Plant-microbial interactions in the rhizosphere: root mediated changes in microbial activity and soil organic matter turnover

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To Tanishka and my whole family

Summary

On a global scale, the soil is a principal repository of terrestrial carbon (C) in the form of soil organic matter (SOM) which is central to soil fertility and ecological functions. The presence of plants changes the SOM dynamics by interacting with soil microorganisms. Biological interactions in rhizosphere – the soil volume in the close vicinity of roots - are pivotal in governing key ecosystem processes such as primary productivity, decomposition of SOM, and nutrient mobilization/immobilization. The input of plant-derived organics via rhizodeposition fuels microorganisms by providing an easily utilizable source of energy and C. Simultaneously, there is strong competition between plants and microorganisms for nutrient acquisition in the rhizosphere affecting dynamics of SOM decomposition. These plant-mediated changes in SOM decomposition are widespread in all terrestrial ecosystems, but remains poorly understood.

In general, the focus of this research is to understand soil biogeochemical processes and their mechanisms in the rhizosphere of agricultural crops. More specifically, the thesis aims to understand SOM decomposition and nutrient cycling in the rhizosphere under field conditions. In this context, rhizosphere priming effects (RPE) – root-induced changes in rates of SOM decomposition through labile C input - and its dependence on soil mineral nutrient status were explored in an arable field with maize. To measure RPE we applied isotopic ^{13}C natural abundance using C_3-C_4 vegetation change. The presence of maize roots increased SOM decomposition compared to the unplanted soils and the application of mineral N decreased the magnitude of priming suggesting nutrient availability modify the SOM decomposition via altering root and microbial activity (Kumar et al. 2016).

Further, the effect of roots on soil aggregate stability and associated enzyme activity (EA) were elucidated in field-grown maize to understand how plants control the microbial activities in distinct aggregate size classes (Kumar et al. 2017). Moreover, a field study was established to understand the effects of maize phenology and soil depth on EA (Kumar et al. 2018). It was shown that EA, and hence rates of SOM decomposition are a function of root activity in addition to microbial biomass. From these studies, we concluded that root activity and microbial activation via root-derived organics are among the major factors governing SOM decomposition and nutrient cycling in the rhizosphere (Kumar et al. 2016 and 2017). Therefore, it becomes crucial to elucidate the effects of root activity and hence, various root traits, on SOM decomposition and nutrient turnover in agroecosystems. The plasticity in root traits in relation to soil nutrient and water limitation, herbivory, and interaction with soil fauna helps plant nutrient acquisition and maximize their growth and development. In response to nutrient shortage, plants manipulate their strategies to maximize the uptake; however, the explicit relationship between specific root trait and nutrient uptake is not fully understood. Therefore, our study focused on the root traits plasticity (morphological and biological trait) for plant phosphorus (P) acquisition in P limited soils and how the availability of P affects the response of such traits. We had an opportunity to understand the plants' P uptake strategies through plasticity of root traits at three distinct plant growth stages (tillering, stem extension, maize heading) of maize. This study highlighted alternative mechanisms of maize for nutrient acquisition in absence of morphological trait (here root hairs). Shifts in root traits (increased root diameter and higher root mycorrhizal colonization by arbuscular mycorrhizal fungi) for P acquisition are important for maintaining plant growth

in absence or poorly developed root hairs and under nutrient limitation (Kumar et al. in preparation).

Collectively, the above-mentioned investigations on plant-soil interactions underpin the idea that microbial mediated SOM decomposition and nutrient cycling in the rhizosphere is fueled by root-derived organics. Changes in EA activity as a function of plant developmental stage highlight plants' regulatory mechanisms on microbial processes. Moreover, the importance of root traits in plant nutrient acquisition should be further understood. Shifts in root traits under environmental changes are important for plant nutrient uptake as well as in driving key ecosystem processes such as C and nutrient cycling. These mechanisms are central to the functioning of agroecosystems and highlight the needs of further research in rhizosphere ecology.

Zusammenfassung

Im globalen Maßstab ist der Boden eine wesentliche Quelle von Kohlenstoff (C) in Form von organischer Bodensubstanz (OBS), die zentral für die Bodenfruchtbarkeit und für ökologische Funktionen ist. Pflanzen verändern die Dynamik des Abbaus der organischen Bodensubstanz durch Interaktionen mit Bodenmikroorganismen. Biologische Interaktionen in der Rhizosphäre – dem Bodenvolumen in enger Umgebung zu den Wurzeln – sind zentral für die Regulation von wichtigen Ökosystemprozessen wie der Primärproduktion, dem Abbau von OBS und der Nährstoffmobilisierung/-immobilisierung. Der Eintrag von pflanzlichen organischen Stoffen über Rhizodeposition aktiviert Mikroorganismen, indem eine leicht verwertbare Energie- und Kohlenstoffquelle bereitgestellt wird. Gleichzeitig existiert eine starke Konkurrenz zwischen Pflanzen und Mikroorganismen um die Nährstoffe in der Rhizosphäre, welche sich auf die Dynamik des Abbaus der OBS auswirkt. Diese durch Pflanzen vermittelten Änderungen im OBS-Abbau sind in allen terrestrischen Ökosystemen weit verbreitet, bislang aber unzureichend verstanden.

Im Fokus dieser Arbeit steht das Verständnis von biogeochemischen Prozessen im Boden und ihrer Mechanismen in der Rhizosphäre von landwirtschaftlichen Kulturpflanzen. Spezieller bezweckt diese Arbeit den Abbau von OBS sowie Nährstoffumsätze in der Rhizosphäre unter Freilandbedingungen zu verstehen. In diesem Zusammenhang wurden Rhizosphären-Priming-Effekte (RPE) - wurzelinduzierte Veränderungen in der Abbaurate der OBS durch Eintrag labilen Kohlenstoffes – und deren Abhängigkeit vom mineralischen Nährstoffgehalt des Bodens in einem landwirtschaftlichen Maisfeld untersucht. Um die RPE zu messen verwendeten wir

natürliche Isotopenhäufigkeiten von ^{13}C in Verbindung mit einem C_3-C_4 Vegetationswechsel. Das Vorhandensein von Maiswurzen führte zu einem erhöhten Abbau der OBS, der Zusatz von mineralischem N verminderte jedoch das Ausmaß des Primings. Dies lässt darauf schließen, dass die Nährstoffverfügbarkeit den Abbau von OBS über veränderte Aktivität der Wurzeln und der Mikroorganismen modifiziert (Kumar et al. 2016).

Zusätzlich wurde die Auswirkung von Wurzeln auf die Stabilität von Bodenaggregaten und auf die Aktivität assoziierter Enzyme (EA) an Proben aus dem Freiland untersucht, um zu verstehen, wie Pflanzen mikrobielle Aktivitäten in unterschiedlichen Aggregatgrößenklassen kontrollieren (Kumar et al. 2017). Weiterhin wurde eine Freilandstudie durchgeführt, um den Einfluss der Phänologie des Maises und der Bodentiefe auf die EA zu verstehen (Kumar et al. 2018). Wir konnten gezeigen, dass EA, und somit die Abbaurate der OBS, nicht nur von der mikrobiellen Biomasse, sondern auch stark von der Wurzelaktivität abhängen.

Aus diesen Studien lässt sich schließen, dass die Wurzelaktivität und die mikrobielle Aktivierung durch wurzelbürtige organische Stoffe zu den Hauptfaktoren gehören, die den Abbau der OBS und die Nährstoffkreisläufe in der Rhizosphäre regulieren (Kumar et al. 2016 and 2017). Dadurch ergibt sich die Notwendigkeit die Einflüsse der Wurzelaktivität, und damit vielfältige Wurzeleigenschaften (traits), auf den Abbau der OBS und den Nährstoffumsatz in Agrarökosystemen weiter aufzuklären. Die Plastizität der Wurzeleigenschaften in Relation zu Nährstoff- und Wasserlimitierung in Böden, zu Herbivorie und zu Interaktionen mit der Bodenfauna begünstigt den Nährstofferwerb der Pflanze und maximiert ihr Wachstum und ihre Entwicklung. Als Antwort auf

Nährstoffknappheit kann die Pflanze ihre Wurzeleigenschaften ändern um die Nährstoffaufnahme zu maximieren. jedoch ist der explizite Zusammenhang zwischen spezifischen Wurzeleigenschaften und der Nährstoffaufnahme noch wenig erforscht. Daher fokussierte sich unsere Studie auf die Plastizität morphologischer und biologischer Wurzeleigenschaften (root traits) für die Phosphat- (P) Aufnahme der Pflanze aus Böden mit unterschiedlicher P-Verfügbarkeit. Wir hatten die Möglichkeit die pflanzlichen P-Aufnahmestrategien im Bezug auf die Plastizität der Wurzeleigenschaften zu drei verschiedenen Wachstumsstadien (Blattentwicklung, Längenwachstum, Rispenschieben) von Mais zu untersuchen. Diese Studie konnte alternative Mechanismen des Maises zum Nährstofferwerb in Abwesenheit von morphologischen Eigenschaften (hier Wurzelhaare) zeigen. Veränderungen der Wurzeleigenschaften (zunehmender Wurzeldurchmesser und höhere Kolonisation der Wurzel mit arbuskulären Mykorrhizapilzen) für den P-Erwerb stellen sich als wichtig für den Erhalt des Pflanzenwachstums in Abwesenheit oder bei schlecht entwickelten Wurzelhaaren sowie unter Nährstofflimitierung dar (Kumar et al. in preparation).

Zusammenfassend untermauern die oben genannten Untersuchungen zu Pflanzen-Boden-Interaktionen die Annahme, dass der mikrobielle Abbau der OBS und die Nährstoffkreisläufe in der Rhizosphäre durch wurzelbürtigen organischen Kohlenstoff verstärkt werden. Änderungen der Enzymaktivität in Abhängigkeit vom pflanzlichen Entwicklungsstadium verdeutlichten regulatorische Mechanismen der Pflanze auf mikrobielle Prozesse. Weiterhin wurde die Bedeutung der Wurzeleigenschaften für den pflanzlichen Nährstofferwerb genauer analysiert. Anpassungen der Wurzeleigenschaften (root traits) unter Umweltveränderungen sind bedeutsam für die pflanzliche Nährstoffaufnahme aber auch treibende Kraft für wichtige Ökosystemprozesse wie C-

und Nährstoffkreisläufe. Diese Mechanismen sind zentral für die Funktionalität von Agrarökosystemen und untermauern die Notwendigkeit für weitere Forschung in der Rhizosphärenökologie.

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Amit Kumar

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Abbreviations

ANOVA	Analysis Of Variance
C	Carbon
C_3	C_3 photosynthetic pathway
C_4	C_4 photosynthetic pathway
Delta ^{13}C	Isotopic ratio of $^{13}C/^{12}C$
cv.	Cultivar
DAP	Days After Planting
DOC	Dissolved Organic Carbon
DN	Dissolved Nitrogen
EA	Elemental Analyzer
GC	Gas Chromatography
GHGs	Green House Gases
h	hour
IRMS	Isotopic Ratio Mass Spectrometry
J	Joule
K	Potassium
K_{EC}	Fraction of extractable carbon
K_{EN}	Fractioni of extractable nitrogen
L.	Linnaeus
LSD	Least Significant Difference
MBC	Microbial Biomass Carbon

MBN	Microbial Biomass Nitrogen
min	minute
N	Nitrogen
P	Phosphorus
rth3	*roothairless3*
RPE	Rphizosphere Priming Effect
sec	second
SOM	Soil Organic Matter
WHC	Water Holding Capacity

I Extended Summary

1 General introduction

1.1 Global climate change and sustainable agriculture

Thanks to the green revolution which began in 1960s to help us keep the pace in food production with global population growth (Stevenson, 2013). This increase in food production was compromised with high environmental costs (Tilman et al. 2001) such as over cultivation and soil erosion (Kumar 2011), greenhouse gas emissions (Matson et al. 1997; Smith et al. 2013), intense use of pesticides and fertilizers polluting surface and groundwater (Arias-Estevez et al. 2008), and a gradual depletion of soil nutrient stocks (McLauchlan 2006) and carbon (C) (Lal 2004; McLauchlan 2006). At present, the global agriculture is facing strong threats to above mentioned consequences of increasing yield to meet the food demand of the ever increasing global population (Godfray et al. 2010). According to United Nations 2013 report, by 2100 the global population is projected to increase by 50% as compared to now, and therefore, the global grain demand is estimated to double (Godfray et al. 2010), suggesting novel and sustainable agricultural practices with reduced environmental costs.

Sustainable agriculture undertakes the reduced applications of mineral fertilizers and pesticides and the efficient utilization of ecosystem services (i.e. land and resources) to enhance yields as well as decreasing the greenhouse gases (GHGs) emissions and increasing the C content and therefore, C sequestration in agricultural soils (Calabi-Floody et al. 2017; Lal 2009). Sustainable agricultural strategies include integrated pest management (Gurr et al. 2003), conservation farming via reduced tillage or no-tillage to reduce C and nutrient losses and reduced GHGs emissions along with building-up of soil organic matter (SOM) (Balesdent et al. 2000; Paustian et al. 2000; Six et al. 2002).

Intercropping, cover cropping, utilization of key nutrients nitrogen (N), phosphorus (P), and potassium (K) using farmyard manures, returning of organic matter to arable fields are among organic farming practices, which are highly efficient and sustainable practice of crop production and help in enhancement of soil structure (via aggregation), diversity and functions of soil biota (Fageria et al. 2005; Nyakatawa et al. 2001; Peixoto et al. 2006; Waddington et al. 2017; Wang et al. 2006; Yildirim and Guvenc, 2005). It becomes very important in agricultural soils to understand the plant-soil-microbial interactions in order to increase the sustainability than in conventional agriculture where such interactions are marginalized by inputs of agrochemicals such as fertilizers and pesticides (Johansson et al. 2004).

1.2 Plant-soil interactions in the rhizosphere

The term ‘rhizosphere’ was coined by Lorenz Hiltner in 1904. Rhizosphere is defined as the soil volume affected by living roots activity (Uren 2007). Ever since this term has been recognized by scientists, numerous studies have been focused on ecology in the rhizosphere (generally called as rhizosphere ecology) relative to the bulk soil (Barea et al. 2005; Philippot et al. 2013). The importance of understanding rhizosphere ecology within the global C and nutrients cycle is tremendous despite the fact that rhizosphere is comprised of less than 1% of total soil volume (Finzi et al. 2015; Pausch and Kuzyakov, 2011). As we know that more C is stored in soil than that present in global vegetation and as CO_2 in the atmosphere together (Schimel 1995), it is essential to understand rhizosphere processes where the process rates are orders of magnitudes higher than bulk soil (Kuzyakov and Blagodatskaya 2015). To approximate the ‘rhizosphere’ contribution to the total fluxes of nutrients and C in the whole soil volume, it is pivotal to

understand the nutrients and C fluxes as a function of root activity, architecture and function (Finzi et al. 2015).

Plant roots release diversity of C compounds in the rhizosphere and based on plants' life span, such rootderived inputs can be broadly categories in two main sources especially in agricultural systems where above ground biomass is harvested most of the times: 1) remains of roots after above ground plant harvest, and 2) exudates and other root-derived organics (collectively called as rhizodeposits) by living roots during plant growth (Kuzyakov and Domanski 2000). The process of living root mediated inputs of organics in the rhizosphere is called as rhizodeposition. In a recent study, Pausch and Kuzyakov (2017) showed that annual crops translocate lower photoassimilated products belowground than pasture plants (mainly perennials) reflecting optimization of crop plants during domestication for above ground products with a consequent reduction in belowground C allocation. The quality and quantity of rhizodeposits also vary with various biotic and abiotic factors as summarized by Jones et al. (2004). For instance, root exudates are mainly comprised of low molecular weight organic compounds such as amino acids, simple sugars, organic acids whereas the high molecular weight organic compounds are comprised of mucilage, sloughed off cells and dying roots (Jones et al. 2004). Root-derived organics provide the soil microorganisms with localized form of energy resulting in unique biological niche that is characterized by high diversity, abundance and activity of microorganisms relative to bulk soil (Bais et al. 2006; Chaparro et al. 2014; Kuzyakov and Blagodatskaya, 2015; Lange et al. 2015; Loeppmann et al. 2015; Pausch and Kuzyakov 2011). Rhizodeposits mediated interactions between free living soil microorganism and roots (Dijkstra et al. 2013), interactions between roots and symbiotic organisms such as mycorrhizal fungi and

rhizobia bacteria or pathogenic microorganisms are among few to mention interactions which are initiated by root-derived signaling molecules (Berendsen et al. 2012; Harman et al. 2004). Such interactions are of numerous importance in sustainable agriculture and recently have been considered for their role in plant health and nutrient acquisition. Various studies have shown that root exudates are mediators of plant nutrient acquisition in nutrient poor soils (Dakora and Phillips 2002; Ohwaki and Hirata, 1992) most likely by enhancing microbial activities. Plant mediated increase in the metabolism and activity of soil microorganisms via rhizodeposits (microbial activation hypothesis, Cheng and Kuzyakov, 2005; Kuzyakov et al. 2007) may accelerate decomposition of SOM and consequently the release of SOM bound nutrients for plant and microbial uptake (Cheng et al. 2003; Dijkstra et al. 2013). The process of change in decomposition of SOM via input of labile compounds is known as ‘priming effect’ (Kuzyakov et al. 2000) and such changes in SOM decomposition via root-derived organics is termed as ‘rhizosphere priming effect’ (Cheng et al. 2003; Kuzyakov 2002; Zhu and Cheng, 2011). However, it is still not fully understood how plants mediate SOM decomposition via altering the abundance and activity of microorganisms in soil. Studies in the literature indicate that SOM decomposition is not a linear function of total microbial biomass and microbial community structures, rather point toward specific activity of certain microbial group relative to others (Bird et al. 2011; Dijkstra et al. 2013). It is important in sustainable agriculture to understand the mechanisms of building up and decomposition of SOM and consequently nutrient mobilization/immobilization determining soil health and plant productivity. Furthermore, to increase the productivity under global climate change and associated impacts on agriculture, it is important to understand the nutrient acquisition strategies and the associated root traits in crop plants. Root traits such as

rooting length, specific root length, root hair length and density, root respiration, symbiotic association with fungi and bacteria, nutrient uptake kinetics, root diameter are among important traits for maximizing nutrient uptake and reduced metabolic costs under certain environmental stress. For instance, plasticity in root traits in low fertility soils (represent most of agricultural area across the globe) may help plants to cope with nutrient limitation by improved nutrient acquisition. Therefore, a better understanding of root traits and their incorporation in breeding programs will certainly help to improve the yield and under future climate change scenario.

1.3 Plant mediated decomposition of soil organic matter

As mentioned in section 1.2, there is a substantial allocation of photoassimilated C to belowground pools, estimation of the exact amount of rhizodeposits is very difficult and vary widely with plant species, plant growth stage, soil properties, nutrient availability. About 17% of the net assimilated C is released via rhizodeposition (Nguyen, 2003). Rhizodeposits act as easily utilizable energy substrates for microorganisms harboring the rhizosphere which as a result breakdown the SOM via mining for nutrients. Microorganisms release a variety of enzymes in their environment depending on their nutrient requirements and coincidently breakdown the SOM. 'Rhizosphere priming effects (RPEs)' are generally expressed as the difference of CO_2 produced by decomposition of native SOM from planted and unplanted soils (Figure I.1:1). Depending on the CO_2 produced from native SOM from planted and unplanted soils, RPEs may either be positive (higher CO_2 produced from SOM in planted than unplanted soils) or negative (lower CO_2 produced from native SOM in planted than unplanted soils) (Kuzyakov 2002). RPEs are widespread in most of terrestrial ecosystems ranging from 50% reduction to 380% increase in the rates of SOM decomposition in planted than

unplanted soils under the same environmental conditions (summarized by Cheng et al. 2014); however, the mechanisms behind this huge variation in RPEs are still poorly understood (Dijkstra et al. 2013). Various hypotheses explaining the relationship between RPEs and soil nutrient availability have been proposed that the soil nutrient status is an important factor for RPEs (Figure I.1:2). Under low nutrient availability, microorganisms may utilize the rhizodeposits as an energy source for the production and release of enzymes to their surroundings that can release the nutrients locked in SOM (microbial nutrient mining hypothesis, Craine et al. 2007, Fontaine et al. 2011), resulting in positive RPEs. It is important to note here that microbial mining for nutrients via SOM decomposition should be accompanied by the production of CO_2 via oxidation of SOM. This is generally the case for microbial N mining; however, microbial P mining is not necessarily via oxidation of SOM. Organic P is mostly released via hydrolysis without CO_2 production rather than oxidation (McGill and Cole, 1981). Under reduced nutrient availability, if plants compete with microorganisms for nutrient uptake, this may result in suppression in microbial growth and activities and therefore reduced or negative RPEs. Moreover, if soil is rich in availability of nutrients, negative RPEs may occur. Under such conditions, plants and microorganisms utilize the available nutrients and do not rely on SOM decomposition to meet their nutrient demand, resulting in negative RPEs. It is noteworthy that RPEs do not necessarily results in reduction of total SOM due to enhanced rates of decomposition because the overall root-derived inputs may compensate for the enhanced decomposition of labile SOM pools (Cheng, 2009). Most of the studies dealing with RPEs are performed under controlled environmental conditions either in climate chambers or greenhouses and their field magnitude and mechanisms are poorly known, which is certainly required to understand the field

relevance of RPEs and to be included in global C models under global climate changes and future predictions.

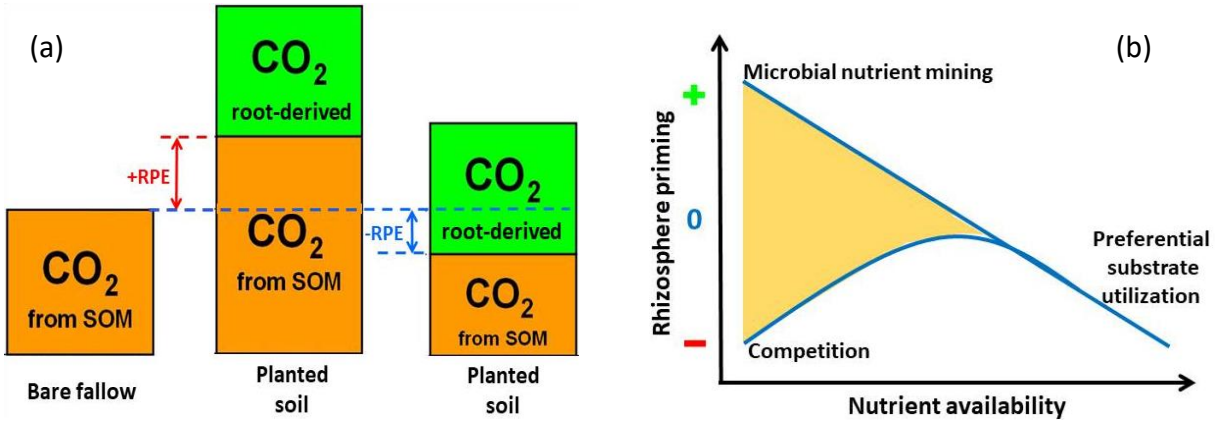

Figure I.1:1: (a) Schematization of rhizosphere priming effects (RPEs). Positive RPEs (double-headed red arrow, accelerated SOM decomposition) and negative RPEs (double-headed blue arrow, retardation of SOM decomposition) (Adapted from Kuzyakov, 2002). (b) Conceptual relationship between RPEs and soil nutrient availability. Microbial nutrient mining: activated microorganisms through rhizodeposits mine for nutrients locked in SOM thereby causingov positive RPEs; Competition: plants and microorganisms compete for nutrients and when plants over compete with microorganisms, negative RPEs occur due to hampering of microbial growth by reduced nutrient availability; Preferential substrate utilization: in presence of alternative easily available resources, microbes switch from decomposing SOM, which result in negative RPEs. Under low nutrient availability, both positive and negative RPEs may occur (Adapted from Dijkstra et al. 2013).

1.4 Spatiotemporal changes in microbial activity in the rhizosphere

Soil microorganisms are key players in ecological functioning and a holistic understanding of microbial mediated processes is therefore very important for sustainable agricultural practices. Decomposition and transformation of SOM, nutrients mobilization/immobilization, and aggregate formation/stabilization are among the most important processes predominantly governed by microorganisms (Nsabimana et al. 2004; Six et al. 2004; Caldwell 2005). Microorganisms secret a myriad of extracellular enzymes in their surroundings to meet their energy and nutritional demands (Schimel

and Weintraub, 2003; Sinsabaugh et al. 2009) The activities of extracellular enzymes are sensitive to changes in their surrounding environment; therefore, they can be used as indicators of microbial mediated processes in the rhizosphere (Romani et al. 2006; Salazar et al. 2011; Wang et al. 2015). Generally, extracellular enzyme activity (EEA) is dependent on various biotic and abiotic factors such as pH (Sinsabaugh 2010), nutrients (Keuskamp et al. 2015; Olander and Vitousek 2000), disturbance (Boerner et al. 2000), succession (Tscherko et al. 2003), microbial community structure and function (Dorodnikov et al. 2009; Tischer et al. 2015), plant species (Caravaca et al. 2005; Razavi et al. 2016), and management practices (Shahbaz et al. 2017). The cycling of major nutrient elements is widely associated with EEA in soil (Burns et al. 2013). The EEA is important in maintaining soil health, as enzymes catalyze the bottleneck steps in SOM decomposition and consequent release of nutrients for plant and microbial uptake (Aon et al. 2001). Depending on the complexity of SOM, various hydrolases and oxido-reductases are produced by microorganisms. For example, β-1,4-glucosidase (BG), cellobiohydrolases (CBH), and β-xylosidase (XYL) are a set of hydrolases produced by microorganisms to acquire C via polysaccharides decomposition. Another widely prevalent enzyme is L-leucine aminopeptidase (LAP), which is associated with the breakdown of amide-linked polypeptides, the primary form of organic N in soils (Finzi et al. 2015; Knicker 2004). β-1,4-N-acetylglucosaminidase (NAG), which predominantly targets chitin and peptidoglycan breakdown, releases both C and N for microbial acquisition. Organic compounds containing ester-linked P are cleaved by phosphomonoesterase (PHO), which releases inorganic P (Finzi et al. 2015; Sinsabaugh and Shah 2011). In rhizosphere, enzyme production is triggered by root exudation, resulting in higher rates of SOM decomposition and consequently release of

nutrients (Kuzyakov and Domanski 2000). The regulation of enzyme activity in rhizosphere is mainly determined by soil nutrient status and plant-microbial competition for the available nutrient elements. In general, when nutrients are not readily available for plant and microbial acquisition, microorganisms as well as plants secrets enzyme to acquire the nutrients locked in SOM via decomposition. But this is not always true. When the nutrient limitation is so severe that microorganisms are unable to carry forward their metabolism and synthesize new enzymes, the EEA decreases. In such cases, availability of the nutrient elements to a threshold level is required for enzyme production and release. Therefore, the regulation of enzyme activity in the rhizosphere is not yet fully understood. Further, when mineral nutrients such as N and P are available in the rhizosphere for easy uptake by plant and microorganisms, there are no requirements for plants and microorganisms to synthesize and secret those enzymes which are involved in N and P acquisition. Presence of mineral nutrients generally results in downregulation of EEA in rhizosphere. EEA is not only dependent on availability of nutrient elements, but also affected by soil depth. As mentioned by Loeppmann et al. 2016, most of studies with enzyme activity in the rhizosphere are confined to topsoil, despite the fact that roots provide the energy subsidy via rhizodeposits throughout the rooting depth. The spatial distribution of roots is heterogeneous in soil and varies with the growth stage of the plant (Chimento and Amaducci 2015), which may impact plant-mediated microbial activity and therefore, EEA at various soil depths. It has previously been demonstrated that there are distinct microbial community compositions and microbial activities along with soil depth (Fierer et al. 2003) and these changes are generally explained by substrate input varying in quality and quantity (Loeppmann et al. 2016). It has been observed that when depth increases, microbial activity decreases, as substrate inputs and gas exchange are

reduced with depth (Stone et al. 2014). Although roots and microbial activity are often linked (Kuzyakov and Blagodatskaya 2015), most of the field studies are conducted only once during a vegetation season (either at the beginning or before harvesting). However, root-mediated effects on microbial activity are taking place throughout the growing season (Bell et al. 2015). It is still unknown from direct field observations how microbial activity is influenced by roots at various plant growth stages, which are characterized by distinct morphological and physiological properties.

Table 1: Examples of soil enzyme activities to assess the functional diversity between and within nutrient cycling. (Adapted from Blagodatskaya and Kuzyakov, 2008; Caldwell, 2005)

Enzyme	Nomenclature	Producer	Important role
β-1,4-glucosidase	EC 3.2.1.21	Fungi, bacteria, and termites	Exocellulases that remove glucan units from the ends of the cellulose chains
Cellobiohydrolase	EC 3.2.1.91	Fungi, bacteria, and protozoans	Hydrolysis of 1,4-β-D-glucosidic linkages in cellulose, releasing cellobiose from the non-reducing ends of the chains
Xylanase	EC 3.2.1.8	Herbivorous microorganisms and fungi	Degrade the linear polysaccharide β-1,4-xylan into xylose, thus breaking down hemicellulose, which in a major component of the cell wall of plants
N-acetyl-β-1,4-glucosaminidase	EC 3.2.1.30	Bacteria, fungi, plants, invertebrates, humans	Hydrolyses the residues from terminal non-reducing ends of chito-oligosacchrides
Acid phosphatase	EC 3.1.3.2	Plants, animals, fungi, and bacteria	Free attached phosphate groups from other molecules during digestion

L-leucine aminopeptidase	EC 3.4.11.1	Occurs naturally in all organisms	Hydrolysis of the peptide bonds, casein hydrolyzing

1.5 Objectives

As mentioned in the introduction, there are clear research gaps about the mechanisms and relevance of microbial-mediated SOM decomposition and nutrient cycling in the rhizosphere under field conditions. Field relevance of such processes will help us in building sustainable practices which are seen as a gateway towards higher food production with reduced negative impact on the environment. Therefore, this thesis focuses on the following objectives:

1) **To estimate rhizosphere priming effects of SOM decomposition under field conditions and its dependence on soil nutrient status (Study 1). Specific hypotheses are that**
   (i) Presence of plants increases SOM decomposition via microbial activation through rhizodeposits.
   (ii) Mineral N application reduces RPE because plants alter their root activities and microorganisms are less dependent on nutrient gains from SOM decomposition.
2) **To investigate the effects of maize roots on the distribution of soil aggregate sizes and associated extracellular enzyme activities (EEA) (Study 2). Specific hypotheses are that**
   (i) EEA is higher in aggregates of planted soil than that of bare fallow, as microorganisms are fueled with C and energy-rich labile substrates by rhizodeposition.
   (ii) EEA is higher in free microaggregates than macroaggregates as the former should be preferentially exposed to root exudates, water and oxygen flow.

3) **To investigate the effects of maize phenology and N fertilization on the distribution of extracellular enzyme activities (EEA) along with soil depth in the field (Study 3). Specific hypotheses are that**

   (i) Actively growing roots during earlier growth stage have higher effects on microbial biomass and EEA via root activities.

   (ii) EEA in rhizosphere varies with soil depth and decrease with increasing soil depth due to reduced substrate inputs and gaseous exchange.

   (iii) Activity of enzymes involved in N acquisition decrease with N fertilization due to preferential substrate utilization.

4) **To investigate the root trait plasticity to maintain plant productivity under phosphorus limitation in soils (Study 4). Specific hypotheses are that**

   (i) In P limited soils, plants compensate for the lack of root hairs by shifting the root trait to higher root mycorrhizal colonization (functionally analogous to hairs) for P acquisition

   (ii) Root mycorrhizal colonization decreases with P fertilization due to strong trade-off for C costs

2 Material and methods

2.1 Description of the agricultural site for field studies

Figure I.2:1 Arial view of experimental location in Reinshof, Goettingen.

The field experiments (Study 1, 2, and 3) were established on an agricultural research field belonging to the Georg-August-University Göttingen, Germany (Figure I.2:1). The soil is characterized as a haplic Luvisol suitable for a broad range of agricultural uses with the following properties: total C content of 1.41 ± 0.04%, total N content of 0.16 ± 0.02%, pH value of 7.2 ± 0.01, and bulk density of 1.2 ± 0.2 g cm^{-3}. The experimental site is under conventional agricultural uses. Conventional tillage practices up to 30 cm of soil depth are performed twice in a year. Maize seeds (*Zea mays* L. cv. Colisee) were sown in the field. The experimental field was divided into 28 plots (5 x 5 m^2) with a 2 m wide buffer strip around each plot to exclude neighbor effects as shown in figure I.2:2. N fertilizer was applied as urea at the soil surface at a rate of 160 kg N ha^{-1} (Weiterer, Landhandel GmbH) 47 days after planting (DAP). Any visible weed growing in the plots was manually removed at regular time intervals throughout the experimental period.

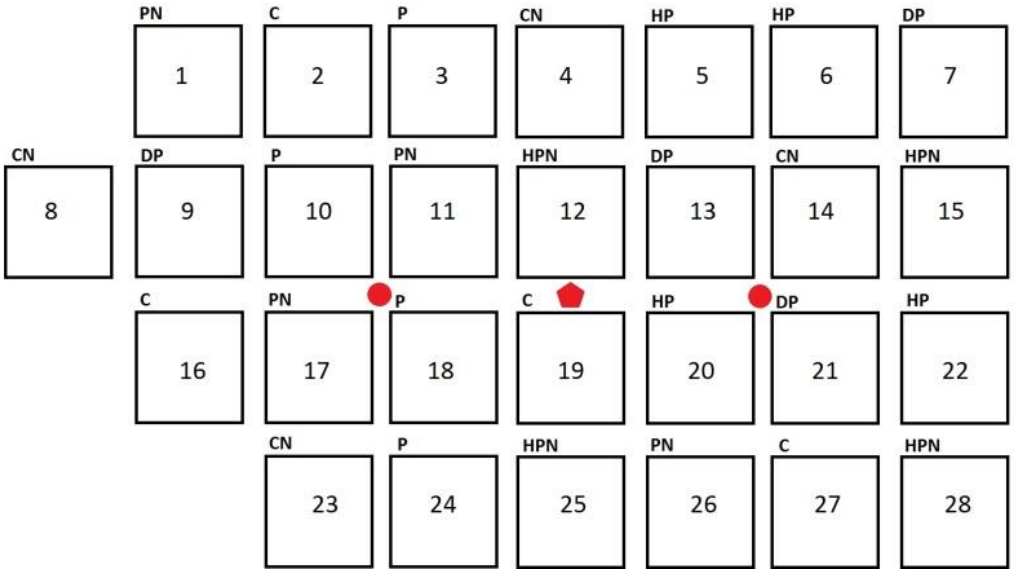

Figure. I.2:2: Experimental plots in Reinshof, Götttingen. C: Bare fallow; CN: Bare fallow with N fertilization; P: Maize with low plant density; PN: Maize with low plant density and with N fertilization; DP: Maize with normal plant density; DPN: Maize with normal plant density and N fertilization; HP: Maize with high plant density; HPN: Maize with high plant density and N fertilization. Low-, Normal-, and High density of maize comprised of 6, 10, and 16 plants m^{-2}.

2.2 Isotope approaches

We applied a C_3-to-C_4 vegetation change in the field to estimate RPE. This approach is based on the discrimination of heavier (^{13}C) and lighter (^{12}C) C isotopes during CO_2 assimilation by C_3 and C_4 plants (Balesdent and Mariotti 1996; Kuzyakov and Domanski 2000). Hence, by planting maize, a C_4 plant, on a soil which developed solely under C_3 vegetation, we introduced a distinct isotopic signal. This enabled partitioning total soil CO_2 efflux for root- and SOM-derived CO_2 and thus to estimate the RPE of field-grown maize. This approach was used in field estimation of RPE in Study 1.

2.3 Soil and plant sampling and analyses

For field incubation in Study 1, CO_2 was trapped in 1M NaOH solution using closed circulation trapping system and total C concentration was measured by SHIMADZU,

TOC-5050 and a subsample was precipitated as $SrCO_3$ with 1M $SrCl_2$ for δ13C analysis using an isotopic ratio mass spectrometer coupled to an elemental analyzer 2000 (Thermo Fischer Scientific, Cambridge UK). Study 4 was performed in the climate chamber under controlled environmental conditions: 16/8-h day/night rhythm with mid-day and night temperatures of 25° C and 15° C respectively, and light intensity at approximately 600 µmol m^{-2} s^{-1} with two-maize (*Zea mays* L.) genotypes, wild type (WT) and *roothairless3* (*rth3*) mutant. Seeds were grown in PVC pots and all the pots were fertilized with inorganic nitrogen (KNO_3, at the rate of 120 kg N h^{-1}) to avoid soil N limitation. Treatments with P-fertilization received inorganic P-fertilizer (KH_2PO_4, at the rate of 60 kg P h^{-1}).

Soil microbial biomass C (MBC) and N (MBN) were analyzed on fresh samples using the chloroform fumigation-extraction method with modifications (Vance et al. 1987). Soil samples were extracted with 0.05 M K_2SO_4 with or without 24 h fumigation using chloroform. Extracts were measured for organic C and N contents with a multi N/C analyzer (multi N/C analyzer 2100S, Analytik Jena). MBC and MBN were calculated by dividing the difference between extracted C and N from fumigated and non-fumigated soil samples with a K_{EC} and K_{EN} factor of 0.45 and 0.54, respectively (Joergensen and Mueller, 1996; Wu et al. 1990). The C and N contents from non-fumigated soil samples were considered as dissolved organic C (DOC) and dissolved N (DN), respectively.

Activities of all the measured enzymes were determined using fluorogenic labeled artificial substrates (Marx et al. 2001). Fresh soil suspension (1g soil + 50 ml distilled H_2O) was prepared and 50 µl of this suspension was dispensed into a black 96-well microplate. Fifty ml of either MES or TRIZMA buffer was added to each well for MUB or AMC based substrates, respectively. A 100 µl of substrate solutions for respective

enzyme activity was added and fluorometric measurements (excitation 360 nm; emission 450 nm) were taken. Fluorescence values were converted to amount of MUB or AMC using specific standard scales based on soil suspension. Enzyme activities were expressed as nanomoles MUB or AMC cleaved per gram dry weighted soil per hour (nmol g^{-1} dry soil h^{-1}).

Aggregates of three size classes were isolated by the method described by Dorodnikov et al. (2009) with modifications using vibratory sieve shaker. From each aggregate size class, soil was weighed to determine the mass distribution and mean weight diameters (MWD) of aggregates (John et al. 2005) as below:

MWD= ∑(Weight % of sample remaining on sieve × Mean inter-sieve size)÷100

where mean inter-sieve size is the average of the two sieve sizes through which the aggregates have passed and on which the aggregates have remained after sieving.

Soil and plant sampling were conducted at defined time period for Study 1, 2, 3, and 4 as mentioned in detail in the respective section of the manuscripts.

3 Results and discussion

3.1 Estimation of rhizosphere priming effects in the field (Study 1)

The rhizosphere priming effect has been widely observed in numerous studies under controlled conditions, but field studies are still rare (Cheng et al. 2014). Here, we provide measurements of RPE of SOM decomposition in a maize field based on SOM-derived CO_2. In agreement with other studies (Dijkstra et al. 2013; Finzi et al. 2015; Mwafulirwa et al. 2016; Pausch et al. 2013), plants accelerated the decomposition of SOM (Figure I.3:1). Higher MBC and MBN in the rhizosphere support the microbial activation by root exudation. This microbial activation is accompanied by increased extracellular enzyme activities, which further confirm that extracellular enzyme production is an important mechanism of SOM decomposition in the rhizosphere (Fontaine et al. 2003; Kuzyakov, 2010).

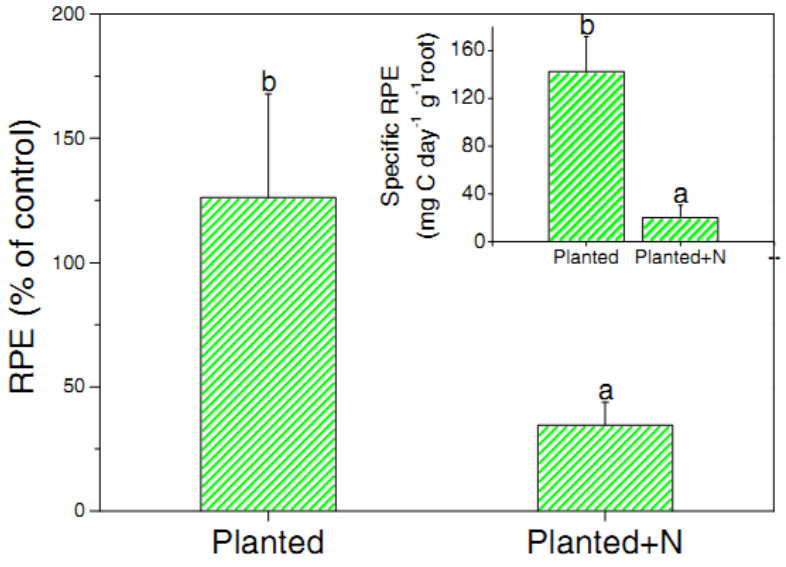

Figure I.3:1: Rhizosphere priming effect (RPE) (±SEM) as % of CO2 efflux from bare fallows for unfertilized (Planted) and N-fertilized (Planted+N) maize plants. The inset shows specific RPE (mg C day-1 g-1 root) (±SEM). Letters indicate the significant differences for RPE ($P < 0.01$) and for specific RPE ($P < 0.05$) between unfertilized and N-fertilized maize planted soils.

The N status of soils largely controls the magnitude of rhizosphere priming. N fertilization substantially reduced rhizosphere priming by lowering SOM decomposition. Lower root-derived CO_2 and enzyme activities in the rhizosphere with N fertilization confirmed that the availability of mineral N weakens the competition between roots and microorganisms. Microorganisms start utilizing exudates and the available mineral N (preferential substrate utilization) (Kuzyakov 2002; Sparling et al. 1982). However, increased root-derived CO_2 and enzyme activities without N fertilization intensify the root and microbial competition for N and the dependence of microorganisms on N mining. These findings suggested that root activity is intimately connected with microbially mediated SOM decomposition (Figure I.3:2).

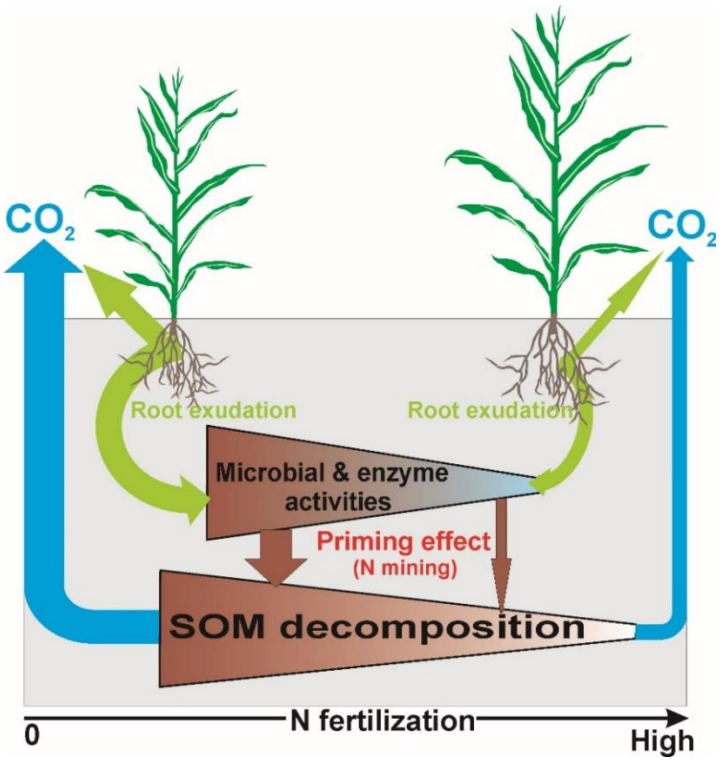

Figure I.3:2: Conceptual figure showing rhizosphere priming on SOM decomposition accompanied by microbial activation and N mining. Arrow thickness indicates process intensity.

3.2 Effects of maize roots on the distribution of soil aggregate classes and associated microbial biomass and extracellular enzyme activities (Study 2)

The influence of roots on aggregate stabilization is well known (Erktan et al. 2015; Six et al. 2004), but very few studies have focused on aggregate disintegration by living roots (Materechera et al. 1994). In our field study, a gradual increase in the portion of microaggregates and a decrease in large macroaggregates with increasing plant density may be due to disintegration of large macroaggregates by growing roots (Figure I.3:3). Mechanistically, the aggregate redistribution may occur through the penetration of living roots into macroaggregates along planes of weakness and through the pores within macroaggregates, thereby decreasing their stability (Materechera et al. 1994).

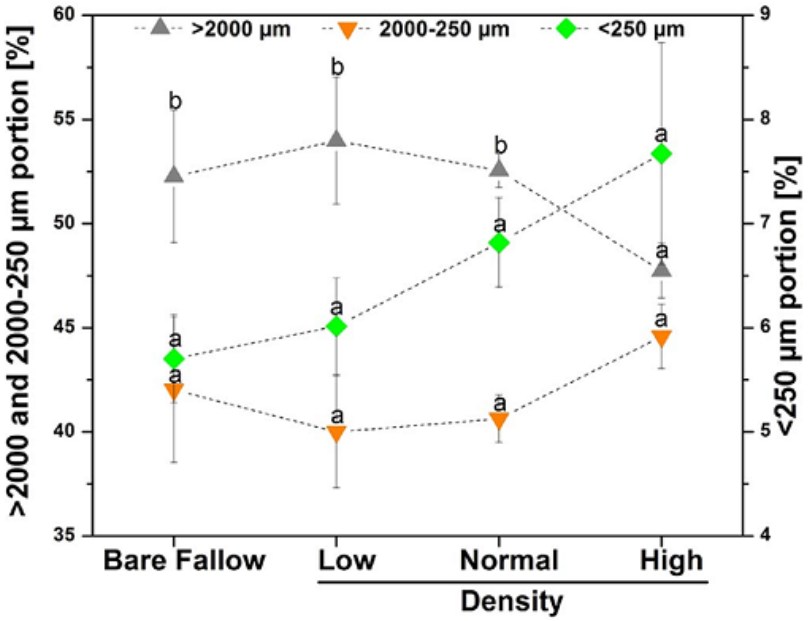

Figure I.3:3: The relative distribution of large and small macroaggregates (left y-axis; mean±SEM) (n=4) and microaggregates (right y-axis; mean±SEM) (n=4) in bare fallow soil and soils with Low, Normal and High maize plant densities. Letters indicate significant differences (Post-hoc LSD test, $P < 0.05$) between bare fallow and three plant densities within the same aggregate size class.

In the present study, microbial biomass C decreased with decreasing aggregate size. Literature is replete with studies showing increased fungal abundance with increasing

aggregate size (Poll et al., 2003; Zhang et al., 2015). The preferential colonization by fungal communities may occur in macroaggregates (Harris et al., 2003) by expanding their biomass through extensive hyphal growth in large pores (De Gryze et al., 2005; Dorodnikov et al., 2009b). In turn, microaggregates are inhabited predominately by bacterial communities (Ranjard and Richaume, 2001; Six et al., 2006). Higher microbial biomass C to N ratio in macroaggregates than microaggregates (although significant only in low plant density) in the present study indicates fungal dominance in macroaggregates as compared to microaggregates. The present study showed that the potential and specific activities of β-1,4-glucosidase, β-1,4-N-acetylglucosaminidase, L-leucine aminopeptidase and acid phosphatase enzymes were higher in rooted than bare fallow soil which is most likely due to microbial activation via rhizodeposits (Kuzyakov and Blagodatskaya, 2015). Enzyme activities increased with decreasing aggregate size as large macroaggregates < small macroaggregates < free microaggregates. Overall higher potential and specific enzyme activities in free microaggregates may result from the location of the latter within soil where root exudations as well as water, nutrient and oxygen flows are higher than in the interior of macroaggregates (Burns et al., 2013; Phillips et al., 2011). In summary, considering microbial activation (Cheng and Kuzyakov, 2005) by growing roots, the present study provides evidence that the influence of roots on microorganism's activities persists in different soil aggregates and such influences are more pronounced in free microaggregates (Figure I.3:4).

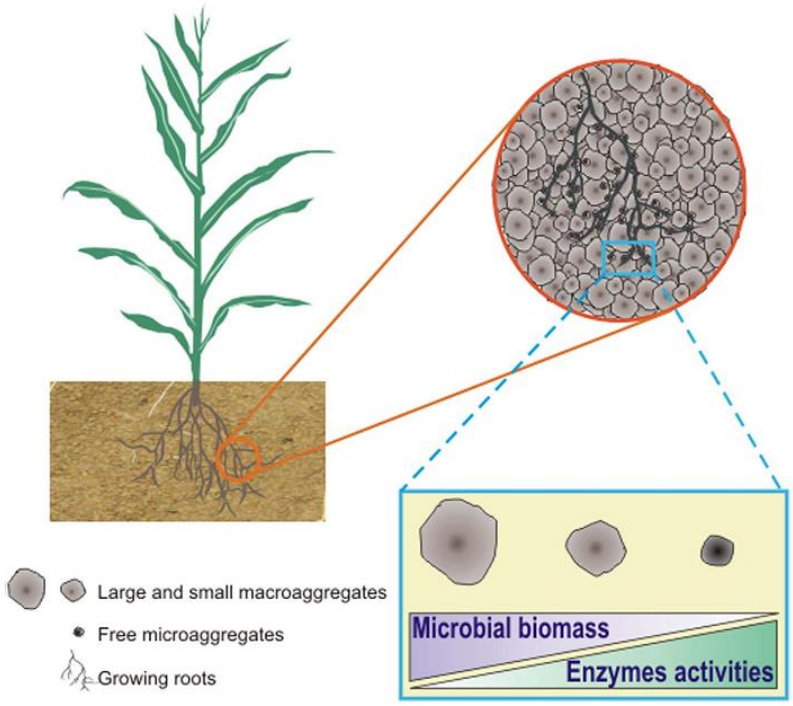

Figure I.3:4: Conceptual figure showing the potential effects of growing roots on extracellular enzyme activities (EEA) and microbial biomass in distinct aggregate size classes in rooted soil. Root induced microbial activities in distinct aggregate size classes are shown by higher EEA and the relations between aggregate size and microbial biomass are illustrated.

3.3 Effects of maize phenology and N fertilization on the distribution of extracellular enzyme activities (EEA) along with soil depth in the field (Study 3)

The present study highlighted regulation of plant phenological stage, soil depth and N fertilization on microbial activity (i.e. EEA). Enhanced activity of all measured enzymes in rooted soil (up to 58% increase in BG activity) as compared to bare fallow at both phenological stages provides evidence of plant-mediated activation of microorganisms (microbial activation hypothesis; Cheng and Kuzyakov 2005). Maize plants grow faster during earlier development stages and allocate a higher amount of photo-assimilated products belowground to roots (Pausch et al. 2013, Pausch and Kuzyakov 2017).

Increased belowground allocation for root development is generally positively related to root exudation (Pausch and Kuzyakov 2017). This increased release of labile substrates by roots (via exudation) at early growth stage facilitates microbial growth, resulting in higher EEA in rooted soil than in bare fallow (Nannipieri et al. 2012; Kuzyakov and Blagodatskaya 2015). In contrast, at maturation stage, when plants have a fully developed root system, the allocation of resources shifts from belowground to aboveground plant tissues (cob formation). As a result, the stimulating effect of roots on EA was reduced at maize maturity (Figure I.3:6). The change in EA of BG, CBH, XYL, NAG, PHO, and LAP in rooted soil depending on plant phenological stage demonstrated that, in the rhizosphere, microorganisms are fueled by root exudation, and their activity is intimately linked to both the quantity and quality of labile substrate inputs via roots (FigureI.3:5).

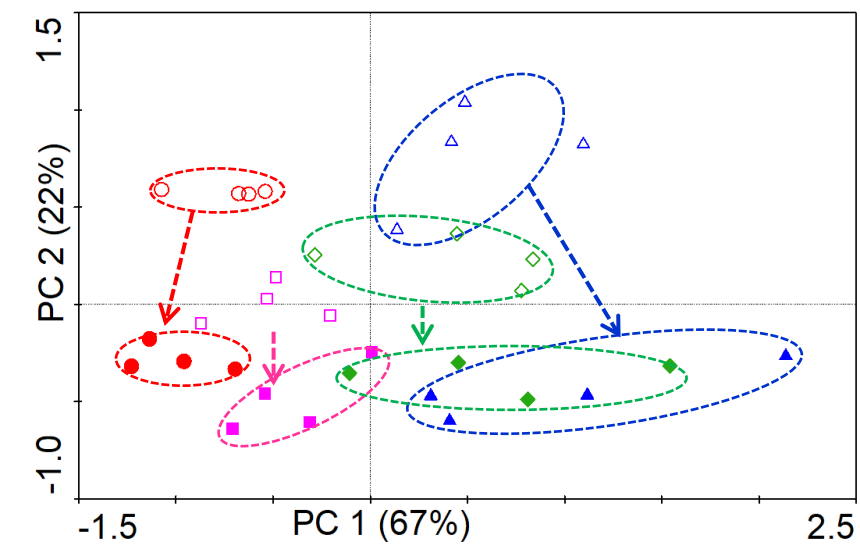

Figure I.3:5: The principal component analysis (PCA) analysis showed distinct enzyme activities at maize silking (unfilled symbols) and maturity (filled symbols) stage. Different colors and shapes indicate each soil depth as follows: 0-5 cm (red circle), 5-15 cm (blue upside triangle), 15-25 cm (green diamond), and 25-35 cm (pink square).

Besides the effect of maize phenology, EA was also altered by soil depth. Regarding soil depth, the highest enzyme activities were centered in the zone of maximum root density (5-25 cm), further supporting plant mediated increases in microbial growth and activity. Reduced Leucine-aminopeptidase and β-1,4-N-acetylglucosaminidase activities with N-fertilization demonstrates reduced resource allocation to N-cycling enzyme synthesis in the presence of alternative N sources (Figure I.3:6).

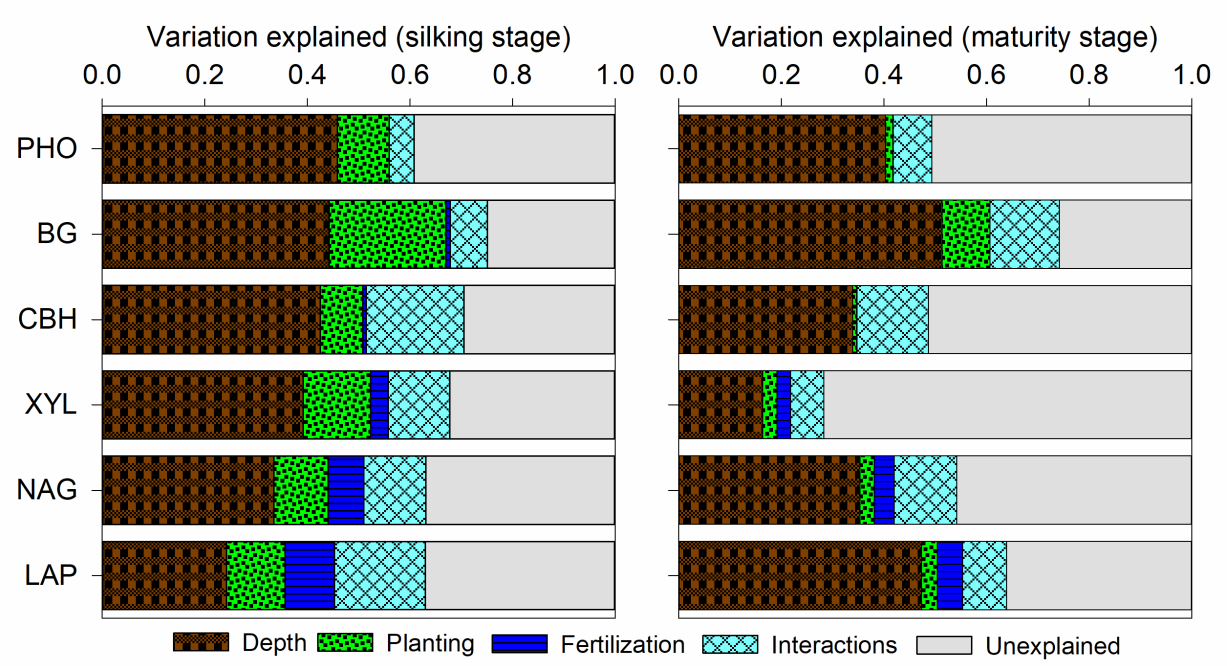

Figure I.3:6: Contribution of three factors: soil depth (0-5 cm, 5-15 cm, 15-25 cm, and 25-35 cm), maize roots (presence or absence of plants), N fertilization (presence or absence of N fertilization), and their interactions on potential activity of phosphomonoesterase (PHO), BG (ß-1,4-glucosidase), CBH (ß-cellobiohydrolase), XYL (ß-xylosidase), NAG (N-acetyl-1,4-glucosaminidase), and LAP (Leucine-aminopeptidase).

To summarize, 1) soil depth had the strongest effect on EA (up to 51% of total variation), 2) the root effect was stronger at the silking versus maturity stage; and 3) N fertilization affected only the enzymes related to N cycle (Figure I.3:7). We conclude that soil depth and plant phenology stage govern EA, and these effects are strongest between 5 and 25 cm soil depth containing silking plants.

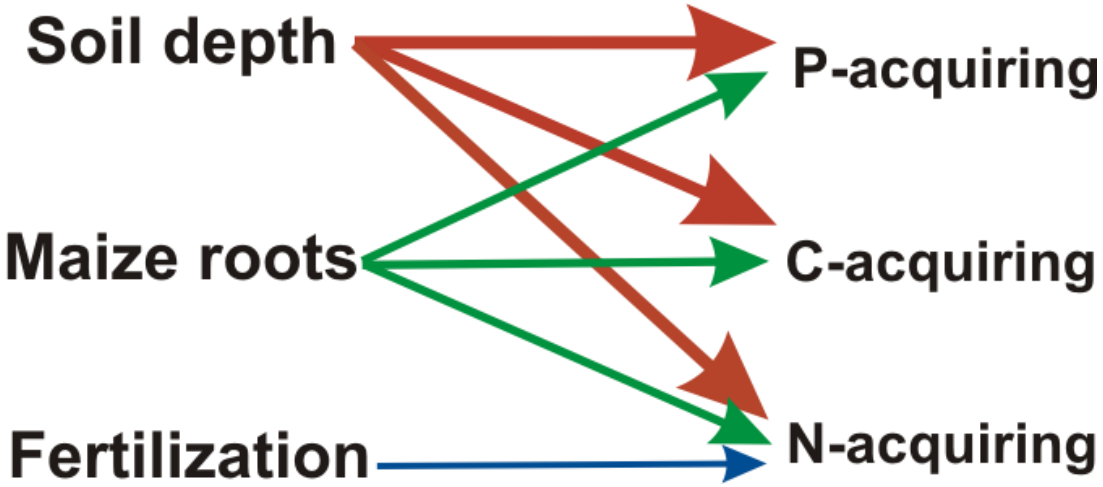

Figure I.3:7: Effects of soil depth, maize roots, and N-fertilization on distribution of activity of P-, C-, and N-acquiring enzymes in maize rhizosphere. Thickness of arrows indicates the strength of the effect on enzyme activities.

3.4 Root trait plasticity to maintain plant productivity under phosphorus limitation (Study 4)

The present study demonstrated that both, root morphological (root hairs) and biological traits (root colonization by AM fungi) are crucial for plant P uptake in P limited soils (Figure I.3:8). Root hairs promoted P uptake most likely by increasing the root surface area for absorption. Presence of root hairs increased the P uptake and decreased the dependency of plants on root mycorrhizal colonization by AM fungi, thereby reducing the C costs for P acquisition. However, the smaller surface area for absorption in absence of root hairs can be counterbalanced by increased root mycorrhizal colonization by AM fungi (Figure I.3:9). This alternative root trait for P uptake by exploring the soil volumes beyond the root depletion zone is important for maintaining plant growth in the absence of root hairs and under nutrient limitation. Plant adaptive strategy in response to higher colonization by increasing the root diameter of fine roots is an efficient policy resulting in lower costs and higher benefits. The present study enhances the understanding of plant

P uptake and interaction-response mechanisms with AM fungi at three major plant growth stages (tillering, stem elongation, and maize heading).

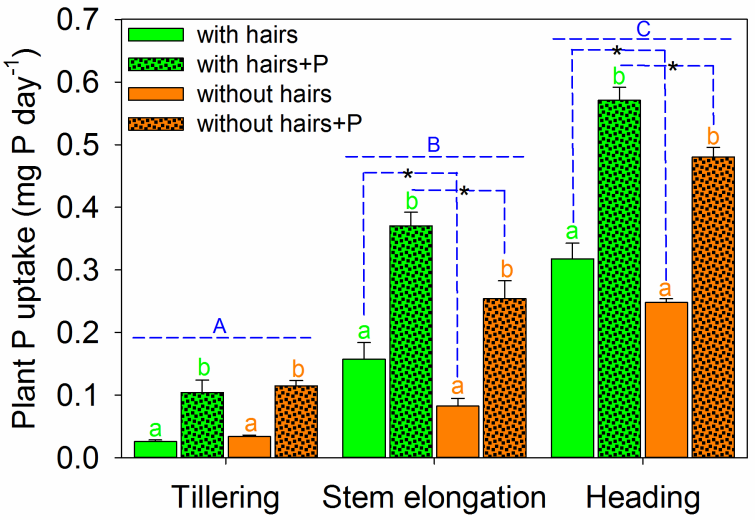

Figure I.3:8: Plant P uptake (mg P day-1±SEM) of unfertilized (without pattern) and P-fertilized (patterned bars) maize plants with (wild type: WT, green bars) and without root hairs (rth3, orange bars) at three growth stages at tillering (30 DAP), stem elongation (44 DAP), and maize heading (64 DAP) (ANOVA, $P < 0.05$). Lower-case letters indicate significant differences of P fertilization on plant P uptake separately for WT and rth3 maize at each plant growth stage (t-test, $P < 0.05$). * indicates significant difference between WT and rth3 maize (t-test, $P < 0.05$) (DAP = Days after planting, n = 4).

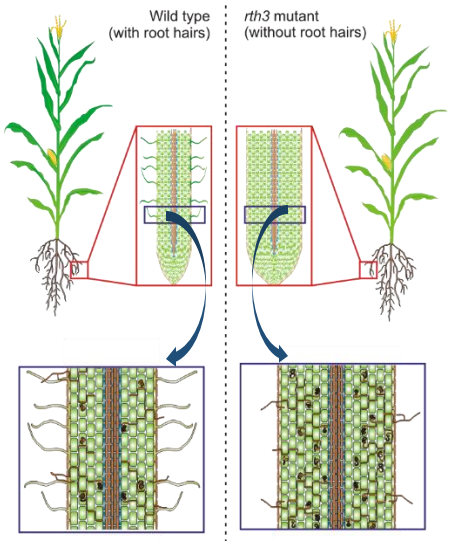

Figure I.3:9: Conceptualized diagram showing plasticity in root traits: increased average fine root diameter and higher root mycorrhizal colonization with AM fungi in rth3 mutant than wild type maize as a mechanism for phosphorus (P) acquisition in P limited soil.

4 Conclusion and outlook

The present thesis leads to the following conclusions:

(1) Rhizosphere priming effects of SOM decomposition are measureable under field conditions and are driven by microbial activation via root-derived organics. The magnitude of RPEs is dependent on soil nutrient status and root activity.

(2) Increased extracellular enzyme activities in all aggregate size classes in rhizosphere as compared to bare fallow are root mediated. Localization dependent conclusions on EEA in various sized aggregates are crucial due to preferential exposure to substrate inputs.

(3) Maize phenology determines the plant-mediated effects on EEA. Moreover, the depth dependent effects on EEA are most likely due to substrate availability and gaseous exchange at deeper soil depths.

(4) Plasticity in root traits for P acquisition is important for maintaining plant growth in absence or poorly developed root hairs and under nutrient limitation.

These conclusions are of particular relevance for future investigations because of following reasons:

(1) Field estimations of RPEs demonstrate the field relevance of plant mediated SOM decomposition. Despite higher root biomass with N fertilization demonstrates that RPEs are not a function of root biomass rather of root and microbial activity. Such mechanisms may vary with plant species and growth stage depending on nutritional demands for plants and microorganisms and therefore, there is need to measure RPEs at distinct plant growth stages as well as for other species.

(2) Higher microbial activity as reflected by higher EEA in rooted soil than bare fallow and in free microaggregates than macroaggregates demonstrates that the hotspots of microbial activity are not homogenously distributed in soil. We fractionated the free microaggregates and the microaggregates residing on the surface of macroaggregates. Future studies should also focus of the aggregate fractionation procedures as these will strongly chance the interpretation of the results.

(3) Plant mediated increase in EEA are dependent on plant growth and thereby root activity. The strength of such changes in EEA depending on plant phenology should be considered for future studies.

(4) Plant mediated changes in rates of SOM decomposition and nutrient cycling via altering microbial activities are central in the context of organic farming and sustainable agricultural practices. It is important to understand the mechanisms of building up and decomposition of SOM with minimal external inputs determining soil health and plant productivity.

5 Contribution to included manuscripts

Contribution (%) of each author to the included manuscripts.

With respect to:

a: concept and experimental design

b: field and laboratory work

c: data evaluation and statistical analyses

d: discussion and interpretation of results

e: manuscript preparation

Manuscripts	Authors	a	b	c	d	e
Maize rhizosphere priming: field estimates using ^{13}C natural abundance. *Published in Plant and Soil (2016). 409:87-97*	A. Kumar	50	90	60	50	70
	Y. Kuzyakov	0	0	0	10	10
	J. Pausch	50	10	40	40	20
Effects of maize roots on aggregate stability and enzyme activities in soil. *Published in Geoderma (2017). 306:50-57*	A. Kumar	60	60	70	55	80
	M. Dorodnikov	0	0	0	20	10
	T. Splettstoesser	0	20	0	5	0
	Y. Kuzyakov	0	0	0	10	0
	J. Pausch	40	20	30	10	10
Maize phenology alters the distribution of enzyme activities in soil: field estimates. *In revision, Applied Soil Ecology (2018). 125:233-239*	A. Kumar	70	70	80	55	80
	M. Shahbaz	0	10	0	10	0
	E. Blagodatskaya	0	0	10	10	0
	Y. Kuzyakov	0	0	0	10	0
	J. Pausch	30	20	10	15	20
Root trait plasticity to maintain plant productivity under phosphorus limitation. *In preparation*	A. Kumar	60	70	60	70	80
	M. Koirala	0	30	20	0	0
	Y. Kuzyakov	10	0	0	0	0
	J. Pausch	30	0	20	30	20

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II Manuscripts

1 Maize rhizosphere priming: field estimates using ^{13}C natural abundance

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Abstract

Root-mediated changes in soil organic matter (SOM) decomposition, termed rhizosphere priming effects (RPE), play crucial roles in the global carbon (C) cycle, but their mechanisms and field relevance remain ambiguous. We hypothesize that nitrogen (N) shortages may intensify SOM decomposition in the rhizosphere because of increase of fine roots and rhizodeposition.

RPE and their dependence on N-fertilization were studied using a C_3-to-C_4 vegetation change. N-fertilized and unfertilized soil cores, with and without maize, were incubated in the field for 50 days. Soil CO_2 efflux was measured, partitioned for SOM- and root-derived CO_2, and RPE was calculated. Plant biomass, microbial biomass C (MBC) and N (MBN), and enzyme activities (β-1,4-glucosidase; N-acetylglucosaminidase; L-leucine aminopeptidase) were analyzed.

Roots enhanced SOM mineralization by 35% and 126% with and without N, respectively. This was accompanied by higher specific root-derived CO_2 in unfertilized soils. MBC, MBN and enzyme activities increased in planted soils, indicating microbial activation, causing positive RPE. N-fertilization had minor effects on MBC and MBN, but it reduced β-1,4-glucosidase and L-leucine aminopeptidase activities under maize through lower root-exudation. In contrast, N-acetylglucosaminidase activity increased with N-fertilization in planted and unplanted soils.

This study showed the field relevance of RPE and confirmed that, despite higher root biomass, N availability reduces RPE by lowering root and microbial activity.

Key words: C_3/C_4 vegetation change, soil CO_2, SOM decomposition, enzyme activities, microbial biomass, N-fertilization.

1.1. Introduction

Agricultural soils are central with regard to global climate change because they may act either as potential C sinks (Smith et al. 2013) or as net sources of greenhouse gases (Lal 2011; Smith 2012). This makes it important to evaluate and balance the C inputs via living roots (rhizodeposits) and dead plants (litter) versus outputs via SOM decomposition. Altered dynamics of SOM decomposition in the rhizosphere play a significant role in the global C cycle (Coleman et al. 1992). This calls for a better understanding of the SOM dynamics in the rhizosphere in the field. In a meta-analysis, Finzi et al. (2015) showed that microbially mediated SOM decomposition is enhanced in the rhizosphere of various vegetation types and concluded that rhizospheric processes in SOM decomposition and subsequent nutrient release are quantitatively important at the ecosystem level. Thus, along with the importance of soil moisture and temperature for SOM decomposition, increasing recognition is being given to biotic processes in the rhizosphere regulating SOM decomposition (Zhu and Cheng 2012). Besides various processes occurring in the rhizosphere, rhizodeposition is the most important link between plant growth and microbially mediated processes in soils (Pausch et al. 2013a). The availability of easily utilizable C substrates is a key limiting factors for microbial activity in soil, and C availability is a main factor controlling SOM turnover (Fontaine et al. 2007; Paterson and Sim 2013). Thus, labile C input, e.g. root exudates, may alter the microbial decomposition of SOM, a process termed ˈrhizosphere priming effectsˈ (Kuzyakov 2002).

Living roots may either inhibit or stimulate the decomposition of SOM (Dijkstra et al. 2006; Fu et al. 2002; Reid and Goss 1982) via synergistic or antagonistic interactions, or both. The RPE of maize on the decomposition of SOM ranged from –30 % to more than

300% considering the effects of soil types, time period, N and CO_2 regimes in various studies (summarized by Cheng et al. 2014). Although RPE has been investigated in many studies, the underlying mechanisms are currently widely debated, but there is evidence that RPE mainly depends on decomposable C (Dormaar 1990; Meier et al. 2015) and the mineral N content in soil (Craine et al. 2007).

Rhizodeposition is an ecologically important part of rhizosphere processes because it serves as the primary energy source for microorganisms. This may enhance the metabolic activity of microorganisms and consequently affects the dynamics of SOM decomposition and, thus, rhizosphere priming (Microbial activation hypothesis, Cheng and Kuzyakov 2005; De Nobili et al. 2001; Kuzyakov et al. 2007; Pausch et al. 2013b). A trace amount of root exudates (µg g^{-1}) may enhance the microbially mediated decomposition of SOM (De Nobili et al 2001). Furthermore, altered root exudation may change the structure and function of microbial communities in the rhizosphere. This subsequently affects the SOM decomposition. Moreover, microbial N mining (Craine et al. 2007) may enhance SOM decomposition when nutrients are limited. Microorganisms as well as plants may thus benefit from nutrients released by extra decomposition of SOM (via RPE).

Here, we investigate the mechanisms of RPE and address the ecological importance of RPE. We applied a C_3-to-C_4 vegetation change in the field to estimate RPE. This approach is based on the discrimination of heavier (^{13}C) and lighter (^{12}C) C isotopes during CO_2 assimilation by C_3 and C_4 plants, which are characterized by distinct photosynthesis types (Balesdent and Mariotti 1996; Kuzyakov and Domanski 2000). Hence, by planting maize, a C_4 plant, on a soil which developed solely under C_3 vegetation, we introduced a distinct isotopic signal. This enabled partitioning total soil

CO_2 efflux for root- and SOM-derived CO_2 and thus to estimate the RPE of field-grown maize. Moreover, the extracellular activity of three enzymes (BG, NAG and LAP) was determined to link rhizosphere priming to microbial activities.

We hypothesized that (i) planting increases SOM decomposition via microbial activation through root exudates, and that (ii) mineral N application reduces RPE because plants alter their root activities and microbes are less dependent on nutrient gains from SOM decomposition.

1.2. Materials and Methods

1.2.1. Experimental setup

The experiment was established on an agriculture field at the experimental research station Reinshof of the Georg-August University, Göttingen, and was solely under C_3 crops. Therefore, the organic C in the soil originated from C_3 vegetation. In this experiment, a vegetation change from C_3 to C_4 (maize) crops was used to introduce a distinct ^{13}C signal into the soil and to partition the total soil CO_2 efflux into root-derived and SOM-derived CO_2.

Four plots (5 x 5 m^2) were established: bare fallow (Unplanted), bare fallow with N-fertilization (Unplanted+N), maize-planted (Planted) and maize-planted with N-fertilization (Planted+N). In both planted plots, maize was grown with a plant density of 6 plants m^{-2}. For N-fertilization, urea (Weiterer, Landhandel GmbH) was applied at the soil surface at a rate of 160 kg N ha^{-1}.

Before the incubation started, maize (*Zea mays* L.) was sown in the field for 10 days. For incubation, mesh pots (height 35 cm, diameter 18 cm) were constructed from stainless metal mesh covered with nylon gauze to avoid soil losses from the pot. The

nylon gauze allowed water and other solute transport across the mesh. Four undisturbed soil cores were collected from each plot (Unplanted, Unplanted+N, Planted, and Planted+N) with a soil corer (height 35 cm, diameter 18 cm) and transferred to the pots. In both planted plots, each soil core contained one 10cm-high maize plant. The pots were then placed back in the holes made with the corer and incubated in the field.

1.2.2. CO_2 trapping

The pots were incubated in the field for 50 days. We have chosen the time point of 50 days after planting in order to sample during the period of maximum growth and root exudation. Afterwards, the pots were removed from the field and brought to laboratory and placed in a growth chamber for 30 h with conditions adapted to those in the field. Total soil respiration was measured using a closed-circulation CO_2 trapping system (Figure II.1:1). Briefly, each pot was placed in a PVC column (KG tubes; height 40 cm, diameter 20 cm). Air inlet tubing at the upper end and outlet tubing at the lower end of the PVC column were connected to a membrane pump. An aliquot of 1 M NaOH solution was inserted between the air outlet tubing and membrane pump (Figure 1). The planted pots were sealed with plastic foil, and at the base of plant stem with a non-toxic gel (Wasserfuhr, GmbH), to avoid any leakage. Prior to CO_2 trapping, CO_2 inside each pot was removed by circulating the isolated air through 1 M NaOH for 2 h. Afterwards, the CO_2 produced in each pot was trapped in 400 ml of 1 M NaOH solution for a period of 24 h by periodic air circulation for 1 h at 6 h intervals. Blanks were included (empty but closed PVC columns) and treated in the same way to correct inorganic C for handling errors. One subsample from each NaOH solution was analyzed for total inorganic C (SHIMADZU,TOC-5050) and another subsample was precipitated as $SrCO_3$ with 1 M $SrCl_2$ for $\delta^{13}C$ analysis using an isotopic ratio mass spectrometer (Delta V Advantage,

Conflo III) coupled to an elemental analyzer 2000 (Thermo Fischer Scientific, Cambridge UK).

Total soil CO_2 efflux (C_{total}) was separated into SOM-derived CO_2 (C_{SOM}) and root-derived CO_2 (rhizosphere respiration) (C_{root}) using a two-source mixing model (Pausch et al. 2013b).

$$C_{SOM} = C_{TOTAL} (\delta^{13}C_{TOTAL} - \delta^{13}C_{ROOT}) / (\delta^{13}C_{SOM} - \delta^{13}C_{ROOT}) \quad (1)$$

$$C_{ROOT} = C_{TOTAL} - C_{SOM} \quad (2)$$

where, δ_{TOTAL}, δ_{SOM} and δ_{ROOT} are the $\delta^{13}C$ values in ‰ for total CO_2 efflux, SOM- and root-derived CO_2. C_{TOTAL}, C_{SOM} and C_{ROOT} are the CO_2 concentrations (mg C day^{-1} kg^{-1} soil).

RPE was calculated as the difference of C_{SOM} between planted and unplanted soils (Pausch et al. 2013b) as shown below:

$$RPE = C_{SOM(Planted)} - C_{SOM(Unplanted)} \quad (3)$$

$$RPE = C_{SOM\ (Planted+N)} - C_{SOM(Unplanted+N)} \quad (4)$$

Note, the ^{13}C isotopic fractionation between root C and root-derived CO_2 was not considered in the present study, which may have affected the calculated RPE. As reviewed by Werth and Kuzyakov (2010), the fractionation for C_4 plants is on average -1.3‰ with variations up to ±2‰. Since the fractionation is unknown for our plant-soil system, we decided not to include the literature value, as this would not add greater certainty to the results.

1.2.3. Harvest

Directly after CO_2 trapping, the total weight of each pot was determined and the pots were destructively harvested. Shoots were cut at the base and dried at 60 °C for 3 days.

The soil cores were pulled out of the pot and the “main” root system was carefully removed. A representative homogenized soil sample (400-500 g) was taken from each pot to determine soil moisture, microbial biomass C and N, and extracellular enzyme activities. Soil moisture was about 12.5 to 14% and did not differ significantly between the planted and unplanted soils.

For root analyses, a soil subsample (300 g fresh soil) was taken and fine roots were picked from the soil for 15 minutes. Afterwards, the “main” roots and the fine roots (from root picking) were scanned by an EPSON (PERFECTION™ V700 PHOTO) scanner and the root length density was determined using WinRhizo (2008) software. All roots with diameters < 2 mm were considered as fine roots. The root length density of roots picked from 300 g soil was up-scaled to the whole pot weight. After scanning, all roots (“main” and picked roots) were dried at 60 °C for 3 days. Roots were analyzed for $\delta^{13}C$ values using the isotopic ratio mass spectrometer and elemental analyzer 2000 noted above. All the isotopic analyses were performed at the Center for Stable Isotope Research Analysis (KOSI) at the University of Göttingen, Germany.

1.2.4. Soil microbial biomass

Soil microbial biomass C (MBC) and N (MBN) were analyzed on fresh samples using the chloroform fumigation-extraction method (Vance et al. 1987). Briefly, a non-fumigated soil sample (8 g fresh soil) was extracted with 40 ml of 0.05 M K_2SO_4 by continuously shaking (Laboratory shaker, GFL 3016) (150 rpm) for 1 h. After shaking, the soil suspension was filtered through Ahlstrom-Munktell filters (Grade: 3hw, diameter 110 mm). The organic C and N contents of filtered solution were measured with a multi N/C analyzer (multi N/C analyzer 2100S, Analytik Jena). The same extraction procedure was

followed for fumigated soil. Fumigation was carried out in a desiccator with 80 ml of ethanol-free chloroform at room temperature for 24 h.

MBC and MBN were calculated by dividing the difference between extracted C and N from fumigated and non-fumigated soil samples with a K_{EC} and K_{EN} factor of 0.45 and 0.54, respectively (Joergensen and Mueller. 1996). The C and N contents from non-fumigated soil samples were considered as dissolved organic C (DOC) and dissolved N (DN), respectively.

1.2.5. Enzyme assays

Extracellular enzymes activities were measured using the method described by Marx et al. (2001). Fluorogenic methylumbelliferone (MU)-based artificial substrates were used to estimate the activities of β-1,4-glucosidase (EC 2.2.1.21) (BG), which catalyzes the terminal reaction in hydrolyzing structural carbohydrates (i.e. cellulose) and the activities of β-1,4-N-acetylglucosaminidase (EC 3.2.1.14) (NAG), which catalyzes the terminal reaction in chitin and other N-acetylglucosamine-containing polymer hydrolysis. Fluorogenic 7-amino-4-methycoumarin (AMC)-based artificial substrate was used to estimate the activity of L-leucine aminopeptidase (EC 3.4.11.1) (LAP), which hydrolyses the terminal reaction in peptide breakdown, releasing leucine and other amino acids (Sinsabaugh and Shah 2012).

Briefly, soil suspension was made by dissolving 1 g fresh soil sample in 50 ml autoclaved water using a low-energy sonication (50 Js^{-1}) for 120 s (Koch et al. 2007; Stemmer et al. 1998). An aliquot of 50 µl was dispensed in a 96-well black microplate (Puregrade, Germany) while stirring the soil suspension to ensure uniformity. Afterwards, 50 µl of MES buffer (pH 6.5) was added to the well. Finally, 100 µl serial concentrations of substrate solutions (20, 40, 60, 80, 100, 200, 400 µmol substrate g

soil^{-1}) were added to the wells. The microplate was rippled and measured fluorometrically (excitation 360 nm; emission 450 nm) at 0, 30, 60, 120 m after substrate addition with an automated fluorometric plate-reader (Victor3 1420-050 Multi-label Counter, PerkinElmer, USA).

To estimate enzyme activity (V), we used the Michaelis-Menten equation for enzyme kinetics (Marx et al. 2001, 2005; Razavi et al. 2015):

$$V = (Vmax \times [S]) / (Km + [S]) \quad (5)$$

where, V_{max} is the maximal rate of enzyme activity; K_m (Michaelis constant) is the substrate concentration at which V_{max} is half; and [S] is the substrate concentration.

Statistics

The experiment was carried out with 4 field replicates for each measured parameter. The values for RPE, microbial biomass C and microbial biomass N, plant biomass, and enzymes activity were expressed as means ± standard errors (mean ± SEM). Prior to analysis of variance (ANOVA), the data were tested for normality (Shapiro-Wilk test, $P > 0.05$) and homogeneity of variance (Levene-test, $P > 0.05$). We used factorial ANOVA to test the effects of plantation and N-fertilization on MBC and MBN, SOM-derived CO_2, and V_{max} of extracellular enzymes. The ANOVAs were followed by post-hoc tests for multiple comparisons using least significant differences (Tukey-test). We used Student's t-test to test the differences in plant biomass (root- and shoot biomass), RPE, root-derived CO_2, specific RPE, and RPE as percent of control in Planted and Planted+N soils. In general, a significance level of $P < 0.05$ was used for ANOVA and t-test if not mentioned specifically. Statistical analyses were performed with STATISTICA for Windows (version 7.0; StatSoft Inc., OK, USA).

1.3. Results

1.3.1. Plant biomass

Total plant biomass (shoot and root biomass) per pot was higher in the N-fertilized plants (Planted+N) (36.3 ± 8.1 g pot^{-1}) than in unfertilized plants (Planted) (20.7 ± 2.2 g pot^{-1}) (Figure II.1:2). The shoot to root ratio was lower in N-fertilized plants (Planted+N) (7.5±0.9) than unfertilized plants (Planted) (10.2±1.3), although the difference was not statistically significant ($P < 0.05$).

1.3.2. Total soil CO_2 efflux and source-partitioning

Plants increased the total soil CO_2 efflux in both N-fertilized and unfertilized soils (Figure II.1:3). However, the CO_2 efflux was lower in N-fertilized treatments (both in Unplanted+N and Planted+N) compared with unfertilized treatments (Unplanted and Planted). Total CO_2 efflux ranged from 27.7±5.9 to 116.0±26.2 mg C day^{-1} kg^{-1} soil, being lowest in bare fallow with N-fertilization (Unplanted+N) and highest in unfertilized soils planted with maize (Planted).

A linear two-source isotopic mixing model was used to calculate the contribution of SOM-derived and root-derived CO_2 to total CO_2 efflux in unfertilized and N-fertilized soils planted with maize. SOM-derived CO_2 was higher (87.4±16.1 mg C day^{-1} kg^{-1} soil) ($P < 0.05$) in unfertilized soils with maize (Planted), whereas N-fertilization resulted in less SOM-derived CO_2 emission (37.2±2.6 mg C day^{-1} kg^{-1} soil) (Planted+N) (Figure II.1:3). Furthermore, specific root-derived CO_2 was calculated by dividing root-derived CO_2 to total root biomass. Specific root-derived CO_2 was higher ($P < 0.05$) in unfertilized plants (Planted) (131.6±22.5 mg C day^{-1} g^{-1} root) than in N-fertilized plants with (Planted+N) (38.4 ±5.8 mg C day^{-1} g^{-1} root) (Figure II.1:3; inset).

1.3.3. Rhizosphere priming effect

Positive RPE was found in both planted soils (Planted and Planted+N), resulting from enhanced decomposition of SOM. Nonetheless, the RPE was lower ($P < 0.1$) in N-fertilized soils with maize (Planted+N) (9.6±2.6 mg C day^{-1} kg^{-1} soil) compared to unfertilized soils with maize (Planted) (48.8±16.1mg C day^{-1} kg^{-1} soil). SOM-decomposition in unfertilized and N-fertilized soils with maize increased by 126.2±41.7% and 34.5±9.2%, respectively, compared to the unplanted soils (Figure II.1:4). Specific RPE was calculated by dividing RPE by total root biomass. Specific RPE was higher ($P < 0.05$) in unfertilized soils (Planted) than N-fertilized soil (Planted+N) (Figure II.1:4; inset).

1.3.4. Microbial biomass and extracellular enzyme activity

Plants had stimulating effects on MBC and MBN. The lowest MBC was in bare fallow (Unplanted), whereas N-fertilization increased MBC (20%) especially in planted soils (Planted+N) versus bare fallow. There was a trend of increasing MBC with N-fertilization and under plants with and without N-fertilization in the sequence: Unplanted < Unplanted+N < Planted < Planted+N (Figure II.1:5). Planting also increased MBN ($P < 0.05$) (Planted and Planted+N) compared to bare fallows (Unplanted and Unplanted+N). N-fertilization, however, had only a minor effect on MBN. The ca. 30% increase in MBN in planted soils reflected microbial activation.

The activities of three enzymes were stimulated by planting (Planted and Planted+N), resulting in increased reaction rates. Planting increased the potential activity of BG (84% and 97% for N-fertilized and unfertilized soils with maize), NAG (80% and 65% for N-fertilized and unfertilized soils with maize), and LAP (27% and 53% for N-fertilized and unfertilized soils with maize) in comparison with N-fertilized and unfertilized bare fallow

(Figure II.1:6). N-fertilization lowered the potential activity of BG and LAP by lowering V_{max} in planted soils, but it increased the activity in bare fallow (not statistically significant). When compared to BG and LAP, NAG showed a different pattern. V_{max} of NAG followed a pattern in ascending order: bare fallow < bare fallow with N-fertilization < unfertilized maize-planted soil < N-fertilized maize-planted soil (Figure II.1:6).

1.4. Discussion

1.4.1. Effects of living roots on SOM decomposition

The rhizosphere priming effect has been widely observed in numerous studies under controlled conditions, but field studies are still rare (Cheng et al. 2014). Here, we provide measurements of RPE of SOM decomposition in a maize field based on SOM-derived CO_2. In agreement with other studies (Dijkstra et al. 2013; Finzi et al. 2015; Mwafulirwa et al. 2016; Pausch et al. 2013b), the plants accelerated the decomposition of SOM.

Positive priming has often been explained by the microbial-activation hypothesis (Chen et al. 2014; Kuzyakov and Cheng, 2005). The secretion of labile C compounds by roots enhances microbial growth and activity, leading to higher extracellular enzyme activities and, hence, accelerated SOM decomposition (Figure II.1:7) (Fontaine et al. 2003; Kuzyakov 2010; Loeppmann et al. 2016; Neumann and Römheld 2007).

SOM-derived CO_2 was about 35% higher for N-fertilized and 126% higher for unfertilized planted soils compared to the bare fallows. Accompanied by positive rhizosphere priming, MBC and MBN were increased through planting (Figure II.1:4). Furthermore, in the rhizosphere; the higher microbial activity in response to root exudation (root-released easily available substrates) was characterized by increased V_{max} for BG, NAG and LAP in comparison with bare fallows. The rhizosphere priming effect increased with activities

of BG, LAP and NAG. In a similar study, BG activity and SOM decomposition were positively correlated (Zhu et al. 2014).

BG is involved in the degradation of structural carbohydrates (i.e. cellulose). It catalyzes terminal hydrolysis in cellulose degradation by producing two moles of glucose per mole of cellobiose, and glucose is an important energy source for microorganisms (Turner et al. 2002). Furthermore, BG synthesis is triggered by the presence of cellobiose, glucose and other metabolites of cellulose degradation (Stewart and Leatherwood 1976). In the rhizosphere, root exudation triggers enhanced synthesis of BG, which is accompanied by RPE in the decomposition of SOM.

When labile C sources with high C/N ratios are available for microorganisms, they start producing N-degrading enzymes to obtain N from SOM (Fontaine et al. 2011). Proteins and chitins are the most abundant organic N sources (Moorhead et al. 2012). For proteins, LAP is involved in the terminal hydrolysis of polypeptides, releasing amino acids. For chitins, NAG hydrolyses N-acetylglucosamine (monosaccharide derivative of glucose) from chito-oligosaccharides (i.e. chitobiose) (Sinsabaugh 1994). LAP and NAG are the most commonly detected N-degrading enzymes for SOM decomposition (Moorhead et al. 2012). Taking into account the microbial activation hypothesis (Cheng and Kuzyakov 2005), the present study provides evidence that, also under field conditions, living roots activate microorganisms for SOM decomposition.

1.4.2. Effect of N-fertilization on SOM decomposition

Planting induced positive priming effects in the rhizosphere (Figure II.1:4). When N was added, however, the extra SOM decomposition (positive RPE) was much lower in N-fertilized soils (35% of bare fallow with N-fertilization) than in unfertilized soils (126% of bare fallow), despite a higher shoot and root biomass of N-fertilized maize plants.

Increased N supply may increase net assimilation, and plants produce higher biomass (Hodge et al. 1996; Warembourg and Estelrich 2001; Zhu et al. 2015). Although the root biomass of N-fertilized maize was about twice as high, the rhizosphere respiration (root-derived CO_2) was lower, indicating lower root activity (Figure II.1:7). It is well known that plants invest more C resources for root exudation under nutrient limitations (Hodge et al. 1996; Kraffczyk et al. 1984; Ratnayake et al. 1978). Root exudation also stimulates microbial activity and nutrient availability (Smith 1976; Yin et al. 2013). Both theoretical (Cheng et al. 2014; Wutzler and Reichstein 2013) and experimental studies (Drake et al. 2013; Phillips et al. 2011) have shown that enhanced root exudation may accelerate RPE for SOM decomposition, thus increasing the flux of nutrients to forms available for plants. Reduced root exudation in N-fertilized maize plants, indicated by lower root-derived CO_2 (consisting of CO_2 from root respiration and CO_2 released by decomposition of exudates), showed that these plants do not rely solely on nutrients from SOM decomposition. RPE can increase with increasing root activity (Zhu et al. 2014). Rhizosphere respiration was positively correlated with RPE ($P < 0.01$). In addition, the specific root length density (fine root length density (< 2 mm) per gram root) was higher ($P < 0.059$) for unfertilized maize plants (data not presented). This altered root architecture of unfertilized maize may help to make the limiting resources accessible by maximizing root surface area and enabling roots to have greater contact with soil surfaces (Paterson and Sim 1999).

These findings suggested that root activity is intimately connected with microbially mediated SOM decomposition. Furthermore, root exudates are characterized by high C to N ratios (Cheng and Kuzyakov 2005). Such high ratios results in higher C availability and a severe N limitation for microorganisms (Kuzyakov and Blagodatskaya 2015;

Kuikman et al. 1990; Liljeroth et al. 1990; Merckx et al. 1987). Moreover, there is a strong competition for mineral N between roots and microorganisms (Kuzyakov and Xu 2013). With addition of N-fertilizer, microorganisms are less dependent on extra N released via priming because the competition between plant roots and microorganisms for mineral N becomes weaker (Van Veen et al. 1989): microorganisms start utilizing exudates and the available mineral N (preferential substrate utilization) (Kuzyakov 2002; Sparling et al. 1982). The present study detected no differences for the effect of N-fertilization on microbial biomass C and N. Nonetheless, N-fertilization altered extracellular enzyme activity differently. Inorganic fertilizers may either maintain or reduce the activities of many extracellular enzymes in planted soils, but increase their activities in unplanted soils (Ai et al. 2012). In the present research, the activities of BG and LAP were generally lowered in maize-planted soils with N-fertilization, whereas their activities increased in bare fallow with N-fertilization. Moreover, the NAG activity increased in both bare fallow and maize-planted soils with N-fertilization. This could be an indirect evidence for shifts in microbial communities in favor of fungi. Various studies showed this shift in microbial taxonomic groups in favor of fungi with N-fertilization (Bardgett et al. 1999; Paul and Clarke. 1996 in Keeler et al. 2009; Weand et al. 2010). In summary, N-fertilization lowered the root release of available C, which subsequently lowered microbial activity by decreasing extracellular enzyme production. The net result is less SOM decomposition in the rhizosphere.

1.5. Conclusions

RPE were measurable in the field. Higher MBC and MBN in the rhizosphere support the microbial activation by root exudation. This microbial activation is accompanied by

increased extracellular enzyme activities, which further confirm that extracellular enzyme production is an important mechanism of SOM decomposition in the rhizosphere. The N status of soils largely controls the magnitude of rhizosphere priming. N fertilization substantially reduced rhizosphere priming by lowering SOM decomposition. Lower root-derived CO_2 and enzyme activities in the rhizosphere with N-fertilization confirmed that the availability of mineral N weakens the competition between roots and microorganisms. However, increased root-derived CO_2 and enzyme activities without N fertilization intensify the root and microbial competition for N and the dependence of microorganisms on N mining.

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1.8. Figures

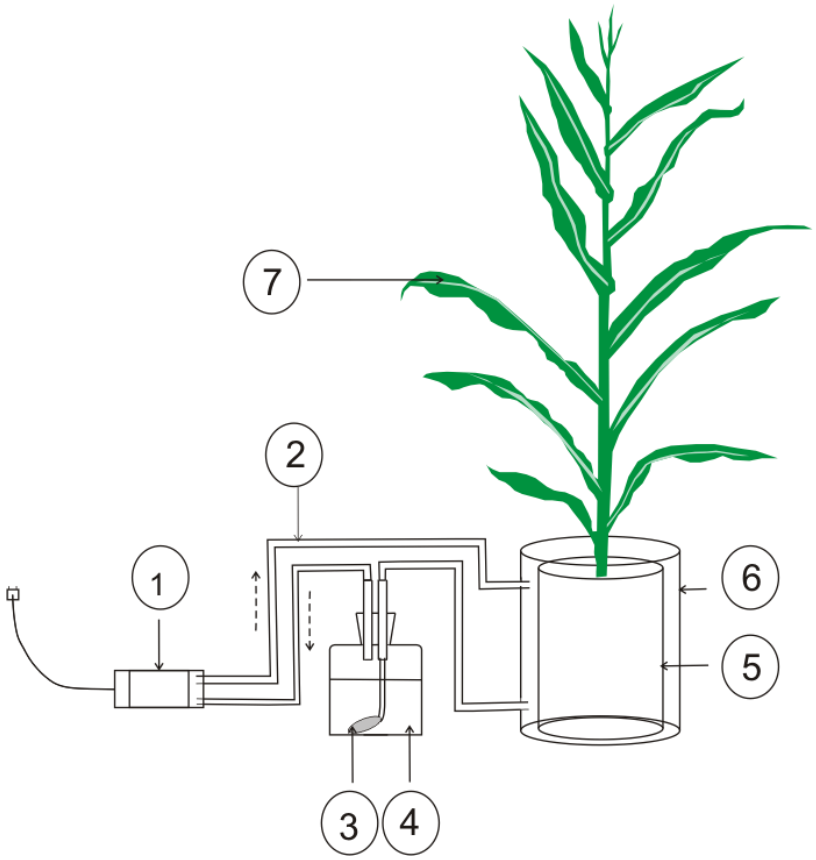

Figure II.1:1: Experimental setup of the CO_2 trapping system. 1 - membrane pump, 2 - PVC tube (diameter 5 mm), 3 - air stone, 4 - NaOH, 5 - pot, 6 - PVC column, 7 - maize plant. Arrows show the direction of air flow in the closed-circulation system.

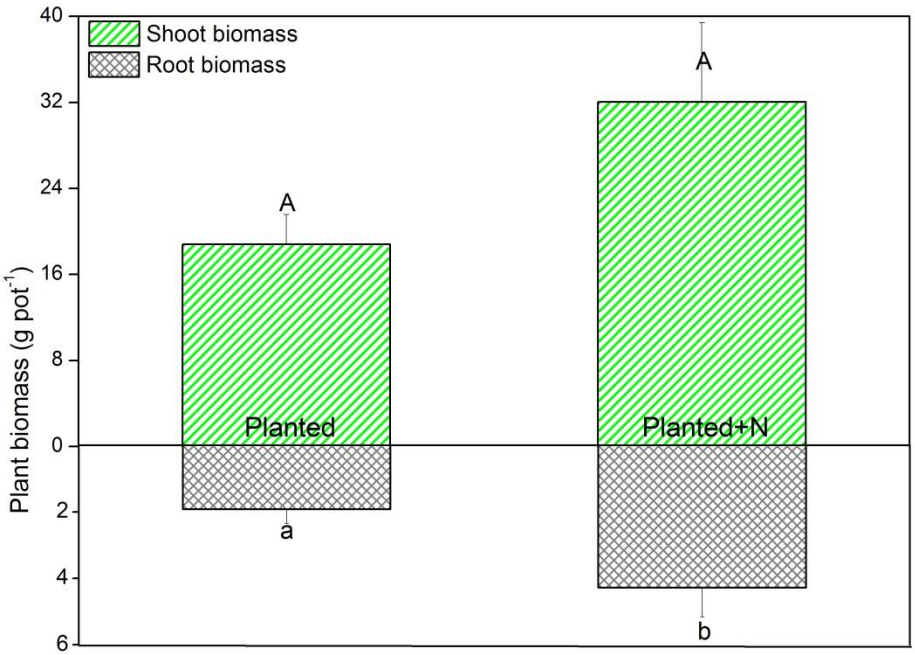

Figure II.1:2: Plant biomass (root and shoot biomass) (g pot^{-1}) (±SEM) for unfertilized and N-fertilized maize plants. Lower-case letters indicate significant differences for root biomass, upper-case letters indicate significant differences for shoot biomass between N-fertilized and unfertilized maize ($P < 0.05$).

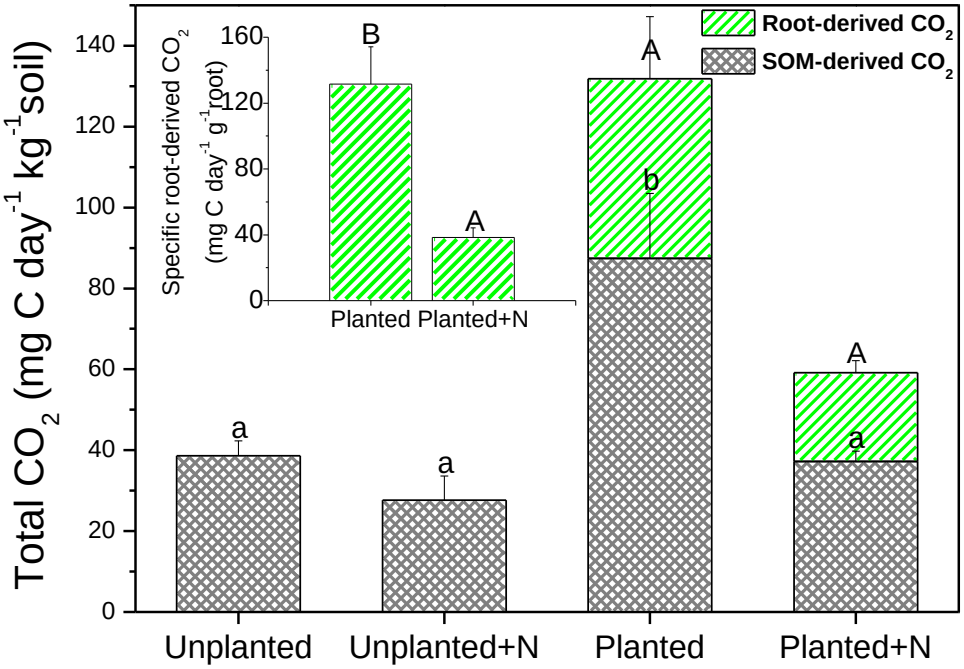

Figure II.1:3: Total CO_2 efflux (mg C day^{-1} kg^{-1} soil) (±SEM) from bare fallow (Unplanted), bare fallow with N-fertilization (Unplanted+N), unfertilized maize-planted (Planted) and N-fertilized maize-planted (Planted+N) soils. Total CO_2 efflux was partitioned by source (SOM-derived and root-derived CO_2). Lower-case letters indicate significant differences between bare faloow, bare fallow with N-fertilization, unfertilized and fertilized maize planted soils (ANOVA, $P < 0.05$). Upper-case letters in root-derived CO_2 and specific root-derived CO_2 (inset) indicate significant differences according to t-test ($P < 0.05$).

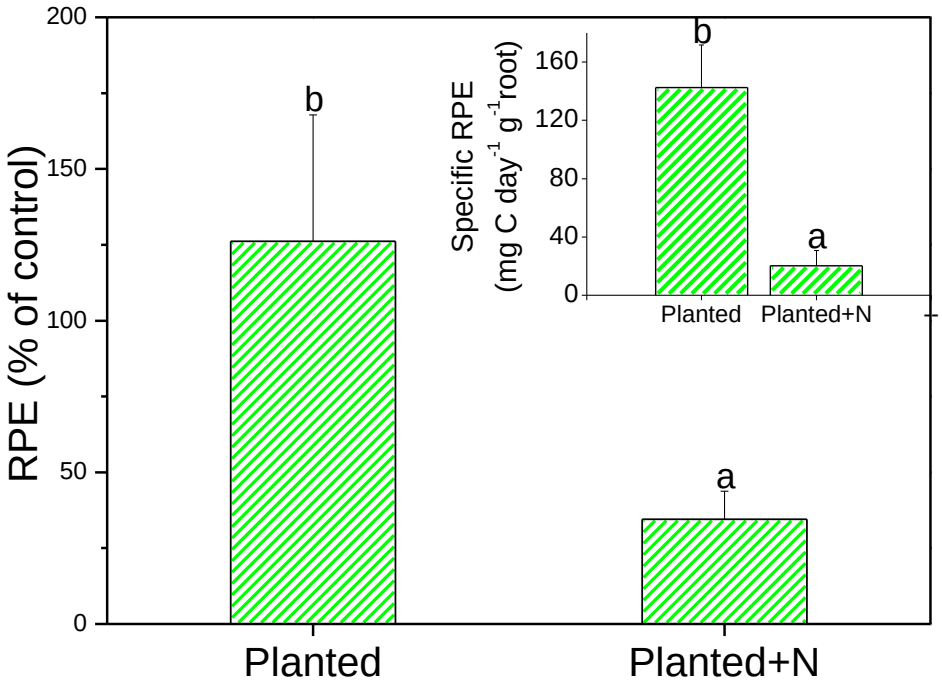

Figure II.1:4: Rhizosphere priming effect (RPE) (±SEM) as % of CO_2 efflux from bare fallows for unfertilized (Planted) and N-fertilized (Planted+N) maize plants. The inset shows specific RPE (mg C day^{-1} g^{-1} root) (±SEM). Letters indicate the significant differences for RPE ($P < 0.01$) and for specific RPE ($P < 0.05$) between unfertilized and N-fertilized maize planted soils.

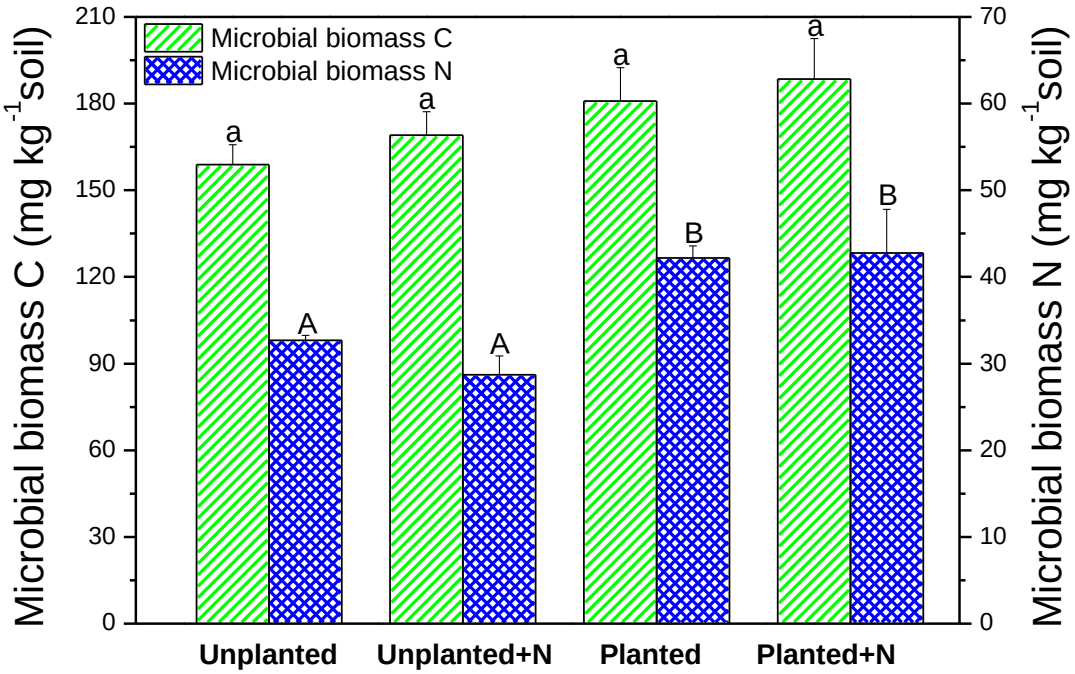

Figure II.1:5: Microbial biomass C (left y-axis; mg C kg^{-1} soil) and N (right y-axis; mg N kg^{-1} soil) (±SEM) in bare fallow (Unplanted), bare fallow with N-fertilization (Unplanted+N), unfertilized maize-planted (Planted) and N-fertilized maize-planted (Planted+N) soils. Lower-case letters indicate significant differences for MBC, upper-case letters indicate significant differences for MBN between bare fallow, bare fallow with N-fertilization, unfertilized and N-fertilized maize planted soils (ANOVA $P < 0.05$).

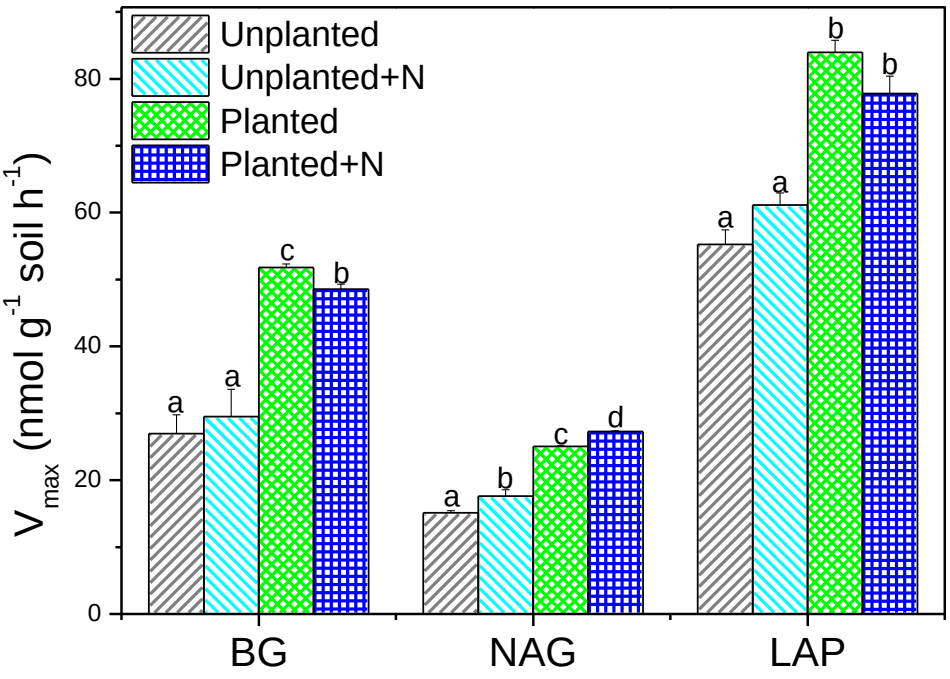

Figure II.1:6: Potential activity (Vmax; nmol g $soil^{-1}$ h^{-1}) (±SEM) of three extracellular enzymes (BG: β-1, 4-glucosidase; NAG: β-1, 4-N-acetylglucosaminidase; LAP: L-leucine aminopeptidase) in bare fallow (Unplanted), bare fallow with N-fertilization (Unplanted+N), unfertilized maize-planted (Planted) and N-fertilized maize-planted (Planted+N) soils. Letters indicate significant differences between bare fallow, bare fallow with N-fertilization, unfertilized and N-fertilized maize-planted soils (ANOVA, $P < 0.05$).

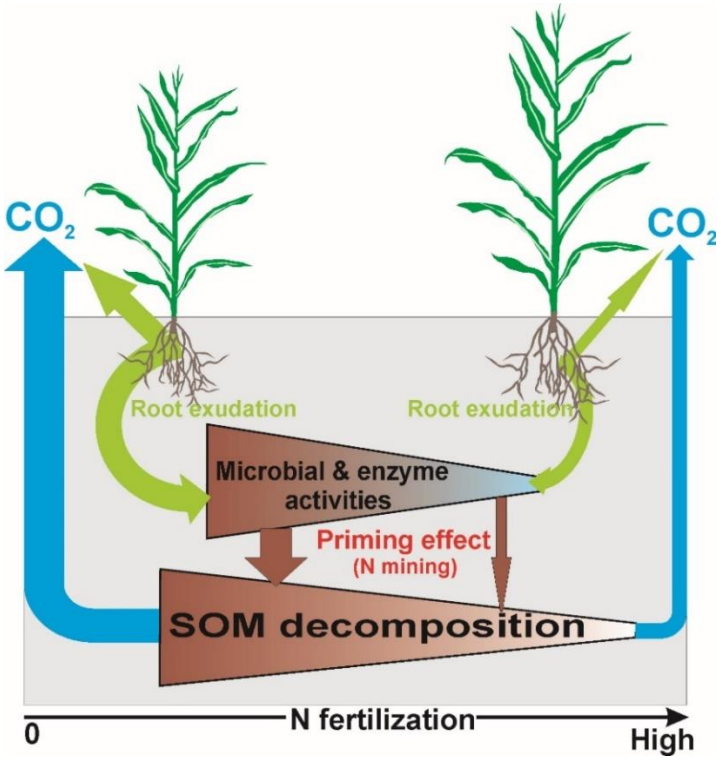

Figure II.1:7: Conceptual figure showing rhizosphere priming on SOM decomposition accompanied by microbial activation and N mining. Arrow thickness indicates process intensity.

2. Effects of maize roots on aggregate stability and enzyme activities in soil

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Abstract

Soil aggregation and microbial activities within the aggregates are important factors regulating soil carbon (C) turnover. A reliable and sensitive proxy for microbial activity is activity of extracellular enzymes (EEA). In the present study, effects of soil aggregates on EEA were investigated under three maize plant densities (Low, Normal, and High). Bulk soil was fractionated into three aggregate size classes (>2000 µm large macroaggregates; 2000-250 µm small macroaggregates; <250 µm microaggregates) by optimal-moisture sieving. Microbial biomass and EEA (β-1,4-glucosidase (BG), β-1,4-N-acetylglucosaminidase (NAG), L-leucine aminopeptidase (LAP) and acid phosphatase (acP)) catalyzing soil organic matter (SOM) decomposition were measured in rooted soil of maize and soil from bare fallow. Microbial biomass C (C_{mic}) decreased with decreasing aggregate size classes. Potential and specific EEA (per unit of C_{mic}) increased from macro- to microaggregates. In comparison with bare fallow soil, specific EEA of microaggregates in rooted soil was higher by up to 73%, 31%, 26%, and 92% for BG, NAG, acP and LAP, respectively. Moreover, high plant density decreased macroaggregates by 9% compared to bare fallow. Enhanced EEA in three aggregate size classes demonstrated activation of microorganisms by roots. Strong EEA in microaggregates can be explained by microaggregates' localization within the soil. Originally adhering to surfaces of macroaggregates, microaggregates were preferentially exposed to C substrates and nutrients, thereby promoting microbial activity.

Keywords: rooted soil, root exudation, free microaggregates, plant density, specific enzyme activity, mean weight diameter.

2.1. Introduction

Intensive agriculture often leads to decreases in soil carbon (C) stocks and reduces the quality of soil organic matter (SOM) (Paz-Ferreiro and Fu, 2016). The alterations to soil C stocks could have further impacts on the global C cycle (Nie et al., 2014). Soil microorganisms are one of the important biotic drivers regulating the soil C cycle. In terrestrial ecosystems, microbially mediated SOM decomposition constitutes a major part of soil C losses along with abiotic factors (Kaiser et al., 2010). Therefore, even minor changes in microbial decomposition of SOM due to intense agricultural practices may substantially impact the global climate via carbon dioxide (CO_2) efflux to the atmosphere.

Extracellular enzyme activities (EEA) are good indicators of microbially mediated SOM decomposition and are highly sensitive to environmental changes (Burns et al., 2013; Mganga et al., 2015; Sinsabaugh et al., 2005). Depending on their functions, enzymes are divided into several groups, of which oxidoreductases and hydrolases are especially relevant for SOM decomposition (Tischer et al., 2015). Among these enzymes, β-1,4-glucosidase (BG) cellulose de-polymerization, releasing two moles of glucose per mole of cellobiose (disaccharide of cellulose) (Turner et al., 2002). Degradation of various organic N compounds in soil, including proteins and chitin, are catalyzed by the hydrolyzing activities of L-leucine aminopeptidase (LAP) and β-1,4-N-acetylglucosaminidase (NAG), respectively (Sanaullah et al., 2011), releasing N for microbial and plant uptake. Extracellular activity of acid phosphatase (acP) in soil is associated with P mineralization through hydrolysis of organic phosphate compounds (Goldstein et al., 1988; Nuruzzaman et al., 2006).

Activities of extracellular enzymes are triggered by the presence of plants and are usually higher than in bulk soil. Release of labile substrates (i.e. root exudation) by living roots into soil enhances EEA (microbial activation hypothesis; Cheng and Kuzyakov, 2005, Kumar et al., 2016, Zhu et al., 2014). Availability of labile C from root exudation increases the microbial demand for other nutrients such as nitrogen (N) and phosphorus (P). The microbial activation enhances SOM decomposition via mining for N and P (Kuzyakov and Xu, 2013).

Soil aggregation is another factor affecting SOM decomposition as well as nutrient cycling because microbial communities and their activities differ between aggregate size classes (Caravaca et al., 2005; Duchicela et al., 2012; Gupta and Germida, 2015). Soil aggregation physically protects SOM by making it inaccessible for microbial mineralization. Aggregation strongly regulates aeration, nutrient retention, and erosion (Blankinship et al., 2016) and controls the sequestration of plant-derived organic matter by occlusion into macro- and microaggregates (Lagomarsino et al., 2012; Tian et al., 2015). Based on observations, it has been identified that C content increase with increasing aggregate size classes from micro- to macroaggregates. Moreover, microaggregates constitute relatively old and recalcitrant C than macroaggregates (Six et al., 2004). Therefore, the quality of C contained within microaggregates or macroaggregates regulates the microbial community structure and associated activity (Bach and Hofmockel, 2014: Hattori 1988).

Soil macro- (>250 µm) and microaggregates (<250 µm) are responsible for the heterogeneous distribution of microorganisms (Blaud et al., 2012) and therefore may affect the associated EEA. The impact of aggregate size class on EEA is inconsistent:

increase, decrease or no change have been obtained. One of the possible reasons may be the methods of aggregate size fractionation (Allison and Jastrow, 2006; Dorodnikov et al., 2009a; Fang et al., 2016; Shahbaz et al., 2016). For instance, application of conventional wet- and dry sieving may substantially modify easily soluble and desiccation-sensitive enzyme molecules, and cause their redistribution from one aggregate size class to another (Dorodnikov et al., 2009a). In contrast, the proposed 'optimal moisture sieving' method was developed to minimize biases from the above-mentioned factors on EEA. The method is based on a moisture content that limits mechanical stress, to induce maximum brittle failure along natural planes of weakness in the bulk soil (Dorodnikov et al., 2009a; Kristiansen et al., 2006). This technique involves neither complete drying nor water saturation, which are respectively necessary for dry and moist sieving. Due to the optimal moisture level, macroaggregates do not disrupt completely and the microaggregates located on surfaces of macroaggregates or along natural planes of weakness are preferentially separated. This fraction comprises the free microaggregate size class, distinct from the microaggregates located inside macroaggregates (Bossuyt et al., 2005; Six et al., 2004).

In the present study, the response of EEA catalyzing the decomposition of C (BG and NAG), N (NAG and LAP), and P (acP) compounds was determined in three aggregate size classes. For this, a modified 'optimal moisture sieving' technique was used to separate bulk soil into large macroaggregates (>2000 µm), small macroaggregates (2000-250 µm), and free microaggregates (<250 µm). Our previous findings have shown increased enzymes activities in the rhizosphere soil as compared to bare fallow, driven by labile C inputs from roots (Kumar et al., 2016). Increase in root density will also change the distribution of the three aggregate size classes. Therefore, the following

research question was addressed: could the optimally fractionated aggregates explain the effects of rhizosphere on microbial biomass distribution and measured EEA? We hypothesized that (i) EEA is higher in aggregates of planted soil than that of bare fallow, as microorganisms are fueled with C and energy-rich labile substrates by rhizodeposition; (ii) EEA is higher in free microaggregates than macroaggregates as the former should be preferentially exposed to root exudates, water and oxygen flows.

2.2. Materials and methods

2.2.1. Experimental setup

The experiment was established on a haplic Luvisol in an agricultural field (51°29'37.2"N and 9°55'36.9"E), which belongs to the research station "Reinshof" of the Georg-August-University Göttingen, Germany. Soil properties are as follow: total C (1.41 ± 0.04%), total N (0.16 ± 0.002%), pH (7.2 ± 0.01), soil bulk density (1.2 ± 0.2 g cm^{-3}).The experimental field was divided into 16 plots, each with an area of 5 x 5 m. To avoid any neighboring effects, the plots were separated by 2 m-wide buffer strips, which were kept vegetation-free throughout the experiment. A gradient of three plant densities (low, normal and high) was established in the field with completely randomized design. For this, maize was sown in plots with a plant density of 16 plants m^{-2}. When the plants were approximately 10 cm high, the plots were thinned according to the plant density gradient. Plots were thinned to 6 plants m^{-2} for low plant density; 10 plants m^{-2} for normal plant density; and 16 plants m^{-2} were left as high plant density. Four plots were kept vegetation-free throughout the experiment as control.

2.2.2. Soil and plant sampling

Soils were collected when the plants entered into the reproductive state (72 days after planting (DAP)) from a depth of 5-15 cm assuming maximum root growth and root exudation during plant vegetative stage (Kumar et al. 2016). This soil depth corresponded to the highest root biomass (data not presented). For soil sampling, the upper 0-5 cm soil layer was carefully removed and soil from 5-15 cm was collected between maize rows with a border spade. After delivery to the laboratory, soils were immediately sieved through an 8-mm sieve. A 5 g sub-sample was dried at 60 °C for 3 days to determine soil moisture content. The remaining soil was used for aggregate size fractionation. To determine shoot biomass, two plants from each plot were cut at the base, dried at 60 °C for 3 days, and weighed. Based on plot size and plant density of the respective treatment, shoot biomass was scaled up to g dry weight m^{-2}. For the total root biomass, which could not be directly quantified, the root-to-shoot ratio was used to scale measured shoot biomass to root biomass in units per area (i.e. g dry weight m^{-2}). The root-to-shoot ratio under normal plant density was 0.11 (97 DAP) and did not differ significantly between low, normal, and high plant densities at the end of the field experiment (130 DAP). The ratio was within the range of the data reported by Amos and Walter (2006), showing that the main changes of root-to-shoot ratio in maize occur within the first 60 days after planting.

2.2.3. Aggregate size fractionation

Aggregates of three size classes were isolated by the method described by Dorodnikov et al. (2009a) with modifications. In order to minimize disturbance to microbial activities, soils were cold dried at 4 °C to approximately 10% gravimetric water content (Bach and Hofmockel, 2015). For this, soil samples were placed in a container and spread into a thin layer. All stones and visible roots were hand-picked. Once the desired condition was

achieved, approximately 700 g soil was transferred to a nest of sieves (2 mm and 0.25 mm). The nest was bolted onto a vibratory sieve shaker AS200 (Retsch, Germany) and shaken for 3 min, 2 times. Aggregates remaining on the 2 mm sieve were classified as large macroaggregates (>2000 µm), aggregates passing through the 2 mm sieve but remaining on the 0.25 mm sieve were classified as small macroaggregates (2000-250 µm), and the remaining soil materials which passed through the 0.25 mm sieve were classified as microaggregates (<250 µm) (Figure II.2:1). From each aggregate size class, soil was weighed to determine the mass distribution and mean weight diameters (MWD) of aggregates. Mean weight diameter was calculated after John et al. (2005):

$$MWD = \sum(Weight\ \%\ of\ sample\ remaining\ on\ sieve\ \times Mean\ inter-sieve\ size) \div 100$$

where mean inter-sieve size is the average of the two sieve sizes through which the aggregates have passed and on which the aggregates have remained after sieving.

Thereafter, post-sieving moisture content, total C and N, microbial biomass C and N, and maximal potential extracellular enzyme activities of C-, N-, and P-degrading enzymes were measured. For moisture content, a soil subsample was dried at 60 °C for 3 days. Total C and N contents were estimated with an Elementar Vario EL analyzer (Elementar Analysensysteme GmbH, Germany).

2.2.4. Soil microbial biomass

The chloroform fumigation-extraction method was used to determine soil microbial biomass C (C_{mic}) and N (N_{mic}) (Vance et al., 1987) with slight modifications. Before microbial biomass determination, aggregates were moisten to field moisture level of 12-15% and incubated for 24 h to assure field conditions. Briefly, an 8 g soil sample (non-fumigated) was extracted with 32 ml of 0.05 M K_2SO_4 for 1 h by continuously shaking

(150 rpm) on a reciprocating shaker (Laboratory shaker, GFL 3016). Afterwards, the soil suspension was filtered (grade: 3 hw, diameter 110 mm, Sartorius) and stored at 4 °C until further analyses. The same extraction procedure was used for fumigated soil. Fumigation was done with 80 ml of ethanol-free chloroform in a desiccator at room temperature for 24 h. The organic C and total N content of the filtered solution was measured with a multi N/C analyzer (multi N/C analyzer 2100S, Analytik, Jena).

Differences between extracted C and N from fumigated and non-fumigated soil were used to calculate microbial biomass C and microbial biomass N. We used K_{EC} and K_{EN} factors of 0.45 and 0.54 for microbial C and N, respectively (Joergensen and Mueller, 1996; Wu et al., 1990).

2.2.5. Enzyme assays

Extracellular enzyme activities were measured with fluorogenically labeled artificial substrates according to Marx et al. (2001). Fluorogenic 4-methylumbelliferone (MUB)-based substrates were used to determine the activities of β-1,4-glucosidase, β-1,4-N-acetylglucosaminidase and acid phosphatase. Fluorogenic 7-amino-4-methylcoumarin (AMC)-based substrate was used to determine the activity of L-leucine aminopeptidase. EEA was determined separately in distinct aggregate size class. For this, distinct aggregates (1 g) were used to make soil suspension by dissolving it in 50 ml distilled and autoclaved water. To release the enzymes trapped on soil clay particles, low-energy sonication (50 Js^{-1}) was applied for 2 min (Loeppmann et al., 2016; Razavi et al., 2015). 50 µl of soil suspension was dispensed into a black 96-well microplate (PureGrade™, GMBH+Co KG, Wertheim, Germany) while stirring the suspension on a magnetic stirrer to maintain uniformity. Thereafter, for MUB-based substrates, 50 µl of MES

($C_6H_{13}NO_4SNa_{0.5}$) buffer (pH6.5) and for AMC-based substrate, 50 µl of TRIZMA ($C_4H_{11}NO_3$.HCl, $C_4H_{11}NO_3$) buffer (pH7.2) was added to each well (Hoang et al., 2016). Finally, 100 µl of substrate solutions of 4-methylumbelliferyl-β-D-glucoside, 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide, L-leucine-7-amido-4-methylcoumarine hydrochloride and 4-methylumbelliferyl phosphate were added to the wells. A substrate concentration of 400 µmol g^{-1} soil was used for the substrate-unlimited maximal potential reaction, as determined in a preliminary experiment using Michaelis-Menten kinetics (by using increasing substrate concentrations to reach V_{max}). Just after substrate addition, the microplate was gently shaken to mix the well contents and measurements were taken fluorometrically (excitation 360 nm; emission 450 nm) at 0, 30, 60, and 120 min after substrate addition with an automated fluorometric plate reader (Victor3 1420-050 Multi-label Counter, PerkinElmer, USA). Fluorescence was converted to amount of AMC or MUB, according to standards. Enzyme activities were expressed as MUB or AMC released in nanomol per gram aggregate dry weight and hour (nmol g^{-1} aggregate h^{-1}).

Statistics

The experiment was conducted with 4 field replicates. The values presented in figures are means of 4 field replicates ± standard errors (mean ± SEM). The data set was checked for normality (Shapiro-Wilk test, $P > 0.05$) and homogeneity of variance (Levene test, $P > 0.05$) prior to analysis of variance (ANOVA). For β-1,4-glucosidase and β-1,4-N-acetylglucosaminidase, the data did not meet the requirement for normality. Therefore, data were square-root transformed and retested for normal distribution with the Shapiro-Wilk test. Afterwards, two-factor ANOVA was performed to test the effects of aggregate size class and plant density on C_{mic} and N_{mic}, and potential and specific EEA.

One-factor ANOVA was used to test the effect of plant density on MWD, relative distribution of aggregates within each aggregate size class, and root biomass. Post-hoc tests for multiple comparisons using least significant differences (Tukey-test, $P < 0.05$) were performed on each measured parameter after ANOVA. STATISTICA for Windows (version 7.0, StatSoft Inc., OK, USA) was used to perform ANOVA analyses. Figures were drawn with OriginPro 8.5G (OriginLab Corporation., Northampton, MA 01060, USA). The level of significance was defined at $P<0.05$ for all statistical analyses, if not mentioned specifically.

2.3. Results

2.3.1. Aggregate size class distribution and mean weight diameter

Large and small macroaggregates dominated in the bare and planted soil, whereas microaggregates accounted for only a small part (Figure II.2:2). The relative distribution (in %) of aggregate size classes were: large macroaggregates (48 - 54%) > small macroaggregates (40 - 45%) > microaggregates (6 - 8%). The C and N content was 1.17 to 1.22% C and 0.13% N, respectively and did not differ significantly across the aggregate size classes.

Plant density had a minor effect on the relative distribution of aggregate size classes. The percentage of large macroaggregates in high plant density was significantly lower ($P < 0.05$) than bare fallow and low- and normal plant density. The percentage of microaggregates showed an increasing trend with increasing plant density. The MWD did not vary between bare fallow and various plant densities, except that high plant density had a minor decrease when compared to normal maize density (Fig. II.2:3).

2.3.2. Plant and microbial biomass

Aboveground biomass was 362 g m^{-2} under low plant density and increased by 88% and 149% under normal and high plant density, respectively. However, the increase was not significantly different ($P > 0.05$) between normal and high plant density (Supplementary Table II.2:1). As total root biomass could not be accurately determined in the field, root biomass per area was calculated based on the measured root-to-shoot ratio. Root biomass increased from 41.2±6.0 g m^{-2} for low plant density to 80.2±6.1 g m^{-2} for high plant density.

Microbial biomass C decreased with decreasing aggregate size and ranged from 106.4±18.5 to 138.7±12.8 mg C kg^{-1} aggregate (large macroaggregates), 79.5±5.4 to 121.1±3.9 mg C kg^{-1} aggregate (small macroaggregates), and 77.8±14.8 to 95.4±8.7 mg C kg^{-1} aggregate (microaggregates) (Figure II.2:4). Planting had minor effects on C_{mic} relative to bare fallow. Comparing between the aggregate size classes, large macroaggregates comprised higher C_{mic}. Microbial biomass N had a tendency to decrease with decreasing aggregate size classes. The content of N_{mic} was on average 27.3±2.6 mg N kg^{-1} aggregate in large macroaggregates, 22.8±0.8 mg N kg^{-1} aggregate in small macroaggregates, and 20.9±1.1 mg N kg^{-1} aggregate in microaggregates under low plant density (Supplementary Table II.2:1).

2.3.3. Extracellular enzyme activities

In contrast to microbial biomass, the potential activities of C, N, and P degrading enzymes (BG, LAP, NAG, and acP) tended to increase with decreasing aggregate size in planted soil (Figure II.3:5). Under bare fallow, the potential activities of BG and LAP were lower in microaggregates than macroaggregates, whereas the potential activity of

NAG remained constant and that of acP was higher in microaggregates than in macroaggregates. Additionally, under bare fallow and low, normal and high plant densities, specific activities of BG, LAP, NAG, and acP remained similar, with a slight increase under high plant density (Figure II.2:6). Effects of planting on specific enzyme activities were strongest in microaggregates (Figure II.2:6). In microaggregates, the specific activity of BG was 0.47 nmol h^{-1} mg^{-1} C_{mic} in bare fallow and increased by 21-73% in the presence of roots. The specific activity of NAG was 0.57 nmol h^{-1} mg^{-1} C_{mic} in bare fallow and increased by 5-31%; specific activity of acP was 6.1±0.3 nmol h^{-1} mg^{-1} C_{mic} in bare fallow and varied by -2% and 26%; and the specific activity of LAP was 0.35 nmol h^{-1} mg^{-1} C_{mic} in bare fallow and increased by about 35-92% in presence of roots under various plant densities. The specific enzyme activities were similar in each of the three aggregate size classes of the bare fallow.

2.4. Discussion

2.4.1. Aggregate fractionation

According to the aggregate hierarchy concept (Elliot and Coleman, 1988), microaggregates are located inside macroaggregates and comprise older C pools (Six et al., 2004). As shown by Dexter (1988), the maximum soil friability (tendency toward segregation of unconfined soil into smaller fragments under certain mechanical stress) occurs at about 38% of water content (field capacity). Soil colloids shrink and cracks appear, defining the boundaries of aggregates. These cracks remain as points of weakness for physical breakdown. Therefore, at this soil water content, aggregate fractionation results in breakdown of macroaggregates along the planes of weakness,

releasing the microaggregates located on surfaces of macroaggregates and along their planes of weakness. Thus, the procedure adopted by Dorodnikov et al. (2009a) for aggregate size fractionation, termed as '*optimal moisture sieving*', accounted for free microaggregates as described in the aggregate hierarchy concept (Bossuyt et al., 2004; Oades, 1984; Simpson et al., 2004; Six et al., 2004). According to the method used in this study, the proportion of micro- to macroaggregates size classes strongly depends on the soil moisture level. Generally, the lower the moisture, the lower would be slicking and therefore, the proportion of macroaggregates is higher (Chenu et al. 2000). However, we aimed to keep the moisture under which EEA would be close to field conditions and the proportion of micro- to macroaggregates in the tested soil corresponded to field moisture conditions. In the present study, the soil moisture content was around 7 to 10% of total weight after sieving. We assume the breakdown of macroaggregates along the planes of weakness was minimal as shown by Dexter (1988). Therefore, we assume that with the aggregate fractionation technique we applied, mainly the free microaggregates and the microaggregates adhering on the surface of macroaggregates were isolated (Figure II.2:1). The small portion of isolated microaggregates in the present study (6-8% of total soil) further supports this concept.

2.4.2. Root effects on aggregate size distribution

The influence of roots on aggregate stabilization is well known (Erktan et al., 2015; Six et al., 2004), but very few studies have focused on aggregate disintegration by living roots (Materechera et al., 1994). In our field study, a gradual increase in the proportion of microaggregates and a decrease in large macroaggregates with increasing plant density may be due to disintegration of large macroaggregates by growing roots (Figure II.2:2). Also, the mean weight diameter, which is an indicator of aggregate stability

(Tripathi et al., 2014) tended to decrease with increasing plant density. This also confirmed the redistribution of aggregate size classes in the presence of growing roots. Mechanistically, the aggregate redistribution may occur through the penetration of living roots into macroaggregates along planes of weakness and through the pores within macroaggregates, thereby decreasing their stability (Materechera et al., 1994). Hence, root morphology (root thickness, root length density, root branching, etc.) is one of the main drivers affecting aggregate redistribution (Carter et al., 1994).

2.4.3. Microbial biomass C in micro- and macroaggregates

Microbial biomass C decreased with decreasing aggregate size. The hierarchical aggregate concept (Elliot and Coleman, 1988) integrates the aggregate categories with the pore structure, which defines microsites of habitability for microorganisms (Gupta and Germida, 2015). Literature is replete with studies showing increased fungal abundance with increasing aggregate size (Poll et al., 2003; Zhang et al., 2015). The preferential colonization by fungal communities may occur in macroaggregates (Harris et al., 2003) by expanding their biomass through extensive hyphal growth in large pores (De Gryze et al., 2005; Dorodnikov et al., 2009b). In turn, microaggregates are inhabited predominately by bacterial communities (Ranjard and Richaume, 2001; Six et al., 2006). Higher C_{mic} / N_{mic} ratio in macroaggregates than microaggregates (although significant only in low plant density) in the present study indicates fungal dominance in macroaggregates as compared to microaggregates (Supplementary Table II.2:2) (Dorodnikov et al., 2009b). The lower microbial biomass in the microaggregates in comparison with large and small macroaggregates could reflect the distribution of fungal and bacterial communities (Gupta and Germida, 2015) as a result of different habitats.

In the short term, labile C inputs from roots did not change overall microbial growth. Such inputs predominantly activate fast-growing microbial communities (Blagodatskaya and Kuzyakov, 2008). The same amounts of microbial biomass in bare fallow and in planted soils (Figure II.2:4) are in line with other studies (Duineveld et al., 1998; Fontaine et al., 2007), highlighting the regulatory effect of living plants on activities rather than on the abundance of microorganisms in agricultural soil.

2.4.4. Effects of roots and aggregate sizes on extracellular enzyme activities

Extracellular enzyme production by microorganisms, which regulates microbially mediated SOM decomposition, may occur under nutrient limitations. In addition, root exudation may trigger extracellular enzyme production (Kumar et al., 2016; Kuzyakov and Blagodatskaya, 2015) via microbial activation. In the presence of root-released organics, which are characterized by higher C/N ratios, the microbial demand for other nutrients (especially N and P) increases (Fontaine et al., 2011). Further, plants exacerbate the nutrient limitations due to competition with microorganisms (Kuzyakov and Xu, 2013). In order to fulfill these extra nutritional demands, microorganisms produce N- and P-degrading enzymes to mine for them from SOM. Along with the P demand, acP activity reflects the overall microbial activity (as it participates in phosphorylation processes within cells and by lysis appears extracellular), which differ between macro- and microaggregates and was the highest among all enzymes tested. The results from the present study corroborate the reported increase in extracellular activities of C-, N- and P-degrading enzymes with decreasing aggregate size class (Nie et al., 2014). Similarly, to the potential EEA, the specific EEA for C-, N-, and P-degrading enzymes also increased in the order: large macroaggregates<small macroaggregates<microaggregates (Figure II.2:6). Overall higher total and specific EEA

in free microaggregates can result from the location of the latter within soil where plant root exudations as well as water, nutrient and oxygen flows are higher than in the interior of macroaggregates (Burns et al., 2013; Phillips et al., 2011). Similarly, an absence of labile substrate inputs in bare fallow soil resulted in lower enzyme activities. In summary, considering microbial activation (Cheng and Kuzyakov, 2005) by growing roots, the present study provides evidence that the influence of roots on microorganism's activities persists in different soil aggregates and such influences are more pronounced in free microaggregates (Figure II.2:7).

2.5. Conclusions

Pronounced effects of aggregate size on C_{mic}, N_{mic} as well as on EEA were demonstrated. Higher EEA in rooted soil than in bare fallow soil for three aggregate size classes highlights plant-mediated microbial activation. The presence of roots stimulated microbial activity (potential and specific EEA), which governs the catalytic reactions of SOM decomposition. Markedly higher specific EEA in free microaggregates than in large- and small macroaggregates may result from the better supply of root exudates, water, nutrients and oxygen to microorganisms. Minimal or no effect of aggregate size on specific EEA under bare fallow indicated microbial inefficiency in enzyme synthesis in the absence of root-released organics.

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2.8. Figures

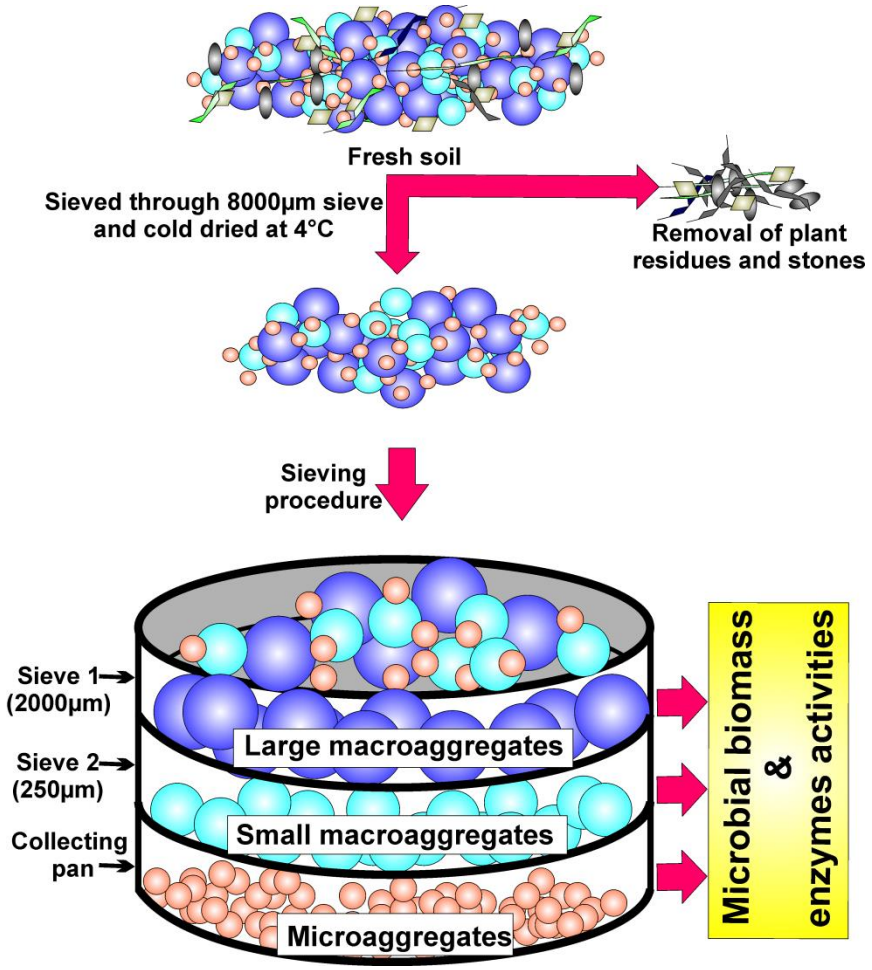

Figure II.2:1: Schematic diagram showing soil preparation and aggregate size fractionation.

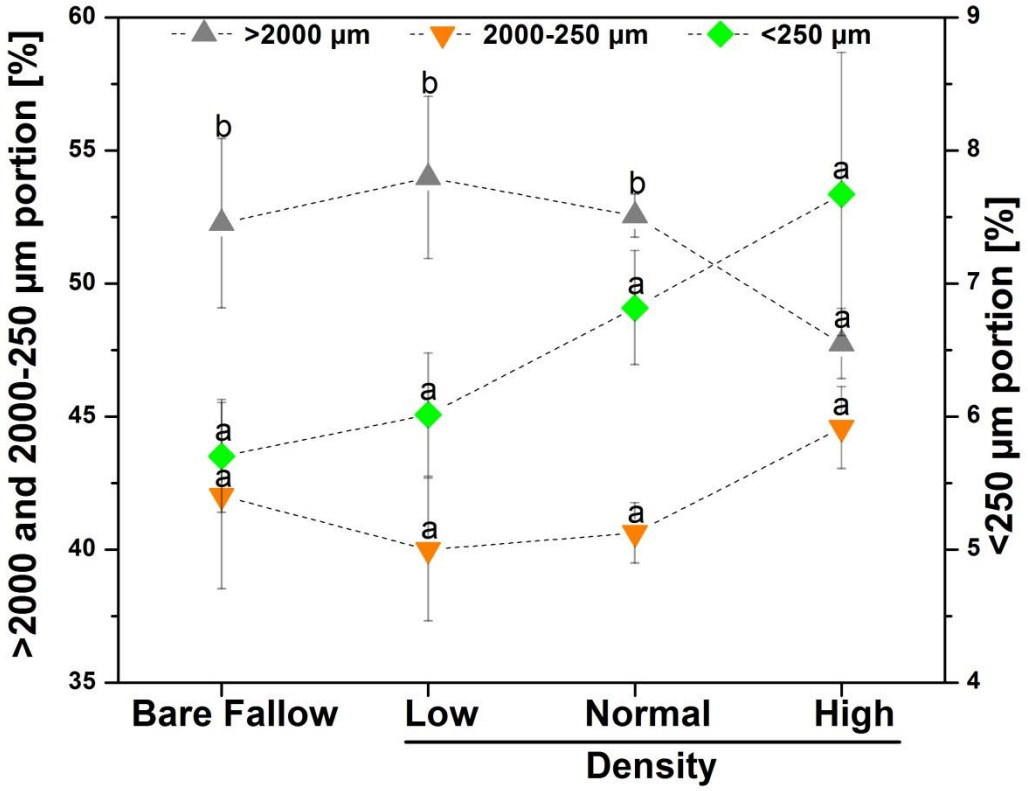

Figure II.2:2: The relative distribution of large and small macroaggregates (left y-axis; mean±SEM) (n=4) and microaggregates (right y-axis; mean±SEM) (n=4) in bare fallow soil and soils with Low, Normal and High maize plant densities. Letters indicate significant differences (Post-hoc test, $P < 0.05$) between bare fallow and three plant densities within the same aggregate size class.

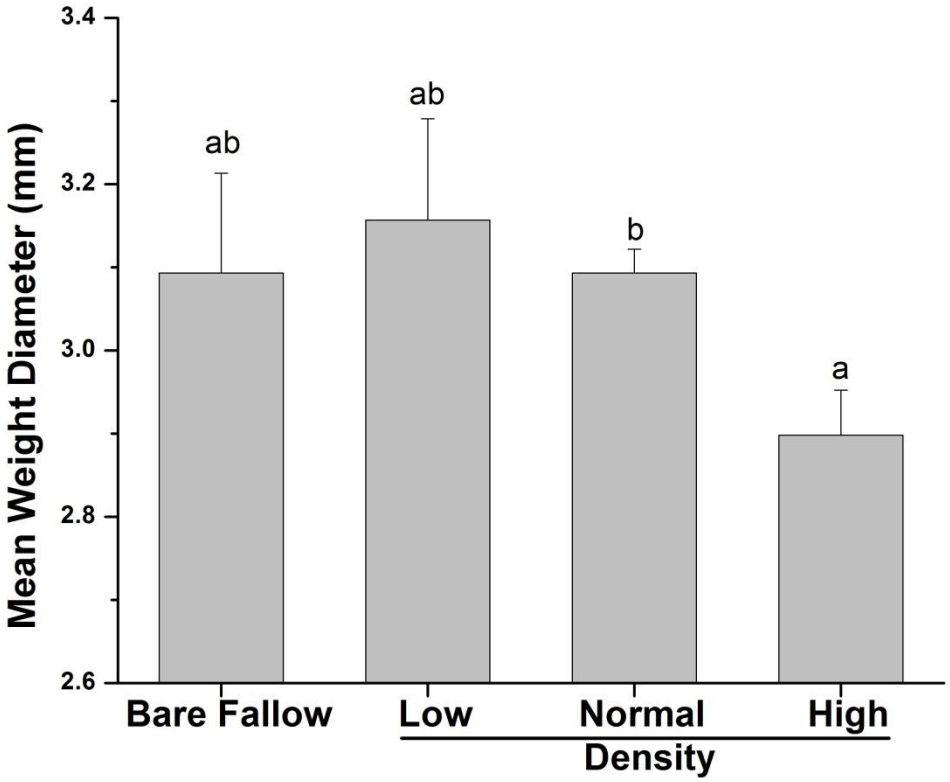

Figure II.2:3: Mean weight diameter (±SEM) (n=4) in bare fallow soil and soils with Low, Normal and High maize plant densities. Letters indicate significant differences (Post-hoc LSD test, $P<0.05$) in MWD between bare fallow soil and soils with low, normal and high maize plant densities.

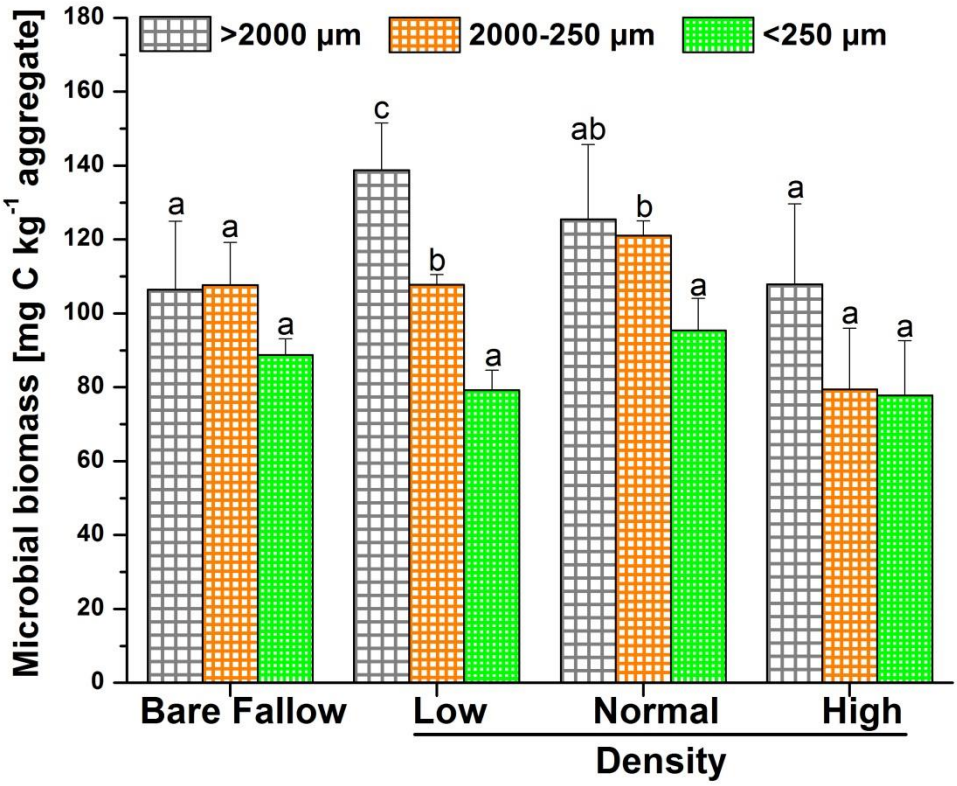

Figure II.2:4: Microbial biomass (mg C kg^{-1} aggregate; mean ± SEM) (n=4) in bare fallow soil and soils with Low, Normal and High maize plant densities. Letters indicate significant differences (Post-hoc LSD test, $P<0.05$) in microbial biomass C between aggregate size classes in bare fallow soil and soils with Low, Normal, and High maize plant densities.

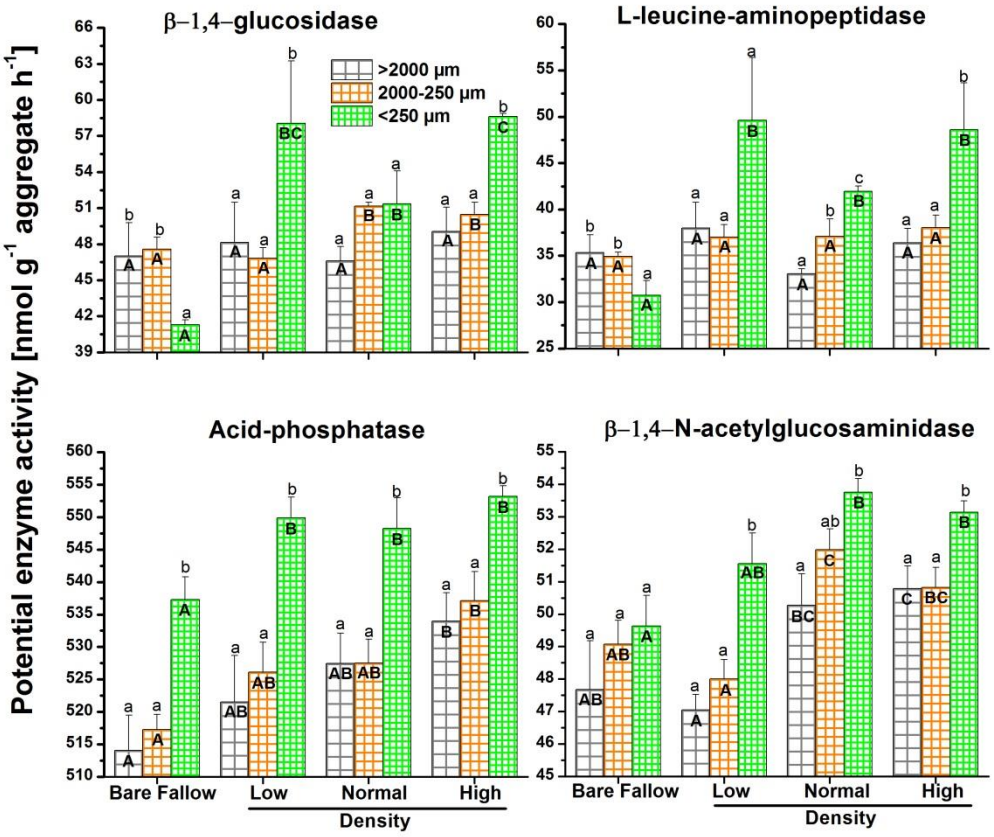

Figure II.2:5: Potential activity of: β-1,4-glucosidase; L-leucine aminopeptidase; acid phosphatase; and β-1,4-N-acetylglucosaminidase (nmol h^{-1} g^{-1} soil) (±SEM) (n=4) in distinct aggregate size classes in bare fallow soil and soils with Low, Normal and High maize plant densities. Upper-case letters indicate significant differences (Posy-hoc LSD test, $P < 0.05$) in potential activity within the same aggregate size class. Lower-case letters indicate significant differences (Post-hoc LSD test, $P < 0.05$) in potential activity between distinct aggregate size classes.

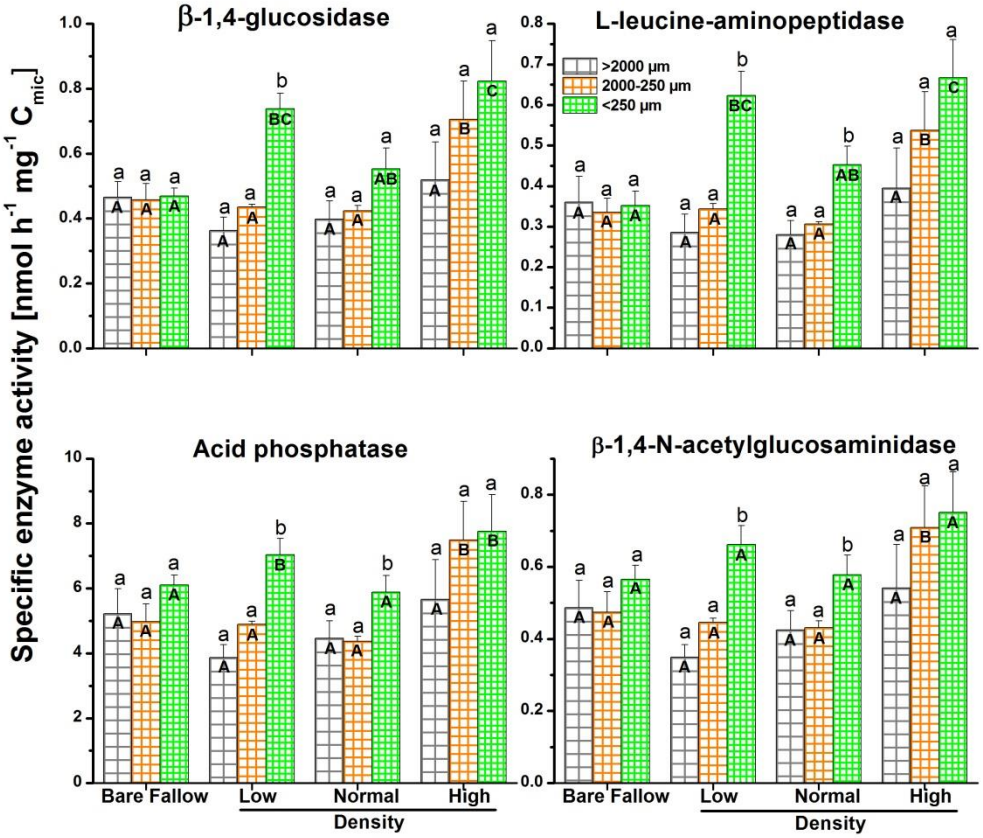

Figure II.2:6: Specific activity (ratio of potential activity and microbial biomass C) of: β-1,4-glucosidase; L-leucine aminopeptidase; acid phosphatase; and β-1,4-N-acetylglucosaminidase (nmol h^{-1} g^{-1} soil) (±SEM) (n=4) in bare fallow soil and soils with Low, Normal and High maize plant densities in distinct aggregate size classes. Upper-case letters indicate significant differences (Post-hoc LSD test, $P < 0.05$) in specific activity within the same aggregate size class. Lower-case letters indicate significant differences (Post-hoc LSD test, $P < 0.05$) in specific activity between distinct aggregate size classes.

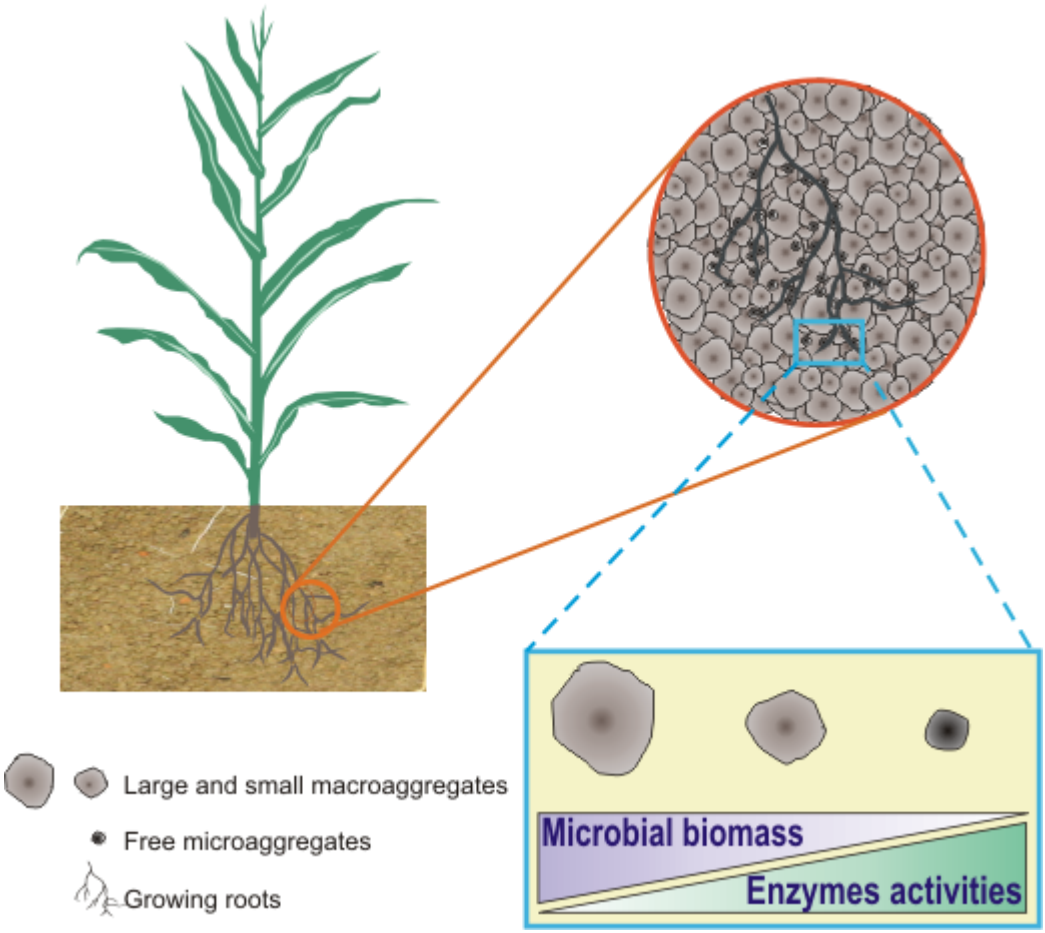

Figure II.2:7: Conceptual figure showing the potential effects of growing roots on extracellular enzyme activities and microbial biomass in distinct aggregate size classes in rooted soil separated by optimal moisture sieving method. Root induced microbial activities in distinct aggregate size classes are shown by higher EEA and the relations between aggregate size and microbial biomass are illustrated.

3. Maize phenology alters the distribution of enzyme activities in soil: field estimates

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Abstract

Microbial processes mediated by soil enzymes are crucial in soil organic matter decomposition, resulting in release of nutrients that become available for plant and microbial uptake. Therefore, it is crucial to know the sensitivity of enzyme activities (EA) along soil depths at distinct plant vegetation stages, and how the availability of mineral nitrogen (N) alters EA. We studied effects of N fertilization (0 and 160 kg N ha^{-1}), soil depth (0 to 35 cm), and plant-phenological stage (silking and maturity) on microbial biomass C (C_{mic}) and potential activities of C-, N- and P-acquiring enzymes in the field under *Zea mays* L.

Nitrogen fertilization increased shoot biomass by more than 80% compared to unfertilized plants. Maize roots triggered increases in C_{mic} and EA for all measured enzymes compared to bare fallow. Stimulating effect of plant roots on EA was enzyme specific and stronger at silking than maturity stage of maize. The down-regulating effect of N fertilization on EA involved in acquiring N was most pronounced on the activity of L-leucine aminopeptidase and β-1,4-N-acetylglucosaminidase. Soil depth was the primary determinant of EA, explaining up to 51% of the variation. Depth-dependent EA changes were stronger in rooted soil.

A pronounced biotic control on EA was demonstrated by higher EA in rooted soil than in bare fallow. This confirmed root-mediated microbial activation. Stronger effect of silking vs. maturity stage on EA indicated that actively growing roots fuel microorganisms via root-derived organics. Thus, soil depth and plant roots were major factors controlling microbial activity in arable soil.

Key words: root exudation, vegetation stage, microbial activation, nutrient cycling

3.1. Introduction

Food security will be a vital issue in meeting the demand of an increasing global population. Thus, there is renewed interest in understanding the biochemical processes in agricultural soils, and how altering these processes may be used to increase agricultural productivity (Johnston et al. 2009). Such sustainable agricultural practices offer tremendous opportunities for maintaining or increasing soil health (i.e. fertility) (Doran and Zeiss 2000). Sustainable agriculture refers to maintenance or enhancement of soil health with minimum disturbance and has laid the foundation for understanding soil ecological functioning (Weiner 2017). Soil microorganisms are central to ecological functioning (Bender et al. 2016). A better understanding of microbial functioning will help to elucidate the biogeochemical processes contributing to nutrient transformations in soils (Nannipieri et al. 1978, 2003). Decomposition and transformation of soil organic matter (SOM), nutrient mobilization/immobilization, and aggregate formation/stabilization are among the most important processes predominantly governed by microorganisms (Nsabimana et al. 2004; Six et al. 2004; Caldwell 2005). The cycling of major nutrient elements is widely associated with enzyme activity (EA) in soil (Burns et al. 2013). EA is important in maintaining soil health, as enzymes catalyze the bottleneck steps in SOM decomposition and consequent release of nutrients for plant and microbial uptake (Aon et al. 2001). Generally, EA is dependent on various biotic and abiotic factors such as pH (Sinsabaugh 2010), nutrients (Keuskamp et al. 2015; Olander and Vitousek 2000), disturbance (Boerner et al. 2000), succession (Tscherko et al. 2003), microbial community structure and function (Dorodnikov et al. 2009; Tischer et al. 2015), plant species (Caravaca et al. 2005; Razavi et al. 2016), and management practices (Renella et al. 2007; Shahbaz et al. 2017).

Depending on the complexity of SOM, various hydrolases and oxido-reductases are produced by microorganisms. For example, β-1,4-glucosidase (BG), cellobiohydrolases (CBH), and β-xylosidase (XYL) are a set of hydrolases produced by microorganisms to acquire C via polysaccharide decomposition. Another widely prevalent enzyme is L-leucine aminopeptidase (LAP), which is associated with the breakdown of amide-linked polypeptides, the primary form of organic N in soils (Finzi et al. 2015; Knicker 2004). β-1,4-N-acetylglucosaminidase (NAG), which predominantly targets chitin and peptidoglycan breakdown, releases both C and N for microbial acquisition. Organic compounds containing ester-linked P are cleaved by phosphomonoesterase (PHO), which releases inorganic P (Finzi et al. 2015; Sinsabaugh and Shah 2011). In rooted soils, enzyme production is triggered by root exudation, resulting in higher rates of SOM decomposition and in a consequent release of nutrients (Kuzyakov and Domanski 2000).

Root exudates provide easily accessible substrates for microorganisms and are an ecologically important contributor to rhizosphere processes. According to the microbial activation hypothesis (Cheng and Kuzyakov 2005; Kuzyakov et al. 2007), root exudation triggers the up-regulation of metabolic activities in microbial cells. Enhanced metabolic demands lead to the production and release of enzymes. Therefore, EA are sensitive indicators of microbial activity (Nannipieri et al. 2002).

Mineral fertilizers, representing another form of easily accessible nutrients, also affect SOM decomposition by altering microbial activities. In the presence of easily accessible nutrients, microorganisms down-regulate resource allocation for enzyme synthesis and release, as they are not solely dependent on nutrient gains via SOM decomposition.

However, attempts to determine the impact of N fertilization on microbial activities have been inconclusive, with studies reporting increases, decreases and even no effect on EA with fertilization (Shen et al. 2010; Ai et al. 2012; Kumar et al. 2016). It is assumed that in nutrient limited soils, microbial growth and activity are constrained due to low availability of C and nutrients and, as a result, the input of resources (via root exudation and N fertilization) will enhance microbial growth and activity (Renella et al. 2006). Increased growth will consequently increase enzyme production to mineralize more SOM to meet microbial nutrient demands. Under nutrient limitations, N addition may stimulate the production of enzymes, as N is essential for enzyme synthesis (Olander and Vitousek 2000). In contrast, when N is not a limiting factor, microorganisms do not allocate their resources to the production of enzymes associated with N acquisition. Therefore, there is a negative feedback between supply and demand for production of enzymes. The addition of one nutrient may alter the EA of not only the enzymes involved in that particular nutrient cycle, but may also alter the activities of other enzymes involved in the cycling of other nutrients. For example, xylanase activity (involved in decomposition of hemi-cellulose) decreased in the presence of mineral N (Chen et al. 2014). Microbial activity relies not only on the availability of nutrients, but is also affected by soil depth. It has been observed that when depth increases, microbial activity decreases, as substrate inputs and gas exchange are reduced with depth (Loeppmann et al. 2016; Stone et al. 2014). The spatial distribution of roots is heterogeneous in soil and varies with the grown stage of the plant (Chimento and Amaducci 2015), which may impact plant-mediated microbial activities at various soil depths. It has previously been demonstrated that there are distinct microbial community composition and their activities along with soil depth (Fierer et al. 2003; Jakson et al. 2009) and these changes are

generally explained by substrate input varying in quality and quantity (Loeppmann et al. 2016).

Although roots and microbial activity are often linked (Kumar et al. 2017, Kuzyakov and Blagodatskaya 2015), most of the field studies are conducted only once during a vegetation season (either at the beginning or before harvesting). However, root-mediated effects on microbial activity are taking place throughout the growing season (Bell et al. 2015). It is still unknown from direct field observations how microbial activity is influenced by roots at various plant growth stages, which are characterized by distinct morphological and physiological properties. Thus, the following research question was addressed: How sensitive is EA to the presence of plants and N fertilization (availability of mineral N) across a range of soil depths at distinct maize phenological stages? To answer this question, potential activities of six enzymes catalyzing the decomposition of organic C compounds (BG, CBH, XYL, NAG), organic N compounds (LAP and NAG), and organic P compounds (PHO) were determined with or without plants at four soil depths (0-5 cm, 5-15 cm, 15-25 cm, and 25-35 cm), at two maize phenological stages (silking and maturity), and with and without N fertilization.

3.2. Materials and methods

3.2.1. Experimental setup

The experiment was established on an agricultural research field belonging to the Georg-August-University Göttingen, Germany. The soil is characterized as a haplic Luvisol suitable for a broad range of agricultural uses with the following properties: total C content of 1.41 ± 0.04%, total N content of 0.16 ± 0.02%, pH value of 7.2 ± 0.01, and

bulk density of 1.2 ± 0.2 g cm^{-3}. The experimental site is under conventional agricultural uses. Conventional tillage practices up to 30 cm of soil depth are performed twice in a year. Maize seeds (*Zea mays* L. cv. Colisee) coated with methiocarb (4(methylthio)3,5-xylyl-N-methyl carba-mate), a pesticide, and thiram (tetramethylthiuram sulphide), a fungicide were sown in the field. The experimental field was divided into 16 plots (5 x 5 m^2) with a 2 m wide buffer strip around each plot to exclude neighbor effects as follows: Bare fallow, bare fallow with N fertilization (Bare fallow+N), maize-planted (Planted), and maize-planted with N fertilization (Planted+N) in a completely randomized design. N fertilizer was applied as urea at the soil surface at a rate of 160 kg N ha^{-1} (Weiterer, Landhandel GmbH) 47 days after planting (DAP). Any visible weed growing in the plots was manually removed at regular time intervals throughout the experimental period.

3.2.2. Soil and plant sampling

Soil and plants were sampled twice during the experimental period at 72 DAP and 130 DAP, which corresponds to the silking and maturity stages of maize plants. Soils were collected from four soil depths at 0-5 cm, 5-15 cm, 15-25 cm and 25-35 cm with a corer (inner diameter 7 cm) between the maize rows in the middle of the diagonal between two plants and transported to the laboratory in cooling boxes. Soil moisture content was estimated as the difference between field moist and oven-dried soil (at 105°C for 48 h). Afterwards, soils were passed through a 2 mm sieve and used for further analyses. For shoot biomass determination, two plants were cut at the base from each plot at randomly selected positions, oven dried at 60°C for 5 days, and weighed. As total root biomass could not be accurately determined in the field, root biomass per unit of area (i.e. g dry weight m^{-2}) was calculated based on the measured shoot to root ratio of maize from the same field (Kumar et al. 2016). The root to shoot ratio was 0.11 and 0.14 for

unfertilized and N fertilized maize at maize silking stage. These ratios were used to calculate the root biomass at maize silking stage (0.04±0.01 kg m^{-2} for unfertilized maize and 0.09±0.01 kg m^{-2} for N fertilized maize). At maize maturity, root to shoot ratio (0.16) and root biomass (0.22±0.04 kg m^{-2}) was determined using the equation derived from Amos and Walters (2006) for unfertilized maize. At maturity, we assume similar effect of N fertilization on root biomass as at maize silking (higher biomass under N fertilization) because very marginal change in root to shoot ratio occurs when the plants are in maturity phase (Amos and Walters, 2006).

3.2.3. Soil microbial biomass

Microbial biomass C was determined by the chloroform fumigation-extraction method (Vance et al. 1987) with slight modifications. Briefly, 10 g of fresh soil (non-fumigated) was extracted with 40 ml of 0.05 M K_2SO_4 for 60 min by continuously shaking on a reciprocating shaker (Laboratory shaker, GFL 3016). Thereafter, the soil suspension was filtered and stored at 4°C until further analyses. The same extraction procedure was used for fumigated samples, where soil was fumigated with 80 ml of ethanol-free chloroform in a desiccator at room temperature for 24 h. The organic C content of the filtered solution was measured with a multi N/C analyzer (multi N/C analyzer 2100S, Analytik, Jena).

Microbial biomass C (C_{mic}) was calculated as follows:

C_{mic} = Extracted $C_{(fumigated\ soil)}$ – Extracted $C_{(non\text{-}fumigated\ soil)}$

A K_{EC} factor of 0.45 was used for the determination of microbial biomass C (Joergensen and Mueller 1996).

3.2.4. Enzyme assays

Activities of β-1,4-glucosidase (BG), cellobiohydrolase (CBH), β-1,4-N-acetylglucosaminidase (NAG), β-xylosidase (XYL) and phosphomonoesterase (PHO) were determined using fluorogenic 4-Methylumbelliferone (MUB)-based substrates (Marx et al. 2001). Activity of L-leucine aminopeptidase (LAP) was determined using a fluorogenic 7-Amino-4-Methycoumarin (AMC)-based substrate. To begin, 1 g of fresh soil was suspended in 50 ml autoclaved sterile water, followed by 2 min of low energy sonication (50 Js^{-1}) to make a soil suspension. A 50 µl aliquot of this suspension was dispensed into a black 96-well microplate (PureGrade^{TM}, GMBH+Co KG, Wertheim, Germany). Thereafter, 50 ml of either MES ($C_6H_{13}NO_4SNa_{0.5}$) buffer (pH 6.5) or TRIZMA ($C_4H_{11}NO_3$.HCl, $C_4H_{11}NO_3$) buffer (pH 7.2) was added to each well for MUB or AMC based substrates, respectively. Finally, 100 µl of substrate solutions of 4-Methylumbelliferyl-β-D-glucoside, 4-Methylumbelliferyl-N-acetyl-β-D-cellobioside, 4-Methylumbelliferyl-N-acetyl-β-D-glucosaminide, 4-Methylumbelliferyl-β-D-xylopyranoside, L-Leucine-7-amido-4-methylcoumarine hydrochloride and 4-Methylumbelliferyl-phosphate were added to the wells for activities of BG, CBH, NAG, XYL, LAP and PHO activities, respectively. Immediately after substrate addition, microplates were gently shaken and fluorometric measurements (excitation 360 nm; emission 450 nm) were taken at 0, 30, 60 and 120 min after substrate addition with a fluorometric plate-reader ((Victor3 1420-050 Multi-label Counter, PerkinElmer, USA). Fluorescence values were converted to amount of MUB or AMC using specific standard scales based on soil suspension. Enzyme activities were expressed as nanomoles MUB or AMC cleaved per gram dry weighted soil per hour (nmol g^{-1} dry soil h^{-1}).

Statistics

The presented values in graphs and tables are averages of four field replicates ± standard errors (mean ± SEM), except for shoot biomass (8 replicates). Data were tested for normality and homogeneity of variance using the Shapiro-Wilk test and Levene test ($P>0.05$), respectively, prior to analysis of variance (ANOVA). Factorial ANOVAs were used to test the effects of roots, N fertilization and soil depths separately at maize silking and maturity stages for all the measured parameters. ANOVAs were followed by post-hoc tests for multiple comparisons using least significant differences (Tukey test). We used Student's t-test for the differences between N fertilized and unfertilized maize shoot biomass at silking and maturity stage. Significance was set at $P<0.05$ for both ANOVAs and t-tests. Principal component analysis (PCA) was used to assess the patterns of EA for both phenological stages (silking and maturity) of maize. We performed PCA on a reduced version of EA data, in which the average values of four replicates for each soil depth, presence or absence of maize plants, and with and without N fertilization were used. Statistical analyses were performed with STATISTICA for windows (version 13.2; StatSoft Inc., OK, USA).

3.3. Results

3.3.1. Plant biomass and soil moisture

N-fertilization increased shoot biomass by 80% and 91% at silking and maturity stages, respectively, compared with unfertilized plants (Supplementary figure II.3:1). The shoot biomass was approximately 3 times higher for both unfertilized and N fertilized plants at maturity vs. silking stage. Overall, the presence of plants resulted in lower soil moisture contents. Soil moisture was lower in rooted soil (with and without N fertilization) at each

depth and at both sampling times. Compared to bare fallow, the moisture content was reduced by 11%, 21%, 15%, and 18% at 0-5 cm, 5-15 cm, 15-25 cm, and 25-35 cm depth, respectively, in rooted soil at silking stage (Supplementary Table II.3:1). Moreover, moisture content was always lower at 0-5 cm than at other soil depths.

3.3.2. Distribution of extracellular enzyme activities

The potential EA of BG, CBH, XYL, NAG, LAP, and PHO showed significantly different distribution patterns with varying depth in bare fallow and rooted soil with or without N fertilization at both phenological stages (silking and maturity) (Figure II.3:4). In general, the potential EA was always higher at middle depths (5-15 cm and 15-25 cm) than in shallow or deep soil. Moreover, the EA involved solely in catalyzing the mineralization of organic C compounds (i.e. BG, CBH, and XYL) responded in a similar fashion with depth and N fertilization as those involved in catalyzing the mineralization of organic N compounds (i.e. LAP) (Supplementary Table II.3:1). For example, the presence of plants increased ($P < 0.05$) the potential activity of BG (C-acquiring enzyme) at both phenological stages, and the stimulating effects of roots were more pronounced at 5-15 cm and 15-25 cm soil depths (Figure II.3:1). Maize roots increased the EA of BG by 57% and 58% at silking stage, but only by 32% and 30% at maturity at 5-15 cm and 15-25 cm depths, respectively. Furthermore, N fertilization did not ($P > 0.05$) change the EA of BG either in bare fallow or in rooted soil at both phenological stages (Figure II.3:1). Similarly, the EA of LAP (N-acquiring enzyme) was affected by both maize roots and N fertilization. In rooted soil, the EA of LAP increased by 25% and 11% at silking stage, and by 19% and 7% at maize maturity at 5-15 cm and 15-25 cm depths, respectively (Figure II.3:2). The decrease in LAP with N fertilization was more prominent in rooted soils than in bare fallow. The activity of NAG (involved in both C- and N-acquiring)

showed a pattern similar to LAP, whereas PHO activity was more similar to C-acquiring enzymes (Figure II.3:3 and Supplementary Table II.3:1).

3.3.3. Predictors of extracellular enzyme activities

Distribution of EA was affected by soil depth (determining nutrient availability and microbial activity), maize phenological stage (determining quality and quantity of resource availability) and application of N fertilization (alleviation of competition between plants and microbes for mineral N), and these effects were enzyme specific. Depth had the strongest effect on EA distribution for all measured enzymes at both sampling times. For example, at silking and maturity stages, soil depth alone explained from 24% (LAP) to 46% (PHO) and from 16% (XYL) to 51% (BG) of total variation in EA, respectively (Figure II.3:3). Followed by depth, the second main predictor of EA distribution was the presence of maize roots. Maize roots increased the EA of all measured enzymes at silking stage (Figure II.3:3). At maturity however, the stimulating effect of roots on EA was significant only for BG activity (Supplementary Table II.3:2). The strongest effects of roots were recorded for EA of BG, explaining 23% and 9% of the total variation at maize silking and maturity stages, respectively. CBH was the least sensitive to the presence of plant roots.

N fertilization reduced the EA of enzymes involved in mineralization of N-containing organic compounds (LAP and NAG) and did not change the EEA of BG, CBH (solely C-acquiring enzymes) and PHO (solely P-acquiring enzymes), except XYL activity (Figure II.3:3, Supplementary Table II.3:2). At silking stage, N fertilization explained a significant portion of EA variation for LAP (10%) and NAG (7%). The effects of N fertilization remained significant even at maturity, although the proportion of variation explained was

reduced by half compared to the silking stage (5% for LAP and 4% for NAG). N fertilization reduced ($P < 0.05$) the EA of XYL only at maize silking stage (Supplementary Table II.3:2). The projection of EA on PCA confirmed that BG, CBH, NAG, and XYL (C-acquiring enzymes) and PHO (P-acquiring enzyme) were more closely related to each other than to LAP (solely N-acquiring enzyme) (Figure II.3:4).

3.4. Discussion

The present study highlighted regulation of plant phenological stage, soil depth and N fertilization on microbial activity (i.e. EA). Shoot biomass was higher in N fertilized plots at both phenological stages due to an increase in net N uptake, resulting in higher leaf biomass and photosynthetic intensity (LeBauer and Treseder 2008). The reduced moisture in rooted soil as compared to bare fallow is due to transpiration by plants (Prieto et al. 2012; Steudle 2000). Higher evaporation at the surface layer leads to more drastic drying, which explains the lower moisture content in the top (0-5 cm) layer as compared to lower soil depths (Mganga et al. 2015).

Enhanced activity of all measured enzymes in rooted soil (upto 58% increase in BG activity) as compared to bare fallow at both phenological stages provides evidence of plant-mediated activation of microorganisms (microbial activation hypothesis; Cheng and Kuzyakov 2005). Maize plants grow faster during earlier development stages and allocate a higher amount of photo-assimilated products belowground to roots (Pausch et al. 2013, Pausch and Kuzyakov 2017). Increased belowground allocation for root development is generally positively related to root exudation (Pausch and Kuzyakov 2018). This increased release of labile substrates by roots (via exudation) at early

growth stage facilitates microbial growth, resulting in higher EA in rooted soil than in bare fallow (Nannipieri et al. 2012; Kuzyakov and Blagodatskaya 2015). In contrast, at maturation stage, when plants have a fully developed root system, the allocation of resources shifts from belowground to aboveground plant tissues (cob formation). As a result, the stimulating effect of roots on EA was reduced at maize maturity (Figure II.3:1 and 3:2). Moreover, there is a shift in the quality and quantity of root exudation with plant growth stage (Badri et al. 2009; Pausch and Kuzyakov 2017). The change in EA of BG, CBH, XYL, NAG, PHO, and LAP in rooted soil depending on plant phenological stage demonstrated that, in the rhizosphere, microorganisms are fueled by root exudation, and their activity (i.e. EA) is intimately linked to both the quantity and quality of labile substrate inputs via roots. Among all the measured EA, only the BG activity was higher in rooted soils at maize maturity, indicating higher microbial demand for easily available substrates as an energy source.

Besides the effect of maize growth stages, EA was also altered by soil depth. The EA of the top layer (0-5 cm) was more driven by moisture than by maize roots. Minimum moisture at 0-5 cm depth across the soil profile corresponded to minimum EA for all measured enzymes at both sampling times. Reduced moisture via higher evaporation at the top soil layer may have limited microbial growth and activity (Schimel et al. 1999). Middle depths (5-15 cm and 15-25 cm) were characterized by higher moisture and root density in comparison to the top layer (0-5 cm), which resulted in root-mediated increases in potential EA. Furthermore, while the moisture content at 25-35 cm depth was similar to the 5-15 cm and 15-25 cm layers, the limited labile C inputs via root exudation as well as reduced gaseous exchange may have hampered microbial growth and consequently EA (Salome et al. 2010). A significant positive correlation between

C_{mic} and enzyme activities (Supplementary Figure II.3:2) provided evidence of the microbial contribution to EA, which is in accordance with other studies (Perucci 1992; Garcia-Gil et al. 2000). The minimal effects of N fertilization on the activities of P- and C-acquiring enzymes (except for XYL at silking stage) and a decrease in activities of N-acquiring enzymes confirmed that microorganisms were not limited in N (Figure II.3:3). When an alternative N source (mineral N) is available, the microorganisms down-regulate production of enzymes responsible for N acquisition through SOM decomposition and shift to utilization of the available N source (preferential substrate utilization) (Kuzyakov et al. 2000). Neutral or negative effects of N addition on enzyme activities and SOM decomposition were demonstrated by Keeler et al. (2009).

3.5. Conclusions

Root-derived organics increased activities of all measured enzymes, indicative of root-mediated microbial activation. In comparison to bare fallow, EA was higher in rooted soil despite possessing lower moisture contents, demonstrating intimate plant-microbial interactions via root-derived organics. Maize phenology mediated changes in EA emphasized regulatory mechanisms of microbial activity. Higher impact of maize silking vs. maturity stage on EA demonstrated that actively growing roots contributed to higher EA most likely by root exudation. Regarding soil depth, the highest enzyme activities were centered in the zone of maximum root density (5-25 cm), further supporting plant mediated increases in microbial growth and activity. Reduced Leucine-aminopeptidase and β-1,4-N-acetylglucosaminidase activities with N-fertilization demonstrates reduced resource allocation to N-cycling enzyme synthesis in the presence of alternative N

sources. To summarize, 1) soil depth had the strongest effect on EA (up to 51% of total variation), 2) the root effect was stronger at the silking versus maturity stage; and 3) N fertilization affected only the enzymes related to N cycle. We conclude that soil depth and plant phenology stage govern EA, and these effects are strongest between 5 and 25 cm soil depth containing silking plants.

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3.8. Figures

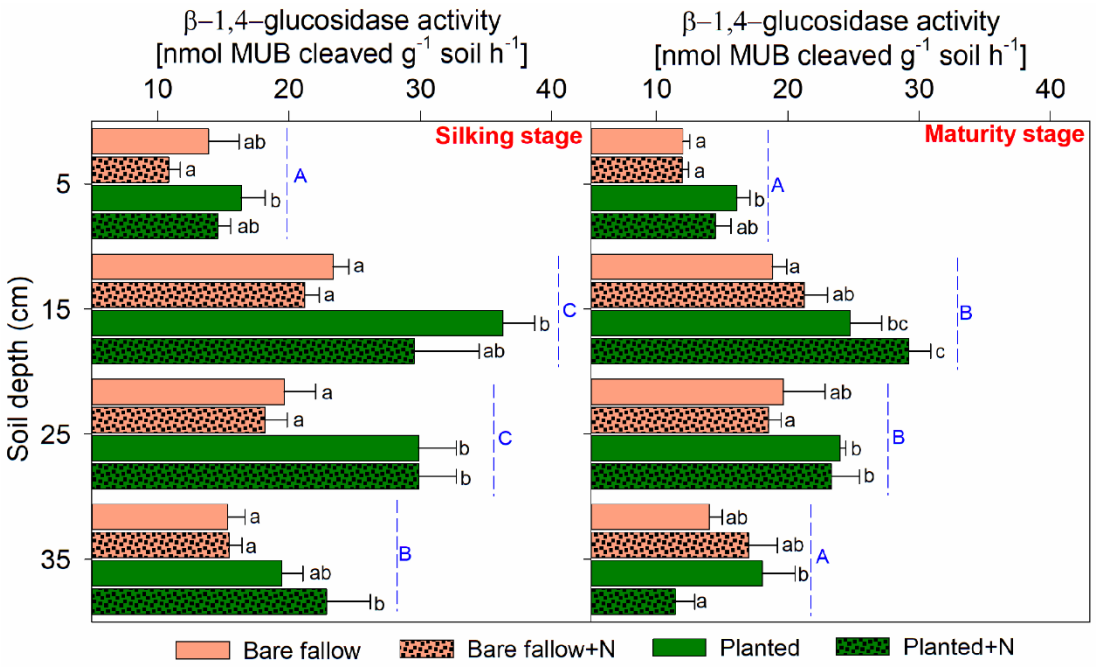

Figure II.3:1: Potential activity of β-1,4-glucosidase (nmol MUB cleaved g^{-1} soil h^{-1}; mean ± SE) in bare fallow, bare fallow with N-fertilization (Bare fallow+N), unfertilized maize-planted (Planted), and N-fertilized maize-planted (Planted+N) soil at four depths at maize silking and maturity stage. Lower-case letters indicate significant differences (Post-hoc LSD test, $P < 0.05$) within each depth whereas the upper-case letters indicate significant differences between the depths (Post-hoc LSD test, $P < 0.05$, blue dashed lines) (n=4).

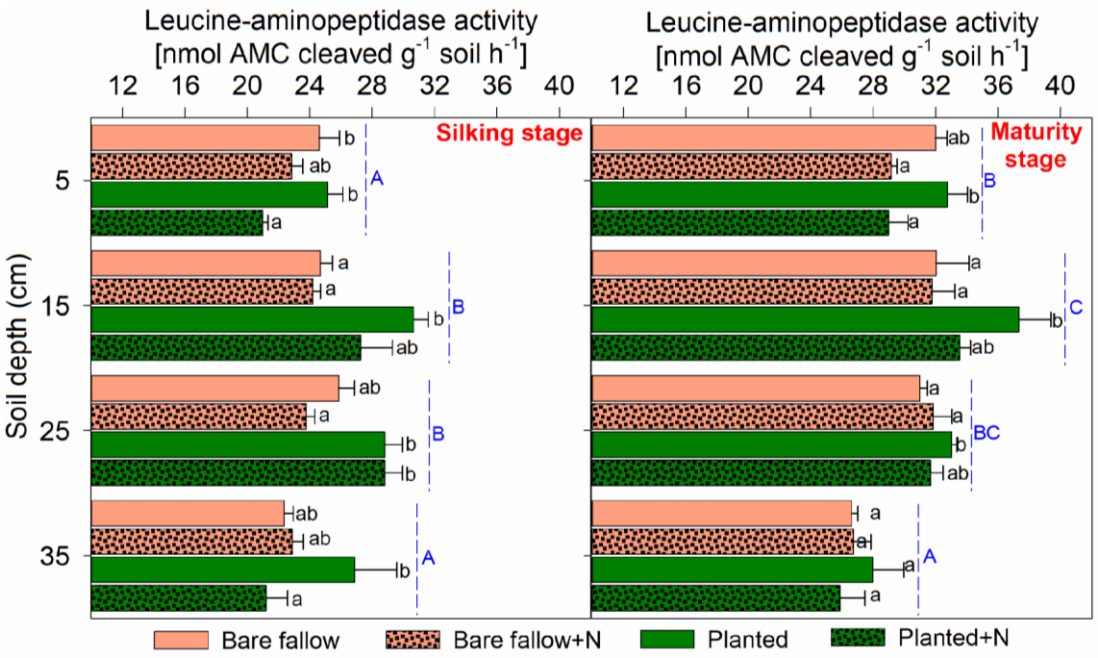

Figure II.3:2: Potential activity of Leucine-aminopeptidase (nmol AMC cleaved g^{-1} soil h^{-1}; mean ± SE) in bare fallow, bare fallow with N-fertilization (Bare fallow+N), unfertilized maize-planted (Planted), and N-fertilized maize-planted (Planted+N) soil at four depths at maize silking and maturity stage. Lower-case letters indicate significant differences (Post-hoc LSD test, $P < 0.05$) within each depth whereas the upper-case letters indicate significant differences (Post-hoc LSD test, $P < 0.05$, blue dashed lines) (n=4).

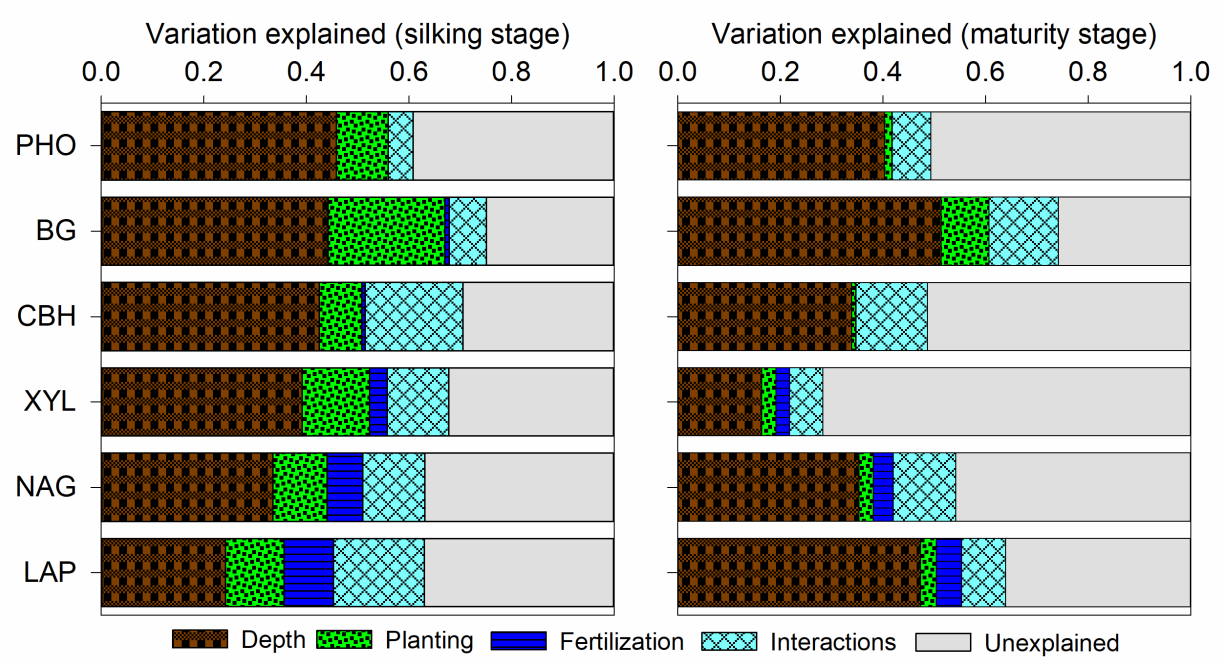

Figure II.3:3: Contribution of three factors: soil depth (0-5 cm, 5-15 cm, 15-25 cm, and 25-35 cm), maize roots (presence or absence of plants), N fertilization (presence or absence of N fertilization), and their interactions on potential activity of phosphomonoester (PHO), BG (β-1,4-glucosidase), CBH (β-cellobiohydrolase), XYL (β-xylosidase), NAG (N-acetly-1,4-glucosaminidase), and LAP (Leucine-aminopeptidase).

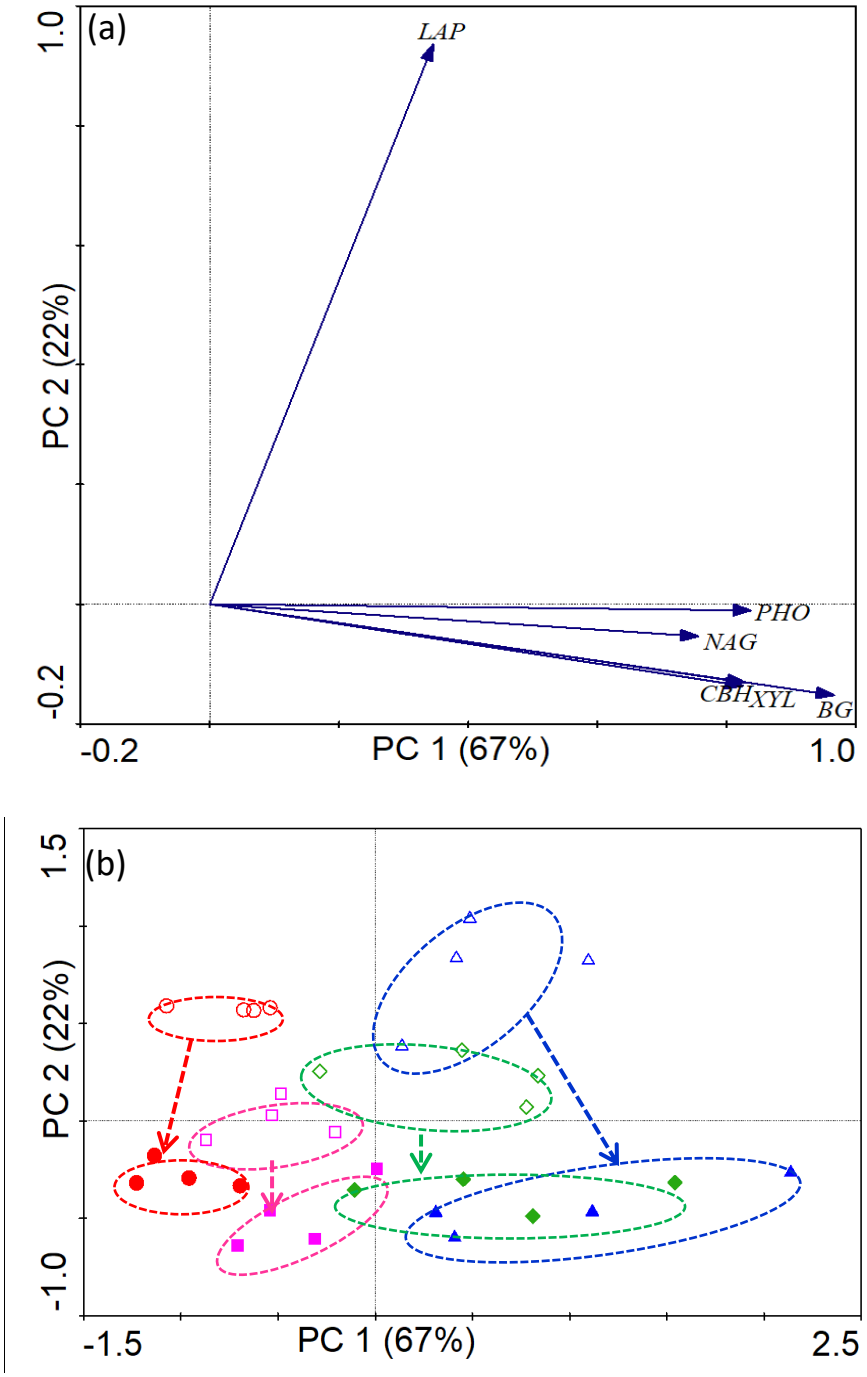

Figure II.3:4: Principal component analysis (PCA) showing (a) the distribution of potential activity of BG (β-1,4-glucosidase), CBH (β-cellobiohydrolase), XYL (β-xylosidase), NAG (N-acetly-1,4-glucosaminidase), and LAP (Leucine-aminopeptidase), and PHO (phosphomonoesterase). The PCA analysis showed (b) distinct enzyme activities at maize silking (unfilled symbols) and maturity (filled symbols) stage. Different colors and shapes indicate each soil depth as follows: 0-5 cm (red circle), 5-15 cm (blue upside triangle), 15-25 cm (green diamond), and 25-35 cm (pink square).

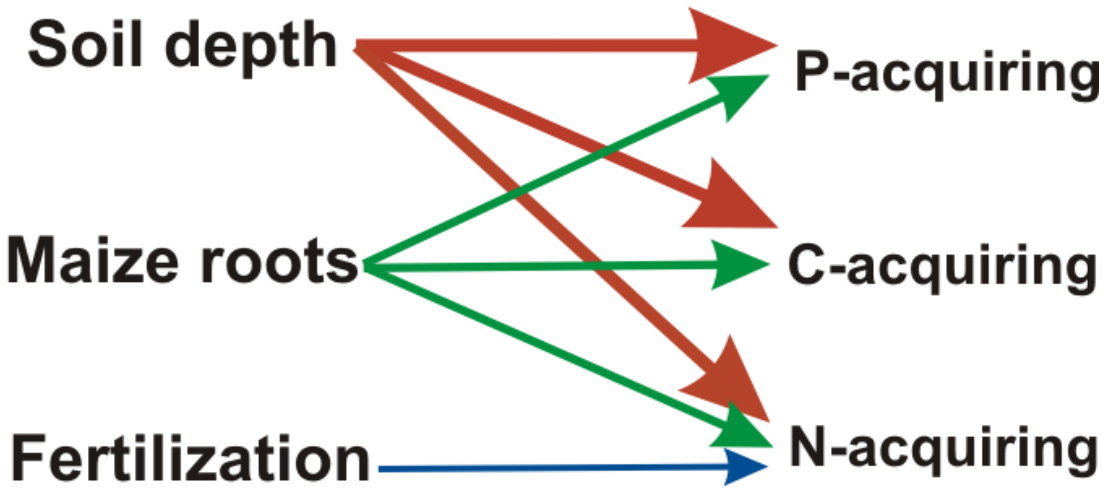

Figure II.3:5: Effects of soil depth, maize roots, and N-fertilization on distribution of activity of P-, C-, and N-acquiring enzymes in maize rhizosphere. Thickness of arrows indicates the strength of the effect on enzyme activities.

4. Root trait plasticity to maintain plant productivity under phosphorus limitation

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Abstract

To combat soil nutrient limitation which is further strengthened by intense competition between plant and microorganisms, plants have developed complex adaptive responses in various root traits. Root hairs and root colonization by arbuscular mycorrhizal fungi (AMF) are two key traits for plant phosphorus (P) acquisition in P limited soil. The main objective of present the study was to understand plants' P acquisition strategies with shifting root traits using two genotypes of maize characterized by presence (wild type) or absence (*roothairless3* mutant) of root hairs. We hypothesized that 1) in P limited soils, plants compensate for the lack of root hairs by shifting to root mycorrhizal colonization (functionally analogous to hairs) for P acquisition; 2) root mycorrhizal colonization decreases with P fertilization due to strong trade-off for C costs. The present study demonstrated that root hairs and AM fungi are crucial for plant P acquisition. Root hairs promoted P uptake most likely by increasing root surface area for absorption. Root hairs decreased the dependency of plants on root mycorrhizal colonization, thereby reducing C costs for P acquisition. However, smaller surface area for absorption in absence of root hairs can be counterbalanced by increased root mycorrhizal colonization. Plasticity in root traits (increased root diameter and higher root mycorrhizal colonization by AMF) for P acquisition is important for maintaining plant growth in absence or poorly developed root hairs and under nutrient limitation.

Key words: nutrient acquisition, *roothairless3* mutant, root morphological trait, root diameter, root mycorrhizal colonization, root hairs

4.1. Introduction

There has been remarkable advance in linking plant traits to ecosystem processes from landscape to global scale (Adler et al. 2014). Trait based understanding of ecosystem functioning is mostly derived from plants' aboveground traits such as total and specific leaf area, maximum plant height, photosynthetic capacity, life span, respiration, regeneration capacity, nutrient status and life span of leaves, wood density, and phenology. However, the belowground traits (root traits) are equally important in driving major ecosystem processes such as carbon (C) and nutrients cycling (Bardgett et al. 2014). Altered response of root traits for instance root respiration, rhizodeposition, nutrient uptake kinetics, root diameter, rooting depth, root length density, and root interactions with mycorrhizas and rhizobia to environmental variables are among main drivers of SOM decomposition. In agroecosystems, plasticity in aforementioned traits in relation to soil nutrient and water limitation, herbivory, and interaction with soil fauna helps plant nutrient acquisition and maximize their growth and development (Faucon et al. 2017). For instance, under P-limitation, primary root growth is suppressed and the root architecture changes to shallower root system bearing more lateral roots and increased root hair density, which enables the roots to explore more soil volumes (Peret et al. 2011).

Root hairs (single cell extension of epidermal cells) are a key morphological root trait for P acquisition in barley (Gahoonia et al. 2001). Presence of root hairs may contribute by up to 80 % to plant P uptake by increasing the root surface area and hence, the contact space between the root and soil for absorption (Jungk 2001). Moreover, root hairs increase the total surface area for exudation as well as their rapid turnover contributes to increased total rhizodeposition (Lambers et al. 2006). Rhizodeposition, an energy

subsidy to microorganisms is supplemented by roots arbitrate the interactions between plants and soil microorganisms such as symbiotic associations with fungi. Most of the crop plant roots are symbiotically colonized with arbuscular mycorrhiza (AM). Plants provide photo-assimilated products to the fungal symbiont and in turn receive nutrients especially N and P via fungal channels from soil volumes beyond the depletion zone of roots. The extraradical mycelium of AM may also enter the very fine soil pores thereby increasing the nutrient and water uptake (Khalvati et al. 2005). One fundamental knowledge gap in understanding rhizosphere ecology of agroecosystems is that most of the previous studies are done at only one plant growth stage and the dynamics of plant-microbial relationship with plant growth stage are still poorly understood. At early plant growth stages, there is an establishment of interactions between roots and microorganisms. At later growth stages, such interactions become more important in rhizosphere as there are plant growth associated changes in root and soil properties (Philippot et al. 2013; Wen et al. 2017) and variation in quality and quantity of rhizodeposits (Chapparo et al. 2013) as well as intense competition between plants and soil microorganisms for limited nutrients (Kuzyakov and Xu, 2013). For example, root exudates are comprised of organic compounds constituting higher C to N ratios ranging from 50-100 (Drake et al. 2013) and their input into soil varies with plant phenology stages (Chapparo et al. 2013). This temporal change in root exudation quality and quantity further changes the stoichiometry of available resources in the rhizosphere. Root-derived C inputs coupled with plant nutrient uptake at earlier growth stages could exert rapid shift in nutrient availability (strong N and P limitation) in rhizosphere soil (Dijkstra et al. 2013; Phillips et al. 2011). In response to nutrient shortage, plants manipulate their strategies to maximize the uptake; however, an explicit relationship is

missing between specific root trait and nutrient uptake (Chen et al. 2016). Moreover, such strategies are species specific and the cause of variations in the same are still poorly understood. Therefore, the present study focuses on the root traits plasticity (morphological and biological trait) for plant P acquisition in P limited soils and how the availability of P (via P fertilization) affects the response of such traits. We hypothesized that 1) in P limited soils, plants compensate for the lack of root hairs by shifting the root trait to higher root mycorrhizal colonization (functionally analogous to hairs) for P acquisition; 2) root mycorrhizal colonization decreases with P fertilization due to strong trade-off for C costs. The present study provides the fundamental opportunity to understand the plants' P uptake strategies through plasticity of root traits at three distinct plant growth stages (tillering, stem extension, maize heading).

4.2. Materials and Methods

4.2.1. Experimental setup

Surface soil (0-20 cm) was collected from an agricultural site at Dikopshof Wesseling station of University of Bonn, Germany. The site belongs to a long-term trial without any input of mineral fertilizers. Soil properties are as follow: total C (7.8±0.02 g C kg^{-1} soil), total N (0.74±0.01 g N kg^{-1} soil), C to N ratio (10.5±0.02), calcium-acetate-lactate extractable P (23.2±0.7 mg P kg^{-1} soil), and soil pH 6.48. Field moist soil was passed through a 2-mm sieve and 1.5 kg dry weight equivalent was filled in Polyvinyl chloride (PVC) pots (KG tubes, height 20 cm, diameter 10 cm). In total, 4 treatments were established (with 4 replicates each): maize with root hairs (with hairs), maize with root hairs with P fertilization (with hairs+P), maize without root hairs (*rth3*; without hairs), and

maize without root hairs with P fertilization (without hairs+P). In order to understand the effects of plant phenological stage on rhizosphere processes, samples were taken at three distinct growth stages (tillering, stem elongation, and maize heading). All soil-filled pots were pre-incubated in a growth chamber for 3 days before sowing. Seeds of a maize (*Zea mays* L.) wild type (WT) and a *roothairless3* (*rth3*) mutant were surface sterilized with 10% H_2O_2 for 3 min, washed 5 times with distilled water and germinated on moist filter paper in petri plates in dark for 5 days. After germination, seedlings were transferred to PVC pots and grown under controlled environmental conditions in a climate-chamber with 16/8-h day/night rhythm with mid-day and night temperatures of 25° C and 15° C respectively, and light intensity at approximately 600 µmol m^{-2} s^{-1}. After establishment of seedling (usually 2-3 days), all the pots were fertilized with inorganic nitrogen (KNO_3, at the rate of 120 kg N h^{-1}) to avoid soil N limitation. Treatments with P-fertilization received inorganic P-fertilizer (KH_2PO_4, at the rate of 60 kg P h^{-1}). The soil water content was checked every day and maintained at 70% water holding capacity (WHC) with distilled water throughout the experimental period. Sixteen pots were destructively harvested after 30 days after planting (DAP), 45 DAP, and 64 DAP constituting tillering, stem extension, and maize heading, respectively.

4.2.2. Pot harvesting

At each harvesting time, maize shoots were cut at the base. The ‘main’ root system was carefully removed after pulling out soil from the pot. Roots were picked with tweezers from each pot for a definite time period (15 min). A subsample of roots was collected for measurements of mycorrhizal colonization (see description below). All roots were scanned with an EPSON (PERFECTION™ V700 PHOTO) scanner and root length density, fine roots, and average root diameter of fine roots were determined using

WinRHIZO (Regents Instruments Inc., Quebec, Canada). Afterwards, roots and shoots were freeze-dried and ball-milled to powder for total elemental analyses. Total P content in plant tissues and soil were measured using inductively coupled plasma-atomic emission spectrometer (iCAP 6300 Duo VIEW ICP Spectrometer, Thermo Fischer Scientific GmbH, Dreieich, Germany).

4.2.3. Root mycorrhiza colonization by AM fungi

Root mycorrhizal colonization by AM fungi was measured after staining the roots with blue ink in lacto-phenol (Phillips and Hayman, 1970). Briefly, fine roots (< 2 mm) were collected manually with tweezers for 2 min. Fine roots were cut into 1 cm segments and washed with distilled water. Root segments were cleared in 2.5% KOH at 90° C for 1 h. Thereafter, root segments were washed in distilled water to remove access KOH and treated with 3% H_2O_2 for 30 min at room temperature. Afterwards, the root segments were washed again with distilled water and stained with ink (lacto-phenol) for 2 min. Root mycorrhizal colonization was observed at 10 x 40 magnification under light microscope (Axionplan, Zeiss, Germany) and the percentage of mycorrhizal colonization was counted using the grid-line intersection method (Giovannetti and Mosse 1980).

Statistics

The experiment was conducted with 4 replicates for each treatment at each harvesting time. The values presented in figures and tables are means ± standard errors of means (± SEM). The data was checked for normality (Shapiro-Wilk test, $P>0.05$) and homogeneity of variance (Levene test, $P>0.05$) at individual plant growth stage prior to analysis of variance (ANOVA). One-way ANOVA was performed to test the effect of plant growth stage on root and shoot biomass, root mycorrhizal colonization, average fine root diameter, and plant P uptake followed by post-hoc HSD test for multiple

comparisons. We used student's t-test to test the effects of P fertilization within the same genotype and effect of genotype at each growth stage. In general, a significance level of $P<0.05$ was used for ANOVA and t-test if not mentioned specifically. All the statistical analyses were performed using STATISTICA for Windows (version 13.2; StatSoft Inc., OK, USA).

4.3. Results

4.3.1. Plant biomass

Total plant biomass (root and shoot biomass) increased from the tillering to maize heading stage for both (wild type and *rth3* mutant) genotypes (Figure II.4:1). The main difference in production of plant biomass was due to P fertilization, where P fertilization increased the total plant biomass by 38% of mutants (*rth3*) and by 43% of wild type (WT) at maize heading. The *rth3* mutant (completely lacking root hairs) had the similar biomass production (shoot and root) as the WT maize (possessing root hairs) (Figure II.4:1).

4.3.2. Root morphology and mycorrhiza colonization

The average fine root diameter (AFRD; in mm) increased with P fertilization for the WT during tillering and stem elongation stages (Figure II.4:2). This increase was up to 21% at tillering and 10% at stem elongation stage (Figure II.4:2). There was a trend for an increase in AFRD for the *rth3* maize with P fertilization up to 8%, 3%, and 10% at tillering, stem elongation and maize heading, respectively, although this increase was not significant. Comparing the two genotypes, *rth3* increased their AFRD in comparison to the WT. The AFRD of unfertilized *rth3* maize was by 16%, 15% and 20% as

compared to WT at tillering, stem elongation, and maize heading, respectively. Moreover, the AFRD of *rth3* increased gradually along with plant growth stages (0.39±0.01 at tillering, 0.45±0.02 at stem elongation, and 0.47±0.02 at maize heading); however, it remained similar for WT (Figure II.4:2). Root mycorrhizal colonization decreased with P fertilization in both WT and *rth3* maize (Figure 3). With P fertilization, root mycorrhizal colonization decreased by 19%, 24%, and 16% in WT and by 17%, 27%, and 19% in *rth3* at tillering, stem elongation, and heading stage, respectively. Moreover, the root mycorrhizal colonization increased in both WT and *rth3* with plant growth stage, however, the increase with growth stage was more pronounced in *rth3* and without P fertilization. The mycorrhizal colonization increased by 14%, 15%, and 21% at tillering, stem elongation, and heading, respectively in unfertilized *rth3* than WT maize roots (Figure II.4:3). Root mycorrhiza colonization by AM fungi and AFRD correlated significantly (r^2 = 0.30, P = 0.005) in *rth3* whereas there was no correlation in WT (r^2 = 0.002, P = 0.80) (Figure II.4:4).

4.3.3. Plant P uptake

P fertilization increased the plant P uptake (mg P day^{-1}) from tillering to stem elongation to maize heading. At maize tillering, P fertilization significantly increased the P uptake by 305% and 242% in WT and *rth3* maize, however, both genotypes (WT and *rth3*) has similar uptake rates (Figure II.4:5). Later from stem elongation to maize heading, both, P fertilization and genotype effects (WT and *rth3*) were evident. In P fertilized WT maize, P uptake was more than twice as higher in comparison to unfertilized WT (0.16±0.03 mg P day^{-1} and 0.37±0.03 mg P day^{-1} for unfertilized and P fertilized WT, respectively) during maize stem elongation. This increase in P uptake with fertilization was evident also at maize heading (0.32±0.02 mg P day^{-1} and 0.57± 0.02 mg P day^{-1} in unfertilized and P

fertilized WT, respectively). Similarly, *rth3* maize had higher P uptake under P fertilization and this increase was up to 4 times and 2 times higher than unfertilized *rth3* maize at stem elongation (0.08±0.01 mg P day^{-1} and 0.25±0.02 mg P day^{-1} for unfertilized and P fertilized *rth3*, respectively) and at heading (0.25 mg P day^{-1} and 0.48±0.01 mg P day^{-1} for unfertilized and P fertilized *rth3*, respectively), respectively. Comparing the genotype effect during maize stem elongation and maize heading, WT maize performed better than *rth3* in terms of P uptake. Without P fertilization, the WT showed a 91% and 28% higher P uptake than the *rth3* during stem elongation and maize heading, respectively (Figure II.4:5). With P fertilization, P uptake increased by 46% and 19% in WT than *rth3* maize during stem elongation and maize heading. A significant correlation between root mycorrhiza colonization by AM fungi and plant P uptake especially in unfertilized WT and *rth3* maize highlights importance of root mycorrhizal colonization with AM fungi for plant P acquisition (Figure II.4:6).

4.4. Discussion

The present study provides further evidence on the importance of root hairs for P uptake and we have discovered that the lack in a functional root trait (here root hairs) causes shifts to other traits (here mycorrhiza) with complementary functions. These traits may however be more C cost intensive and their development may hence be down regulated if the respective function is not required. For instance, functional traits for P uptake are down regulated in soils with high P availability as shown in this study. In nutrient limited soils, increase in total plant biomass (root and shoot biomass) after P fertilization is a well-observed response of plants. Such a response has been reported by various studies on grasses (Haines et al. 2015; Sundqvist et al. 2014), agricultural crops

(Bakhshandeh et al. 2017; Chen et al. 2004; Gahoonia et al. 1999), and trees (Lavigne and Krasowski, 2007). The availability of extra P via fertilization increases the net P uptake resulting in higher photosynthetic activity and consequently higher biomass production.

P fertilization increased the total plant biomass; however, it resulted in reduced root colonization by arbuscular mycorrhizal (AM) fungi as compared to unfertilized plants. This highlights the importance of mycorrhizal symbiosis for plant P acquisition. Moreover, at higher nutrient availability when plants are not limited by nutrients, the higher C costs by plants for P acquisition exceeding the mycorrhizal benefits may also downregulate the root colonization by AM fungi (Carbonnel and Gutjahr, 2014). The inhibitory mechanisms of P fertilization on spore germination, growth and development of mycorrhizal hyphae, and root mycorrhiza colonization have been observed in previous studies in pure cultures (Hepper, 1983) as well as in soils (Jakobsen et al. 2005; Treseder and Allen 2002). Moreover, there are reports showing a decrease in AM fungi abundance with increasing nutrient availability across chronosequences (Dickie et al. 2013), natural gradients of mean annual rainfall (Bohrer et al. 2001), successional and environmental gradients (Zangaro et al. 2014). A gradual increase in root colonization by AM fungi along with plant growth stages highlights that when P become limited (due to plant and microbial uptake), the symbiotic association of plant roots with AM fungi becomes increasingly important for plant P acquisition. The present study also highlighted that *rth3* maize (completely lacking root hairs) had higher root mycorrhizal colonization than WT maize (possessing root hairs) indicating that in absence of root hairs (a key morphological trait for nutrient and water uptake), mycorrhiza counteracts for plant P acquisition. Such an increase in root mycorrhizal colonization in absence of

root hairs demonstrates the relative importance of fungal partner which is in accordance with Jakobsen et al. 2005 who showed higher root mycorrhiza colonization of *brb* (root hairless mutant) than its wild type (possessing root hairs) in *Hordeum vulgare* cv Pallas.

Mycorrhizal symbiosis may cause various adaptive strategies such as changes in root-to-shoot ratio (Veresoglou et al. 2012), root architecture and longevity (Hooker and Atkinson, 1996), root length (Camenzind et al. 2016), and root diameter (Comas et al. 2014). Such allometric changes are plant specific and depend on experimental duration as well as on plant and their fungal partner identities (Veresoglou et al. 2012). Many of these evidences are derived from plant phylogeny by determining changes in root morphological and architectural traits using phylogenetically independent contrasts (Comas et al. 2014), therefore an in-depth understanding require empirical evidences. For the first time, the present study demonstrates empirically that in the absence of root hairs (*rth3* maize), plants increase their average diameter of fine roots (< 1mm) to facilitate colonization by AM fungi (Figure 3). This increase in average fine root diameter (AFRD) of *rth3* maize with growth stage and a significant correlation ($P = 0.005$) between AFRD and root mycorrhizal colonization by AM fungi in *rth3* maize highlights the requirement of more root volume for increased mycorrhiza colonization. This could be beneficial for *rth3* maize for a couple of reasons such as 1) increased AFRD will have more space to be colonized by AM fungi (Reinhardt and Miller, 1990); 2) increased AFRD will comparatively increase the root longevity and therefore slower turnover (Comas et al. 2012; Eissenstat, 1992), which is beneficial for plant to maintain and carry forward the active exchange of nutrients and C between AM fungi and plants; 3) increased AFRD in *rth3* maize will comparatively increase the root surface area for a given unit of root length as compared to WT maize (Haling et al. 2013); and 4) increase

in AFRD in *rth3* maize will reduce the metabolic costs such as root respiration (Lynch and Ho, 2005).

A significant correlation between plant P uptake and root colonization by AM fungi particularly in unfertilized maize (in both, *rth3* and WT) highlighted the importance of AM fungi for plant P acquisition in P limited soils. Moreover, it was demonstrated that for the given unit of P uptake, *rth3* maize possessed higher root colonization by AM fungi emphasizing the compensation for absence of root hairs, which has previously been shown by Jakobsen et al. 2005 and Li et al. 2014. The total P uptake along with maize phenological stages showed that at tillering stage, when the nutrients are still abundant, there was no difference in P uptake between *rth3* and WT maize.

In a similar study with two genotypes of *Hordeum vulgare* L. characterized by presence (WT) and absence (*brb*) of root hairs, Pausch et al. 2016 suggested preferential utilization of root-derived organics by microorganisms at tillering stage and reduced competition for nutrients between plants and microorganisms. Once the plants advance in their growth stages, the nutrients level in soil decreases due to plant and microbial uptake resulting in strong competition between them (Mwafulirwa et al. 2016; Veresoglou et al. 2012). In the present study, at stem elongation and maize heading, P uptake increased in WT as compared to *rth3* maize. This increased P uptake in WT maize is most likely due to extension of the rhizosphere through root hairs. Literature is replete with studies demonstrating empirically (Haling et al. 2013; Holz et al. 2017) as well as theoretically (Itoh and Barber, 1983; Nye 1966) a rhizosphere extension with root hairs by increasing the total surface area. In summary, the present study highlighted that in the absence of a single morphological trait (root hairs), plants intensify their

interactions with AM fungi to maximize P uptake. We also showed that the lack of root hairs is not fully compensated for by higher mycorrhization likely due to higher C costs for maintaining the symbiosis with AM fungi.

4.5. Conclusions

The present study demonstrated that both, root morphological (root hairs) and biological traits (root colonization by AM fungi) are crucial for plant P uptake in P limited soils. Root hairs promoted P uptake most likely by increasing the root surface area for absorption. Presence of root hairs increased the P uptake and decreased the dependency of plants on root mycorrhizal colonization by AM fungi, thereby reducing the C costs for P acquisition. However, the smaller surface area for absorption in absence of root hairs can be counterbalanced by increased root colonization by AM fungi. This alternative root trait for P uptake, by exploring the soil volumes beyond the root depletion zone, is important for maintaining plant growth in the absence or reduced growth of root hairs and under nutrient limitation. Plant adaptive strategy in response to higher colonization by increasing the root diameter of fine roots is an efficient policy resulting in lower costs and higher benefits. The present study enhance the understanding of plant P uptake and interaction-response mechanisms with AM fungi at three major plant growth stages (tillering, stem elongation, and maize heading).

4.6. Acknowledgement

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4.7. References

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4.8. Figures

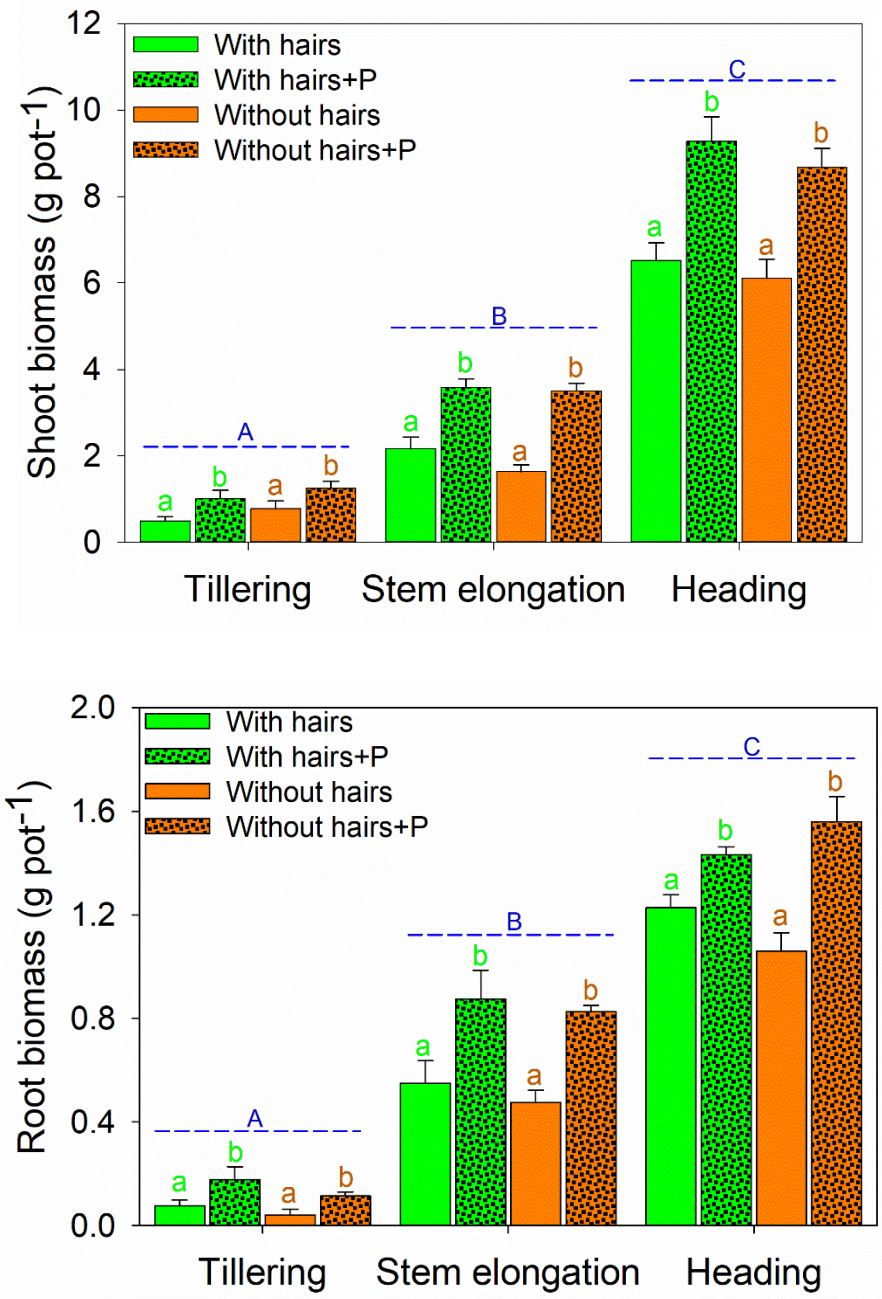


Figure II.4:1: Plant biomass: (upper) shoot biomass (g pot^{-1}±SEM) and (lower) root biomass (g pot^{-1}±SEM) of unfertilized (without pattern) and P-fertilized (patterned bars) maize plants with (wild type: WT, green bars) and without root hairs (*rth3* mutant, orange bars). Upper-case letters indicate significant differences of plant growth stages at tillering (30 DAP), stem elongation (44 DAP) and heading (64 DAP) (ANOVA, $P<0.05$). Lower-case letters indicate significant differences of P fertilization on maize shoot and root biomass separately for WT and *rth3* maize at each plant growth stage (t-test, $P<0.05$). (DAP = Days after planting, n = 4).

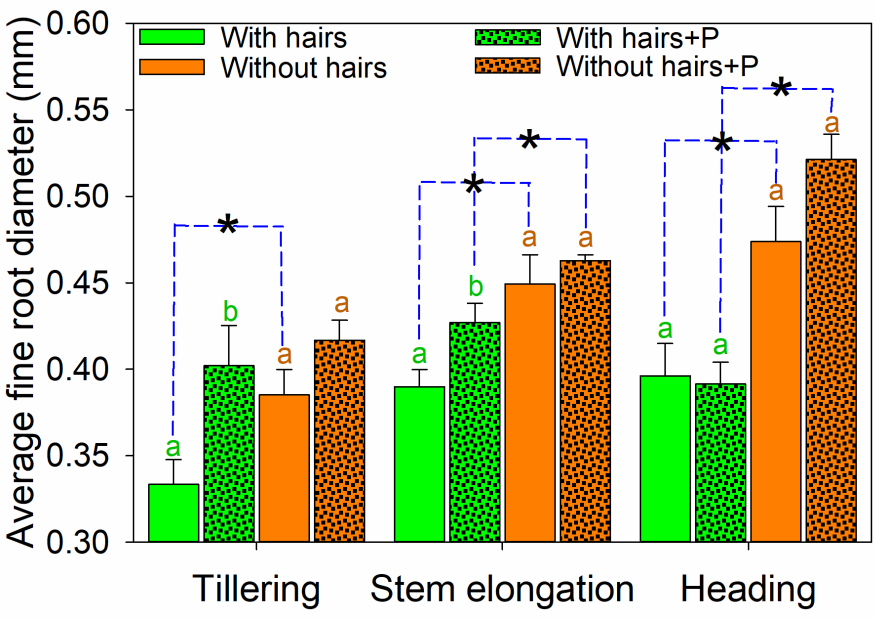


Figure II.4:2: Average fine root diameter (mm±SEM) of unfertilized (without pattern) and P-fertilized (patterned bars) maize plants with (wild type: WT, green bars) and without root hairs (*rth3* mutant, orange bars) at three plant growth stages at tillering (30 DAP), stem elongation (44 DAP), and heading (64 DAP). Lower-case letters indicate significant differences of P fertilization on average fine root diameter separately for WT and *rth3* maize at each plant growth stage (t-test, $P<0.05$). * indicates significant differences between WT and *rth3* maize (t-test, $P<0.05$). (DAP = Days after planting, n = 4).

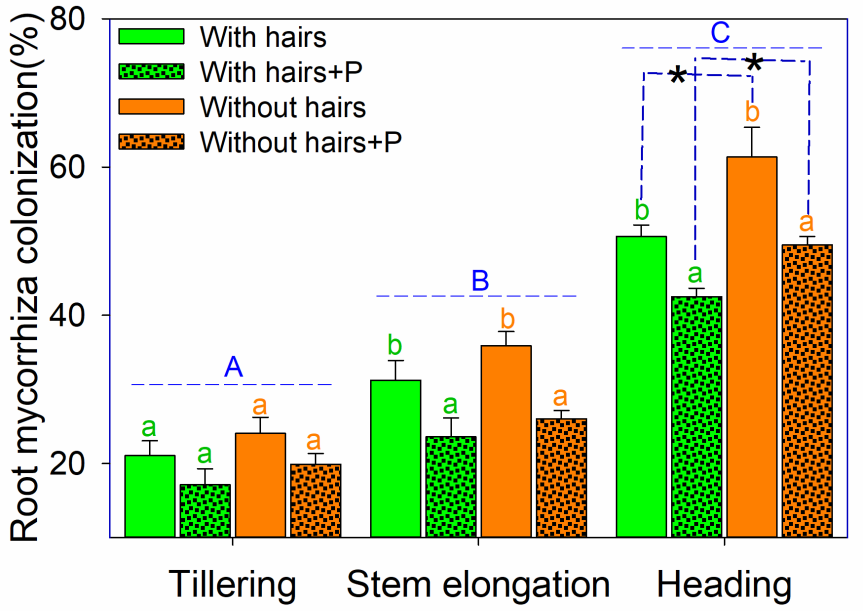


Figure II.4:3: Percentage of roots with mycorrhizal colonization by AM fungi (%±SEM) of unfertilized (without pattern) and P-fertilized (patterned bars) maize plants with root hairs (WT, green bars) and without root hairs (*rth3*, orange bars). Upper-case letters indicate significant differences of plant growth stages at tillering (30 DAP), stem elongation (44 DAP) and heading (64 DAP) (ANOVA, $P<0.05$). Lower-case letters indicate significant differences of P fertilization on root mycorrhiza colonization by AM fungi separately for WT and *rth3* maize at each plant growth stage (t-test, $P<0.05$). * indicates significant differences between WT and *rth3* maize (t-test, $P<0.05$). (DAP = Days after planting, n = 4).

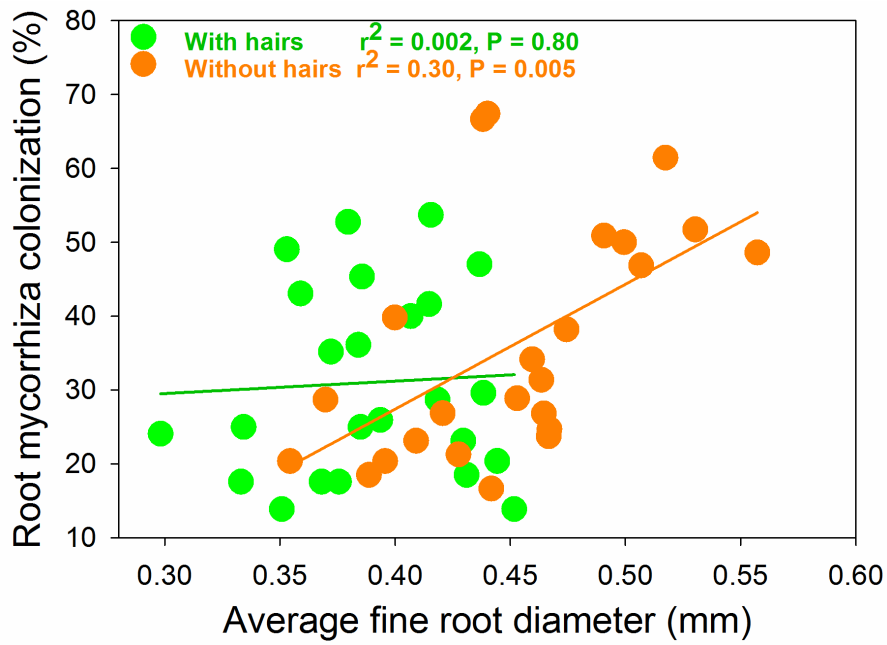


Figure II.4:4: Correlation between average fine root diameter (mm) and percentage of roots with mycorrhizal colonization by AM fungi of maize with root hairs (WT, green circles) and without root hairs (*rth3*, orange circles).

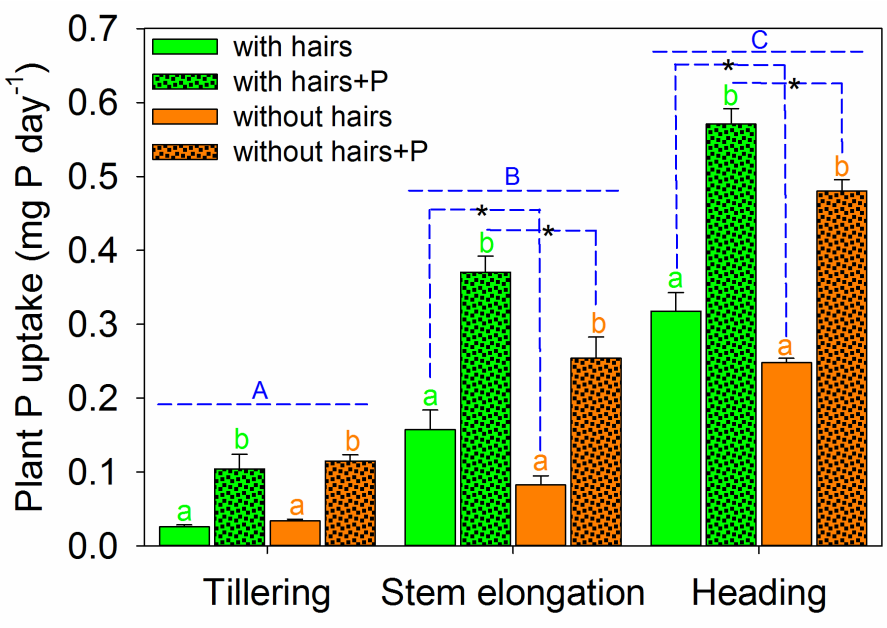


Figure II.4:5: Plant P uptake (mg P day^{-1}±SEM) of unfertilized (without pattern) and P-fertilized (patterned bars) maize plants with (wild type: WT, green bars) and without root hairs (*rth3*, orange bars) at three growth stages at tillering (30 DAP), stem elongation (44 DAP) and heading (64 DAP). Upper-case letters indicate significant differences of plant growth stages at tillering (30 DAP), stem elongation (44 DAP) and heading (64 DAP) (ANOVA, $P<0.05$). Lower-case letters indicate significant differences of P fertilization on plant P uptake separately for WT and *rth3* maize at each plant growth stage (t-test, $P<0.05$). * indicates significant differences between WT and *rth3* maize (t-test, $P<0.05$). (DAP = Days after planting, n = 4).

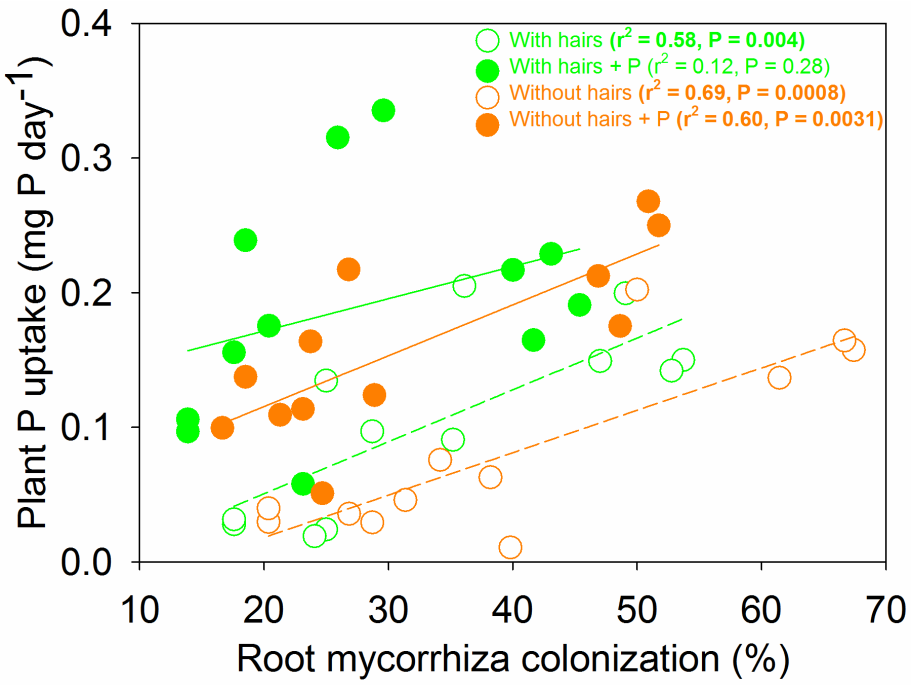


Figure II.4:6: Correlation between maize P uptake (mg P day^{-1}) and percent root mycorrhiza colonization by AM fungi of maize with root hairs (WT, green circles) and without root hairs (*rth3*, orange circles) with P fertilization (filled circles).

Additional studies

Shahbaz M, Kuzyakov Y, Sanaullah M, Heitkamp F, Zelenev V, **Kumar A**, Blagodatskaya E. (2017) Microbial decomposition of soil organic matter is mediated by quality and quantity of crop residues: mechanisms and thresholds. *Biology and Fertility of Soil*. DOI: 10.1007/s00374-016-1174-9

Shahbaz M, **Kumar A**, Kuzyakov Y, Borjesson G, Blagodatskaya E. (2018) Priming effects induced by glucose and decaying plant residues on SOM decomposition: A three-source 13C/14C partitioning study. *Soil Biology and Biochemistry. 121:138-146*

Sun Y, **Kumar A**, Kuzyakov Y, Pausch J. *In-preparation.* Effects of glucose and N addition on priming effects in forest soils: estimations using ^{13}C and ^{15}N isotopic approach.

Supplementary material

II.2: Effects of maize roots on aggregate stability and enzyme activities in soil

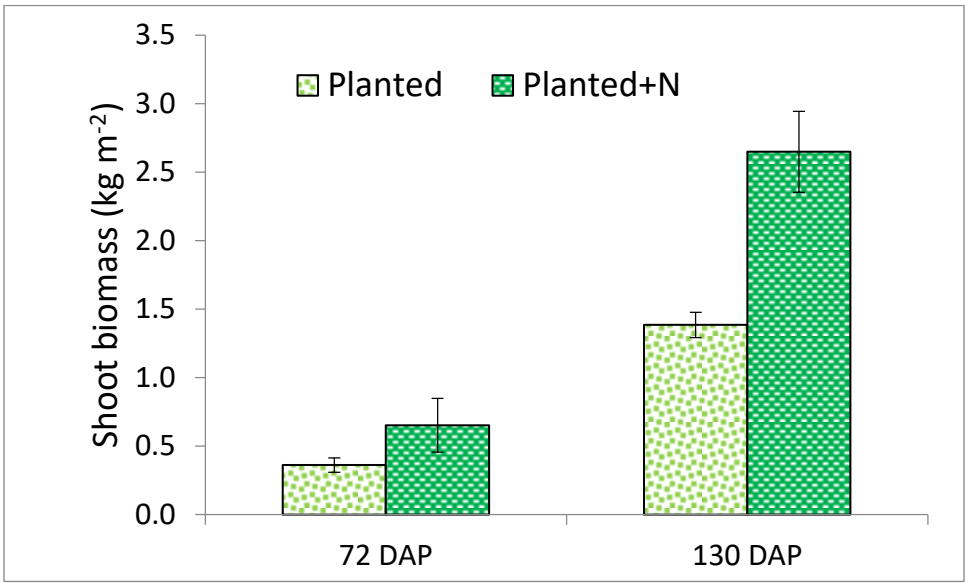
Supplementary Table II.2:1: Total plant biomass (g m^{-2}) and microbial biomass N (mg N kg^{-1} aggregate) in bare fallow soil and soils with Low, Normal and High maize plant densities in distinct aggregate size classes. Upper-case letters indicate significant differences (Post-hoc LSD test, P<0.05) in plant biomass and in microbial biomass N between bare fallow and Low, Normal and High maize plant densities. Lower-case letters indicate significant differences (Post-hoc LSD test, P<0.05) in microbial biomass N between distinct aggregate size classes.

	Plant biomass (g m^{-2})		Microbial biomass N (mg N kg^{-1} aggregate)		
	Shoot	Root	>2000 µm	2000-250 µm	<250 µm
Bare fallow	-	-	23.4±2.4Aab	25.5±2.7Ab	17.9±1.3Aa
Low density	361.7±52.5A	41.2±6.0A	27.3±2.6Ab	22.8±0.9Aab	20.9±1.1ABa
Normal density	568.1±46.0B	64.7±5.2B	24.8±3.5Aa	25.1±1.8Aa	23.9±2.2Ba
High density	704.7±53.8B	80.2±6.1B	25.2±4.2Aa	22.0±2.7Aa	22.6±2.0ABa

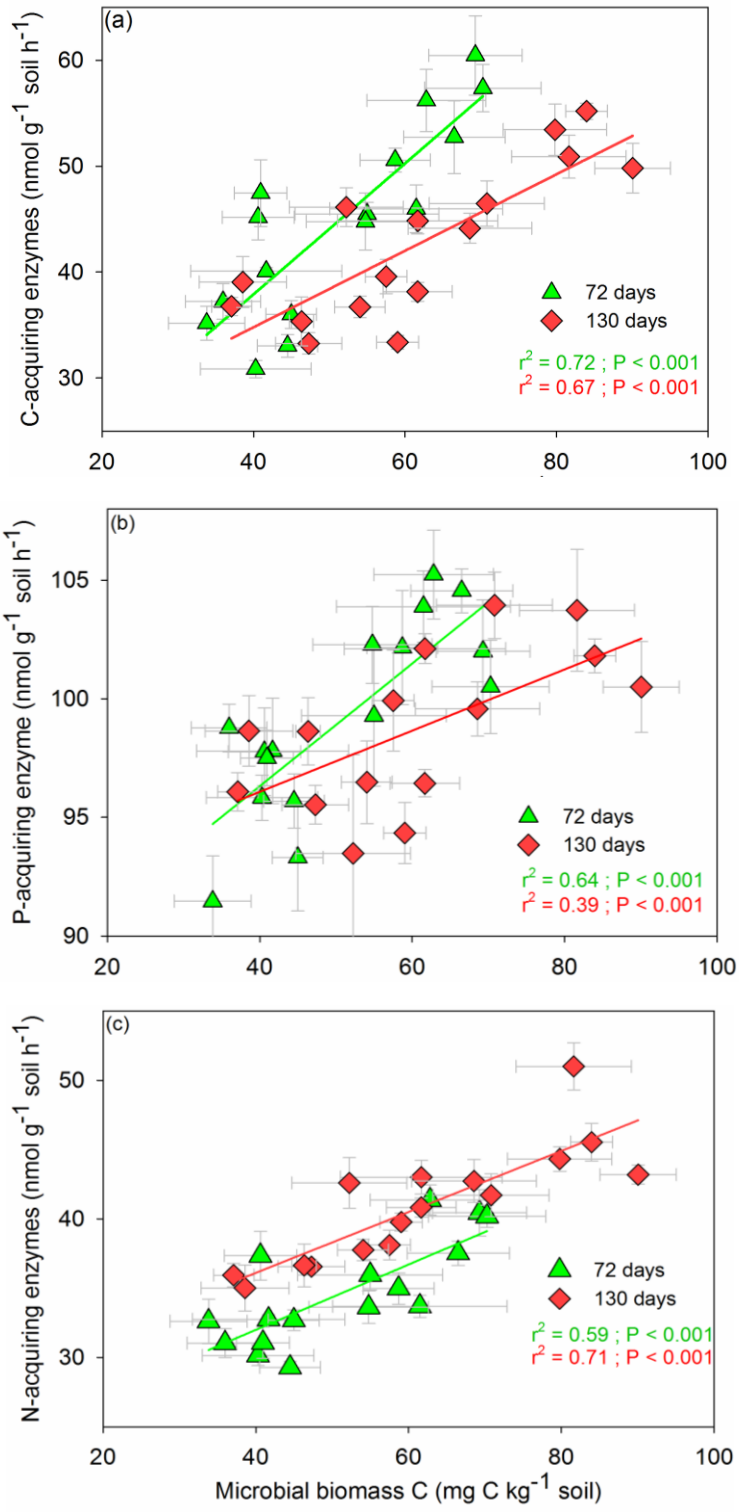
Supplementary Table II.2:2: Ratio of microbial biomass C (mg C kg^{-1} aggregate) to microbial biomass N (mg N kg^{-1} aggregate) (C_{mic}/N_{mic}) in bare fallow soil and soils with Low, Normal and High maize plant densities in distinct aggregate size classes. Upper-case letters indicate significant differences (Post-hoc LSD test, P<0.05) in C_{mic}/N_{mic} between bare fallow and Low, Normal and High maize plant densities. Lower-case letters indicate significant differences (Post-hoc LSD test, P<0.05) in C_{mic}/N_{mic} between distinct aggregate size classes.

	C_{mic} / N_{mic}		
	>2000 µm	2000-250 µm	<250 µm
Bare fallow	4.56±0.62Aa	4.32±0.46ABa	5.10±0.66Aa
Low density	5.11±0.32Aa	4.73±0.11Aba	3.82±0.31ABb
Normal density	5.12±0.48Aa	4.89±0.38Aa	4.16±0.59ABa
High density	4.57±1.17Aa	3.59±0.44Ba	3.42±0.49Ba

II.3. Maize phenology alters the distribution of enzyme activities in soil: field estimates



Supplementary Figure II.3:1: Maize shoot biomass (kg m^{-2}) at silking (72 days) and maturity (130 days) stages.



Supplementary Figure II.3:2: Correlation between microbial biomass C (mg C kg^{-1} soil) and potential activities (nmol g^{-1} soil h^{-1}) of (a) C-acquiring enzymes; (b) P-acquiring enzyme; and (c) N-acquiring enzymes at silking (72 days) and maturity (130 days) stages of maize.

Supplementary Table II.3:1: Soil moisture (%), CBH (Cellobiohydrolase; nmol MUB cleaved g^{-1} soil h^{-1}), NAG (β-1,4-glucosaminidase; nmol MUB cleaved g^{-1} soil h^{-1}), XYL (β-xylosidase; nmol MUB cleaved g^{-1} soil h^{-1}), and PHO (phosphomonoesterase; nmol MUB cleaved g^{-1} soil h^{-1}) at four soil depth intervals (0-5 cm, 5-15 cm, 15-25 cm, and 25-35 cm) at two sampling times (silking and maturity stages) under bare fallow and planted soil in presence or absence of N-fertilization.

Treatment variables	Soil depth (cm)	Soil moisture (%)		CBH		NAG		XYL		PHO	
		72 DAP	130 DAP	72 DAP	130 DAP	72 DAP	130 DAP	72 DAP	130 DAP	72 DAP	130 DAP
Bare fallow	0-5	8.4±0.4	12.6±0.5	6.7±0.3	6.8±0.4	8±0.4	7.8±0.3	6.5±0.4	6.8±0.3	91.5±1.9	94.3±1.3
	5-15	15.1±0.5	16.2±0.3	9±0.2	8.1±0.3	10.3±0.5	11±1.5	8±0.4	7±0.2	102.2±2.4	102.1±0.6
	15-25	16.9±0.5	18±0.7	8.6±0.6	7.6±0.8	10.1±0.3	11.6±1.5	7.2±0.5	7.3±1.3	99.3±3	93.5±4.2
	25-35	16.7±0.7	16.5±0.4	7.8±0.7	7.2±0.6	10.4±0.7	9.3±0.7	6.6±0.4	6.3±0.1	97.8±2.2	96.1±0.8
Bare fallow+N	0-5	9.5±1	13±0.4	6.7±0.5	7.6±0.5	7.3±0.3	7.4±0.1	6±0.3	6.3±0.1	93.3±2.2	96.4±0.6
	5-15	16.2±0.5	15.9±0.1	7.7±0.3	8.2±0.6	9.5±0.7	9.9±0.7	7.6±0.3	7±0.2	102±2.2	103.7±2.6
	15-25	17.1±0.4	18.2±0.3	9.3±0.7	7.9±0.4	9.9±0.8	10.9±0.6	7.5±0.1	6.8±0.4	100.5±2	92.9±3.3
	25-35	15.8±0.6	16.9±0.4	7.1±0.4	7.3±0.2	8.1±0.4	8.3±0.5	6.6±0.1	6.4±0.1	97.8±1.9	99.9±2.1
Planted	0-5	7.5±0.6	14.6±0.4	7.8±0.6	7.1±0.3	7.5±0.4	8.2±0.2	7.2±0.3	6.8±0.2	96.3±1.5	93.6±1.4
	5-15	11.9±1.4	16±0.2	8.8±0.5	8.6±0.3	13.5±0.8	15±2.2	8.7±0.4	8.2±0.8	106.1±1.6	105.8±4
	15-25	14.4±0.2	16.5±0.1	10±0.4	9±0.2	12.6±0.8	10.8±0.9	9.3±0.6	7.1±0.2	107.6±2.6	97.8±0.4
	25-35	13.7±0.5	17.2±0.6	9±0.8	7.4±0.5	13.1±2.4	9.7±1	7.6±0.4	6.8±0.2	99.5±2.9	99.9±1.2
Planted+N	0-5	7.3±0.4	14.5±0.4	6.2±0.2	6.9±0.3	7.5±0.6	8.6±0.4	6.1±0.1	6.5±0.3	95.1±1.6	93.5±2.8
	5-15	13.2±0.2	16.2±0.3	11.3±0.5	8.9±0.4	10.9±1.3	10.4±0.3	7.5±0.4	7.2±0.1	105.4±1.6	103.4±0.3
	15-25	13.9±0.9	16.7±0.3	10±0.4	8.1±0.3	12.6±0.8	11.1±0.6	9.3±0.6	7.3±0.4	107.6±2.6	98.2±3.3
	25-35	13.6±0.6	16±0.3	7.2±0.7	6.3±0.3	9±0.9	8.8±1.3	7.1±0.5	6.3±0.2	97.8±2.3	97.7±2.3

Supplementary Table II.3:2: Factorial ANOVA showing effect of soil depth, planting, and N fertilization on soil moisture (%), microbial biomass C (Cmic; mg C kg-1 soil), BG (β-1,4-glucosidase; nmol MUB cleaved g^{-1} soil h^{-1}) CBH (Cellobiohydrolase; nmol MUB cleaved g^{-1} soil h^{-1}), NAG (β-1,4-glucosaminidase; nmol MUB cleaved g^{-1} soil h^{-1}), XYL (β-xylosidase; nmol MUB cleaved g^{-1} soil h^{-1}), LAP (Leucine-aminopeptidase; nmol AMC cleaved g^{-1} soil h^{-1}) and PHO (phosphomonoesterase; nmol MUB cleaved g^{-1} soil h^{-1}) at four soil depth intervals (0-5 cm, 5-15 cm, 15-25 cm, and 25-35 cm) at two sampling times (silking and maturity stages) under bare fallow and planted soil in presence or absence of N-fertilization. Provided are the P-values and bold numbers indicate significant effect (P<0.05).

Variables	Soil moisture	C_{mic}	BG	CBH	XYL	NAG	LAP	PHO
72 DAP								
Treatment	**0.000**	0.135	**0.000**	**0.001**	**0.000**	**0.001**	**0.000**	**0.001**
Depth	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
Fertlization	0.465	0.478	0.211	0.291	**0.028**	**0.004**	**0.001**	0.937
Depth*Fertilization	0.286	0.737	0.286	**0.036**	0.243	0.067	0.702	0.965
Depth*Treatment	0.384	0.856	0.051	0.236	**0.015**	0.143	**0.013**	0.192
Fertilization*Treatment	0.708	0.618	0.874	0.845	0.185	0.430	0.059	0.469
Fertilization*Depth*Treatment	0.718	0.835	0.649	**0.002**	0.968	0.620	0.129	0.982
130 DAP								
Treatment	0.800	**0.000**	**0.000**	0.418	0.182	0.098	0.052	0.247
Depth	**0.000**	**0.000**	**0.000**	**0.000**	**0.019**	**0.000**	**0.000**	**0.000**
Fertlization	0.863	0.153	0.991	0.800	0.183	**0.047**	**0.013**	0.768
Depth*Fertilization	0.621	**0.018**	0.137	0.571	0.939	0.164	0.359	0.970
Depth*Treatment	**0.000**	**0.027**	**0.020**	0.151	0.770	0.312	0.226	0.257
Fertilization*Treatment	0.333	0.883	0.200	0.057	0.661	0.701	0.086	0.230
Fertilization*Depth*Treatment	0.238	0.418	0.088	0.661	0.468	0.319	0.913	0.743

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Bayreuth,19.04.2018, Amit Kumar