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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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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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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

360 nm wavelength. A picture of the membrane was taken with a digital camera (DSC-HX10V, Sony). The distribution of alkaline phosphatase activity was analyzed in the same sample by the same technique directly after the determination of the distribution of the acid phosphatase activity.

A calibration line was prepared from membranes that were soaked in solutions of 4-methylumbelliferone (MUF) of different concentrations (0, 35, 70, 130, 200 μM) and oven dried at 30 °C. These calibration membranes were cut into strips of 2 cm, and photographed under the UV light in the same way as the zymogram membranes. The amount of MUF on an area basis was calculated from the volume of solution taken up by the membrane and by the size of the membrane.

2.3. ^{14}C labeling and imaging

The distribution of photosynthates was analyzed with ^{14}C imaging after labeling the lupines in ^{14}C atmosphere. ^{14}C imaging (which is also called autoradiography) is a semi-quantitative method, in which the radioactivity of the ^{14}C is transformed into light flashes that can be seen on a light-sensitive screen (Pausch and Kuzyakov, 2011; Wichern et al., 2011). The lupines were labeled with ^{14}C (according to Kuzyakov and Cheng, 2001) starting 12 h after the end of the zymography measurement. The plants in the rhizoboxes were placed in a transparent plastic chamber. One pulse of 5 MBq ^{14}C–CO_2 was released into the chamber by injecting H_2SO_4 into $Na_2^{14}CO_3$. The air inside the chamber was circulated by a fan, and total CO_2 concentration was monitored by a CO_2 analyzer. The plants were allowed to take up the ^{14}C–CO_2 for 10 h. Subsequently, the lower side of the rhizoboxes was opened and the boxes were placed on the imaging plates (BAS-MS 2040, Fujifilm). After 22 h of exposition, the plates were scanned by a ^{14}C imager (FLA-5100, Fujifilm). Subsequently, roots were removed from soil. The soil attached to the roots was collected by shaking the root and is from now on referred to as rhizosphere soil, while the soil remaining in the rhizobox is termed bulk soil. The soil was freeze dried. Roots and aboveground biomass were oven dried at 60 °C. Soil and above- and belowground plant biomass were burned using an oxidizer (Ox500, Harvey Instrumental Cooperation) and ^{14}C activity in the samples was determined with a multi-purpose scintillation counter (6500 Beckman–Coulter).

2.4. Image analysis and statistics

Image processing and analysis was done using the open source software imageJ. The digital images were transformed to 8-bit, i.e. grayscale images. The ^{14}C images were inverted, so that the size of the measured parameter (in this case ^{14}C activity) increased with gray value as in the images of the zymogram. The matrices of the zymogram images and the images of the calibration membranes were multiplied by a factor of 7.0 in order to increase their contrast. To illustrate the results, we depicted the values of the grayscale image in color. The linear correlation between the MUF concentration and the mean of grayscale in an area of 4 cm^2 of each calibration gel was calculated using the software Origin 6.0. The zymogram and the ^{14}C images were divided into segments of 10 gray values, and the areas of these segments were calculated as percentage of the area of the entire image. This was done for the whole image and for the part of the zymogram or ^{14}C image that had been attached to the tap root. Homogeneity of variance of the sizes of the areas was tested by the Levene-test. Significance of differences between areas was tested by ANOVA followed by the Duncan-test using the software SPSS18.0, where $\alpha < 0.05$ was considered as the threshold value for significance.

3. Results

L. albus grew well in the control soil and in the P fertilized soil, and no significant differences were found in above- and belowground biomass between the two treatments after 26 days (Table 1). The gray values of the calibration membranes were linearly correlated with their substrate concentration and hence with the phosphatase activity ($R^2 = 0.95$, Fig. 1). Activity of both alkaline and acid phosphatases in the control soil was up to 34 pmol mm^{-2} h^{-1}, which is up 5.4-times higher than in the bulk soil (Fig. 2A and B). While acid phosphatases were highly active in the P-fertilized (Fig. 2A a–d) and in the control soils (Fig. 2A e–h), alkaline phosphatase was only found in the control soils (Fig. 2B e–h), and not in the P-fertilized soils (Fig. 2B a–d). Both alkaline and acid phosphatase activity were associated with the presence of roots. However, alkaline phosphatase activity could be detected at a larger distance from the root (Fig. 2B e–h), leading to larger areas of alkaline phosphatase activity than areas of acidic phosphatase activity (Fig. 3A). The area of high activity – between 11.6 and 21.7 pmol mm^{-2} h^{-1} – was between 2- and 2.5-times larger for alkaline than for acid phosphatase activity, while this factor decreased to 0 in the range of the activities higher than 26 pmol mm^{-2} h^{-1} (Fig. 3A). The area of enzyme activity in the part of the image that was associated with the tap root (Fig. 3B) decreased with enzyme activity as observed for the total image (Fig. 3A) showing that enzyme activity was only high in a small area around the tap root.

Significantly more ^{14}C was allocated aboveground than aboveground (Table 1). The ^{14}C images showed that most ^{14}C allocated belowground was found in the tap roots, while significantly less ^{14}C was allocated in the lateral roots (Fig. 4). No significant differences were found in the size of the areas of increased ^{14}C allocation between the P-fertilized (Fig. 4 a–d) and the control soils (Fig. 4 e–h). While the area of ^{14}C activity in the total image decreased strongly with gray value, i.e. with the concentration of recent photosynthates (Fig. 5A) it decreased only little with gray value in the part of the image that was associated with the tap root (Fig. 5B), indicating that ^{14}C activity was high in all parts of the tap root. On average, recent photosynthates were allocated in the rhizosphere 30–40 times more strongly than in the bulk soil, and 8500-times less than in the roots (Table 1). Alkaline phosphatase activity in the control soils (Fig. 2B e–h) was also high in the rhizosphere of lateral roots, where relatively little recent photosynthates were allocated (Fig. 4 e–h).

Table 1
Below- and aboveground biomass, ^{14}C activity in below- and aboveground biomass, ^{14}C activity in bulk and rhizosphere soil, and soil pH for P fertilized and control soils/plants. The values represent means calculated from four independent replicates per treatment. The numbers in the brackets represent standard deviations. Letters indicate the results of the Duncan-test; n.s. indicates that the data do not differ significantly between the two treatments.

Treatment	Biomass [g]		^{14}C activity in biomass [kBq mg^{-1}]		^{14}C activity in soil [kBq mg^{-1}]		pH in bulk soil
	Above-ground	Below-ground	Above-ground	Below-ground	Bulk soil	Rhizosphere soil	
+P	0.21 ($\pm$0.03)$^{n.s.}$	0.60 ($\pm$0.28)$^{n.s}$	3.7 ($\pm$2.8)$^{n.s.}$	1.8 ($\pm$1.5)$^{n.s.}$	7.8×10^{-6} ($\pm 4.7 \times 10^{-6}$)$^{n.s}$	296.2×10^{-6} ($\pm 201.5 \times 10^{-6}$)$^{n.s}$	6.3 ($\pm$0.0)^{b}
Control	0.22 ($\pm$0.04)$^{n.s.}$	0.58 ($\pm$0.28)$^{n.s}$	9.7 ($\pm$5.3)$^{n.s.}$	2.7 ($\pm$0.7)$^{n.s.}$	11.7×10^{-6} ($\pm 4.7 \times 10^{-6}$)$^{n.s}$	235.4×10^{-6} ($\pm 37.0 \times 10^{-6}$)$^{n.s}$	6.4 ($\pm$0.0)^{a}

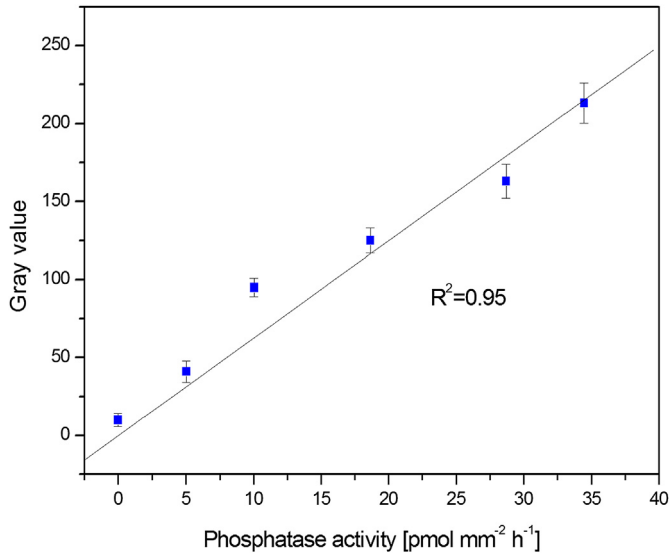

Fig. 1. Linear correlation between the gray value of the calibration membranes (shown in color in Fig. 2C) and the phosphatase activity calculated from the concentration of methylumbelliferone and the incubation time of the zymograms.

4. Discussion

4.1. Soil zymography using fluorescent substrates

We developed the zymography technique presented by Spohn et al. (2013a), by integrating fluorescent substrates. Previously, natural substrates such as gelatin and starch were used for protease and amylase activity instead of fluorescent substrate. However, the use of fluorescent substrate has two advantages. First, coloring of the gels is not necessary when fluorescent substrates are used. Second, fluorescent MUF substrates are available for many enzymes, and consequently it is possible to determine the distribution of various enzymes based on the same calibration line. As we showed here, the correlation between substrate concentration on an area basis and the gray value is linear at least up to 40 pmol mm^{-2} MUF (Fig. 1). A linear correlation is advantageous because it does not require transformations of the images of the zymograms for the visualization of enzyme activity as in the case of a non-linear relationship between substrate concentration and gray value (Spohn et al., 2013a).

Recently, there have also been other attempts to use fluorescent substrates to analyze the distribution of enzyme activity in soils and in biological specimens such as fungal cell colonies (Baldrian and Vetrovský, 2012). The authors dissolved the fluorescent substrates in agarose solution that was then directly poured onto the sample. According to our experience, this approach does not work properly for two reasons. First, many MUF substrates are not heat stable, which is problematic since a 1% agarose solution is only liquid, i.e. can only be cast at temperatures above 52 °C. Below this temperature it becomes a gel. MUF-phosphate, for example, already thermally hydrolyses at 40 °C (Appendix 1). Changing the matrix of the gel to sodium dodecyl sulfate polyacrylamide (SDS) – another gel matrix commonly used in biochemistry, which solutes without heating – is not possible since acrylamide strongly quenches fluorescence (Phillips et al., 1986). The second reason why dissolving the fluorescent substrates directly in agarose solution does not work properly, is that the MUF-substrates are not fixed in agarose gels. Hence, due to diffusion of the substrate, the resolution of this enzyme mapping method is extremely poor (Appendix 2). We showed that soaking membrane filters (pore diameter of 0.45 μm) with the MUF-solution and drying them at 30 °C fixes the MUF substrates on the membrane filters. This can be seen in the zymograms by the sharp borders between areas of high and of low enzyme activity, as seen in the zymograms of the acidic phosphates (Fig. 2A–H). These sharp borders reflect the increase in phosphatase activity by more than 5.4-times within 2 mm of soil. In the original soil zymography method (Spohn et al., 2013a), the dissolution of the substrate in the agarose did not decrease the resolution because polymers such as starch and gelatin were used. Polymers, in contrast to the monomer MUF, do not move in the agarose gel due to their much larger size.

Dong et al. (2007) developed a semi-quantitative approach to map the distribution of enzyme activity with chromatography paper that was soaked with MUF-substrate solution and directly incubated on soil. The problem with this approach is that soil that attaches to the filter quenches fluorescence (Appendix 2). According to our experience, these soil smears cannot be cleaned with water since rinsing the membrane also removes MUF. This problem is overcome in our approach, as the membrane filter is not directly in contact with the soil, but is protected by an agarose gel (Appendix 2).

4.2. Distribution of acid and alkaline phosphatase activities

We found that both acid and alkaline phosphatase activity were associated with the presence of roots. However, alkaline phosphatase activity was more widely distributed in the control soils (Fig. 2B e–h), leading to larger areas of higher activity of alkaline phosphatase than of acid phosphatase (Fig. 3). Acid phosphatases are produced by plants and microorganisms, whereas alkaline phosphatases are exclusively produced by microorganisms (Juma and Tabatabai, 1988; Nannipieri et al., 2011). The results indicate a spatial separation of different groups of organic P mineralizing organisms. The spatial differentiation could be between either microorganisms and *L. albus* or between microorganisms that produce exclusively alkaline phosphatases on the one hand, and *L. albus* and microorganisms that produce acid phosphatases (and are intimately associated with the root) on the other hand. The spatial separation of different phosphatase producing organisms might alleviate a potential competition between them. It can be speculated that at a moderate distance from the root, microorganisms can still feed on rhizodeposits, but do not have to directly compete with the plant for P. This is possible since the diffusion distance of root exudates in soil (Kuzyakov et al., 2003; Sauer et al., 2006) is larger than that of phosphate (Chen et al., 2002; Wang et al., 2005). Marschner et al. (2011) also suggested a spatial differentiation between microbial and plant P acquisition. However, they stated that this would be between the root tip and the root hair zone. By analyzing enzyme activity in soil slices, Tarafdar and Jungk (1987) showed that the extension of enzyme activity as a function of distance to root increases with plant development. Hence, it seems likely that the distribution of acid and alkaline phosphatase activity would increase during further growth of the lupines.

4.3. Effect of P fertilization of phosphatase activity

We showed that P fertilization strongly decreased alkaline phosphatase activity, but had no effect on acid phosphatase activity (Fig. 3). A negative correlation between P availability and phosphatase activity has been found in fertilization experiments in several ecosystems (Spiers and McGill, 1979; Olander and Vitousek, 2000). In their meta-analysis, Sinsabaugh et al. (2008) concluded that an inverse relationship between extracellular phosphatase activity and relative P availability is a general phenomenon. Our observation that alkaline phosphatase activity was strongly reduced due to P fertilization suggests that microorganisms that produce alkaline phosphatases react more strongly to change in P

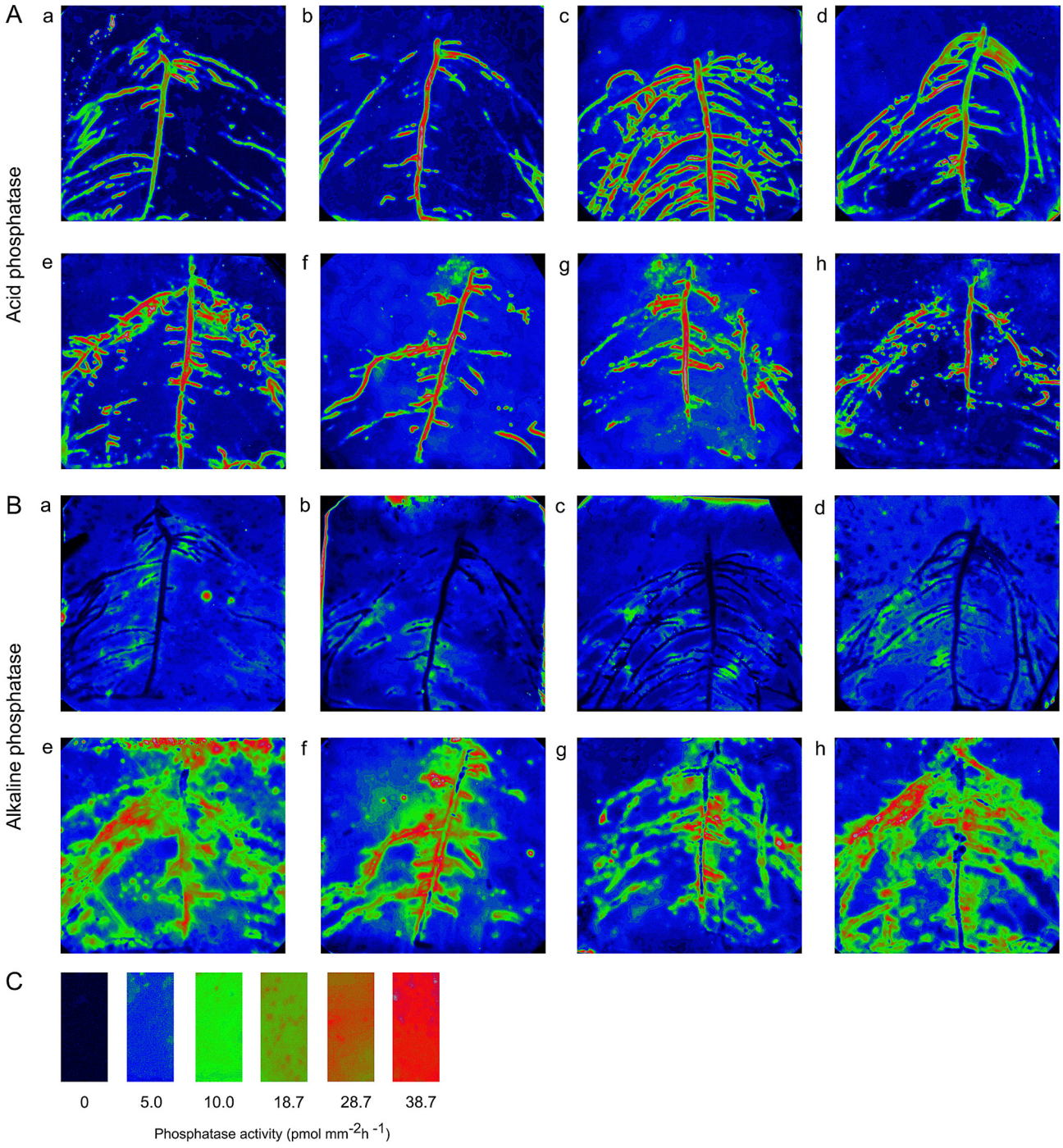

Fig. 2. Zymograms showing acid phosphatase (A) and alkaline phosphatase (B) together with the calibration line (C) that is composed of six calibration membranes. Images a–d show P amended soils and images e–h show control soils.

availability than *L. albus* and acid phosphatase producing microorganisms. In the P amended soils, microorganisms reduced extracellular alkaline phosphatase production most likely since they could draw on the easily available inorganic P. *L. albus*, in contrast, did not respond to P fertilization, which might be because it is well adapted to nutrient poor soils (Huyghe, 1997), and sufficient P was available in the soil in the time frame of the study. The interpretation that *L. albus* did not experience P deficiency is supported by the finding that neither the above- and belowground biomass nor the ^{14}C allocation in above- and belowground biomass (Table 1) were affected by P fertilization; since herbaceous plants usually allocate more C belowground when affected by nutrient limitation (Müller et al., 2000).

4.4. Phosphatase activity and ^{14}C allocation

The 30- to 40-times higher recovery of ^{14}C in the rhizosphere than in the bulk soil (Table 1) shows that the roots released C assimilated 22–32 h before the measurement. While the enzymes had similar levels of activity along the tap root and along lateral roots (Figs. 2 and 3), photosynthates were mostly allocated in the tap root (Fig. 4), leading to high ^{14}C activity in nearly all parts of the tap

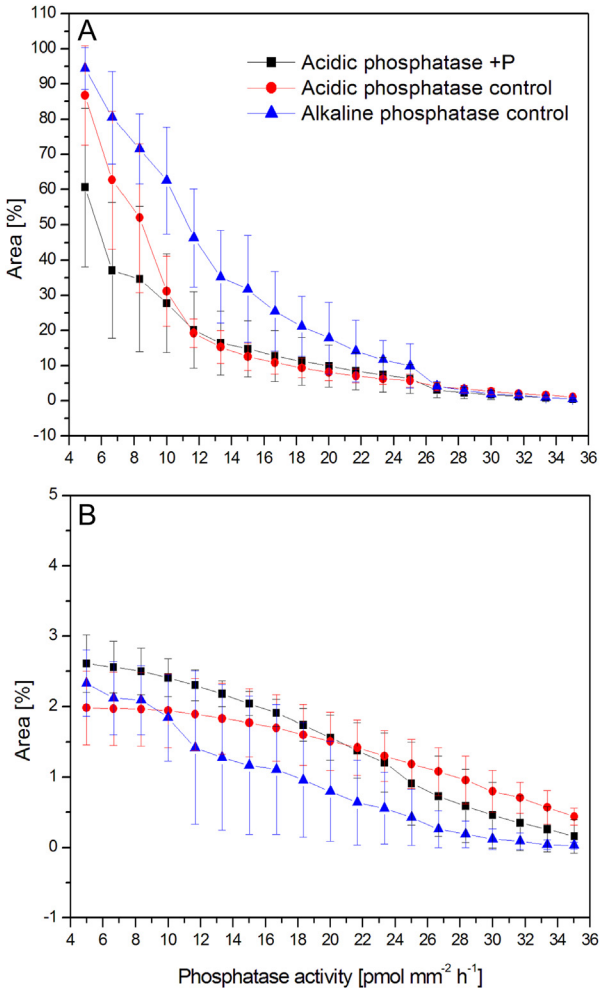

Fig. 3. Proportion of the area of enzyme activity in the total image (A) and in the part of the image that was associated with the tap root (B) as a function of the phosphatase activity. While acid phosphatase activity is shown for the P fertilized and for the control soils, alkaline phosphatase activity is only shown for the control soils since no activity could be detected in the P fertilized soils. Data points depict means calculated from four independent replicates, error bars depict standard deviations.

root (Fig. 5B). Hence, our study shows that alkaline phosphatase activity was also high in parts of the rhizosphere where relatively little recent photosynthates had been allocated and released during the last 32 h prior to analysis (Fig. 2). The reason for this could be that these parts of the rhizosphere had received more photosynthates during earlier stages of plant growth prior to the labeling. In this case, either the microorganisms and the alkaline phosphatases or only the extracellular phosphatases still maintained high activity at the time of analysis. The latter would be in accordance with studies that emphasized that the activity of extracellular enzymes can be independent of microbial activity once they have been released (Schimel and Weintraub, 2003). Another reason why alkaline phosphatase along the lateral roots was high in areas that did not receive recent photosynthates could be that alkaline-phosphatase producing microorganisms, in contrast to acid-phosphatase producing microorganisms, fed on C sources other than rhizodeposits, which might alleviate a potential competition between them.

4.5. Conclusions

A further development of the soil zymography technique that integrates fluorescent enzymes allowed us to study the distribution of extracellular acid and alkaline phosphatases in the rhizosphere of *L. albus* at a high resolution. While acid phosphatase activity was very closely associated with the presence of roots, alkaline phosphatase activity was more widely distributed in soil. This indicates a spatial differentiation of different organic P mineralizing organisms. It could be that there is a spatial separation between microorganisms and the plant. The other possible interpretation is a separation between alkaline-phosphatase producing microorganisms on the one hand and *L. albus* and acid phosphatase-producing microorganisms that are intimately associated with the root on the other hand. The spatial separation of different organic P mineralizing organisms might alleviate a potential competition between them. Alkaline phosphatase activity was also high in the 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. While acid phosphatase activity was not affected by fertilization, alkaline (microbial) phosphatase activity decreased strongly due to P fertilization, suggesting that alkaline phosphatase-producing microorganism react more strongly to

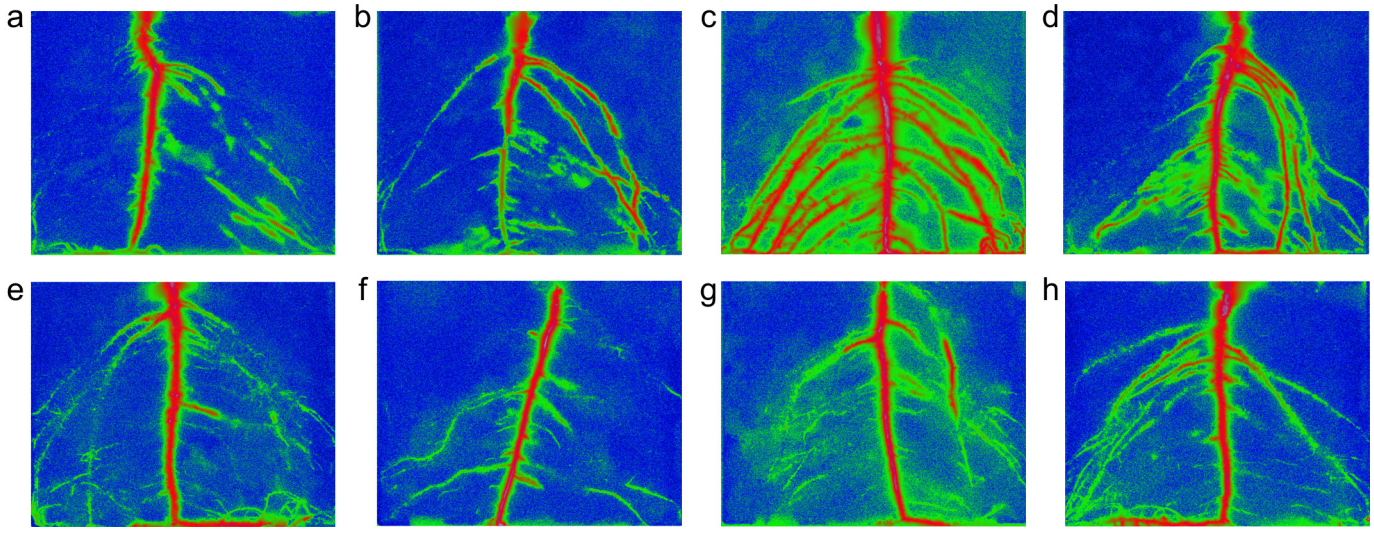

Fig. 4. ^{14}C images of P amended soils (a–d) and control soils (e–h). The lighter the color of the ^{14}C image, the more ^{14}C was exposed to this part of the ^{14}C image. In the color images, the ^{14}C activity increases from blue over green to red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

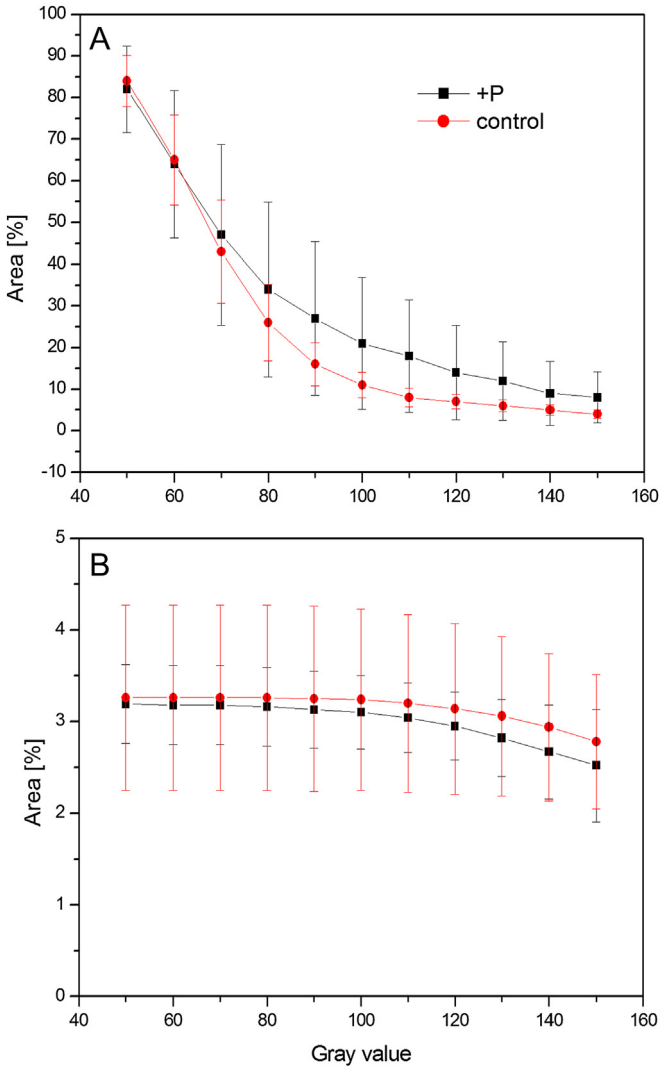

Fig. 5. Proportion of the area of ^{14}C allocation in the total image (A) and in the part of the image that was attached to the tap root (B) as a function of gray value. The higher the gray value, the larger was the ^{14}C activity, i.e. the concentration of recent photosynthates. Results are shown for the P fertilized soils and for the control soils. Data points depict means calculated from four independent replicates, error bars depict their standard deviations.

inorganic P fertilization than other organic P mineralizing organisms. Finally, we conclude that soil zymography with fluorescent substrates is a very promising approach for studying soil ecological questions at microscales.

Author contributions

MS designed and conducted the experiment, developed the zymogram technique, analyzed the data and wrote the manuscript; YK contributed to the manuscript.

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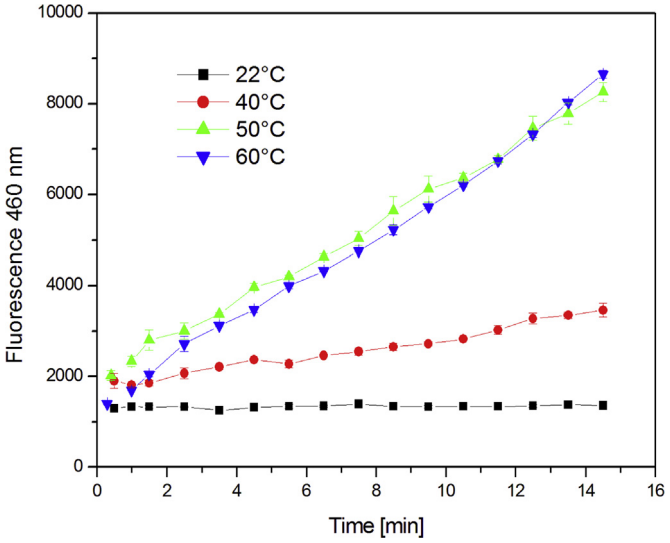
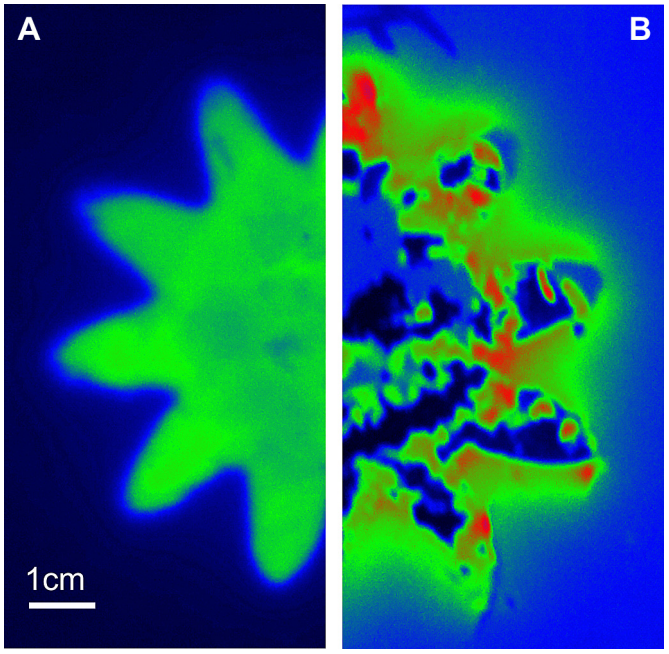
Appendix



Appendix 1. Fluorescence of a solution of methylumbelliferyl-phosphate (MUF-P) in water at four temperatures as a function of time. The MUF-P solution was kept on a heatable magnetic stirrer with a temperature controller. Four samples were taken simultaneously once a minute. Fluorescence was determined with a multilabel counter (Victor^3, Perkin Elmer). Data points represent means calculated from four measurements and error bars show standard deviations.



Appendix 2. Phosphatase activities in a soil that was pressed into a flower-shaped aluminum mold measured using methylumbelliferyl-phosphate (MUF-P) with the technique used in this study (A), in which a membrane is coated with MUF-P and with a second technique (B), in which the MUF-P is contained in an agarose gel. Images were transformed as described in Section 2.4. The comparison shows that the resolution of the second technique (B) is lower than the resolution of the technique used in this study (A) since the methylumbelliferone diffuses in the gel (B), while it is fixed on the membrane, leading to a much higher resolution of the technique used in this study (A). The dark patches in image B are derived from soil stains that cannot be removed without destroying the gel, while in the first technique a gel prevents soils stains on the membrane (A). The gel containing MUF-P (B) was casted at 55 °C, which lead to a higher background fluorescence as in the membrane (A) due to thermal degradation of the molecule (see Appendix 1).

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