Effects of biotic and abiotic factors on the spatial distribution of enzyme activities in the soil

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Summary

Enzymes produced by plant and microorganisms are crucial to soil functions as they decompose insoluble macromolecules organic compound to smaller, soluble molecules which can be assimilated by cells. The enzymes are spatial and temporal heterogeneously distributed in soil as affected by microbial and root activity. It is imperative to measure biotic and abiotic factors affected the spatial and temporal distribution of enzyme activity in order to reveal complex interactions between enzymes, macromolecules organic compound decomposition and plant, microbial nutrient acquisitions. Among various factors we selected three biotic factors: root morphology (root hairs and root radius), root architectures (lateral root and taproot), and plant development and two abiotic controls: phosphorus availability and temperature. Therefore, this thesis aimed to visualize and localize distribution of various enzymes activities in two hotspheres in soil: rhizosphere, root-detritusphere depending on biotic and abiotic controls.

Directly soil zymography were established to investigate: 1) the root morphology (root hairs and root radius) and root architectures (taproots and lateral roots) effects on the spatial distribution of enzyme activity in the rhizosphere; 2) plant development (reproductive stage and vegetative stage) effects on the spatial distribution and temporal dynamic of enzyme activity in two legume species; 3) the temperature effects on hotspot areas of enzyme activity and the duration of hot moments in the root-detritusphere. Beside, zymography was combined with enzyme kinetics to test the substrate turnover in the rhizosphere of maize with and without root hairs zone and in the root-detritusphere under a series of temperatures. Moreover, zymography was combined with pH planar optode to investigate the spatial distribution phosphatase activity and pH in the rhizosphere of lupine in response to phosphorus availability and cluster root formation.

The abiotic effects: root morphology (root hairs and root radius) and architectures (lateral root and taproots) and plant development influenced the *in situ* and the spatial distribution of enzyme activity as well as the substrate turnover in the rhizosphere: 1) the rhzosphere extent of enzyme activity and the substrate turnover depended on the root hairs: the rhizosphere extent of β-glucosidase activity in wheat root regions with

hairs was 1.5-fold broader and substrate turnover was 2 times faster than in root regions without hairs; 2) the rhizosphere extent relative to root radius and the enzyme activity per root surface area in plant with thin root (wheat) were higher than in plant with thick root (maize): the rhizosphere extents relative to root radius of β-glucosidase and phosphatase activity in wheat were nearly 2 times broader than in maize; and the leucine aminopeptidase activity per root surface area in wheat was 7 times higher than that in maize; 3) the rhizosphere extent relative to root radius and enzyme activity per root surface area in plant with long and dense root hairs (lupine) was significantly higher than in plant with short and sparse root hairs (lentil): the rhizosphere extent relative to root radius of acid phosphatase in lupine was 1.5 times broader than in lentil; the β-glucosidase and cellobiohydrolase activity per root surface area in lupine was 2-3 times higher than in lentil; 4) the rhizosphere volume per root length was 30-70 times higher and enzyme activity per root surface area was 6-14 fold higher for lateral roots than for taproots; 5) the spatial distribution of enzyme activity *in situ* in the rhizosphere depended on plant development and growth stage: Lentil kept as vegetative growth and the rhizosphere extent was constant; lupine entered reproductive growth in the 7^{th} week after planting companied with broader rhizosphere extent, however, the enzyme activity decreased by 10-50% compared to the vegetative stage.

Moreover, the spatio-temporal patterns of phosphatase activities and pH in the rhizosphere depending on biotic and abiotic interaction effects such as P availability and cluster root formation: 1) before cluster root formations, phosphorus deficiency increased acid phosphatase activities by 20%, decreased pH by 0.8 units and broadened the rhizosphere extent by about 0.4 mm around taproot; 2) after cluster root formation, the rhizosphere extent of phosphatase activity around taproot of lupine was 0.2 mm narrower, while the hotspot areas of alkaline phosphatase activity was 40% larger for lupine grown under P-deficiency than amended with $Ca(H_2PO_4)_2$. These indicate that lupine used various strategies to conquer P deficiency during growth: increased phosphatase activity, soil acidification and broaden their rhizosphere extent around taproot are the mechanisms before cluster root formation. After cluster root development, the main mechanism is increase of hotspot area of phosphatase activity to explore larger soil volume for P acquisition.

Beside, abiotic factors such as temperature affected the enzyme activity hotspot area formation, the duration of hot moment, enzyme kinetics and the substrate turnover: 1) the hotspot areas increased by 2-24 times from 10 to 30 ℃, however, the hotspots area decreased by 5-73% for all enzymes at 40 ℃ compared to at 30 ℃; 2) V_{max} increased with temperature from 10 to 30 ℃ by 1.5-6.6 times but decreased at 40 ℃ ; 3) The turnover time of all substrates were shorter at warm compared to cold temperatures: the turnover time of substrates decomposed by phosphatase, cellobiohyrolase and leucine-amino peptidase at 30 ℃ were 1.7-6.7 folds faster than at 10 ℃.

Overall, this thesis developed new concepts and developed numbers of approaches dedicated to investigate the abiotic and biotic effects on spatial distribution, hotspot area formation of soil enzyme activities. The effect of abiotic and biotic controls on spatial distribution of the enzyme hotspots has important consequences not only for soil science, but also for ecology, plant-soil-microbial interactions, nutrients and element cycles.

Zusammenfassung

Enzyme, die von Pflanzen und Mikroorganismen produziert werden, sind für die Bodenfunktionen entscheidend, da sie unlösliche organische Verbindungen von Makromolekülen zu kleineren, löslichen Molekülen zersetzen, die von Zellen assimiliert werden können. Die Enzyme sind räumlich und zeitlich heterogen im Boden verteilt und werden von mikrobiellen und Wurzelaktivitäten beeinflusst. Es ist unerlässlich, biotische und abiotische Faktoren zu messen, die die räumliche und zeitliche Verteilung der Enzymaktivität beeinflussen, um komplexe Wechselwirkungen zwischen Enzymen, der Zersetzung organischer Verbindungen von Makromolekülen und der mikrobiellen Nährstoffaufnahme von Pflanzen aufzudecken. Unter verschiedenen Faktoren wählten wir drei biotische Faktoren: Wurzelmorphologie (Wurzelhaare und Wurzelradius), Wurzelarchitekturen (Seitenwurzel und Pfahlwurzel) und Pflanzenentwicklung und zwei abiotische Kontrollen: Phosphorverfügbarkeit und Temperatur. Daher war es das Ziel dieser Arbeit, die Verteilung verschiedener Enzymaktivitäten in zwei heißen Böden im Boden zu visualisieren und zu lokalisieren: Rhizosphäre, Wurzel-Detritussphäre, abhängig von biotischen und abiotischen Kontrollen.

Es wurde eine direkte Boden - Zymographie zur Untersuchung von: 1) der Wurzelmorphologie (Wurzelhaare und Wurzelradius) und Wurzelarchitekturen (Pfahlwurzeln und Seitenwurzeln) auf die räumliche Verteilung der Enzymaktivität in der Rhizosphäre; 2) Pflanzenentwicklung (Reproduktionsstadium und vegetatives Stadium) Auswirkungen auf die räumliche Verteilung und zeitliche Dynamik der Enzymaktivität in zwei Leguminosenarten; 3) die Temperatureinflüsse auf die Hotspot-Bereiche der Enzymaktivität und die Dauer der heißen Momente in der Wurzel-Detritus-Sphäre. Außerdem wurde die Zymographie mit Enzymkinetiken kombiniert, um den Substratumsatz in der Rhizosphäre von Mais mit und ohne Wurzelhaarzone und in der Wurzeldetritussphäre unter einer Reihe von Temperaturen zu testen. Darüber hinaus wurde die Zymographie mit einer planaren pH-Optode kombiniert, um die Aktivität der Phosphatase in der räumlichen Verteilung und den pH-Wert in der Rhizosphäre der Lupine als Reaktion auf Phosphorverfügbarkeit und Clusterwurzelbildung zu untersuchen.

Die abiotischen Effekte: Wurzelmorphologie (Wurzelhaare und Wurzelradius) und Architekturen (Seitenwurzel und Pfahlwurzeln) und Pflanzenentwicklung beeinflussten die in situ und die räumliche Verteilung der Enzymaktivität sowie den Substratumsatz in der Rhizosphäre: 1) das Rhsosphärenausmaß Die Enzymaktivität und der Substratumsatz waren abhängig von den Wurzelhaaren: das Rhizosphären-Ausmaß der β-Glucosidase-Aktivität in Weizenwurzelregionen mit Haaren war 1,5-fach breiter und der Substratumsatz war 2-mal schneller als in Wurzelregionen ohne Haare; 2) die Rhizosphärenausdehnung in Bezug auf den Wurzelradius und die Enzymaktivität pro Wurzeloberfläche in Pflanzen mit dünner Wurzel (Weizen) waren höher als in Pflanzen mit dicker Wurzel (Mais): die Rhizosphärenausdehnung relativ zum Wurzelradius von β-Glucosidase und Phosphatase Aktivität in Weizen war fast 2 mal breiter als in Mais; und die Leucin-Aminopeptidase-Aktivität pro Wurzeloberfläche in Weizen war 7 mal höher als die in Mais; 3) das Rhizosphärenausmaß bezogen auf den Wurzelradius und die Enzymaktivität pro Wurzeloberfläche in Pflanzen mit langen und dichten Wurzelhaaren (Lupine) war signifikant höher als in Pflanzen mit kurzen und spärlichen Wurzelhaaren (Linse): die Rhizosphärenausdehnung relativ zum Wurzelradius der Säurephosphatase in Lupine war 1,5 mal breiter als in Linsen; die β-Glucosidase- und Cellobiohydrolase-Aktivität pro Wurzeloberfläche in Lupine war 2-3 mal höher als in Linsen; 4) das Rhizosphärenvolumen pro Wurzellänge war 30 bis 70 mal höher und die Enzymaktivität pro Wurzeloberfläche war 6-14 mal höher für seitliche Wurzeln als für Pfahlwurzeln; 5) Die räumliche Verteilung der Enzymaktivität in situ in der Rhizosphäre hing vom Pflanzenentwicklungs- und Wachstumsstadium ab: Die Linse blieb als vegetatives Wachstum erhalten, und das Ausmaß der Rhizosphäre war konstant; Lupine trat in der 7. Woche nach dem Anpflanzen zusammen mit einem breiteren Rhizosphären-Ausmaß in das reproduktive Wachstum ein, jedoch nahm die Enzymaktivität verglichen mit dem vegetativen Stadium um 10-50% ab.

Darüber hinaus sind die raum-zeitlichen Muster der Phosphataseaktivitäten und der pH-Wert in der Rhizosphäre abhängig von biotischen und abiotischen Interaktionseffekten wie P-Verfügbarkeit und Clusterwurzelbildung: 1) vor Clusterwurzelbildung erhöhte Phosphormangel die Aktivität der sauren Phosphatase um 20%, pH-Wert ab um 0,8 Einheiten und verbreiterte die Rhizosphären-Ausdehnung um etwa 0,4 mm um die Pfahlwurzel herum; 2) Nach der Bildung der

Clusterwurzel war das Ausmaß der Phosphataseaktivität um die Pfahlwurzel der Lupine um 0,2 mm enger, während die Hotspot-Bereiche der alkalischen Phosphataseaktivität für Lupine, die unter P-Mangel gezüchtet wurden, um 40% größer als mit $Ca(H_2PO_4)_2$. Dies deutet darauf hin, dass Lupine verschiedene Strategien zur Überwindung von P-Mangel während des Wachstums verwendet: erhöhte Phosphatase-Aktivität, Bodenversauerung und Erweiterung ihrer Rhizosphären-Ausdehnung um Pfahlwurzeln sind die Mechanismen vor der Clusterwurzelbildung. Nach der Clusterwurzelentwicklung besteht der Hauptmechanismus in der Zunahme der Aktivität der Phosphatase im Hotspotbereich, um ein größeres Bodenvolumen für die P-Akquisition zu erforschen.

Nebenbei beeinflussten abiotische Faktoren wie die Temperatur die Enzymaktivität Hotspot-Bereich-Bildung, die Dauer des heißen Moments, die Enzymkinetik und den Substratumsatz: 1) Die Hotspot-Bereiche erhöhten sich jedoch von 2 bis 24 mal von 10 auf 30 °C die Fläche nahm bei 40 °C für alle Enzyme um 5 bis 73% ab, verglichen mit 30 °C; 2) Vmax erhöhte sich mit der Temperatur von 10 bis 30 °C um das 1,5-6,6-fache, verringerte sich jedoch bei 40 °C; 3) Die Umsatzzeit aller Substrate war im Vergleich zu kalten Temperaturen kürzer: Die Umsatzzeit der Substrate, die bei 30 °C durch Phosphatase, Cellobiohydrolase und Leucin-Aminopeptidase zersetzt wurden, war 1,7 bis 6,7 mal schneller als bei 10 °C.

Insgesamt wurden in dieser Arbeit neue Konzepte entwickelt und Ansätze entwickelt, um die abiotischen und biotischen Effekte auf die räumliche Verteilung, die Entstehung von Enzymaktivitäten im Hotspot-Bereich, zu untersuchen. Die Wirkung von abiotischen und biotischen Kontrollen auf die räumliche Verteilung der Enzym-Hotspots hat wichtige Konsequenzen nicht nur für die Bodenkunde, sondern auch für Ökologie, Pflanzen-Boden-Mikroben-Wechselwirkungen, Nährstoffe und Elementenzyklen.

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Abbreviations

C	Carbon
N	Nitrogen
P	Phosphorus
SOM	Soil organic matter
CO_2	Carbon dioxide
ANOVA	Analysis of variance

1. Extended summary

1.1 Introduction

The enzymes excreted by both microorganisms and roots, catalyze the decomposition of root exudate and other rhizodeposits into absorbable forms (Nannipieri et al., 2007; Sinsabaugh et al., 2008), and provide nutrients for microorganisms and plants (Henry, 2013). Therefore, quantification of enzyme activity in the rhizosphere is a sensitive indicator of changes in the plant-root-microbe interactions (Aon et al., 2001; Nannipieri et al., 2007), and determination of spatial distribution of enzyme activities can provide complementary information on the microbial activity and root nutrient availability (Wallenstein and Weintraub, 2008). The enzymes are spatial and temporal heterogeneously distributed in soil as affected by biotic and abiotic factor. Among various factors we selected three biotic factors: root morphology (root hairs and root radius), root architectures (lateral root and taproot) and plant development stage (vegetative and reproductive growth) and two abiotic controls: phosphorus availability and temperature.

1.1.1 Biotic factors (root morphology, architectures and plant development) and spatial distribution of enzyme activity

Nutrients are very often limited in soils (Hodge, 2004), and this limitation is extremely high in the rhizosphere as microorganisms and plants compete for the same nutrients (Kuzyakov and Xu, 2013). Plants use root morphological strategies to overcome nutrient limitation, such as development of the roots with large surface area and long length (Jungk, 2001; Ma et al., 2001). Root hairs, the tubular-shaped outgrowths from root epidermal cells (Peterson and Farquhar, 1996), dramatically increase the root surface area, and play important roles in nutrient and water acquisition as well as in the interactions with microbes (Gilroy and Jones, 2000). Apart from these functions, root hairs also play an important role in modulating the properties and composition of the rhizosphere through exudation, and in some species, exudates appear to be produced solely by root hairs (Czarnota et al., 2003; Datta et al., 2011). Because, the lifespan of root hairs is short (at most a few days), dead root hairs released a lot of C into the soil (Nguyen, 2003). These large amounts of labile carbon

and other rhizodeposits released by root hairs stimulate microbial activity (Parkin, 1993; Asmar et al., 1994) and further influence enzyme dynamic such as accelerated substrate turnover. Moreover, root hairs actively participate in the interactions between plants and nitrogen-fixing microorganisms and symbiotic mycorrhizal fungi by providing nutrients, hormones and signaling molecules (Peterson and Farquhar, 1996; Libault et al., 2010). However, root hairs are highly variable in numbers, length, density and longevity, depending on both genetic potential of plants and environmental conditions (Jungk, 2001). It is widely accepted that the exudation rate and the ability of nutrient acquisition are positively correlated with the length and density of root hair (Yan et al., 2004).

Root radius is another root morphology parameter that influences exudation and nutrient acquisition. Thin and thick roots have distinctive nutrient absorption strategies (Kong et al., 2016). The quantity of exudation and ability of absorption are proportional to the root radius (Lambers et al., 2006). Exudates are also heterogeneously distributed between root architectural structures such as young lateral roots and old taproots. Lateral roots and root hairs increase the root surface area, which elevates exudation and facilitates nutrient uptake (Jungk, 2001; McCormack et al., 2015). In contrast, older thicker taproot exhibit low uptake ability and exudation but high transport capacity (Guo et al., 2008; Gambetta et al., 2013; McCormack et al., 2015).

Moreover, the amount of root exudates is dependent on plant development (Farrar et al., 2003), which increases with plant growth until the reproductive stage (Gransee and Wittenmayer, 2000; Aulakh et al., 2001; Odell et al., 2008). Therefore, Microbial activity and the spatial pattern enzyme activities are were affected by the quality and quantity of root exudate, and are depended on root morphologies (root hairs and root radius), root architectures (lateral root and taproot) and plant growth stage (vegetative and reproductive growth). However, the interactive effects of root hairs and root radius on spatial distribution and in situ enzymes activity are completely unknown. Specifically, the effects of root hairs on the rhizosphere extent of enzyme activity and enzyme mediated turnover of various substrates in the rhizosphere remain unclear. Besides, the effects root architectures, plant development on the spatial distribution of enzyme activity in rhizosphere remained to be clarified.

1.1.2 Abiotic factors (phosphorus availability and temperature) and spatial distribution of enzyme activity

Enzyme activities in soil are controlled by abiotic factors such as temperature, water potential, pH, soil texture and nutrient availability (Burns et al., 2013). Phosphorus (P) availability is an important abiotic factor influence the spatial distribution of enzyme activity, which has attracted considerable interest. This is due to P is the second quantitatively important major nutrient for plant growth (Bieleski, 1973; Raghothama, 1999), as it plays an important role in various metabolic pathways as well as a key component in nucleic acids, ATP and phospholipids (Vance et al., 2003). Although phosphorus is abundant in soil (Dalai, 1977), they are often unavailable for plants due to rapid formation of insoluble complexes with minerals (Al, Fe and Ca) and organic compounds (Fox and Comerford, 1992; Yao et al., 2018). As a result, about a third of terrestrial soils contain insufficient available P for optimum crop production (Batjes, 1997; Li et al., 2007). Therefore, the application of organic (eg., phytate is abundant in animal manure) and mineral fertilizers kept rising to increase food production globally (Cordell et al., 2009).

To maximize phosphorus absorption efficiency, plants have evolved a range of mechanisms: 1) altering root morphology such as intensified branching and increase the density and length of root hairs to increase the soil volume explored by root (Lynch, 2005; Hill et al., 2006); 2) by symbiotic association with microorganisms such as mycorrhizal fungi to enhance phosphate availability and uptake ability (Richardson et al., 2001; Jakobsen et al., 2005); 3) by acidification of the rhizosphere to improve phosphate solubility; 4) exudation of chelating organic acid to reduce concentration of polyvalent cations in soil solution and thus reduce P precipitation and 5) secretion of phosphatases to mobilize organic P (Hinsinger, 2001; Hocking, 2001). The rhizosphere (a small volume of soil around living root) is strongly influenced by root and microbial activity (Hilmer, 1904; Darrah, 1993), which is one of one of the most dynamic habitats on Earth (Hinsinger et al., 2009). Therefore, these adaptations not only improved phosphorus availability but also lead to chemical, physical and biological alteration in the rhizosphere.

Most of these mechanisms are exploited by white lupine (*Lupinus albus*. L) a model crop for phosphorus study, which can be grown on a broad range of soils (Weisskopf

et al., 2006). Under phosphorus deficiency, the bottlebrush-like cluster or proteoid root will grow from the pericycle along the lateral root of lupine (Zobel, 1991; Neumann et al., 2000). The cluster roots are rows of rootlets covered with the dense root hairs, which efficiently increase soil volume and absorptive surface area (Watt and Evans, 1999; Gilroy and Jones, 2000). Additionally, cluster roots release large amount of acid phosphatase, proton and phosphatase to increase phosphorus availability (Gerke et al., 1994 Gilbert, 2000 #317). The exudation of organic acid and phosphatase is depended on cluster root development stage: they are high in mature than in senescent clusters (Neumann and Römheld, 1999). This is attributed to short lifespan of cluster root and decrease of exudation at the onset of senescence (Dinkelaker et al., 1995). Lupine should firstly develop embryonic taproot before forming cluster roots (Dinkelaker et al., 1995). However, the spatial and temporal aspects of taproot response to P-deficiency are much less investigated than cluster root, but as the early phase of plant nutrition is pivotal important. Therefore, we tackle some elemental question regarding root types of white lupine: 1) what is the ability of taproots to release phosphatase and protons, i.e. how is enzyme activity enhanced and rhizosphere acidified around taproot due to P deficiency activity. To which extent are these mechanisms higher or lower expressed in taproots versus cluster root: 2) does lupine exploit different strategies to improve P acquisition during root development as a results of shifts in root morphology.

Besides plant root, microorganisms also play a key role in providing phosphorus to plant by production of phosphatase to hydrolyze organic P (Illmer et al., 1995). Phosphorus availability in soil can directly and indirectly influence microbial phosphatase production ability (Harder and Dijkhuizen, 1983). The availability of inorganic P can directly suppress microbial production of phosphatase (Olander and Vitousek, 2000; Sinsabaugh et al., 2008). Plant phosphorus availability can influence plant growth and via that root exudation (Grierson and Comerford, 2000) which indirectly affect microbial activity and microbial phosphatase production (Philippot et al., 2013). The quality and quantity of root exudates vary spatially and temporally and are especially strongly dependent on root development and soil nutrient status (Neumann and Römheld, 1999; Badri and Vivanco, 2009). Therefore, phosphatase activity reflecting microbial activity and phosphorus availability should also change over root growth (Li et al., 2007; Marschner et al., 2011).

Phosphatases can be separated for acid and alkaline phosphatase based on the pH optima of their activity. While both root and microorganism can produce acid phosphatase, alkaline phosphatase originates exclusively from microorganisms (Juma and Tabatabai, 1988; Nannipieri et al., 2011). Acid and alkaline phosphatase activity and their rhizosphere extent vary in response to mineral P supply (Spohn and Kuzyakov, 2013). This is an effective strategy to alleviate competition between root and microorganism, since they occupied different spatial niches for nutrient acquisition (Marschner et al., 2011). However, it is reminded unknown, the temporal dynamics of acid and alkaline phosphatase activity and as well as pH in response to P availability over root development. It is imperative to quantitative analysis of these parameters in order to better understand phosphorus acquisition strategies.

The temperature sensitivity of enzyme activity is another abiotic factor has received considerable interest because of its potential feedback to climate change (Davidson et al., 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 et al., 2006; Steinweg et al., 2008). Thus, soil warming increases the breakdown and assimilation of organic matter, enhancing microbial growth and enzyme synthesis (Davidson et al., 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 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., 2011). Therefore, hot moments - the events that accelerate processes as compared to the average rates (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 et al., 2006) and usually

increase with temperature (Stone et al., 2012; Baldrian et al., 2013). It remains unresolved, however, whether temperature affects the dynamics and localization of enzyme activity hotspots. This calls for *in situ* monitoring of 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)

1.2 Objective

This thesis aims to estimate biotic (root morphology, root architectures, plant development, plant species) and abiotic (P availability, temperature) factors on the spatial distribution of enzyme activity.

1) To investigate the root morphology (root hairs and root radius) and root architectures (taproots and lateral roots) effects on the spatial distribution of enzyme activity in the rhizosphere (study 1 and 2)

2) To clarify plant development (reproductive stage and vegetative stage) effects on the spatial distribution and temporal dynamic of enzyme activity of two legume species (study 2)

5) To investigate the spatial distribution and temporal dynamic of acid and alkaline phosphatase activity and pH in the rhizosphere of lupine before and after cluster root development in response to P availability (study 3)

4) To quantified the substrate turnover in the rhizosphere of maize with and without root hairs zone and in the root-detritusphere under a series of temperatures (study 1 and 4)

3) To evaluate the temperature effects on hotspot areas of enzyme activity and the duration of hot moments in the root-detritusphere (study 4)

1.3 Materials and Methods

1.3.1 Soil sampling

Soil from Ap horizon of an arable loamy haplic Luvisol located on a terrace plain of the Leine River north-west of the city of Göttingen (Holtensen, Germany) as a temperate agroecosystem, were used for study1, 2, 4. The calcareous loess subsoil strongly limited in nutrients located in Hohenheim was used for study 3.

1.3.2 Experiments setup

Maize (*Zea mays*. L), wheat (*Triticum aestivum*. L), lupine (*Lupinus albus*. L) and lentil (*Lens culinaris*. L) were used for these studies. Seeds were germinated on filter paper for 72 h. 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. During the growth period, 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 ℃ and a daily light period of 14 h with a photosynthetically active radiation intensity of 250 -300 µmol m^{-2} s^{-1}. During the growth period, the soil water content was maintained at 60-70% of the water holding capacity.

1.3.3 Analytical methods

Soil zymography

Direct soil zymography (Sanaullah et al., 2016) was applied when maize, wheat, lupine and lentil were one week old (study 1); lupine and lentil 1, 4, 8 week old respectively (study 2); 11 and 24 days old lupine under different P availability (study 3); in the rhiosphere of 2 week old maize (at a climate chamber temperature of 20 ± 1 ℃) and 7 and 14 days after the cutting shoots (root - detritusphere) (for samples kept at 10, 20, 30 and 40 ℃) (study 4);

Thin polyamide membrane filters (Tao Yuan, China) with diameter of 20 cm was adjusted to the rhizobox size and a pore size of 0.45 mm were saturated with the following substrates: 1) 4-methylumbelliferyl-β-D-glucoside to detect β-glucosidase activity, 2) 4-methylumbelliferyl-β-D-cellobioside to detect cellobiohydrolase activity, 3) 4-methylumbelliferyl-phosphate to detect acid phosphatase activity, and 4) L-

leucine-7-amido-4-methylcoumarin hydrochloride to detect leucine-aminopeptidase activity (Koch et al., 2007; Razavi et al., 2015). Each of these substrates was dissolved to a concentration of 12 mM in buffers, MES buffer for 4-methylumbelliferyl (MUF) based substrate and TRIZMA buffer for 7- amido-4-methylcoumarin (AMC) based substrate. All substrates and chemicals were purchased from Sigma Aldrich (Germany). Under UV light, the MUF and AMC become fluorescent when the respective specific enzyme hydrolyzes the substrate. 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. After incubation for 1 h, the membranes were carefully lifted off the soil surface and any attached soil particles were gently removed using tweezers and a soft brush (Razavi et al., 2016). The membranes were placed under ultraviolet (UV) illumination with an excitation wavelength of 355 nm in a dark room. The camera (EOS 5D, Canon), the sample, and the distance between the UV light were fixed, and a photograph of the membrane was taken. 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 (Spohn and Kuzyakov, 2014). The membranes used for calibration were imaged under UV light in the same way as described for the rhizoboxes

Enzyme kinetic and substrate turnover

Enzyme activities were measured one week after cultivating of wheat, soil was collected from the rhizosphere with and without root hairs (study 1) and 14 days after cutting shoots, soil was collected for root - detritusphere under a series of temperature (10, 20, 30 and 40 ℃) (study 4).

Suspensions of with 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 emissionwavelength 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 All enzyme activities were measured 30 min, 1 h and 2 h after adding soil solution, buffer and substrate solution (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 V_{max} were determined for each enzyme using the Michaelis-Menten equation:

$$v = \frac{V_{max} \times [S]}{K_m + [S]}$$

where v is the reaction rate (as a function of enzyme concentration), [S] is the substrate concentration, K_m is the substrate concentration at half-maximal rate, and V_{max} is the maximum reaction rate.

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 detritusphere and labile substances released from roots are abundant in the rhizosphere, 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.

pH measurement by planar optode

The pH was visualized by a sensor foil embedded with fluorescent indicator dye (study 3) (Blossfeld and Gansert, 2007). The foils contain both analyte-sensitive and analyte insensitive dyes. Green fluorescence is declined at lower pH, whereas the red fluorescence is unaffected. A camera connected to PC was used to detect these fluorescence signals. Subsequent data analysis by the open source software imageJ created the ratio of the red and the green channel (R values), a quantitative two-dimensional map of pH values (Blossfeld and Gansert, 2007). The pH sensitive foil (SF-HP5R), the camera (VisiSens TD), the LED light with wavelength of 470 nm and the image capture software (VisiSens AnalytiCal) all purchased from company

(PreSens GmbH, Regensburg, Germany). The rhizoboxes were opened from the lower, rooted side, the images of the root was taken by camera (EOS 5D, Canon) and the pH foil (10 × 14 cm) was applied to the soil surface. After incubated on the soil surface for 2 hours, a snap shot image was taken in the dark room. The positions of the camera, the sample and the LED light were fixed throughout the experiment.

A calibration line was prepared from 1 cm^2 pH foils that were soaked in mixed buffer solution of NaH_2PO_4 • H_2O and Na_2HPO_4• 2 H_2O in a range of pH (7.74, 7.48, 7.12, 6.78, 6.44, 6.12, 5.58) for two hours. The calibration image was taken under the same setup as described above.

1.4 Results and Discussion

1.4.1 Overview of objectives and main results of the studies

Objectives and main conclusions of individual studies are presented in Table ES1.

Table ES 1 Objectives and main results of the studies.

Study	Objectives	Main results and conclusion
Study 1 Spatial patterns of enzyme activities in the rhizosphere depended on root hairs and root radius	● To investigate the role of root hairs and root radius and their interactive effects on the *in situ* and spatial distribution of enzymes of various plants ● To study the effects of root hairs on the rhizosphere extent of enzyme activity and on the enzyme mediated turnover of various substrates	✓ roots with long and dense hairs (lupine) have a higher enzyme activity per root area and a broader rhizosphere extent relative to root radius than roots with short and sparse hairs (lentil) ✓ the rhizosphere extent relative to root radius was broader and enzyme activity per surface area was higher around thin roots (wheat) than around thick roots (maize) ✓ root hairs broaden the rhizosphere extension up to 50% and facilitated the substrate turnover by 2 times
Study 2 Spatio-temporal patterns of enzyme activities	● To investigate the root architectures (taproots and lateral roots) effects on the spatial	✓ lateral roots have much larger rhizosphere volume per unit root length and higher enzyme activity per root surface area

in the rhizosphere: Effects of plant development and root architecture	distribution of enzyme activity in the rhizosphere ● To clarify plant development (reproductive stage and vegetative stage) effects on the spatial distribution and temporal dynamic of enzyme activity	than the taproots ✓ the enzyme activity in the rhizosphere increased with plant growth until reproductive stage
Study 3 Spatio-temporal patterns of phosphatase activities and pH in the rhizosphere depending on P availability and root development: Coupling zymography with planar optode	● To investigate spatio-temporal patterns of phosphatase activities and pH in the rhizosphere of lupine before and after cluster root formation. ● To test whether lupine exploits different strategies to improve phosphorus acquisition as root development and root morphology change.	✓ Phosphorus deficiency increased acid phosphatase activities by 20%, decreased pH by 0.8 units and broadens the rhizosphere extent by about 0.4 mm around taproot, while phytate addition smoothed these changes on before cluster root formations ✓ The rhizosphere extent of phosphatase activity around taproot of lupine was narrower under P-deficiency than amended with $Ca(H_2PO_4)_2$ after cluster root formation ✓ The hotspot areas of alkaline phosphatase activity was 40% larger while the shoot biomass and shoot P concentration were about 35% lower for lupine grown under P-deficiency than amended with $Ca(H_2PO_4)_2$ after cluster root formation ✓ Lupine used different strategies to conquer phosphorus

		deficiency during growth: increased phosphatase activity, soil acidifcation and broaden their rhizosphere extent around taproot are the mechanisms before cluster root formation. After cluster root development, the main mechanism is increase of hotspot area of phosphatase activity to explore larger soil volume for P acquisition.
Study 4 temperature effects on the spatial distribution of enzyme activity hotspots and the duration of hot-moment	● To investigate temperature effect on the spatial distribution of enzyme activity hotspot and the duration of hot-moment in the root-detritusphere ● To clarify catalytic properties (K_m and V_{max}) and substrate turnover in respond to temperature	✓ Warming increased hotspot areas of enzyme activities and shorten the duration of hot-moment ✓ Vmax increased with temperature up to 30 ℃, the substrate turnover was fast under warm temperature ✓ The response of Km to temperature was enzyme specific

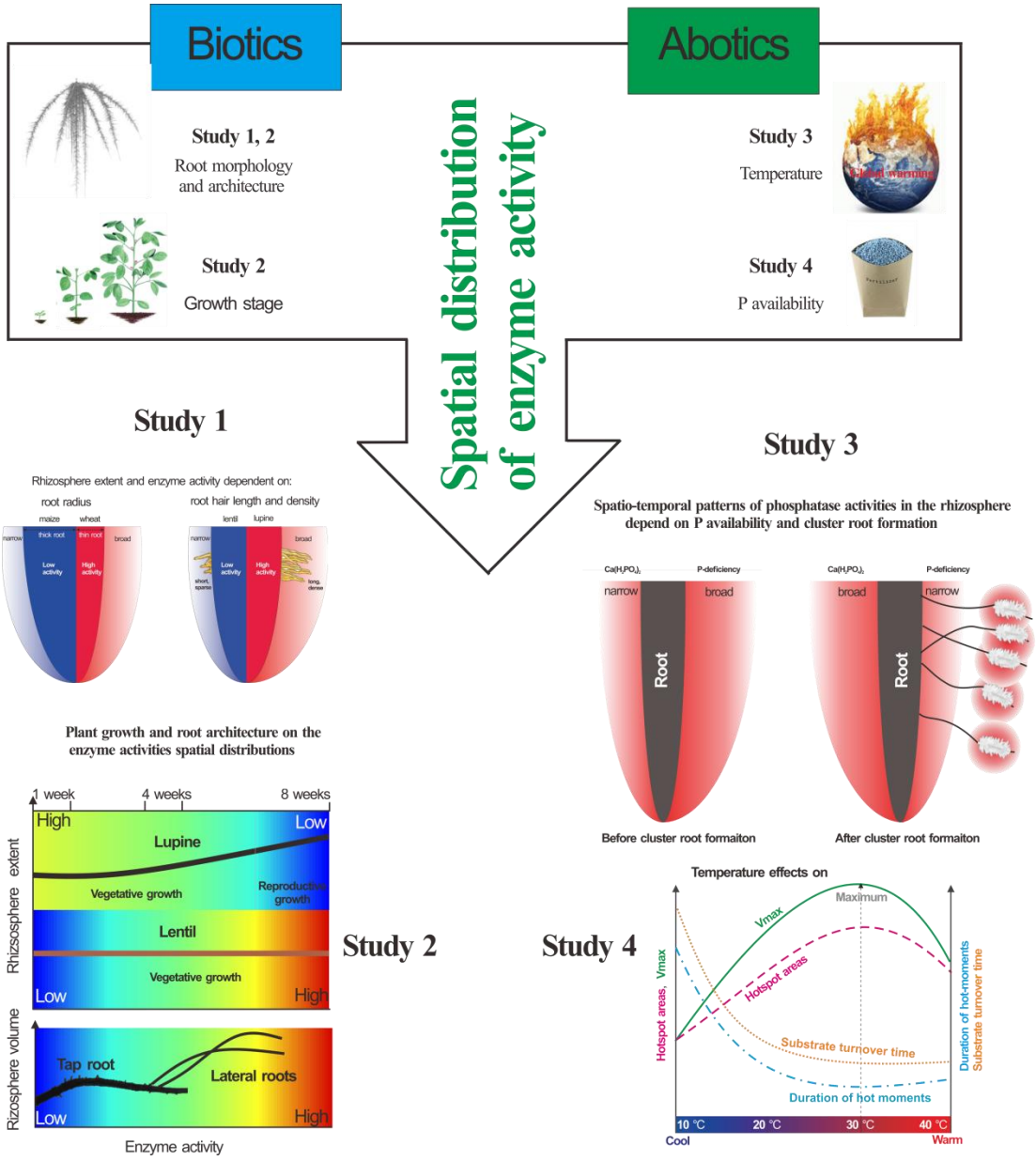

Fig. ES1 Synthesis of main results of the studies

1.4.2 Biotic factors (root morphology, architectures and plant development) on the spatial distribution of enzyme activity

1.4.2.1 Root hairs broadened the rhizosphere extent and facilitated substrate turnover

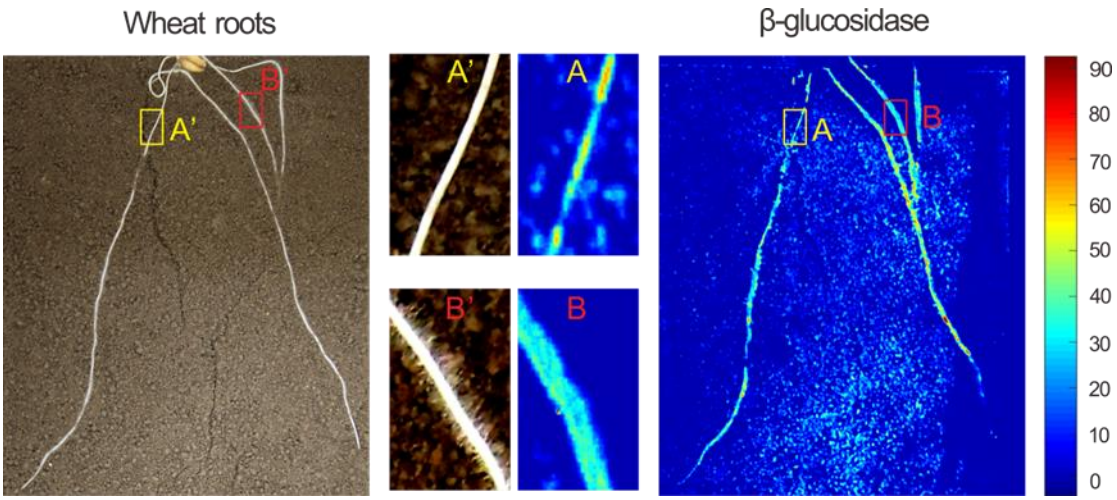

Fig. ES2 Examples of wheat roots grown in a rhizobox and the spatial distribution of β-glucosidase. A' and B' indicate root regions without and with hairs, respectively. A and B indicate the spatial distribution of β-glucosidase in regions without and with hairs, respectively. Side color maps are proportional to the enzyme activities (pmol mm^{-2} h^{-1})

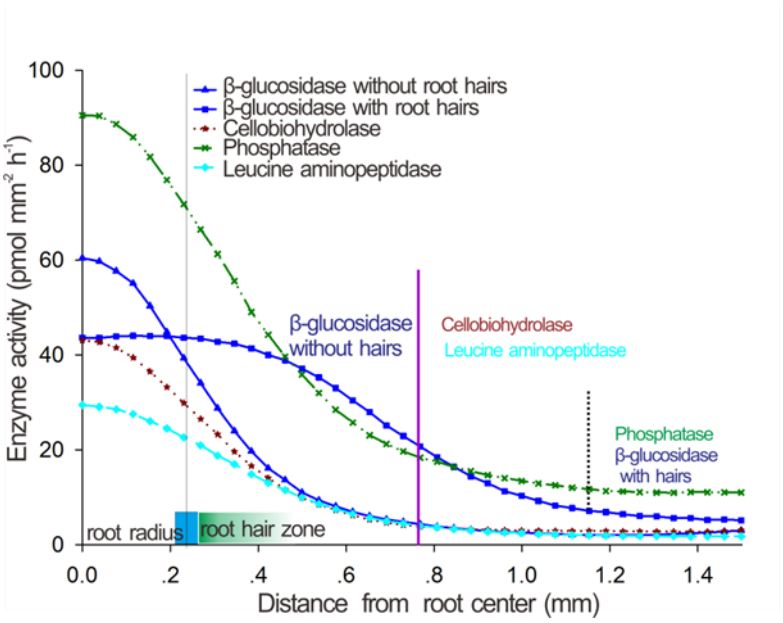

Fig. ES3 Profiles of enzyme activity distribution as a function of the distance from the wheat root center towards the surrounding soil. Vertical graylines vertical: position of

average root radius. Blue shading: standard deviation of root radius. Vertical lines on the curves: rhizosphere extent for individual enzymes. Green shading: root hair zone (gradual fading means the boundary of root hairs is not sharp). Each line refers to the mean value of six roots. Error bars of enzyme activities are omitted to improve visualization; the standard errors were always less than 10% of the activity values.

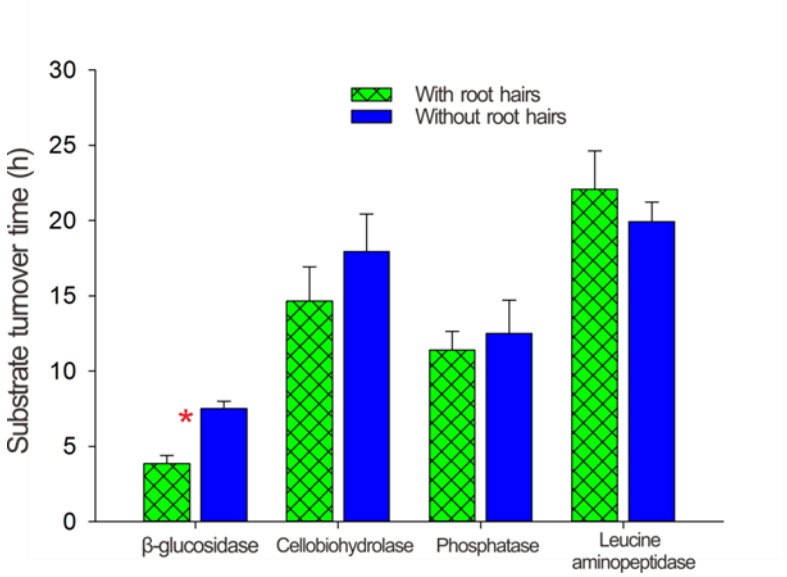

Fig. ES4 Substrate turnover time for βglucosidase, cellobiohydrolase, phosphatase and leucine aminopeptidase in the rhizosphere of wheat with and without root hairs. Bars:means of four replicates (±SE). Asterisks: significant differences ($p < .05$ after Duncan test) between regions with and without root hairs.

The rhizosphere extent of β-glucosidase activity in wheat root regions with hairs was 1.5-fold broader than in root regions without hairs. With the exception of leucine aminopeptidase, we observed a tendency of shorter substrate turnover time in wheat root regions with versus without root hairs for all tested enzymes. In the case of β-glucosidase, the substrate turnover in the root hair region was 2 times faster than in the hairless root zone ($p < .05$). Root hairs modulate the chemical properties and composition of the rhizosphere (Datta et al., 2011) by releasing large quantities of exudates and other rhizodeposits (Czarnota et al., 2003; Nguyen, 2003; Jones et al., 2009). Abundant exudates released by root hairs are quickly consumed by fast-growing microorganisms and increase their activities (Blagodatskaya et al., 2009; Chen et al., 2014). These active microbes produce more enzymes that can decompose

rhizodeposits, which are abundant in the root-hair zone due to the very short lifespan of tiny hairs (Kuzyakov and Xu, 2013), and further influences enzyme dynamics such as substrate turnover. This is explanation confirmed by the faster substrates turnover of β-glucosidase, cellobiohyrolase and acid phosphatase in root regions with hairs than without hairs (Fig. ES4).

1.4.2.2 Rhizosphere extent and enzyme activity in the rhizosphere depend on root radius, dense and length of root hairs

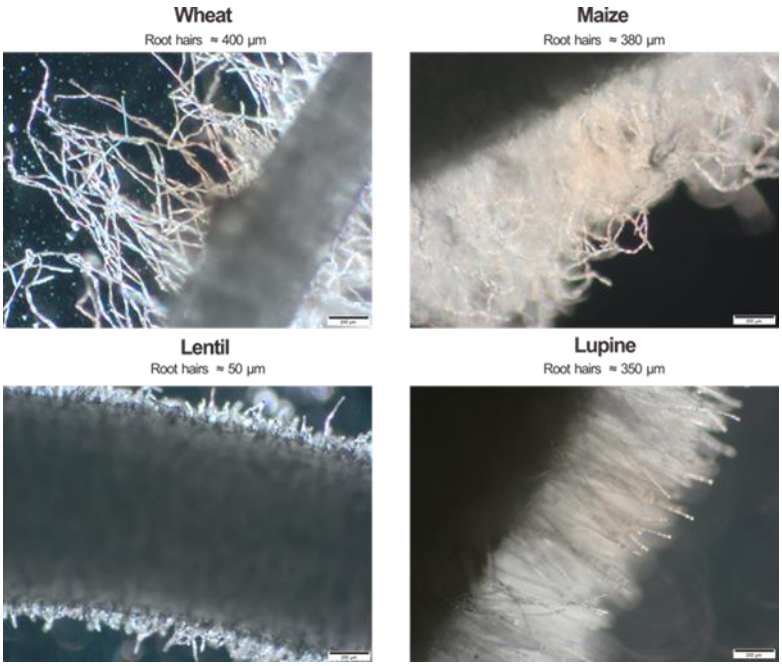

Fig. ES5 Microscopy images of root hairs of lentil, lupine, wheat and maize germinated on filter paper. Bar on bottom right: 200 µm. Average root hair length indicated above the image.

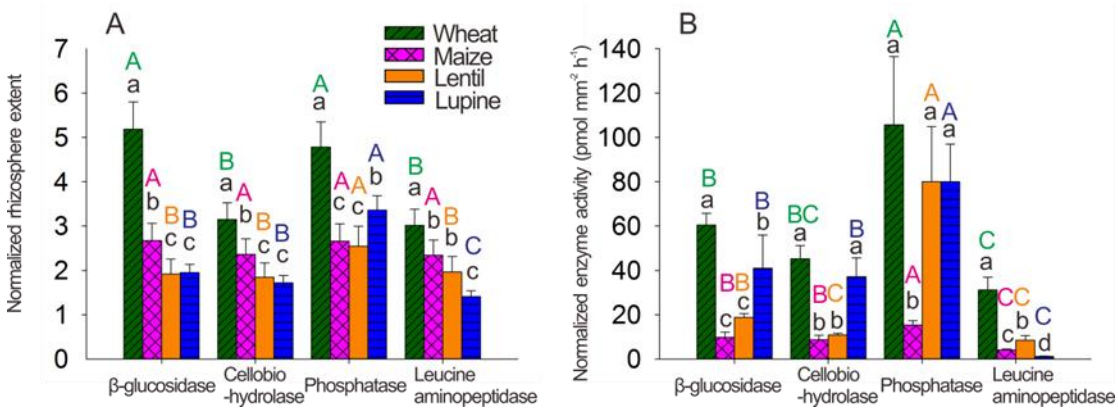

Fig. ES6 Normalized rhizosphere extent (extent of rhizosphere divided by root radius) (A); normalized enzyme activity (sum of the activity of an individual enzyme in the rhizosphere divided the root surface area) (B). Capital letters in colors (same color indicates one plant species): significant differences ($p < .05$ after Duncan test) between enzymes. Small letters black indicate significant differences ($p < .05$ after Duncan test) between plants species

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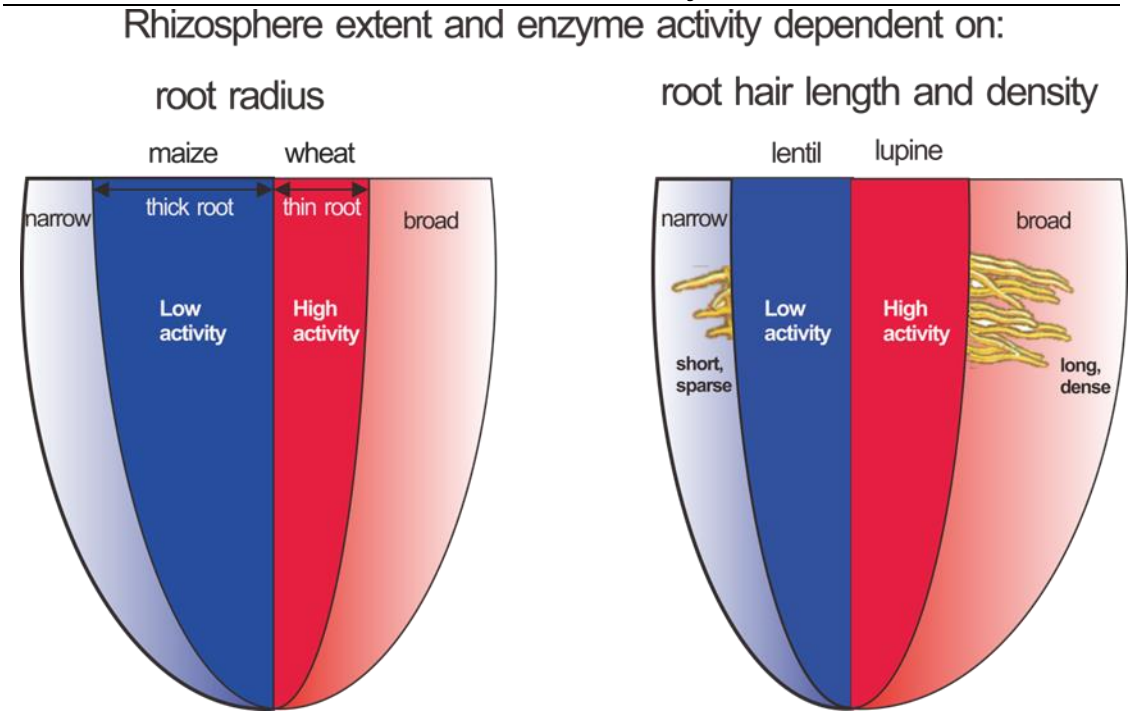

Fig. ES7 The extent and the total enzyme activity in the rhizosphere are strongly affected by the root size. To exclude the effect of root size, the rhizosphere extent was normalized by the root radius (rhizosphere extent relative to root radius); the total activity of an individual enzyme was normalized by the root surface area (enzyme activity per root area), and is referred to hereafter as the normalized rhizosphere extent and normalized enzyme activity.

The normalized rhizosphere extent and normalized enzyme activity in wheat (thin roots) was significantly higher than in maize (thick roots) (Fig. ES5 and 6). For example, the normalized rhizosphere extents of β-glucosidase and phosphatase in wheat were nearly 2 times broader than in maize ($p < .05$) (Fig. ES6A), and the normalized leucine aminopeptidase activity in wheat was 7 times higher than that in maize ($p < .05$) (Fig. ES6B). Accordingly, thin roots are more efficient in determining the rhizosphere extent and the enzyme activity per area than the thick roots, confirming that fine roots acquire nutrient better than coarse roots (Gambetta et al., 2013; McCormack et al., 2015)

However, the normalized extent of acid phosphatase in lupine (thick) was 1.5 times broader than in lentil (thin roots) ($p < .05$) (Fig. ES6A). Similarly, the normalized

enzyme activity of β-glucosidase and cellobiohydrolase in lupine was 2-3 times higher than in lentil ($p < .05$) (Fig. ES6B). This contradiction is explained by considering the length and density of root hairs in both plants. Lupine has long and dense root hairs as compared to the short and sparse hairs of lentil (Fig. ES5). This contrasting length and density of root hairs will affect the quantities of C released (Nguyen, 2003). Moreover, root hair length and density directly and indirectly affect the enzyme release to facilitate nutrient mineralization (Brechenmacher et al., 2009; Libault et al., 2010). In summary, the rhizosphere extent relative to root radius was broader and enzyme activity per surface area was higher around thin roots (wheat) than around thick roots (maize); roots with long and dense hairs (lupine) have a higher enzyme activity per root area and a broader rhizosphere extent relative to root radius than roots with short and sparse hairs (lentil) (Fig. ES7).

1.4.2.3 Rhizosphere volume and enzyme activities around lateral and taproots of lupine

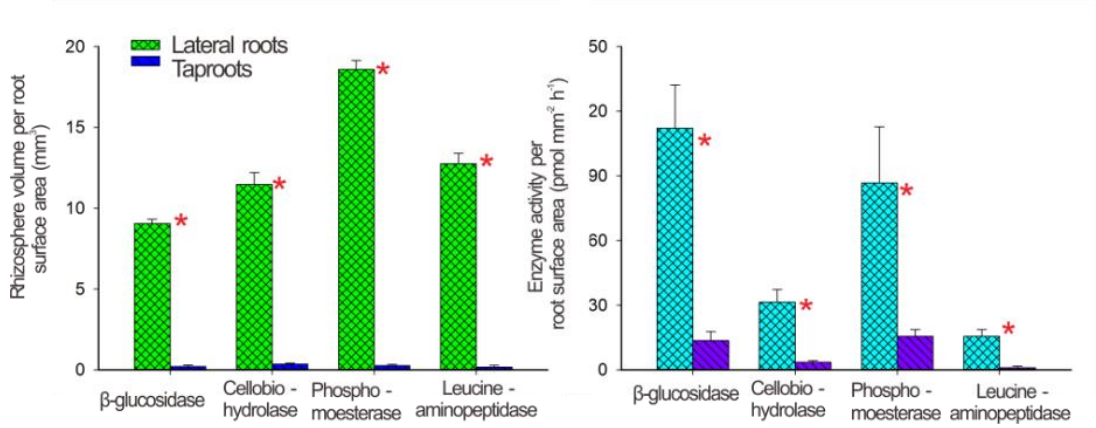

Fig. ES8 Rhizosphere volume per root surface area, which was calculated as the rhizosphere volume divided by the root surface area; enzyme activity per root surface area, which was calculated by summing all activity in the rhizosphere and dividing by the root surface area. Bars: means calculated from four replicates (±SE). Red asterisks: significant differences ($p < .05$, Duncan test) between lateral roots and taproots.

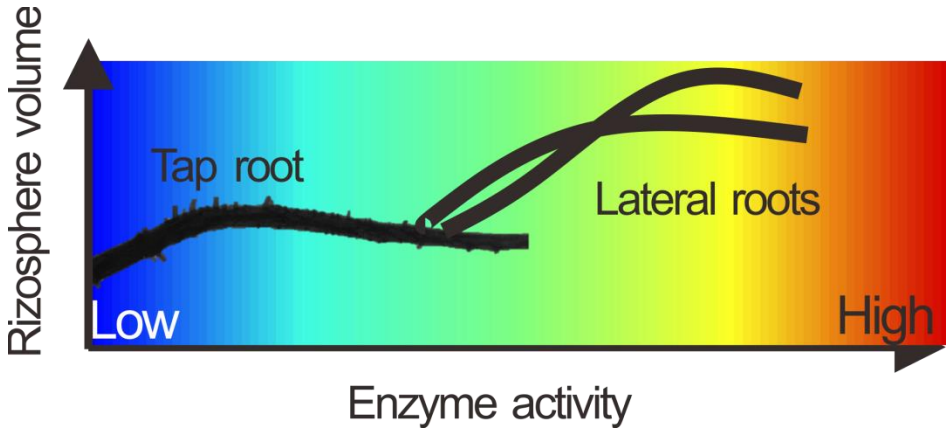

Fig. ES9 The enzyme activity (per root surface area) was higher and the rhizosphere volume was broader around lateral roots than around older taproots.

The rhizosphere volumes per root length and enzyme activity per root surface area in lateral roots were respectively 30-70 times and 6-14 higher roots than in taproots (Fig. ES8). Thus, lateral roots and its surrounding microorganism have higher enzyme synthesis and lateral roots have higher rhizosphere volume efficiency than taproots do. This can be related to the changes in root anatomy: when roots become old, cork periderm will develop, cell walls thicken, and suberin will be deposited (McCormack et al., 2015). All these factors reduce the uptake of water and ions as well as exudate release (Steudle and Peterson, 1998; Gambetta et al., 2013) and so, narrow the rhizosphere extent. In contrast, young lateral roots with smaller diameter have high exudation rates (McCormack et al., 2015), which can stimulate microbial activity and enzyme production (Asmar et al., 1994). Therefore, lateral roots have much higher enzyme activity per root surface area and larger rhizosphere volume per root length compared to taproots (Fig. ES9).

1.4.2.4 Rhizosphere extent and dynamics of enzyme activities around the roots of lentil and lupine depend on growth stage

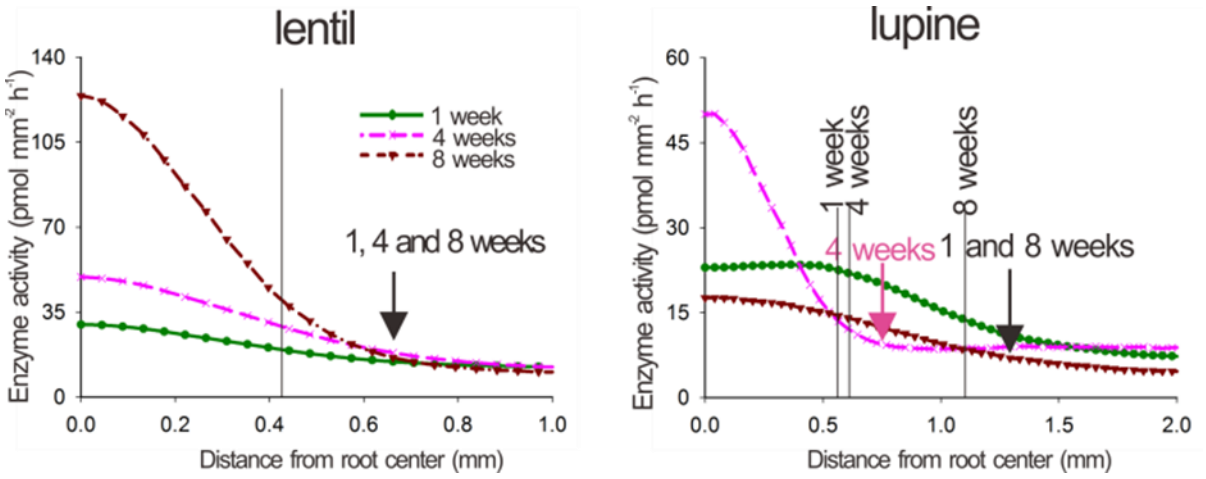

Fig. ES10 Acid phosphomonoesterase activity as a function of distance from root center for 1, 4 and 8-week -old lentil and lupine. Each line refers to the mean activity around six roots. Vertical gray lines indicate the average root radius. Small vertical arrows show the development of rhizosphere size overtime. Error bars of enzyme activities are omitted to improve visualization, but the standard errors are all times less than 10% of the activity values.

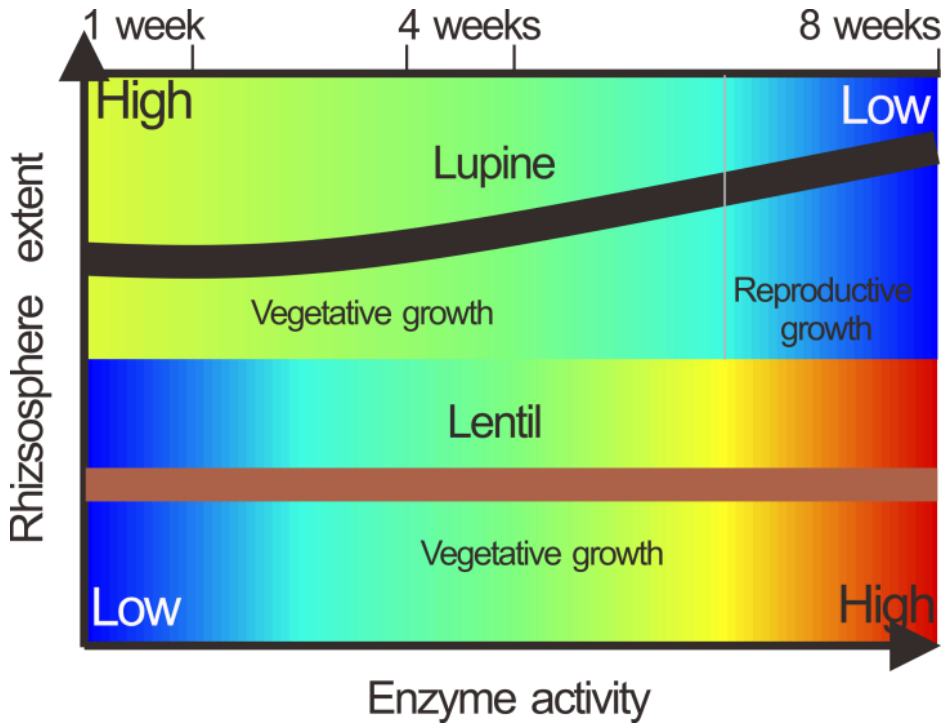

Fig. ES11 During vegetative growth of lentil the rhizosphere extent remained constant, while enzyme activities on the root surface increased. Lupine entered reproductive growth in the 7th week, which was accompanied by broader rhizosphere extent and decreased enzyme activity around the roots.

Lentil kept as vegetative growth and the rhizosphere extent was constant, while the enzyme activities at the root surface kept increasing (Fig. ES10). The spatial stability of the rhizosphere extent reflects the equilibrium between enzyme input (synthesis by roots and microorganisms) and output (decomposition, turnover and other deactivation processes) of both extracellular and intracellular (Miralles et al., 2012; Schimel et al., 2017; Nannipieri et al., 2018). Rhizosphere microorganisms can be mutualistic as well as competitive with plant (Richardson et al., 2009). This stable pattern of rhizosphere extent is an efficient strategy for plants to mobilize and acquire nutrients in competition with rhizosphere microorganisms by enhancing available nutrient acquisitions (Kuzyakov and Xu, 2013). Lupine entered reproductive growth in the 7th week after planting; the rhizosphere extent was broader in the 8th week than in 1st and 4th weeks. However, enzyme activity at the root surface of lupine decreased

by 10-50% in comparison to the preceding vegetative stage (1st and 4th weeks). The stable rhizosphere extent lasted longer for lentil than for lupine: for lentil it was constant during the whole period, but for lupine it broadened in the 8th week (Fig. ES10). This is directly related to the root radius: root radius of lentil remained constant, while the radius of lupine roots increased with age. The larger the radius, the more exudates will be released into the soil (Lambers et al., 2006), and therefore a broader rhizosphere develops around thicker roots (lupine). Moreover, enzyme activity in the rhizosphere depends on plant age and physiological state. Root exudation increases during vegetative growth, but it decreases when reproductive growth starts (Aulakh et al., 2001; De-la-Peña et al., 2010). This reduces the abundance of active microorganisms (Chaparro et al., 2014; Schmidt and Eickhorst, 2014) and consequently leads to a down-regulation of enzyme production (Allison et al., 2010). Therefore, the enzyme activity in the rhizosphere increased with plant growth until reproductive stage (Fig. ES11).

1.4.3 Abiotic factors (phosphorus availability and temperature) on the spatial distribution and temporal dynamic of enzyme activity

1.4.3.1 *Sptial and temporal dynamics of rhizosphere extent in the taproot zone depends on phosphorus availability and cluster root formation*

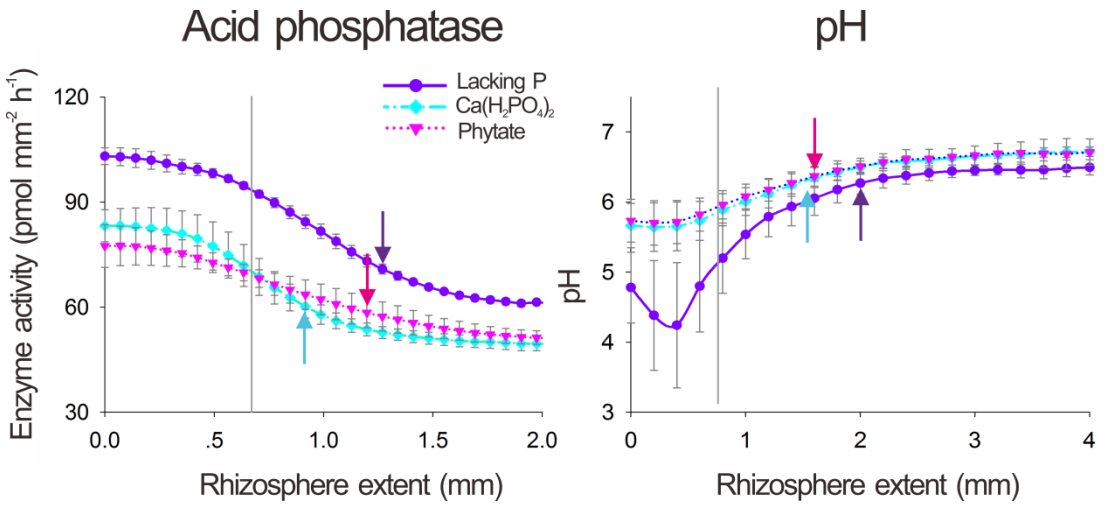

Fig. ES12 Acid phosphatase and pH as a function of distance from taproot center of 11 day-old lupine (before cluster root formation) grown in soil lacking P, amended

$Ca(H_2PO_4)_2$ or with phytate, respectively. Vertical gray lines indicate average root radius. Vertical arrows on the curves show rhizosphere extent for enzyme activity or pH. Data points indicate means calculated from four replicates (±SE).

The rhizosphere extent of acid phosphatase and pH around taproot were 0.35-0.52 mm broader for lupine grown in soil under P deficiency than in soil added with $Ca(H_2PO_4)_2$ before cluster roots formation (11^{th} day) (Fig. ES12). This indicated that before cluster root formation, taproot can release more phosphatase and protons and conversion of P from non-available into available to conquer P scarcity. Under P deficiency, roots exude a wide range of organic acid anions to keep P mobile by binding bivalent cations via ligand exchange (Neumann and Römheld, 1999; Rengel, 2002). In order to maintain charge balance, protons should be excreted in exchange to cations and so, acidify the rhizosphere (Hinsinger, 2001; Hinsinger et al., 2003), which further enhances Ca-phosphates solubilization (Neumann et al., 2000). Moreover, P deficiency inhibits nitrate but enhances ammonium uptake, leading even to a higher H+ release and thus promoting Ca-phosphates dissolution (Neumann et al., 1999).

Besides rhizosphere soil acidification, roots can enhance the availability of organic P forms either directly by a higher release of acid phosphatase and indirectly through higher root exudation and other rhizodeposition, which further stimulates microbial activity and the production of microbial phosphatases to mineralize organic-P (Jones et al., 2009).

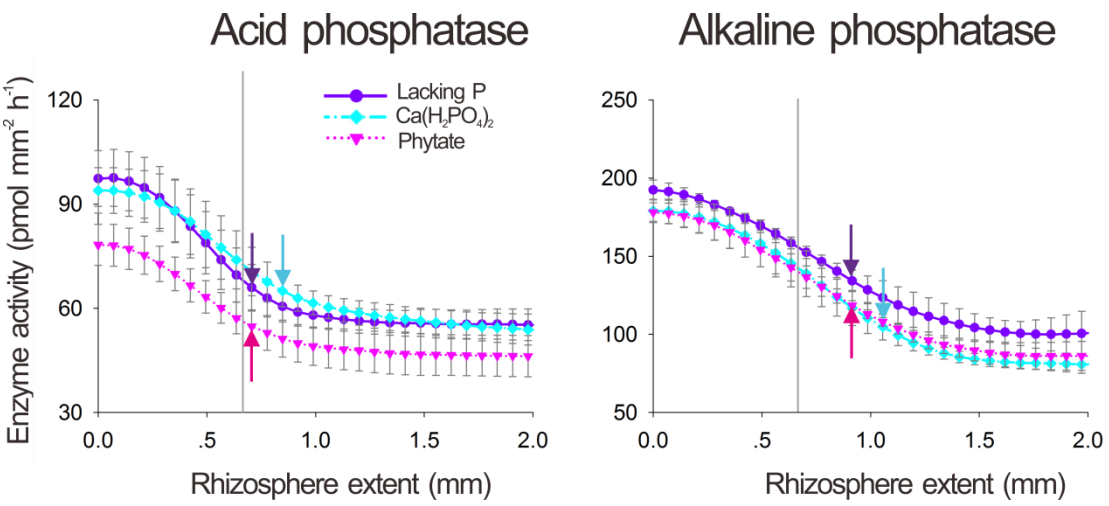

Fig. ES13 Acid and alkaline phosphatase as a function of distance from taproot center of 24 day-old lupine (after cluster root formation) grown in soil lacking P, amended $Ca(H_2PO_4)_2$ or with phytate, respectively. Vertical gray lines indicate average root radius. Vertical arrows on the curves show rhizosphere extent for enzyme activity. Data points indicate means calculated from four replicates (±SE).

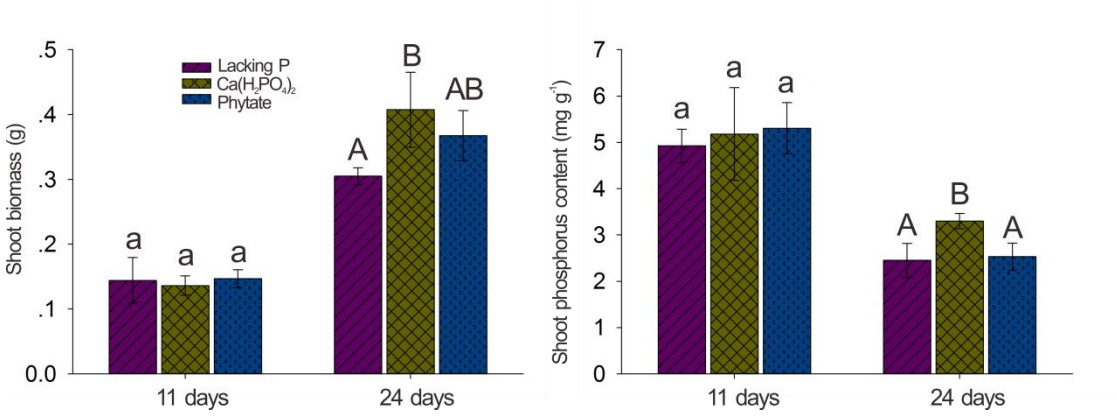

Fig. ES14 shoot biomass (C) and shoot P content (D) of 11 and 24-old day (before and after cluster root formation) lupine grown in soil lacking P, amended with $Ca(H_2PO_4)_2$ or with phytate, respectively. Small and capital letters indicates significant differences ($p < 0.05$ after Duncan test) of these parameters between treatments on 11 and 24 days, respectively.

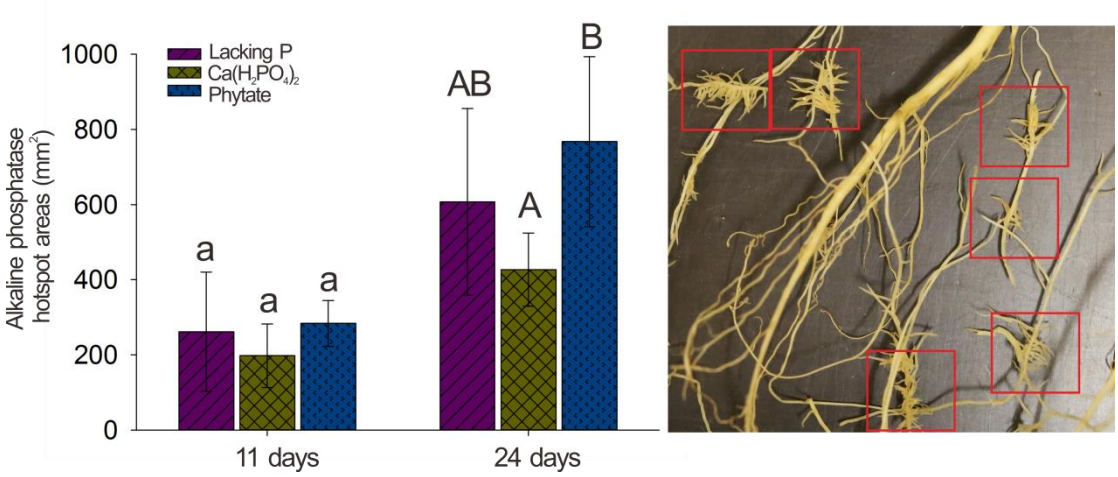

Fig. ES15 Alkaline phosphatase activity hotspot area of 11 and 24-old day lupine grown in soil lacking P, amended with $Ca(H_2PO_4)_2$ or with phytate, respectively. Small and capital letters indicates significant differences ($p < .05$ after Duncan test) of these parameters between treatments on 11 and 24 days, respectively. The roots of lupine grown in soil under phosphorus deficiency. Red rectangles show the cluster roots.

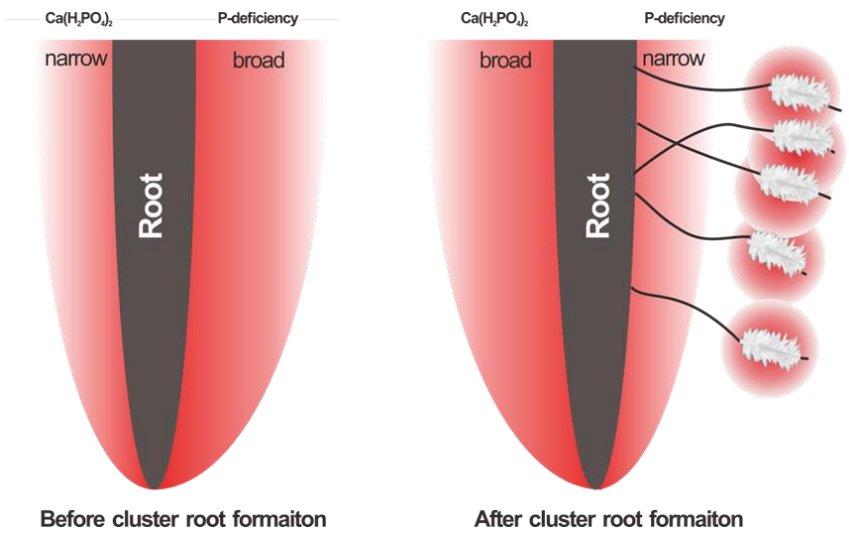

Fig. ES16 Spatial distribution of pH and phosphatase activity in the rhizosphere of lupine in response to P availability. before and after cluster root formation. The red show indicated the rhizosphere extent of pH or phosphatase activity.

In contrast, the rhizosphere extent of both acid and alkaline phosphatase around taproot for lupine grown in soil amended with phytate or absent of P were about 0.2 mm narrower than amended with $Ca(H_2PO_4)_2$ after cluster roots formation (24^{th} day) (Fig. ES13). This is an inverse effect compared to observation before cluster root formation. This phenomenon can be explained by two mechanisms. Firstly, the endogenous P from seed will be exhausted as plant growing. Consequently longtime exposure to P deficiency environment can impede plant photosynthesis and decrease exudation, which will inhibit microbial and enzyme activity (Schimel and Weintraub, 2003). In contrast, adequate P supply stimulated plant growth and the carbon allocation belowground (Kuzyakov and Cheng, 2001). Therefore, amended with $Ca(H_2PO_4)_2$ more exudates will be released, which can stimulate microbial growth and enzyme synthesis (Kuzyakov et al., 2002; Nannipieri et al., 2007). This explanation can be confirmed by higher shoot P content and larger shoot biomass for lupine grown in soil amended with $Ca(H_2PO_4)_2$ than in P-deficient soil (Fig. ES14). Secondly, the root morphology was altered under P-deficiency, lupine invested energy to the cluster root formation rather than to the taproots exudation. This explanation supported by the larger phosphatase activity hotspot area under P-deficiency on 24^{th} day, which mainly attributed to the cluster root formation (Fig. ES15). Therefore,

lupine used various strategies to conquer P deficiency during growth: increased phosphatase activity and decreased pH, both with a large rhizosphere extent around taproot, ensure co-mobilization of organic as well as mineral P along taproot (Fig. ES16). After cluster root development, the plant clearly allocates its main resource invested for P mobilization towards the cluster roots to explore a maximal large soil volume (Fig. ES16)

1.4.3.2 Enzyme kinetics and hotspot areas response to temperature

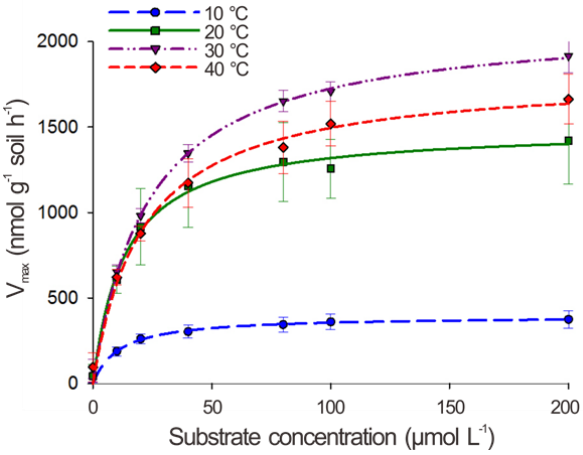

Fig. ES17 Michaelis-Menten kinetics (enzyme activity as a function of substrate concentration) for cellobiohydrolase in response to increasing temperature: 10, 20, 30, 40 ℃. Values are means of four replicates (±SE).

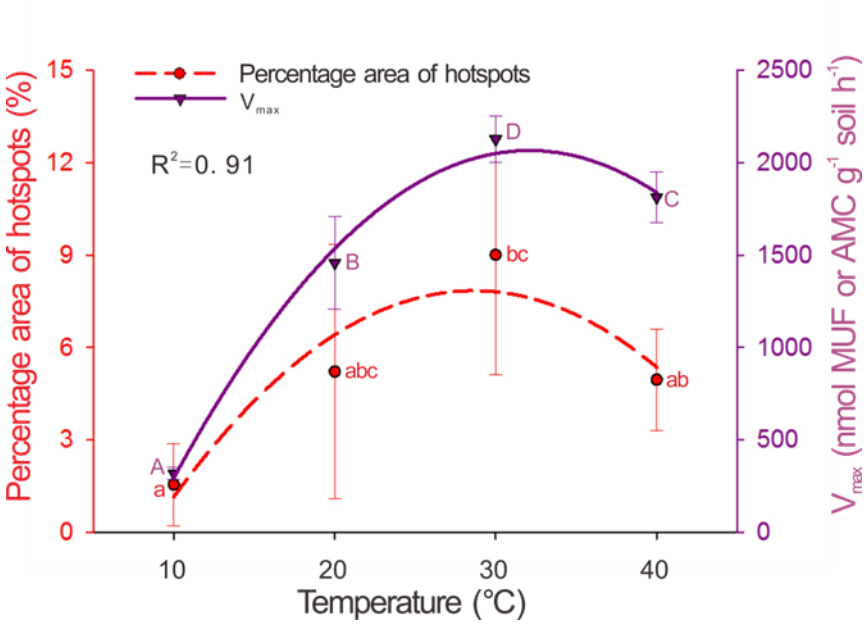

Fig. ES18 Percentage area of hotspots (with 25% highest activity) for cellobiohydrolase in the root-detritusphere (14days after cutting shoots) depending on temperature (10, 20, 30, 40 ℃) (red dashed lines). V_{max} values of the three enzymes in the Michaelis-Menten equation (purple line). Data points indicate means of four replicates (±SE). The differences of percentage area of hotspotsand V_{max} at four temperatures were tested by ANOVA followed by the Duncan-test ($p < 0.05$). Letters show significant differences between temperatures. The R^2 values are coefficients of determination of V_{max} and hotspot areas.

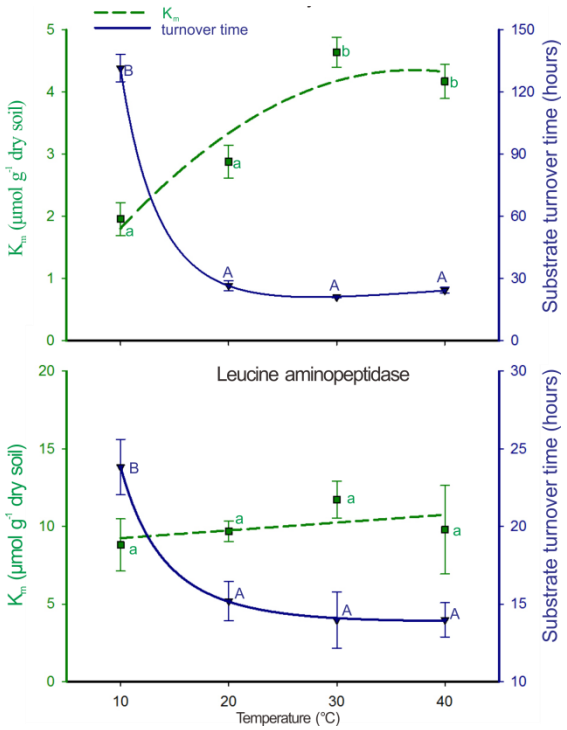

Fig. ES19 K_m values (green dashed lines) and substrate turnover time (blue lines) of cellobiohydrolase and leucine aminopeptidase at four temperatures (10, 20, 30, 40 ℃). Values are means of four replicates (±SE). The differences of K_m and substrate turnover time at four temperatures were tested by ANOVA followed by the Duncan-test ($p < .05$). Letters show significant differences between temperatures.

V_{max} and hotspot areas showed the same trend regarding temperature and they were increased with temperature from 10 to 30 ℃, however, they significantly decreased at 40 ℃ compared with at 30 ℃ (Fig. ES17 and 18). This indicated that the more abundant the enzymes, the larger the areas that will be required and occupied in the soil profile. The decreases of Vmax and hotspot area at 40 ℃ are due to enzyme activity responds positively up to an optimum temperature, beyond which enzymes start to denature (Berry and Raison, 1981; Atkin and Tjoelker, 2003)}. The K_m values of cellobiohydrolase were significantly higher at 30 and 40 ℃ than at 10 and 20 ℃ (Fig. ES19); in contrast, the K_m values of leucine-aminopeptidase were nearly constant over the whole temperature range (Fig. ES19), indicating the temperature response pattern of K_m was enzyme-specific.

1.4.3.2 Warming increases hotspot areas of enzyme activity and shortens the duration of hot moments

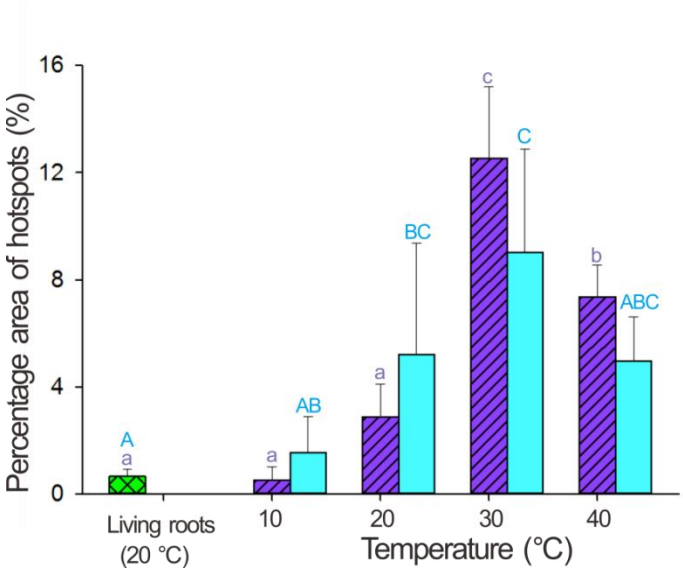

Fig.ES20 Hotspots as a percentage of total area for cellobiohydrolase in the rhizosphere of living roots and root-detritusphere (7 and 14 days after cutting shoots) at four temperatures (10, 20, 30, 40 ℃). Bars: means calculated from four replicates (±SE). Small letters: significant differences ($p < 0.05$ 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.

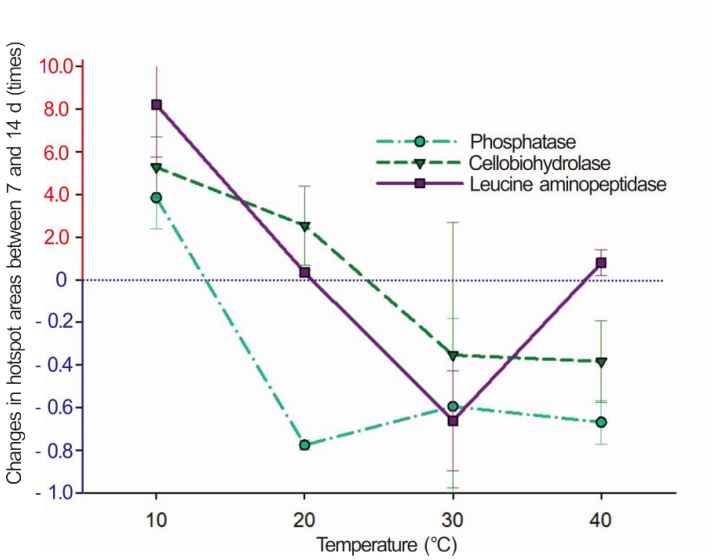

Fig. ES21 Changes in enzyme hotspot (with 25% highest activity, see Fig. 1.) areas between 7 and 14 days after cutting of shoots for phosphatase, cellobiohydrolase and leucine aminopeptidase at four temperatures (10, 20, 30, 40 ℃). Positive values show increased, whereas, negative values show decreased hotspot areas at 14 days versus 7 days after cutting. Data points indicate means calculated from four replicates (±SE).

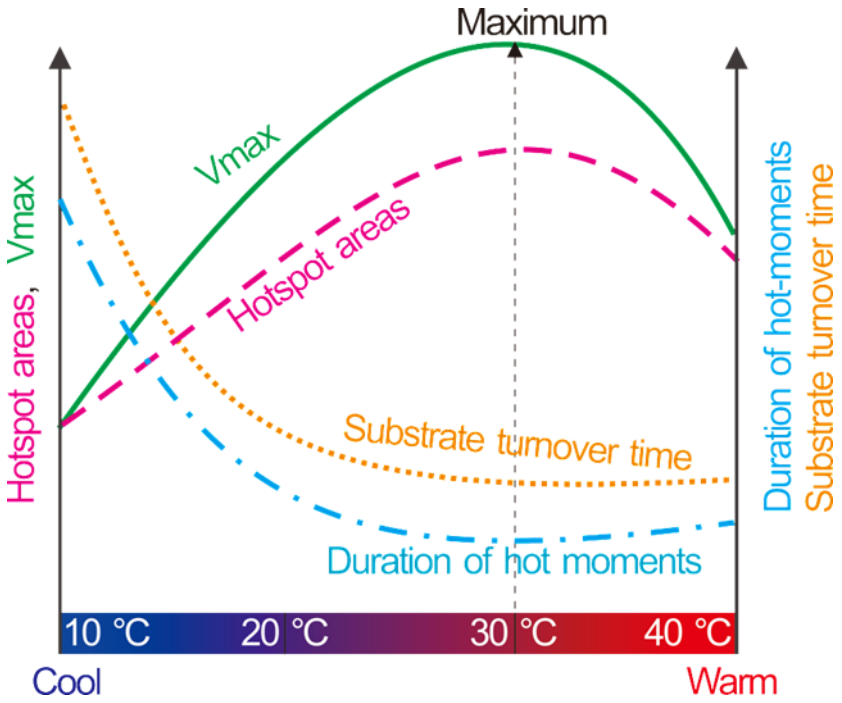

Fig. ES22 Temperature effects on hotspot areas of enzyme activity, maximum enzyme activities (Vmax), substrate turnover time and the duration of hot moments.

From 10 to 30 ℃, the hotspot areas increased by 2-24 times (Fig. ES20). Such increments were due to faster organic matter decomposition (Wallenstein et al., 2009; Wallenstein et al., 2011), 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 for enzymes at 10 ℃ increased by 3.8-8.2 times in the root-detritusphere 14 days versus 7 days after cutting (Fig. ES21). The hotspot areas of cellobiohydrolase and leucineaminopeptidase at 20 ℃ 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 ℃ (except leucine aminopeptidase at 40 ℃) 14 days compared to 7 days after cutting (Fig. ES21). The

decreased of hotspot areas at warm temperatures (30 and 40 ℃), indicating 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., 2011). 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. ES19). 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 down regulation of enzyme production (Schimel and Weintraub, 2003; Knorr et al., 2005; Allison et al., 2010). Therefore, we conclude that warming increases hotspot areas of enzyme activity, Vmax, facilities the enzyme substrate turnover and shortens the duration of hot moments in the root-detritusphere (Fig. ES22).

1.5 Conclusions

1) The spatial distribution of enzyme activity depend on biotic factors (root morphology, architectures and plant development), namely

- The exist of root hairs broadened the rhizosphere extent and facilitated substrate turnover
- The rhizosphere extent relative to root radius was broader and enzyme activity per surface area was higher around thin roots (wheat) than around thick roots (maize); roots with long and dense hairs (lupine) have a higher enzyme activity per root area and a broader rhizosphere extent relative to root radius than roots with short and sparse hairs (lentil).
- Lateral roots have much higher enzyme activity per root surface area and larger rhizosphere volume per root length area compared to taproots.
- The enzyme activity in the rhizosphere increased with plant growth until reproductive stage

2) The spatial distribution of enzyme activity depend on abiotic factors (phosphorus availability and temperature), namely

- Lupine used various strategies to conquer P deficiency during growth: increased phosphatase activity, soil acidifcation and broaden their rhizosphere extent around taproot are the mechanisms before cluster root formation. After cluster root development, the main mechanism is increase of hotspot area of phosphatase activity to explore larger soil volume for P acquisition
- Warming increases hotspot areas of enzyme activity and shortens the duration of hot moments

Overall, this thesis developed new concepts and developed numbers of approaches dedicated to investigate the abiotic and biotic effects on spatial distribution, hotspot area formation of soil enzyme activities. The effect of abiotic and biotic controls on spatial distribution of the enzyme hotspots has important consequences not only for

soil science, but also for ecology, plant-soil-microbial interactions, nutrients and element cycles.

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1.7 Contributions to the included manuscripts

The PhD. thesis comprises publications and manuscripts which were elaborated in cooperation with various coauthors. The coauthors listed in these publications and manuscripts contributed as follows:

Study 1 Spatial pattern of enzyme activities in the rhizosphere: Effects of root hairs and root radius

Status: Published in *Soil Biology and Biochemistry* (2018)

Xiaomin Ma	experimental design and execution, data preparation and interpretation, manuscript preparation
Mohsen Zarebanadkouki	data preparation and interpretation; comments to improve the manuscript
Yakov Kuzyakov	discussion of experimental design; comments to improve the manuscript
Evgenia Blagodatskaya	discussion of results, comments to improve the manuscript
Johanna Pausch	discussion of results, comments to improve the manuscript
Bahar S. Razavi	discussion of results, comments to improve the manuscript

Study 2 Spatio-temporal patterns of enzyme activities in the rhizosphere: Effects of plant growth and root morphology

Status: Published in *Biology and fertility of soils* (2018)

Xiaomin Ma	experimental design and execution, data preparation and interpretation, manuscript preparation
Yuan Liu	discussion of results, comments to improve the manuscript
Mohsen Zarebanadkouki	data preparation and interpretation

Bahar S. Razavi	discussion of results, comments to improve the manuscript
Evgenia Blagodatskaya	discussion of results, comments to improve the manuscript
Yakov Kuzyakov	discussion of results, comments to improve the manuscript

Study 3 Coupling of zymography with pH optode reveals changes in P acquisition strategies of white lupine with growth depending on P supply

Status: *in preparation*

Xiaomin Ma	experimental design and execution, data preparation and interpretation, manuscript preparation
Kyle Mason-Jones	experimental discussion, discussion of results, comments to improve the manuscript
Yuan Liu	experimental discussion and execution, discussion of results, comments to improve the manuscript
Evgenia Blagodatskaya	comments to improve the manuscript
Yakov Kuzyakov	comments to improve the manuscript
Michaela A. Dippold	comments to improve the manuscript
Bahar S. Razavi	comments to improve the manuscript

Study 4 Warming increases hotspot areas of enzyme activity and shortens the duration of hot moments in the root-detritusphere

Status: Published in *Soil Biology and Biochemistry* (2017)

Xiaomin Ma	experimental design and execution, data preparation and interpretation, manuscript preparation
Bahar S. Razavi	discussion of results, comments to improve the manuscript

Maire Holz	discussion of results, comments to improve the manuscript
Evgenia Blagodatskaya	discussion of results, comments to improve the manuscript
Yakov Kuzyakov	discussion of results, comments to improve the manuscript

2 Manuscripts

Study 1 Spatial patterns of enzyme activities in the rhizosphere: Effects of root hairs and root radius

Xiaomin Ma [a *], Mohsen Zarebanadkouki [b], Yakov Kuzyakov [a, e], Evgenia Blagodatskaya [a, c *], Johanna Pausch [d], Bahar S. Razavi [a]

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Ma, X., Zarebanadkouki, M., Kuzyakov, Y., Blagodatskaya, E., Pausch, J.,Razavi, B.S., 2018. Spatial patterns of enzyme activities in the rhizosphere: Effects of root hairs and root radius. Soil Biology and Biochemistry, 118, 69-78.

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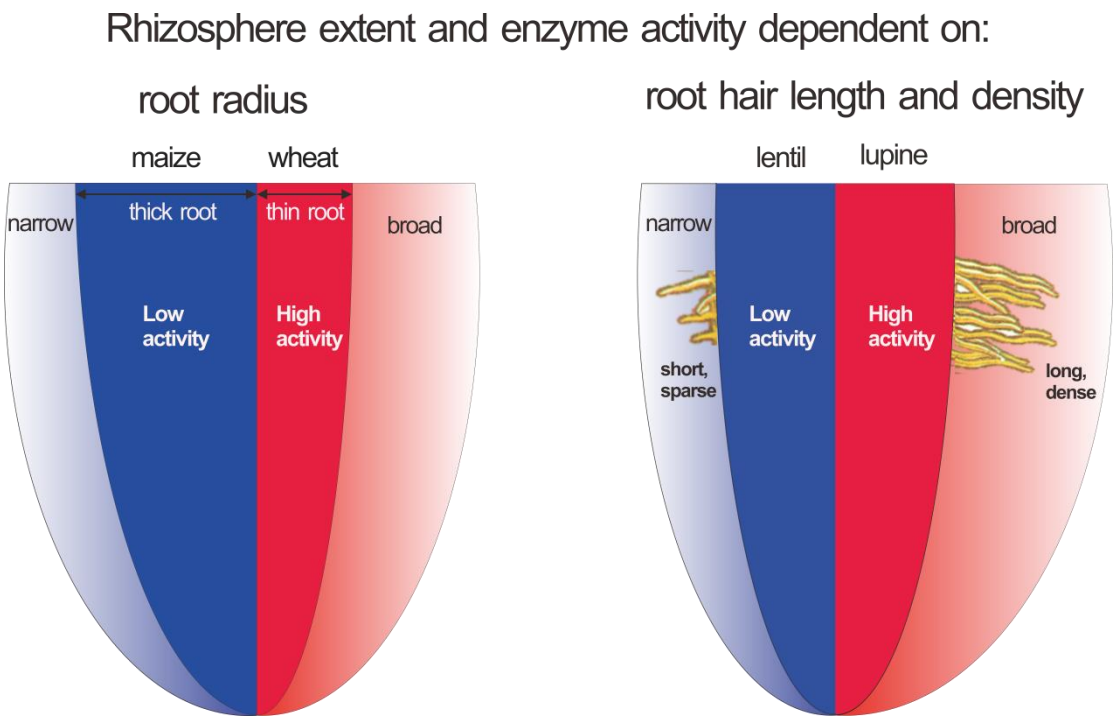
Graphic abstract



Highlights

1. Root hairs broaden the rhizosphere extent and facilitated the substrate turnover
2. The normalized rhizosphere extent was broader in thin than in thick roots
3. The normalized enzyme activity was higher in thin roots than in thick roots
4. Long and dense hairs heighten the normalized enzyme activity
5. Roots with long and dense hairs broaden the normalized rhizosphere extent

Abstract

The importance of root hairs and root radius for exudation and nutrient acquisition by plants is known mainly from nutrient solution studies. The *in situ* effects of root hairs and root radius on the spatial distribution of enzyme activity in the rhizosphere of various plants are unknown. Four plants with contrasting root morphology (maize, wheat, lentil and lupine) were chosen to test the effects of root hairs and root radius on the spatial distribution of β-glucosidase, cellobiohydrolase, leucine aminopeptidase and acid phosphatase. We combined zymography with enzyme kinetics to evaluate the effects of root hairs on the rhizosphere extent and on substrate turnover. The extent of enzyme activity in the rhizosphere of four plants ranged from 0.55 to 2 mm. The extent of β-glucosidase was 1.5 times broader (1.2 mm versus 0.8 mm) and the substrate turnover was 2-fold faster around wheat root regions with hairs than hairless locations. The rhizosphere extent relative to root radius and the enzyme activity per root surface area were plant and enzyme specific: the rhizosphere extent was 1.5-2 times broader and the enzyme activity was 2-8-fold higher in wheat (with thin roots and long root hairs) compared to maize, lentil and lupine. The rhizosphere extent of acid phosphatase (1.1-2.0 mm) was 1.5-2-fold broader than that of other enzymes (0.5-1.0 mm). For the first time, we showed that the rhizosphere extent relative to root radius was 20-100% broader and enzyme activity per surface area was 4-7-fold higher around thin roots (wheat) than around thick roots (maize). Moreover, the rhizosphere extent relative to root radius was 10-30% broader and enzyme activity per root area was 2-7 times higher around roots with long and dense hairs (lupine) than around roots with short and sparse hairs (lentil). We conclude that root hairs and root radius shape the rhizosphere: root hairs contributed mainly to the rhizosphere extent, while root radius more strongly affected the enzyme activity per root surface area.

Key words*: Rhizosphere extent; Enzyme spatial distribution; Zymography; Root hairs; Root radius; Nutrient mobilization*

Introduction

Plant roots exude a very broad range of compounds into the soils (Jones et al., 2009), stimulating microbial and enzyme activities (Parkin, 1993; Asmar et al., 1994) and forming one of the most chemically, physically and biologically complex spheres with very intensive interactions - the rhizosphere (Bertin et al., 2003). The rhizosphere is one of the most important enzymatic hotspots (Kuzyakov and Blagodatskaya, 2015), where extracellular enzymes are produced by both microorganisms and living roots. Moreover, a considerable amount of intracellular enzymes is continuously released into the rhizosphere by lysis and damage of root and microbial cells (Bais et al., 2004).

The production and spatial distribution of enzymes in soils are a dynamic function of microbial and root properties, including microbial physiology (Henry et al., 2005; Allison and Treseder, 2008), root morphology, root exudation and rhizodeposition (Kuzyakov, 2002). Microorganisms synthesize and release enzymes largely as a nutrient acquisition strategy and roots may express more enzymes when nutrients are scarce (Harder and Dijkhuizen, 1983). The release of labile rhizodeposits varies spatially and temporally and depends on plant physiology and root morphology (Nguyen, 2003). Furthermore, the quality and composition of exudates vary between plant species, cultivars, over plant development and even along the root segments (Aulakh et al., 2001; Badri and Vivanco, 2009). Accordingly, the enzymes decomposing various components of rhizodeposits and SOM change in quantity and quality over time and between plant species. Thus, enzyme activity, reflecting microbial and roots activities, is also heterogeneously distributed in soil (Grierson and Comerford, 2000; Wallenstein and Weintraub, 2008).

Nutrients are very often limited in soils (Hodge, 2004), and this limitation is extremely strong in the rhizosphere because microorganisms and plants compete for the same nutrients (Kuzyakov and Xu, 2013). Plants use root morphological strategies to overcome nutrient limitation, such as the development of the roots with large surface area and long length (Jungk, 2001; Ma et al., 2001). Among these, root hairs, the tubular-shaped outgrowths from root epidermal cells (Peterson and Farquhar, 1996), strongly increase the root surface area, and play important roles in nutrient and water acquisition as well as in the interactions with microbes (Gilroy and Jones, 2000).

Apart from these functions, root hairs are also essential in modulating the properties and composition of the rhizosphere through exudation, and, in some species, exudates are apparently produced solely by root hairs (Czarnota et al., 2003 ; Datta et al., 2011). Root hairs have a short lifespan (at most a few days), and dead root hairs therefore released abundant C into the soil (Nguyen, 2003). These large amounts of labile carbon and other rhizodeposits released by root hairs stimulate microbial activity (Parkin, 1993; Asmar et al., 1994) and further influences enzyme dynamics such as accelerated substrate turnover. Moreover, root hairs actively participate in the interactions between plants and nitrogen-fixing microorganisms and symbiotic mycorrhizal fungi by providing nutrients, hormones and signaling molecules (Peterson and Farquhar, 1996; Libault et al., 2010). Root hairs, however, vary highly in number, length, density and longevity, depending on both the genetic potential of plants and on environmental conditions (Jungk, 2001). It is widely accepted that exudation rates and nutrient acquisition capacities are positively correlated with root hair length and density (Yan et al., 2004).

Root radius is another root morphology parameter that influences exudation and nutrient acquisition. Thin and thick roots have distinctive nutrient absorption strategies (Kong et al., 2016). Exudation quantity and nutrient absorption ability are proportional to the root radius (Lambers et al., 2006). Therefore, the spatial pattern and dynamics of enzyme activities might be influenced by root hairs and root radius, dependent on root exudation, rhizodeposition and root hair morphology.

The role of root hairs and root radius and their interactive effects on the spatial distribution of enzymes and their *in situ* activity are completely unknown. Specifically, the effects of root hairs on the rhizosphere extent of enzyme activity and on the enzyme mediated turnover of various substrates in the rhizosphere remain unclear. The rhizosphere extent of enzyme activities can be estimated by a novel approach: zymography, which enables visualizing the spatial distribution of enzyme activities in the soil and rhizosphere (Spohn et al., 2013; Sanaullah et al., 2016). The estimation of enzyme-mediated substrate turnover (T_t) can be calculated based on enzyme kinetics considering the parameters V_{max} (maximum reaction rate) and K_m (half-saturation constant) in the Michaelis-Menten equation (Michaelis and Menten, 1913; Tischer et al., 2015).

We used zymography to visualize the spatial distributions of β-glucosidase, cellobiohydrolase, leucine aminopeptidase and acid phosphatase, which are responsible for C, N, and P cycles in the rhizosphere of four plants with contrasting root morphology. We combined zymography with enzyme kinetics to test the effect of root hairs on the spatial distribution of the rhizosphere and on substrate turnover. Maize (*Zea mays*) and wheat (*Triticum aestivum*) are gramineous plants with fibrous root system, with contrasting root morphologies: while both have long root hairs, maize roots are thicker than wheat roots. Lentil (*Lens culinaris*) and lupine (*Lupinus polyphyllus*) are members of the *Fabaceae*; both have a tap-root system and are nitrogen-fixing legume crops (Dinkelaker et al., 1989; Tovar, 1996). Both also have contrasting root morphology: the roots of lentil are thin with short hairs, whereas the roots of lupine are thicker with longer root hairs. All selected plants are key agricultural crops for food and fodder production and can be grown on a broad range of soils. We hypothesized that 1) due to intensive root exudation by root hairs, the rhizosphere extent of enzyme activities is broader in root regions with hairs than without hairs, 2) root hairs influence enzyme dynamics and lead to accelerated substrate turnover in the rhizosphere, and 3) the spatial pattern of enzyme activity is plant species- and enzyme- specific and depends on root morphology (root hairs and root radius).

2 Material and methods

2.1. Soil and plant 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 Gottingen, Germany. The soil was then passed through a sieve with a mesh radius of 2 mm. The soil had the following 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 rhizoboxes with inner dimensions of 21.2 × 10.8 × 3.3 cm were filled with soil to a final density of 1.2 g cm^3.

Maize (*Zea mays*), wheat (*Triticum aestivum*), lupine (*Lupinus polyphyllus*) and lentil (*Lens culinaris*) seeds were germinated on filter paper for 72 h and thereafter one seedling was planted in a depth of 5 mm in each rhizobox. Each species had 6 replications in separate rhizoboxes. The rhizoboxes were kept in a climate chamber at a controlled temperature of 20 ± 1 ℃ and a daily light period of 14 h with a photosynthetically active radiation intensity of 250 μmol m^{-2} s^{-1}. During the growth period, the rhizoboxes were kept inclined at an angle of 45 ° so that the roots grew along the lower wall of the rhizobox. The rhizoboxes were irrigated with distilled water to maintain water content at 60% of the water holding capacity.

2.2. Direct soil zymography

When plants were one week old, direct zymography (Sanaullah et al., 2016) was used to visualize the activity of four enzymes in the rhizosphere. Thin polyamide membrane filters (Tao Yuan, China) with a size of 20 × 10.8 cm and a pore size of 0.45 mm were saturated with the following substrates: 1) 4-methylumbelliferyl-β-D-glucoside to detect β-glucosidase activity, 2) 4-methylumbelliferyl-β-D-cellobioside to detect cellobiohydrolase activity, 3) 4-methylumbelliferyl-phosphate to detect acid phosphatase activity, and 4) L-leucine-7-amido-4-methylcoumarin hydrochloride to detect leucine-aminopeptidase activity (Koch et al., 2007; Razavi et al., 2015). Each of these substrates was dissolved to a concentration of 12 mM in buffers, MES buffer for 4-methylumbelliferyl (MUF) based substrate and TRIZMA buffer for 7-amido-4-methylcoumarin (AMC) based substrate. All substrates and chemicals were purchased from Sigma Aldrich (Germany). Under UV-light, the MUF and AMC become fluorescent when the respective specific enzyme hydrolyzes the substrate. 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 β-glucosidase, secondly acid phosphatase, thirdly cellobiohydrolase and lastly leucine-aminopeptidase activity were measured. This order was maintained throughout the experiments. After incubation for 1 h, the membranes were carefully lifted off the soil surface and any attached soil particles were gently removed using tweezers and a soft brush (Razavi et

al., 2016). The time interval between each measurement (different enzymes) was 1 hour. Based on preliminary tests we considered the residue fluorescence of the previous enzyme to be negligible (Ma et al., 2017). The membranes were placed under ultraviolet (UV) illumination with an excitation wavelength of 355 nm in a dark room. The camera (EOS 5D, Canon), the sample, and the distance between the UV light were fixed, and a photograph of the membrane was taken.

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 mM). 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 (Spohn and Kuzyakov, 2014). The membranes used for calibration were imaged under UV light in the same way as described for the rhizoboxes.

2.3. Image processing and analysis

Images were processed in 4 steps: 1) transformation of projected signal (fluorescence) on the images to grayvalues, 2) background adjustment, 3) root segmentation, root skeletonization and root radius calculation, and 4) conversion of grayvalues to enzyme activity.

Fluorescence on the zymograms under UV light shows the areas of enzyme activities, and the intensity of fluorescence is proportional to the activity of the enzyme. To obtain quantitative information, we processed the zymograms using the image processing toolbox in Matlab 2015. Zymograms were transformed to 16-bit grayscale images as matrices and corrected for light variations and camera noise (Razavi et al., 2016). The grayvalue of blank sides of samples was used as a referencing signal. After referencing the zymograms, the average grayvalues of referencing point were subtracted from the zymograms. To this end, a linear function was used to relate the grayvalues of the calibration membranes in an area of 4 cm^2 to their substrate concentrations.

Roots segmentation was simple because of the remarkable contrast between the soil and roots. Single root segments that did overlap with others and were entirely visible in zymograms were selected for further analyses. The selected roots were then skeletonized with a thinning algorithm (Lam et al., 1992) and their radiuses were calculated using the Euclidean distance map function in Matlab (Zarebanadkouki and Carminati, 2014). Then, assuming an asymmetric radial geometry around the roots, the average grayvalue as a function of the distance from the root center was calculated for each individual root. For further quantifications, we defined the rhizosphere extent as the distance of a region with at least 30 % higher enzyme activity than the bulk soil from the center of root. Enzyme activity in the bulk soil was defined as the absence of a decreasing trend of enzyme activity. The total activity of an individual enzyme in the rhizosphere was calculated as the sum up pixel-wise enzyme activity in a region between the root center and the extent of the rhizosphere. Indeed, the extent and the total enzyme activity in the rhizosphere are strongly affected by the root size. To exclude the effect of root size, the rhizosphere extent was normalized by the root radius (rhizosphere extent relative to root radius); the total activity of an individual enzyme was normalized by the root surface area (enzyme activity per root area), and is referred to hereafter as the normalized rhizosphere extent and normalized enzyme activity. This enables comparing the plant species.

2.4. Enzyme kinetics and substrate turnover in wheat root regions with and without hairs

One week after cultivating of wheat, 0.1 g soil was collected from the rhizosphere with and without root hairs from each rhizobox. The soil was collected carefully with a needle to avoid mixing with bulk soil. Suspensions of 0.1 g soil in 10 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, 100 μL a range of substrate concentrations from low to high (0, 5, 10, 20, 40, 80, 100, 150 μM) and 50 μL of buffer (MES or TRIZMA, the same buffers as for zymography) was added to 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 measured 30 min, 1 h and 2 h after adding soil solution, buffer and substrate solution. Enzyme activities were expressed as MUF or AMC release in nmol per g dry soil per hour (nmol g^{-1} dry soil $^{-1}$). The assay of each enzyme at each substrate concentration was performed in four analytical replicates. The Michaelis-Menten constant K_m and V_{max} were determined for each enzyme using the Michaelis-Menten equation:

$$v = \frac{V_{max} \times [S]}{K_m + [S]}$$

where v is the reaction rate (as a function of enzyme substrate concentration), [S] is the substrate concentration, K_m is the substrate concentration at half-maximal rate, and V_{max} is the maximum reaction rate.

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). As labile substances released from roots are abundant in the rhizosphere, 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.

2.5 Statistical analyses

Normality and homogeneity of the rhizosphere extent, total activity of an individual enzyme in the rhizosphere, normalized rhizosphere extent, and normalized activity of an individual enzyme and substrate turnover time were analyzed using Shapiro-Wilk's test and Levene test. Significance of differences of rhizosphere extent, total activity of an individual enzyme in the rhizosphere, normalized rhizosphere extent and normalized activity of an individual enzyme between plants and enzymes, as well as substrate turnover time in the rhizosphere with and without root hairs were tested by ANOVA followed by the Duncan-test using the software SPSS18.0, at $\alpha < 0.05$. The significant effects of root hairs and root radius on the normalized rhizosphere extent and normalized enzyme activity were determined by redundancy

analysis (RDA) and variation partitioning analysis (VPA) using the vegan package in R 3.4.0.

3 Results

Wheat and maize are gramineous; the roots of wheat were thinner than the roots of maize, with an average root radius of 0.24 and 0.47 mm, respectively (Figs. 1, Appendix 1, 3A and 3B). The main roots of maize had root hairs along the entire root length, whereas only some regions of the main roots of wheat had root hairs (proximal parts) (Fig. 2 and Appendix 1). For the leguminous crops, the roots of lentil (radius=0.40mm) were thinner than the roots of lupine (root radius=0.60mm) (Figs. 3C, 3D and Appendix 1). Root hairs of lupine were 5-7 times longer than those of lentil (Fig. 1).

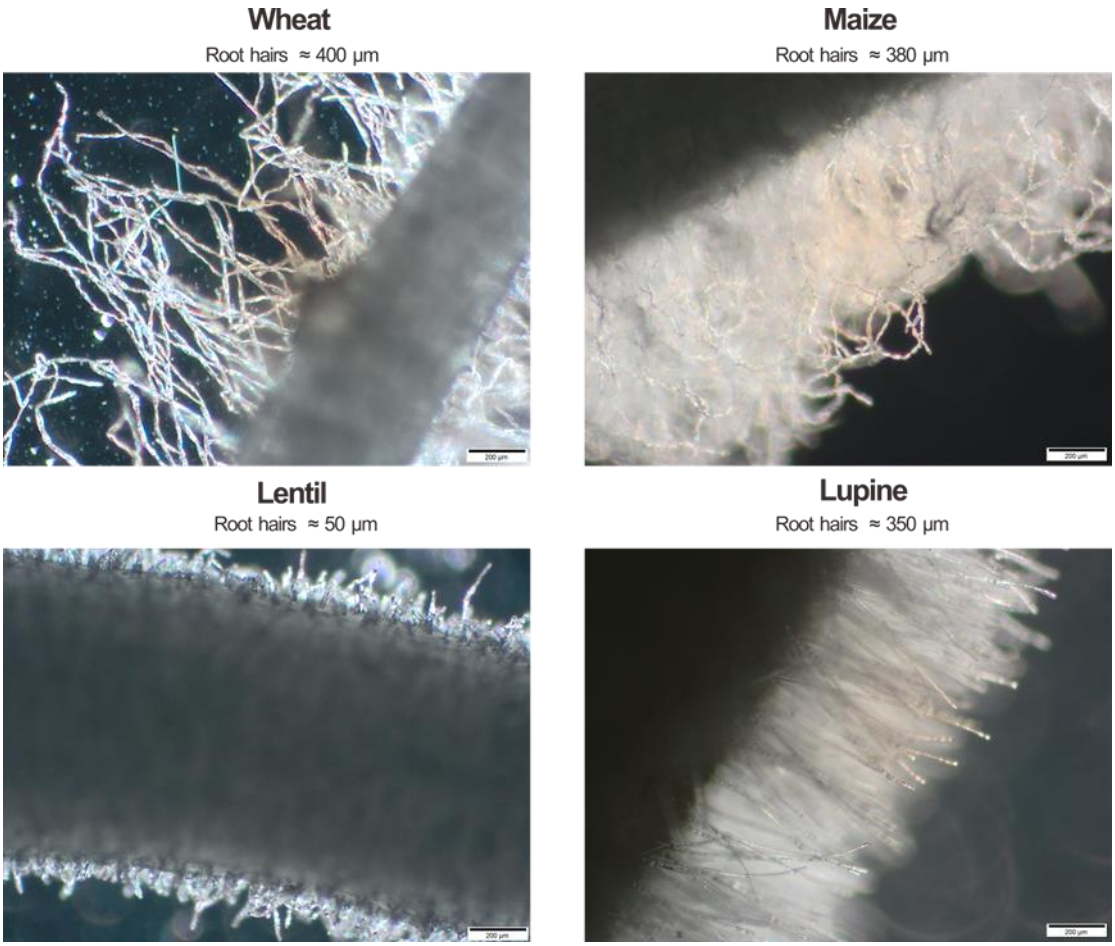


Fig. 1 Microscopy images of root hairs of lentil, lupine, wheat and maize germinated on filter paper. Bar on bottom right: 200 µm. Average root hair length indicated above the images.

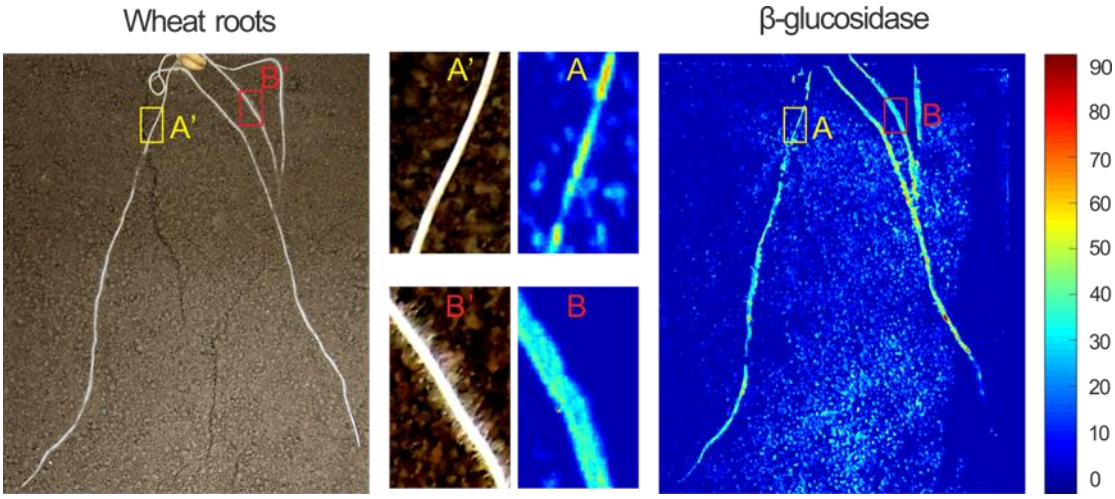


Fig. 2 Examples of wheat roots grown in a rhizobox and the spatial distribution of β-glucosidase. A' and B' indicate root regions without and with hairs, respectively. A and B indicate the spatial distribution of β-glucosidase in regions without and with hairs, respectively. Side color maps are proportional to the enzyme activities (pmol mm^{-2} h^{-1}).

3.1 Rhizosphere extent and total enzyme activity in the root zone

The rhizosphere extent of enzyme activity ranged from 0.55 to 2 mm and was plant and enzyme specific. Generally, the rhizosphere extent of enzyme activity was broader for plants with thick than with thin roots: for maize 1.1-1.4 time broader than for wheat, for lupine 1.2-1.5 fold broader than for lentil (Figs. 3 and 4A). The rhizosphere extent of β-glucosidase activity in wheat root regions with hairs was 1.5-fold broader than in root regions without hairs (Fig. 3A). The rhizosphere extent of phosphatase in wheat, lentil and lupine was 1.5-2 times broader than that of the other three enzymes ($p<0.05$ for lentil and lupine) (Fig. 3A, 3C, 3D and 4A). The activity of β-glucosidase, cellobiohydrolase and leucine aminopeptidase in the rhizosphere of lupine was generally 2-6-fold higher than that in the other three plants ($p<0.05$) (Fig. 4B). In summary, the rhizosphere extent was broader and total enzyme activity was higher in thick than in thin roots.

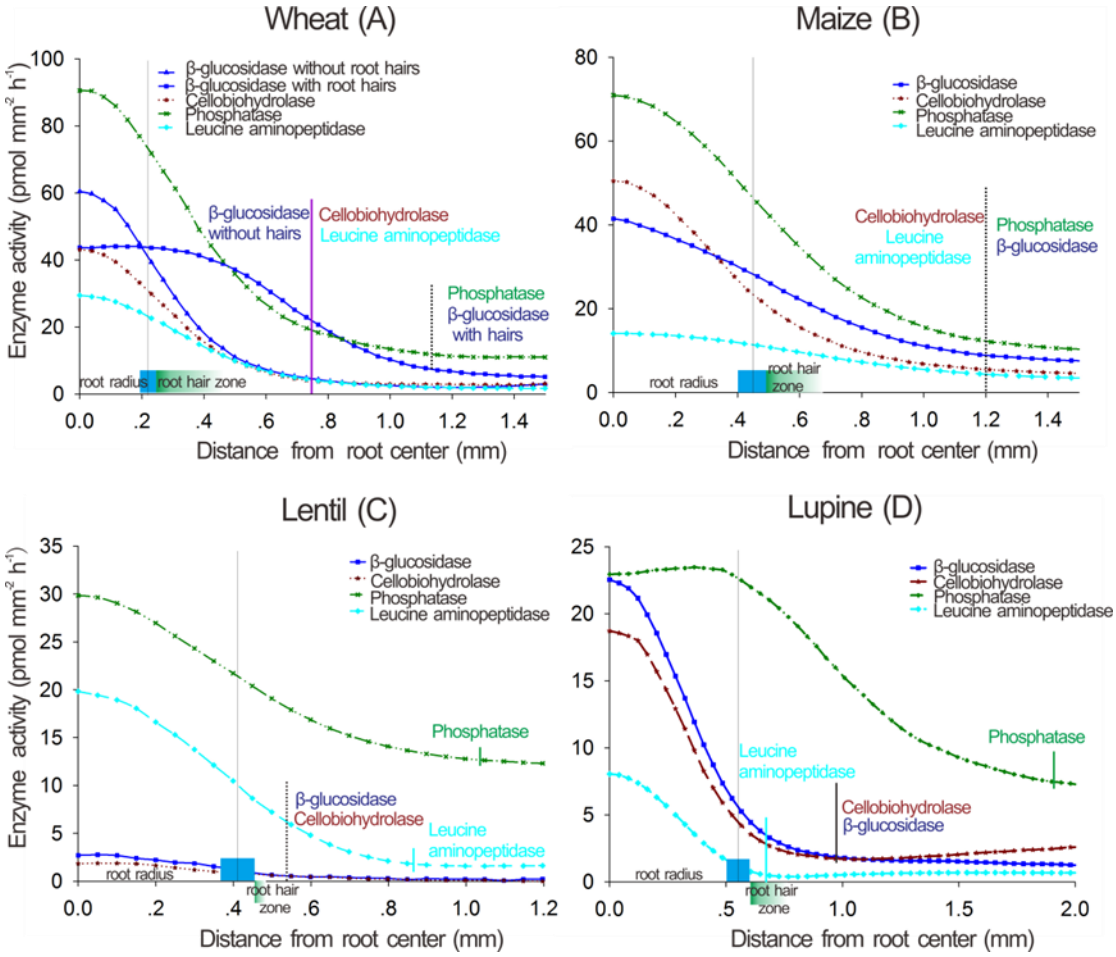


Fig. 3 Profiles of enzyme activity distribution as a function of the distance from the root center towards the surrounding soil: wheat (A), maize (B), lentil (C) and lupine (D). Vertical gray lines vertical: position of average root radius. Blue shading: standard deviation of root radius. Vertical lines on the curves: rhizosphere extent for individual enzymes. Green shading: root hair zone (gradual fading means the boundary of root hairs is not sharp). Each line refers to the mean value of six roots. Error bars of enzyme activities are omitted to improve visualization; the standard errors were always less than 10% of the activity values.

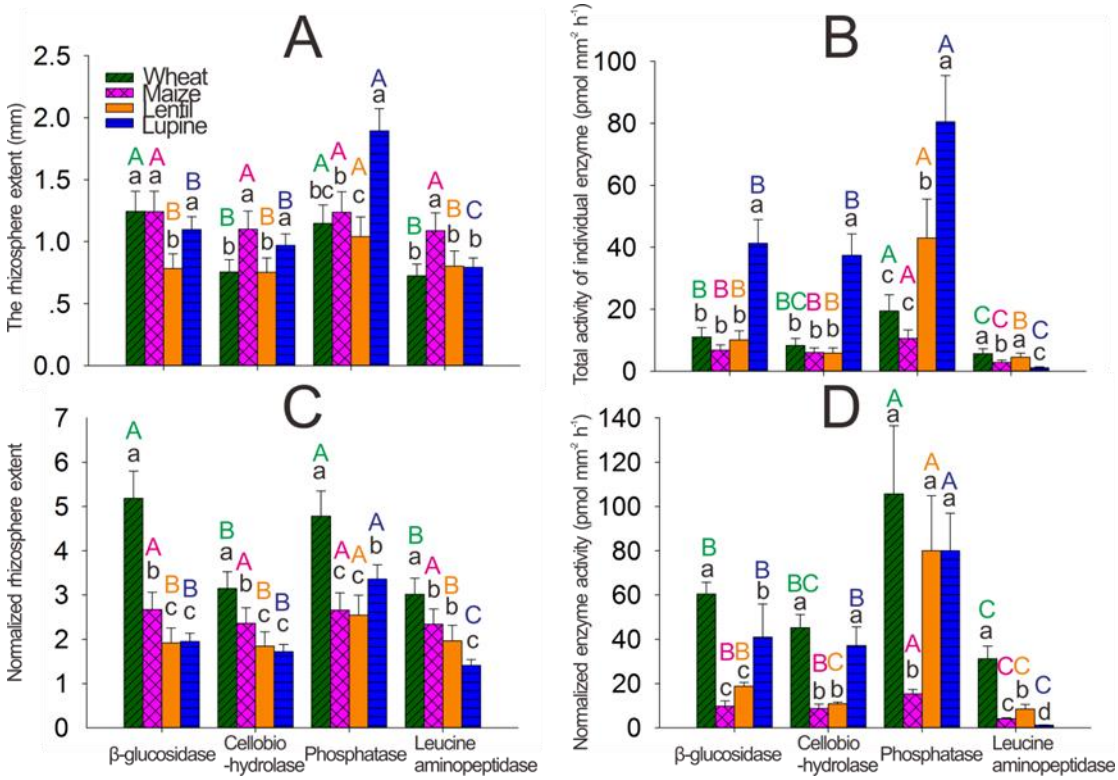


Fig. 4 Rhizosphere extent of enzyme activities (A); total activity of individual enzymes (B); normalized rhizosphere extent (extent of rhizosphere divided by root radius) (C); normalized enzyme activity (sum of the activity of an individual enzyme in the rhizosphere divided the root surface area) (D). Capital letters in colors (same color indicates one plant species): significant differences ($p < 0.05$ after Duncan test) between enzymes. Small letters in black indicate significant differences ($p < 0.05$ after Duncan test) between plants species.

3.2 Normalized rhizosphere extent and normalized enzyme activity in the rhizosphere

The normalized rhizosphere extent and normalized enzyme activity in wheat (thin roots) was significantly higher than in maize (thick roots). For example, the normalized rhizosphere extents of β-glucosidase and phosphatase in wheat were nearly 2 times broader than in maize ($p<0.05$) (Fig. 4C), and the normalized leucine aminopeptidase activity in wheat was 7 times higher than that in maize ($p<0.05$) (Fig. 4D). However, the normalized extent of acid phosphatase in lupine (thick roots with

long and dense root hairs) was 1.5 times broader than in lentil (thin roots with short and sparse root hairs) ($p<0.05$). Similarly, the normalized enzyme activity of β-glucosidase and cellobiohydrolase in lupine was 2-3 times higher than in lentil ($p<0.05$). In contrast, the normalized extent of the rhizosphere and normalized activity of leucine aminopeptidase in lentil were 1.3 times broader and 7-fold higher than in lupine ($p<0.05$). The normalized rhizosphere extents of phosphatase were 1.2-1.5 times broader than the other three enzymes (Fig. 4C). In summary, the extent of rhizosphere relative to root radius was broader in thin than in thick roots. With the exception of leucine aminopeptidase, the enzyme activity per root surface area was higher in roots with long and dense root hairs (lupine) than in roots with short and sparse root hairs (lentil).

3.3 Effect of root morphology on substrate turnover, rhizosphere extent and enzyme activity

With the exception of leucine aminopeptidase, we observed a tendency of shorter substrate turnover time in wheat root regions with versus without root hairs for all tested enzymes. In the case of β-glucosidase, the substrate turnover in the root hair region was 2 times faster than in the hairless root zone ($p<0.05$) (Fig. 5).

Root hairs play a significant role and contributed 16.5%, 14.3% and 17.6% to the variability of β-glucosiadase, cellobiohydrolase and phosphatase normalized rhizosphere extent, respectively ($p<0.05$) (Fig. 6). In contrast, the effect of root radius was more pronounced for the normalized enzyme activity. The root radius contributed 13%, 37.2%, 38.3% to the variability of β-glucosidase, phosphatase and leucine aminopeptidase activity, respectively ($p<0.05$) (Fig. 6). Thus, root hairs play more important role in the rhizosphere extent, while root radius plays a more significant role in the enzyme activity per root surface area.

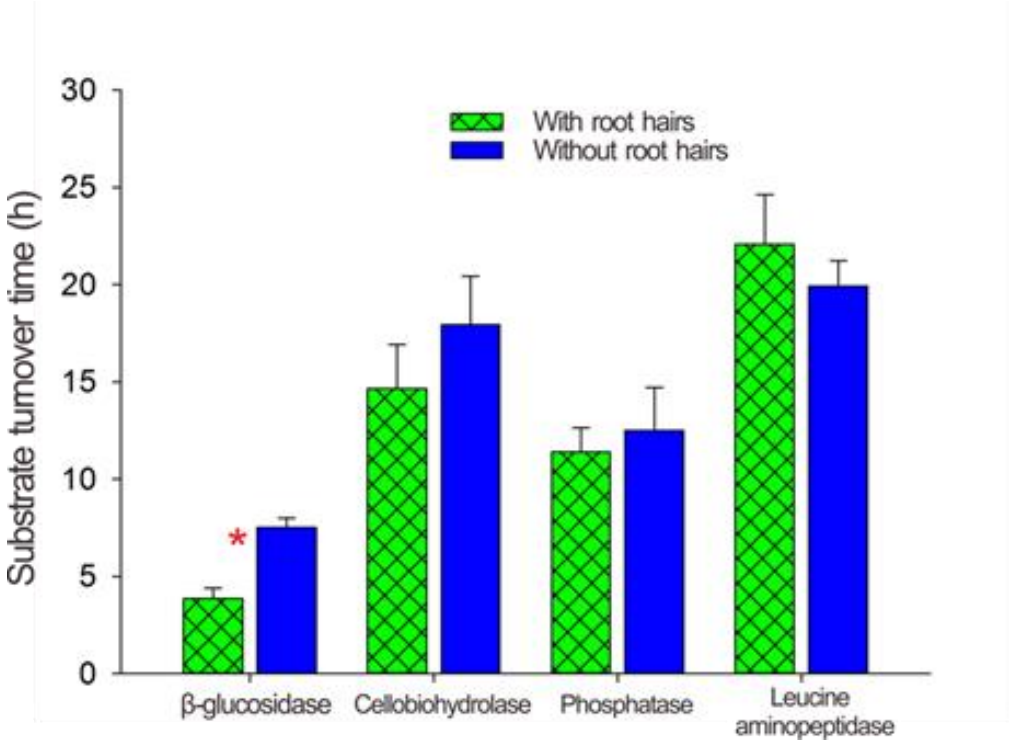


Fig. 5 Substrate turnover time for β-glucosidase, cellobiohydrolase, phosphatase and leucine aminopeptidase in the rhizosphere of wheat with and without root hairs. Bars: means of four replicates (±SE). Asterisks: significant differences ($p < 0.05$ after Duncan test) between regions with and without root hairs.

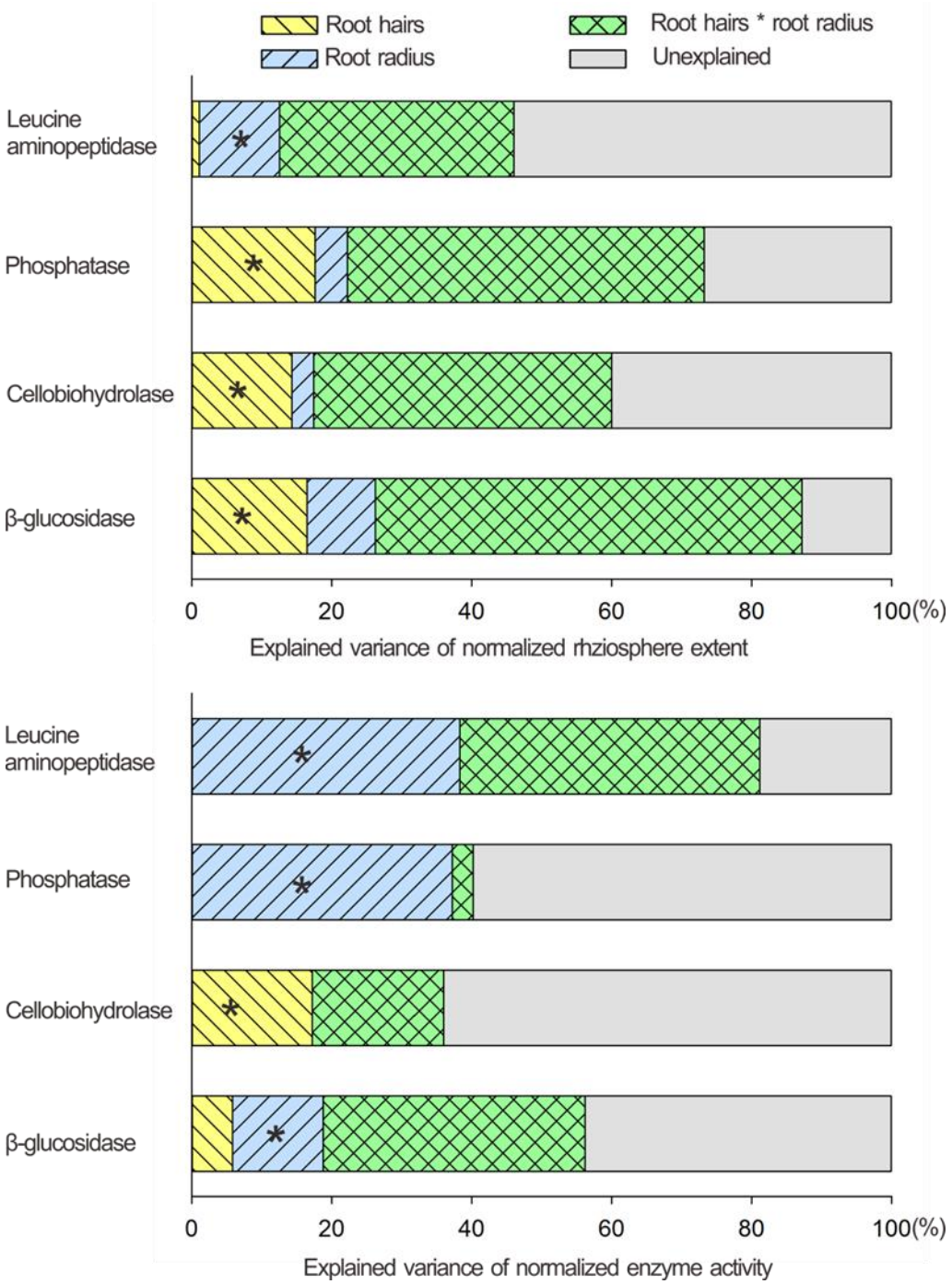


Fig. 6 Contributions of the two factors: root hairs and root radius to the variability of normalized rhizosphere extent and normalized enzyme activity. The analysis involved redundancy analysis (RDA) and variation partitioning analyses (VPA). Asterisks indicate significant factors (root radius, root hairs).

4 Discussions

The rhizosphere extent in terms of enzyme activities was very narrow, ranging from 0.55 to 2 mm from the root center in four plants species to the surroundings. Similar rhizosphere extents were obtained by visualizing oxygen consumption, pH and redox changes (Blossfeld et al., 2011; Schmidt et al., 2011; Rudolph et al., 2013). The rhizosphere extents of enzyme activities reflect the equilibrium between enzyme inputs (synthesis and release from roots and microorganisms) and output (microbial decomposition and other deactivation of the enzymes) (Asmar et al., 1994; Bais et al., 2004; Miralles et al., 2012). Such narrow extent is an efficient strategy for plants to acquire nutrients in the competitive rhizosphere environment (Kuzyakov and Xu, 2013).

4.1 Effects of root hairs on rhizosphere extent and substrate turnover

In line with the first hypothesis root hairs expand the rhizosphere extent, for example the rhizosphere extent of β-glucosidase was 50% broader around wheat root regions with hairs than without hairs (Fig. 3A). For other three enzymes, the extent of rhizosphere was also broader around regions with hairs than without hairs, but this effect was less pronounced as compared with β-glucosidase (Table. S1). Root hairs modulate the chemical properties and composition of the rhizosphere (Datta et al., 2011) by releasing large quantities of exudates and other rhizodeposits (Czarnota et al., 2003; Nguyen, 2003; Jones et al., 2009). Abundant exudates released by root hairs are quickly consumed by fast-growing microorganisms and increase their activities (Blagodatskaya et al., 2009; Chen et al., 2014). These active microbes produce more enzymes that can decompose rhizodeposits, which are abundant in the root-hair zone due to the very short lifespan of tiny hairs (Kuzyakov and Xu, 2013), and further influences enzyme dynamics such as substrate turnover. This is explanation confirmed by the faster substrates turnover of β-glucosidase, cellobiohyrolase and acid phosphatase in root regions with hairs than without hairs (Fig. 5), which supports our second hypothesis.

4.2 The rhizosphere extent and enzyme activity depend on root hairs and root radius

In general, the rhizosphere extent of enzyme activities from the root center was broader for plants with thick roots (maize and lupine) than with thin roots (wheat and lentil): the rhizosphere extent of cellbiohydrolase and leucine aminopeptidase was 1.5 times broader around maize than around wheat ($p<0.05$); the rhizosphere extent of enzymes contributed to C, and P cycles were significantly broader around lupine than around lentil roots ($p<0.05$) (Figs. 3 and 4A). Lupine, with the thickest roots, had the highest activity for all tested enzymes (except for leucine aminopeptidase) ($p<0.05$) (Fig. 4B). This is due to larger rhizosphere volume around roots with a bigger radius. The bigger the radius is the more exudates will be distributed in the soil volume. Therefore, a broader rhizosphere and higher total enzyme activity are expected around thick roots.

After normalization by root radius and surface area, the rhizosphere extent was broader and enzyme activity was higher around thin (wheat) than around thick (maize) roots ($p<0.05$) (Fig. 4C and 4D). Accordingly, thin roots are more efficient in determining the rhizosphere extent and the enzyme activity per area than the thick roots, confirming that fine roots acquire nutrient better than coarse roots (Gambetta et al., 2013; McCormack et al., 2015). In contrast, the normalized rhizosphere extents of phosphatase ($p<0.05$) and β-glucosidase were broader, and the normalized enzyme activity of β-glucosidase and cellobiohydrolase were higher ($p<0.05$) around lupine (thick roots) than around lentil (thin roots) (Figs. 4C and 4D). This contradiction is explained by considering the length and density of root hairs in both plants. Lupine has long and dense root hairs as compared to the short and sparse hairs of lentil (Fig. 1). This contrasting length and density of root hairs will affect the quantities of C released (Nguyen, 2003). Moreover, root hair length and density directly and indirectly affect the enzyme release to facilitate nutrient mineralization (Brechenmacher et al., 2009; Libault et al., 2010). In contrast, lentil (thin roots) has a broader normalized rhizosphere extent and higher normalized activity of leucine aminopeptidase than lupine (thick roots) ($p<0.05$) (Fig. 5), indicating that lentil produced more N-acquiring enzymes. This reflects the crucial role of legume root hairs in attracting N-fixing rhizobia (Libault et al., 2010). Without long and dense root hairs, lentil needs to produce more N mineralization enzymes directly by roots.

4.3 The rhizosphere extent and enzyme activity is enzyme and plant specific

In line with the last hypothesis, the rhizosphere extent and enzyme activities were plant and enzyme specific. Acid phosphatase has the broader rhizosphere extent and higher activity ($p<0.05$) than other enzymes. This is because phosphatases are produced by both roots and microorganisms (Dick et al., 1983; Juma and Tabatabai, 1988; Nannipieri et al., 2011) to meet the strongly demand for P (a component of key molecules such as nucleic acids, ATP and phospholipids) (Marschner et al., 2011). Nonetheless, P mobility is slow due to strong adsorption. This requires a broader rhizosphere extent and high activity phosphatase to provide P for roots in comparison to other nutrients (Barber, 1962; Heuer et al., 2017). Therefore, roots release protons and organic ligands; this acidifies the rhizosphere and thus promotes the activity of acid phosphatase (Šarapatka et al., 2004; Lambers et al., 2006). The normalized rhizosphere extent and normalized enzyme activities of β-glucosidase, cellobiohydrolase and leucine aminopeptidase were plant species-specific due to different root morphologies (root hairs, root radius) (Jungk, 2001) and root exudation patterns (Badri and Vivanco, 2009). For instance, due to different root exudation patterns, the enzyme activity in legumes evenly distributed along the roots, while in grasses high activity associated with root tips (Razavi et al., 2016; Pausch and Kuzyakov, 2017). Variation partitioning analysis of the normalized rhizosphere extent and of normalized enzyme activity showed that root hairs contributed more to the rhizosphere extent, whereas root radius more strongly affects the enzyme activity per root surface area (Fig.6). Moreover, due to the different demand for nutrients, even the normalized enzyme activity responsible for acquiring of mineral elements also varies between plants (Farrar and Jones, 2000). For example, maize had a lower phosphatase activity than other three tested plants, ($p<0.05$) (Fig. 4B and 4D), may due to low P demand. Such an explanation, however, requires experimental confirmation by simultaneous determination of the specific nutrient acquisition along with enzyme activity in a variety of plant species. Correspondence of the rhizoboxes experiments with limited space and sieved soil to natural condition is not fully evident, this calls for the field studies to test the roots behavior, development and their influence on the spatial distribution of enzyme activity.

5 Conclusions

We visualized the spatial distribution of enzyme activities in the rhizosphere of four plant species with contrasting root and root hair morphology. We found that root hairs broaden the rhizosphere extension up to 50% and facilitated the substrate turnover by 2 times. For the first time, we report that 1) the rhizosphere extent relative to root radius was broader and enzyme activity per surface area was higher around thin roots (wheat) than around thick roots (maize); 2) roots with long and dense hairs (lupine) have a higher enzyme activity per root area and a broader rhizosphere extent relative to root radius than roots with short and sparse hairs (lentil) (concept Fig. 7). In conclusion, root hairs and root radius shape the rhizosphere: root hairs contributed mainly to the rhizosphere extent, whereas root radius mainly affected the enzyme activity per root surface area. Breeding species with long and dense root hair and more tiny secondary roots is a promising way to increase rhizosphere extension and nutrient acquisition.

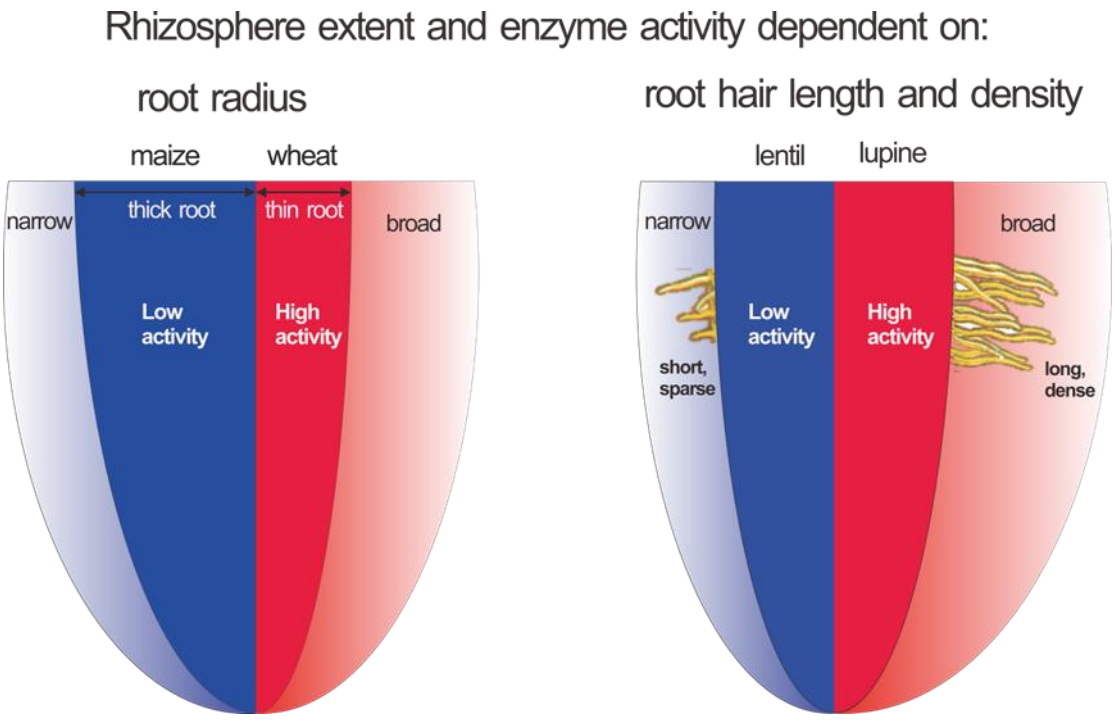


Fig. 7 Effects of root radius (left) and root hair length and density (right) on rhizosphere extent (standardized to root radius) and enzyme activity (standardized to root surface area). Roots presented in dark (blue or red), rhizosphere extend in light

(blue or red). Left: Thin roots with high enzyme activities (dark red) lead to larger rhizosphere extend (light red) compared to large roots with low enzyme activities (dark blue) having a short rhizosphere range (light blue). Right: Roots with long and dense hairs (dark red) have higher enzyme activity and broader rhizosphere extend (light red) than roots with short and sparse hairs (dark blue).

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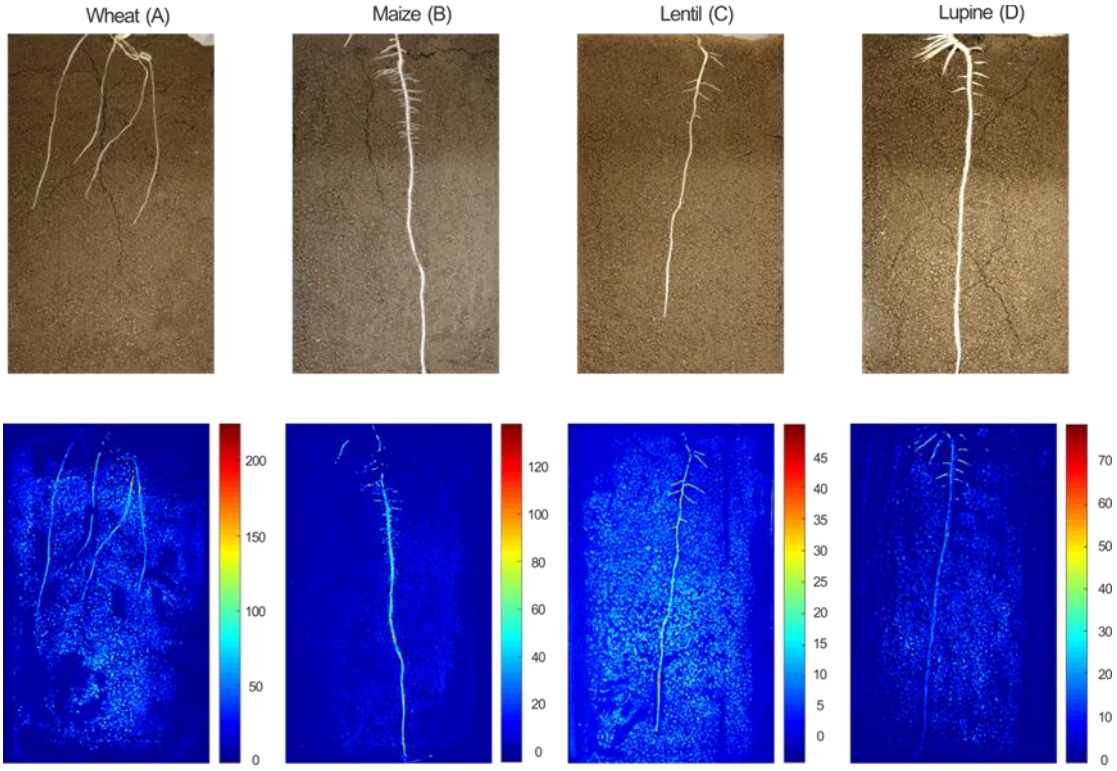
Supplement Information



Appendix 1 Examples of root grown in rhizoboxes: wheat (A), maize (B), lentil (C) and lupine (D) and spatial distribution of acid - phosphatase activity. Side color maps are proportional to the enzyme activities (pmol mm^{-2} h^{-1}).

Table S1 The rhizosphere extent of β-glucosidase, cellobiohydrolase, leucine aminopeptidase and acid phosphatase around wheat root with and without hairs regions.

	With root hairs (mm)	Without root hairs (mm)
β-glucosidase	1.07 ±0.04	0.78 ±0.01
cellobiohydrolase	0.78 ±0.02	0.74 ±0.02
leucine aminopeptidase	0.78 ±0.03	0.72 ±0.01
acid phosphatase	1.07 ±0.02	0.99 ±0.03

Study 2 Spatio-temporal patterns of enzyme activities in the rhizosphere: Effects of plant growth and root morphology

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Abstract

Lentil and lupine, having contrasting root morphologies were chosen to investigate the effects of plant growth and root morphology on the spatial distribution of β-glucosidase, cellobiohydrolase, leucine aminopeptidase and acid phosphomonoesterase activities. Lentil kept as vegetative growth and the rhizosphere extent was constant, while the enzyme activities at the root surface kept increasing. Lupine entered reproductive growth in the 7th week after planting, the rhizosphere extent was broader in the 8th week than in 1st and 4th weeks. However, enzyme activity at the root surface of lupine decreased by 10-50% in comparison to the preceding vegetative stage (1st and 4th weeks). Lupine lateral roots accounted for 1.5-3.5 times more rhizosphere volume per root length than taproots, with 6-14 folds higher enzyme activity per root surface area. Therefore, we conclude that plant growth and root morphology influenced enzyme activity and shape the rhizosphere as follows: the enzyme activity in the rhizosphere increased with plant growth until reproductive stage; lateral roots have much larger rhizosphere volume per unit root length and higher enzyme activity per root surface area than the taproots.

Keywords: Rhizosphere extent • Enzyme spatial distribution • Zymography • Plant growth stage • Root morphology • Visualization approaches

1. Introduction

Living roots release labile root exudates and other rhizodeposits into the soil over the growing season, which stimulate microbial activities and enzyme synthesis, and form one of most dynamic habitats-the rhizosphere (Hinsinger et al. 2009; Kuzyakov and Blagodatskaya 2015). The rhizosphere effect started from root surface and extends several millimeters out into the soil-rhizosphere extent (Dazzo and Gantner 2012), which is great importance for plant performance as well as for nutrient cycling and ecosystem functioning (Bertin et al. 2003; Singh et al. 2004). The extent and spatial distribution of the rhizosphere is dynamic functions depending on soil and root properties. Because the diffusion of the rhizodeposition depends on both soil physical properties such as soil particle size and water content (Hallett and Bengough 2013), and the root exudation profile and root morphology change during root development (Dazzo and Gantner 2012; Neumann and Römheld 2000). It is assumed that enzymes involved in SOM decomposition and nutrient mineralization in the rhizosphere also vary spatially and temporally during plant development (Badalucco and Kuikman 2001; Tarafdar and Jungk 1987).

The enzymes excreted by both microorganisms and roots, catalyze the decomposition of root exudate and other rhizodeposits into absorbable forms (Nannipieri et al. 2007; Sinsabaugh et al. 2008) and provide nutrients for microorganisms and plants (Henry 2012). Root exudates contain a great variety of compounds including carbohydrates, carboxylic and amino acids, phenolics, fatty acids, sterols, enzymes, vitamins, hormones and nucleosides (Dakora and Phillips 2002; Grayston et al. 1997; Read et al. 2003). The amount, composition and distribution of root exudates in the rhizosphere depend on root morphology, plant development and plant species (Badalucco and Kuikman 2001; Jones et al, 2009). Exudates are heterogeneously distributed along the root due to differing morphological and architectural structures such as young lateral roots, root hairs (Ma et al. 2018; Pausch et al. 2016) and old taproots (Nguyen 2003). Lateral roots and root hairs increase the root surface area, which elevates exudation and facilitates nutrient uptake (Jungk 2001; McCormack et al. 2015). In contrast, older thicker taproot exhibit low uptake ability and exudation but high transport capacity (Gambetta et al. 2013; Guo et al. 2008; McCormack et al. 2015). Moreover, the amount of root exudates

depends on plant development (Farrar et al. 2003), which increases with plant growth until the reproductive stage (Aulakh et al. 2001; Gransee and Wittenmayer 2000; Odell et al. 2008). Plant species also influences root exudates: individual plants release different root exudate to facilitate growth of beneficial microorganisms in the rhizosphere (Philippot et al. 2013). Microbial and enzyme activities in the rhizosphere are affected by the quality and quantity of root exudates, and therefore change during plant and root development (Remenant et al. 2009; Schmidt and Eickhorst 2014) and vary between plant species (Güsewell and Schroth 2017; Proctor and He 2017).

Quantification of enzyme activity in the rhizosphere is also a sensitive indicator of changes in the plant-root-microbe interactions (Aon et al. 2001; Nannipieri et al. 2007), and determination of spatial distribution of enzyme activities in the rhizosphere can provide complementary information on the microbial activity in the vicinity of root surface (Wallenstein and Weintraub 2008). A recently developed two-dimensional imaging technique, termed zymography, allows quantitative visualization of the spatial and temporal distribution of enzyme activities in soil (Sanaullah et al. 2016), biopores (Hoang et al. 2016), the rhizosphere of young plants (Ma et al. 2018; Razavi et al. 2016) and the detritusphere (Ma et al. 2017). The technique is suited to tracing the effects of plant age, root development and morphology on rhizosphere enzyme distribution, yet has not been previously applied for this purpose. Lentil (*Lens culinaris*) and lupine (*Lupinus albus*) are members of the fabaceae, are nitrogen-fixing legume crops, and have tap-root systems. Both are important agricultural food crops and can be grown on a broad range of soils (Dinkelaker et al. 1989; Tovar 1996). However, the crops differ in some characteristics. Firstly, the vegetative growth period of lentil is longer than lupine 55-130 d versus 35-60 d, respectively (Croser et al. 2016; Roberts et al. 1986). Second, young seedlings of the two species have contrasting root morphologies: lentil seedling roots are thin with short and sparse root hairs, while those of lupine are thicker with long and dense root hairs (Ma et al. 2018). Using the innovative zymographic technique, for the first time, we quantitatively imaged the *in situ* activity of various hydrolytic enzymes in the rhizosphere of lentil and lupine plants during root development and root morphology (lateral root and taproot). We hypothesized that 1) due to the decrease of root exudation, the rhizosphere extent could be narrower and the enzyme activity could be lower at

reproductive stage than at vegetative stage, 2) due to different root morphology, the enzyme activity could be higher and the rhizosphere extent is broader around lateral roots than around taproots, and 3) the spatial pattern of enzyme activity could be specific for the plant species and enzyme activities, and also dependent on root morphology and plant growth stage.

2. Material and methods

2.1 Soil and plant 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 Goettingen, Germany. The soil was sieved (< 2 mm) and had the following 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). Rhizoboxes with inner dimension of 21.2 × 10.8 × 3.3 cm were filled with soil to reach a final density of 1.2 g cm^{-3}. Lupine and lentil seeds were germinated on filter paper for 72 h and thereafter one seedling was planted at a depth of 5 mm in each rhizobox. Each plant species had 6 replications in separate rhizoboxes. The rhizoboxes were kept in a climate chamber with a controlled temperature of 20 ± 1 ℃ and a daily light period of 14 h with a photosynthetically active radiation intensity of 250 μmol m^{-2} s^{-1}. During the growth period, the rhizoboxes were kept inclined at an angle of 45 ° so that the roots grew along the lower wall of the rhizobox. The rhizoboxes were irrigated with distilled water to maintain water content at 60% of water holding capacity.

2.2 Direct soil zymography

When plants were 1, 4 and 8 weeks old, direct zymography (Sanaullah et al. 2016) was used to visualize the activities of four enzymes following Ma et al. (2017). β-glucosidase, cellobiohydrolase, acid phosphomonoesterase were detected by fluorescent methylumberrliferyl (MUF)-substrates and leucine aminopeptidase was detected by detected by fluorescent L-leucine-7-amido-4-methylcoumarin (AMC)-substrate (Koch et al. 2007). Each of these substrates was dissolved to a concentration of 12 mM in MUF or AMC buffers. The rhizoboxes were opened from the lower, rooted side and the membrane saturated with substrate solution was applied directly to

the soil surface. Soil zymography was performed for each enzyme separately on the same rhizobox in the order: β-glucosidase, acid phosphomonoesterase, cellobiohydrolase, leucine aminopeptidase. Each membrane was incubated on the soil surface for 1 h, after which the membranes as taken to a dark room, photography were taken under UV light (355 nm). The calibration was conducted and calculated following Ma et al. (2017). 4 cm^2 membranes squares that were soaked in solutions of increasing concentrations of MUF or AMC, then were photographed under UV light. The amount of MUF or AMC per unit area was calculated from the membrane size, the volume of solution taken up and the concentration of the solution.

2.3 Image processing and analysis

Zymogram fluorescence corresponds to the areas of enzyme activity, with the intensity of fluorescence proportional to the activity of the enzyme. Quantification was performed with the image processing toolbox in Matlab 2015. Image processing consisted of 4 steps: 1) transformation of signal (fluorescence) of the images to grayvalues, 2) background adjustment, 3) root segmentation, root skeletonization and root radius calculation, and 4) conversion of gray values to enzyme activity. Zymograms were transformed to 16-bit grayscale images as matrices and corrected for light variations and camera noise (Razavi et al. 2016). Gray values of blank sides of the images were used as background signal and subtracted. A linear function was used to relate the gray values of the calibration membranes to their substrate concentrations, and consequently to enzyme activities.

The roots were easily segmented due to strong contrast between soil and roots. Single, non-overlapping root segments that were entirely visible in zymograms were selected for further analyses. The selected roots were then skeletonized with a thinning algorithm (Lam et al. 1992) and their radii were calculated using the Euclidean distance map function in Matlab (Zarebanadkouki and Carminati 2014). Then, assuming an asymmetric radial geometry around the roots, the average gray value as a function of distance from root center was calculated for each individual root. We defined the rhizosphere extent as the distance from the root boundary with at least 30% higher enzyme activity than the bulk soil. The rhizosphere volume per unit root length was calculated according to the following equation:

$$Vr\ (mm^3) = \pi(RE^2 - RR^2) \times 1(mm)$$

RE and RR is rhizosphere extent and root radius, respectively. The total activity of individual enzymes in the rhizosphere was calculated as the pixel-wise enzyme activity in the region between the root center and the rhizosphere limit. To compare lateral and taproots, the rhizosphere volume and total activity of individual enzymes were normalized to the root surface area, which will be referred to as rhizosphere volume per root surface area and enzyme activity per root surface area.

2.4 Statistical analyses

Normality and homoscedasticity of rhizosphere volume, total activity of individual enzymes in the rhizosphere, and enzyme activity per root surface area were checked using Shapiro-Wilk's test and Levene tests. Significance of the differences in these parameters between tap and lateral roots were tested by ANOVA followed by the Duncan-test using the software SPSS18.0, at $\alpha = 0.05$.

3. Results

Examples of acid phosphomonoesterase zymograms clearly demonstrated the spatio-temporal distribution of enzyme activity in the rhizosphere of two plant species (Fig. 1). Lentil roots developed slowly and the plants remained in vegetative throughout the incubation period. In contrast, lupine roots penetrated the rhizoboxes rapidly and the plants started to flower in the 7th week (Fig. 1).

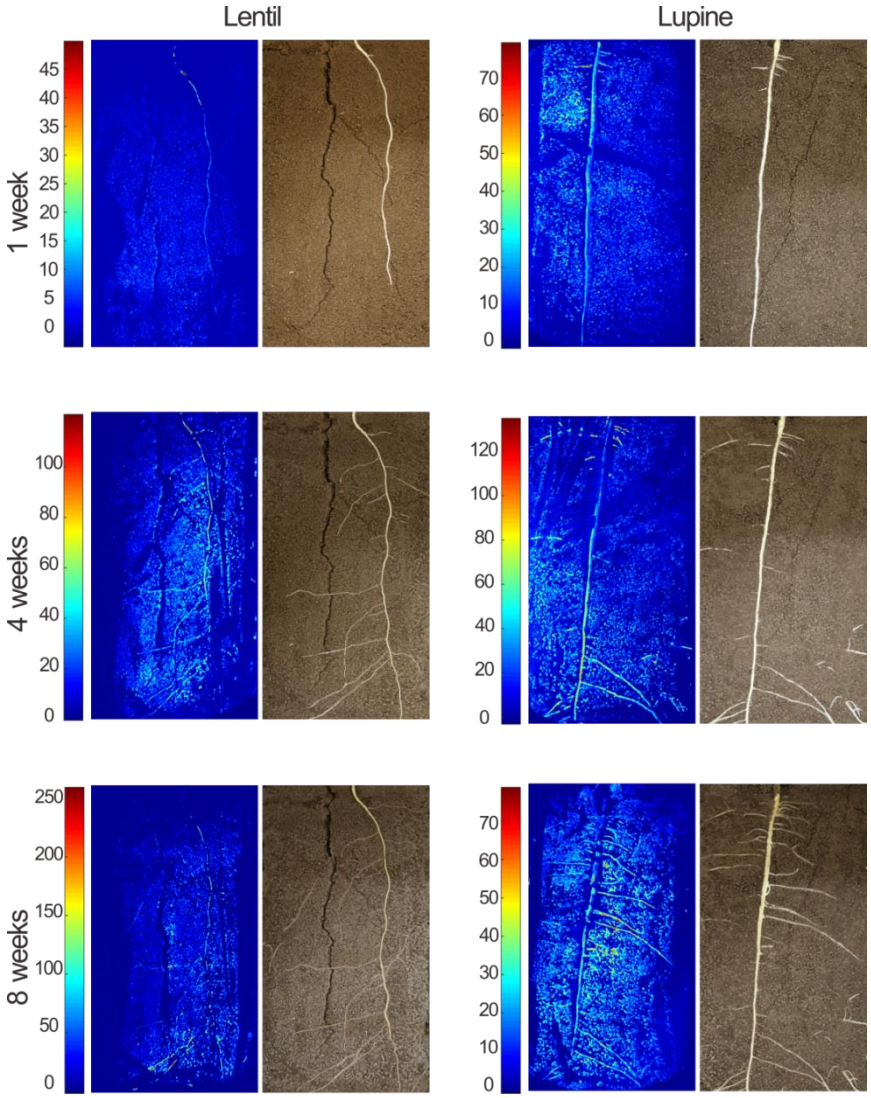

Fig. 1 Examples of roots grown in rhizoboxes and the spatial distribution of acid phosphomonoesterase activity in soil and rhizosphere of 1, 4 and 8-week-old lentil and lupine. Side color maps are proportional to the enzyme activities (pmol mm^2 h^{-1}). Please note different scaling of the color bars.

3.1 Rhizosphere extent and dymanics of enzyme activity in lentil and lupine

The rhizosphere extent of β-glucosidase, cellobiohydrolase and acid phosphomonoesterase activities around lentil roots was constant throughout the growth period (0.6 mm) (Fig. 2a, b and c). For leucine aminopeptidase activity, however, the rhizosphere was broader in the 4th week (about 0.9 mm) than in the 1st and 8th weeks (about 0.6 mm) (Fig. 2d). Around lupine roots, the rhizosphere extent of β-glucosidase, cellobiohydrolase, and leucine aminopeptidase activities was stable only during the first 4 weeks (about 0.6 mm), and thereafter it expanded 50-100% (1.2-1.3 mm) (Fig. 3a, b and d). In contrast, the extent of acid phosphomonoesterase activity in the rhizosphere of lupine was nearly twice as broad in the 1st and 8th weeks than in the 4th week (Fig. 3c). In general, the rhizosphere extent of lentil was temporally constant, while it got broader in lupine as the plant became older.

With exception of leucine aminopeptidase, the activities of enzymes in the rhizosphere of lentil increased over time. For instance, the activities of β-glucosidase and acid phosphomonoesterase in the 8th week were 3 - 8 times higher than in the 1st week (Figs. 2 and S2). In contrast, enzyme activities in the rhizosphere of lupine decreased after entering reproductive growth: they were 10-50% lower in the 8th week (reproductive growth) as compared to in the 1st and 4th weeks (Figs. 3 and S3). In summary, *in situ* enzyme activity increased during vegetative growth but decreased when reproductive growth started.

Remarkably, enzyme activities in the rhizosphere of lentil increased during 8 weeks of growth, but the rhizosphere extent remained constant (Fig. S2). Similarly, the rhizosphere extent of lupine was stable over the first 4 weeks, while the enzyme activities changed (Fig. S3). Thus, the rhizosphere extent and enzyme activities were independent.

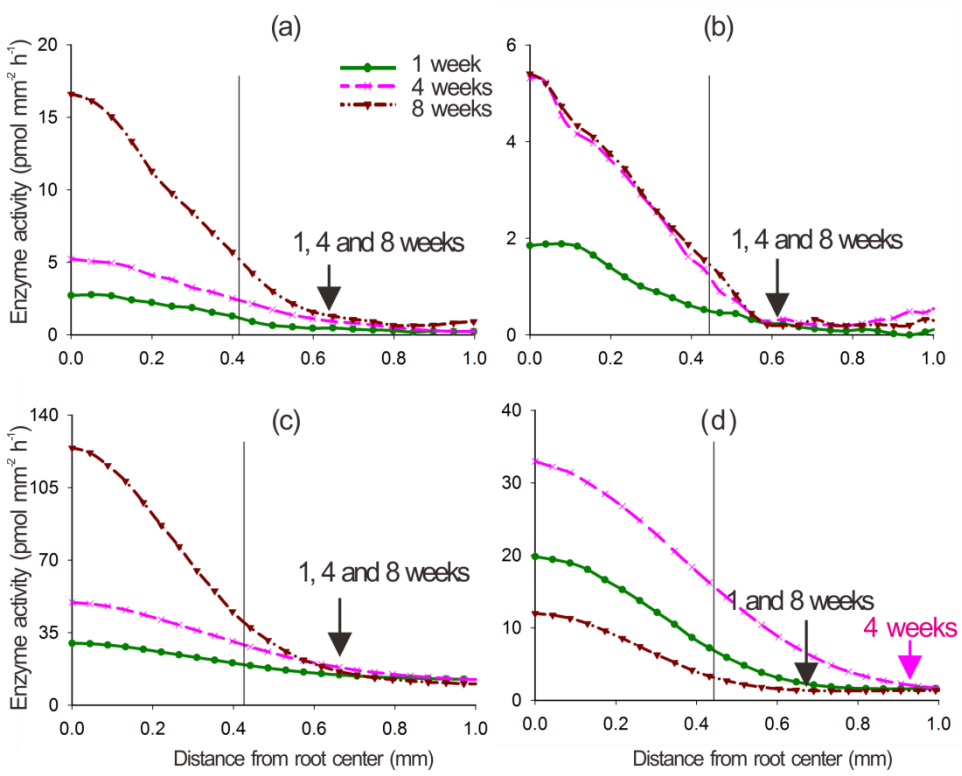

Fig. 2 Enzyme activity as a function of distance from root center for 1, 4 and 8-week - old lentil: a) β-glucosidase, b) cellobiohydrolase, c) acid phosphomonoesterase, d) leucine aminopeptidase. Each line refers to the mean activity around six roots. Vertical gray lines indicate the average root radius. Small vertical arrows show the development of rhizosphere size over time. Error bars of enzyme activities are omitted to improve visualization, but the standard errors are at all times less than 10% of the activity values

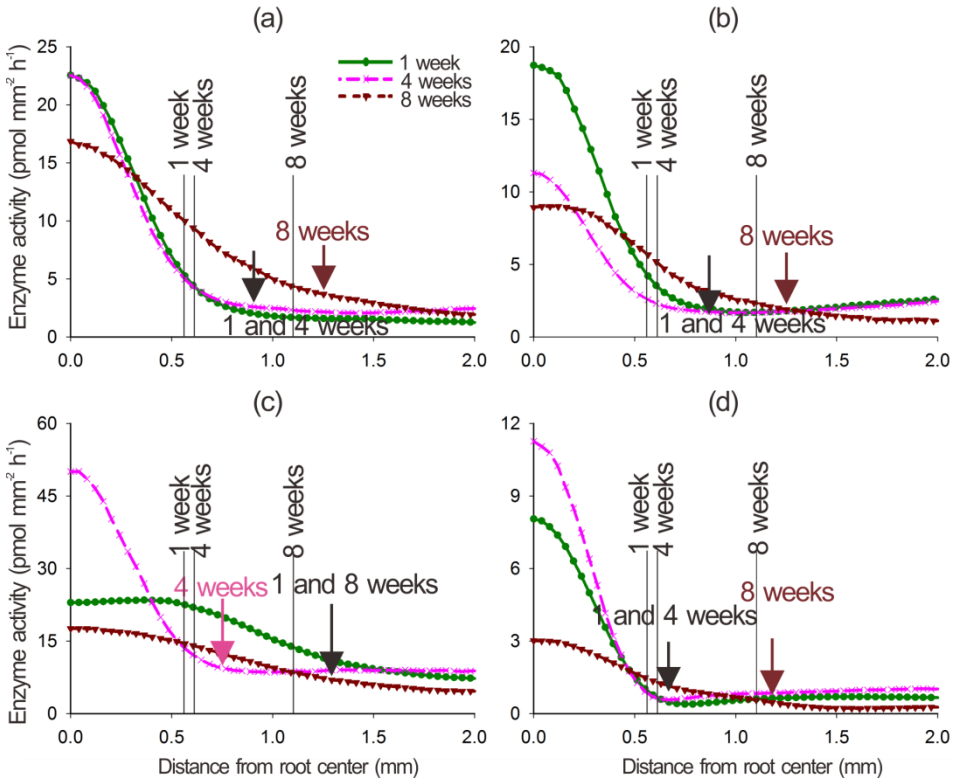

Fig. 3 Enzyme activity as a function of distance from root center for 1, 4 and 8-week-old lupine: a) β-glucosidase, b) cellobiohydrolase, c) acid phosphomonoesterase, d) leucine aminopeptidase. Each line refers to the mean activity around six roots. Vertical gray lines indicate the average root radius. Small vertical arrows show the development of rhizosphere size overtime. Error bars of enzyme activities are omitted to improve visualization, but the standard errors are all times less than 10% of the activity values

3.2 Rhizosphere volume and enzyme activities around lateral and taproots of lupine

Lupine taproots were 4-5 times thicker than lateral roots in the 8th week, with average root radii of 1.1 and 0.24 mm, respectively (Fig. 4). The rhizosphere volume and the rhizosphere volume per root surface area in lateral roots were respectively1.5-3.5 times and 30-70 times higher for lateral roots than for taproots (Fig. 5a and b). The total activity of individual enzymes in the rhizosphere of taproots was 2-3 fold higher than that of lateral roots (Fig. 5c). In contrast, normalized by root surface area, enzyme activity in the rhizosphere of lateral roots was 6-14 fold higher than for

taproots (Fig. 5d). Therefore, while taproots were 4-5 times thicker than lateral roots, lateral roots had larger rhizosphere volumes and higher enzyme activity per root surface area.

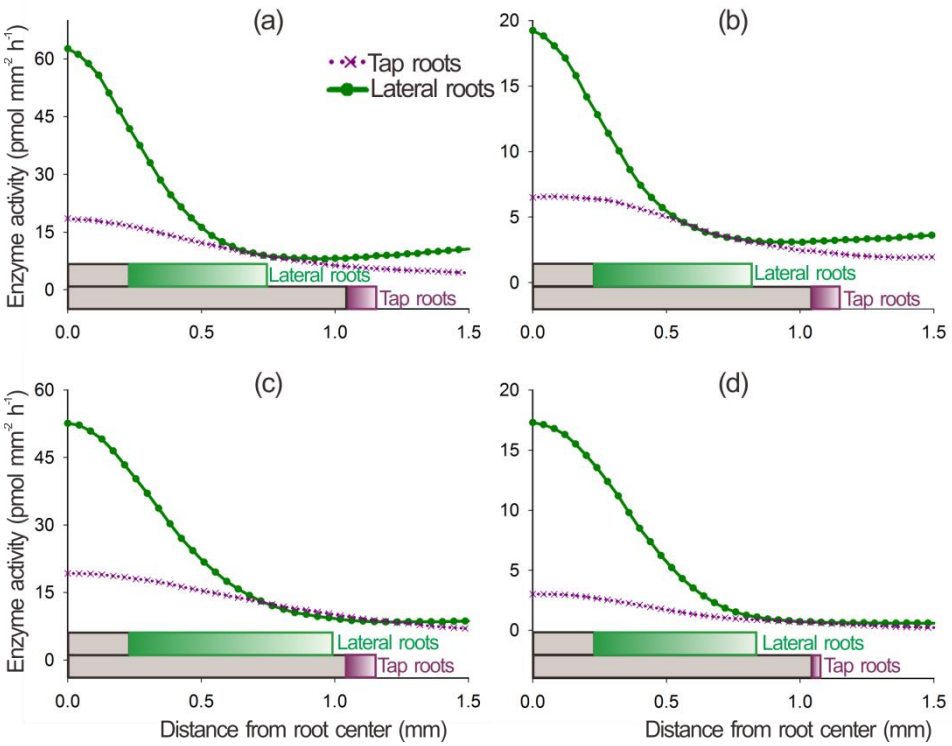

Fig. 4 Enzyme activity as a function of distance from root center for 8 week-old lupine lateral and tap roots: a) β-glucosidase, b) cellobiohydrolase, c) acid phosphomonoesterase, d) leucine aminopeptidase. The white bars above the x-axis indicate the radius of lateral and taproots. The color shadows indicate the rhizosphere extent for each enzyme activity (excluding root radius). Each line refers to the mean activity around six roots. Error bars of enzyme activities are omitted to improve visualization, but the standard errors are all times less than 10% of the activity values

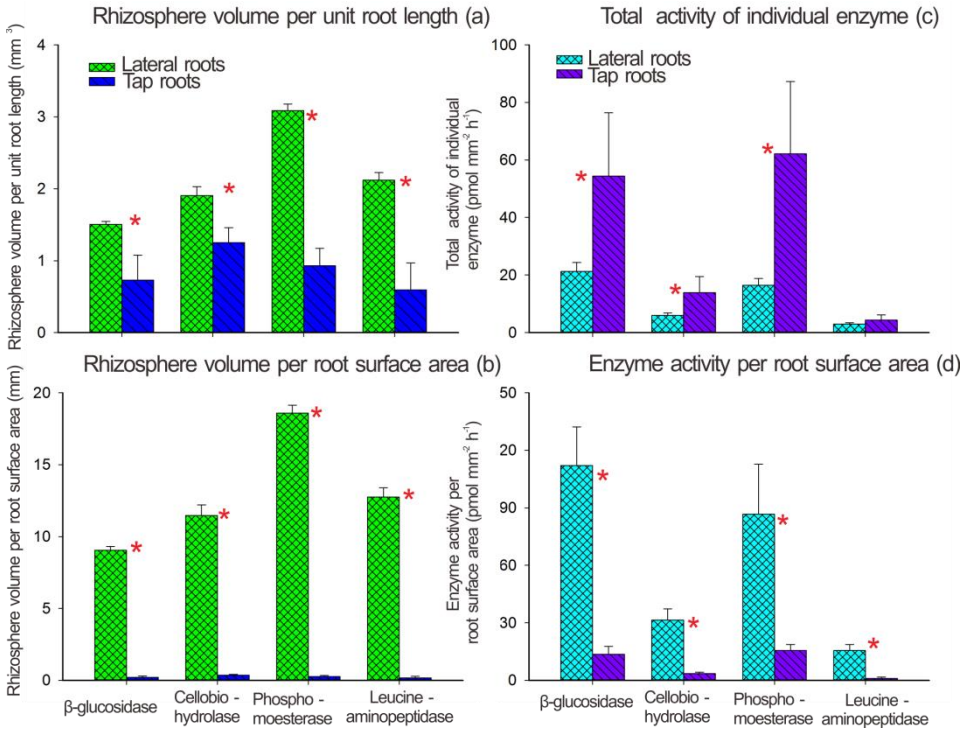

Fig. 5 Rhizosphere volume per unit root length (a); rhizosphere volume per root surface area, which was calculated as the rhizosphere volume divided by the root surface area (b); total activity of individual enzymes in the rhizosphere (c); enzyme activity per root surface area, which was calculated by summing all activity in the rhizosphere and dividing by the root surface area (d). Bars: means calculated from four replicates (±SE). Red asterisks: significant differences ($p < 0.05$, Duncan test) between lateral roots and taproots (all differences between lateral and taproots are highly significant, except for total activity of leucine aminopeptidase)

4. Discussion

4.1 Rhizosphere extent and dynamics of enzyme activities around the roots of lentil and lupine

Although enzyme activity in the rhizosphere of lentil increased during root growth, the rhizosphere extent remained constant (Fig. 2). The spatial stability of the rhizosphere extent reflects the equilibrium between enzyme input (synthesis by roots and microorganisms) and output (decomposition, turnover and other deactivation processes) of both extracellular and intracellular (Miralles et al. 2012; Nannipieri et al. 2018; Schimel et al. 2017). Rhizosphere microorganisms can be mutualistic as well as competitive with plant (Richardson et al. 2009). This stable pattern of rhizosphere extent is an efficient strategy for plants to mobilize and acquire nutrients in competition with rhizosphere microorganisms by enhancing available nutrient acquisitions (Kuzyakov and Xu 2013). The stable rhizosphere extent lasted longer for lentil than for lupine: for lentil it was constant during the whole period, but for lupine it broadened in the 8th week (Figs. 2a and b, 3a and b). This is directly related to the root radius: root radius of lentil remained constant, while the radius of lupine roots increased with age (Figs. 1, 2 and 3). The larger the radius, the more exudates will be released into the soil (Lambers et al. 2006), and therefore a broader rhizosphere develops around thicker roots (lupine).

The rhizosphere extent of leucine aminopeptidase activity for lentil was broader in the 4th than in the 1st week (Fig. 2d). This reflects an increased demand for N after 1 month of growth and hence enhanced production of N-acquiring enzymes to obtain more N from soil organic matter (Kelley et al. 2011). The rhizosphere extent of acid phosphomonoesterase was constant around lentil roots, while for lupine it was time-dependent: it was 2 times broader in the 1st and 8th weeks than in the 4th week (Figs.2c and 3c). Comparing the root morphologies of lentil and lupine explains this phenomenon: lentil has short and sparse root hairs, while the root hairs of lupine are relatively long and dense (Fig. S1). Acid phosphomonoesterase can be released into the rhizosphere directly by root hairs (Gahoonia et al. 2001; López-Bucio et al. 2003) and the activity increases with root hair' density and length (Tarafdar and Jungk 1987; Yan et al. 2004). Moreover, root hairs can indirectly enhance acid

phosphomonoesterase activity by releasing protons and organic acid to provide an acidic environment (Lambers et al. 2006; Šarapatka et al. 2004). However, because the lifespan of root hairs is short (few days) (Nguyen 2003), the decrease of hairs leads to the reduction of acid phosphomonoesterase activity rhizosphere extent of the 4 week-old lupine. Furthermore, soil microorganisms are other primary acid phosphomonoesterases producers (Nannipieri et al. 2011, 2012), and their biomass and community composition in the rhizosphere changed as plant growth (Berg and Smalla 2009; Lagos et al. 2016). These changes might have influenced the phosphomonoesterase gene expression and further affected the relative activity in the rhizosphere (Acuña et al. 2016; Luo et al. 2017). Such an explanation, however, required further studies to investigate the temporal links between microbial community composition, acid phosphomonoesterases genes expression and the acid phosphomonoesterases activity rhizosphere extent over plant development.

The activities of all enzymes except leucine aminopeptidase consistently increased during the whole growth period of lentil because of ongoing vegetative growth throughout the 8 weeks. In contrast, enzyme activities on the root surface decreased in the 8th week in the rhizosphere of lupine (Figs. 3 and S3) because lupine turned to reproductive growth in the 7th week. This is in line with the first and third hypothesis: enzyme activity in the rhizosphere depending on plant age and physiological state. Root exudation increases during vegetative growth, but it decreases when reproductive growth starts (Aulakh et al. 2001; De-la-Peña et al. 2010). This reduces the abundance of active microorganisms (Chaparro et al. 2014; Schmidt and Eickhorst 2014) and consequently leads to a down-regulation of enzyme production (Allison et al. 2010). Although the plants continued with vegetative growth, the activity of leucine aminopeptidase in the rhizosphere of lentil also sharply decreased in the 8th week (Fig. 2d). The microbial proteolytic community composition and their proteolytic genes expression and the protease activity were correlated with plant N acquisition (Baraniya et al. 2016). Therefore, the decrease of leucine aminopeptidase activity may be due to microorganisms shifting their enzyme production from enzymes responsible for degradation of relatively labile polypeptides (leucine aiminopeptidase) to enzymes decomposing relatively recalcitrant substrates (e.g. chitinase) (Kelley et al. 2011; Schimel and Schaeffer 2012) in order to acquire

more N, since N may be exhausted by the plant without regularly nutrient supplement (Giagnoni et al. 2016). Another possible explanation is that N acquired by lentil is already sufficient to cover its N demand, so N degrading enzymes activity decreased. Such explanations, however, require experimental confirmation by simultaneous determination of N acquisition and the activities of other enzymes responsible for N decomposition in the lentil rhizosphere, as N mineralization is a processes including several enzyme reactions (Nannipieri et al. 2018).

4.2 Rhizosphere volume and enzyme activities around lateral and tap roots of lupine

Root morphology has a strong impact on the rhizosphere volume and enzyme activities. The total activity of individual enzymes was higher around the taproots than around lateral roots (Fig. 5c), due to the greater radius of the taproots (Fig. 4). However, the lateral roots have larger rhizosphere volume and higher enzyme activity per root surface area (Fig. 5c and d). Thus, lateral roots and its surrounding microorganism have higher enzyme synthesis and lateral roots have higher rhizosphere volume efficiency than taproots do. This can be related to the changes in root anatomy: when roots become old, cork periderm will develop, cell walls thicken, and suberin will be deposited (McCormack et al. 2015). All these factors reduce the uptake of water and ions as well as exudate release (Gambetta et al. 2013; Steudle and Peterson 1998) and so, narrow the rhizosphere extent. In contrast, young lateral roots with smaller diameter have high exudation rates (McCormack et al. 2015), which can stimialted micrbial activity and enzyme production (Asmar et al. 1994). Therefore, lateral roots have much higher enzyme activity per root surface area and larger rhizosphere volume per root surface area compared to taproots. Generally, enzymes activity can change spatio-temporally in the rhizosphere being related to root morphology as well as plant, microbial physiology. However, zymography alone was not allowed to distinguish the contributions of root and microorganisms to these changes. Future research should combine zymography with detection and expression of enzyme encoding genes as well as other visualization technics such as *in situ* hybridization (FISH) to investigate the origins of enzymes (Nannipieri et al. 2012; Schmidt and Eickhorst 2014).

5. Conclusions

We quantitatively visualized the spatial distribution of enzyme activity *in situ* in the rhizosphere of lentil and lupine during the growth period. Lentil kept as vegetative growth and the rhizosphere extent was constant, while the enzyme activities at the root surface kept increasing. Lupine entered reproductive growth in the 7th week after planting companied with broader rhizosphere extent, however, the enzyme activity decreased by 10-50% compared to the vegetative stage. Lateral roots accounted for 1.5-3.5 times more rhizosphere volume per root length and 6-14 folds more enzyme activity per root surface area than taproots. Therefore, we conclude that plant growth and root morphology influenced enzyme activity and shape the rhizosphere as follows: the enzyme activity in the rhizosphere increased with plant growth until reproductive stage; lateral roots have much larger rhizosphere volume per unit root length and higher enzyme activity per root surface area than the taproots (Fig. 6). Future researches to investigate enzyme activity in the rhizosphere should consider the effects of plant development and root morphology. Zymogrpahy should combine with other techniques to elucidate the origin of enzymes in the rhizosphere.

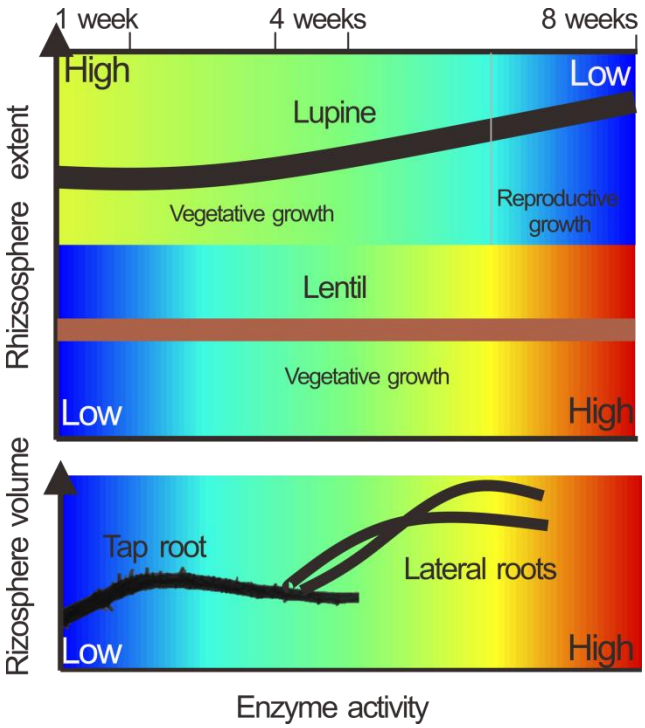

Fig. 6 Rhizosphere extent and enzyme activity dynamics of lentil and lupine roots (upper). Rhizosphere volume and enzyme activity for lateral and taproots of lupine (bottom). Upper: During vegetative growth of lentil the rhizosphere extent remained constant, while enzyme activities on the root surface increased. Lupine entered reproductive growth in the 7th week, which was accompanied by broader rhizosphere extent and decreased enzyme activity around the roots. Bottom: the enzyme activity (per root surface area) was higher and the rhizosphere volume was broader around lateral roots than around older taproots

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Supplement information

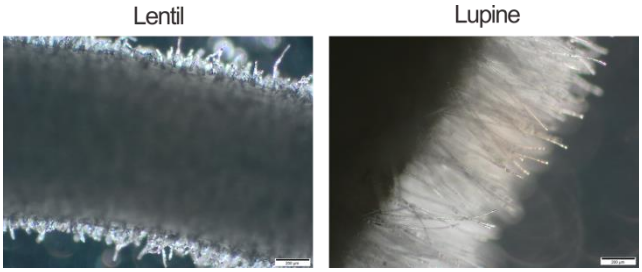

Fig. 1 Microscopy images of root hairs of lentil and lupine germinated on filter paper. The bar on the bottom right corner is indicates 200 µm

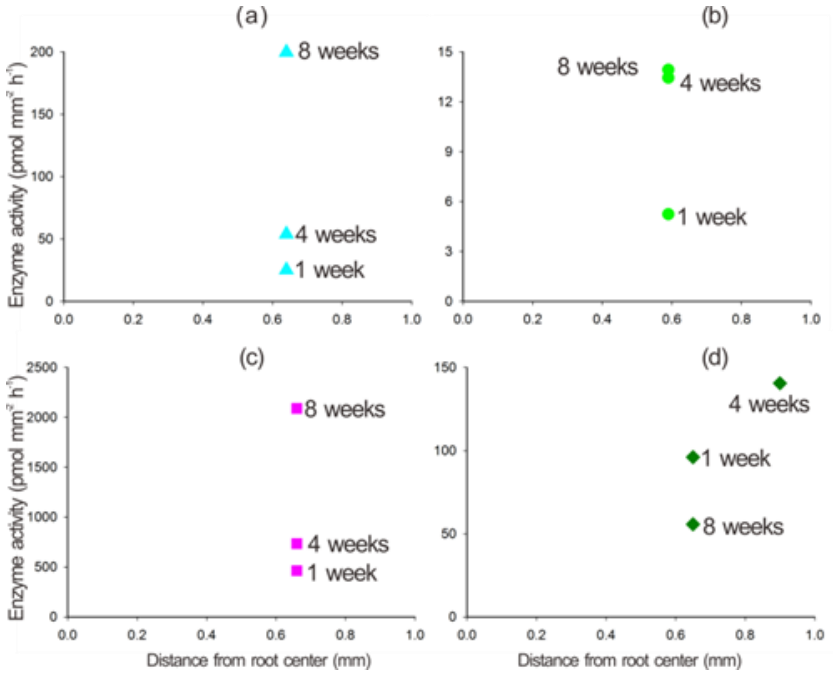

Fig. 2 Relationship between rhizosphere extent and enzyme activity for lentil

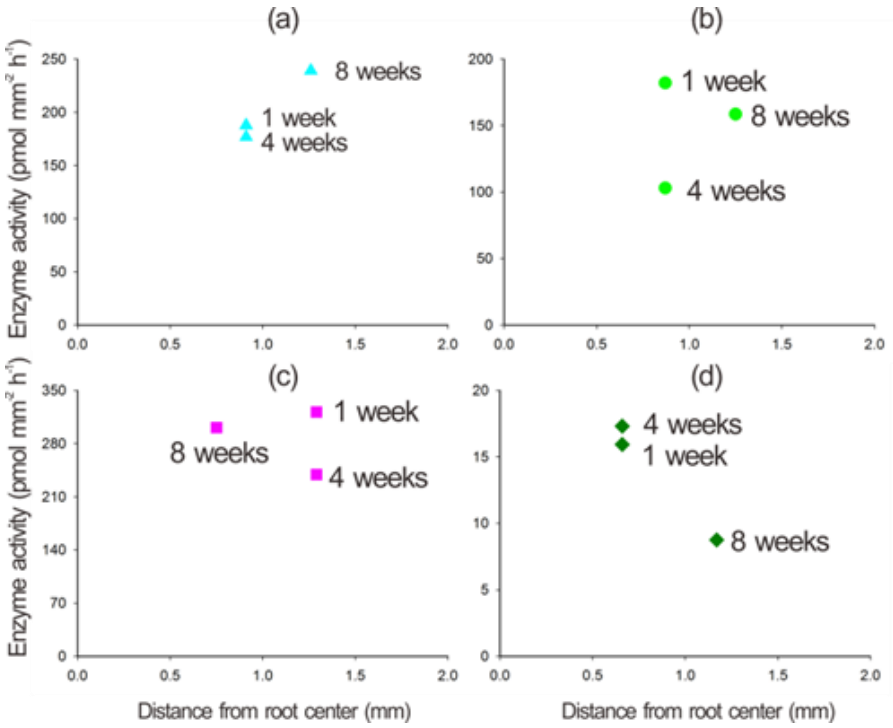

Fig. 3 Relationship between rhizosphere extent and enzyme activity for lupine

Study 3 Coupling of zymography with pH optode reveals changes in P acquisition strategies of white lupine with growth depending on P supply

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Abstract

Phosphorus availability effects on the spatial and temporal dynamics of phosphatase activity and pH in the rhizosphere are importance for the understanding plant nutrient acquisition strategies. For the first time, we combined zymography with pH planar optodes to visualize the effects of P-availability (lacking P, amended with phytate or ($Ca(H_2PO_4)_2$) on the spatial distribution of phosphatase activity, pH and their rhizosphere extent around taproot of lupine before and after cluster root formation. Phosphorus deficiency increased acid phosphatase activities by 20%, decreased pH by 0.8 units and broadens the rhizosphere extent by about 0.4 mm around taproot, while phytate addition smoothed these changes on before cluster root formations (on the 11^{th} day). In contrast, the rhizosphere extent of phosphatase activity around taproot of lupine was 0.2 mm narrower under P-deficiency than amended with $Ca(H_2PO_4)_2$ after cluster root formation (on the 24^{th} day). The hotspot areas of alkaline phosphatase activity was 40% larger while the shoot biomass and shoot P concentration were about 35% lower for lupine grown under P-deficiency than amended with $Ca(H_2PO_4)_2$ on the 24^{th} day. Therefore, we conclude lupine used different strategies to conquer phosphorus deficiency during growth: increased phosphatase activity and decreased pH and broaden their rhizosphere extent around taproot is importance before cluster root formation, while develop cluster root to increase phosphatase activity hotspot area and explore more soil volume played a vital role as plant grown older

Key words: Phosphorus availability; Rhizosphere extent; Zymography; Planar optode, Phosphatase activity; pH; Nutrient mobilization; Cluster root fomation

1 Introduction

Phosphorus (P) is the second quantitatively important major nutrient for plant growth (Bieleski, 1973; Raghothama, 1999), as it plays an important role in various metabolic pathways as well as a key component in nucleic acids, ATP and phospholipids (Vance et al., 2003). Although phosphorus is abundant in soil (Dalai, 1977), they are often unavailable for plants due to rapid formation of insoluble complexes with minerals (Al, Fe and Ca) and organic compounds (Fox and Comerford, 1992; Yao et al., 2018). As a result, about a third of terrestrial soils contain insufficient available P for optimum crop production (Batjes, 1997; Li et al., 2007). Therefore, the application of organic (eg., phytate is abundant in animal manure) and inorganic-P fertilizers kept rising to increase food production (Cordell et al., 2009).

To maximize phosphorus absorption efficiency, plants have evolved a range of mechanisms: 1) by altering root morphology through facilitate root growth and branching and increase the dense and length of root hairs to increase the soil volume explored by root (Lynch, 2005; Hill et al., 2006); 2) by symbiotic association with microorganisms such as mycorrhizal fungi to enhance phosphate availability and uptake ability (Richardson et al., 2001; Jakobsen et al., 2005); 3) by acidification of the rhizosphere to improve phosphate solubility; 4) by exudation of chelating organic acid and phosphatase to mobilize phosphorus (Hinsinger, 2001; Hocking, 2001). The rhizosphere (a small volume of soil around living root) is strongly influenced by root and microbial activity (Hilmer, 1904; Darrah, 1993), which is one of the most dynamic habitats on Earth (Hinsinger et al., 2009). Therefore, these adaptations not only improved phosphorus availability but also lead to chemical, physical and biological alteration in the rhizosphere.

Most of these mechanisms are exploited by white lupine (*Lupinus albus*.L) a model crop for phosphorus study, which can be grown on a broad range of soils (Weisskopf et al., 2006). Under phosphorus deficiency, the bottlebrush-like cluster or proteoid root will grow from the pericycle along the lateral root of lupine (Zobel, 1991; Neumann et al., 2000). The cluster roots are rows of rootlets covered with the dense root hairs, which efficiently increase soil volume and absorptive surface area

(Watt and Evans, 1999; Gilroy and Jones, 2000). Additionally, cluster roots release large amount of organic acid, proton and phosphatase to increase phosphorus availability (Gerke et al., 1994; Gilbert, 2000 #317). The exudation of organic acid and phosphatase is depended on cluster root development stage: they are high in mature than in senescent clusters (Neumann and Römheld, 1999). This is attributed to short lifespan of cluster root and decrease of exudation at the onset of senescence (Dinkelaker et al., 1995). Lupine should firstly develop embryonic taproot before forming cluster roots (Dinkelaker et al., 1995). However, the spatial and temporal aspects of taproot response to P-deficiency requires clarification in terms of: 1) the ability of taproots to release phosphatase, protons, leading to higher enzyme activity and acidification in the rhizosphere before and after cluster root formation, and 2) whether lupine exploits different strategies to improve phosphorus acquisition as root development and root morphology change.

Microorganisms also play a key role in providing phosphorus to plant by production of phosphatase to hydrolyze organic P (Illmer et al., 1995). Phosphorus availability in soil can directly and indirectly influence microbial phosphatase production ability (Harder and Dijkhuizen, 1983). The availability of inorganic P can directly suppress microbial production of phosphatase (Olander and Vitousek, 2000; Sinsabaugh et al., 2008). Phosphorus availability could influence plant growth and the root exudates (Grierson and Comerford, 2000), which further indirectly affect microbial activity and its phosphatase production (Philippot et al., 2013). The quality and amount of root exudates vary spatial and temporally and are dependent on root development and soil nutrient status (Neumann and Römheld, 1999; Badri and Vivanco, 2009). Therefore, phosphatase activity reflecting microbial activity and phosphorus availability should also change over root growth (Li et al., 2007; Marschner et al., 2011). Phosphatase can be separated into acid and alkaline phosphatase base on the activity pH optima. While both root and microorganism can produce acid phosphatase, alkaline phosphatase originates exclusively from microorganisms (Juma and Tabatabai, 1988; Nannipieri et al., 2011). Acid and alkaline phosphatase activity and their rhizosphere extent vary in response to inorganic-P supply (Spohn and Kuzyakov, 2013). This is an effective strategy to alleviate nutrient competition between root and microorganism, since they occupied

different spatial niche (Marschner et al., 2011). However, it is reminded unknown, the *temporal dynamics* of acid and alkaline phosphatase activity and as well as pH in response to P availability over root development. It is imperative to quantitative analysis of these parameters in order to better understand phosphorus acquisition strategies.

Zymography, allows quantitative, two dimensional visualization of enzyme activities, which has applied in the rhizosphere (Razavi et al., 2016; Ma et al., 2018), the detritusphere (Ma et al., 2017) and biopores (Hoang et al., 2016). pH planar optodes had been successfully used for gaining insights into dynamics and distributions of protons at the interface between water and sediments in marine systems (Hulth et al., 2002; Stahl et al., 2006) and the rhizosphere (Faget et al., 2013; Rudolph et al., 2013). For the first time, we coupled the zymography with pH planar optodes to test the spatial distribution and temporal dynamics of acid and alkaline phosphatase as well as pH in response to P availability (lacking P, amended with phytate or $(Ca(H_2PO_4)_2)$. We hypothesized that 1) before cluster root formation P-deficiency strongly stimulated phosphatase activity, increases soil acidification and broadens their rhizosphere extent around taproot of lupine, while the effects declined after cluster root formation; 2) acid and alkaline phosphatases activity varied in spatial and temporally in response to P availability and over root development, due to different origination and pH optima.

2. Material and method

2.1 Soil and plant preparation

The calcareous loess subsoil strongly limited in nutrients was selected and had following properties: $CaCO_3$ = 21.5%, calcium acetate lactate (CAL) extractable P 2.5 mg kg^{-1}, H_2SO_4 extractable P 332 mg kg^{-1}, organic C 0.1 %, total N 0.02 %, K = 40 mg kg^{-1}, soil solution pH = 7.1 . Air-dried soil was sieved to 2 mm and was mixed with quartz sand in a ratio of 7:3. After mixing, dissolved fertilizers were supplied before transplanting of the seedlings by application of 100 mg N kg^{-1} as NH_4NO_3, 150 mg K kg^{-1} as K_2SO_4, 50 mg Mg kg^{-1} as $MgSO_4$, 8.44 mg Fe kg^{-1} as Fe-EDTA, 2.60 mg Zn kg^{-1} as $ZnSO_4$, 1.00 mg Cu kg^{-1} as CuSO4 and 2.20 mg Mn kg^{-1} as $MnSO_4$. Three P treatments were (1) no P, (2) 80 mg P kg^{-1} as $Ca(H_2PO_4)_2$ and (3) 80

mg P kg^{-1} as sodlium phytate ($C_6H_6O_{24}P_6Na_{12}$). Rhizoboxes with inner dimensions of 21.2 × 10.8 × 3.3 cm were filled with the prepared soil to reach a final density of 1.2 g cm^{-3}.

Lupine (*Lupinus albus.* L) seeds were germinated on filter paper for 72 h and thereafter one seedling was planted at depth of 5 mm in each rhizobox. Each treatment had 4 replications in separate rhizoboxes. A total of 24 rhizoboxes were prepared in the experiment (4 replicates ×3 treatments ×2 harvest times (11^{th} and 24^{th} day)). The rhizoboxes were kept in a climate chamber at a controlled temperature of 20 ± 1 ℃ and a daily light period of 14 h with a photosynthetically active radiation intensity of 250 μmolm^{-2} s^{-1}. During the growth period, the rhizoboxes were kept inclined at an angle of 45° so that the roots grew along the lower wall of the rhizobox. The rhizoboxes were irrigated every day with distilled water to maintain at 70% water holding capacity. The spatial distribution of phosphatase activity and pH were monitored when lupine was 11 and 24 days old. After visualization of pH and phosphatase activity, the shoots from each rhizobox were harvested and dried in oven at 60 ℃ for 3 days for estimation of biomass and total P content. Total P was extracted by pressure digestion in concentrated nitric acid and determined by inductively coupled plasma atomic emission spectroscopy (ICP) (Crosland et al., 1995).

2.2 pH measurement by planar optodes

The pH was visualized by a sensor foil embedded with fluorescent indicator dye (Blossfeld and Gansert, 2007). The foils contain both analyte-sensitive and analyte insensitive dyes. Green fluorescence is declined at lower pH, whereas the red fluorescence is unaffected. A camera connected to PC was used to detect these fluorescence signals. Subsequent data analysis by the open source software imageJ created the ratio of the red and the green channel (R values), a quantitative two-dimensional pH map (Blossfeld and Gansert, 2007). The pH sensitive foil (SF-HP5R), the camera (VisiSens TD), the LED light with wavelength of 470 nm and the image capture software (VisiSens AnalytiCal) all purchased from company (PreSens GmbH, Regensburg, Germany). The rhizoboxes were opened from the lower, rooted side, the images of the root was taken by camera (EOS 5D, Canon) and the pH foil (10 × 14

cm) was applied to the soil surface. After incubated on the soil surface for 2 hours, a snap shot image was taken in the dark room. The positions of the camera, the sample and the LED light were fixed throughout the experiment.

A calibration line was prepared from 1 cm^2 pH foils that were soaked in mixed buffer solution of NaH_2PO_4 • H_2O and Na_2HPO_4• 2 H_2O in a range of pH (7.74, 7.48, 7.12, 6.78, 6.44, 6.12, 5.58) for two hours. The calibration image was taken under the same setup as described above.

2.3 Direct soil zymography

After pH measurement, direct zymography (Sanaullah et al., 2016) was used to visualize the acid phosphatase and alkaline phosphatase activity in soil. Methylumbelliferyl (MUF)-phosphate (substrate) was dissolved in buffer to a concentration of 12 mM (MES buffer, pH 6.5, for acid phosphatase and TRIZMA-Base buffer, pH 11.0, for alkaline phosphatase). When the substrate is hydrolyzed by enzyme, MUF is released and becomes fluorescent and can be visualized under UV light (Spohn and Kuzyakov, 2013). The membrane saturated with substrate solution was applied directly to the rooted soil surface. Soil zymography was performed first for acid phosphatase, then for alkaline phosphatase on the same rhizobox. Each membrane was incubated on the soil surface for 1 h, thereafter the membranes were carefully lifted off the soil surface and any attached soil particles were gently removed using soft brush. The membranes were placed under ultraviolet (UV) illumination with an excitation wavelength of 355 nm in a dark room and a photograph of the membrane was taken. The camera (EOS 5D, Canon), the sample, and the distance to the UV light were fixed for all rhizoboxes .

A calibration line was prepared from 4 cm^2 membranes squares 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). The amount of MUF per unit area was calculated from the volume of solution taken up by the membrane and its size (Spohn and Kuzyakov, 2014). The membranes used for calibration were imaged under UV light in the same way as described for the zymography.

2.4 Image analysis

Image processing and analysis were done using the open source software imageJ. Based on the number of pixels and known distance, all the images were adjusted to the scale. The root images were converted to 8-bits gray values. Five 200 × 200 pixels areas per image were placed in patches of root-free soil to calculate bulks soil gray values. The center of root radius was defined as the middle position of two boundaries. The root radius was calculated by the Euclidean distance from the middle position to the boundary of root on one side (Danielsson, 1980).

The images of zymograms were converted to 8-bit gray values and corrected for light variations and camera noise. A linear function was used to relate the grayvalues of the calibration membranes to the MUF concentrations, and consequently to enzyme activities. Base on calibration line gray values at the MUF concentration of zero used as background signal and was subtracted. The bulk soil enzyme activity was calculated as the mean activity of five 200 × 200 pixels areas per image were placed in patches of root-free zymogram. The zymographies were segmented; the mean phosphatase activity in the rhizosphere was calculated. The rhizosphere extent of enzyme activity was defined as the distance from root center to the position at least 20% higher than in the bulk soil. Enzyme activity 5% higher than the average activity in the rhizosphere was defined as hotspots, the hotspot area was calculated based on the pixel size.

For the pH images, digital images were split in to R (red), G (green) and B (blue) channels. After splitting, the red and green channels were transformed to 32-bit gray scale images. The ratio between red and green channel was calculated. The ratios were converted to pH values based on the calibration curve. The relationship between the measured ratio and the corresponding pH value is sigmoidal, and can mathematically be described by the Boltzmann Equation (Blossfeld and Gansert, 2007; Faget et al., 2013):

$$Y = y_0 + \frac{a}{1 + e^{-\left(\frac{x - x_0}{b}\right)}}$$

Where Y is the measured ratio, x is the corresponding pH, y_0 is the maximum ratio value, a is the maximum ratio minus minimum ratio value, b is the slope of the curve and x_0 is the inflexion point of the sigmoidal curve. The bulk soil and rhizosphere pH values were calculated similarly to enzyme activity as described above. The rhizosphere extent of pH was defined as the distance from the root center toward a location at least 0.3 pH units lower than in the bulk soil.

2.5. Statistical analyses

Normality and homogeneity of enzyme activity and pH values in the bulk soil and rhizosphere, hotspot area, root and shoot biomass and shoot P content were analyzed using Shapiro-Wilk's test and Levene test. Significance of differences between P-treatments were tested by one way ANOVA followed by the Duncan-test using the software SPSS18.0, at $\alpha < 0.05$

3. Results

The acid and alkaline phosphatase activity were 50-100% higher in the rhizosphere (Figs. 1, 2 and 4), and decreased from root center toward bulk soil (Fig. 3). In contrast, the pH values were 0.4-1.6 unit lower in the rhizosphere (Figs. 1, 2 and 4), and kept increasing toward bulk soil (Fig. 3). Cluster roots formed for lupine grown in soil lacking P or amended with phytate on the 24^{th} day (Figs. 2 and S1).

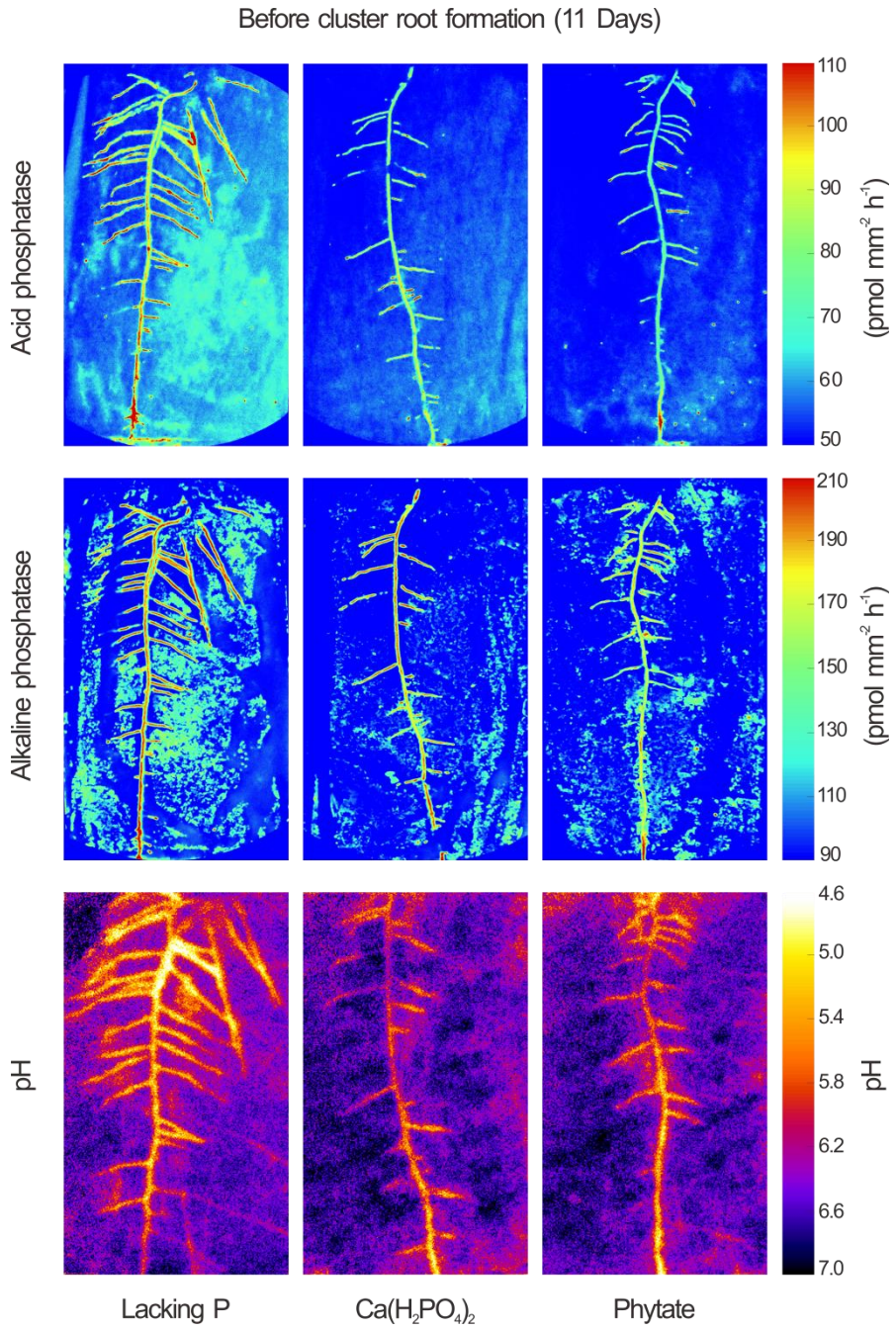

Fig. 1 Examples of acid phosphatase activity, alkaline phosphatase activity and pH in the rhizosphere of lupine before cluster root formation (11 days old). Columns from left to right represent lupine root grown in soil lacking P, amended with $Ca(H_2PO_4)_2$ and added with phytate, respectively. Side color scale is proportional to enzyme activities (pmol mm^{-2} h^{-1}).

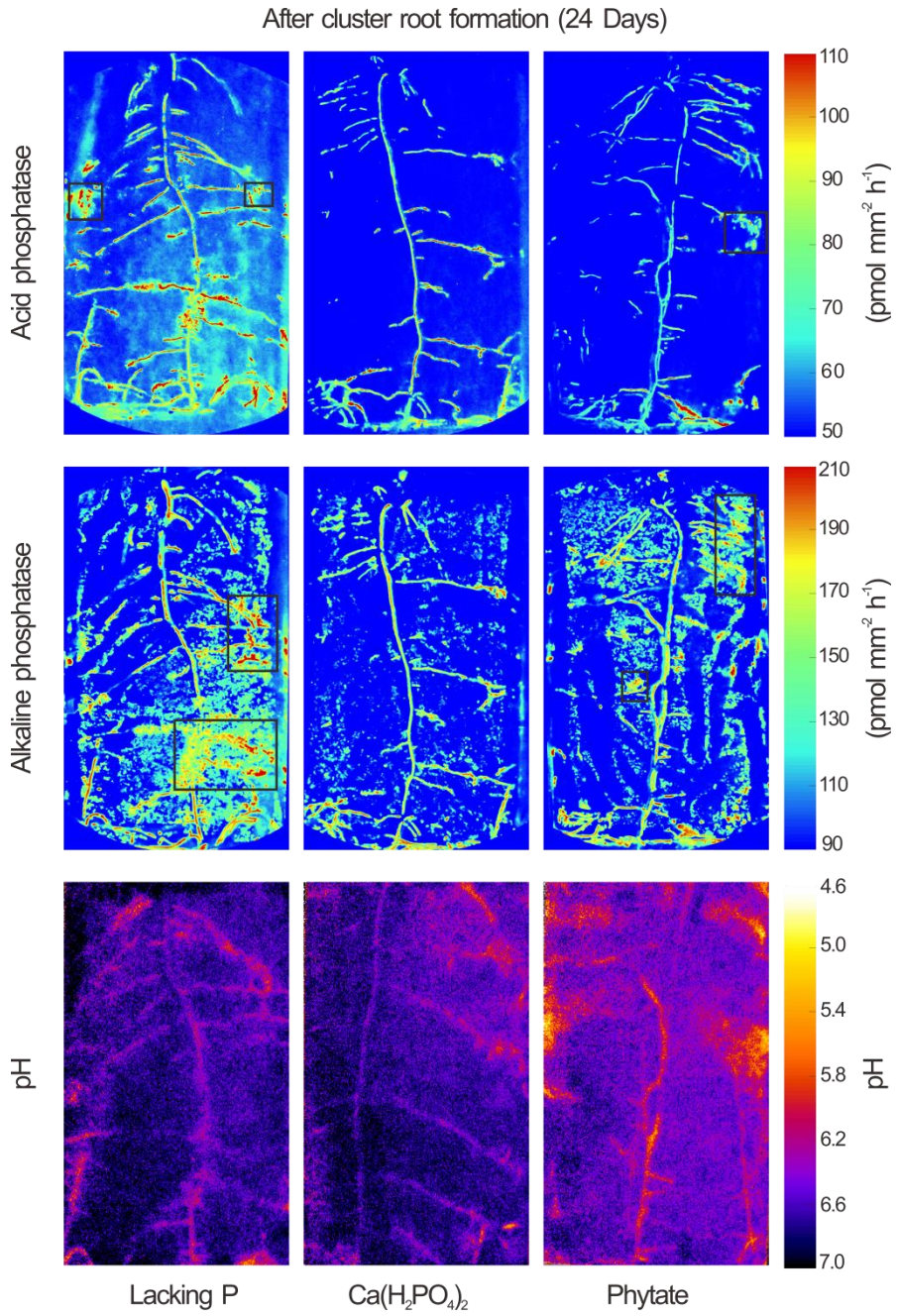

Fig. 2 Examples of acid phosphatase activity, alkaline phosphatase activity and pH in the rhizospshere of lupine after cluster root formation (24 days old). Columns from left to right represent lupine root grown in soil lacking P, amended with $Ca(H_2PO_4)_2$ or with phytate, respectively. Black rectangles on the zymograms indicated the cluster root images. Side color scale is proportional to enzyme activities (pmol mm^{-2} h^{-1})

3.1 Dynamics of rhizosphere extent in the taproot zone

The rhizosphere extent of acid phosphatase and pH around taproot were 0.35-0.52 mm broader for lupine grown in soil under P deficiency than in soil added with $Ca(H_2PO_4)_2$ before cluster roots formation (11^{th} day) (Fig. 3A and 3E, Table. S1). The rhizosphere extent of alkaline phosphatase was similar between treatments about 1.10 mm (Fig. 3C, Table.S1). In contrast, the rhizosphere extent of both acid and alkaline phosphatase around taproot for lupine grown in soil amended with phytate or absent of P were about 0.2 mm narrower than amended with $Ca(H_2PO_4)_2$ after cluster roots formation (24^{th} day) (Fig. 3B and 3D, Table.S1). The rhizospshere extents of pH were similar between treatments around 0.8 mm on the 24^{th} day, and were 0.7-1.2 mm narrower than on 11^{th} day (Fig. 3F, Table. S1). In summary, P deficiency facilitated rhizosphere extension of acid phosphatase activity and pH around taproot of lupine before cluster root formation (11^{th} day), while inhibited their extensions after cluster root formation (24^{th} day).

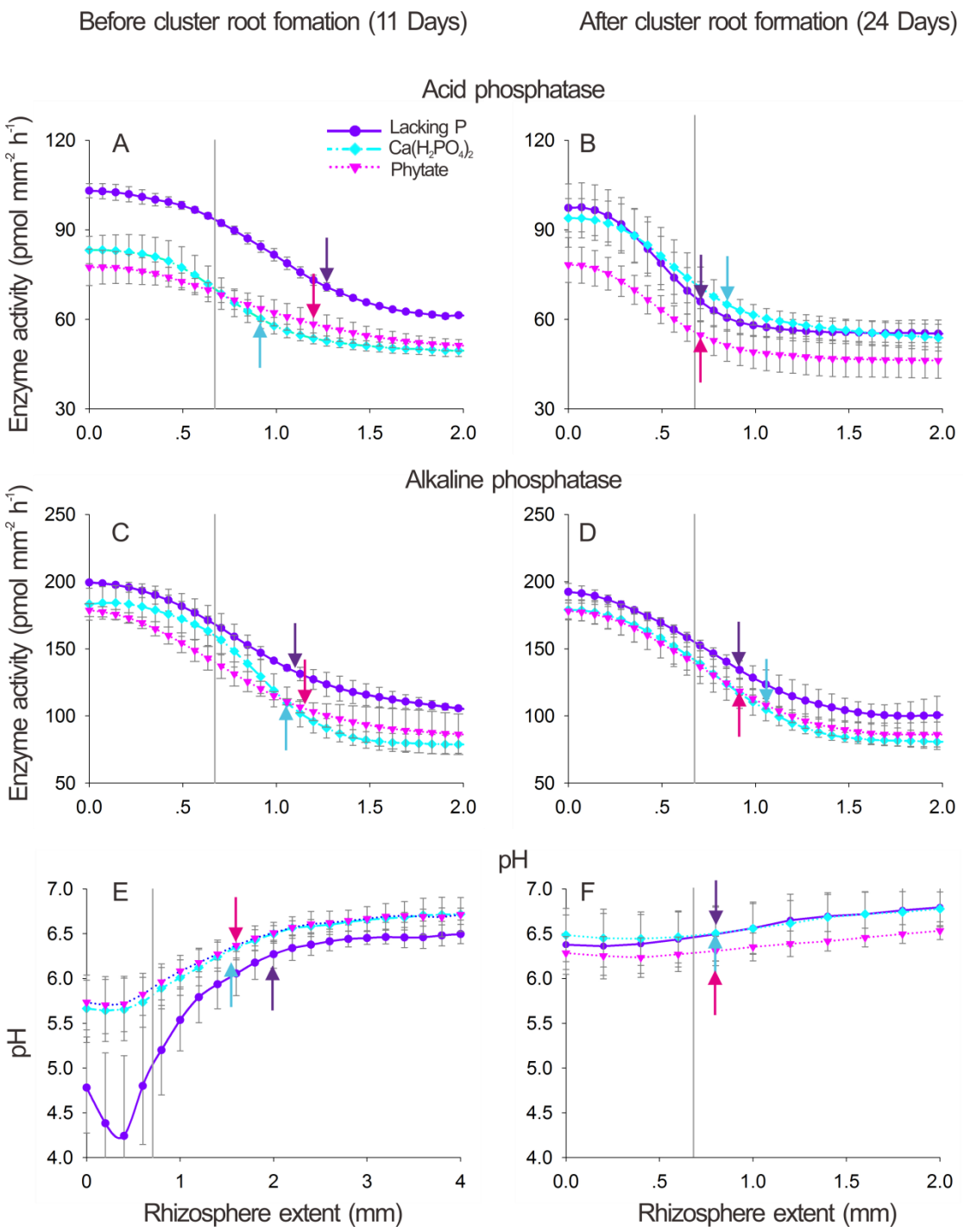

Fig. 3 Acid phosphatase (A and B), alkaline phosphatase(C and D) and pH (E and F) as a function of distance from taproot center of 11 (left) and 24 (right) day-old lupine grown in soil lacking P, amended $Ca(H_2PO_4)_2$ or with phytate, respectively. Vertical gray lines indicate average root radius. Vertical arrows on the curves show rhizosphere extent for enzyme activity or pH. Data points indicate means calculated from four replicates (±SE).

3.2 The dynamics of phosphatase activity, pH, hotspot areas and biomass response to phosphorus treatments

Phosphorus deficiency increased the acid and alkaline phosphatase activity and acidification in the rhizosphere of lupine before cluster root formation (11^{th} day) (Fig. 4A, B and C). For example, the acid phosphatase activity was 20% higher and the pH was 0.8 units lower ($p<0.05$) in the rhizospshere of taproot for lupine grown in soil lacking of P than in other two treatments (Fig. 4A and 4C). However, the difference of these parameters (except alkaline phosphatase activity) between lupine grown in soil under P deficiency and in soil amended with $Ca(H_2PO_4)_2$ was absent after cluster root formation (24^{th} day) (Fig. 4B and 4F). The alkaline phosphatase activity was still 5% higher ($p<0.05$) in the rhizosphere taproot for lupine grown under P-deficiency than other two treatments on the 24^{th} day (Fig. 4D). To summarize, P-deficiency increased phosphatase activity and decreased the pH around taproot for lupine before cluster root formations (11^{th} day), these effect weakened or even disappeared after cluster root appeared (24^{th} day).

The difference of phosphatase activity hotspot areas, shoot biomass and shoot total P content between treatments was absent on 11^{th} day (Fig. 5 A-D). However, the hotspot areas of alkaline phosphatase for lupine grown in soil either lacking P or amended with phytate were up to 40% higher than added with $Ca(H_2PO_4)_2$ on 24^{th} day (Fig. 5B). In contrast, the shoot biomass and shoot total P concentration of lupine grown in soil under P-deficiency were about 35% lower than amended with $Ca(H_2PO_4)_2$ on 24^{th} day ($p<0.05$) (Fig. 5D). In summary, P-deficiency depressed lupine shoot biomass and shoot P concentration, but stimulated phosphatase activity hotspot areas formation.

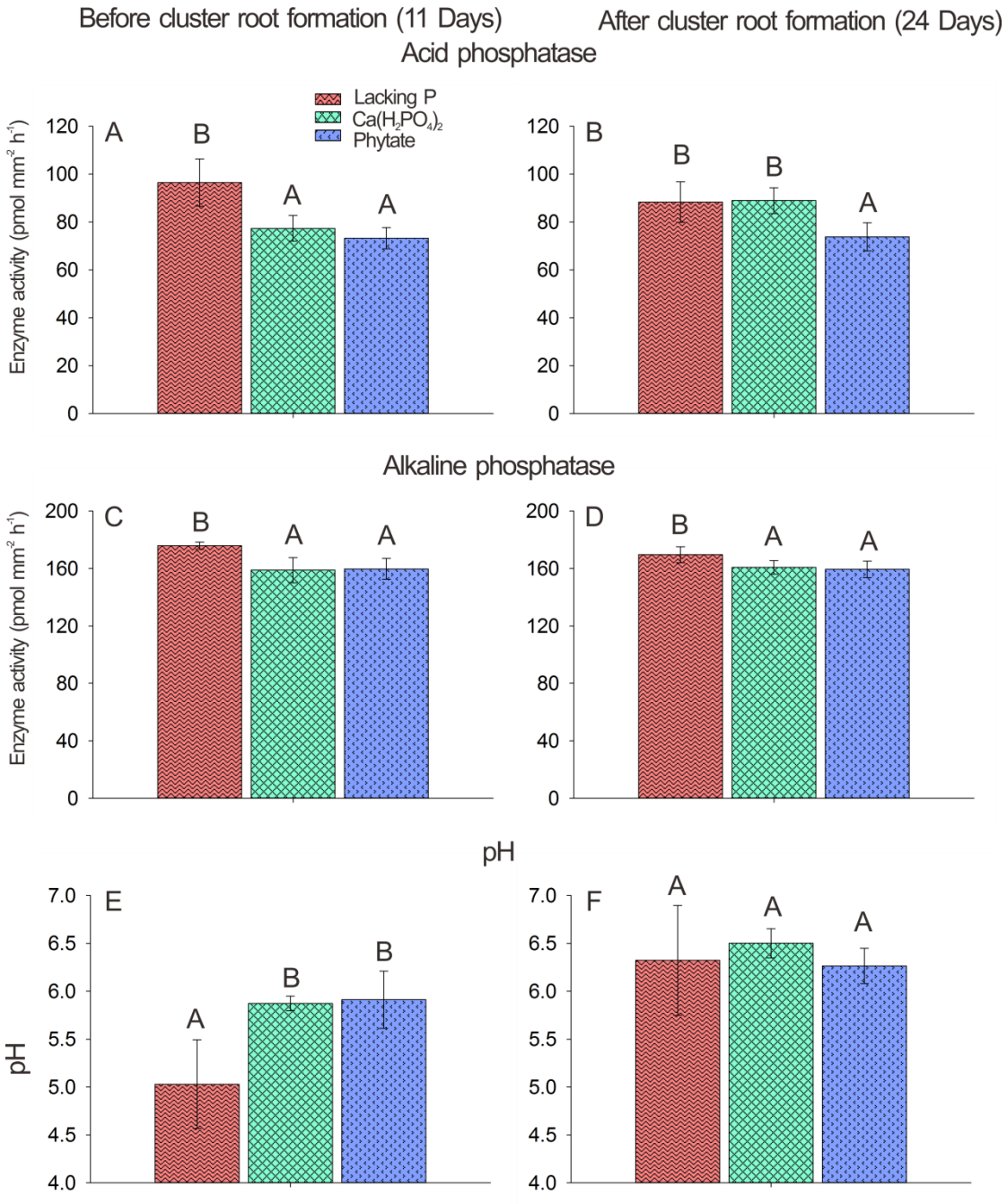

Fig. 4 Activities of acid phosphatase (A and B) and alkaline phosphatase (C and D), and pH (E and F) in rhizosphere of lupine grown in soil lacking of P, added with $Ca(H_2PO_4)_2$ and added with phytate, respectively, before (11 days) and after (24 days) cluster root formation. Letters indicate significant differences ($p < 0.05$ after Duncan test) of enzyme activity and pH in the rhizosphere between levels.

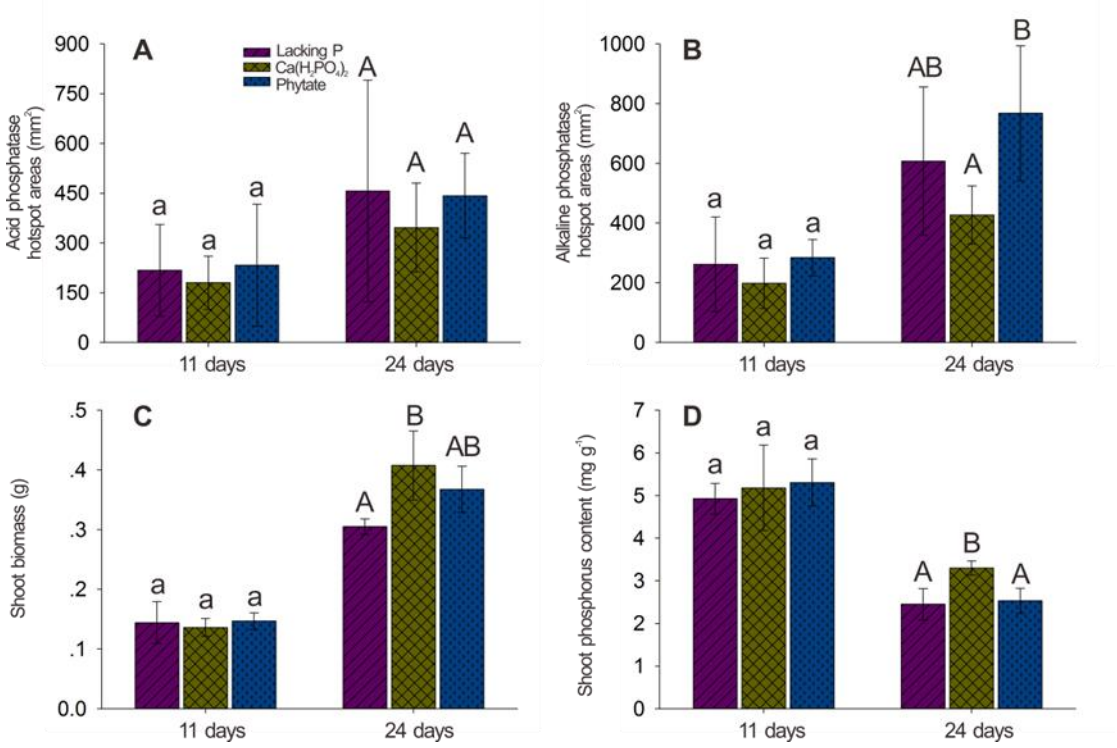

Fig. 5 Acid and alkaline hotspot area (A and B), shoot biomass (C) and shoot P content (D) of 11 and 24-old day lupine grown in soil lacking P, amended with $Ca(H_2PO_4)_2$ or with phytate, respectively. Small and capital letters indicates significant differences ($p < 0.05$ after Duncan test) of these parameters between treatments on 11 and 24 days, respectively.

4. Discussions

4.1 Response to P availability before cluster root formation

Consistent with our first hypothesis, phosphorus deficiency stimulated acid phosphatase activity, increased soil acidification and broaden their radial extent around taproot of lupine before cluster root formation (Figs. 3A, C, E, 4A, C, E and Table.S1). This indicated that before cluster root formation, taproot can release more phosphatase and protons and conversion of P from non-available into available to conquer P scarcity. Under P deficiency, roots can enhance phosphorus availability either directly by release more acid phosphatase and indirectly through release more root exudates and other rhizodeposits which further stimulate microbial activity and the production of phosphatase to mineralize organic-P (Jones et al., 2009). Besides,

roots can exude a wide range of organic acid anions to mobilize P by ligand exchange (Neumann and Römheld, 1999; Rengel, 2002). In order to maintain charge balance, cations such as protons should be excreted together with the anions, which leads to rhizosphere acidification (Hinsinger, 2001; Hinsinger et al., 2003), and further enhance Ca-phosphates solubilization (Neumann et al., 2000). Moreover, P deficiency inhibits nitrate but enhances ammonium uptake, which leading to more H^+ release and Ca-phosphates dissolution (Neumann et al., 1999). The more H^+ and phosphatase released, the larger areas are required and the broader rhizosphere extents are established. The P-deficiency also increased phosphatase activity and soil acidification in the bulk soil (Fig.4). However, phosphorus treatments did not affect the rhizosphere extent of alkaline phosphatase (Fig. 3C). This can be attributed to the suitable pH condition for alkaline phosphatase activity is alkaline (Nannipieri et al., 2011), and the P-deficiency leaded acid circumstance in the rhizosphere offset the alkaline phosphatase production and its activity (Dick et al., 1983; Weisskopf et al., 2006). Although organic phosphorus cannot be used directly by plant, the phytate addition reduced phosphatase activity, pH decrease and their extension (Fig.3 A, C, E and Fig.4 A, C, E). This is because phosphorus solubility is a key factor influencing organic acid and H^+ release (Gerke, 2015). Amended with abundant dissolved phytate, the amount of organic acid and protons release by root to exchange and solubilize bounded-P will be reduced. Corresponding microbe activity and its phosphatase production ability will decline due to decrease of available carbon.

4.2 Temporal dynamics response to P treatments

The pH on the taproot surface was more neutral, its rhizosphere extent was narrower and the response to phosphorus treatments was disappeared after cluster root formation (24^{th} day) (Figs. 3F and 4F). The spatial and temporal different patterns of rhizosphere acidification can be attributed to root anatomy changes in the course of root aging, e.g., development of sclerenchymatous in the outer cortex, thickening of the cell walls and deposition of suberin (McCormack et al., 2015). All these factors hamper proton exudation (Colmer and Bloom, 1998; Gambetta et al., 2013), thus leading the soil acidification disappearance and rhizosphere extent narrowness.

Interesting, the rhizosphere extent of phosphatase activity surround the taproot of lupine grown under P-deficiency was narrower than amended with reduced $Ca(H_2PO_4)_2$ on the 24th day (Fig.3B and 3D), while inverse effect was observed on the 11th day. This phenomenon can be explained by two mechanisms. Firstly, plants require more phosphorus as growing; the endogenous P from seed will be exhausted. Consequently longtime exposure to P deficiency environment can impede plant photosynthesis and decrease exudation, which will inhibit microbial and enzyme activity (Schimel and Weintraub, 2003). In contrast, adequate phosphorus supply stimulated plant growth, the carbon allocation belowground positive correlated with photosynthesis ability (Kuzyakov and Cheng, 2001). Therefore, amended with $Ca(H_2PO_4)_2$ more exudates will be released, which can stimulate microbial growth and enzyme synthesis (Kuzyakov et al., 2002; Nannipieri et al., 2007). This explanation can be confirmed by higher shoot P concentration and larger shoot biomass for lupine grown in soil amended with $Ca(H_2PO_4)_2$ than in P-deficient soil (Fig. 5D). Secondly, the root morphology was altered under P-deficiency, lupine invested energy to the cluster root formation rather than to the taproots exudation. This explanation supported by the larger phosphatase activity hotspot area under P-deficiency on 24th day, which mainly attributed to the cluster root formation (Figs. 2 and 2S).

Contrasting to acid phosphatase, the alkaline phosphatase activity still significantly higher ($p<0.05$) in the rhizosphere of taproot for lupine grown under P deficiency than amended with $Ca(H_2PO_4)_2$ on the 24th day (Fig. 4B). This indicated that the duration of increased enzyme activity caused by P-deficiency last longer for alkaline phosphatase than for acid phosphatase. This can be explained by considering the producers of these two enzymes: alkaline phosphatase can be produced by both plant and microbes, while acid phosphatase can only produce by plants (Dick et al., 1983; Juma and Tabatabai, 1988; Nannipieri et al., 2011). On one hand, root may reduce the acid phosphatase release when aging (Yadav and Tarafdar, 2001). On the other hand, the pH in the rhizosphere are more neutral on the 24th day (Fig. 3F and 4F), microorganism may shift their community to produce more alkaline phosphatase under neutral and alkaline condition (Renella et al., 2006). Furthermore, the increased

of pH will inhibit acid phosphatase but stimulated alkaline phosphatase activity (Dick et al., 2000).

5. Conclusions

For the first time, we combined zymography with pH planar optodes to visualize the effects of P-availability on the *in situ* phosphatase activity, pH and their rhizosphere extent around taproot of lupine before and after cluster root formaiton. Phosphorus deficiency increased acid phosphatase activities by 20%, decreased pH by 0.8 units and broadens the rhizosphere extent by about 0.4 mm around taproot, while phytate addition smoothed these changes on before cluster root formations (on the 11^{th} day). In contrast, the rhizosphere extent of phosphatase activity around taproot of lupine was narrower under P-deficiency than amended with $Ca(H_2PO_4)_2$ after cluster root formation (on the 24^{th} day). The hotspot areas of alkaline phosphatase activity was 40% larger while the shoot biomass and shoot P concentration were about 35% lower for lupine grown under P-deficiency than amended with $Ca(H_2PO_4)_2$ on the 24^{th} day. Therefore, we conclude lupine used different strategies to conquer phosphorus deficiency during growth: increased phosphatase activity and decreased pH and broaden the rhizosphere extent around taproot is importance before cluster root formation (Fig. 6), while develop cluster root to increase phosphatase activity hotspot area and explore more soil volume played a vital role as plant grown older (Fig.6).

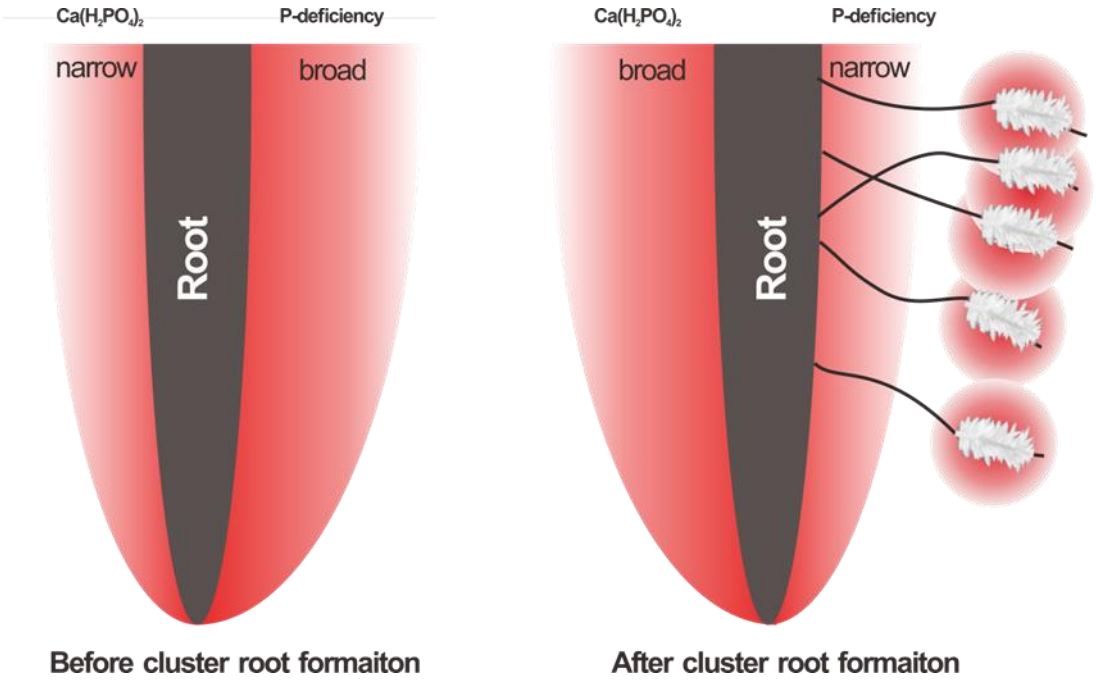

Fig. 6 Spatial distribution of pH and phosphatase activity in the rhizosphere of lupine in response to P availability before and after cluster root formation. The red show indicated the rhizosphere extent of pH or phosphatase activity.

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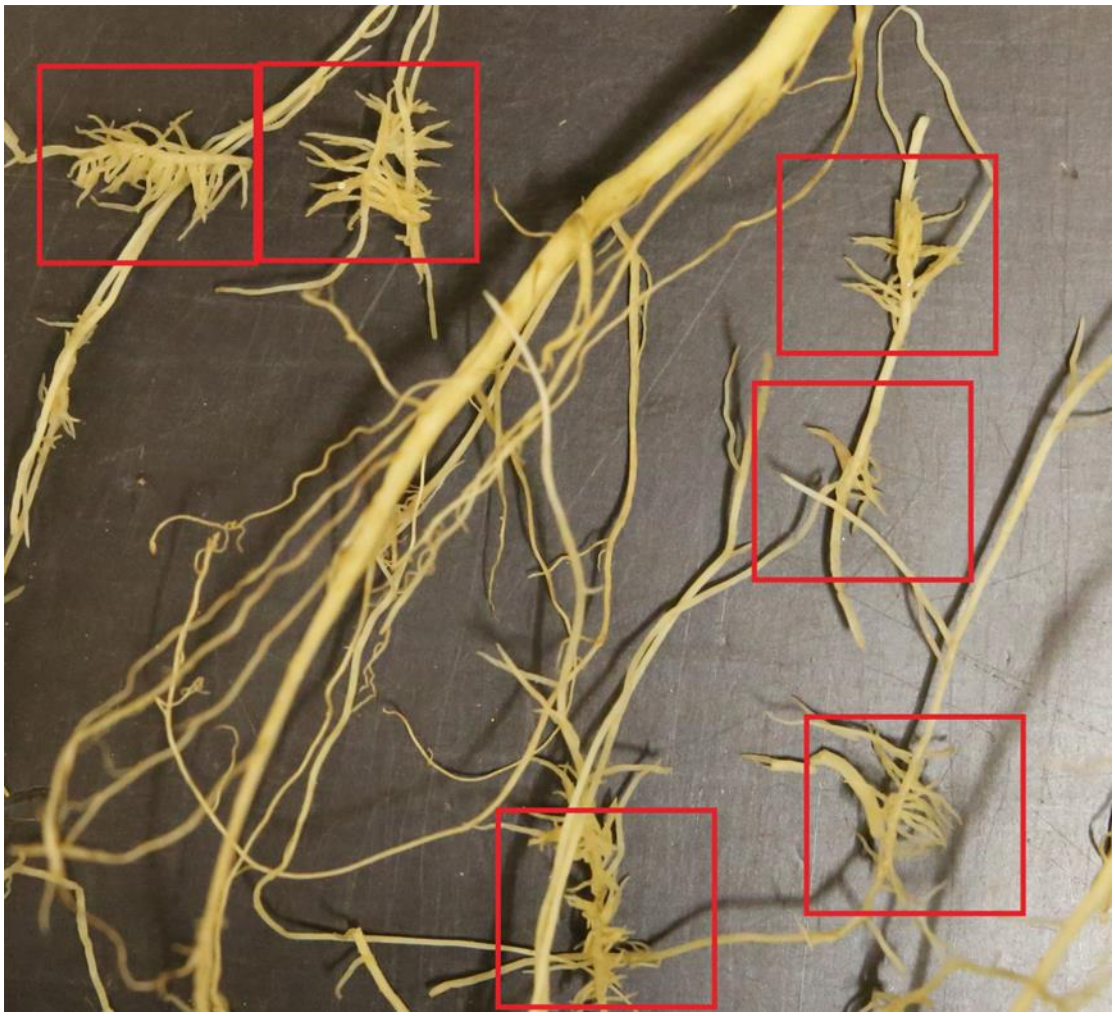

Fig. S1 The roots of lupine grown in soil under phosphorus deficiency. Red rectangles show the cluster roots.

Table S1 Rhizosphere extent of acid phosphatase , alkaline phosphatase and pH around taproot of 11 and 24 day-old lupine grown in soil lacking P, amended $Ca(H_2PO_4)_2$ or with phytate, respectively

P- treatments	Rhizosphere extent (mm) 11 day			Rhizosphere extent (mm) 24 day		
	Acid phosaphatase	Alkaline phospha tase	pH	Acid phosaphtase	Alkaline phospha tase	pH
Lacking P	1.25 ±0.14	1.11±0.10	2.05± 0.50	0.68 ±0.12	0.91 ±0.09	0.80± 0.10
$Ca(H_2PO_4)_2$	0.90± 0.08	1.05±0.09	1.53± 0.30	0.88±0.23	1.05 ±0.06	0.85± 0.25
Phytate	1.13 ±0.20	1.16±0.09	1.60± 0.16	0.68 ±0.15	0.92 ±0.03	0.85± 0.1

Study 4 Warming increases hotspot areas of enzyme activity and shortens the duration of hot moments in the detritusphere

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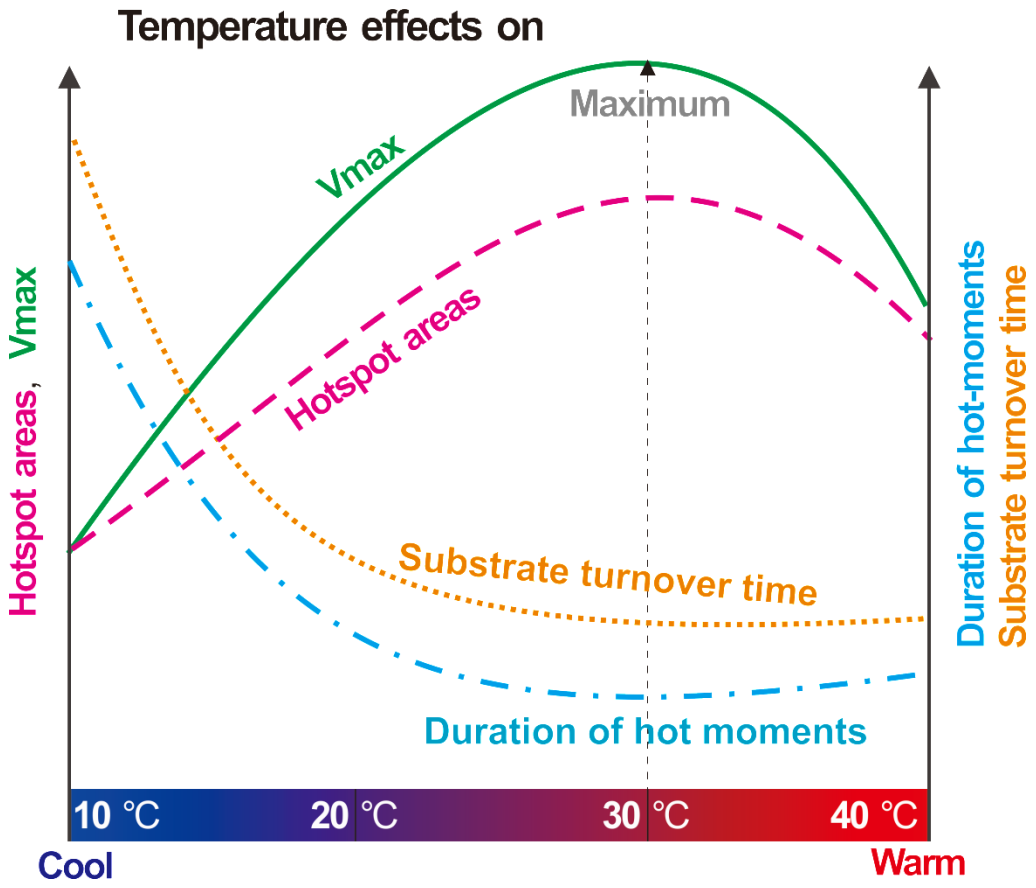
Graphic abstract



Highlights

1. Hotspot areas and Vmax increased with temperature up to 30 ℃, but decreased at 40 ℃.

2. Warming shortened enzymatic hot moments and decreased the substrates' turnover time in the detritusphere.

3. Hotspot areas of enzyme activity closely correlated with Vmax.

4. Hotspot areas of enzyme activity are larger in the detritusphere than rhizosphere.

Abstract

Temperature effects on enzyme kinetics and on the spatial distribution of microbial hotspots are important because of their potential feedback to climate change. We used direct zymography 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 detritusphere (7 and 14 days after cutting shoots). Soil zymography was coupled with enzyme kinetics to test temperature effects (10, 20, 30 and 40 ℃) on the dynamics and localization of these three enzymes in the detritusphere. Total hotspot areas of enzyme activity were 1.9-7.9 times larger and their extension was broader in the detritusphere compared to rhizosphere. From 10 to 30 ℃, 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 ℃. 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 ℃ were 1.7-6.7-fold faster than at 10 ℃. The K_m of cellobiohydrolase and phosphatase significantly increased at 30 and 40 ℃, indicating high enzyme conformational flexibility, or isoenzyme production at warm temperatures. We conclude that soil warming (at least up to 30 ℃) increases hotspot areas of enzyme activity and the maximum reaction rate (V_{max}) in the detritusphere. This, in turn, leads to faster substrate exhaustion and shortens the duration of hot moments.

Key words: Soil warming; Enzyme kinetics; Soil zymography; Rhizosphere, Detritusphere

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 detritusphere (i.e. decaying roots and shoot residues) also forms the hotspots of microbial and enzyme activity because dead litter releases highly polymeric recalcitrant as well as low molecular weight organics (Kögel-Knabner, 2002; Bastian et al., 2009). The rhizosphere and 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 detritusphere versus 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 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). Therefore, hot moments - the events that accelerate processes as compared to the average rates (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, however, whether temperature affects the dynamics and localization of enzyme activity hotspots. Especially due to the high heterogeneity in the rhizosphere and detritusphere, the substrate availability varies in time and space (Ekschmitt et al., 2005). This calls for *in situ* monitoring of 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).

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). Nonetheless, the temperature effect on temporal changes in the spatial distribution and kinetic parameters of soil enzymes remains to be tested. Therefore, for the first time, zymography was coupled with Michaelis-Menten kinetics in the detritusphere to test temperature effects (10, 20, 30 and 40 ℃) 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 of enzyme activities in the rhizosphere and detritusphere. We hypothesized that 1) due to the concentrated input of substrate in the detritusphere, the hotspot areas of enzyme activities in the detritusphere are larger than in the rhizosphere, 2) initially, the hotspot areas in the detritusphere increase faster at warm temperatures than at cold temperatures, 3) due to faster substrate decomposition, the duration of hot moments is shorter at warmer temperatures, 4) 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}, and water content 30% of water holding capacity (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 ℃ 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 ℃, 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 ℃), before the cutting shoots, as well as 7 and 14 days after the cutting (for samples kept at 10, 20, 30 and 40 ℃). 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 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 detritusphere (7 and 14 days after shoot cutting) was tested by ANOVA followed by the Duncan-test using the software SPSS18.0, at $\alpha < 0.05$.

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 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 ℃, 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

$$v = \frac{V_{max} \times [S]}{K_m + [S]} \qquad (1)$$

where v is the reaction rate (as a function of enzyme concentration), [S] is the substrate concentration, K_m is the substrate concentration at half-maximal rate, and V_{max} is the maximum reaction rate. 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 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.

3. Results

3.1. Distribution of enzyme activity in the rhizosphere and detritusphere

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

(Fig. 2).

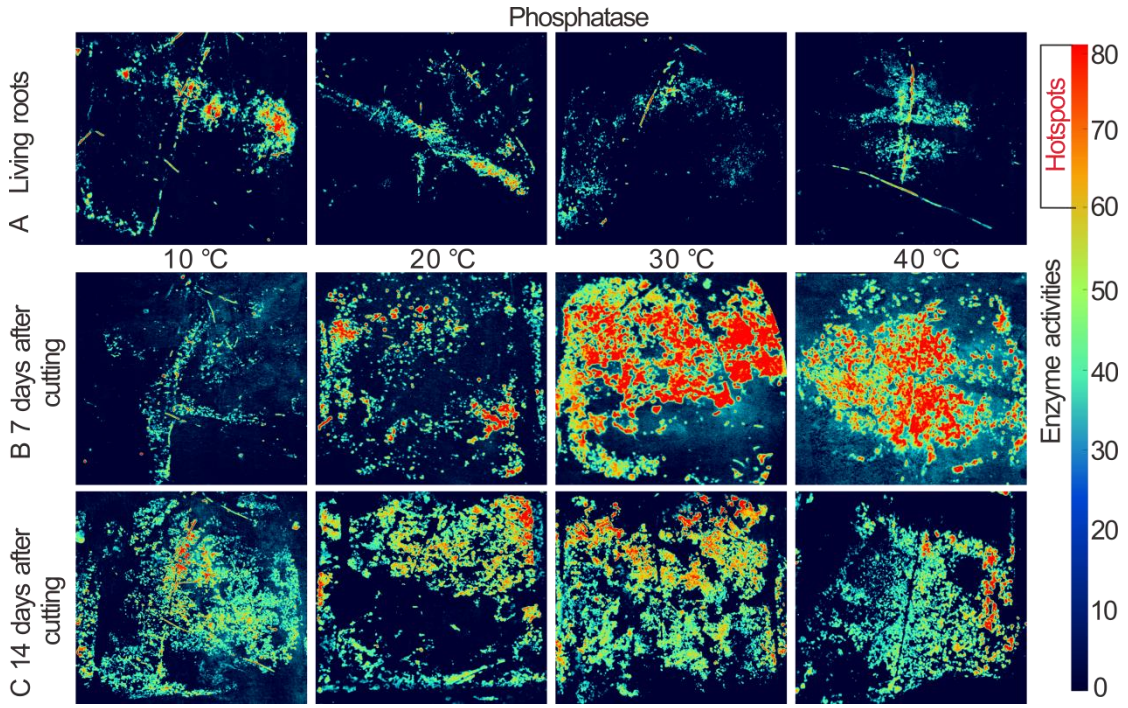


Fig. 1 Phosphatase activity distribution measured by zymography, A) Rhizosphere (Living root) 14 days after sowing; B) Root-detritusphere 7 days after cutting of shoots; C) Root-detritusphere 14 days after cutting of shoots. Columns of rows 2 and 3 (Root-detritusphere) indicate four temperatures (10,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

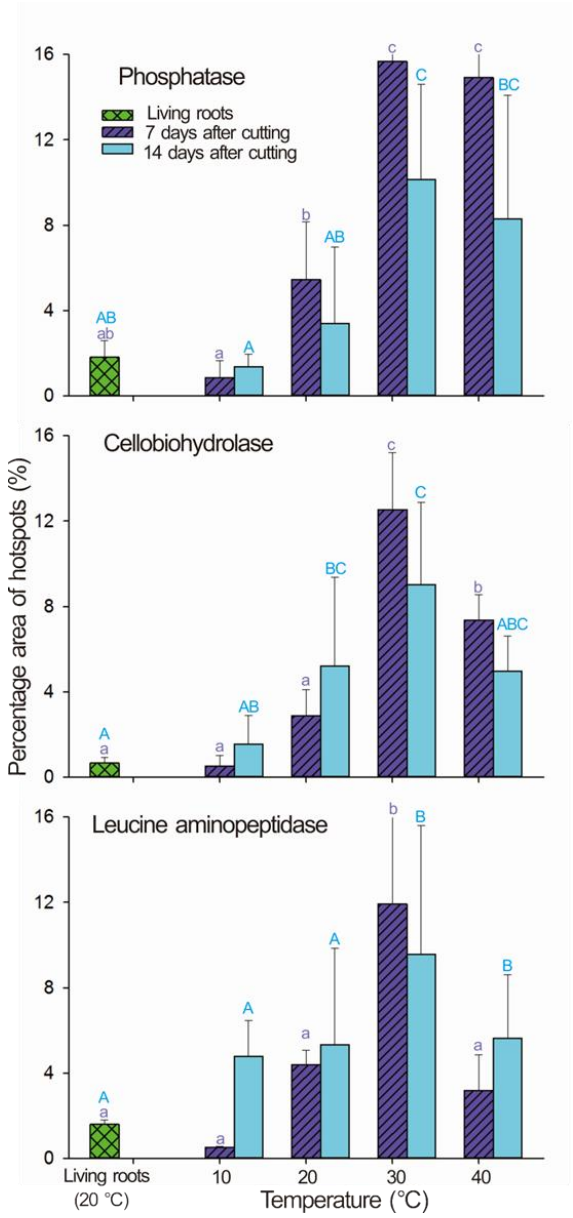


Fig. 2 Hotspots (with 25% highest activity, see Fig.1.) as a percentage of total area for phosphatase, cellobiohydrolase and leucine aminopeptidase in the rhizosphere of living roots and root-detritusphere (7 and 14 days after cutting shoots) at four temperatures (10, 20, 30, 40 ℃). Bars: means calculated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

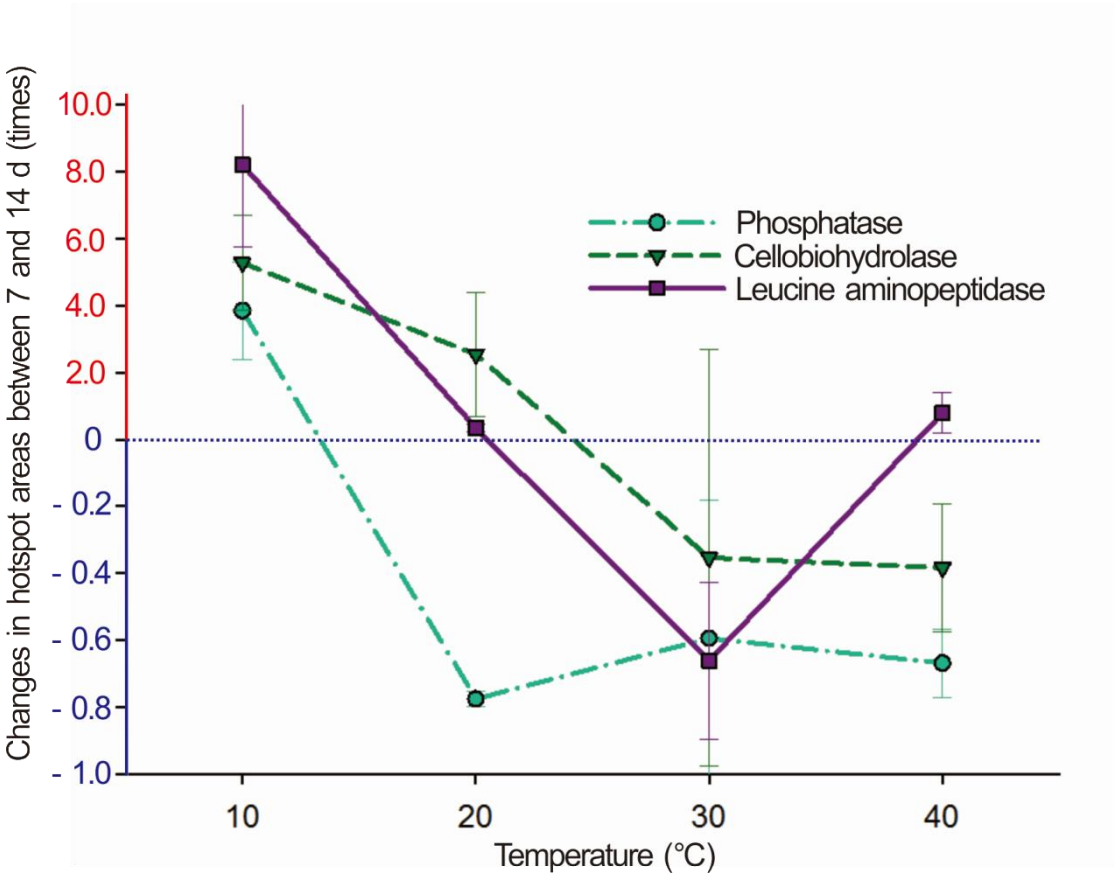


Fig. 3 Changes in enzyme hotspot (with 25% highest activity, see Fig.1.) areas between 7 and 14 days after cutting of shoots for phosphatase, cellobiohydrolase and leucine aminopeptidase at four temperatures (10, 20, 30, 40 ℃). Positive values show increased, whereas, negative values show decreased hotspot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

3.2. 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 ℃ by 1.5-6.6 times. At 40 ℃, the V_{max} of phosphatase and cellobiohydrolase significantly decreased. No significant decrease of V_{max} was recorded for leucine-aminopeptidase (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 ℃. The K_m values of cellobiohydrolase were significantly higher at 30 and 40 ℃ than at 10 and 20 ℃ (Fig. 5). The K_m values of leucine-aminopeptidase were nearly 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 ℃ were 3.2, 6.7 and 1.7 folds faster than at 10 ℃, respectively.

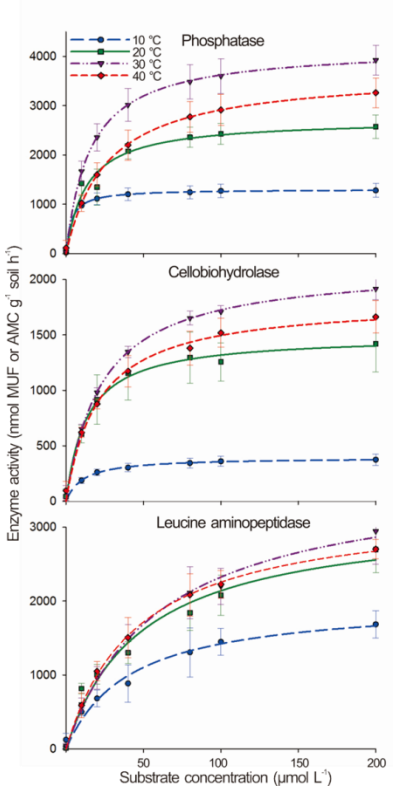


Fig. 4 Michaelis-Menten kinetics (enzyme activity as a function of substrate concentration) for phosphatase, cellobiohydrolase and leucine aminopeptidase in response to increasing temperature: 10, 20, 30, 40 ℃. Values are means of four replicates (±SE).

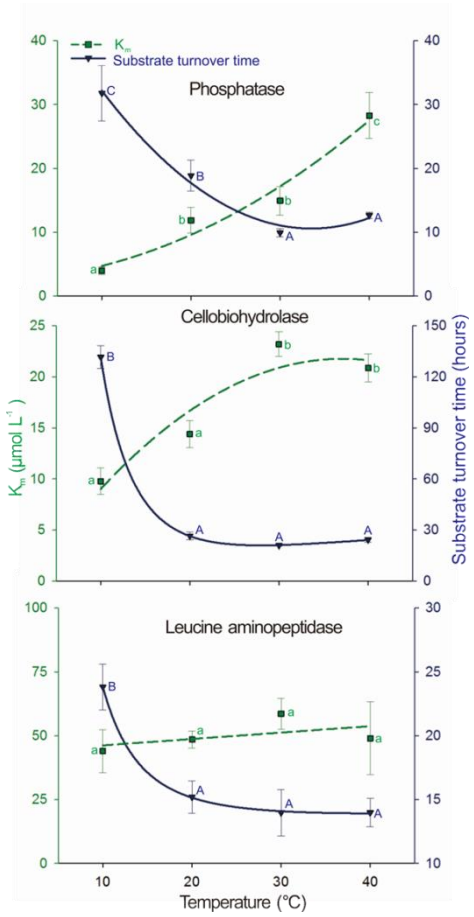


Fig. 5 K_m values (green dashed lines) and substrate turnover time (blue lines) of phosphatase, cellobiohydrolase and leucine aminopeptidase at four temperatures (10, 20, 30, 40 ℃). Values are means of four replicates (±SE). The differences of K_m and substrate turnover time at four temperatures were tested by ANOVA followed by the Duncan-test ($p < 0.05$). Letters show significant differences between temperatures.

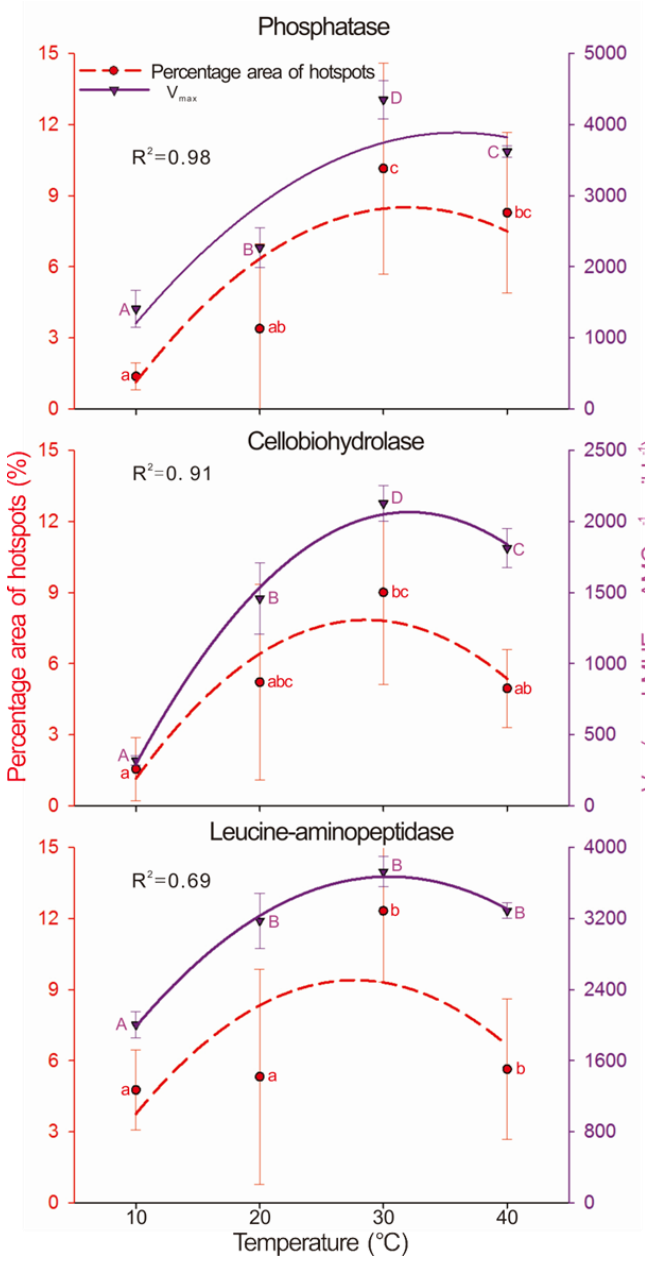


Fig. 6 Percentage area of hotspots (with 25% highest activity, see Fig. 1) for phosphatase, cellobiohydrolase and leucine aminopeptidase in the root-detritusphere (14 days after cutting shoots) depending on temperature (10, 20, 30, 40 ℃) (red dashed lines). V_{max} values of the three enzymes in the Michaelis-Menten equation (purple line). Data points indicate means of four replicates (±SE). The differences of percentage area of hotspots and V_{max} at four temperatures were tested by ANOVA followed by the Duncan-test ($p < 0.05$). Letters show significant differences between temperatures. The R^2 values are coefficients of determination of V_{max} and hotspot areas.

4. Discussion

In line with our first hypothesis, the hotspot areas in the 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 (Davidson and Janssens, 2006). 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 ℃) compared to low temperatures. Such increments were due to faster organic matter decomposition (Wallenstein et al., 2009; Wallenstein et al., 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 area of hotspots at warm temperatures.

The hotspot areas at warm temperatures (30 and 40 ℃) decreased two weeks after shoot cutting (Fig. 3), supporting our third hypothesis on the shorter duration of hot moments at warmer temperatures. This is explained by 1) faster substrate exhaustion and consequently the reduction of enzyme production (Wallenstein et al., 2010), 2) increase of enzyme degradation (Ten Hulscher and Cornelissen, 1996) and 3) thermal adaptation of microorganisms to elevated temperatures (Bradford et al., 2008). High microbial and enzyme activities at warm temperatures accelerate substrate decomposition rates (German et al., 2011). These accelerated rates are further supported by shorter substrates turnover time (Fig. 5). Therefore, labile substrates are rapidly lost (Sinsabaugh and Shah, 2010) without the regular 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). 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 (Ågren, 2010). Moreover, enzyme decomposition increases under warm temperatures (Ten Hulscher and Cornelissen, 1996) because degradation is partially controlled by protease activity (Conant et al., 2011).

Another possible explanation for the transient response of hotspot areas is the shift in microbial community structure as well as conformation changes of enzymes at high temperatures (Bradford et al., 2008; Allison et al., 2010). This explanation is further supported by an increase of the kinetic parameter K_m for cellobiohydrolase and for phosphatase at 30 and 40 °C (Fig. 5). Increasing K_m values with temperature indicate high enzyme conformational flexibility (Somero, 1978) and a change in microbial physiology. The latter led to isoenzyme production at warm temperatures (Baldrian et al., 2013). Contrasting to phosphatase and cellobiohydrolase, the hotspot areas of leucine aminopeptidase did not decline at 40 °C two weeks after shoot cutting. A static enzyme system confirmed by stable K_m at increasing temperatures explains this observation (Fig. 5) (Razavi et al., 2016a).

Contrary to our hypothesis 4, 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). This can be explained by an expression of multiple isoenzymes, each with a different temperature optimum (Bradford, 2013). 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).

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 Fig. 6). This is due to faster microbial growth (Rousk and Bååth, 2011), large microbial biomass (Pietikäinen et al., 2005), and higher enzyme activity (German et al., 2012). At 40 °C, both the V_{max} and percentage area of hotspots decreased for all enzymes (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, a decrease in substrate availability (Gershenson et al., 2009) and changes in microbial physiology (Davidson and Janssens, 2006) at very high temperatures as discussed above, lead to a downregulation of enzymes (López-Urrutia and Morán, 2007; Allison et al., 2010).

5. Conclusions

The hotspot areas of enzyme activities and their distribution in the 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 with temperature, indicating high enzyme conformational flexibility or a change in microbial physiology. The latter led to isoenzyme production 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 detritusphere, leading to faster substrate exhaustion and thus to shorter durations of hot moments.

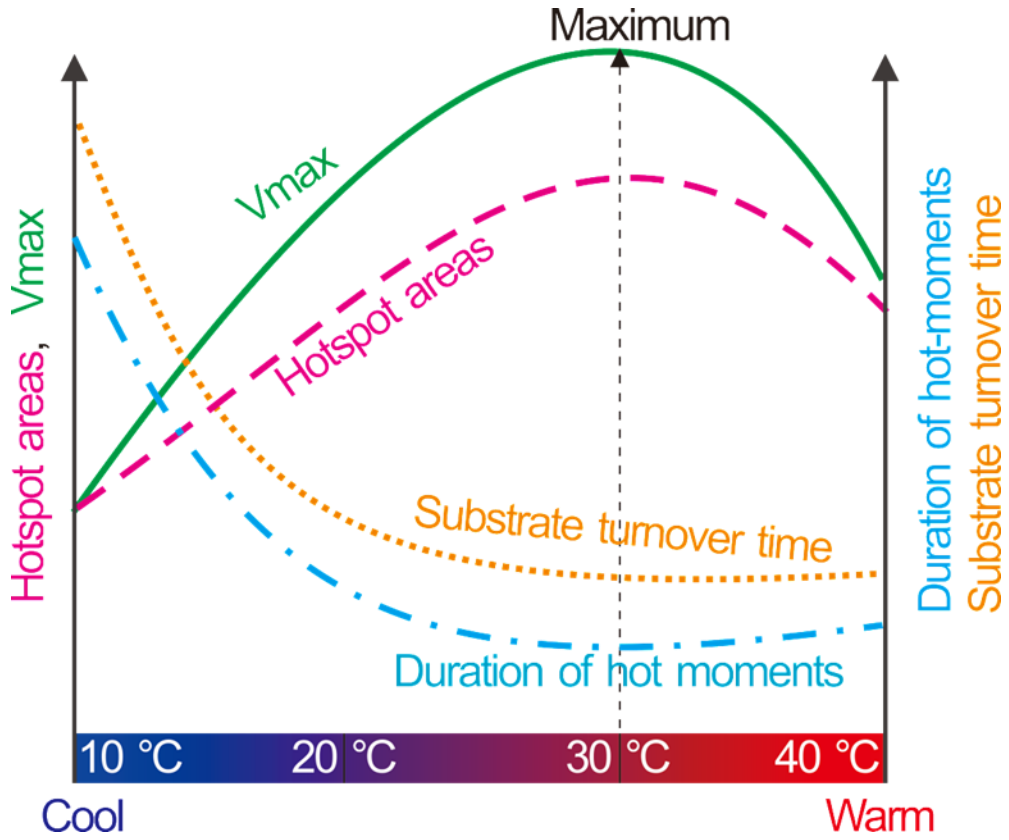


Fig. 7 Temperature effects on hotspot areas of enzyme activity, maximum enzyme activities (Vmax), substrate turnover time and the duration of hot moments.

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Supplement information

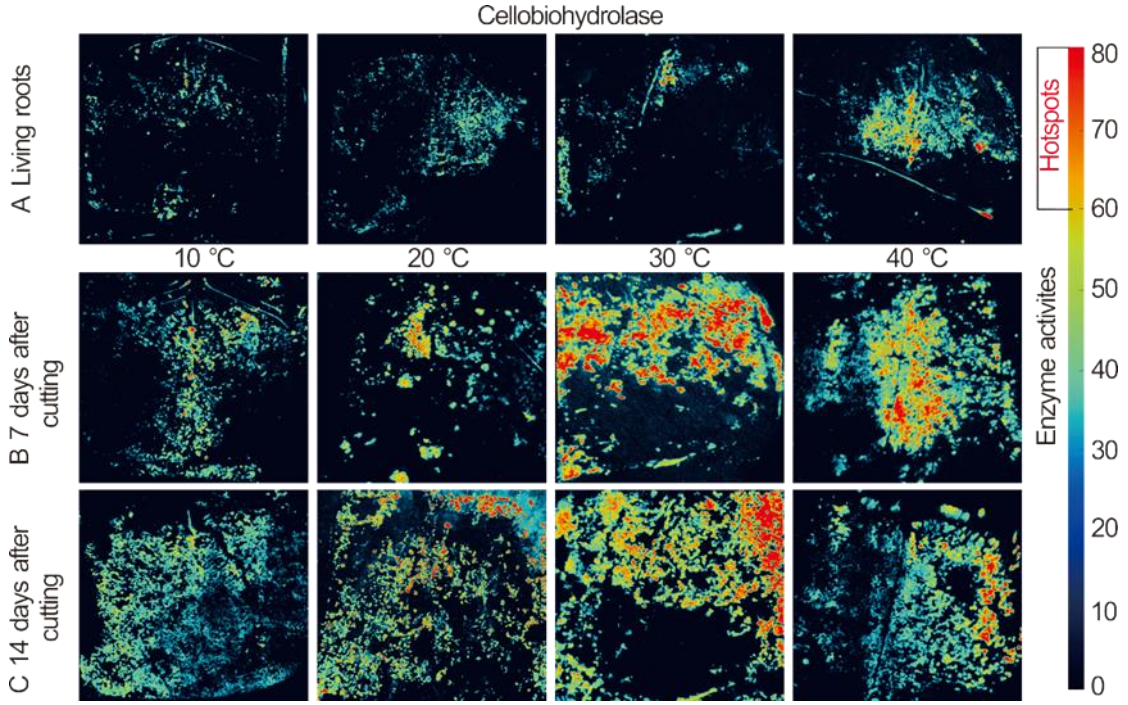

Fig. S1 Cellobiohydrolase activity distribution measured by zymography, A) Rhizosphere (Living root) 14 days after sowing; B) Root-detritusphere 7 days after cutting shoots; C) Root-detritusphere 14 days after cutting the shoots. Columns of rows 2 and 3 (Root-detritusphere) indicate four temperatures (10, 20, 30, 40 ℃). Rhizosphere (the top row) represents 4 replications (all conducted at 20 ℃). Side color scale is proportional to enzyme activities (pmol mm^{-2} h^{-1}).

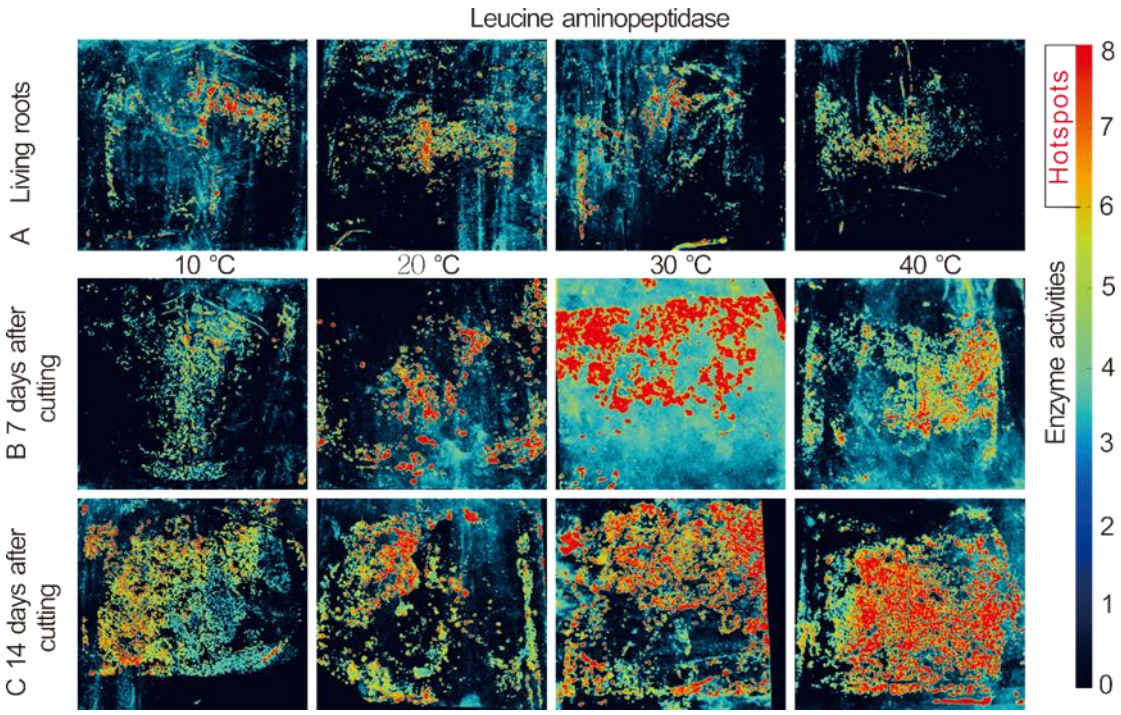

Fig. S2 Leucine aminopeptidase activity distribution measured by zymography, A) Rhizosphere (Living root) 14 days after sowing; B) Root-detritusphere 7 days after cutting shoots; C) Root-detritusphere 14 days after cutting the shoots. Columns of rows 2 and 3 (Root-detritusphere) indicate four temperatures (10, 20, 30, 40 ℃). Rhizosphere (the top row) represents 4 replications (all conducted at 20 ℃). Side color scale is proportional to enzyme activities (pmol mm^{-2} h^{-1})

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Ma, X., Zarebanadkouki, M., Kuzyakov, Y., Blagodatskaya, E., Pausch, J., Razavi, B.S., 2018. Spatial patterns of enzyme activities in the rhizosphere: Effects of root hairs and root radius. Soil Biology and Biochemistry, 118, 69-78.

Ma,X., Liu, Y., Zarebanadkouki, M., Razavi, B.S., Blagodatskaya, E., Kuzyakov, Y., 2018. Spatio-temporal patterns of enzyme activities in the rhizosphere: Effects of plant growth and root morphology, Biology and fertility of soils DOI: 10.1007/s00374-018-1305-6

Conference papers:

Ma, X., Razavi, B.S., Holz, M., Blagodatskaya, E.,Kuzyakov, Y., 2017. Warming increases hotspot areas of enzyme activity and shortens the duration of hot moments in the root-detritusphere. Geograpysical Research Abstract, (oral presentation)

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Ma,X., Kuzyakov, Y., 2018. Spatio patterns of enzyme activities in the rhizosphere: Effect of root hairs and root raidus., EGU (Poster presentation)

Declaration

1. I, hereby, declare that this Ph.D. dissertation has not been presented to any other examining body either in its present or a similar form.

Furthermore, I also affirm that I have not applied for a Ph.D. at any other higher school of education.

Göttingen,

马晓敏
..... Xiaomin Ma .....

(Signature)

2. I, hereby, solemnly declare that this dissertation was undertaken independently and without any unauthorized aid.

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