dark, and exposed to light with