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Warming increases hotspot areas of enzyme activity and shortens the duration of hot moments in the root-detritusphere

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ABSTRACT

Temperature effects on enzyme kinetics and on the spatial distribution of microbial hotspots are important because they are crucial to soil organic matter decomposition. We used soil zymography (*in situ* method for the two dimensional quantification of enzyme activities) to study the spatial distributions of enzymes responsible for P (phosphatase), C (cellobiohydrolase) and N (leucine-aminopeptidase) cycles in the rhizosphere (living roots of maize) and root-detritusphere (7 and 14 days after cutting shoots). Soil zymography was coupled with enzyme kinetics to test temperature effects (10, 20, 30 and 40 °C) on the dynamics and localization of these three enzymes in the root-detritusphere. The percentage area of enzyme activity hotspots was 1.9–7.9 times larger and their extension was broader in the root-detritusphere compared to rhizosphere. From 10 to 30 °C, the hotspot areas enlarged by a factor of 2–24 and V_{max} increased by 1.5–6.6 times; both, however, decreased at 40 °C. For the first time, we found a close positive correlation between V_{max} and the areas of enzyme activity hotspots, indicating that maximum reaction rate is coupled with hotspot formation. The substrate turnover time at 30 °C were 1.7–6.7-fold faster than at 10 °C. The K_m of cellobiohydrolase and phosphatase significantly increased at 30 and 40 °C, indicating low affinity between enzyme and substrate at warm temperatures. We conclude that soil warming (at least up to 30 °C) increases hotspot areas of enzyme activity and the maximum reaction rate (V_{max}) in the root-detritusphere. This, in turn, leads to faster substrate exhaustion and shortens the duration of hot moments.

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1. Introduction

The rhizosphere is one of the most dynamic habitats on Earth (Hinsinger et al., 2009) because living plants stimulate microbial and enzyme activity (Parkin, 1993; Asmar et al., 1994) by releasing labile carbon and other rhizodeposits (Jones et al., 2009). Enzymes excreted by both plants and microbes are the main mediators of organic matter decomposition (Nannipieri et al., 2007; Sinsabaugh et al., 2008). The root-detritusphere (soil around dying and dead roots) also forms the hotspots of microbial and enzyme activity because carbon inputs through the dying root after harvest releases highly polymers as well as low molecular weight organics (Kögel-Knabner, 2002; Bastian et al., 2009). The rhizosphere and root-detritusphere are both considered to be hotspots of enzyme activity (Kuzyakov and Blagodatskaya, 2015); nonetheless, they differ in the composition of their substances (Kögel-Knabner, 2002; Jones et al., 2004) and in nutrient exudation dynamics (Bastian et al., 2009; Poll et al., 2010). Whereas living roots release abundant readily available monomers such as monosaccharides and amino acids (Hinsinger et al., 2009), root detritus mainly contains macromolecular compounds such as cellulose and xylan (Rahn et al., 1999). Moreover, while rhizodepositions represent a continuous flow of substances during plant growth (Kuzyakov and Domanski, 2000), the death of roots is a temporally concentrated C input (Spohn and Kuzyakov, 2014). Due to the concentrated input of available organics from dead roots, it is generally accepted that microorganisms are more abundant (Marschner et al., 2012) and that the hotspots' areas of enzyme activity are larger in the root-

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detritusphere than in the rhizosphere (Spohn and Kuzyakov, 2014).

Enzyme activities in soil are controlled by abiotic factors (temperature, water potential, pH, soil texture) and biotic factors (enzyme synthesis and secretion) (Burns et al., 2013). Among abiotic factors, the temperature sensitivity of enzyme activity has received considerable interest because of its potential feedback to climate change (Davidson and Janssens, 2006). Temperature directly affects enzyme activity by changing the conformational flexibility of enzymes, indirectly by causing shifts in the microbial community (Bárcenas-Moreno et al., 2009; Rousk et al., 2012).

Both microbial and enzyme activities increase with temperature (Davidson and Janssens, 2006; Steinweg et al., 2008). Thus, soil warming increases the breakdown and assimilation of organic matter, enhancing microbial growth and enzyme synthesis (Davidson and Janssens, 2006). Nonetheless, long-time experiments showed that warming initially stimulated soil respiration, microbial biomass and enzyme activity, but the effect diminished over time - a phenomenon frequently termed acclimation (Allison and Treseder, 2008; Frey et al., 2008). This can be attributed to faster depletion of easily accessible organic matter and changes in carbon use efficiency at warm temperatures (Kirschbaum, 2004; Eliasson et al., 2005). The depletion of substrate further results in microorganism starvation (Bradford et al., 2008) and enzyme pool reduction (Wallenstein et al., 2010). The decrease of carbon use efficiency lead to more C to waste with increasing temperature (Ågren, 2010). Therefore, the duration of hot moments - (periods of high microbial and enzyme activities) (Kuzyakov and Blagodatskaya, 2015) are shorter at high temperatures.

Substrate-dependent enzyme activity is described by the Michaelis-Menten function (Michaelis and Menten, 1913). Both parameters of the Michaelis-Menten equation-V_{max} (maximum reaction rate) and K_m (half-saturation constant indicating the affinity of enzyme to substrate) - are temperature sensitive (Davidson and Janssens, 2006) and usually increase with temperature (Stone et al., 2012; Baldrian et al., 2013). It remains unresolved, whether temperature affects the temporal and spatial distribution of enzyme activity hotspots. It is imperative to measure the spatial distribution of enzyme activity as affected by temperature in order to reveal complex interactions between microorganisms, enzymes, and SOM decomposition (Wallenstein and Weintraub, 2008). Especially in the rhizosphere and root-detritusphere, due to the high heterogeneity, the substrate availability varies in time and space (Ekschmitt et al., 2005).

The recently developed imaging technique termed zymography (Spohn et al., 2013) offers an opportunity to analyze the two-dimensional spatial distribution of enzyme activity in soil (Vandooren et al., 2013; Spohn and Kuzyakov, 2014). Combining soil zymography with enzyme kinetics enabled relating the distribution of hotspots to enzyme catalytic properties in bulk soil, in the rhizosphere (Sanaullah et al., 2016) as well as in biopores (Hoang et al., 2016). For the first time, zymography was coupled with Michaelis-Menten kinetics in the root-detritusphere to test temperature effects (10, 20, 30 and 40 °C) on the dynamics and spatial distribution of enzyme activity. Cellobiohydrolase, leucine-aminopeptidase and acid-phosphatase (involved in C, N, and P cycling, respectively) were used to study the kinetic parameters (V_{max} and K_m) and to localize enzyme activities in the rhizosphere and root-detritusphere. We hypothesized that 1) due to the concentrated input of substrate in the root-detritusphere, the areas of enzyme activities hotspots are larger in the root-detritusphere than in the rhizosphere, 2) the hotspot areas of enzyme activates in the root-detritusphere response faster but the duration of hot moment is shorter at warm temperatures than at cold temperatures, 3) catalytic properties (K_m and V_{max}) respond positively to increasing temperature.

2. Material and methods

2.1. Sample preparation

The soil was collected from the top 10 cm of the Ap horizon of an arable loamy Haplic Luvisol located on a terrace plain of the Leine River north-west of Göttingen, Germany. The soil had the following physiochemical properties: 7% sand, 87% silt, 6% clay, pH 6.5, organic carbon 12.6 g C kg^{-1}, total nitrogen 1.3 g N kg^{-1} (Kramer et al., 2012; Pausch et al., 2013). The soil was passed through a 2 mm sieve before the experiments.

Maize (*Zea mays* L.) seeds were germinated on filter paper for 72 h. Sixteen pre-germinated maize seedlings were selected. One seedling was planted in a depth of 5 mm in each rhizobox, which was filled with soil to a final density of 1.4 g cm^3. The rhizoboxes had an inner size of 12.3 × 12.5 × 2.3 cm. During 2 weeks of growth, the rhizoboxes were kept inclined at an angle of 45° so that the roots grew near the lower wall of the rhizobox. The rhizoboxes were kept in a climate chamber with a controlled temperature of 20 ± 1 °C and a daily light period of 14 h with a photosynthetically active radiation intensity of 300 μmol m^{-2} s^{-1}. During the growth period, the soil water content was maintained at 60% of the water holding capacity by irrigating the soil from the bottom with distilled water. Soil water content was kept constant during the experiments. After growing the plants for 2 weeks, the shoots were cut at the surface of the soil and the rhizoboxes were incubated at 10, 20, 30 and 40 °C, for 14 more days, i.e., 4 rhizoboxes (replicates) at each temperature.

2.2. Soil zymography

Direct soil zymography (Sanaullah et al., 2016) was applied after cultivating the plants for 2 weeks (at a climate chamber temperature of 20 ± 1 °C), before the cutting shoots (living roots - rhizosphere), as well as 7 and 14 days after the cutting shoots (root - detritusphere) (for samples kept at 10, 20, 30 and 40 °C). Enzyme activities were visualized using membranes saturated with 4-methylumbelliferone (MUF)-substrates and 7-amino-4-methylcoumarin (AMC)-substrates. The MUF and AMC become fluorescent when substrates are enzymatically hydrolyzed by a specific enzyme (Spohn et al., 2013). Cellobiohydrolase was detected by 4-methylumbelliferyl-β-D-cellobioside, phosphatase by 4-methylumbelliferyl-phosphate, and leucine-aminopeptidase by L-leucine-7-amido-4-methylcoumarin hydrochloride (Koch et al., 2007; Razavi et al., 2015). Each substrate was dissolved to a concentration of 12 mM in universal buffers (MES buffer for MUF substrate and TRIZMA buffer for AMC substrate. All substrates and chemicals were purchased from Sigma Aldrich (Germany). Polyamide membrane filters (Tao Yuan, China) with a diameter of 20 cm and a pore size of 0.45 μm were cut into sizes adjusted for the rhizobox. The cut membranes were saturated with the substrates for each enzyme. The rhizoboxes were opened from the lower, rooted side and the saturated membranes were applied directly to the soil surface. Soil zymography was performed for each enzyme separately on the same rhizobox; firstly acid-phosphatase, secondly cellobiohydrolase and thirdly leucine-aminopeptidase activity were measured. This order was maintained throughout the experiments. After incubation for 1 h at the given temperature, the membranes were carefully lifted off the soil surface and any attached soil particles were gently removed using tweezers (Razavi et al., 2016b). The time span between each measurement was 30 min. Based on the preliminary test we considered the residue of fluorescence of the previous enzyme to be negligible. The membranes were placed under ultraviolet (UV) illumination with an excitation wavelength of 355 nm in a dark room. The camera (EOS

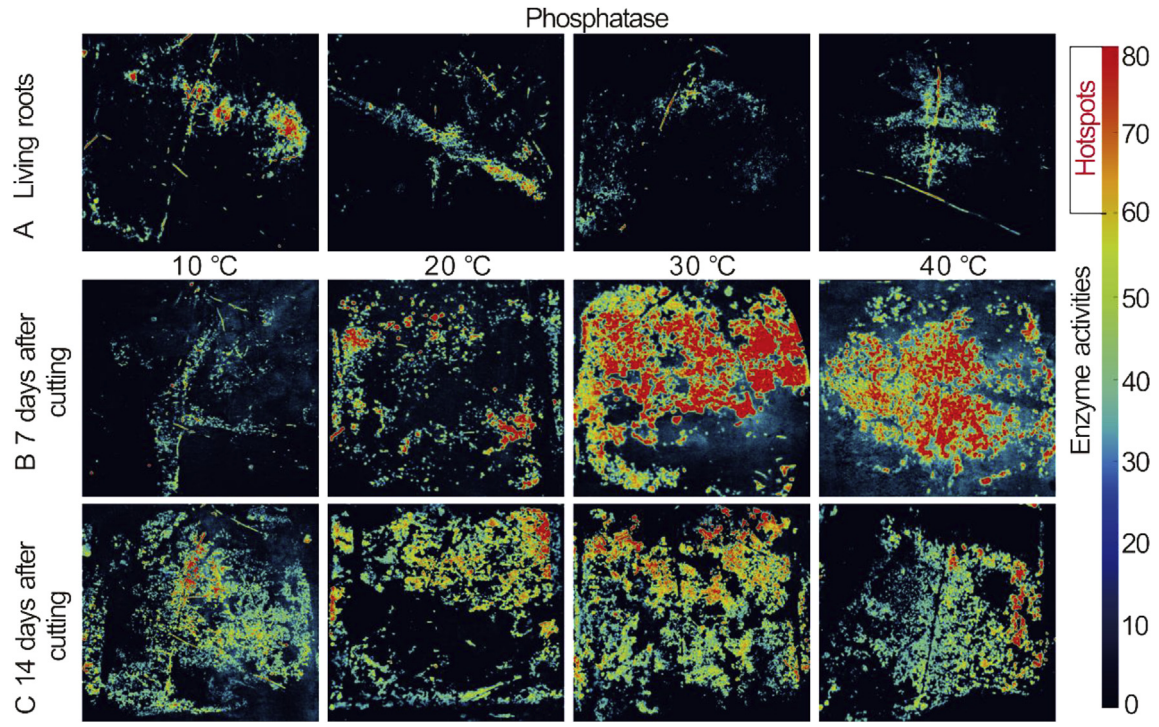

Fig. 1. Phosphatase activity distribution measured by zymography, A) Rhizosphere (Living root) 14 days after sowing; B) Root-detritusphere 7 days after cutting of shoots; C) Root-detritusphere 14 days after cutting of shoots. Columns of rows 2 and 3 (Root-detritusphere) indicate four temperatures (10, 20, 30, 40 °C). Rhizosphere (the top row)represents 4 replications (all conducted at 20 °C). Side color scale is proportional to enzyme activities (pmol mm^{-2} h^{-1}).

5D, Canon), the sample, and the distance between the UV light were fixed, and a photograph of the membrane was taken as described in Razavi et al. (2016b).

A calibration line was prepared from membranes that were soaked in solutions of increasing concentrations of MUF (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 mM) and AMC (0, 10, 20, 40, 50, 60, 70 and 80 μM). These calibration membranes were cut into pieces of 4 cm^2. The amount of MUF or AMC on an area basis was calculated from the volume of solution taken up by the membrane and its size (Razavi et al., 2016b). The membranes used for calibration were imaged under UV light in the same way as described for the samples.

2.3. Image analysis

Image processing and analysis were done using the open source software imageJ. The digital images were transformed to 32-bit grayscale images. We calculated the linear correlation between the MUF and AMC concentration and the mean grayscale in an area of 4 cm^2 of each calibration membrane. The background was calculated based on the calibration line at a concentration of zero by subtracting this value from all the zymographs. Enzyme activities in the upper quartile (top 25%) were defined as hotspots (red color). The total hotspot areas were calculated as a percentage area of the entire image (background subtracted based on the calibration line). Normality and homogeneity of variance were checked using Shapiro-Wilk's test and Levene tests. Significance of differences between the percentage area of hotspots in the rhizosphere (living roots) and root-detritusphere (7 and 14 days after shoot cutting) was tested by A8.0, at $\alpha < 0.05$. The changes in hotspot areas between 7 and 14 days after cutting shoots were calculated by fowling equation.

$$\text{Changes in hotspot areas} = \frac{\text{HsA 14d} - \text{HsA 7d}}{\text{HsA 7d}} \quad (1)$$

where HsA14d and HsA7d are hotspot areas in 14 and 7 days after cutting shoots, respectively.

2.4. Enzyme kinetics and statistical analyses

Enzyme activities were measured 14 days after cutting shoots in a range of substrate (the same substrates as for zymography) concentrations (0–200 μmol L^{-1}). Half a gram (dry weight equivalent) of root-detritusphere soil (soil attached to dead decaying roots) was collected from each rhizobox. Suspensions of 0.5 g soil with 50 mL deionized water were prepared using low-energy sonication (40 J s^{-1} output energy) for 2 min (Koch et al., 2007). 50 μL of soil suspension was added to 100 μL substrate solutions and 50 μL of buffer (MES or TRIZMA, the same buffers as for zymography) in a 96-well microplate. Fluorescence was measured in microplates at an excitation wavelength of 355 nm and an emission wavelength of 460 nm, and a slit width of 25 nm, with a Victor 1420-050 Multi label Counter (Perkin Elmer, USA). All enzyme activities were determined and incubated at 10, 20, 30 and 40 °C, respectively, for 2 h. After each fluorescence measurement (30 min, 1 h and 2 h) the microplates were promptly returned to the climate chambers, so that the measurement time did not exceed 2 min (Razavi et al., 2015). Enzyme activities were expressed as MUF or AMC release in nmol per g dry soil per hour (nmol g^{-1} dry soil h^{-1}). The assay of each enzyme at each substrate concentration was performed in three analytical replicates (12 wells in the microplate).

The Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) were determined for each enzyme and each temperatures using the Michaelis-Menten equation

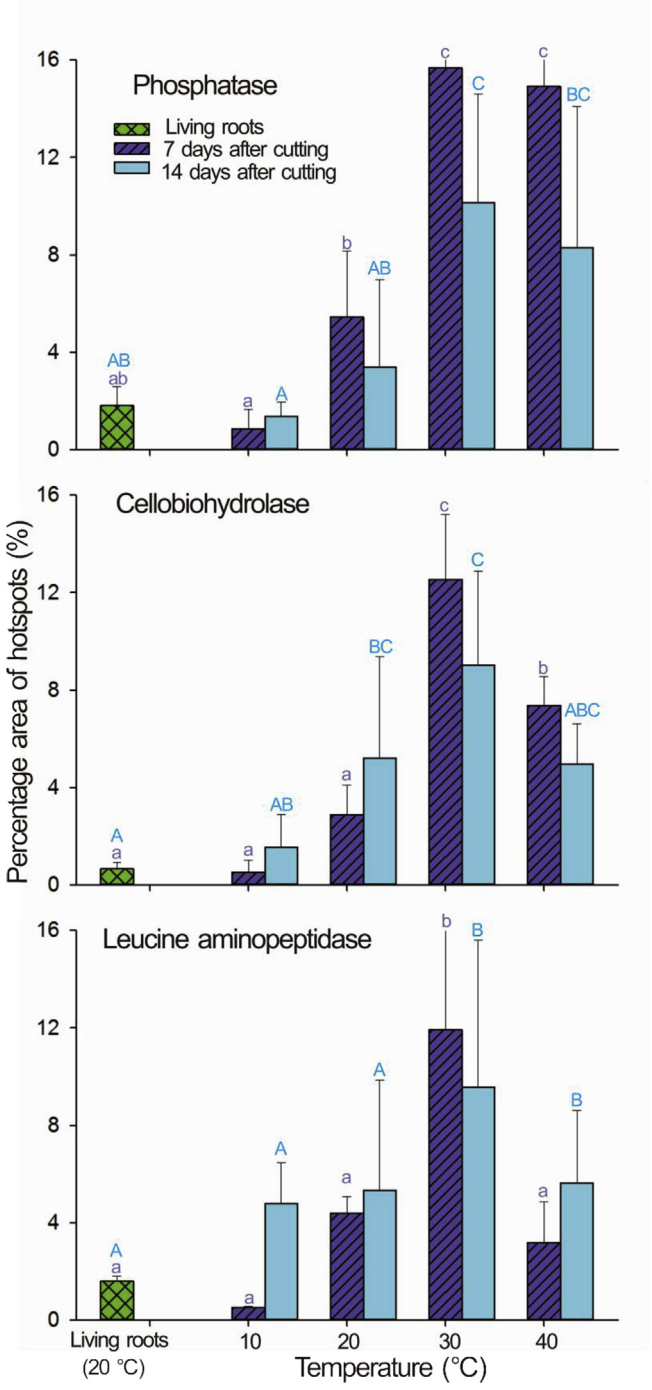

Fig. 2. Hotspots (with 25% highest activity, see Fig. 1.) as a percentage of total area for phosphatase, cellobiohydrolase and leucine aminopeptidase in the rhizosphere of living roots and root-detritusphere (7 and 14 days after cutting shoots) at four temperatures (10, 20, 30, 40 °C). Bars: means calculated from four replicates (±SE). Small letters: significant differences ($p < 0.05$ after Duncan test) between living rootsand root-detritusphere of 7 days after cuttingat each temperature; capital letters: differences between living rootsand root-detritusphereof14 days after cuttingat each temperature.

$$v = \frac{V_{max} \times [S]}{K_m + [S]} \quad (2)$$

where v is the reaction rate, [S] is the substrate concentration. K_m (the substrate concentration at half-maximal rate) is related to the formation and break-down reaction rate constants of the enzyme-substrate complex (Michaelis and Menten, 1913). It is dependent on different factors such as temperature (Stone et al., 2012). V_{max} is directly a function of the enzyme activity, with V_{max} = kcat*ET, where kcat is the rate constant of the catalytic breakdown of enzyme-substrate complex to enzyme and product and ET is the total (active) enzyme concentration (Keleti and Welch, 1984). The turnover time (T_t) of the added substrates was calculated according to the following equation: T_t (hours) = $(K_m + S)/V_{max}$ (Panikov et al., 1992; Larionova et al., 2007). Since cutting the shoots leads to a concentrated input of available organics in the root-detritusphere, the high-substrate concentration was chosen to calculate the turnover time of added substrates ($S = 200$ μmol L^{-1} which equals 40 μmol g^{-1} dry soil). The K_m values were also converted to μmol g^{-1} dry soil for T_t calculations. Normality and homogeneity of variance were checked using Shapiro-Wilk's test and Levene tests. ANOVA followed by the Duncan-test at a probability level of $p < 0.05$ was used to define temperature ranges with significantly different V_{max}, K_m and T_t. The relationship between V_{max} and hotspot areas at various temperatures was tested by linear regression.

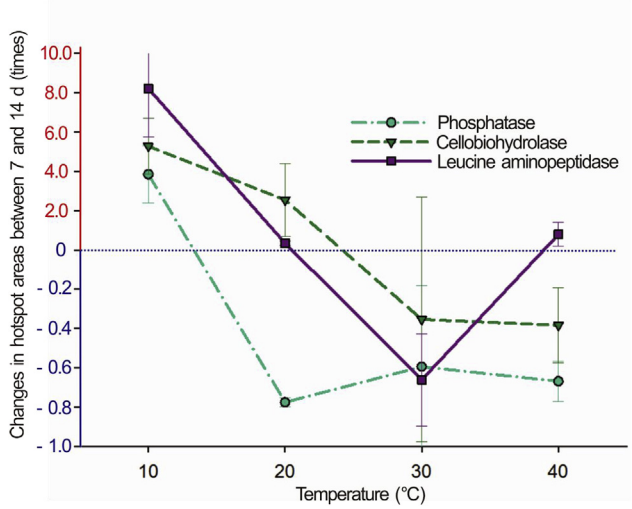

Fig. 3. Changes in enzyme hotspot (with 25% highest activity, see Fig. 1.) areas between 7 and 14 days after cutting of shoots for phosphatase, cellobiohydrolase and leucine aminopeptidase at four temperatures (10, 20, 30, 40 °C). Positive values show increased, whereas, negative values show decreased hotspot areas at 14 days versus 7 days after cutting. Data points indicate means calculated from four replicates (±SE).

3. Results

3.1. Distribution of enzyme activity in the rhizosphere and root-detritusphere

Total hotspot areas in the root-detritusphere were 1.9–7.9 times larger than in the rhizosphere (at same temperature: 20 °C) (Figs. 1 and 2). The extensions of hotspot areas were broader at 30 and 40 °C than at 10 and 20 °C (Fig. 1, Figs. S2 and S3). From 10 to 30 °C, the hotspot areas increased by 2–24 times (Fig. 2). At 40 °C, however, the percentage area of hotspots decreased by 5–73% for all enzymes compared to at 30 °C (Fig. 2).

The hotspot areas for enzymes at 10 °C increased by 3.8–8.2 times in the root-detritusphere 14 days versus 7 days after cutting (Fig. 3). The hotspot areas of cellobiohydrolase and leucine-aminopeptidase at 20 °C only increased by 2.5 and 0.35 (not significant) times 14 days versus 7 days after cutting. The hotspot areas decreased by 0.35–0.66 times for all enzymes at 30 and 40 °C (except leucine aminopeptidase at 40 °C) 14 days compared to 7

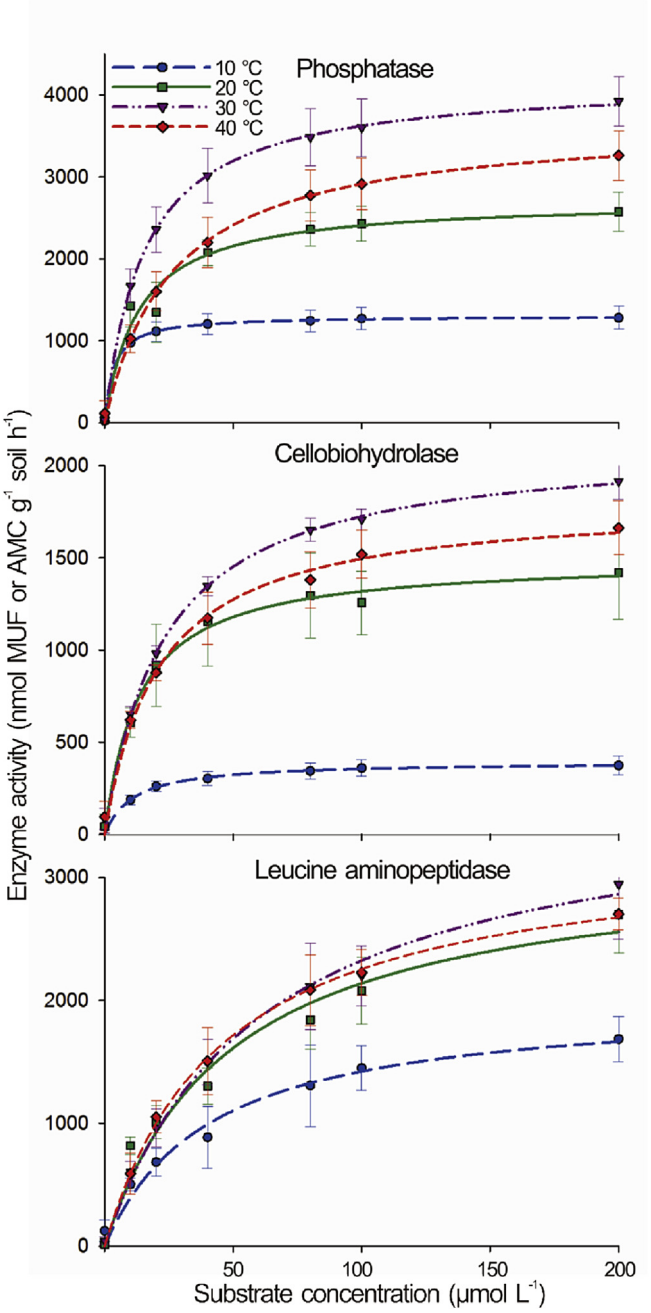

Fig. 4. Michaelis-Menten kinetics (enzyme activity as a function of substrate concentration) for phosphatase, cellobiohydrolase and leucine aminopeptidase in response to increasing temperature: 10, 20, 30, 40 °C. Values are means of four replicates (±SE).

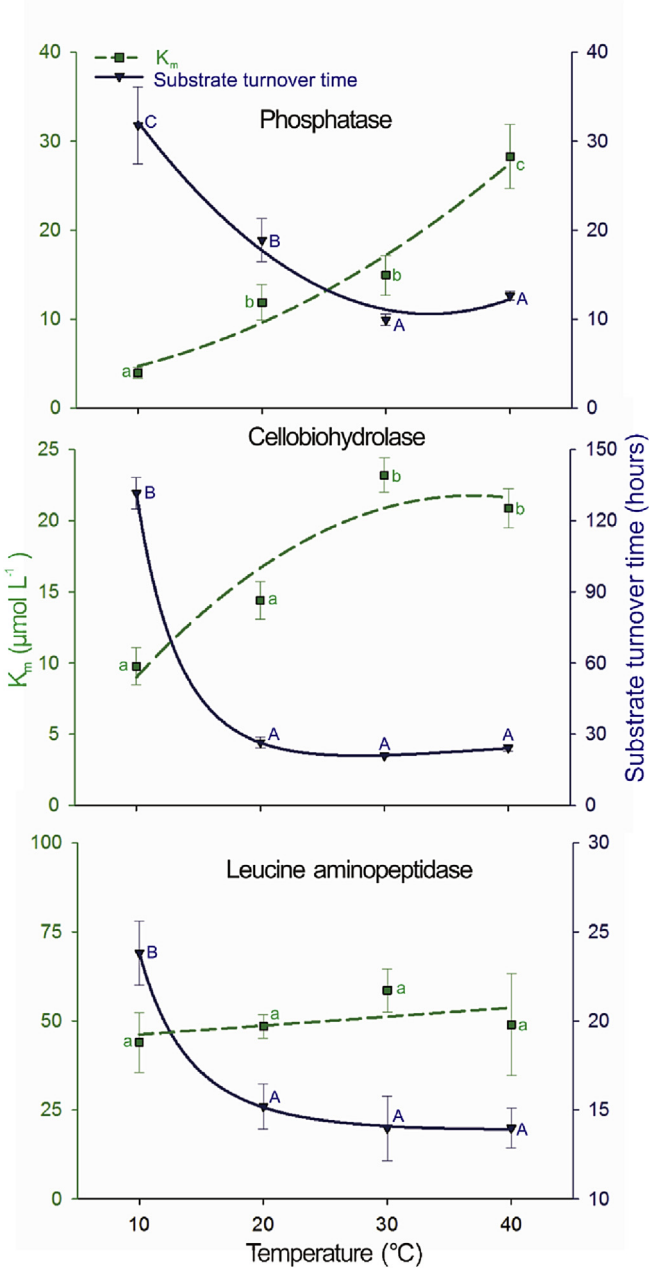

Fig. 5. Km values (green dashed lines) and substrate turnover time (blue lines) of phosphatase, cellobiohydrolase and leucine aminopeptidase at four temperatures (10, 20, 30, 40 °C). Values are means of four replicates (±SE). The differences of Km and substrate turnover time at four temperatures were tested by ANOVA followed by the Duncan-test ($p < 0.05$). Letters show significant differences between temperatures. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

days after cutting (Fig. 3), however, due to large variation between rhizoboxes, the difference was not statistically significant.

3.2. Temperature response of enzyme kinetics and substrate turnover time

The substrate-dependent enzyme activities were fitted well by the Michaelis-Menten kinetics (Fig. 4). For all enzymes, V_{max} increased with temperature from 10 to 30 °C by 1.5–6.6 times. The V_{max} of phosphatase and cellobiohydrolase significantly decreased at 40 °C compared with at 30 °C. However, such significant decrease of V_{max} at 40 °C was absent for leucine -aminopeptidasethe (Figs. 4 and 6). Remarkably, the V_{max} and hotspot areas showed the same trend regarding temperature (Fig. 6) and they were positively correlated (linear regression coefficients: $R^2 = 0.98, 0.91, 0.69$ for phosphatase, cellobiohydrolase and leucine-aminopeptidase, respectively).

The temperature response pattern of K_m was enzyme-specific. The K_m of phosphatase consistently increased within the entire temperature range up to 40 °C. The K_m values of cellobiohydrolase were significantly higher at 30 and 40 °C than at 10 and 20 °C (Fig. 5). The K_m values of leucine-aminopeptidase were nearly

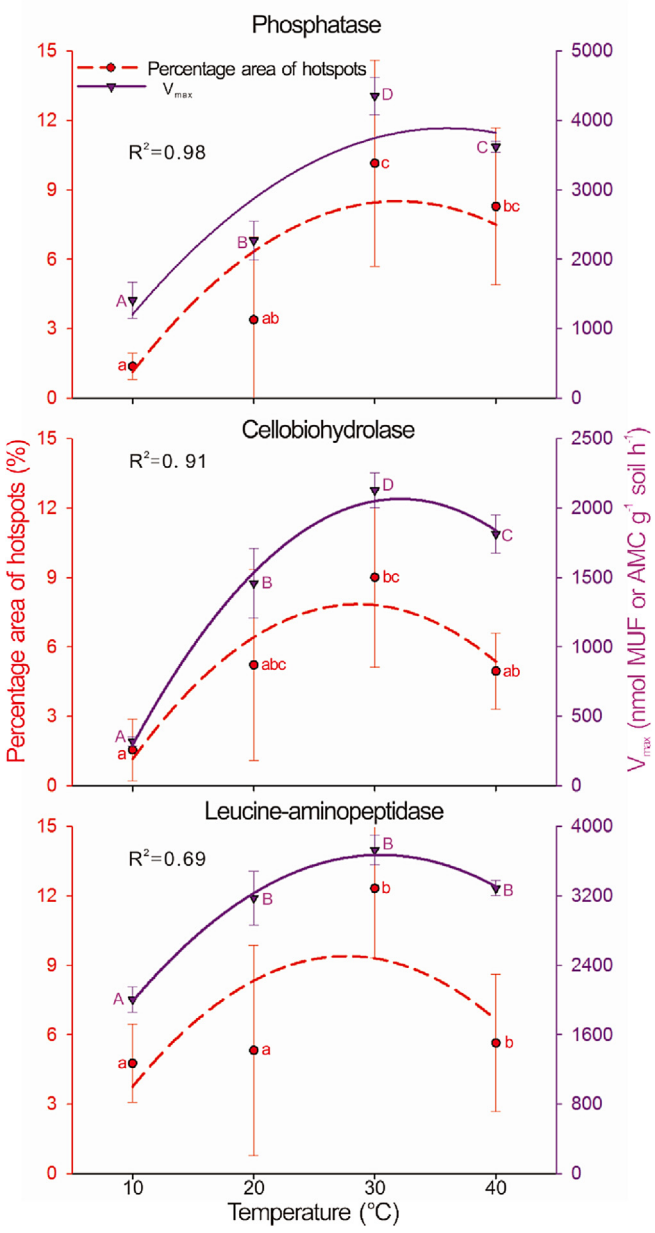

Fig. 6. Percentage area of hotspots (with 25% highest activity, see Fig. 1) for phosphatase, cellobiohydrolase and leucine aminopeptidase in the root-detritusphere (14 days after cutting shoots) depending on temperature (10, 20, 30, 40 °C) (red dashed lines). Vmax values of the three enzymes in the Michaelis-Menten equation (purple line). Data points indicate means of four replicates (±SE). The differences of percentage area of hotspotsand Vmax at four temperatures were tested by ANOVA followed by the Duncan-test ($p < 0.05$). Letters show significant differences between temperatures. The R2 values are coefficients of determination of Vmaxandhotspot areas. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

constant over the whole temperature range (Fig. 5).

The turnover time of all substrates were shorter at warm compared to cold temperatures (Fig. 5). For example, the turnover time of substrates decomposed by phosphatase, cellobiohyrolase and leucine-amino peptidase at 30 °C were 3.2, 6.7 and 1.7 folds faster than at 30 °C, respectively.

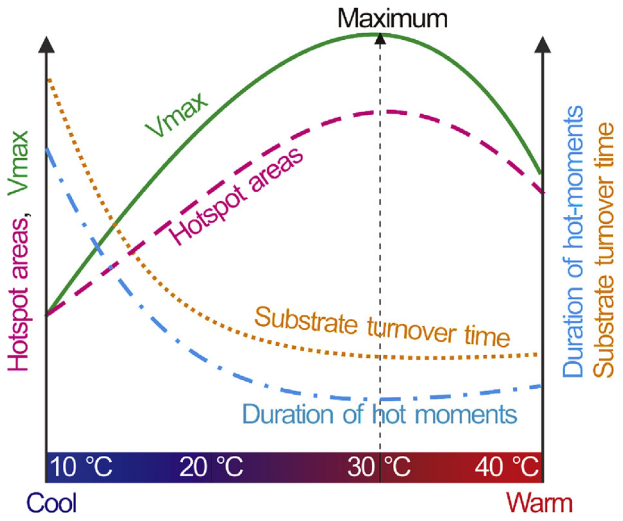

Fig. 7. Temperature effects on hotspot areas of enzyme activity, maximum enzyme activities (Vmax), substrate turnover time and the duration of hot moments.

4. Discussion

In line with our first hypothesis, the hotspot areas in the root-detritusphere were larger than in the rhizosphere (Fig. 2). This finding is attributed to increased exudation from roots directly after shoot cutting (Guitian and Bardgett, 2000; Kuzyakov et al., 2002). This temporally concentrated input of various organics from dying roots into the soil strongly stimulates microbial activity and therefore enzyme activity (Allison et al., 2010; Spohn and Kuzyakov, 2014). The organic matter broken down to soluble units by enzymes (Conant et al., 2011) will be taken up by microorganisms (Fischer et al., 2010), which in turn increases microbial growth and enzyme synthesis (Blagodatskaya and Kuzyakov, 2013). Thus, larger hotspot areas of enzyme activities corresponded to a higher and broader input of substrates from decomposing roots.

According to our second hypothesis, hotspot areas of enzyme activities increased faster and extended more broadly at high temperatures (up to 30 °C) compared to low temperatures. Such increments were due to faster organic matter decomposition (Wallenstein et al., 2009, 2010), stimulated by microbial (Bradford et al., 2008; Steinweg et al., 2008) and enzymatic activities (Kirschbaum, 2006) at warm temperatures. Additionally, the diffusion of enzymes and substrates is faster at warm temperatures (Hu et al., 1992) due to acceleration of Brownian motion with temperature and the increasing probability of collision between substrate and enzyme (Burns et al., 2013; Blagodatskaya et al., 2016). All these factors lead to a broader extension and large areas of hotspots at warm temperatures.

The hotspot areas at warm temperatures (30 and 40 °C) decreased two weeks after shoot cutting (Fig. 3), supporting our second hypothesis on the shorter duration of hot moments at warmer temperatures. This is explained by faster substrate exhaustion and consequently the reduction of enzyme production (Wallenstein et al., 2009, 2010). High microbial and enzyme activities at warm temperatures accelerate substrate decomposition rates (German et al., 2011). These accelerated rates are supported by shorter substrates turnover time at warm temperatures (Fig. 5). The faster substrates turnover rate coupled with no regular substrate input (since shoots cutting is a temporally concentrated substrate input), which results in a local reduction of microbial growth due to starvation, ultimately leading to a downregulation of enzyme production (Schimel and Weintraub, 2003; Knorr et al.,

2005; Allison et al., 2010). Another possible explanation for the transient response of hotspot areas is the thermal adaptation of microorganisms to elevated temperatures (Allison and Treseder, 2008). The adaptation may lead to microbes produce enzymes with different affinity to substrates (Bradford, 2013). This explanation is supported by an increase of the kinetic parameter K_m for cellobiohydrolase and phosphatase at 30 and 40 °C (Fig. 5). A further explanation is the decreased carbon use efficiency at higher temperatures (López-Urrutia and Morán, 2007), which would result in reduced allocation of assimilated C towards enzyme production in response to warming (30 and 40 °C) (Ågren, 2010). Moreover, enzyme decomposition increases under warm temperatures (Ten Hulscher and Cornelissen, 1996) because decomposition is partially controlled by other proteases which may have high activity at warm temperatures (Conant et al., 2011).

Contrasting to phosphatase and cellobiohydrolase, the hotspot areas of leucine aminopeptidase did not decline at 40 °C two weeks after shoot cutting. Nearly constant K_m values at increasing temperatures indicating static enzyme system explains this observation (Fig. 5). Alternatively, K_m values remain stable based on the high structural stability of enzymes which catalyze reactions within a broad temperature range (Razavi et al., 2016a). Similarly, cellobiohydrolase demonstrated relatively stable K_m values at 10 and 20 °C (Fig. 5), showing a temperature-independence of enzyme affinity to substrate (Koch et al., 2007; Razavi et al., 2016a).

Hotspot areas and V_{max} showed a very close positive correlation, indicating that maximum activities were coupled with the spatial distribution of enzymes. The more abundant of the enzymes, the larger the areas that will be required and occupied in the soil profile. Within 10–30 °C, the V_{max} and the hotspot areas increased (Figs. 4 and 6). Both the V_{max} and percentage area of hotspots decreased for all enzymes at 40 °C compared to at 30 °C (Figs. 5 and 6). Two mechanisms can explain this phenomenon. First, enzyme activity responds positively up to an optimum temperature, beyond which enzymes start to denature (Berry and Raison, 1981; Atkin and Tjoelker, 2003). Second, decrease in substrate availability at high temperatures as discussed above (López-Urrutia and Morán, 2007; Allison et al., 2010).

5. Conclusions

The hotspot areas of enzyme activities and their distribution in the root-detritusphere were broader than in the rhizosphere, especially at warm temperatures. We attribute this to the concentrated release of C from dying roots. The substrate turnover time was shorter and the hotspot areas of enzyme activities decreased faster at warm temperatures. This indicates fast substrate consumption and thus a shorter duration of hot moments (Fig. 7). The K_m of cellobiohydrolase and phosphatase increased at 30 and 40 °C, indicating low affinity between enzyme and substrate at warm temperatures. V_{max} and hotspot areas responded positively up to an optimum temperature of 30 °C, but both of them decreased at 40 °C (Fig. 7). For the first time, we found a positive correlation between V_{max} and hotspot areas of enzyme activity. This indicates that maximum reaction rates were coupled with hotspots formation. In conclusion, soil warming (at least up to 30 °C) increases hotspot areas of enzyme activity and the maximum reaction rate (V_{max}) in the root-detritusphere, leading to faster substrate exhaustion and thus to shorter durations of hot moments.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2017.01.009.

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