Mycorrhization and Warming Modulate Soil Organic Matter Stability

Dissertation

to obtain the Dr. rer. nat. degree

of the Faculty of Agricultural Sciences

Georg-August-University Göttingen

Submitted by

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Göttingen, January 2021

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Date of oral examination: 26, March, 2021

Summary

Soil organic matter (SOM) is the primary source of plant-available nutrients, as well as a substantial carbon (C) reservoir in terrestrial ecosystems. Maintenance of SOM levels is therefore critical for ecosystem sustainability. SOM stocks mainly depend on the balance between uptake (by photosynthesis) and release (via SOM decomposition). Mycorrhizal colonization increases net photosynthesis within the host plant for 4-30%, thus stimulates microbial activity and accelerate or retard SOM decomposition, consequently leading to SOM destabilization and losses. Furthermore, this SOM destabilization is also depends strongly on temperature, which are predicted to rise by 1.0 to 4.8 °C at the end of twenty-first century, with high risk to activate microorganisms and accelerate their turnover, thus promoting terrestrial C cycle. Therefore, this thesis aims to evaluate the effects of mycorrhization and warming on SOM stability and its underlying microbial mechanisms.

In Study 1, we used continuous $^{13}CO_2$ labeling to quantify the C allocation and rhizosphere priming effect (RPE) of a mycorrhizal wild type progenitor and its mycorrhiza defective mutant (reduced mycorrhizal colonization) of tomato. Arbuscular mycorrhizal fungi (AMF) increased the net rhizodeposition by 25-72% in soils, and lowered the RPE on SOM decomposition by 24-38%. This indicated a higher potential for C sequestration by MYC plants because the reduced nutrient availability restricted the activity of free-living decomposers. The RPE and N-cycling enzyme activities decreased by N fertilization 8 and 12 weeks after transplanting, suggesting a lower microbial N demand from SOM mining.

Based on the ^{13}C profile of microbial phospholipid fatty acids (PLFAs) (Study 2), the ^{13}C incorporation into fungal biomarker (PLFA and NLFA 16:1ω5c) increased with sampling time, indicated that AMF was prominent in the plant-soil system. The preferential C allocation to AMF was at the expense of C flow to other microbial groups, thus resulting in a lower ^{13}C incorporation into bacteria and saprotrophic fungi. Even more, high N availability negatively impacted on AMF growth and further rhizodeposited C recovered in the AMF, which resulted from higher C immobilization in the aboveground and higher rhizosphere respiration. Overall, AMF facilitates soil C sequestration by retaining more plant rhizodeposits in soils and by reducing the RPE on SOM decomposition, which is mainly dependent on the N availability.

Together with AMF, ectomycorrhizal fungi (ECM) are the two most widespread mycorrhizal types on Earth. Therefore, ^{14}C imaging coupled with zymography was used to investigate the spatial distribution of rhizodeposite C and enzyme activities in response to ECM and another major soil fungal guilds in temperature forests-non-mycorrhizal rhizosphere fungi (NMRF) in Study 3. Plants inoculated with ECM and NMRF allocated more C to soils compared to uninoculated control. When NMRF is co-existence with ECM (MIX), ECM competed with NMRF and thus the growth of ECM was suppressed, as a consequence less assimilated C was allocated to the

rhizohyphosphere for MIX compared to ECM. Furthermore, we observed 57% higher chitinase and 49% higher leucine-aminopeptidase in the rhizohyphosphere with ECM compared with the control, whereas NMRF showed a higher production of β-glucosidase. Therefore, *Picea abies* colonized with ECM and NMRF both induced an increased root exudation and promote enhanced enzyme activities, but ECM focused on nutrient mobilization, whereas NMRF presence stimulates enzymes of the C cycle.

Besides rhizodeposits via mycorrhizal pathway, temperature is a another crucial factor enhancing soil microbial activity, and thus threatening SOM stability. Based on microbial and enzymatic functional traits under 8-year long-term warming agricultural field (Study 4), soil organic C (SOC) and total nitrogen (TN) remained stable at warming below 2 °C, while higher warming (by 2-4 °C) did not affect SOC but it increased TN content. Possible explanation of increased TN was linked to unbalanced process of necromass formation and enzymatic decomposition. 2-4 °C warming induced faster microbial growth and turnover, whereas it reduced catalytic efficiency and slowed down the enzyme-mediated turnover of oligosaccharides and polypeptides. Lower enzymatic efficiency and slower turnover of organic residues under 2-4 °C warming thus may cause accumulation of N-related necromass. Consequently, the responses of microbial functional traits to climate warming were dependent on warming magnitudes, above 2 °C warming would exceed a threshold that changes the predicted temperature effect on soil C and N pools in the future, which might in its feedback reaction rather a further future CO_2 source feeding into the atmosphere globally

Furthermore, the response of soil C cycle to climate warming is also dependent on the ecosystem types. Given that montane grasslands are expected to be exposed to intensive warming, the response of microbial functions may be different from agroecosystems. Therefore, intact plant-soil mesocosms were translocated downslope spanning a temperature increase of 7 °C (from 13, 15, 17 to 20 °C) in the European Alps (Study 5). Microbial community in lower elevation shifted toward to slow-growing *K*-strategists due to the decreased availability of C substrates. Further, the increase of C-degrading enzymes, accompanied by the decrease of substrate turnover time of β-glucosidase, implied a stronger microbial C turnover because of the C limitation in the lower versus higher elevation soils. This, in turn, presumably leads to potential C losses under climate warming due to the significantly increased C and nutrients cycling of montane grassland soils.

Since enzymes are closely linked to SOM decomposition, knowledge on temperature sensitivity (Q_{10}) of enzyme activities is required to predict the future soil C release to the atmosphere. Soil samples from eight-years warming field sites (ambient, +1.6 °C, +3.2 °C) were incubated at a short-term constant temperature (from 5 to 25 °C with 5 °C intervals) under microbial steady-state and activated mode (Study 6). We found a legacy effect of eight-year field warming which facilitated the consumption of labile organics due to faster microbial growth and turnover. Thus, it caused a lower Q_{10} of enzyme activities in warmed soils. Additional labile C inputs caused a higher Q_{10}-V_{max} in warmed versus ambient soil, which demonstrated a reduced microbial

memory effect due to thermophilic nature of activated microorganisms. Consequently, the microbial memory is strongly dependent on microbial physiological state, which can be quickly altered by substrate supply.

Overall, this thesis suggests that temperature effect on soil C and N pools are mainly dependent on warming magnitudes and ecosystem types. Specifically, if future climate warming beyond the aim of Paris Climate Agreement (> 2 ℃), it would accelerates SOM mineralization and threaten SOM stability. For example, is may induce a severe alteration of N cycle with several potential negative feedbacks-from unhealthy net primary productivity increase in natural ecosystems via groundwater NO_3- accumulation up to increased N_2O emission with feedback on related climate change. However, mycorrhizae is ubiquitous in terrestrial ecosystems because it can stimulate belowground C inputs and inhibit the growth of saprotrophic fungi, consequently facilitate soil C sequestration. Therefore, mycorrhization may act as a positive mitigation strategy buffer against the predicted increases in SOM decomposition in the future warmer world.

Zusammenfassung

Organische Bodensubstanz (SOM) ist die Hauptquelle für pflanzenverfügbare Nährstoffe sowie ein bedeutendes Kohlenstoff (C)-Reservoir in terrestrischen Ökosystemen. Die Aufrechterhaltung der SOM-Werte ist daher für die Nachhaltigkeit des Ökosystems von entscheidender Bedeutung. SOM-Bestände hängen hauptsächlich vom Gleichgewicht zwischen Aufnahme (durch Photosynthese) und Freisetzung (über SOM-Zersetzung) ab. Die Mykorrhizenkolonisation erhöht die Nettophotosynthese innerhalb der Wirtspflanze um 4 bis 30%, stimuliert somit die mikrobielle Aktivität und beschleunigt oder verzögert die SOM-Zersetzung, was zu SOM-Destabilisierung und -Verlusten führt. Darüber hinaus hängt diese SOM-Destabilisierung auch stark von der Temperatur ab, die voraussichtlich Ende des 21. Jahrhunderts um 1,0 bis 4,8 ° C ansteigen wird, wobei ein hohes Risiko besteht, auch Mikroorganismen zu aktivieren und ihren Umsatz zu beschleunigen, wodurch der terrestrische C-Zyklus gefördert wird. In dieser Arbeit sollen daher die Auswirkungen von Mykorrhisierung und Erwärmung auf die SOM-Stabilität und die zugrunde liegenden mikrobiellen Mechanismen untersucht werden.

In Studie 1, verwendeten wir eine kontinuierliche $^{13}CO_2$-Markierung, um die C-Allokation und den Rhizosphären-Priming-Effekt eines Mykorrhiza Wildtyp Vorläufers und seiner Mykorrhiza defekten Mutante von Tomaten zu quantifizieren. AMF erhöhte die Netto-Rhizodeposition in Böden um 25 bis 72% und senkte die RPE bei SOM-Zersetzung um 24 bis 38%. Dies deutete auf ein höheres Potenzial für die C-Sequestrierung durch mit AMF besiedelte Pflanzen hin, da die verringerte Nährstoffverfügbarkeit die Aktivität frei lebender Zersetzer einschränkt. Die RPE- und N-zyklischen Enzymaktivitäten nahmen 8 und 12 Wochen nach der Transplantation durch N-Befruchtung ab, was auf einen geringeren mikrobiellen N-Bedarf aus dem SOM-Abbau hinweist.

Basierend auf dem ^{13}C-Profil von mikrobiellen Phospholipidfettsäuren (PLFAs) (Studie 2), der ^{13}C-Einbau in Pilzbiomarker (PLFA und NLFA 16: 1ω5c) mit der Probenahmezeit zu, was darauf hinweist, dass AMF im Pflanzenboden hervorragend ist System Rollenspiel. Die bevorzugte C-Zuordnung zu AMF ging zu Lasten des C-Flusses zu anderen mikrobiellen Gruppen, was zu einem geringeren 13C-Einbau in Bakterien und saprotrophe Pilze führte. Darüber hinaus wirkte sich eine hohe N-Verfügbarkeit negativ auf AMF aus, und das rhizodepositivierte C im AMF erholte sich weiter, was auf eine geringere C-Zuordnung zum Untergrund aufgrund einer höheren C-Immobilisierung im oberirdischen Bereich und einer höheren Rhizosphärenatmung zurückzuführen war. Insgesamt erleichtert AMF die Sequestrierung von Boden C, indem mehr Pflanzen-Rhizodeposits (Netto-Rhizodeposition) in Böden erhalten bleiben und die RPE während der SOM-Zersetzung verringert wird, was hauptsächlich von der Verfügbarkeit von N abhängt.

Ektomykorrhizapilze (ECM) sind zusammen mit AMF die weltweit am weitesten

verbreiteten mykohhizalen Pilze. Daher wurde die ^{14}C-Bildgebung in Verbindung mit der Zymographie verwendet, um die räumliche Verteilung der Rhizodeposit C- und Enzymaktivitäten als Reaktion auf ECM und andere wichtige Bodenpilzgilden in Temperaturwäldern - nicht mykorrhizale Rhizosphärenpilze (NMRF) - in Studie 3 zu untersuchen. Mit ECM und NMRF inokulierte Pflanzen wiesen den Böden im Vergleich zur nicht inokulierten Kontrolle mehr C zu. Wenn NMRF neben ECM (MIX) existiert, konkurrierte ECM mit NMRF und somit wurde das Wachstum von ECM unterdrückt, was dazu führte, dass der Rhizohyphosphäre für MIX im Vergleich zur ECM-Behandlung weniger assimiliertes C zugeordnet wurde. Darüber hinaus beobachteten wir 57% höhere chitinase und 49% höhere leucine-aminopeptidase in der Rhizo-Hyphosphäre mit ECM im Vergleich zur Kontrolle, während NMRF eine höhere Produktion von β-glucosidase zeigte. Daher induzieren mit ECM und NMRF kolonisierte *Picea-Abies* beide eine erhöhte Wurzelexsudation und fördern verstärkte Enzymaktivitäten, aber ECM konzentrierte sich auf die Nährstoffmobilisierung, während die Anwesenheit von NMRF Enzyme des C-Zyklus stimuliert.

Neben Rhizodeposits über den Mykorrhisierungsweg ist die Temperatur ein weiterer entscheidender Faktor, der die mikrobielle Aktivität des Bodens erhöht und damit die SOM-Stabilität gefährdet. Basierend auf mikrobiellen und enzymatischen Funktionsmerkmalen unter 8-jähriger langfristiger Erwärmung des landwirtschaftlichen Feldes und kombinierter erweiterter Literaturrecherche (Studie 3), Das organische C (SOC) des Bodens und der Gesamtstickstoff (TN) blieben bei Erwärmung unter 2 °C stabil, während eine höhere Erwärmung (um 2-4 °C) den SOC nicht beeinflusste, aber den TN-Gehalt erhöhte. Eine mögliche Erklärung für eine erhöhte TN war mit der gegensätzlichen Reaktion der kinetischen Parameter der funktionellen Merkmale des enzymatischen und mikrobiellen Wachstums verbunden. Eine höhere Erwärmungsgröße induzierte ein schnelleres Wachstum und einen schnelleren Umsatz von Mikroben, während die katalytische Effizienz verringert und der durch Enzyme vermittelte Umsatz von Oligosacchariden und Polypeptid-ähnlichen Verbindungen verlangsamt wurde. Dies führte zu einer langsameren Zersetzung organischer Rückstände und zu Ansammlungen von mikrobieller Nekormasse. Daher stieg der N-Gehalt in gelösten organischen und SOM-Pools unter einer höheren Erwärmungsgröße an, was zu einem verringerten stöchiometrischen Ungleichgewicht zwischen der mikrobiellen Biomasse und ihrer Verfügbarkeit labiler Ressourcen führte, was zu einer SOM-Zersetzung führte. Folglich waren die Reaktionen mikrobieller Funktionsmerkmale auf die Klimaerwärmung von den Erwärmungsgrößen abhängig. Sie sollten in die Modelle aufgenommen werden, um die Vorhersage der Rückkopplungen von Boden C und N auf die Klimaerwärmung zu verbessern.

Darüber hinaus hängt die Reaktion der Boden-C-Funktionen auch von den Ökosystemtypen ab. Angesichts der Tatsache, dass montanes Grasland einer intensiven Erwärmung ausgesetzt sein dürfte, können biogeochemische Zyklen des Bodens anfällig für zukünftige klimatische Bedingungen sein und die Reaktion mikrobieller Funktionen kann sich von denen von Agrarökosystemen unterscheiden. Daher wurden intakte Pflanzen-Boden-Mesokosmen drei Jahre lang entlang eines

Höhengradienten übertragen (Studie 5). Die mikrobielle Gemeinschaft in geringerer Höhe verlagerte sich aufgrund der verringerten Verfügbarkeit von C-Substrat zu langsam wachsenden K-Strategen. Ferner implizierte die Zunahme von C-abbauenden Enzymen, begleitet von der Abnahme der Substratumsatzzeit von β-glucosidase, einen stärkeren mikrobiellen C-Umsatz aufgrund der C-Begrenzung in den Böden mit niedrigerer oder höherer Höhe.

Da Enzyme für die SOM-Zersetzung verantwortlich sind, sind Kenntnisse über enzymatische Mechanismen der Temperaturanpassung erforderlich, um die Auswirkungen der Erwärmung auf den Boden-C-Zyklus vorherzusagen. So wurden Bodenproben von acht Jahre wärmenden Feldstandorten (Umgebungstemperatur, +1,6 °C, +3,2 °C) bei einer kurzfristigen Inkubation bei konstanter Temperatur (von 5 bis 25 °C in Intervallen von 5 °C) unter mikrobiellem Steady inkubiert -Zustand und aktivierter Modus (Studie 6) zur Messung der Temperaturempfindlichkeit (Q_{10}) von hydrolytischen Enzymaktivitäten. Wir fanden einen Legacy-Effekt der achtjährigen Felderwärmung, der den Verbrauch labiler organischer Stoffe aufgrund des schnelleren mikrobiellen Wachstums und Umsatzes erleichterte und somit zu einer langfristigen Erwärmung des Bodengedächtnisses führte, was zur Folge hatte, dass Q_{10} der Enzymaktivitäten in erwärmten Böden geringer war. Labile C-Einträge verursachten einen höheren Q_{10}-V_{max} in erwärmtem Boden im Vergleich zu Umgebungsboden und zeigten aufgrund der thermophilen Natur aktivierter Mikroorganismen einen verringerten mikrobiellen Gedächtniseffekt. Folglich ist das mikrobielle Gedächtnis stark vom mikrobiellen physiologischen Zustand abhängig, der durch die Substratversorgung schnell verändert werden kann.

Deutet diese These darauf hin, dass die Klimaerwärmung, die über das Ziel des Pariser Klimaabkommens (> 2 °C) hinausgeht, das mikrobielle Wachstum und die Produktion von Enzymen stimuliert, wodurch die SOM-Mineralisierung beschleunigt und der SOC verringert wird. Mykorrhizen sind jedoch in terrestrischen Ökosystemen allgegenwärtig, da sie unterirdische C-Einträge stimulieren und das Wachstum von saprotrophen Pilzen hemmen können, wodurch die Sequestrierung von Boden C erleichtert wird. Folglich könnte die Mykorrhizierung als positiver Puffer für die Abschwächungsstrategie gegen die vorhergesagten Erhöhungen der SOM-Zersetzung in der zukünftigen erwärmten Welt wirken.

Acknowledgements

Time files and it has been four years since I came to Göttingen and started my PhD work in the Biogeochemistry of Agroecosystems, University of Göttingen. The present work would not have been possible without the support of many people to whom I am very grateful. Thanks to China scholarship council for support me to study in here.

I would like to acknowledge everyone I met here. First of all, I would like to express sincere thanks to my supervisors Prof. Dr. Johanna Pausch, Prof. Dr. Michaela A. Dippold and Dr. Matthias Gube for the patient and professional guidance during my PhD study. I appreciate they gave me a large extent of flexibility and independence on my PhD work, and I will keep going. I am thanks Prof. Dr. Johanna Pausch for the possibility to carry out this thesis under her supervision. Despite some geographical distances from Bayreuth and Göttingen during my PhD, she was always helping me and keep weekly scientific discussions.I thank her from bottom of my heart. I also would like to thank Prof. Dr. Michaela Dippold for her strong and great support both in science and life in Göttingen. I would like to say I have a very happy time in the 'Biogeochemistry of Agroecosystems' in Göttingen. I am also grateful that I have a chance to do the mycorrhizal experiment with Dr. Matthias Gube. Many thanks for your comments to the manuscript and interesting knowledge about mushrooms. I also thank Prof. Dr. Klaus Dittert for willing to spare precious time to attend my defense and give suggestions on my work.

My sincere gratitude goes to Prof. Dr. Yakov Kuzyakov who welcomed me in Göttingen in 2016 for pursuing my doctoral studies. I also sincerely thank PD Dr. Evgenia Blagodatskaya for her willingness to discussion, great and creative suggestions on the manuscripts.

My gratitude goes out to all the technical staff, in particular Karin Schmidt, for their help in sample preparation and analysis. I am also grateful to the laboratory manager Dr. Callum C. Banfield. When I had questions about isotopic experiments and had problems during running experiments, I can always get a resolution from your side. Without your help and support to my lab work, I could not finish my experiments successfully.

Prof. Dr. Huadong Zang, you act like my other 'supervisor'. Every time I ask you questions, you can reply me in time and are always patient and explain clearly. A special thanks to Dr. Lingling Shi for her brilliant suggestions and full help on my PhD work.

Sincere thanks to my colleagues of the Department of Soil Science of Temperate Ecosystems, Agricultural Soil Science, Biogeochemistry of Agroecosystems. Specifically, I want to thank my Chinese friends for their inspiration and company during my time in Göttingen. Special thanks for my roommate (Guodong Shao), thanks for his patience and help in life. I also thanks for my neighbors (Xuechen Zhang and Xiaona Song), thanks for your sudden coming to have lunch without any

plans. Although sometimes I were unwilling to make food, I thought this interesting and fun ‘food time’ with you and Guodong Shao make my PhD some colors. I would like to say this is the fate that let us meet in Göttingen, and it wouldn’t have been the same without you.

In the end, I also would like to thank my family for their continuously support, kindness, and motivation in the last year.

Table of Contents

List of Figures

Extended Summary:

List of Figures

Study 1:

Study 2:

List of Figures

Study 3:

List of Figures

Study 4:

Study 5:

Study 6:

List of Tables

Abbreviations

C	Carbon
N	Nitrogen
SOM	Soil organic matter
SOC	Soil organic carbon
RPE	Rhizosphere priming effect
AMF	Arbuscular mycorrhizal fungi
ECM	Ectomycorrhizal fungi
NMRF	Non-mycorrhizal rhizosphere fungi
SAP	Saprotrophic fungi
^{13}C	Stable carbon isotope with atomic mass 13
^{14}C	Radioactive carbon isotope with atomic mass 14
CO_2	Carbon dioxide
SIGR	Substrate-induced growth respiration
μ	Specific growth rate
TMB	Total microbial biomass
GMB	Growing microbial biomass
PLFA	Phosphor lipid fatty acid
NLFA	Neutral lipid fatty acid
Q_{10}	Temperature sensitivity
LSC	Liquid scintillation counter
IRMS	Isotope ratio mass spectrometry
ANOVA	Analysis of variance

1. Extended summary

1.1 Introduction

Globally, soils store 1500 Pg carbon (C), twice as much as the atmosphere and biosphere combined together (Todd-Brown et al., 2013; Wieder et al., 2013). The global soil organic carbon (SOC) storage depend mainly on CO_2 uptake (photosynthesis) and release (microbial soil organic matter (SOM) decomposition) (Song et al., 2018). Even small changes in the soil C pool (e.g., plant-derived C input, SOM decomposition) will affect the maintenance of soil fertility, the stability of ecosystem, and the regulation of greenhouse gas emission (Swift et al., 2004). Mycorrhization and warming are important biotic and abiotic factors affecting SOM stability (Bardgett et al., 2008; Schmidt et al., 2011; Frey, 2019; Zhou et al., 2020a).

1.1.1 Mycorrhization and soil organic matter stability

Mycorrhiza is one of the most common inter-species interactions on Earth, involving 90% of plant species (Smith and Read, 2008). There is increasing evidence that a significant part of rhizodeposition (4-30% of the net photosynthesis) enters the soil through the mycorrhizal network, contributing to microbial biomass build-up and subsequent soil C storage (Finlay and Rosling, 2006; Finlay, 2008; Zhou et al., 2020a). However, this additional C supply by mycorrhizaes also provides energy for rhizosphere microbial communities, thereby shaping their structures and functions and thus driving distinct microbial nutrient cycling (Eisenhauer et al., 2010; Pausch and Kuzyakov, 2018). Therefore, mycorrhizal fungi may serve as a useful predictor of C cycling processes in ecosystems around the world (Frey, 2019).

Arbuscular mycorrhizal fungi (AMF) can form symbiotic associations with about 71% of all flowering plants, including many important crops such as wheat and barley (Brundrett and Tedersoo, 2019). AMF are obligate symbionts and, there is no evidence that they have the saprotrophic capacity to degrade SOM (Talbot et al. 2008; Bödeker et al. 2014). However, AMF can promote free-living microorganisms by releasing labile substrates via exudation and hyphal turnover, and providing energy for enzyme production and SOM decomposition (Schmidt et al., 2011). Although several studies have reported that AMF can enhance or inhibit litter decomposition (Hodge, 2001; Leifheit et al., 2015), the mechanisms underlying the effects of AMF on SOM decomposition still remain unsolved. What's more, numerous studies have demonstrated that AMF are sensitive to N fertilization (Treseder, 2004; Johnson et al., 2013). This is an important issue because N fertilization is widely used as a common agricultural management strategy to increase crop yield in agroecosystems (Galloway et al., 2008). The extent to which AMF contribute to ecosystem C storage may therefore change, if soil N availability increases (Johnson et al., 2013). However, the mechanisms underlying the effects of AMF on C allocation and SOM decomposition under N fertilization are still an open question. This knowledge gap was addressed in

Study 1, which aimed to investigate the interactive effect of AMF and N fertilization on rhizodeposition and SOM decomposition.

Although recent progress has been made in our understanding of C fluxes from the plant to AMF, and rhizosphere microorganisms, knowledge is still scare with respect to the relative flow of C to specific biological groups in plant-AMF-soil systems (Olsson and Johnson, 2005; Drigo et al., 2010). This knowledge is important for the understanding of terrestrial C dynamics and storage, as groups of soil biota (in particular bacteria, mycorrhizal fungi and saprotrophic fungi) function differently in the incorporation and turnover of C, in the chemical nature of their respective byproducts, and in their respective effects on soil biogeochemical cycles (Six et al., 2006; Strickland and Rousk, 2010). This knowledge gap was addressed in Study 2, which aimed to study the influence of AMF symbiosis and N fertilization on the incorporation and fate of rhizodeposit-C into microorganisms.

Together with AMF, ectomycorrhizal fungi (ECM) are the two most widespread mycorrhizal fungi in the world (Brundrett, 2009). For example, ECM accounts for approximately one-third of the living biomass in forest soils (Högberg and Högberg, 2002). ECM assist plants' uptake of nutrients from soil via the decomposition of the organic N pools and receive C from the host plant in return (Read and Perez-Moreno, 2003; Smith and Read, 2010). It is evidenced that up to 30% of plant's total assimilates is consumed by their ECM partner for the development of the extraradical mycelium and exudations (Hobbie et al., 2013). Therefore, ECM plays a major role in soil C cycles in forests and it requires to further research to quantify the C allocation to belowground via ECM and its' role in the subsequent microbial functions. However, non-mycorrhizal rhizosphere fungi (NMRF), exhibits diverse lifestyles, from plant-beneficial endophytes over saprophytic fungi to parasitic organisms thriving on fungal, plant or animal biomass (Peršoh, 2015), is another major soil fungal guilds in forests (Read and Perez-Moreno, 2003; Kohout et al., 2013). NMRF living in the rhizosphere could compete with ECM for root exudates (Cairney and Meharg, 2002; Esperschütz et al., 2009), thus indirectly affect belowground C allocation when ECM is in co-existence with NMRF. Thus, NMRF abundance is expected to be a major determinant of the ECM-induced microbial functions and deserves much more attention. This knowledge gap was addressed in Study 3, which aimed to disentangle the effects of ECM and NMRF on plant C allocation belowground and enzyme activities, especially in co-existence with ECM and NMRF.

1.1.2 Warming and soil organic matter stability

Elevated temperature is projected to activate microorganisms and accelerate their turnover, thus promoting terrestrial C cycle with potential feedbacks to future climate change (Davidson and Janssens, 2006; Bardgett et al., 2008; Alvarez et al., 2018). Warming-induced acceleration of SOM mineralization is the result of microbial growth on heterogeneous organic substrates and is strongly dependent on the size and functional traits of the active microbial fraction (Schlesinger and Andrews, 2000; Chen et al., 2014). However, the predictions on whether microorganisms will grow faster or slower under climate warming remain controversial. As an immediate

response, warming may accelerates the turnover of the fast-growing microbial population (Blagodatskaya et al., 2010), whilst the microbial growth rate may reduces due to the rapid exhaustion of available organic C under long-term warming (Bradford et al., 2008). As exoenzymes produced and released by microorganisms are the engine of SOM decomposition and labile organic compounds production (Koch et al., 2007; Wallenstein et al., 2009), shifts in enzyme activities lead to changes in SOM decomposition, C storage, and other biological processes (Waldrop et al., 2004). Alterations in soil C and nutrient cycles not only cause a positive feedback on atmospheric CO_2 and exacerbate climate change (Heimann and Reichstein, 2008), but affects nutrient limitation and plant growth (Laganière et al., 2010), especially in agroecosystem which could influence global food productions. Previous experimental studies also reported that the responses of microbial functional traits to climate warming were complex and depended on warming magnitudes, methods, and ecosystem types. Taken together, mountain grassland systems are of great ecologically importance and are particularly sensitive to global warming (Sala et al., 2000). This leads to uncertainty for future soil C cycle in terrestrial ecosystems. Climate warming responses of microbial growth and enzyme kinetics thus was determined under different warming magnitude and methods in agroecosystems and mountain grasslands in Study 4 and 5.

Additionally, soil C dynamics are also discussed from the point of view of their sensitivity to climate warming (Heimann and Reichstein, 2008). And the responses of enzyme kinetics to short- and long-term duration of warming changes may feed with empiric data to better predict the vulnerability of soil C stocks in a future warmer world (Alvarez et al., 2018; Chen et al., 2020). Given that the substrate supply is a key factor regulating microbial growth and enzyme production (Wallenstein et al., 2009; Karhu et al., 2014), ignoring microbial activation by increasing C inputs due to increased net primary productivity under climate warming (Heimann and Reichstein, 2008; Yin et al., 2013), could result in a underestimation of SOM decomposition as well as in the missing feedbacks between the climate and nutrient turnover (Salazar-Villegas et al., 2016). Therefore, we aimed to study the temperature sensitivity of enzymes in response to labile substrate input with future climate warming in Study 6.

1.2 Aims and hypotheses

As mentioned in the introduction, SOM stability is based on the rhizodeposition and microbial activities, which can be affected by mycorrhization (i.e. AMF and ECM). Therefore, this thesis focuses on the following objectives:

(1) To assess the C input via AMF as well as its impact on SOM stabilization and C sequestration depending on N fertilization (Study 1 and 2). We hypothesize that AMF would increase C allocation to the soils due to the direct linkage with their hosts (Study 1). Further, we expect that AMF would be most benefited in the plant-soil interactions, and the stimulated growth of AMF is at the expense of other free-living decomposers, and thus less C is incorporated into saprotrophic microorganisms, consequently decrease RPE on SOM decomposition (Study 1

and 2). However, N fertilization applied in agroecosystems would decrease C allocated to belowground, suppress AMF growth, and thus reduce RPE on SOM decomposition (Study 1 and 2).

(2) To reveal the relative C flow towards soils and the corresponding spatial rearrangement of microbial functions, specifically those involved in nutrient cycling as affected by ECM and NMRF, two major soil fungal guilds in forest soils (Study 3). We hypothesize that s higher amount of photosynthetic C is allocated to the soils inoculated with ECM as compared to inoculation with NMRF, and ECM colonization would increase soil N- and P-related enzyme activities, while NMRF would increase predominantly C-degrading enzyme activities. Furthermore, ECM colonization would extend the spatial distribution of rhizodeposited C and this would induce an increase in the spatial dispersal of nutrient-mobilizing enzymes. When competition between ECM and NMRF, it would reduce plant C allocation to the soils (Study 3).

Besides rhizodeposition via mycorrhization pathway, global warming is a another crucial factor enhancing soil microbial activity and thus threatening SOM stability. Thus, this thesis also focuses on the following objectives:

(3) To estimate the microbial functioning and enzyme kinetics change with climate warming *in situ* (Study 4 and 5). We hypothesize that climate warming would stimulate microbial growth rate, as well as enzyme activities, reduce soil C and N pools, and thereby shrinking soil C sequestration due to plant-derived labile C inputs under warming. We also predict that this positive response of soil C and N pools would depend on the warming magnitudes and ecosystem types (Study 5 and 6).

(4) To investigate the temperature sensitivity (Q_{10}) of enzyme activities after long-term warming, and to see whether Q_{10} would be altered by the labile C input (Study 6). We hypothesize that under long-term warming, faster microbial turnover would reduce labile organic compounds, which restrict the energy available for enzymes production and their Q_{10}, thus, retarding decomposition of SOM. This can be destroyed by the input of available substrate (e.g., as a result of increased rhizodeposition under warming) due to altering microbial physiological state, accelerating enzyme activity and consequently, reducing soil C stocks.

1.3 Materials and methods

1.3.1 Soil sample

Soil collected from the Ap horizon (0-20 cm) of an experimental field at the Reinshof Research Station of the Georg-August University of Göttingen, Germany, was used for Study 1 and 2. Soil collected from northern part of Göttingen, Lower Saxony, Germany, was used for Study 4 and 6. The soil was air-dried and sieved (< 2 mm) to achieve a high degree of homogeneity and reduce the variability among replicates. A sterilized 1:1:1:1 w/w mixture of nutrient-poor soil, fine sand, coarse sand and perlite was used for Study 3.

1.3.2 Experiment set-up

Two tomato genotypes (*Lycopersicon esculentum L.*): a mutant tomato with highly

reduced AMF symbiosis, termed rmc (reduced mycorrhizal colonization), and a closely related wild type hereafter termed MYC (Barker et al., 1998), were were grown in PVC pots with and without N fertilization. N-treatments received 344 mg N per pot, equivalent to a rate of 150 kg N ha^{-1} (Study 1 and 2).

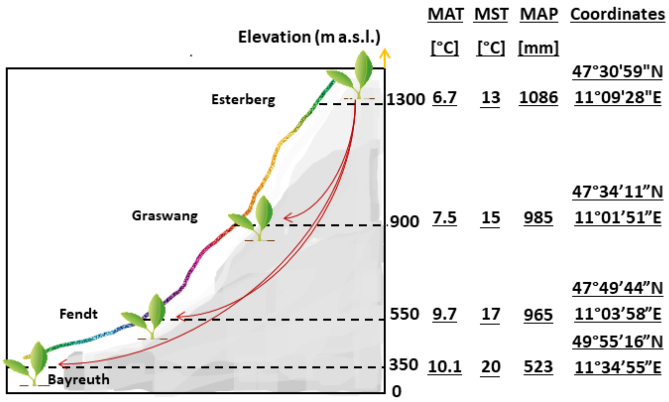
For the Study 3, European spruce (*Picea abies L.*) seedlings were grown in rhizoboxes, which were divided into rhizohyphosphere and hyphosphere by a nylon mesh. Spruce seedlings were inoculated with a mixture of three ECM fungal species (ECM), a mixture of three non-mycorrhizal rhizosphere fungal species (NMRF), and a mixture of all ECM and NMRF fungal species (MIX), respectively, as listed in Table. ES1.

Table. ES1 Fungal types used in the experiment. ECM: ectomycorrhizal fungi, NMRF: non-mycorrhizal rhizosphere fungi.

Fungal type	Taxa		
ECM	*Amanitamuscaria*	*Hebeloma crustuliniforme*	*Cenococcum geophilum*
NMRF	*Trichoderma asperellum*	*Trichoderma viride*	*Cryptococcus terricola*

The Study 4 was conducted on an on-going (since August 2010) long-term warming experiment. The three heating regimes included: (1) ambient soil temperature, (2) ambient +1.6 °C, and (3) ambient +3.2 °C. The experimental site consisted of 12 plots (2 m × 2.5 m each) arranged in two rows. Soil samples were collected from the upper 10 cm of ambient, +1.6 °C and +3.2 °C plots in October 2018 (8-year warming).

The study 5 was conducted conducted on four grassland sites along an elevational gradient ranging from 350 m to 1300 m a.s.l. in the European Alps (Fig. ES1). In the summer 2016, the intact plant-soil community monoliths were extracted from the pre-alpine grassland in Esterberg, and then translocated within site as a control and downslope to each site with a lower elevation than the original. During peak growing season of 2018, soil samples were collected from field and brought back to the laboratory to do further analyse.



	MAT [°C]	MST [°C]	MAP [mm]	Coordinates
Esterberg	6.7	13	1086	47°30'59"N 11°09'28"E
Graswang	7.5	15	985	47°34'11"N 11°01'51"E
Fendt	9.7	17	965	47°49'44"N 11°03'58"E
Bayreuth	10.1	20	523	49°55'16"N 11°34'55"E

Fig. ES1 Geographic and climatic characteristics of the research sites along the elevational gradient in the European Alps. MAT, MST and MAP are mean annual temperature, mean summer temperature and mean annual precipitation, respectively.

The soils sampled from long-term warming field were used to incubate in Study 6. Soil samples were incubated in a Rapid Automated Bacterial Impedance Technique bioanalyser (RABIT; Microbiology International Ltd, Frederick, MD, USA), at 5, 10, 15, 20 and 25 °C, added with water (microbial steady-state) and substrates (a mixture of glucose and nutrition solution, microbial activation).

1.3.3 Analytical methods

1.3.3.1 Isotope approaches

To gain a better understanding of rhizosphere processes, stable and radioactive isotope based labeling techniques were applied to differentiate between plant-derived C and native SOM. For the Study 1 and 2, the tomatoes were exposed to ^{13}C enriched CO_2 in a growth chamber equipped with a continuous $^{13}CO_2$ labeling system, from the emergence of the first leaf till harvest. For the Study 3, the trees were exposed to the radioactive tracer (^{14}C-CO_2) in a plastic chamber for a short period of time (10 h).

Analysis of ^{13}C

In order to determine the $\delta^{13}C$ value, CO_2 trapped in NaOH was precipitated as $SrCO_3$ after the addition of $SrCl_2$ solution, and analyzed with an Elemental Analyzer (EA, Eurovector) coupled to an IRMS (Delta Plus XL IRMS, Thermo Finnigan MAT, Bremen, Germany) (Study 1). Roots, shoots and soil samples were dried at 60 °C for 3 days and, homogenized in a ball mill and analyzed for $\delta^{13}C$ and C content, as well as for N content by EA-IRMS. For the MBC and DOC, the K_2SO_4-extracted solutions were freeze-dried (Beta 1-8 LSCplus, Martin Christ Gefriertrocknungsanlagen GmbH, Harz, Germany) and analyzed using IRMS. The $^{13}C/^{12}C$ isotope ratios of the single fatty acids were determined by an IRMS Delta PlusTM coupled to a gas chromatograph (GC; Trace GC 2000) via a GCII/III-combustion interface (all units from Thermo Fisher, Bremen, Germany).

Analysis of ^{14}C

To determine the ^{14}C activity of plants (roots and shoots) and soils (Studies 3), the samples were combusted in a combustion unit and the released CO_2 was trapped in NaOH. The ^{14}C activity was measured with a LSC and the total C content with a TOC analyzer. The ^{14}C activity of fumigated and non-fumigated extracts was measured with scintillation cocktail and determined with a liquid scintillation counter Tricarb™ B3180 TR/SL (PerkinElmer Inc., Waltham, MA, U.S.A.). The ^{14}C-MBC was estimated as the difference in K_2SO_4-extractable ^{14}C between fumigated and non-fumigated soils without a correction factor (Zang et al., 2020) (Study 3). To determine the spatial distribution of plant-derived C (Study 3), ^{14}C phosphor image was taken per rhizobox for 17 h, and were scanned (FLA 5100 scanner, Fujifilm). The ^{14}C images were converted to ^{14}C activities by a regression describing the relation between pixel-wise photo-stimulated luminescence and known imaging activities of ^{14}C according to Banfield et al. (2017).

1.3.3.2 CO_2 emission, microbial biomass and DOC

Soil CO_2 was trapped from seal pots by circulating the soil air through NaOH solution at 8, 12, and 16 weeks transplanting (Study 1). The total C concentration of the NaOH samples was measured by titration with HCl against phenolphthalein after addition of $BaCl_2$. MBC and DOC was determined by the chloroform-extraction-extraction methods (Study1, 2, 3, 4, 5).

1.3.3.3 AMF abundance

The magnified intersection method described by McGonigle et al. (1990) was used to determine the percentage of root length colonization by AMF (Study 1).The neutral lipid fatty acid (NLFA) 16:1ω5c was measured as a biomarker for extraradical AMF biomass in the soil (Olsson, 1999), following the protocol described by Frostegard et al. (1991).

1.3.3.4 Enzyme activity

Enzyme activities were measured using fluorogenically labeled substrates (Marx et al., 2001; Study 1, 4, 5, 6). Fresh soil suspension (1g soil + 50 ml sterile water) was prepared and 50 µl of this suspension was dispensed into a microplate. Afterwards, 50 µl of buffer (MES for MUF; Trizma for AMC based substrates, respectively) and 100 µl of the corresponding substrates were added, fluorometric measurements (excitation 360 nm; emission 450 nm) were taken. In order to determine the spatial distribution of specific enzymes involved in C, N and P cycling (Study 3), zymography was used to visualize according to the methodology of Zhou et al. (2021). The images obtained from zymography were transferred into a 16-bit gray scale using the open source software ImageJ. Then the calibration line obtained for each enzyme was used to convert gray values of each zymography pixel into enzyme activities.

1.3.3.5 Kinetics of substrate-induced growth respiration

The kinetics of substrate-induced growth response (SIGR) (Study 4, 5, 6) in the soil was analyzed according to Blagodatskaya et al. (2010) and Zhou et al. (2020b). Soil

samples were incubated in a RABIT system, and the measurement of CO_2 release was based on the conductivity changes. Microbial growth respiration in the glucose amended soil was used to model the specific growth rates of microorganisms (μ), the growing microbial biomass (GMB) (Blagodatskaya et al., 2010).

1.3.3.6 Phospholipid fatty acid analysis of microbial communities

Phospholipid (PLFA) lipid fatty acids (Study 2) were extracted and analyzed according to the protocol described by Frostegard et al. (1991). Briefly, 6 g of soil was extracted with a 25 mL one-phase mixture of chloroform, methanol and 0.15 M aqueous citric acid (1:2:0:8, *v/v/v*, pH 4.0) with two extraction steps. Phospholipids were separated from neutral lipids and glycolipids by solid phase extraction using a activated Silica gel (Silica gel Merck, particle size 0.063-0.200 mm). Alkaline saponification of the purified phospholipids was performed with NaOH dissolved in dried MeOH, followed by methylation with BF3 dissolved in methanol, purified by liquid–liquid extraction with hexane. All PLFA samples were analyzed by GC.

1.4 Results and Discussion

1.4.1 Overview of objectives and main results of the studies

Objectives and main conclusions of individual studies are presented in Table ES2.

Table. ES 2 Objectives and main results of the studies.

Study	Objectives	Main results and conclusions
Study 1 Arbuscular mycorrhiza enhances rhizodeposition and reduces the rhizosphere priming effect on the decomposition of soil organic matter	● To assess the effect of AMF symbiosis on C allocation within the plant-soil system depending on N fertilization ● To study the RPE and its underlying microbial mechanisms by AMF symbiosis and N fertilization ● To estimate the soil C balance and RPE induced by AMF symbiosis and N fertilization	✓ AMF symbiosis decreased the relative C allocation to roots, in turn increased the net rhizodeposition. ✓ Net rhizodeposition was higher for MYC than rmc plants 16 weeks after transplanting, the RPE was comparatively lower. This indicated a higher potential for C sequestration by plants colonized with AMF because the reduced nutrient availability restricts the activity of free-living decomposers. ✓ N fertilization lowered the magnitude of RPE, because of decreased enzyme activities that indicated a lowered microbial N demand. ✓ N fertilization decreased the net C rhizodeposition induced by AMF symbiosis, which may partly reflect the more restricted mycorrhizal abundance.
Study 2 Arbuscular mycorrhizae prominents in the rhizosphere carbon uptake and saprotrophic microorganism shift	● To assess the effect of AMF symbiosis and N fertilization on the incorporation and fate of rhziodeposit-C into microorganisms ● To identify predominant microbial	✓ Although less ^{13}C was incorporated into AMF hyphal biomass (PLFA 16:1ω5c, 0.12-0.25%), there was significant allocation of ^{13}C into AMF storage compounds (NLFA 16:1ω5c, 3.09-4.07%) in the soil with AMF symbiosis. This suggested that AMF

	groups utilizing newly rhizodeposit-C	symbiosis play a main role in the utilization of rhizodeposits. ✓ AMF symbiosis shifted the microbial community composition, resulting in a lower ^{13}C incorporation into the bacteria, SAP fungi compared to plants without AM. ✓ High N availability negatively impacted on AMF symbiosis and further rhizodeposited C recovered in the AMF.
Study 3 Ectomycorrhizal and non-mycorrhizal rhizosphere fungi increase root exudation and enzyme activities: a ^{14}C pulse labeling of *Picea abies seedlings*	● To quantify the role of ECM and NMRF and their interactive effects on plant C allocation belowground and spatial distribution of rhizodeposit-C ● To investigate the role of ECM and NMRF and their interactive effects on spatial distribution of microbial enzyme activities	✓ ECM and NMRF allocated more C to soils compared to uninoculated control soil. ✓ ECM competed with NMRF and thus the growth of ECM was suppressed, which was supported by the plant C allocated to the rhizohyphosphere for MIX was 110% higher than for the control, but 64% lower than that for ECM treatment. ✓ A higher chitinase and leucine-aminopeptidase in the rhizohyphosphere with ECM compared with the control and NMRF inoculation was observed, which suggests that enzyme activities are regulated by the supply of photosynthates from the hosts. ✓ NMRF showed a higher production of β-glucosidase, probably because NMRF consumed rhizodeposits efficiently.
Study 4 Restricted power: is microbial pool still able to maintain	● To estimate whether warming exceeding 2 ℃ affects soil C and N pools	✓ A low magnitude of temperature increase (< 2 ℃) only altered microbial traits (i.e. microbial biomass),

the stability of soil organic matter under warming exceeding 2 degrees?	● To quantify the changes of soil microbial and enzymatic process, as well as their consequences for C and N pools depending on the magnitude of warming (< 2 ℃ and > 2 ℃)	but neither enzyme functioning nor soil basic properties. However, a higher warming (2-4 ℃) was sufficient to change almost all soil, microbial pools, and enzyme-related processes in the long-term. ✓ Microorganisms grow and turnover faster under higher warming, and enzyme systems shift towards lower affinity to the substrate, i.e. slower glucose or amino-N production. ✓ The SOC and total nitrogen (TN) remained stable at warming below 2 ℃, while stronger warming (by 2-4 ℃) did not affect SOC but it increased the TN content.
Study 5 Response of microbial growth and enzyme kinetics to climate change in montane grassland	● To estimate the microbial functioning and change with climate warming *in situ* ● To investigate the response of enzymatic kinetic to climate change	✓ Climate warming shifted microbial community towards slow-growing microorganism. ✓ Substrate turnover time of C-degrading enzymes was lower in the soil at lower elevations, implies a stronger and faster C turnover in warmer than in colder soils. ✓ Warming can lead to proportionally high soil C and N losses when increased N mineralization rates at warmer temperatures are not compensated by rapid plant N uptake and plant-derived C inputs to the soil due to lower root biomass production of less diverse plant communities.
Study 6 The soil memory: Long-term field warming controls	● How does long-term warming affect the temperature responses of potential	✓ At steady-state, V_{max} of C- and N-degrading enzymes was lower in historically warmed compared to

short-term temperature responses of soil microbial functions	enzyme activities ● Whether or not soil microbial memory occurs persistently when microorganisms were activated by the labile C input with future climate warming	ambient soil. ✓ Native available substrates for β-glucosidase, chitinase, and leucine aminopeptidase in historically warmed was 2-times lower than in ambient soil. Thus it caused a lower Q_{10}-V_{max} in warmed soils. ✓ At activation mode, V_{max} and Q_{10}-V_{max} was higher in historically warmed compared to ambient soil, suggested that input of labile substrate activated dormant microorganisms in historically warmed soils.

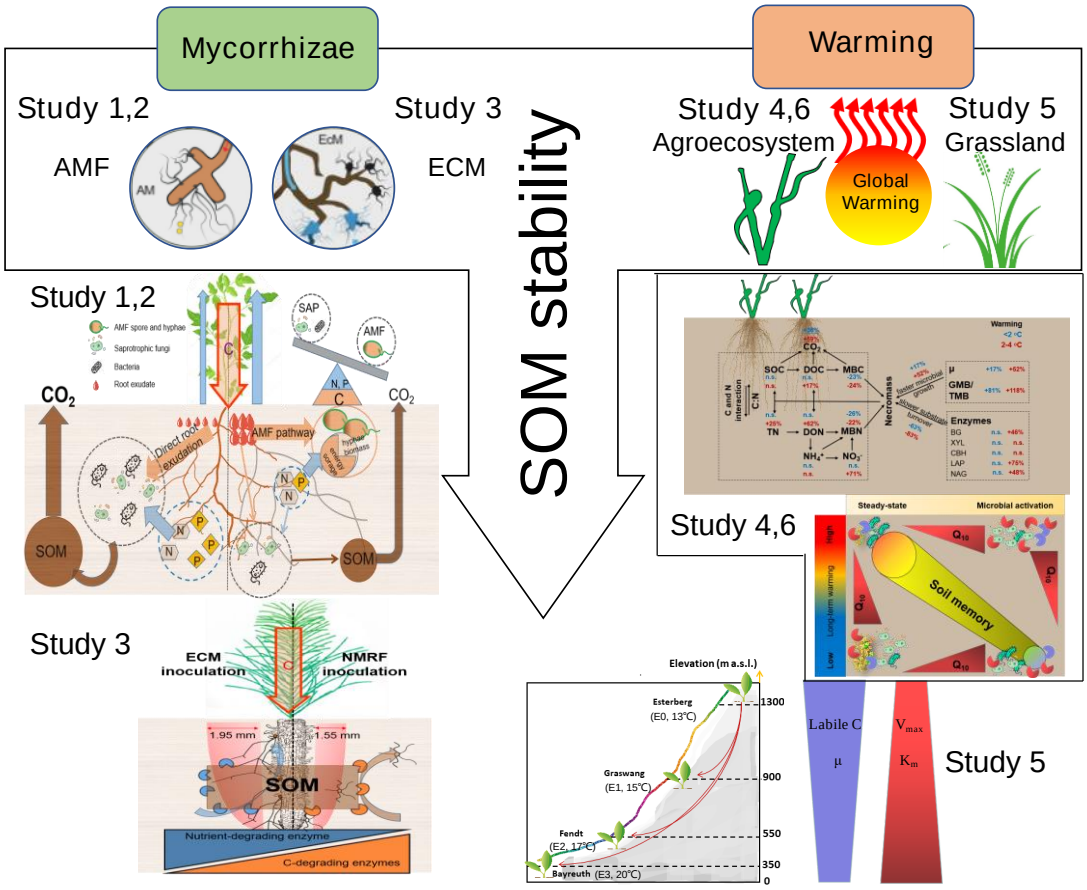


Fig. ES2 Synthesis of main results of the thesis.

1.4.2 Mycorrhization and soil organic matter stabilization

1.4.2.1 Arbuscular mycorrhizal fungi enhances rhizodeposition and decreases soil organic matter decomposition

Plants with AMF had 33% higher net rhizodeposition than plants without mycorrhization in the soil (rhizosphere + bulk) (Fig. ES3). This is in accordance with a study from Kong and Fridley (2019) who reported mutualistic microorganisms increased C allocation to soil. Higher C inputs under plants with AMF are explained by higher amounts of assimilates allocated to the development of extraradical mycelium and spores (Olsson and Johnson, 2005), and by the extremely long residence time of glomalin-related soil protein released from AMF (Rillig, 2004). The symbiotic AMF interactions are known to transfer C away from the rhizosphere to the bulk soil (less microbial activity) (Zhu and Miller, 2003; Hafner et al., 2014), and thus to facilitate C sequestration. In summary, the AMF symbiosis increased net rhizodeposition in the rhizosphere and the bulk soil, due to the important pathway of C inputs through AMF hyphae.

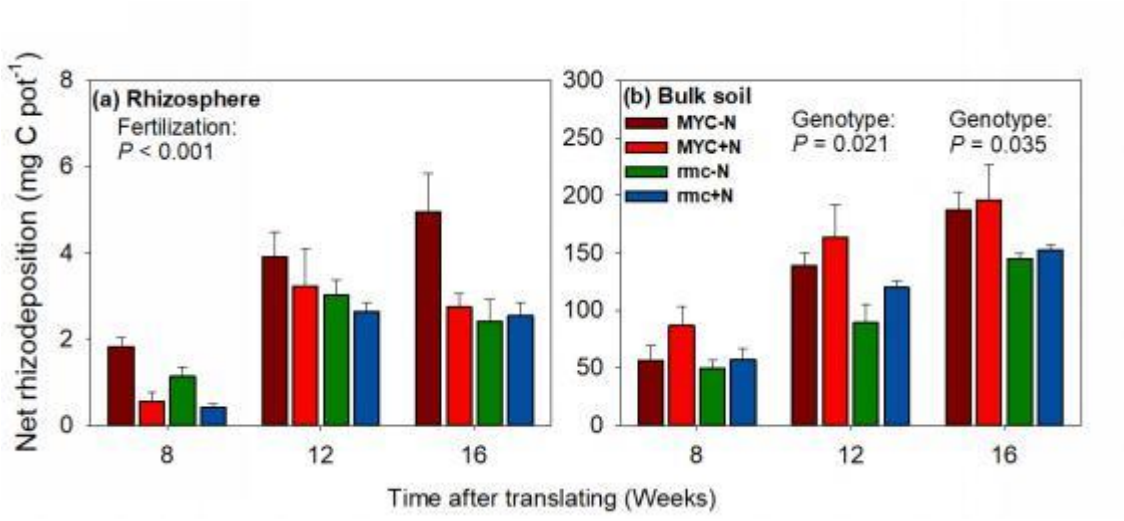


Fig. ES3 Rhizodeposition remaining in rhizosphere (a) and bulk soil (b) at harvest (net rhizodeposition) of mycorrhizal wild type (MYC) and mutant (rmc) tomato with reduced mycorrhizal colonization with and without N fertilization over a 16 weeks growth period. Fertilization: with and without N; Genotype: MYC and rmc. Net rhizodeposition means the total amount of ^{13}C remaining in soil at the time of harvest.

Compared with unfertilized soil, we recorded a reduced C flow to the belowground pools and less AMF abundance (NLFA 16:1ω5c) for MYC plants with N fertilization (Fig. ES6). This suggested that N fertilization has reduced AMF fungal activity as a result of a decreased transport of photosynthate-C to roots and further to their AMF symbiosis. Firstly, plants invested relatively more resources to aboveground biomass under N fertilization, thus resulted in reduced relative belowground C inputs and less C allocation to the symbiotic fungi. Secondly, high soil N availability stimulated plant growth (higher aboveground biomass) and thus increased the competition for essential resources between roots, including AMF and neighboring microorganisms (Kuzyakov and Xu, 2013; Konvalinková et al., 2017). This, in turn, may have reduced the

development of AMF. Accordingly, AMF growth and their C sink strength are reduced by N fertilization.

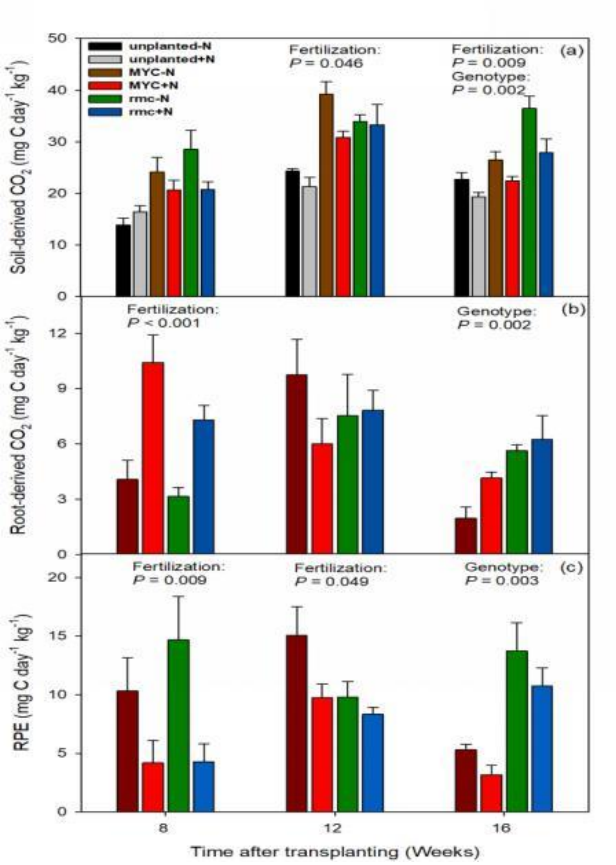


Fig. ES4 Soil-derived (a), root-derived CO_2 (b), and rhizosphere priming effect (RPE) (c) of mycorrhizal wild type (MYC) and mutant (rmc) tomato with reduced mycorrhizal colonization with and without N fertilization over a 16 weeks growth period.

Rhizosphere priming effect was lower in soil under MYC versus rmc plants. The lower rhizosphere respiration of MYC plants represented less readily available plant-derived C (Fig. ES4), which further decreased the activated microorganisms and thus the demand for nutrients from SOM mining (Fontaine et al., 2011). In the present study, AMF symbiosis actively scavenge soil for nutrients, making them highly efficient for nutrient uptake (Verbruggen et al., 2016). The reduced N and P availability further imposed nutrient limitation for free-living decomposers and reduced their activities in bulk soil (Fig. ES4; Brzostek et al., 2015). AMF are less limited by C than saprotrophic fungi due to direct C allocation from the plant hosts (Fig. ES6). Thus, AMF may produce secondary metabolites that are antagonistic against free-living saprotrophic fungi (Keller et al., 2005; Fernandez and Kennedy, 2016). The efficient substrate uptake of nutrients by AMF may have restricted the activity and nutrient use of free-living decomposers (Fig. ES5). This is the most plausible mechanism for increased C retention by AMF symbiosis.

The intensity of RPE on SOM decomposition decreased by two thirds in fertilized versus unfertilized pots at earlier stages (e.g., 8 and 12 weeks; Fig. ES4). This could be explained by the reduced nutrient demand of soil microbes and to their preferential

utilization of root-released C (Phillips et al., 2011; Zang et al., 2017).

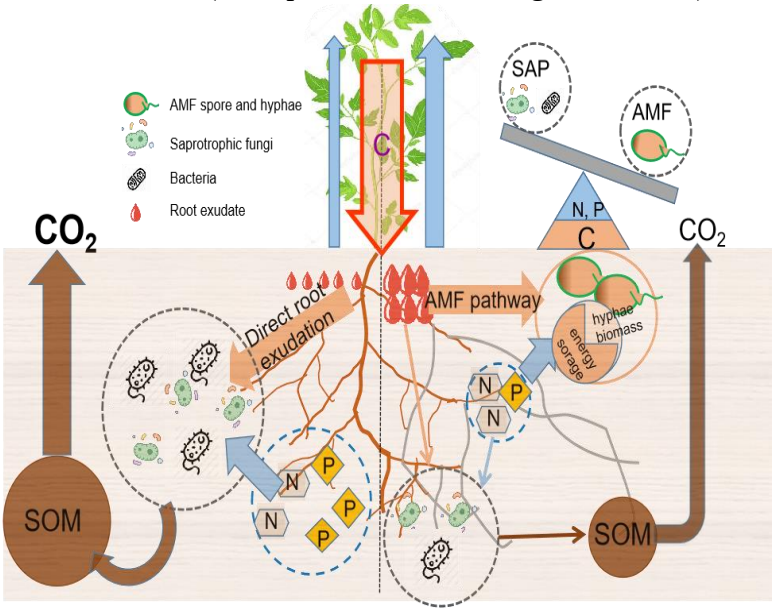


Fig. ES5 Conceptual figure showing rhizosphere priming on SOM decomposition accompanied by microbial substrate (i.e. C and nutrient) competition. Arrow thickness indicates process intensity.

1.4.2.2 The predominant role of AMF in rhizosphere C flow and microbial community shift

In the present study, the direct incorporation of plant-derived C was much greater for AMF than bacteria and SAP (Fig. ES6). The ^{13}C incorporation in the AMF-specific biomarker (NLFA and PLFA 16:1ω5c) increased over the growth period and was still prominent at 16 weeks after transplanting (Fig. ES6). Increased ^{13}C labeling of NLFA 16:1ω5c suggested the enhanced production of AMF storage compounds, and increased ^{13}C labeling of PLFA 16:1ω5c implied AMF growth stimulation (Olsson and Johnson, 2005). These data confirmed earlier results on the dominant role of AMF in the flow of C from plants into the soil and AMF hyphae (Johnson et al., 2002; Olsson and Johnson, 2005; Drigo et al., 2010).

By contrast, SAP fungi exhibited 20-30 times lower ^{13}C enrichment than in bacterial PLFAs (Fig. ES6). This is in line with Wardle et al. (2004) who suggested fungi dominate the turnover of C bound in complex structures. SAP fungal assimilation of C derived from necromass or dead root material rather than from labile root exudates might form an alternative explanation for the lower incorporation of ^{13}C into the fungal PLFAs (Rousk and Frey, 2015).

When conditions became less suitable for AMF colonization of the root (e.g., under N fertilization), there is a lower surplus of carbohydrates that can be used by the fungus for storage structures (Fig. ES6). In other words, in the case of greater nutrient limitation in plants without fertilization, AMF symbiosis thus could be more important, promoting preferential C allocation to AMF, at the expense of SAP. This could be an important explanation of the decreased ^{13}C enrichment in SAP in the

unfertilized soil. Overall, the general explanation is that the N-limited plant consumes more of the newly fixed C to the root that reduce the C availability to the free living saprotrophic microorganisms, whereas a high N availability negatively impact on AMF symbiosis results from less C allocation. The mechanism behind this is an increased C immobilization in the aboveground biomass as well as the stimulated rhizosphere respiration.

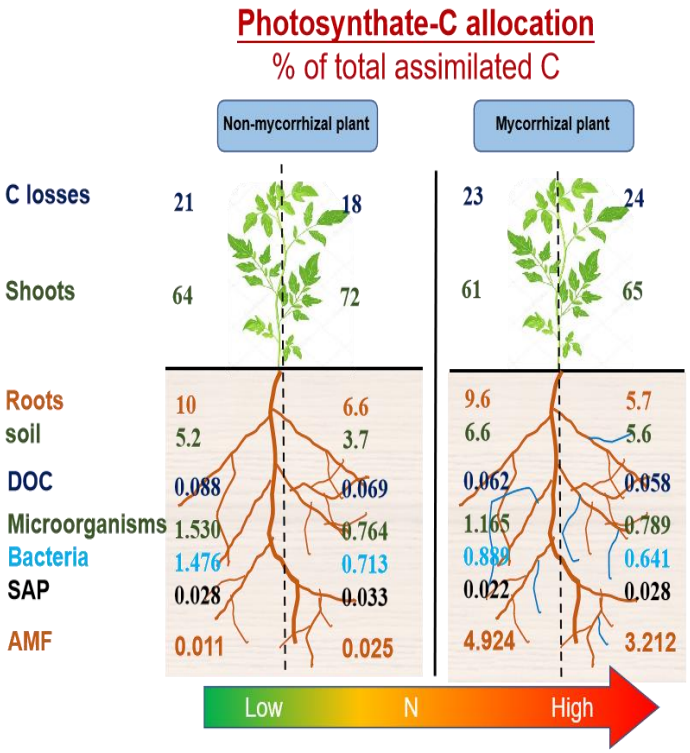


Fig. ES6 Carbon (C) budget within a plant-soil system of mycorrhizal and non-mycorrhizal plants with and without N fertilization presented as % of total assimilated C into each C pool of plant and soil during 16-weeks continuous ^{13}C labelling. The C losses includes root respiration and CO_2 released from soil organic matter decomposition.

1.4.2.3 Ectomycorrhizal and non-mycorrhizal rhizosphere fungi increase root exudation and enzyme activities

Based on ^{14}C imaging, we found that both ECM and NMRF increased plant C allocation belowground relative to Control, as indicated by the higher specific ^{14}C activities in the rhizohyphosphere with NMRF (Fig. ES7). This agrees with earlier studies which found that NMRF could incorporate recently fixed plant C and thus be major consumers of root exudates (Hannula et al., 2012; Pausch et al., 2016).

Although both ECM and NMRF increase plant C allocation belowground, they exerted various influences on the radial patterns of exudates (Fig. ES8). Although most of the recently assimilated C was allocated to infected root tips (Wu et al., 2002),

mycorrhizal hyphae can displace plant C beyond the root zone (Smith and Read, 2008), leading to a wider spread and even distribution of C and, hence, to a smaller ^{14}C hotspot area in soil inoculated with ECM. As a consequence, the rhizosphere extent was broader for plants inoculated with ECM compared to others (Fig. ES8). Overall, both ECM and NMRF increased the belowground C sink strength but exerted different effects on the spatial distribution of exudates in the rhizosphere.

When co-inoculated with ECM and NMRF, the specific ^{14}C activity decreased by 35% compare to ECM inoculation (Fig. ES7). This points to a competitive relationship between these fungal groups. In the context of our experiment, this means that ECM potentially limit their growth due to the necessity to share substrates with NMRF, especially with regard to N resources. As a consequence,^{14}C activities were lower in the hyphosphere soil of MIX compared with ECM treatment.

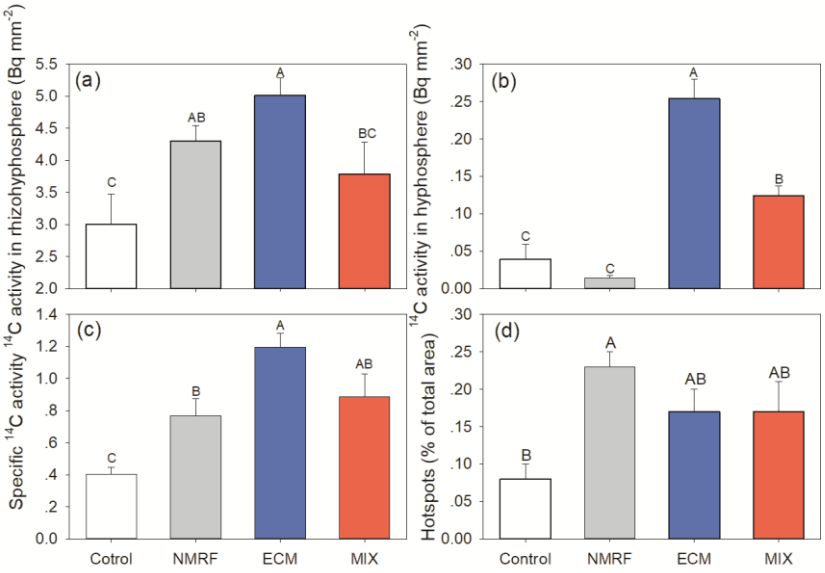


Fig. ES7 ^{14}C activity in the rhizohyphosphere soil (a), hyphosphere soil (b), specific ^{14}C activity (c), and percentage of hotspots in rhizohyphosphere soil (b) of European spruce (*Picea abies* L.) without inoculation (Control), inoculated with non-mycorrhizal rhizosphere fungi (NMRF), inoculated with ectomycorrhizal fungi (ECM), and inoculated with ECM and NMRF (MIX).

Besides rhizodeposited C, enzyme activities and their spatial extension in the rhizosphere were strongly affected by the type of fungi present (Fig. ES8). ECM are supplied with C by their host and are generally rather nutrient- than C-limited (Smith and Read, 2010; Franklin et al., 2014). Accordingly, the use of these exudates by microorganisms as C and energy resources implies an increase in the soil volume for nutrient uptake. Besides, the higher C content in the extraradical mycelium of ECM (Trudell and Edmonds, 2004; Trocha et al., 2016) also suggests a higher nutrient demand for ECM itself compared with NMRF.

Although NMRF increased belowground C inputs, the C allocated to soils may be insufficient to meet the growth demand of the entire hyphal network of NMRF

(Hobbie and Horton, 2007) because of the high hyphal density in soil (Söderström, 1979). Therefore, more β-glucosidase (and presumably also other C-cycle involved enzymes) were released to mineralize native SOM and dead root tissue to acquire energy for their hyphal growth (Fig. ES8).

Overall, the target enzymes released by ECM and NMRF were different due to the acquisition of their most limited or required elements - either for their own tissue (Allison and Vitousek, 2005) or for maintaining their symbiotic relationship (Read and Rerez-Moreno, 2003).

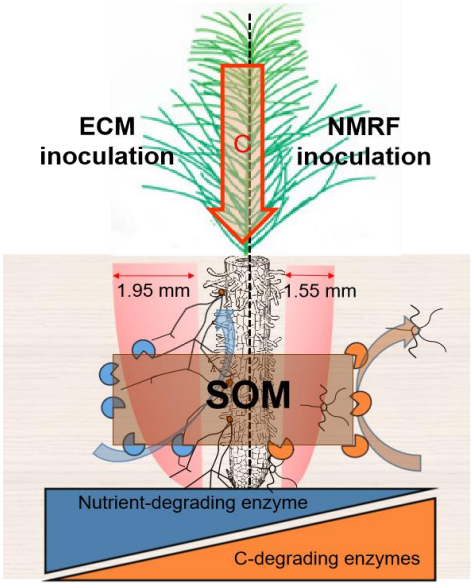


Fig. ES8 General conceptual pattern of C allocation belowground, specific enzyme production, and spatial distribution of rhizodeposit-C and enzyms from plants inoculated with ectomycorrhizal (ECM) and non-mycorrhizal rhizosphere fungi (NMRF).

1.4.3 Warming and soil organic C storage

1.4.3.1 Self-regulatory mechanism of soil organic carbon stability under warming in agroecosystems

Remarkably, the SOC remained stable even with higher warming magnitude (2-4 °C) in long term, as proved by both case study and literature review (Fig. ES9). The stable SOC was attributed to the faster microbial growth and necromass formation, which was counterbalanced by the slower enzyme-mediated substrate turnover. The increased fraction of DOC with 2-4 °C warming, accompanied by an essential decrease in microbial biomass (Fig. ES10), implying that necromass (dead microbial biomass) was a possible source of the higher labile organic matter with higher warming magnitude (Miltner et al., 2012). This further favored fast-growing microorganisms, and consequently accelerated microbial turnover. However, fast-growing microorganisms with *r*-strategy are very sensitive to any limitation (e.g.,

energy and resource availability), and commonly shift to dormancy or even die when substrate becomes limiting (Salazar-Villegas et al., 2016; Shahbaz et al., 2017). After consumption of labile C, the *r*-strategists therefore reduce their biomass rather than mine available organic compounds from recalcitrant SOM pools. As a result of such self-regulation, the SOC pool remained unaffected.

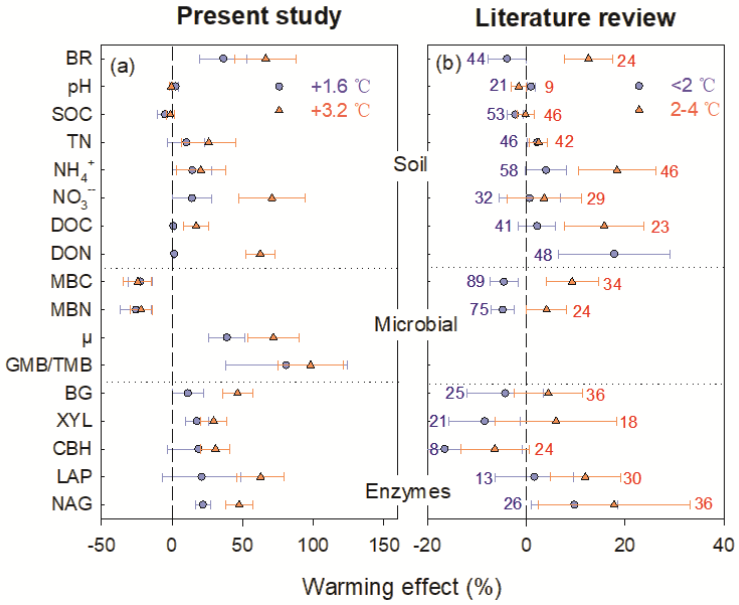


Fig. ES9 Effect of warming on soil parameters (SOC, TN, IN, DOC, DON), basal respiration (BS), microbial parameters (MBC, MBN, μ, GMB/TMB), and enzyme activities (BG, XYL, CBH, LAP, NAG) based on our study (panel a) or from a review of the literature (panel b). The details for data selection can be found in the text.

Although 8 years warming did not change SOC, TN was increased by 26% (+3.2 °C) (Fig. ES9). The 2-4 °C warming changed enzyme systems towards lower affinity to the substrate, i.e. slower glucose or amino-N production, but lower turnover time and catalytic efficiency of β-glucosidase and leucine aminopeptidase (Fig. ES10). Thus, it caused a slower decomposition of organic residues compared with faster necromass production. Specifically, the generation time of microbial population was 3 h faster but the turnover time of peptides (cleaved by leucine aminopeptidase) was 7 h slower at higher warming (+2-4 °C) compared with ambient soil (Fig. ES10), resulting in increased total N. Moreover, the ambient C:N of soil (~19) was close to the theoretic threshold (20-25, Fig. ES10), i.e., the C:N value above which the N will be immobilized and below which N will be mobilized by microorganisms (Mooshammer et al., 2014). The possible consequences of decreased soil C:N to a value of 16 by 2-4 °C warming, is net N mobilization for improving N availability for plant growth (Manzoni et al., 2012). This corroborates with the relatively stable microbial C:N ratios with warming (~5.5), which means microorganisms did not immobilize more N

even when it was available (Fig. ES10). Therefore, the stoichiometric imbalance between microbial decomposers and their labile resources (dissolved organic pool) may also result in enhanced N releases from necromass under the higher magnitude of warming.

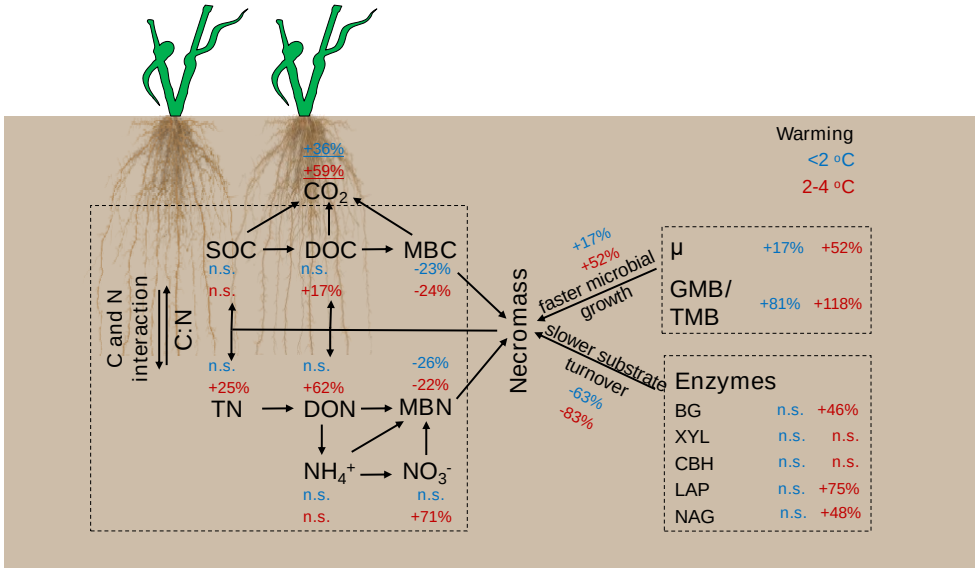


Fig. ES10 The fundamental microbial mechanistic framework with soil temperature increases of ≤ 2 °C or 2-4 °C.

1.4.3.2 Response of microbial growth and enzymatic kinetics to climate warming in montane grassland

Due to decreased root and shoot biomass with climate warming, less rhizodeposition was released into soil with lower elevation, thus induced a strong competition for easily degradable C sources which favors the *K*-selected microorganisms (Blagodatskaya et al., 2014). Under lower C availability as indicated by lower DOC, active but starving microorganisms, the *K*-strategists contribute to microbial growth (Chen et al., 2014), as a result the μ values decreases as compared with soil under higher elevation (Fig. ES11).

As microorganisms regulate the production of enzymes in response to environmental resource availability (Allison and Vitousek, 2005), and warming decreased plant C supply as mentioned above, it is possible that microorganisms allocate more resources to the acquisition of C (Sinsabaugh and Follstad, 2012). Hence, these findings indicate that warming-induced decreased C availability stimulated C-degrading enzymes and increased microbial C limitation. There was a clear stoichiometry shift to higher investment in C acquisition in soils with lower elevation, as indicated by higher enzymatic ratio of C- to N-degrading and C- to P-degrading enzymes in soil under lower elevation. The decreased DOC and DN content with climate warming further suggested that translocation could decrease C availability and stimulate labile C-acquired enzyme activity, which reflects the aggravation of C limitation in lower

elevation. Furthermore, a shorter substrate turnover time and higher K_a of β-glucosidase in soil at lower elevation compared with higher elevation was observed, which suggests that the microbial community was more limited by C rather than nutrient in the warmer and drier soils at lower elevation.

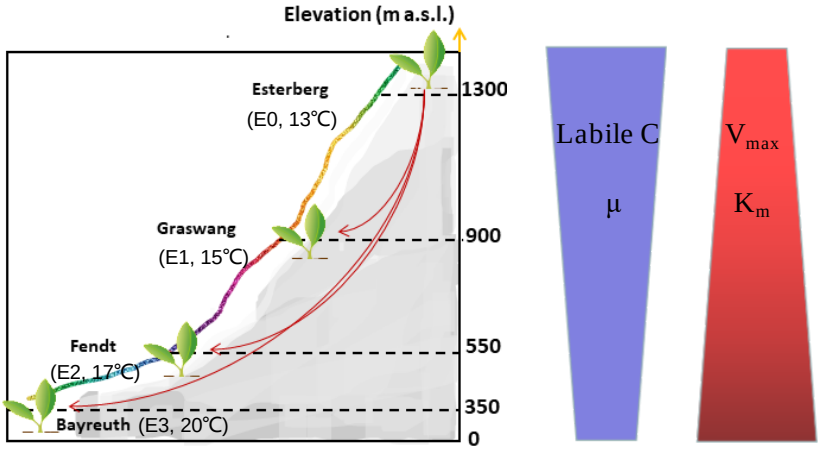


Fig. ES11 Conceptual figures of microbial functional traits in soil at four elevations (E0 with 13°C, E1 with 15°C, E2 with 17°C, E3 with 20°C).

1.4.3.3 Temperature sensitivity of enzyme activities under steady-state and microbial activation mode

The temperature sensitivity (Q_{10}-V_{max}) of β-glucosidase, chitinase, and leucine aminopeptidase decreased with historical warming (Fig. ES12), because of the microbial memory effect (thermal acclimation) (Bradford et al., 2008; Walker et al., 2018), which could result from limitations by labile organic substrates (Davidson and Janssens, 2006). Eight years of accelerated C cycling substantially depleted the easily available C pool in the historically warmed soils. This was further proved by the lower content of native substrates for β-glucosidase, chitinase, and leucine aminopeptidase in the historically warmed compared with ambient soils (Fig. ES12). The low amount of available substrates precludes slow microbial metabolic activity, and thus many soil microorganisms shifted to dormancy (Lennon and Jones, 2011). Given that active microorganisms are more sensitive to the fluctuating environment (Blagodatskaya and Kuzyakov, 2013), and therefore showed a higher temperature sensitivity in the ambient soils compared with dormant microorganisms under long-term warming.

After activating microorganism, we observed an increase both in all the tested enzyme activities and Q_{10}-V_{max} (Fig. ES12) compared with steady-state. Altered C availability in soil are well known to affect microbial physiological status (Cleveland et al., 2007). Stronger limitation by the labile native substrate in the historically warmed soil reduced metabolic activity, and thus caused a memory effect in response to further

short-term increasing temperature. In contrast, the surplus of glucose increased native labile substrates, induced microbial growth, as well as increased both enzyme activity and catalytic efficiency, as a consequence eliminated the microbial memory effect in the historically warmed soils. Thus, a sudden increase in C availability can induce a short-term fluctuation in community composition shifting the domination from slow-growing oligotrophic microorganisms (favored by low substrate availability) to temporal dominance of copiotrophic microorganisms (Cleveland et al., 2007). Shifts towards more active microorganisms at warmer temperatures combined with increasing labile C input (e.g., microbial activation) from enhanced vegetation productivity at higher mineralization rates can thus result in higher temperature sensitivity (Hartley et al., 2008). Therefore, the Q_{10}-V_{max} was higher in the historically warmed compared with ambient soils after substrate addition. However, due to the limited binding between enzymes and soil organics because of the increased absorption rate between organic-mineral surface with elevated incubation temperature (Wallenstein et al., 2011), the apparent increase of Q_{10}-V_{max} after microbial activation between historical warmed and ambient soil was only observed at low incubation temperatures.

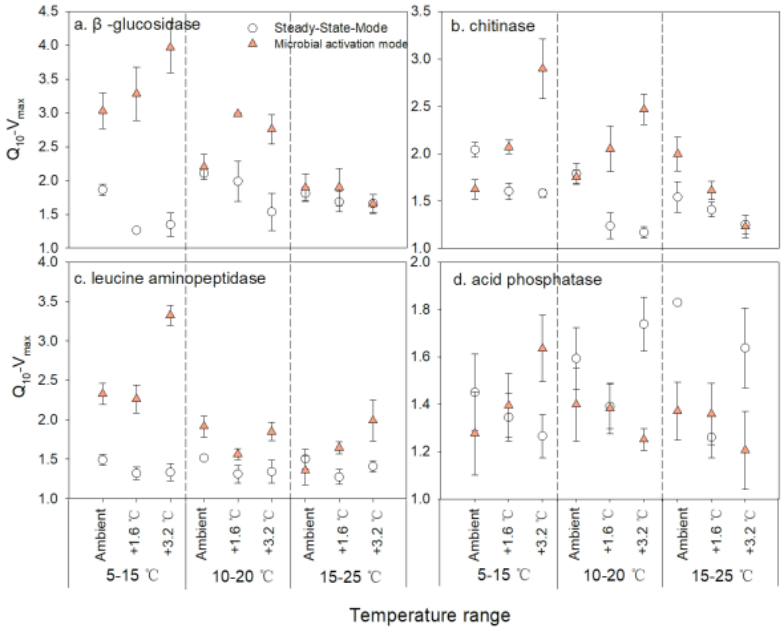


Fig. ES12 Temperature sensitivity of potential enzyme activities (Q_{10}-V_{max}) of β-glucosidase (a), chitinase (b), leucine aminopeptidase (c), and acid phosphomonoesterase (d) for soil incubated with temperature increasing from 5 to 25 °C at 5 °C increments. Soil was sampled after 8 years of field warming (at: ambient; +1.6 °C; and +3.2 °C).

1.5 Conclusions and outlook

Based on ^{13}C labeling, this thesis demonstrated for the first time that AMF symbiosis facilitate soil C sequestration by retaining more plant rhizodeposits in soil (net rhizodeposition) and by reducing SOM decomposition. The latter was demonstrated by restricted growth and activities of saprotrophic microorganisms due to the sufficient C and nutrient uptake by AMF. However, the AMF growth and C sink was dependent on N fertilization in agroecosystems. Although ECM also stimulated belowground C inputs which was similar with AMF, it boosted enzyme activities to degrade organic N pools to cover the nutrient demand of ECM. In contrast, NMRF showed a higher investment in enzymes involved in maintaining their C supply, thus boosting C cycling. When co-existence between ECM and NMRF, ECM could inhibit the growth of NMRF, thus suppressed SOM decomposition in forest soils. Therefore, we highlighted the effect of mycorrhization on rhizodeposition has important consequences for SOM decomposition, which is dependent on N fertilization and fungal competition for substrates.

Besides rhizodeposits via mycorrhization pathway, temperature is a another crucial factor enhancing soil microbial activity and thus threatening SOM stability. However, we demonstrated that changes in SOM decomposition strongly depends on warming magnitudes. A low magnitude of temperature increase (< 2 °C) only altered microbial traits (i.e. microbial biomass), but neither enzyme functioning nor soil basic properties. However, a higher warming (2-4 °C) that beyond the Paris Climate Agreement aim was sufficient to activate microbial growth and turnover, enzyme production, thus accelerated SOM mineralization. Furthermore, increasing labile C input from enhanced vegetation productivity in the future warmer world would shift microbial physiological status, thus resulted in higher temperature sensitivity of enzyme activities, consequently enhancing SOM decomposition. These findings inferred that higher warming magnitude have a profound impact on global soil C cycling processes with implications of a positive feedback on atmospheric CO_2 and exacerbate climate change.

Given their multiple potential impacts on SOM formation, stabilization and decomposition, mycorrhization may be a factor contributing to the observed SOM stabilization and decomposition and furthermore, it may help explain the variation in responses of soil climate-C feedback. Given that climate warming may affect abiotic and biotic factors influencing mycorrhizal symbiosis directly and indirectly, whether this C sequestration via mycorrhization will persistent under climate warming, as well as whether it could act as a positive mitigation strategy buffer against the predicted increases in SOM decomposition in the future warmer world requires further research. Altogether, this indicates a clear need for further investigation of the warming-plant-mycorrhization-soil C link, for which empirical tests are lacking. Further, the Paris Climate Agreement is pursuing efforts to limit the increase in global temperature to below 2 °C above the pre-industrial level. However, there is increasing evidence that climate change will be faster than projected, indicating that soils face accelerating damage. Therefore, it is important to examine mycorrhizal abundance

under the 2 ℃ target and to evaluate what will happen if the soils bear greater warming. Filling up this critical knowledge gap will help predict the ecological consequences of changes in mycorrhizal abundance under global change scenarios.

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1.7 Contribution to the included manuscripts

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

Study 1 Arbuscular mycorrhiza enhances rhizodeposition and reduces the rhizosphere priming effect on the decomposition of soil organic matter

Status: Published in *Soil Biology & Biochemistry* (2020)

Jie Zhou: experimental design and execution, data preparation and interpretation, manuscript preparation

Huadong Zang: discussion of results, comments to improve the manuscript

Sebastian Loeppmann: experimental design, comments to improve the manuscript

Matthias Gube: comments to improve the manuscript

Yakov Kuzyakov: comments to improve the manuscript

Johanna Pausch: experimental design, discussion of results, comments to improve the manuscript

Study 2 Arbuscular mycorrhizae prominent in the rhizosphere carbon uptake and shift the function of saprotrophic microorganisms

Status: In preparing

Jie Zhou: experimental design and execution, data preparation and interpretation, manuscript preparation

Hongcui Dai: data analysis, comments to improve the manuscript

Sebastian Loeppmann: experimental design, comments to improve the manuscript

Matthias Gube: comments to improve the manuscript

Lingling Shi: comments to improve the manuscript

Yakov Kuzyakov: comments to improve the manuscript

Johanna Pausch: experimental design, discussion of results, comments to

improve the manuscript

Michaela A Dippold: discussion of results, comments to improve the manuscript

Study 3 Ectomycorrhizal and non-mycorrhizal rhizosphere fungi increase root exudation and enzyme activities: a ^{14}C pulse labeling of *Picea abies seedlings*

Status: Under review in *Soil Biology & Biochemistry*

Jie Zhou: experimental design and execution, data preparation and interpretation, manuscript preparation

Matthias Gube: comments to improve the manuscript

Bin Song: experimental design, data preparation

Immo Shan: data preparation

Lingling Shi: experimental design, discussion of results, comments to improve the manuscript

Yakov Kuzyakov: comments to improve the manuscript

Michaela A Dippold: discussion of results, comments to improve the manuscript

Johanna Pausch: discussion of results, comments to improve the manuscript

Study 4 Restricted power: is microbial pool still able to maintain the stability of soil organic matter under warming exceeding 2 degrees?

Status: In preparing

Jie Zhou: experimental design and execution, data preparation and interpretation, manuscript preparation

Yuan Wen: discussion of results, comments to improve the manuscript

Lingling Shi: comments to improve the manuscript

Michaela A. Dippold: comments to improve the manuscript

Yakov Kuzyakov: comments to improve the manuscript

Huadong Zang: experimental design, discussion of results, comments to improve the manuscript

Davey L. Jones: comments to improve the manuscript

Evgenia Blagodatskaya: discussion of results, comments to improve the manuscript

Study 5 Response of microbial growth and enzyme kinetics to climate change: a soil translocation study along an elevational gradient across montane grasslands

Status: In preparing

Jie Zhou: experimental design and execution, data preparation and interpretation, manuscript preparation

Yue Sun: experimental design and execution, data preparation and interpretation,comments to improve the manuscript

Evgenia Blagodatskaya: comments to improve the manuscript

Anke Jentsch: comments to improve the manuscript

Johanna Pausch: discussion of results, comments to improve the manuscript

Study 6 The soil memory: Long-term field warming controls short-term temperature responses of soil microbial functions

Status: Submitted to Global Change Biology

Jie Zhou: experimental design and execution, data preparation and interpretation, manuscript preparation

Bahar S. Razavi: comments to improve the manuscript

Sebastian Loeppmann: comments to improve the manuscript

Miles R. Marshall: comments to improve the manuscript

Yakov Kuzyakov: comments to improve the manuscript

Huadong Zang: experimental design, discussion of results, comments to improve the manuscript

Michaela A. Dippold: discussion of results, comments to improve the manuscript

Evgenia Blagodatskaya: discussion of results, comments to improve the manuscript

2 Manuscript

Study 1 Arbuscular mycorrhiza enhances rhizodeposition and reduces the rhizosphere priming effect on the decomposition of soil organic matter

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Status: Published in Soil Biology & Biogeochemistry

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Abstract

Arbuscular mycorrhizal fungi (AMF) represent an important route for plant carbon (C) inputs into the soil. Nonetheless, the C input via AMF as well as its impact on soil organic matter (SOM) stabilization and C sequestration remains largely unknown. A mycorrhizal wild type progenitor (MYC) and its mycorrhiza defective mutant (reduced mycorrhizal colonization: rmc) of tomato were continuously labeled with $^{13}CO_2$ to trace root C inputs into the soil and quantify rhizosphere priming effects (RPE) as affected by AMF symbiosis and N fertilization. Mycorrhizal abundance and ^{13}C incorporation into shoots, roots, soil and CO_2 were measured at 8, 12 and 16 weeks after transplanting.

AMF symbiosis decreased the relative C allocation (% of total assimilated C) to roots, in turn increased the net rhizodeposition. Positive RPE was recorded for both MYC and rmc plants, ranging from 16-71% and 25-101% of the unplanted control, respectively. Although net rhizodeposition was higher for MYC than rmc plants 16 weeks after transplanting, the RPE was comparatively lower. This indicated a higher potential for C sequestration by plants colonized with AMF (MYC) because the reduced nutrient availability restricts the activity of free-living decomposers. Although N fertilization decreased the relative C allocation to roots, rhizosphere and bulk soil, it had no effect on the absolute amount of rhizodeposition to the soil. The RPE and N-cycling enzyme activities decreased by N fertilization 8 and 12 weeks after transplanting, suggesting a lower microbial N demand from SOM mining. The positive relationship between enzyme activities involved in C cycling, microbial biomass C and SOM decomposition underlines the microbial activation hypothesis, which explains the RPE. We therefore concluded that AMF symbiosis and N fertilization increase C sequestration in soil not only by increasing root C inputs, but also by lowering native SOM decomposition and RPE.

Keywords: Arbuscular mycorrhizal fungi (AMF), Carbon balance, Continuous labeling, N fertilization, Rhizodeposition, Rhizosphere priming effect (RPE)

1. **Introduction**

Globally, soils store 500-3000 Pg carbon (C), more than the atmosphere and biosphere together (Todd-Brown et al., 2013; Wieder et al., 2013). The global C storage depends on the balance between newly formed soil organic matter (SOM) and C lost through the decomposition of old SOM (Song et al., 2018). About half of total plant assimilated C is translocated from above- to below-ground pools, either as root and shoot litter or as rhizodeposits released from living roots (Zang et al., 2019). The soil CO_2 efflux is one of the largest fluxes in the global C cycle, with 50% controlled by plant-soil interactions (Hopkins et al., 2013).

There is increasing evidence that a significant part of this C enters and leaves the soil through the mycorrhizal network (Finlay and Rosling, 2006; Finlay, 2008).

Arbuscular mycorrhizal fungi (AMF) are the dominant mycorrhizal type, forming symbiotic associations with about 71% of all flowering plants including many important crops such as wheat, barley, corn and soybean (Brundrett and Tedersoo, 2019). Since the 1990s it has been recognized that AMF symbiosis often increase the allocation of photosynthates to both roots and AMF (Jakobsen and Rosendahl, 1990; Olsson et al., 2005; Drigo et al., 2010). In fact, C allocation to AMF ranges between 5% and 25% of the available photoassimilates (Jakobsen and Rosendahl, 1990; Johnson et al., 2002; Konvalinková et al., 2017; Řezáčová et al., 2018). This contributes to microbial biomass build-up (Olsson and Johnson, 2005). Accordingly, AMF are important regulators of the C flux from above- to below-ground pools (Zhu and Miller, 2003).

AMF symbiosis contributes to soil C flux by incorporating C into the intra- and extraradical mycelium (Leake et al., 2004), transporting and exuding C through the extraradical mycelium (Godbold et al., 2006), stabilizing the soil structure (e.g., aggregation) (Rillig, 2004), as well as by providing C to the microbial community (Jones et al., 2009). Furthermore, AMF can promote free-living microbial communities by releasing labile substrates via exudation and hyphal turnover, stimulating microbial growth (Toljander et al., 2007) and providing energy for enzyme production and SOM decomposition (Schmidt et al., 2011). The change of SOM decomposition in the presence of living roots is termed rhizosphere priming effect (RPE) (Kuzyakov, 2002; Cheng et al., 2014). Compared to root-free soil, the SOM decomposition with living roots can be altered by as much as -70% to 380% (Zhu and Cheng, 2011; Cheng et al., 2014). Although there is general consensus that roots stimulate microbial activity (Blagodatskaya et al., 2009; Kuzyakov and Blagodatskaya, 2015; Loeppmann et al., 2016a; 2018), it is unclear how interactions between roots, AMF and saprotrophic microorganisms alter the belowground C inputs and, therefore SOM decomposition, and consequently C storage (Fierer et al., 2009; Phillips, Brzostek and Midgley, 2013). Several studies have reported that AMF symbiosis can enhance litter decomposition and support plant N capture, assuming enhanced C-flow to microbial communities (Hodge et al., 2001; Cheng et al., 2012). On the contrary, AMF symbiosis did not accelerate (Nottingham et al., 2013; Shahzad et al., 2015) or inhibit litter decomposition (Leifheit et al., 2015). So far, the mechanisms underlying the effects of AMF symbiosis on SOM decomposition still remain unsolved (Carrillo et al., 2016; Paterson et al., 2016). This emphasis of the AMF on soil C storage and C balance remains an open question (Averill et al., 2014). Answering this question can help to better predict how AMF symbiosis will affect pools and fluxes of the C cycle when vascular plants become more abundant.

Numerous studies have demonstrated that AMF are sensitive to N fertilization (Treseder, 2004; Reay et al., 2008; Lilleskov et al. 2011; Johnson et al., 2013; Mohan et al., 2014). Nitrogen fertilization can affect the allocation pattern of photosynthesized C in various belowground C pools (Kuzyakov, 2002; Zang et al., 2017). The abundance of AMF (percentage of root length colonization) in general declines by 15% in ecosystems exposed to mineral N fertilization (Treseder, 2004), which may change the magnitude of C flow from plants to AMF and to soil C pools, subsequently altering the magnitude of RPE and soil C storage.

Here, we investigated the dynamics of C allocation and transformation in the plant-soil system as affected by AMF symbiosis and N fertilization based on continuous $^{13}CO_2$ labeling of a mycorrhizal wild type progenitor (MYC) and its mycorrhizal defective mutant of tomato (rmc). Continuous $^{13}CO_2$ labeling was conducted to trace plant C inputs into the soil to quantify RPE by partitioning soil CO_2 efflux into root-derived and soil-derived CO_2. The objectives of this study were to 1) assess the effect of AMF symbiosis on C allocation within the plant-soil system depending on N fertilization; 2) quantify C inputs by AMF symbiosis into the soil; 3) estimate the soil C balance and RPE induced by AMF symbiosis and N fertilization.

2. Materials and Methods

2.1 Soil origin and plant growth

Soil samples were collected from the Ap horizon (0-20 cm) of an experimental field at the Reinshof Research Station of the Georg-August University of Göttingen, Germany (28°33´26´´N, 113°20´8´´E). The soil was air-dried and sieved (< 2 mm) to achieve a high degree of homogeneity and reduce the variability among replicates. Fine roots and visible plant residues were carefully removed manually. The soil contained 1.3% total C, 0.14% total N, had a pH of 6.8, and a $\delta^{13}C$-value of organic C of -25.78‰, $\delta^{15}N$-value of total N of 5.69‰, and a bulk density of 1.30 g cm^{-3}.

Two tomato genotypes (*Lycopersicon esculentum L.*) were grown in this experiment: 1) mutant tomato with highly reduced AMF symbiosis, termed rmc (reduced mycorrhizal colonization), and 2) a closely related wild type hereafter termed MYC (Barker et al., 1998). The growth of the two genotypes is very similar under a range of circumstances, including non-mycorrhizal conditions, suggesting that the mutation affecting colonization of rmc has no pleiotropic effects on other plant processes (Cavagnaro et al., 2004). The use of genotypes enabled studying the impacts of AMF symbiosis on C allocation and RPE, without soil sterilization to establish a non-mycorrhizal control, thereby maintaining an intact soil microbial community. Both tomato types were grown with and without N fertilization (e.g. MYC-N, MYC+N, rmc-N, and rmc+N). In addition, unplanted pots with and without N fertilization (unplanted-N; unplanted+N) were prepared as controls. Each treatment was replicated 12 times, yielding a total of 72 pots, of which 4 replicates per treatment were harvested at each of the three harvests (8, 12, and 16 weeks after transplanting). N-treatments received 344 mg N per pot (60% of N from NH_4^+ and 40% of NO_3^-), equivalent to a rate of 150 kg N ha^{-1}.

PVC pots (7.5 cm diameter, 21 cm height), equipped with an inlet tube at the bottom and outlet tube at the top, were filled with 1 kg air-dried, sieved soil. To potentially improve AMF colonization, the soil was inoculated with *Rhizophagus irregularis* by mixing 1 kg soil with 500 mg of microgranulate (100 spores g^{-1} microgranulate) (BIOFA AG, Münsingen, Germany). The soil was kept at 20% gravimetric soil moisture content (equivalent of 60% of the water holding capacity) with deionized water. After pre-incubation at room temperature for two weeks, the seedlings in pots were moved to a growth chamber (day time of 14 h and 25 °C; night time of 10 h and

15 ºC). The relative humidity was 40% and plants received artificial light with 800 µmol m^{-2} s^{-1} photosynthetic active radiation (PAR). The locations of the pots in the growth chamber were changed weekly by mixing them randomly to guarantee similar growing conditions for the plants. The soil water content of all pots was maintained at 60% water holding capacity by deionized water addition (every 1-3 days).

2.2 Set-up of $^{13}CO_2$ continuous labeling of plants

The growth chamber was equipped with a continuous $^{13}CO_2$ labeling system. Briefly, the $^{13}CO_2$ used in the experiment was generated through the reaction of $Na_2{}^{13}CO_3$ (2.9 atom% ^{13}C, 0.5 mol L^{-1}) and excess of lactic acid outside of the chamber. The tracer solution was prepared by mixing 1g of 99 atom% ^{13}C enriched Na_2CO_3 (Cambridge Isotope Laboratories, Tewksbury, MA, USA) with 52 g of unlabeled Na_2CO_3 in 1 L of deionized water. The concentration was continuously monitored by an infrared gas analyzer (LI-830, LI-COR, USA) and ranged from 350 to 700 ppm. Lactic acid was added to the tracer solution when the CO_2 concentration inside the chamber dropped below 350 ppm. Chamber CO_2 was sampled periodically in order to validate its ^{13}C enrichment (mean $\delta^{13}C$ value was slightly fluctuating at 700‰). The plants were labeled from the emergence of the first leaf until harvest. They were watered during the dark period to avoid assimilation of unlabeled CO_2. After closing the chamber, the chamber air was pumped through external 50-L tight soda lime to remove unlabeled CO_2 and was then flushed with ^{13}C-enriched CO_2 before the light switched on and plants started photosynthesis.

2.3 Measurements

2.3.1 Soil CO_2 efflux

The soil CO_2 efflux and the $\delta^{13}C$–signature of soil CO_2 efflux were measured 3 times during the continuous labeling experiment (8, 12 and 16 weeks after transplanting) using a closed-circulation CO_2 trapping system (Cheng et al., 2003; Pausch et al., 2013; 2016). Briefly, a Plexiglas lid was placed on the soil surface, containing a hole for the tomato shoots. Each pot was the sealed with nontoxic silicon (Wasserfuhr GmbH) at the base of the plant 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 1 h. Afterwards, CO_2 produced during a 48-h period in each sealed pot was trapped in 100 ml of 1 M NaOH solution by periodic air circulation for 1 h at a 6-h interval. Blanks were included (empty, sealed pot) and treated in the same way to correct inorganic C for handling errors. An aliquot of each NaOH solution was measured by titration of 0.25 ml with 0.01 M HCl against phenolphthalein after addition of 0.5 M $BaCl_2$. Another aliquot was precipitated as $SrCO_3$, washed several times with deionized water to reduce the pH to 7.0 (Blagodatskaya et al., 2011). After drying at 60 ºC for 3 days the $\delta^{13}C$ of the $SrCO_3$ was analyzed at the Center for Stable Isotope Research and Analysis (KOSI) of the University of Goettingen with an Elemental Analyzer (EA, Eurovector) coupled to an IRMS (Delta Plus XL IRMS, Thermo Finnigan MAT, Bremen, Germany).

2.3.2 Plant and soil sampling

Plants and soil were sampled immediately after each CO_2 trapping. Shoots were cut at the base, and roots were pulled out of the pot. The main root system was manually removed with tweezers for root biomass and colonization analyses. The rhizosphere soil was collected by quickly shaking off soil adhered to the main roots and passing it through a 2-mm sieve to remove broken root fragments. In addition, roots were picked from the subsample to upscale root dry weight to the total soil per pot. Fine root subsamples (~ 1 g fresh fine roots) were randomly picked and cut into 1 cm length to measure AMF colonization. A representative homogenized soil subsample of each pot was stored at 4 °C to determine microbial biomass C, mineral N (NH_4^+ and NO_3^-), available P, neutral lipid fatty acid (NLFA) 16:1ω5c, and enzyme activities.
Roots, shoots and soil samples were dried at 60 °C for 3 days and, after weighing the dry biomass, the samples were homogenized in a ball mill and analyzed for $\delta^{13}C$ and C content, as well as for N content by EA-IRMS (see above). To determine the soil water content, 10 g fresh soil were dried at 105 °C for 1 day.

2.3.3 AMF abundance assessment

Roots were washed with distilled water, cleared in 10 % KOH (60 °C, 40 min), acidified with 1% HCl (20 °C, 5 min), stained with ink in lactoglycerol (60 °C, 20 min) and destained in lactoglycerol. All stained roots were arranged lengthwise in lactoglycerol on slides and mounted under a light microscope (100×) (Axionplan, Zeiss, Germany). The magnified intersection method described by McGonigle et al. (1990), was used to determine the percentage of root length colonization by AMF.
In addition to root staining, the neutral lipid fatty acid (NLFA) 16:1ω5c was measured as a biomarker for extraradical AMF biomass in the soil (Olsson, 1999), following the protocol described by Frostegard et al. (1991). Briefly, lipids were extracted from 6 g soil in a one-phase mixture of chloroform, methanol and 0.15 M citric acid (1:2:0.8, v/v/v, pH 4.0). The lipids were fractionated into neutral lipids with activated Silica gel (Silica gel Merck, particle size 0.063-0.200 mm) by eluted with 10 mL chloroform. The fatty acid residues in neutral lipids was transformed into free fatty acid methyl esters and analyzed by a Hewlett Packard 5890 gas chromatograph.

2.3.4 Microbial biomass C

Soil microbial biomass C (MBC) was determined by the chloroform fumigation extraction method (Vance et al., 1987) with modification. After destructive sampling, the soil was carefully mixed and 8 g soil was directly extracted for 1 h using 32 ml of 0.05 M K_2SO_4. Another 8 g soil was fumigated with chloroform for 24 h and extracted in the same manner. The samples were filtered and the extracts were frozen until analysis. Total C concentration was measured using a 2100 TOC/TIC analyzer (Analytik Jena, Germany).
MBC was calculated by dividing the difference between extracted C from fumigated and non-fumigated soil samples with a k_{EC} factor of 0.45 (Wu et al. 1990). After freeze-drying (Beta 1–8 LSCplus, Martin Christ Gefriertrocknungsanlagen GmbH, Harz, Germany), the $\delta^{13}C$ of MBC was analyzed by an elemental analyzer (Eurovector) coupled to an IRMS (Delta Plus XL IRMS, Thermo Finnigan MAT,

Bremen, Germany) at the Center for Stable Isotope Research and Analysis (KOSI) located at the Georg-August University of Göttingen.

2.3.5 Enzyme kinetics

Enzyme 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 (BG) (EC 2.2.1.21), cellobiohydrolase (CBH) (EC 3.2.1.91), xylanase (XYL) (EC 3.2.2.27), β-1, 4-N-acetylglucosaminidase (NAG) (EC 3.2.1.52), leucine aminopeptidase (LAP) (EC 3.4.11.1) and acid phosphatase (ACP) (EC 3.1.3.2) (Sinsabaugh and Follstad 2012). Briefly, 1g soil (dry weight equivalent) was suspended in 50 mL sterile water by shaking for 30 min and then dispersing with an ultrasonic disaggregator for 2 min using low-energy sonication (50 Js^{-1}) (Loeppmann et al., 2016a). 50 mL of the soil suspension was pipetted into 96-well black microplates (Puregrade, Germany) while stirring the soil suspension to ensure uniformity. Afterwards, 50 µL of buffer (MES, pH 6.5 or TRIZMA, pH 7.8) and 100 µL of the corresponding substrates at concentrations of 20, 40, 60, 80, 100, 200 and 300 µmol L^{-1} were added. Immediately after substrate addition, the microplates were measured fluorometrically (excitation wavelength 360 nm; emission 450 nm) at 0, 60 and 120 min with an automated fluorometric plate-reader (Victor3 1420 050 Multi-label Counter, PerkinElmer, USA).

To calculate the potential enzyme activity (V), we used the Michaelis-Menten equation for enzyme kinetics (Marx et al. 2001):

$$V = (V_{max} \times [S]) / (K_m + [S]) \quad (1)$$

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

2.3.6 Soil N and P analysis

Soil mineral N (N_{min}: NO_3^- + NH_4^+) was extracted from 5 g fresh soil with 10 ml of 2 M KCl solution. Samples were shaken for 2 h, filtered, and the extracts were analyzed for NO_3^- and NH_4^+ based on absorbance measurements (Mulvaney et al., 1996).

Soil inorganic P was extracted from 1 g fresh soil with 30 ml of 0.5 M $NaHCO_3$ (pH 8.5) in a 50 ml Falcon tube. Samples were shaken for 16 h, centrifuged and filtered. The P concentration in the extracts of the samples and standards were determined by the malachite green colorimetric method (Yevdokimov et al., 2016).

2.4 Calculations

2.4.1 ^{13}C isotopic composition of MBC/ metabolic quotient

The ^{13}C isotopic composition of the microbial biomass C ($\delta^{13}C_{MBC}$) was determined by a mass balance equation:

$$\delta^{13}C_{MBC} = (\delta^{13}C_f \times C_f - \delta^{13}C_{uf} \times C_{uf}) / (C_f - C_{uf}) \quad (2)$$

where $\delta^{13}C_f$ and C_f are the $\delta^{13}C$ values and amount of C in fumigated samples and $\delta^{13}C_{uf}$ and C_{uf} are the $\delta^{13}C$ values and amount of C in non-fumigated samples, respectively.

Based on the continuous ^{13}C labelling method, we calculated the metabolic quotient (qCO_2) by obtaining the rate of soil-derived CO_2 production per unit of soil-derived microbial C and unit of time.

2.4.2 Contribution of root-derived and soil-derived C to rhizosphere and bulk soil, CO_2 and MBC

The contributions of soil-derived (%soil-C) and root-derived C (%root-C) to rhizosphere soil (RS), bulk soil (BS), MBC and soil CO_2 were calculated using linear two-source mixing models (Kuzyakov and Bol, 2006):

$$\%Soil\text{-}C_t = (\delta^{13}C_t - \delta^{13}C_{root}) / (\delta^{13}C_{soil} - \delta^{13}C_{root}) \quad (3)$$

$$\%Root\text{-}C_t = 100 - \%Soil\text{-}C_t \quad (4)$$

where $\delta^{13}C_t$ represents the C isotopic ratio of rhizosphere soil, bulk soil, soil CO_2 efflux, or MBC. $\delta^{13}C_{root}$ is the C isotopic ratio of the respective root and $\delta^{13}C_{soil}$ is the mean C isotopic ratio of the respective pool/flux in the unplanted control.

To calculate absolute soil- and root-derived CO_2 fluxes, the percentage was multiplied with the total C in the efflux.

2.4.3 Rhizosphere priming effect

The rhizosphere priming effect (RPE, mg C kg^{-1} soil day^{-1}) on SOM decomposition was calculated as the difference in absolute soil-derived CO_2 between planted and unplanted soils. The calculations were done separately for soils with and without N fertilization.

$$[RPE]_{fertilized} = [C_{soil\text{-}planted}]_{fertilized} - [C_{soil\text{-}control}]_{fertilized} \quad (5)$$

$$[RPE]_{unfertilized} = [C_{soil\text{-}planted}]_{unfertilized} - [C_{soil\text{-}control}]_{unfertilized} \quad (6)$$

where $[C_{soil\text{-}planted}]_{fertilized}$ and $[C_{soil\text{-}planted}]_{unfertilized}$ (mg C kg^{-1} soil day^{-1}) were CO_2 emitted by bare control soil with and without N fertilization, respectively.

2.5 Statistical analysis

The experiment was carried out with 4 replicates of each treatment harvested at each of the 3 time points. The values presented in the figures are given as means ± standard error (mean ± SE). AMF colonization was analyzed using a generalized linear model (GLM) following a binomial distribution in R studio (v 3.4.1). Prior to analysis of variance (ANOVA), where data from each sampling time were analyzed separately, the data sets have been tested for normality and homogeneity of variance by Shapiro-Wilk ($P > 0.05$) and Levene-test ($P > 0.05$), respectively. Any data that were not fit for normal distribution were log transformed. A two-way ANOVA was used to

assess the effects of tomato genotypes and N fertilization, as well as their interaction for all measured parameters separately for each sampling time. Significant ($P < 0.05$) differences between means were identified using the *post hoc* Tukey HSD test. Residuals were checked for a normal distribution using the Shapiro-Wilk test. All ANOVA analyses were performed using SPSS version 19.0 (SPSS Inc., USA).

3. Results

3.1 Plant biomass and isotope enrichment

Plant growth was unaffected by AMF colonization; both genotypes had similar shoot and root biomass, especially in the unfertilized soil (Fig. 1). N fertilization suppressed root biomass at early stages (e.g., 8 and 12 weeks after transplanting) (Fig. 1, $P < 0.05$) and increased shoot biomass 12 and 16 weeks after transplanting by 33-68% (Fig. 1, $P < 0.01$). The C/N ratio of shoot and root was not affected by AMF colonization, indicating stable steady-state plant nutrition. However, N fertilization decreased the C/N ratio of shoot and roots, except at the last sampling (Table. S1, S2, $P < 0.05$).

The plants were successfully labeled with $^{13}CO_2$ (Table. S1). The δ ^{13}C values ranged from 638‰ to 698‰ in shoots and from 604‰ to 680‰ in roots.

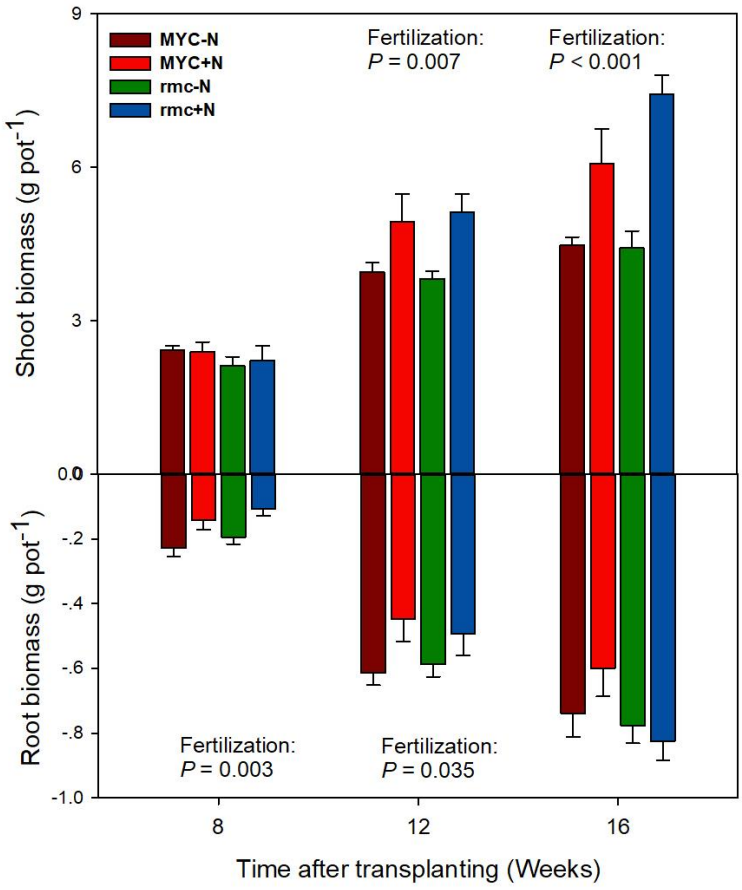

Fig. 1 Shoot and root biomass of mycorrhizal wild type (MYC) and mutant (rmc) tomato with reduced mycorrhizal colonization with and without N fertilization over a 16 weeks plants´ growth period. Values shown are presented as means (n=4) ± standard error (SE). *P* values were calculated by using a two-way ANOVA for each sampling time (e.g. 8, 12, 16 weeks after transplanting). N reflects the N fertilization. Fertilization: with and without N; Genotype: MYC and rmc.

3.2 Abundance of arbuscular mycorrhizal fungi

The AMF colonization for MYC and rmc tomato roots was similar and less than 1% of root length (data not shown) at 8 weeks after transplanting. However, the root lengths colonized by AMF for MYC plants were 21-37% and 34-58% at 12 and 16 weeks, respectively (P < 0.001). Only 2.5% and 7% of rmc root length were colonized at 12 and 16 weeks, respectively. N fertilization strongly decreased AMF colonization of MYC plants by 69% after 12 weeks and by 66% after 16 weeks following transplanting.

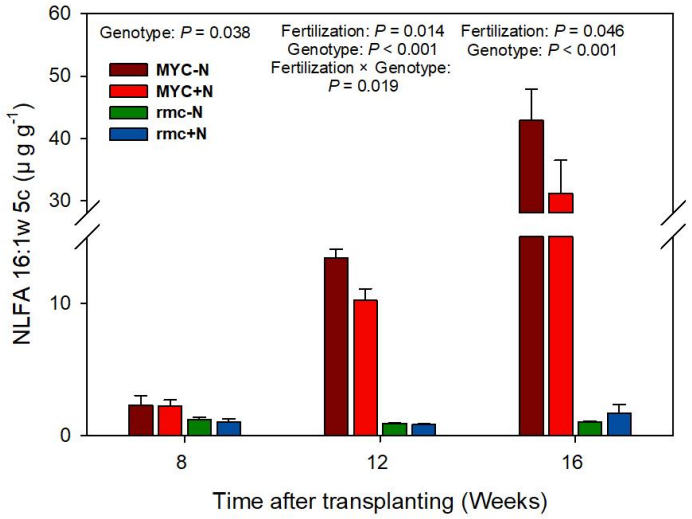

Fig. 2 Neutral lipid fatty acid (NLFA) 16:1ω5c of mycorrhizal wild type (MYC) and mutant (rmc) tomato with reduced mycorrhizal colonization with and without N fertilization across 16 weeks of plant growth. Values shown are means (n=4) ± standard error (SE). P values were calculated by using a two-way ANOVA for each sampling time (e.g. 8, 12, 16 weeks after transplanting). N reflects the N fertilization. Fertilization: with and without N; Genotype: MYC and rmc.

Eight weeks after transplanting, the neutral lipid fatty acid (NLFA) 16:1ω5c in soil under MYC plants accounted 2.2 µg g^{-1}, reflecting 1-fold higher values than that in soil under rmc plants (1.15 µg g^{-1}) (Fig. 2, P < 0.05). At later growth stages (12 and 16 weeks after transplanting), the amount of NLFA 16:1ω5c was 11-39 times higher in soil under MYC than under rmc plants (Fig. 2; P < 0.05). N fertilization showed a negative effect on NLFA 16:1ω5c in the soil under MYC plants (P < 0.05), whereas there was no significant difference in soil under rmc plants.

3.3 Soil mineral N and available P

Mineral N was reduced by 90% in planted versus unplanted soil over the 16 weeks of plant growth. Not expected was the lower NH_4^+ concentration in soil under MYC than that for rmc plants at 16 weeks (Fig. S1a, P < 0.05). N fertilization significantly increased the soil NO_3^- concentration solely 8 weeks after transplanting (Fig. S1b, P < 0.001).

Planting decreased soil-available P throughout the experiment (Fig. S1c). Soil

available P was 25% lower in soil planted with MYC than for rmc plants at the latest sampling date ($P < 0.01$). Here, N fertilization did not change the available P concentrations during the plant growth period.

3.4 Carbon input into the soil

Eight weeks after transplanting, root-derived C in the rhizosphere soil was 1.7 to 2.3 times higher in unfertilized versus fertilized soil (Fig. 3a, $P < 0.001$). At later growth stages, there was no significant effect of fertilization on rhizodeposition for rmc plants. The net rhizodeposition of MYC plants, however, decreased by 21% and 75% in the fertilized compared with unfertilized soil at 12 and 16 weeks, respectively. At 12 and 16 weeks, the symbiosis with AMF (MYC plants) increased the amount of assimilated C remaining in the bulk soil (Fig. 3b, $P < 0.05$).

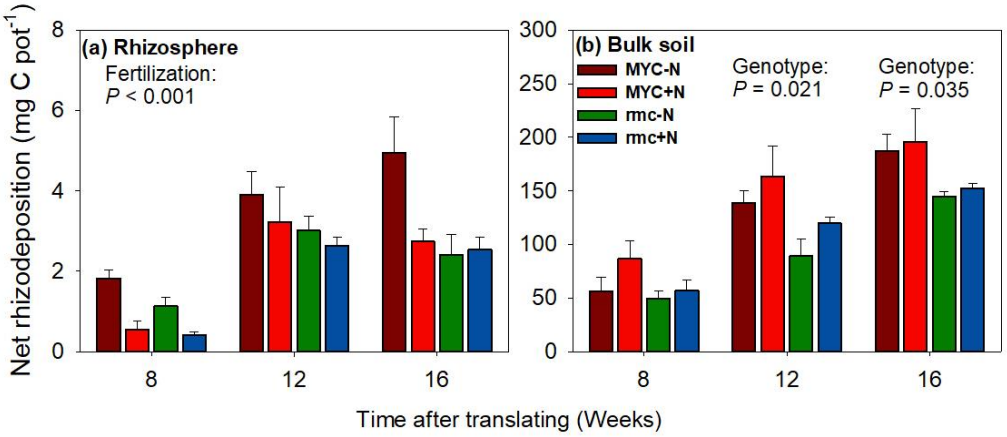

Fig. 3 Rhizodeposition remaining in rhizosphere (a) and bulk soil (b) at harvest (net rhizodeposition) of mycorrhizal wild type (MYC) and mutant (rmc) tomato with reduced mycorrhizal colonization with and without N fertilization over a 16 weeks growth period. Values shown are means ± standard error (SE) (n=4). *P* values were calculated from two-way ANOVA for each sampling (e.g. 8, 12, 16 weeks after transplanting). Fertilization: with and without N; Genotype: MYC and rmc. Net rhizodeposition means the total amount of ^{13}C remaining in soil at the time of harvest.

The ratio of net rhizodeposition in the soil (rhizosphere + bulk) to roots was higher for MYC (0.23-0.37) than for rmc plants (0.27-0.34) after 12 and 16 weeks (Fig. S2a, $P < 0.05$). N fertilization increased the ratio of net rhizodeposition-to-roots by 1.0 and 0.5 times compared to unfertilized soil at 8 and 12 weeks, respectively ($P < 0.01$).

Overall, MYC plants had 33% higher net rhizodeposition than rmc plants in the soil (rhizosphere + bulk) without fertilization at 16 weeks despite similar root biomass (Fig. 1, 3).

3.5 Carbon output from the soil

Total soil CO_2 efflux was influenced by the presence of plants, genotype and N fertilization. Planting increased total CO_2 efflux by 25-150% during the growth period, while N fertilization decreased the total CO_2 efflux by 6-22%. Moreover, the CO_2 efflux was not affected by plant genotypes at early growth stages (e.g., 8 and 12 weeks) whereas lower efflux in MYC compared with rmc plants was observed at 16

weeks (Fig. 4, $P < 0.001$).

Similarly, soil-derived CO_2 was unaffected by plant genotypes at 8 and 12 weeks, although that of MYC (~ 25 mg C kg^{-1} day^{-1}) was lower than rmc plants (~ 32 mg C kg^{-1} day^{-1}) (Fig. 4a, $P < 0.01$). In the planted pots, N fertilization decreased soil-derived CO_2 by 17-37%, 2-26% and 17-31% compared to unfertilized plants at 8, 12 and 16 weeks, respectively (Fig. 4a, $P < 0.05$).

Root-derived CO_2 decreased by 34-65% for MYC compared to rmc plants within 16 weeks (Fig. 4b, $P < 0.05$). Root-derived CO_2 of N-fertilized soil was 56-61% and 10-52% higher than that from unfertilized soil 8 and 16 weeks after transplanting, respectively, but it was similar between N-fertilized and unfertilized soil at 12 weeks (Fig. 4b).

3.6 Rhizosphere priming effect

Plants induced positive RPE regardless of plant genotypes (MYC and rmc), N fertilization and plant growth stages. RPE did not differ between MYC and rmc plants at 8 weeks. At 12 weeks, however, RPE was 54% higher for MYC than for rmc plants without fertilization ($P < 0.05$). The patterns changed at 16 weeks, where the rmc showed a significantly higher RPE than the MYC genotype with AMF colonization (Fig. 4c, $P < 0.01$). The RPE increased by 16-23% for MYC plants compared to the unplanted soil. The greatest positive RPE（44-60%）was measured for rmc plants with reduced AMF colonization. This was obviously accompanied with N fertilization, which reduced the RPE, especially at earlier plant growth stages (e.g., 8 and 12 weeks) (Fig. 4c, $P < 0.05$).

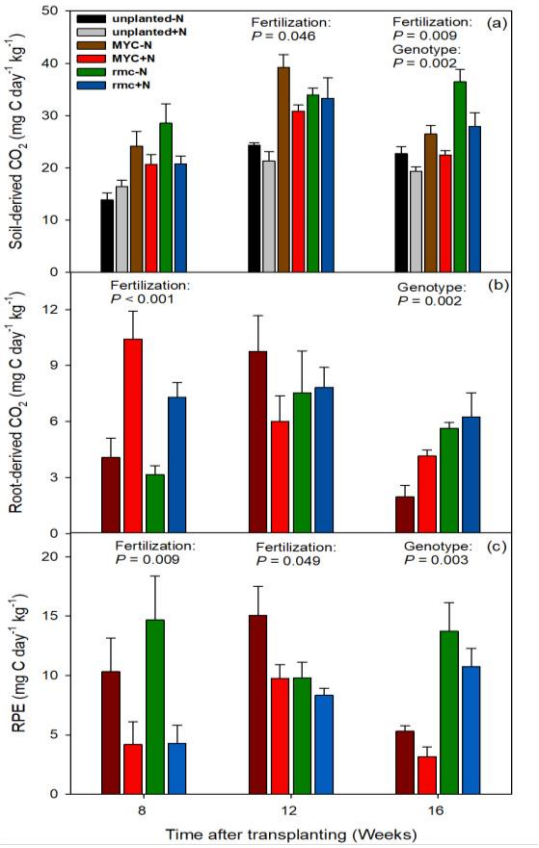

Fig. 4 Soil-derived (a), root-derived CO_2 (b), and rhizosphere priming effect (RPE) (c) of mycorrhizal wild type (MYC) and mutant (rmc) tomato with reduced mycorrhizal colonization with and without N fertilization over a 16 weeks growth period. Values shown are means (n=4) ± standard error (SE). *P* values were calculated by two-way ANOVA for each sampling (e.g. 8, 12, 16 weeks after transplanting). Fertilization: with and without N; Genotype: MYC and rmc.

3.7 Microbial metabolic quotient

The rate of soil-derived CO_2 production per unit of soil-derived MBC and unit of time (qCO_2) was 28% higher in MYC than in rmc soil 12 weeks after transplanting. At 16 weeks, a 36-44% reduction was recorded for MYC plants (Fig. S2b, $P < 0.01$). qCO_2 declined (~45%) with N fertilization at 8 weeks (Fig. S2b, $P < 0.05$), then the negative effect diminished in the later periods.

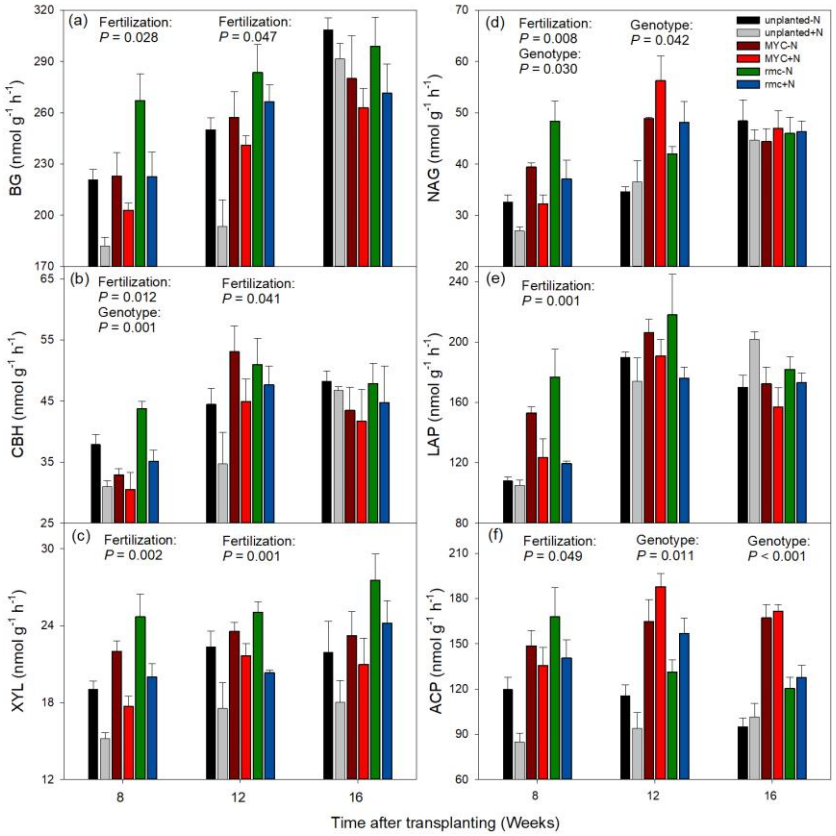

Fig. 5 Potential enzyme activities (V_{max}; nmol g^{-1} soil h^{-1}) of BG (β-1, 4-glucosidase, a), CBH (Cellobiohy-drolase, b), XYL (xylanase, c), NAG (β-1, 4-N-acetyglucosaminidase, d), LAP (leucine aminopeptidase, e), ACP (acid phosphatase, f) of mycorrhizal wild type (MYC) and mutant (rmc) tomato with reduced mycorrhizal colonization with and without N fertilization over a 16 weeks growth period. Values shown are means (n=4) ± standard error (SE). *P* values were calculated by using a two-way ANOVA for each sampling time (e.g. 8, 12, 16 weeks after transplanting). Fertilization: with and without N; Genotype: MYC and rmc.

3.8 Enzyme activities

The potential activities of BG, XYL, CBH, NAG, LAP and ACP increased by planting (except at 16 weeks) (Fig. 5), reflecting plants as a meaningful additional source of enzyme production. This points to strong rhizosphere activity for balanced nutrient cycling. Similar BG, XYL and LAP activities were observed between plant genotypes during the growth period. The potential activity of ACP for MYC versus rmc plants was 20-26% and 34-39% higher at 12 and 16 weeks, respectively (Fig. 5f, $P < 0.05$). All the tested enzyme activities decreased after N fertilization at earlier plant growth stages ($P < 0.05$).

4. Discussion

4.1 Belowground plant carbon inputs

4.1.1 Effect of arbuscular mycorrhizal fungi on belowground plant C inputs

The response of plant biomass to AMF symbiosis was neutral or even negative in this study (Fig. 1). The stimulation of plant growth through mutualistic interaction with AMF is controlled by the balance between C costs and nutritional benefits, and hence, depends on climatic and edaphic conditions (Friede et al., 2016). In our study, the C cost for construction and maintaining the AMF association possibly exceeded the mycorrhizal growth benefits of enhanced nutrient acquisition as reported earlier (Jakobsen et al., 2016; Konvalinková et al., 2017). It has to be considered, that the plants were grown in pots, which may have increased the C cost compared to field trials due to nutrient constraints. The literature has also revealed that arbuscular mycorrhizal associations comprise a broad spectrum of plant growth responses, with positive (mutualism), neutral (commensalism), or even negative (parasitism) effects (Johnson et al., 1997; Klironomos, 2003; Friede et al., 2016).

The C allocation into belowground pools under MYC (42-46% of total belowground C) was higher than that under rmc plants (33-34%) during the 16 weeks of plant growth. This is in accordance with a study from Kong and Fridley (2019) who reported mutualistic microbes reduced the allocation of C to root mass but increased C allocation to soil. Higher C inputs under plants with AMF are explained by higher amounts of assimilates allocated to the development of extraradical mycelium and spores (Fig. 2; Olsson and Johnson, 2005), and by the extremely long residence time of glomalin-related soil protein released from AMF (Rillig, 2004). Importantly, net rhizodeposition in the current experiment also includes AMF hyphae and their exudation. The symbiotic AMF interactions are known to transfer C away from the rhizosphere to the bulk soil (less microbial activity) (Zhu and Miller, 2003; Herman et al., 2012; Hafner et al., 2014), and thus to facilitate C sequestration. In summary, the AMF symbiosis increased net rhizodeposition in the rhizosphere and the bulk soil, due to the important pathway of C inputs through AMF hyphae, an interpretation supported by the higher net rhizodeposition per unit of root biomass under MYC plants.

4.1.2 Effect of N fertilization on plant C inputs and allocation

N fertilization increased aboveground biomass and decreased root biomass, especially during the earlier growth periods (e.g., 8 and 12 weeks; Fig. 1), reflecting less of plants´ C was used to built-up root structure. Compared to fertilized plants, unfertilized plants generally allocated relatively more C into the belowground: roots as well as rhizosphere and bulk soil (Fig. S3). This result is consistent with the negative correlation between N fertilization and the allocation of newly plant-assimilated C to belowground pools and fluxes (Kuzyakov, 2001; Pausch and Kuzyakov, 2018). The higher belowground C inputs without fertilization reflected predominant responses on a stimulated root biomass production under N-deficiency, implying optimal partitioning of resources between shoots and roots (Farrar and Jones, 2000). Maintaining high microbial activity in unfertilized soil (e.g., enzyme activity, Fig. 4) requires more available C and energy input by roots into the soil for microbial mineralization of SOM and nutrient mining to meet the nutrient demand of the plants (Phillips et al., 2011).

N fertilization enhanced the net rhizodeposition per unit of root biomass (Fig. S2a), boosting plants´ C for N and P trading potential. At the same time, however, less root

biomass and promoted mineralization of newly rhizodeposited C by N inputs (Zang et al., 2019) led to similar absolute amounts of net C remaining in the soil irrespective of N fertilization (Fig. 3). The fast turnover of rhizodeposits under N fertilization is connected with the stimulation of enzymes to degrade labile C (i.e. rhizodeposited C) (Loeppmann et al., 2016b) and with the inhibition of enzyme activities for the decomposition of more recalcitrant SOM (Fig. 6; Dijkstra et al., 2013; Zang et al., 2017). This resulted in a lower SOM decomposition but higher root and rhizomicrobial CO_2 emissions (Fig. 4). Therefore, the overall effect of N fertilization on net rhizodeposition is difficult to predict due to the complex processes affected by N availability, including belowground C allocation, turnover and stabilization.

4.1.3 The interactive effects of arbuscular mycorrhizal fungi and N fertilization on plant C allocation and belowground C inputs

We recorded a reduced C flow to the belowground pools and less AMF abundance (NLFA 16:1ω5c), as well as mycorrhizal colonization of roots for MYC plants with N fertilization (Fig. 2; Fig. S3; Table. S1). The difference in net rhizodeposition of MYC and rmc plants was smaller in fertilized than in unfertilized soil (Fig. 3), suggesting that the balance between plant and AMF symbiosis largely depends on soil nutrient availability. Firstly, plants invested relatively more resources to aboveground biomass under N fertilization. This resulted in reduced relative belowground C inputs (Fig. S3) and less C allocation to the symbiotic fungi, as indicated by lower AMF colonization and NLFA 16:1ω5c (Fig. 2; Table. S1). This confirms the proposed assumption that plants prefer to allocate more C to their symbionts in order to be able to trade for nutrients under scarcity conditions (Schleuss et al., 2015). Secondly, N fertilization reduced the C availability to the AMF symbiosis (Olsson et al. 2005) due to the decrease of root biomass (Fig. 1). Thirdly, high soil N availability stimulated plant growth (higher aboveground biomass) and thus increased the competition for essential resources between roots, including AMF and neighboring microorganisms (Kuzyakov and Xu, 2013; Konvalinková et al., 2017). This, in turn, may have reduced the development of AMF. Accordingly, AMF growth and their C sink strength are reduced by N fertilization.

4.2 Rhizosphere priming effect on soil organic matter decomposition

RPE was consistently positive (16-101%) during the 16 weeks of plant growth (Fig. 4c). We explain this by the “microbial activation hypothesis”. It assumes that the activity and growth of microorganisms are enhanced through metabolizing labile substrates (e.g., root exudates), leading to an accelerated SOM turnover (Kuzyakov, 2002; Cheng and Kuzyakov, 2005). This was supported by the positive correlation between MBC and SOM decomposition (Fig. S4a, $P < 0.001$), and between BG, XYL, CBH, NAG and LAP activities and SOM decomposition (Fig. S4b, c, d, e, f, $P < 0.05$). The positive correlation suggests that rhizodeposit-induced microbial enzyme production as an important mechanism for RPE (Phillips et al., 2011; Blagodatskaya et al., 2014).

The intensity of RPE on SOM decomposition decreased by two thirds in fertilized versus unfertilized pots at earlier stages (e.g., 8 and 12 weeks), but did not differ at 16 weeks (Fig. 4c). Microorganisms accelerated SOM mineralization for nutrients under N-deficiency to meet their stoichiometric ratios (Dijkstra et al., 2013; Kirkby et al., 2013) and here, consequently induced positive RPE. By contrast, soil microorganisms could have down-regulated the synthesis of enzymes in response to higher N supply (Fig. 5), leading to a suppressed RPE (Craine et al., 2007; Phillips et al., 2011). The N reduced SOM decomposition and corresponded with decreased C-related enzyme activities (Fig. 5a, b, c). This suggests that N fertilization increased available plant C sources to support surrounding microbes, thus reducing the production of carbohydrate hydrolase (Chen et al., 2014). The higher C/N ratio of unfertilized roots (Table. S1) reflects a large N demand, yielding a higher RPE (Pausch et al., 2016). Additionally, the decreased RPE after N fertilization could reflect microbial preferential utilization of root-released labile C stimulated by fertilization (Zang et al., 2017). This was also supported by the increased root-derived CO_2 in the rhizosphere (Fig. 4b) under fertilization, especially in the earlier growth periods. Accordingly, N fertilization suppressed RPE due to the reduced nutrient demand of soil microbes and to their preferential utilization of root-released C.

At the end of the experiment, more root-derived C was retained in soil under MYC versus rmc plants, reflecting the increased C use efficiency as supported by the decreased qCO_2 (Fig. S2b). The lower rhizosphere respiration of MYC plants (16 weeks after transplanting) represented less readily available plant-derived C (Fig. 4b), which further decreased the activated microorganisms and thus the demand for nutrients from SOM mining (Fontaine et al., 2011). Moreover, AMF symbiosis is known to increase the abundance of large macroaggregates by temporary binding agents, such as glomalin-related soil protein (Rillig, 2004; Rillig and Mummey, 2006), enabling physically protected rhizodeposited-C against microbial degradation. Therefore, a weaker positive RPE was revealed in soil planted with MYC compared with rmc 16 weeks after transplanting.

Although AMF probably cannot decompose SOM directly, due to a lack of saprotrophic capacity (Read and Perez-Moreno, 2003), they may still be involved in decomposition processes. These include the stimulation or the inhibition of saprotrophic fungal activity via the release of labile C by AMF, or by affecting the competition for nutrients between AMF and saprotrophs (Talbot et al., 2008). In the current study, the higher NLFA 16:1ω5c is associated with higher external hyphae production in soil planted with MYC (Fig. 2). These hyphae actively scavenge soil for nutrients, making them highly efficient for nutrient uptake (Verbruggen et al., 2016). This is indicated by both, the reduced NH_4^+ and available P content in soil under MYC plants (Fig. S1), as well as by the slightly higher C/N of soil under MYC versus rmc plants (Table. S1, 2). The reduced N and P availability further imposed nutrient

limitation for free-living decomposers and reduced their activities in bulk soil (Nottingham et al., 2013; Brzostek et al., 2015). In turn, this process contributed to soil C build-up in the respective areas. AMF are less limited by C than saprotrophic fungi due to direct C allocation from the plant hosts (Drigo et al., 2010). Thus, AMF may produce secondary metabolites that are antagonistic against free-living saprotrophic fungi, retarding saprotrophic activity (Keller et al., 2005; Fernandez and Kennedy, 2016). The efficient substrate uptake of nutrients by AMF may have restricted the activity and nutrient use of free-living decomposers. This is the most plausible mechanism for increased C retention by AMF symbiosis.

5. Conclusions

Plants´ symbiosis with arbuscular mycorrhizal fungi (AMF) (MYC plants) decreased the relative carbon (C) allocation to roots compared to plants with reduced mycorrhizal colonization (rmc plants). However, the absolute amount of net rhizodeposition increased after 12 and 16 weeks. AMF declined the rhizosphere priming effect (RPE) compared to rmc plants, which reflects the increasing nutrient limitation with time, especially the available soil P. These conditions likely restricted activity of free-living microorganisms in the bulk more than in the rhizosphere soil. Despite the reduced root biomass, N fertilization facilitated C sequestration by increased net rhizodeposition and decreased soil organic matter decomposition. Moreover, N fertilization lowered the magnitude of RPE, because of decreased enzyme activities that indicated a lowered microbial N demand. Without fertilization, the amount of net rhizodeposition in the rhizosphere and bulk soil for MYC plants increased by 20% and 30% compared to rmc plants, respectively. N fertilization decreased the net C rhizodeposition induced by AMF symbiosis, which may partly reflect the more restricted mycorrhizal abundance. In conclusion, AMF symbiosis and N fertilization increase soil C sequestration by retaining more plant rhizodeposits (net rhizodeposition) and by reducing the rhizosphere priming effect on the decomposition of soil organic matter.

Acknowledgments

We thank the China Scholarship Council (CSC) for funding to Jie Zhou in Germany. This study was financially supported by the German Research Foundation (DFG) within the project PA 2377/2-1, GU 1309/5-1. The continuous ^{13}C labeling was conducted in the Gabriele Lehmann and Rainer Schulz of the Laboratory for Radioisotopes (LARI), Göttingen. The isotopic analyses were performed at the Centre for Stable Isotope Research and Analysis (KOSI), Göttingen. The seeds for the experiment were kindly supplied by Dr. Susan Barker (Faculty of Sciences, School of Agriculture and Environment, The University of Western Australia, Australia). Furthermore, we want to acknowledge Dr. Bernd Horneburg,

Georg-August-University Göttingen, Dept. of Crop Science for his comments to the topic. The authors also would like to thank Karin Schmidt and Bernd Kopka for their laboratory assistance. Special thanks to Jun. Prof. Dr. Michaela A. Dippold, Biogeochemistry of Agroecosystems, Göttingen, for the help in lipid analysis. We further thank three unknown reviewers and the editor for detailed comments and suggestions on this manuscript.

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Supplementary

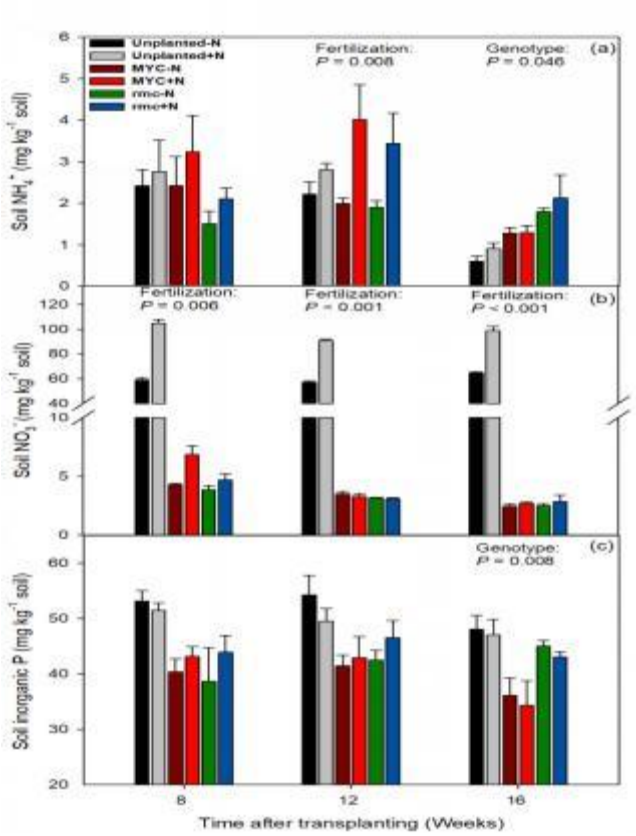

Fig. S1 Soil NH_4^+ (a), NO_3^- (b) and inorganic P (c) content of mycorrhizal wild type (MYC) and mutant (rmc) tomato with reduced mycorrhizal colonization with and without N fertilization.

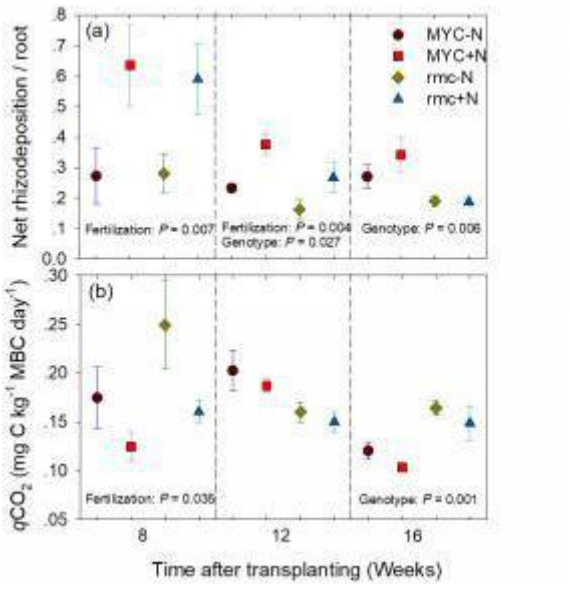

Fig. S2 The ratio of net rhizodeposition (rhizosphere+bulk soil) and root biomass (a), and microbial metabolic quotient (qCO_2) (b) of mycorrhizal wild type (MYC) and mutant (rmc) tomato with reduced mycorrhizal colonization with and without N fertilization.

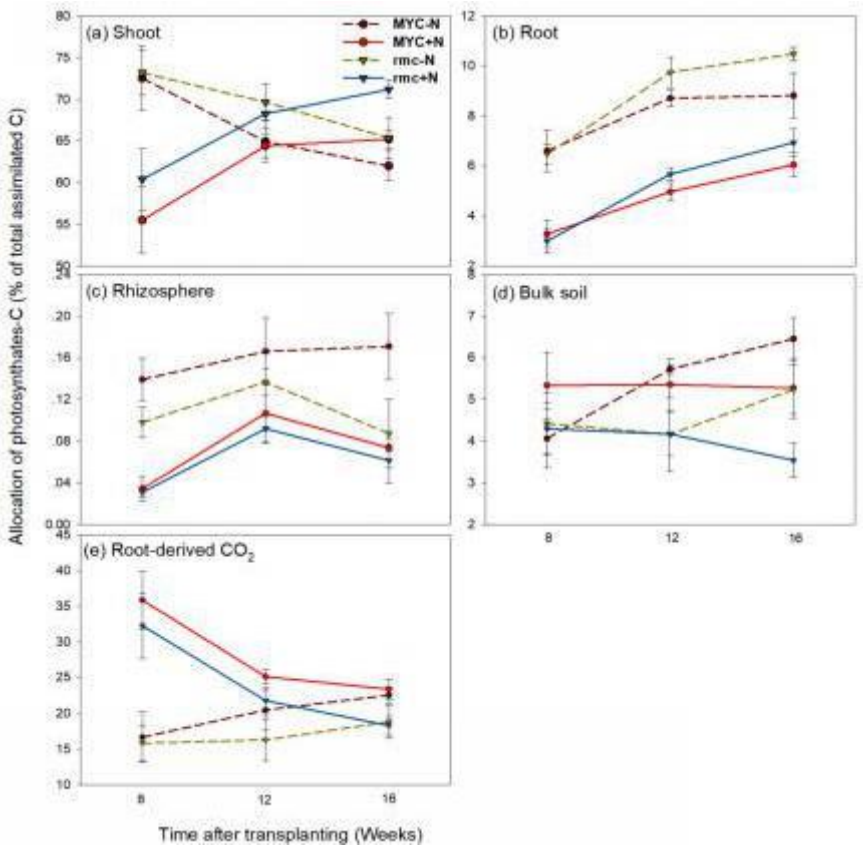

Fig. S3 Allocation of photosynthate-C (% of total assimilated C) into shoots (a), roots (b), rhizosphere (c), bulk soil (d), and root-derived CO_2 (e) of mycorrhizal wild type (MYC) and mutant (rmc) tomato with reduced mycorrhizal colonization with or without N fertilization over a 16-weeks growth period. Values shown are means (n=4) ± standard error (SE).

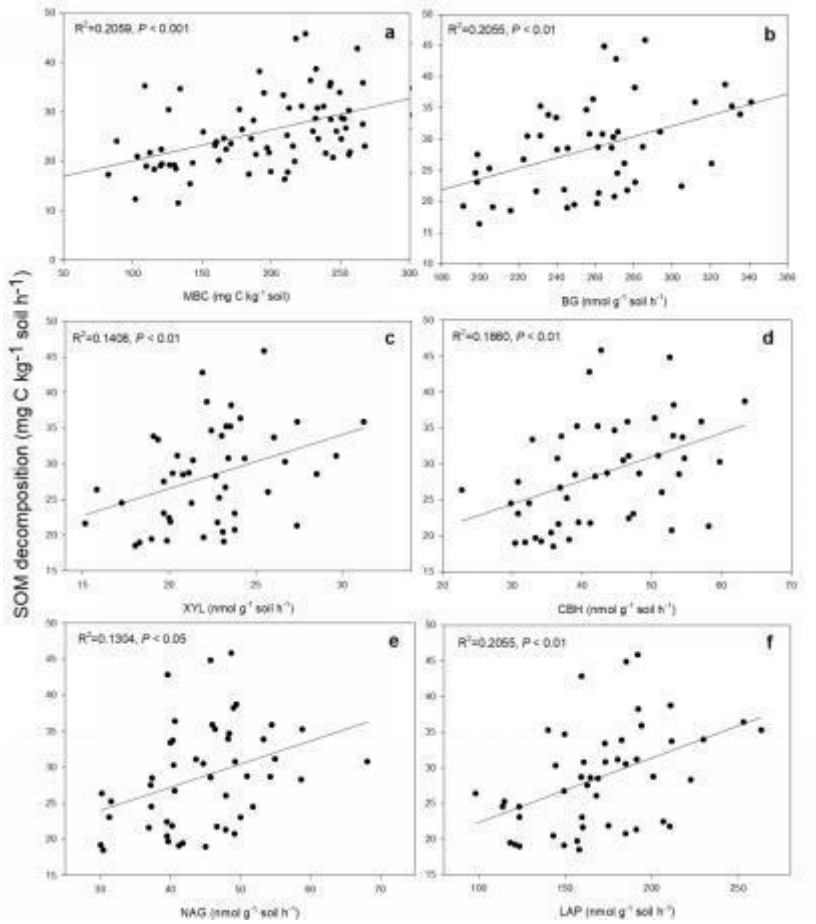

Fig. S4 Relationship between soil organic matter (SOM) decomposition and microbial biomass carbon (MBC) (a), β-1,4-glucosidase(BG) (b), xylanase (XYL) (c), cellobiohydrolase (CBH) (d), β-1,4-N-acetyglucosaminidase (NAG) (e), and leucine aminopeptidase (LAP) (f) of mycorrhizal wild type (MYC) and mutant (rmc) tomato with reduced mycorrhizal colonization with or without N fertilization over a 16-weeks growth period. Values shown are means (n=4) ± standard error (SE).

Study 2 Arbuscular mycorrhizae prominent in the rhizosphere carbon uptake and shift the function of saprotrophic microorganisms

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Abstract

The overall processes by which carbon (C) is fixed by plants in photosynthesis then released into the soil by rhizodeposition and subsequently utilized by soil microorganisms, links the atmospheric and soil C pools. Taken together, large fraction of C stored in the soil was allocated through arbuscular mycorrhizal fungi (AMF). However, little is known about the path of rhizodeposited-C into soil-borne communities in response to AMF symbiosis and nitrogen (N) fertilization. A mycorrhiza defective mutant of tomato (reduced mycorrhizal colonization: rmc) and its mycorrhizal wild type progenitor (MYC) were used to control for the formation of AMF symbiosis. Continuous $^{13}CO_2$ labeling was performed to quantify the photosynthetic C allocation in the specific active microbial communities via ^{13}C profile of microbial neutral (NLFA) and phospholipid fatty acids (PLFAs) depending on AMF symbiosis and N fertilization.

Based on the abundance and ^{13}C enrichment results, the ^{13}C incorporation into fungal biomarker (PLFA 16:1ω5c, NLFA 16:1ω5c, and PLFA 18:2ω6, 9) was increased with sampling time over 16-weeks incubation, and AMF was prominent in the plant-soil system in the end of experiment (4.62% of total assimilated C). Although only less ^{13}C was incorporated into AMF hyphal biomass (PLFA 16:1ω5c, 0.12-0.25%), there was significant allocation of ^{13}C into AMF storage (NLFA 16:1ω5c, 3.09-4.07%) in the soil with AMF symbiosis. This suggested that AMF symbiosis play a main role in rhizodeposited C uptake, as well as long-term retention of rhizodeposited C in AMF spores, which could be of importance to soil organic C sequestration. However, high N availability negative impacted on AMF symbiosis and further rhizodeposited C recovered in the AMF, which resulted from less C allocation to belowground because of higher C immobilization in the aboveground and higher rhizosphre respiration. Followed by AMF, gram-negative bacteria also took up a significant proportion of the belowground allocated C. However, SAP fungi showed a less reliance on rhizodeposited C throughout the growth period which may due to a lower use of rhizodeposits or a preference to older C compounds as energy sources. AMF symbiosis shifted the microbial community composition, resulting in a lower ^{13}C incorporation into the bacteria, SAP fungi compared to the soil with rmc plants. These results suggested that plant-derived C was more allocated to AMF storage for later use rather than translocated to free-living microorganisms or respiration, as supported by the higher ratio of NLFA and PLFA. The preferential C allocation to AMF was at the expense of C flow to other microbial groups may form a alternative explanation for the lower enrichment of ^{13}C in the other free-living microorganisms. Overall, our results confirmed that mycorrhizal plant exerting a greater influence on both bacterial and SAP fungal communities, which was highly dependent on N fertilization. We also concluded that root C is predominantly transloacted by AMF symbiosis, which will be most favoured without fertilization.

Keywords: arbuscular mycorrhizal fungi (AMF), microbial utilization, N fertilization, rhizodeposits, bacteria and fungi, soil carbon assimilation, ^{13}C-phospholipid fatty acid

1. Introduction

Plants are the primary source of carbon (C) for soil microorganisms and soil organic C (SOC) (Ge et al., 2017). Living roots release approximately 5-20% of photosynthesized C into soil via rhizodeposition (Kuzyakov and Domanski, 2000; Jones et al., 2009; Pausch and Kuzyakov, 2018). Rhizodeposits are used by rhizomicrobial communities to fuel metabolic processes and build up microbial biomass (Kindler et al., 2009), which eventually contributes to (SOC stabilization through this microbial transformation (Cotrufo et al., 2013; Sokol and Bradford, 2019). However, knowledge with respect to the relative flow and translocation of plant-derived C among microbial functional groups, is still limited (Drigo et al., 2010; De Deyn et al., 2011; Birgander et al., 2017), as groups of soil biota (in particular bacteria, mycorrhizal fungi and saprotrophic fungi) function differently in the incorporation and turnover of C, in the chemical nature of their respective byproducts, and in their respective effects on soil biogeochemical cycles (Six et al., 2006; Strickland and Rousk, 2010). This limited understanding is a critical uncertainty of soil C formation and stabilization.

Next to direct root exudation, plants influence soil microorganisms through symbiotic relationships in which microorganisms receive C directly from the plants (Jones et al., 2009). Arbuscular mycorrhizal fungi (AMF) form symbioses with 71% of all flowering plants (Brundrett and Tedersoo, 2019), and have been recognized as a potentially important functional group involved in the sequestration of plant-derived C (Frey-Klett et al., 2007; Averill et al. 2014; Wurzburger and Brookshire 2017; Zhou et al., 2020). Given that large quantities of recently fixed C can be transferred to AMF from their autotrophic symbiont (Johnson et al., 2002), AMF have a competitive advantage over other free-living microorganisms in the acquisition of soil resource with such a large of energy (Nottingham et al., 2013). Competition among AMF and other free-living microorganisms for soil nutrients can lead to increased soil C stocks as microbial activity is suppressed (Averill et al., 2014; Zhou et al., 2020). On the other hand, AMF can facilitate free-living microbial communities by releasing organic compounds, which stimulate microbial growth more than non-mycorrhizal plants (Barea et al. 2005). As a feedback, alterations in soil microbial composition could have significant consequences for C and N transformations (Singh et al., 2010). For instance, it has been postulated that bacteria-dominated food webs lead to higher short-term mineralization rates of organic C and N (Wardle et al., 2004), whereas fungal stimulation, in particular of AMF, may enhance C uptake (Treseder and Allen, 2000), and N immobilization through hyphal translocation (Frey et al., 2000). Although recent progress has been made in our understanding of C fluxes from the plant to AMF, and rhizosphere microorganisms, knowledge is still scare with respect to the relative flow of C to specific biological groups in plant-AMF symbiosis-soil systems (Olsson and Johnson, 2005; Drigo et al., 2010). Therefore, precise identification of the microbial groups involved in C utilization and sequestration is the key to explore the functional roles of microorganisms improving soil fertility.

Up to 30% of C fixed during photosynthesis can be allocated to the AMF (Drigo et al., 2010), which is not constant, but depends on soil N availability (Treseder, 2004; Mohan et al., 2014). The abundance of AMF in general declines by 15% in ecosystems exposed to mineral N fertilization (Treseder, 2004; Zhou et al., 2020), thereby affecting AMF functions as well as other free-living microbial community structures and functions (Toljander et al., 2007). However, there is still a general lack of knowledge with respect to the relative responses of different specific microbial groups in response to AMF and N fertilization.

Phospholipid fatty acids (PLFAs) are essential components of cellular membranes,

several of which have been used as biomarkers for specific microbial groups (Frostegård et al., 1993; Zelles, 1997). Determination of neutral (NLFA) and PLFAs in combination with stable isotope probing have provided an efficient approach to follow the C flow to soil microorganisms, as well as study the relative importance of rhizodeposits in determining specific microorganisms (Butler et al., 2003; Paterson et al., 2011; Drigo et al., 2010). In this study, we performed a continuous ^{13}C-CO_2 labeling experiment of a mycorrhizal wild type progenitor (MYC) and its mycorrhizal defective mutant of tomato (rmc) with or without N fertilization during 16-weeks incubation. We expected that AMF should be most benefited in the soil plated with MYC plants, which is dependent on the N availability. Because AMF have the most direct connection with plant rhizosphere allocation and should thus respond more than other free-living microorganisms (e.g., bacteria and SAP fungi). We also anticipated that SAP fungi is less affected by the AMF symbiosis than the bacteria, since it has been reported that they are less dependent on the rhizodeposits in the soil (Birgander et al., 2017). Moreover, we aimed to (i) assess the effect of AMF symbiosis and N fertilization on the incorporation and fate of rhziodeposit-C into microorganisms; (ii) identify predominant microbial groups utilizing newly rhizodeposit-C. Hence, we hypothesized that: (1) root C is predominantly transloacted by AMF symbiosis, which will be most favoured without fertilization and will obtain significantly more C from plants than when soil is fertilized; (2) SAP fungi will be least affected by AMF symbiosis with regard to C flow from plants.

2. Materials and methods

2.1 Soil preparation, plant growth, and continuous ^{13}C labeling

Soil samples were collected from the Ap horizon (0-20 cm) of an experimental field at the Reinshof Research Station of the Georg-August University of Göttingen, Germany (28°33´26´´N, 113°20´8´´E). The soil was air-dried and sieved (< 2 mm) to achieve a high degree of homogeneity and reduce the variability among replicates. Fine roots and visible plant residues were carefully removed manually. The soil contained 1.3% total C, 0.14% total N, had a pH of 6.8, and a $\delta^{13}C$-value of organic C of -25.78‰, $\delta^{15}N$-value of total N of 5.69‰, and a bulk density of 1.30 g cm^{-3} (Zhou et al., 2020).

Two tomato genotypes (*Lycopersicon esculentum L.*) were grown in this experiment: 1) mutant tomato with highly reduced AMF symbiosis, termed rmc (reduced mycorrhizal colonization), and 2) a closely related wild type hereafter termed MYC (Barker et al., 1998). The use of genotypes enabled studying the impacts of AMF symbiosis on microbial C utilization, without soil sterilization to establish a non-mycorrhizal control, thereby maintaining an intact soil microbial community. Both tomato types were grown with and without N fertilization (e.g. MYC-N, MYC+N, rmc-N, and rmc+N). N-treatments received 344 mg N per pot (60% of N from NH_4^+ and 40% of NO_3^-), equivalent to a rate of 150 kg N ha^{-1}.

PVC pots (7.5 cm diameter, 21 cm height) were filled with 1 kg air-dried, sieved soil. The soil was kept at 20% gravimetric soil moisture content (equivalent of 60% of the water holding capacity) with deionized water. After pre-incubation at room temperature for two weeks, the seedlings in pots were moved to a growth chamber (day time of 14 h and 25 °C; night time of 10 h and 15 °C). The relative humidity was 40% and plants received artificial light with 800 μmol m^{-2} s^{-1} photosynthetic active radiation (PAR). The locations of the pots in the growth chamber were changed weekly by mixing them randomly to guarantee similar growing conditions for the plants. The soil water content of all pots was maintained at 60% water holding capacity by deionized water addition (every 1-3 days).

The growth chamber was equipped with a continuous $^{13}CO_2$ labeling system. Briefly, the $^{13}CO_2$ used in the experiment was generated through the reaction of $Na_2{}^{13}CO_3$ (2.9 atom% ^{13}C, 0.5 mol L^{-1}) and excess of lactic acid outside of the chamber. The tracer solution was prepared by mixing 1g of 99 atom% ^{13}C enriched Na_2CO_3 (Cambridge Isotope Laboratories, Tewksbury, MA, USA) with 52 g of unlabeled Na_2CO_3 in 1 L of deionized water. The plants were labeled from the emergence of the first leaf until harvest. They were watered during the dark period to avoid assimilation of unlabeled CO_2. After closing the chamber, the chamber air was pumped through external 50-L tight soda lime to remove unlabeled CO_2 and was then flushed with ^{13}C-enriched CO_2 before the light switched on and plants started photosynthesis.

Soil were sampled immediately after each harvest. A representative homogenized soil subsample of each pot was stored at 4 ^oC to determine microbial biomass C. Another soil sub-sample was stored at -20 ^oC to neutral lipid fatty acid (NLFA) 16:1ω5c, and phospholipid lipid fatty acids (PLFA).

2.3 Measurements

2.3.1 *Microbial biomass C*

Soil microbial biomass C (MBC) was determined by the chloroform fumigation extraction method (Vance et al., 1987) with modification. After destructive sampling, the soil was carefully mixed and 8 g soil was directly extracted for 1 h using 32 ml of 0.05 M K_2SO_4. Another 8 g soil was fumigated with chloroform for 24 h and extracted in the same manner. The samples were filtered and the extracts were frozen until analysis. Total C concentration was measured using a 2100 TOC/TIC analyzer (Analytik Jena, Germany). MBC was calculated by dividing the difference between extracted C from fumigated and non-fumigated soil samples with a k_{EC} factor of 0.45 (Wu et al. 1990). The extracted C contents from non-fumigated soil samples were considered as dissolved organic C (DOC). After freeze-drying (Beta 1–8 LSCplus, Martin Christ Gefriertrocknungsanlagen GmbH, Harz, Germany), the $\delta^{13}C$ of MBC was analyzed by an elemental analyzer (Eurovector) coupled to an IRMS (Delta Plus XL IRMS, Thermo Finnigan MAT, Bremen, Germany) at the Center for Stable Isotope Research and Analysis (KOSI) located at the Georg-August University of Göttingen.

2.3.2 Lipid biomarker and stable isotope analysis

Neutral (NLFA) and phospholipid (PLFA) lipid fatty acids were extracted and analyzed according to the protocol described by Frostegård et al. (1993) with some modifications (Gunina et al., 2014). Briefly, 6 g of soil was extracted with a 25 mL one-phase mixture of chloroform, methanol and 0.15 M aqueous citric acid (1:2:0:8, *v/v/v*, pH 4.0) with two extraction steps. The 19:0-phospholipid (dinonadecanoylglycerolphosphatidylcholine, Larodan Lipids, Malmö, Sweden) was used as internal standard one (IS1) and was added directly to soil before extraction (25 µL with 1 µg μL^{-1}). Additional chloroform and citric acid were added to the extract to achieve a separation of two liquid phases, in which the lipid fraction was separated from other organics. Phospholipids were separated from neutral lipids and glycolipids by solid phase extraction using a activated Silica gel (Silica gel Merck, particle size 0.063-0.200 mm). Alkaline saponification of the purified phospholipids was performed with 0.5 mL of 0.5 M NaOH dissolved in dried MeOH, followed by methylation with 0.75 mL of BF3 dissolved in methanol. The resulting fatty acid methyl esters (FAMEs) were purified by liquid–liquid extraction with hexane (three times). Before the final quality and quantity measurements, internal standard two (IS2) (13: 0 FAME) (15 µL with 1 µg μL^{-1})

was added to the samples (Knapp, 1979). All PLFA and NLFA samples were analyzed by gas chromatograph (GC) (Hewlett Packard 5890 GC coupled to a massselective detector 5971A) (Gunina et al., 2014).

The $^{13}C/^{12}C$ isotope ratios of the single fatty acids were determined by an IRMS Delta PlusTM coupled to a gas chromatograph (GC; Trace GC 2000) via a GCII/III-combustion interface (all units from Thermo Fisher, Bremen, Germany) (Gunina et al., 2014). A 15 m HP-1 methylpolysiloxane column coupled with a 30 m HP-5 (5 % phenyl)-methylpolysiloxane column (both with an internal diameter of 0.25 mm and a film thickness of 0.25 µm) was used.

To classify PLFA to corresponding microbial groups, a factor analysis based on principal component analysis with varimax standard rotation was performed on C contents of individual PLFA for the entire dataset. Fatty acids with a factor loading of > 0.5 (absolute value) on the same factor were grouped together, considering their known association to microbial groups as described by previous studies based on pure cultures (Zelles, 1999). The results of the factor analysis are presented in Supplementary Table S1.

The amount of the PLFA 16:1ω5c and NLFA 16:1ω5 were used as signatures for AMF biomass to confirm that genotype plants significantly reduced AMF across the treatments and control. The PLFA 16:1ω5c is not specific to AMF since it can occur in some bacterial groups, but a considerable proportion of this PLFA is considered to originate from AMF when the ratio of NLFA to PLFA 16:1ω5c is > 1 (Olsson, 1999). The ratio of saturated to monounsaturated PLFAs was used in conjunction with the ratios of the sum of cyclopropyl PLFAs to the sum of their monoenoic precursors (cy17:0 + cy19:0)/ (16:1ω7+18:1ω7; abbreviated cy/pre) as indicators of physiological or nutritional stress in bacterial communities (Kaur et al., 2005). Total PLFA concentration was the sum of single identified PLFA. The PLFA ratios relative to fungal to bacteria, Gram (+) to Gram (-) bacteria were calculated by using the sum of the respective fatty acid biomarkers and were assumed to represent the relative abundance of these groups.

2.4 Calculations

Obtained $\delta^{13}C$ data were used to calculated $^{13}C_{atom\%}$. The amount of ^{13}C incorporated into individual C pools were calculated as follows:

$$^{13}C_x = \frac{(^{13}C_{atom\%})_{x,L} - (^{13}C_{atom\%})_{x,UL}}{100} * C_x \qquad (1)$$

where $(^{13}C_{atom\%})_{x,L}$ and $(^{13}C_{atom\%})_{x,UL}$ are $^{13}C_{atom\%}$ in the labeled and control samples, respectively, and C_x is the C content in the individual pool (i.e. DOC) in the labeled sample.

^{13}C in microbial biomass was estimated as the difference in ^{13}C between fumigated and non-fumigated extracted K_2SO_4 solutions and divided by a factor of 0.45 (Ge et al., 2017):

$$^{13}C_{MBC} = \frac{\left[(^{13}C_{atom\%})_{f,L} - (^{13}C_{atom\%})_{f,UL}\right] \times C_f - \left[(^{13}C_{atom\%})_{f,L} - (^{13}C_{atom\%})_{f,UL}\right] \times C_{uf}}{100 \times 0.45} \qquad (2)$$

where f is the fumigated soil extract and nf means non-fumigated soil extract; C_f and C_{uf} are the amount of C in fumigated and non-fumigated samples.

The ^{13}C incorporation into individual PLFA (µg ^{13}C g^{-1} soil) was determined using a mass balance approach:

$$^{13}C_{PLFA} = \frac{(^{13}C_{atom\%})_{PLFA,L} - (^{13}C_{atom\%})_{PLFA,UL}}{100} * C_{PLFA} \qquad (3)$$

where $(^{13}C_{atom\%})_{PLFA,L}$ and $(^{13}C_{atom\%})_{PLFA,UL}$ are PLFA extracts from labeled and control

samples, respectively. C_{PLFA} was the content of individual PLFA in the labeled samples. Then we calculated the relative ^{13}C distribution (%) in each specific microbial group according:

$$\%^{13}C = \frac{^{13}C_{PLFA-group}}{\sum ^{13}C_{PLFA}} \times 100 \qquad (4)$$

where $^{13}C_{PLFA\text{-}group}$ is the amount of ^{13}C-PLFA incorporated into the specific microbial group, and $\sum ^{13}C_{PLFA}$ is the total amount of ^{13}C-PLFA incorporated into soil microorganism excepts the unclassified biomarkers.

The percentage of root-derived C that was incorporated into DOC, MOC, SOC, and microbial groups in the soil and plant-soil system was expressed as the percentage of ^{13}C recovery on each sampling time (e.g., 8, 12, 16 weeks after transplanting) according to:

$$\%^{13}C_{recovery} = \frac{^{13}C_{sample}}{\sum ^{13}C_{x}} \times 100 \qquad (5)$$

where $^{13}C_{sample}$ is the amount of ^{13}C in the shoot, root, soil, MBC, DOC, bacteria, SAP fungi, AMF (PLFA+NLPF). $\sum ^{13}C$ is the total amount of ^{13}C in the soil or plant-soil systems. For calculation of the C content of AMF hyphal biomass and SAP fungal C, PLFA 16:1ω5c and 18:2ω6, 9 were multiplied with the conversion factor of 107 and 366, respectively (Johansen et al., 1996; Olsson and Johansen, 2000; Olsson and Wilhelmsson, 2000; Klamer and Bååth, 2004; Korkama et al., 2007). Since the microbial biomass C consists mainly of fungal and bacterial C, the bacterial C was calculated by subtracting AMF hyphal and SAP fungal C values from mean MBC values of soil.

2.5 Statistical analysis

The experiment was carried out with three replicates of each treatment. The values presented in the figures are given as means ± standard error (mean ± SE). Prior to analysis of variance (ANOVA), the data were tested for normality (Shapiro-Wilk, $p > 0.05$) and homogeneity of variance (Levene-test, $p > 0.05$). Any data that appear non-normal and percentage data were log transformed so that they conform to the assumption of normality before further statistical analysis. A two-way ANOVA was used to assess the effects of AMF colonization (tomato genotype) and N fertilization, as well as their interaction for all measured parameters separately for each sampling time. Significant ($p < 0.05$) differences between means were identified using the *post hoc* Tukey HSD test. Residuals were checked for a normal distribution using the Shapiro-Wilk test. All statistical analyses were performed using SPSS version 19.0 (SPSS Inc., USA).

3. Results

3.1 Abundance of microbial community

After 16 weeks, total PLFA biomass was 50-54 μg g^{-1} soil for MYC, which was 10-20% higher compared to rmc plants ($p < 0.05$, Fig. S1). After 12 and 16 weeks, PLFA 16:1ω5c was accounted for 4.5-5.2% for MYC, higher than rmc plants ($p < 0.05$, Fig. 1; Table. S1). In contrast, the relative abundance of PLFA 18:2ω6, 9 was lower for MYC than that for rmc (2.46% vs 3.49%, $p < 0.05$). N fertilization decreased the relative abundance of AMF but increased the abundance of SAP ($p < 0.05$, Fig. 1; Table. S1).

3.2 ^{13}C incorporation into soil microbial community

Compare with rmc plants, total ^{13}C incorporated into PLFAs increased by 10-42% for

MYC after 8 and 16 weeks, whereas ^{13}C-PLFA was 73% lower in soil with MYC after 12 weeks ($p < 0.05$, Fig. 2). The ^{13}C incorporated into PLFA 16:1ω5c ranged from 0.02-0.32 nmol g^{-1} soil for MYC and gradually increased with incubation time. After 16 weeks, the amount of ^{13}C in the PLFA 18:2ω6, 9 was 39-68% lower for MYC than that for rmc plants ($p < 0.05$), whereas there were no difference between tow genotype plants after 8 and 12 weeks. After 16 weeks, N fertilization decreased the amount of ^{13}C in the total PLFAs, 16:1ω5c, but increased the ^{13}C incorporation into 18:2ω6, 9 compared to the unfertilized soil ($p < 0.05$, Fig. 2).

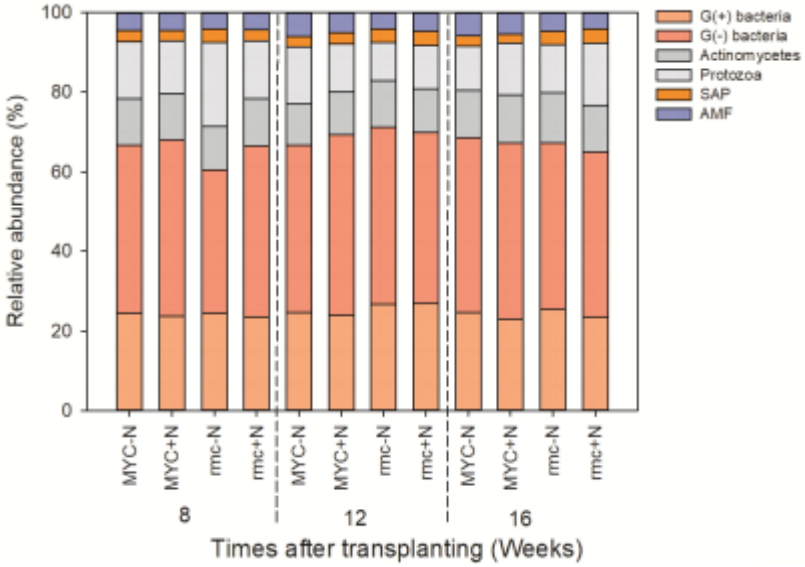

Fig. 1 Relative abundance of specific microbial groups of mycorrhizal wild type (MYC) and mutant tomato with reduced mycorrhizal colonization (rmc) with and without N fertilization over a 16 weeks growth period. Values shown are means ± standard error (SE) (n = 3). G(+) bacteria, gram positive bacteria; G(-) bacteria, gram negative bacteria; SAP, saprotrophic fungi (18:2ω6,9); AMF, arbuscular mycorrhizal fungi (16:1ω5c). Here, we summed up the amount of G(+) bacteria, G(-) bacteria, actinomycete, protozoa, SAP and AMF as total.

The relative incorporation of ^{13}C into specific biomarker was AMF symbiosis and N fertilization dependent, especially after 16 weeks (Table. S1). After 8 weeks, there were no difference in ^{13}C distribution in G(+), G(-), Actinomycete and 16:1ω5c irrespective of N fertilization or genotype plants, whereas N fertilization increased the relative abundance of SAP in fertilized than in unfertilized soil (6% vs 1%, Fig. 3). After 12 weeks, N fertilization did not change the ^{13}C distribution in microbial specific groups. The relative abundance of ^{13}C in 16:1ω5c and 18:2ω6,9 for MYC was accounted for around respective 16% and 14%, higher than that for rmc at 12 weeks after transplanting, whereas it was lower in G(+) and G(-) bacteria for MYC compared with rmc ($p < 0.05$, Fig. 3; Table. S1). After 16 weeks, AMF symbiosis increased the ^{13}C distribution in PLFA 16:1ω5c, but decreased the relative abundance of ^{13}C in the G(+), G(-) bacteria, and PLFA 18:2ω6,9 ($p < 0.05$). In the fertilized soil, the ^{13}C distribution of PLFA 18:2ω6,9 was significant higher compared with unfertilized soil (16% vs 9%, $p < 0.05$, Fig. 3), whereas it was lower for PLFA 16:1ω5c, G(+) and G(-) bacteria than that in the unfertilized soil after 16 weeks. For all three sampling time and in all four treatments more than 40% of the ^{13}C recovered from the PLFA was incorporated into G(-) bacteria (Fig. 3).

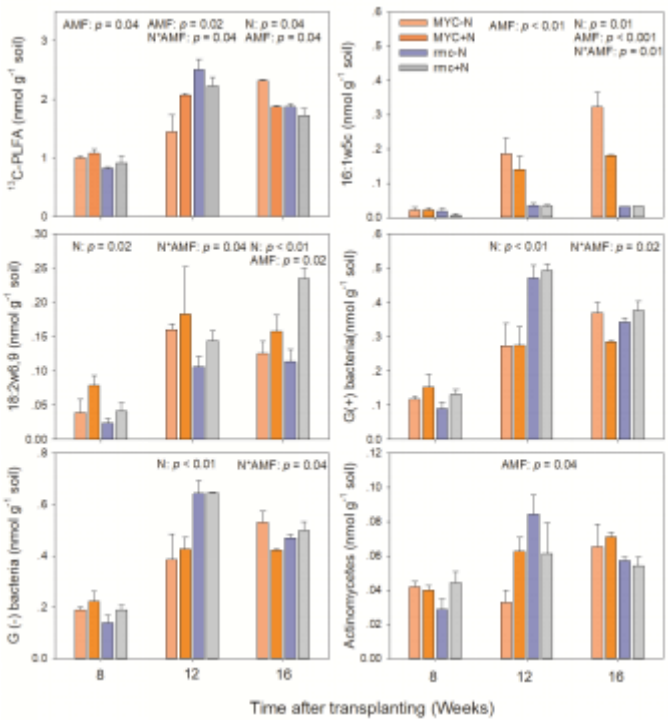

Fig. 2 Absolute amount of plant-derived carbon incorporation into specific microbial groups f mycorrhizal wild type (MYC) and mutant tomato with reduced mycorrhizal colonization (rmc) with N and without N fertilization over a 16 weeks growth period. Values shown are means ± standard error (SE) (n = 3). G(+) bacteria, gram positive bacteria; G(-) bacteria, gram negative bacteria; SAP, saprotrophic fungi (18:2 ω 6,9); AMF, arbuscular mycorrhizal fungi (16:1 ω 5c). P values were calculated from two-way ANOVA for each sampling (e.g., 8, 12, 16 weeks after transplanting). N: with and without N; AMF: with highly and low arbuscular mycorrhizal fungi colonization for MYC and rmc tomato.

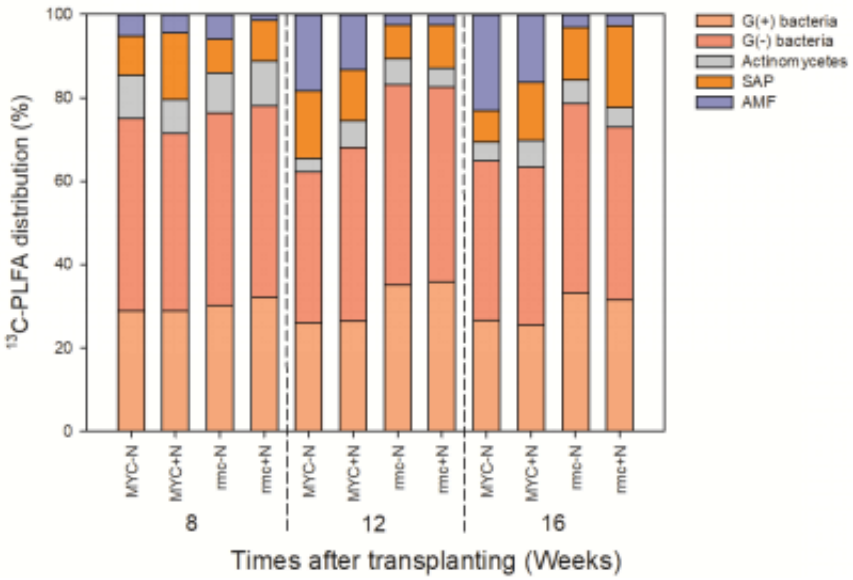

Fig. 3 Relative abundance of 13C distribution in the specific microbial groups of mycorrhizal wild type (MYC) and mutant tomato with reduced mycorrhizal colonization (rmc) with N and without N fertilization over a 16 weeks growth period. Values shown are means ± standard error (SE) (n = 3). G(+) bacteria, gram positive bacteria; G(-) bacteria, gram negative bacteria; SAP, saprotrophic fungi (18:2 ω 6,9); AMF, arbuscular mycorrhizal fungi (16:1 ω 5c). Here, we summed up the amount of G(+) bacteria, G(-) bacteria, actinomycete, SAP and AMF as total.

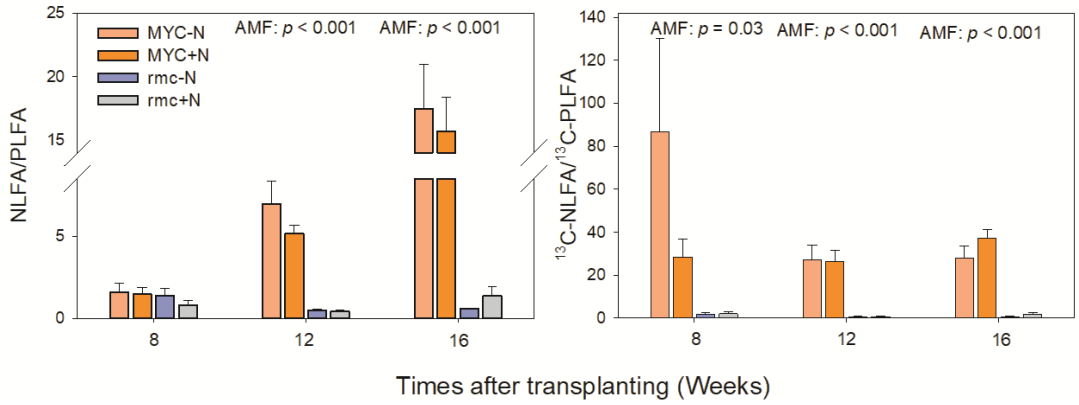

Fig. 4 The ratio of neutral and phosphorous fatty acid 16:1ω5c in microbial abundance (NLPF/PLFA) and ^{13}C amount (^{13}C-NLFA/^{13}C-PLFA) of mycorrhizal wild type (MYC) and mutant tomato with reduced mycorrhizal colonization (rmc) with and without N fertilization over a 16 weeks growth period. Values shown are means ± standard error (SE) (n = 3). N: with and without N; AMF: with highly and low arbuscular mycorrhizal fungi colonization for MYC and rmc tomato.

3.3 The ratio of neutral and phosphorus fatty acid

The NLFA/PLFA ratio for MYC plants ranged from 1.48 to 17.48 and increased with incubation time, whereas the ratio of NLFA and PLFA was around 0.42 to 1.39 for rmc plants (Fig. 4a). This suggested a well-developed AMF growth in soil with MYC plants, especially at 12 and 16 weeks after transplanting. Similarly, the ratio of ^{13}C-NLFA and ^{13}C-PLFA ranged from 26-86 for MYC, which was significant higher than that for rmc plants (0.49-1.89, $p < 0.05$, Fig. 4b), indicating that AMF allocated proportionally more C to storage structure for MYC compared with rmc plants. However, both ratios remained unchanged between fertilized and unfertilized soils over growth period ($p > 0.05$, Fig. 4).

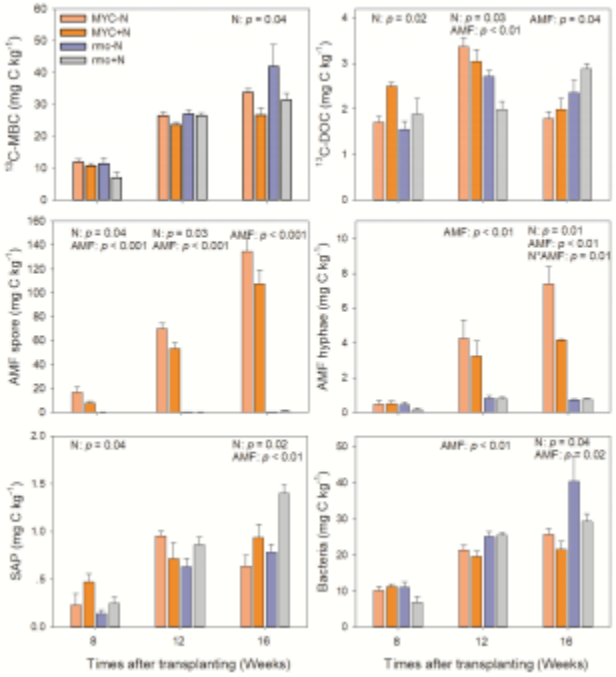

Fig. 5 The amount of ^{13}C in microbial biomass (^{13}C-MBC), dissolved organic carbon (DOC), storage compound of arbuscular mycorrhial fungi (AMF spore), AMF hyphae, saptrophic fungi (SAP), and bacteria biomass of mycorrhizal wild type (MYC) and mutant tomato with reduced mycorrhizal colonization (rmc)

with and without N fertilization over a 16 weeks growth periods. Values shown means ± standard error (SE) (n=3). N: with and without N; AMF: with highly and low arbuscular mycorrhizal fungi colonization for MYC and rmc tomato.

3.4 Carbon budget in the soil pools

The ^{13}C-MBC increased from 9.5 after 8 weeks to 43.2 mg C kg^{-1} after 16 weeks, and N fertilization decreased the amount of ^{13}C-MBC by 26-33% compared with unfertilized soil ($p < 0.05$, Fig. 5a). AMF spores and hyphal biomass in soil with MYC plants also increased with incubation time, and up to 120 and 6 mg C kg^{-1}, respectively (Fig. 5c, d). After 16 weeks, AMF symbiosis decreased the amount of ^{13}C in SAP fungal biomass by 23-48% compared with rmc plants, whereas N fertilization increased the ^{13}C incorporated into SAP fungal biomass by 47-78% ($p < 0.05$, Fig. 5e). Moreover, the amount of ^{13}C in the bacteria biomass was lower for MYC compared with rmc plants at 12 and 16 weeks after transplanting, whereas it was higher in the fertilized than that in the unfertilized soil after 16 weeks ($p < 0.05$, Fig. 5f).

The recovery of ^{13}C-MBC in the soil pools with MYC was around 13-18%, lower than that for rmc plants (22-35%) after 12 and 16 weeks ($p < 0.05$, Fig. 6a). Similarly, the ^{13}C recovered in the DOC pools was also lower for MYC compared with rmc plants in the last two sampling time ($p < 0.05$, Fig. 6b). The percentage of rhizodeposited C recovered in the AMF spore and hyphal biomass were much greater for MYC (65% and 3.5%, respectively) than for rmc plants (0.4% and 0.5%, respectively). Further, 0.35-0.64% of rhizodeposited C was recovered in the SAP fungal biomass for MYC, whereas 0.54-0.91% of ^{13}C was recovered in the SAP for rmc plants at 12 and 16 weeks after transplanting ($p < 0.05$, Fig. 6e). Similarly, more ^{13}C was recovered in the bacteria biomass for rmc compared with MYC plants after 12 and 16 weeks ($p < 0.01$, Fig. 6f). Moreover, N fertilization increased the ^{13}C recovery in SAP at 8 and 16 weeks ($p < 0.05$). After 8 and 12 weeks, N fertilization decreased the rhizodeposited C recovered in the bacteria biomass compared with unfertilized soil (14% vs 22%, Fig. 6).

Overall, the percentage of total assimilated C enriched in the bacteria and AMF in the unfertilized soil with MYC plants increased from 0.78 and 1.29 to 0.89% and 4.92% with time, respectively (Fig. 7). However, N fertilization decreased the assimilated C enriched in the bacteria and AMF. With incubation time, the ^{13}C recovered in the SAP fungi increased from 0.017 to 0.028%, and N fertilization increased the assimilated C incorporated into SAP for MYC plants after 16 weeks (Fig. 7).

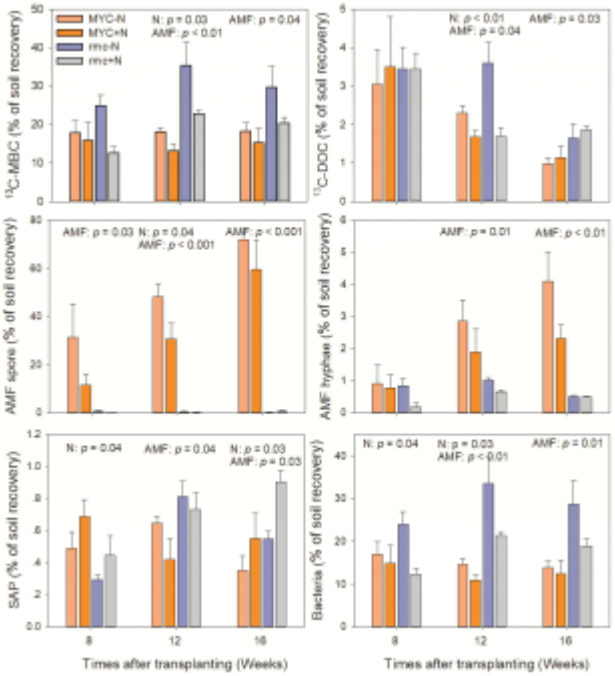

Fig. 6 The ^{13}C recovery (percent of soil recovery) in the microbial biomass (^{13}C-MBC), dissolved organic carbon (DOC), storage compound of arbuscular mycorrhial fungi (AMF spore), AMF hyphae, saptrophic fungi (SAP), and bacteria biomass of mycorrhizal wild type (MYC) and mutant tomato with reduced mycorrhizal colonization (rmc) with and without N fertilization over a 16 weeks growth periods. Values shown means ± standard error (SE) (n=3). Here, we summed up the ^{13}C in the soil (rhizosphere + bulk soil) as total. N: with and without N; AMF: with highly and low arbuscular mycorrhizal fungi colonization for MYC and rmc tomato.

4. Discussion

4.1 Microbial community dynamics as affected by AMF symbiosis

The AMF symbiosis are known to affect exudation patterns (Frey-Klett et al., 2007), thereby shaping the distinct size and structure of soil-borne communities (Fig. 1; Table. S1). As reported in the same experiment, AMF symbiosis enhanced C translocation to belowground via AMF symbiosis for MYC compared with rmc plants (Zhou et al., 2020), and this increased in C availability could explain the higher total PLFAs in soil with MYC plants (Fig. S1). The proportion of Gram (-) bacteria was the highest and this group increased in the soil planted with MYC compared with rmc plants (Fig. 1), which preferably take up easily available substances (Paterson, 2003; Dippold et al. 2014). Since rhizodeposits mostly consists of rather bioavailable neutral sugars, Gram-negatives showed the strongest preference for rhizodeposite-C utilization, especially at the higher C input for MYC plants with AMF symbiosis. In contrast, higher rhizodeposites decreased the abundance of Gram (+) bacteria in soil with MYC and N fertilization. This was supported by Buyer et al. (2010) who showed that Gram (+) bacteria decreased with increasing soil organic C availability. As SAP fungi is more capable of colonizing in nutrient poor soils with their wide-range enzymatic capabilities (Frey et al., 2003), AMF symbiosis resulted in relative smaller abundance of PLFA 18:2ω6,9c, due to the nutrient deficiency because of the uptake by AMF hyphae as shown in the same experiment (Zhou et al., 2020).

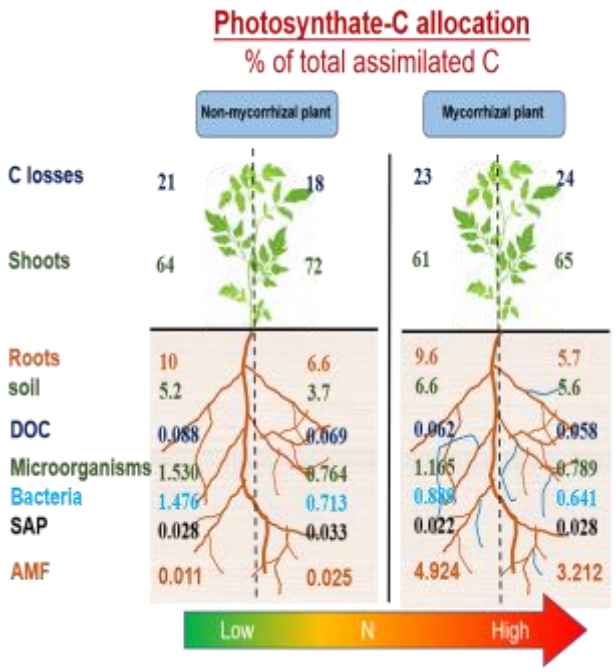

Fig. 7 Carbon (C) budget within a plant-soil system of mycorrhizal tomato (MYC) with and without N fertilization presented as % of total assimilated C into each C pool of plant and soil during 16-weeks continuous ^{13}C labelling. The percentage data of C losses, shoot, root and soil were collected from the same experiment (Zhou et al., 2020). The C losses includes root respiration and CO_2 released from soil organic matter decomposition

4.2 Distinct ^{13}C incorporation into microbial functional groups

Consistent with our first hypothesis, the direct incorporation of plant-derived C was much greater for AMF than bacteria and SAP. The ^{13}C enrichment in the AMF-specific NLFA and PLFA biomarker (16:1ω5c) increased over the growth period and was still prominent at 16 weeks after transplanting (Fig. 2). Increased ^{13}C labeling of NLFA 16:1ω5c suggested enhanced production of AMF storage organs, and increased ^{13}C labeling of PLFA 16:1ω5c implied AMF growth stimulation (Olsson and Johnson, 2005). Because NLFA 16:1ω5c is usually stored in intraradical vesicles or in spores and make up a large proportion of AMF biomass (Olsson and Johnson, 2005), whilst fine absorptive AMF hyphae turnover rapidly, which are known to live only 5-6 days (Staddon et al., 2003). Therefore we observed a lower retention of C in the PLFA 16:1w5c fraction (4% of soil recovery), whereas much of the C contained within AMF is translocated into spores(60% of soil recovery) that may remain in the soil for long periods (Fig. 6). These data confirmed earlier results on the dominant role of AMF in the flow of C from plants into the soil and AMF are consistently the first to profit from plant derived photosynthates (Johnson et al., 2002; Olsson and Johnson, 2005; Drigo et al., 2010).

Followed by AMF, bacteria was more active in cycling newly fixed plant-derived C within the rhizosphere. Indeed, other studies have shown rapid allocation of C to rhizosphere inhabiting bacteria (Elias et al., 2017), with Gram (-) bacteria particularly utilize labile C compounds such as exudates in the rhizosphere (Bird et al., 2011; Koranda et al., 2014). Although Gram (-) bacteria take up root exudates fast and show a rapid turnover, they do not exchange their biomass as rapidly as AMF (Apostel et al., 2013; Sommer et al., 2017). None of the other microorganisms reached a similarly high and fast C replacement as the mycorrhizal fungal groups. Therefore, the direct C flux into AMF is highly efficiency and

dominates the C nutrition of these fungal groups. Gram (-) bacteria are less important in ^{13}C uptake than AMF, but more important than Gram (+) bacteria. Similar results were also observed by Drigo et al. (2010) in a $^{13}CO_2$ pulse labeling experiment who showed that Gram (-) and mycorrhizal fungi appeared to be most important actively assimilating root derived C. Our results further illustrated that rather low amounts of actinomycete fatty acid were present in soil of both genotypes with or without N fertilization (Fig. 3). This indicated that actinomycete is less active or that have a preference for other C substrates with the soil system in the presence of rhizodeposits (Kramer and Glexner, 2008; Mellado-Vázquez et al., 2016). Actinomycetes can degrade complex organic polymers and are positioned late in the microbial reaction chain (Lacey, 1973). Accordingly, they might not compete well for the large amount of easily available plant-derived C (Sommer et al., 2017).

SAP fungi exhibited 20-30 times lower ^{13}C incorporation than in bacterial PLFAs. This is in line with Wardle et al. (2004) who suggested that bacteria is more important for the turnover of easily available C, while fungi dominate the turnover of C bound in complex structures. As a result of the difference between fungal and bacterial lifespans, the storage of C in microbial biomass is highly persistent in fungi, while it turns over rapidly in bacteria (Rousk and Bååth, 2011). Previous studies have indicated that bacterial biomass turns over 2-3 times during a growing season, while fungi regenerate only 75% of their biomass over the same period (Moore et al., 2005). Thus characteristic of bacteria causes an accelerated transfer of labile C derived from the lysis of cells through the movement of soil solution or the re-use of bacteria (Liu et al., 2019). Alternatively, SAP fungal assimilation of C derived from necromass or dead root material rather than from labile root exudates might form an alternative explanation for the lower incorporation of ^{13}C into the fungal PLFAs (Paul, 2007; Rousk and Frey, 2015). Additionally, previous studies also showed that soil bacteria could active feeding on living SAP through weaken the living fungal exterior (cell wall and cell membrane) via the release of a combination of lytic enzymes (chitinaes) and fungicides (Rudnick et al., 2015; Ballhausen and Boer, 2016), and thus less ^{13}C was incorporated into SAP fungal rather than bacterial groups.

The pattern of ^{13}C incorporation by different microbial groups observed in our study are consistent with observations in earlier studies (Paterson et al., 2007): AMF are the major C pools in the belowground (Fig. 7). High ^{13}C incorporation in the AMF represented by the NLFA 16:1ω5c signature was observed which indicates that the AMF are important in the C transfer from plants into other soil microorganisms (Treonis et al., 2004; Olsson and Johnson, 2005; Drigo et al., 2010).

4.3 Microbial carbon utilization as mediated by AMF symbiosis and N fertilization

Following AMF colonization, root exudation patterns may be altered with the fungi acting as a C sink (Jones et al., 2004), retaining plant-derived C in AMF biomass and spores and controlling its time of release to other soil microorganisms (Olsson and Johnson, 2005). In our study, more rhizodeposits were stored in the AMF spores (NLFA 16:1ω5c), subsequently the activity of soil microorganisms reduced because of the lack of sufficient C and energy. Due to that the C cost for construction and maintaining the AMF association possibly exceeded the mycorrhizal growth benefits of enhanced nutrient acquisition (Jakobsen et al., 2016), the response of plant biomass to AMF symbiosis was neutral or even negative in this study as reported by Zhou et al. (2020). Therefore, the ^{13}C distribution of PLFA signatures cy17:0 and cy19:0, which was respective recognized as plant growth promoting rhizobacteria *Pseudomonas* spp. and *Burkholderia* spp. (Artursson et al., 2005; Jalili et al.,

2009), was relative lower in soil with MYC compared with rmc plants. Furthermore, the modification of *cis*-monounsaturated fatty acids to the more stable cyclo fatty acids could help in maintaining a functional living membrane during stress (e.g., less available rhizodeposits for rmc plants than for MYC plants), as indicated by the concomitant increase in cyclopropyl (cy17:0 and cy19:0) fatty acids, together with an increase in the trans:cis ratio of unsaturated fatty acids ((cy17:0+cy19:0)/(16:1ω7c+18:1ω7c)). *Bacillus* spp. has been recognized as typical bulk-soil inhabitants (Smalla et al., 2001). Using PLFA signature i17:0 for *Bacillus* spp. (Kaneda, 1991), we detected less plant-derived C for this group in soil with AMF symbiosis throughout the experiment, which was consistent with previous studies that slow-growing soil microorganism were less affected by AMF symbiosis (Drigo et al., 2007). Therefore, AMF-associated microorganisms generally assimilated less plant-derived C than the microbial communities for rmc plants.

While AMF symbiosis are not known to produce antibiotics, the presence of AMF symbiosis has been shown to repress some members of SAP fungi and bacteria (Fig. 2), which may resulted from the direct or indirect manipulation of the community through hyphal exudates (Toljander et al., 2007; Welc et al., 2010). As our previous study shown, AMF symbiosis reduced the available N and P thus depleted microorganisms of shared limiting nutrients, suppressed the activities of free-living saprotrophs (Veresoglou et al., 2011; Leifheit et al., 2015). Further, AMF symbiosis may parasitize nutrient-rich saprotrophic fungi as an alternate resource-acquisition strategy (Fernandez and Kennedy, 2016). Alternatively, AMF symbiosis may occupy the same niche as these microorganisms and compete with them during nutrient acquisition (Veresoglou et al., 2011). For example, some bacterial strains such *Pseudomonas* spp. are able to colonize both plant roots and AMF hyphae (Miransari, 2011). However, whether the suppression is based on qualitative changes of rhizodeposits, modifications of the soil abiotic environment, competition for nutrients or reflects direct interactions between compounds released by the fungi and certain bacterial taxa should be an issue for future investigations.

In particular, the C retention in the AMF fraction was significantly lower under N fertilization (Fig. 5, 6), may be triggered as plants respond to N availability in the soil by changing their C allocation patterns (Johnson et al., 2003). When conditions became less suitable for AMF colonization of the root, the fungi invested proportionally less in storage lipids, that is, spores. One interpretation of this could be that at higher N availability, when less C is allocated to the roots (Fig. 7), there is less of surplus of carbohydrates that can be used by fungi for storage structures (Olsson and Johnson, 2005). This suggested that N fertilization has reduced AMF fungal activity as a result of a decreased transport of photosynthate-C to roots and reduced C allocation by plants to their AMF symbiosis. In the case of greater nutrient limitation in plants without fertilization, AMF symbiosis could be more important, promoting preferential C allocation to AMF, at the expense of SAP fungi. This could be an important explanation of the decreased ^{13}C incorporation in SAP fungi in the unfertilized soil. On the other hand, a stimulated root growth and AMF colonization and hence nutrient acquisition in unfertilized soil were believed to suppress the ^{13}C uptake of SAP. Furthermore, increased ^{13}C incorporation into SAP fungi in the fertilized compared with unfertilized was observed by Steer and Harris (2000), due to that the increased nutrient availability enable fungi to utilize C resources unavailable to other bacteria. Overall, the general explanation has been that the N-limited plant consumes more of the newly fixed C to the root that reduce the C availability to the free living saprotrophic microorganisms, whereas a high N availability negative impact on AMF symbiosis results from less C allocation and

that the mechanism behind this is an increased C immobilization in the aboveground biomass as well as the stimulated rhizosphere respiration (Zhou et al., 2020).

4.4 Carbon budget in the plant-AMF symbiosis-soil interaction

Total photosythate allocation to AMF symbiosis has been estimated to be as high as 20-30% (Drigo et al., 2010), but we only measured 3.22-4.92% of total assimilated C in the AMF (Fig. 7). On the one hand, a large proportion of neutral lipids in AMF are found in intraradical vesicles (Olsson and Johansen, 2000). On the other hand, the transfer from intraradical mycelium to extraradical may be rapid (Drigo et al., 2010), and respiration and turnover in the soil may be high (Staddon et al., 2003). Alternatively, PLFA are rapidly degraded upon cell death and are not found in storage lipids (Zelles et al., 1992), therefore more plant-derived C were recovered in the microbial necormass pools, as a consequence contribution the SOC sequestration in soil with AMF symbiosis. Compared to the soil with rmc plants, approximately 60% of plant-derived C was stored in fungi storage lipids in soil with AMF symbiosis. This lipid reserve is probably sequestered in structures with a low turnover rate (i.e. extraradical spores), but also provide a source of lipids for translocation to the extraradical mycelia or other free-living microorganisms (Bago et al., 2002). However, the ^{13}C incorporated into AMF hyphal biomass increased with incubation, and in the end up to 0.12-0.25% of total assimilated C in the plant-soil system, which accounted for around 16-22% of MBC (Fig. 6, 7). This was consistent with Leake et al. (2004) who found AMF accounts for 20-30% of soil microbial biomass, thus contributes largely in soil C cycling.

5. Conclusions

With simultaneous AMF symbiosis and N fertilization manipulations, our results highlight the importance of microbial communities and especially AMF for belowground C fluxes in agroecosystem. According to the results of this study, AMF are particularly important for C flow in soils. This is in accordance with the fact that AMF are receive all of their C from the plant host, thus the AMF biomarker (PLFA- and NLFA- 16:1w5c) should have the highest rhizodepsoits. After AMF biomarkers, the highest label occurred in biomarkers of gram-negative bacteria, which showing them to be the major rhizodeposits utilizers together with AMF. Together, our results suggest that AMF symbiosis affects the composition of soil microbial communities. The major functional changes included decreased SAP and bacterial abundance. The preferential C allocation to AMF also appears to reduce the C flow to other free-living microorganisms, particularly SAP fungi.

Acknowledgments

We thank the China Scholarship Council (CSC) for funding to Jie Zhou in Germany. This study was financially supported by the German Research Foundation (DFG) within the project PA 2377/2-1, GU 1309/5-1. The continuous ^{13}C labeling was conducted in the Gabriele Lehmann and Rainer Schulz of the Laboratory for Radioisotopes (LARI), Göttingen. The isotopic analyses were performed at the Centre for Stable Isotope Research and Analysis (KOSI), Göttingen. The seeds for the experiment were kindly supplied by Dr. Susan Barker (Faculty of Sciences, School of Agriculture and Environment, The University of Western Australia, Australia).

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Supplementary

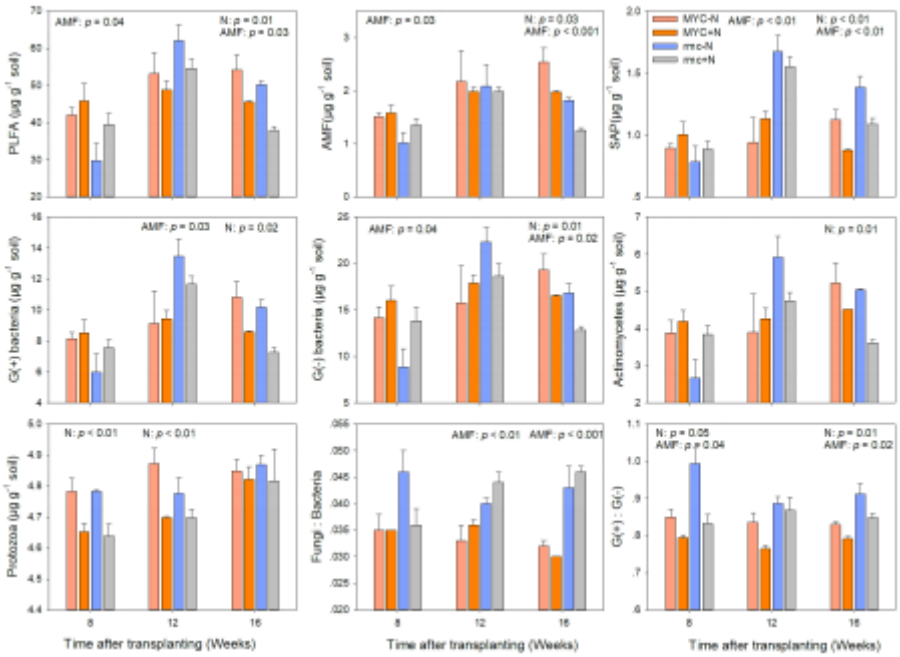

Fig. S1 Total microbial biomass (PLFA) and specific microbial groups (AMF, SAP, G(+), G(-) bacteria, Actinomycetes, protozoa), as well as ratios of fungi and bacteria, G(+) and G (-) of mycorrhizal wild type (MYC) and mutant tomato with reduced mycorrhizal colonization (rmc) with N and without N fertilization.

Table. S1 Principle component analysis of phosphorous fatty acid.

	1	2	3	4	5	6	Specific group
i14:0	0.052	0.901	-0.052	0.024	0.057	0.108	G(+) bacteria1
i15:0	0.882	0.008	0.151	0.004	0.155	0	G(+) bacteria 2
a15:0	0.805	0.352	0.091	0.04	0.068	-0.021	G(+) bacteria 2
i16:0	0.405	0.502	0.041	-0.045	-0.007	-0.405	G(+) bacteria 1
a16:0	0.22	0.898	0.059	0.076	0.033	-0.052	G(+) bacteria 1
16:1ω7c	-0.029	-0.145	0.263	0.196	0.734	-0.182	G(-) bacteria 2
16:1ω5c	-0.004	0.189	0.087	0.905	-0.022	0.064	AMF
10Me16:0	0.335	-0.277	0.479	0.044	-0.105	-0.435	Actinomycets 1
i17:0	0.544	0.331	0.204	-0.327	-0.038	-0.251	G(+) bacteria 2
a17:0	0.365	0.573	0.233	0.045	0.066	-0.186	G(+) bacteria 1
cy17:0	0.362	0.17	0.462	-0.012	0.024	-0.416	G(-) bacteria 1
10Me17:0	-0.303	0.936	-0.01	0.009	-0.048	-0.296	Actinomycets 2
18:2ω6,9	0.485	0.485	0.001	-.0507	-0.259	0.039	SAP fungi
18:1ω9c	0.021	-0.02	0.958	0.109	-0.025	0.047	G(-) bacteria 1
18:1ω7c	-0.099	-0.431	0.549	0.214	0.015	-0.487	G(-) bacteria 1
10Me18:0	-0.15	0.203	0.075	-0.075	0.119	-0.931	Actinomycets 1
cy19:0	0.138	-0.018	0.849	0.037	0.038	-0.165	G(-) bacteria 1
20:4ω6c	-0.565	0.114	0.592	-0.327	-0.129	0.165	Prorozoa
20:1ω9c	0.033	0.039	0.017	-0.078	0.999	-0.007	G(-) bacteria 2

Manuscript

Study 3 Ectomycorrhizal and non-mycorrhizal rhizosphere fungi increase root exudation and enzyme activities: a 14C pulse labeling of Picea abies seedlings

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Abstract

Consequences of interactions between ectomycorrhizal fungi (ECM) and non-mycorrhizal rhizosphere fungi (NMRF) for plants carbon (C) allocation belowground and nutrient cycles in soil remain unknown. To address these questions, we performed a mesocosm study with Norway spruce seedlings (*Picea abies* L.) inoculated with ECM, NMRF, or a mixture of both (MIX). $^{14}CO_2$ pulse labeling of spruce was applied to trace and visualize the ^{14}C incorporation into roots, rhizohyphosphere and hyphosphere. Activities and localization of enzymes involved in the C, nitrogen (N) and phosphorus (P) cycling were visualized using zymography.

Spruce seedlings inoculated with ECM and NMRF allocated more C to soils (ECM: 10.7%; NMRF: 3.5% of total recovered C) compared to uninoculated control seedlings. The ^{14}C activity in the hyphosphere was highest for ECM and lowest for NMRF. In the presence of both, NMRF and ECM (MIX), the ^{14}C activity was 64% lower compared with ECM inoculation alone. This suggests a suppressed C allocation via ECM likely due to the competition between ECM and NMRF for nutrients. Furthermore, we observed 57% higher chitinase and 49% higher leucine-aminopeptidase in the rhizohyphosphere of ECM compared to Control treatment. In contrast, β-glucosidase activity (14.3 nmol cm^{-2} h^{-1}) was highest with NMRF. In support of this, enzyme stoichiometry in soil with ECM shifted to a higher investment of nutrient acquisition enzymes (e.g., chitinase, leucine-aminopeptidase, acid phosphatase) compared to NMRF inoculation, where investment in β-glucosidase increased. In conclusion, the alleviation of ECM from C limitation promotes higher activities of enzymes involved in the N and P cycle to cover the nutrient demand of ECM and host seedlings. In contrast, C limitation of NMRF probably led to a shift in investment towards higher activities of C cycling enzymes.

Keywords: ^{14}C imaging, Carbon allocation, Rhizodeposits, Ectomycorrhizal fungi, Non-mycorrhizal rhizosphere fungi, Enzyme activity

1. Introduction

Up to half of the carbon (C) fixed by plants through photosynthesis is translocated to the roots and associated fungi (Orwin et al., 2011; Clemmensen et al., 2013). About 50% of this translocated C is subsequently transferred into the soil (Kuzyakov & Domanski, 2000) through root exudation and turnover of roots and fungal hyphae (Zhou et al., 2006; Strand et al., 2008). Root-released C provides energy for soil microbial communities, consequently shaping their structure and function and thus driving microbial nutrient cycling (Eisenhauer et al., 2010; Cheng et al., 2014; Pausch & Kuzyakov, 2018). However, with respect to different trophic modes of fungi, knowledge about the relative C flow to soil and subsequent microbial functioning is still limited.

Ectomycorrhizal fungi (ECM) and free-living non-mycorrhizal rhizosphere fungi (NMRF) are two major soil fungal guilds in boreal temperate forests with contrasting strategies in C resource capture (Read & Perez-Moreno, 2003; Kohout et al., 2013). Carbon used for ECM growth and extraradical hyphae extension is mainly plant derived (Högberg et al., 2001; Nehls, 2008), although some ECM can absorb organic nutrients from soil solution (Chalot et al., 1996; Teramoto et al., 2016). Up to 30% of total assimilates of a plant is consumed by their ECM partner (Wu et al., 2002). Most of this C is used for the development of the extraradical mycelium and for respiration, while only a small amount is released into soil as exudates (Högberg et al., 2001; Ekblad et al., 2013). In contrast to ECM, the impact of NMRF on C allocation to the soil remains unclear, although some studies showed that NMRF can rapidly metabolize organic compounds released from living roots which may probably affect plant C allocation to soil (Hannula et al., 2012; Pausch et al., 2016).

ECM acquire C directly from the host plant and do therefore not depend on soil organic matter (SOM) as a C source (Lindahl & Tunlid, 2015; Verbruggen et al., 2017). The assumption is that ECM play a major role in the soil organic N and P degradation to obtain nutrients for their own growth but also to supply their host plant (Read & Perez-Moreno, 2003; Smith & Read, 2010; Fernandez & Kennedy, 2016). Hence, ECM-associated plants exhibit enhanced exoenzyme activities, specifically chitinase and phosphatase compared with non-mycorrhizal plants (Talbot et al., 2008; Kluber et al. 2010). In contrast, NMRF are commonly co-limited by C and nutrients (Bödeker et al., 2016) due to the rapid utilization of easily available C in soils (Högberg et al., 2003; Hopkins et al., 2014). To meet their C and nutrient demand, NMRF rely on the excretion of a broad variety of hydrolases and oxidases, including proteinases and cellulases, which enables them to access SOM-derived C and nutrients (Baldrian, 2008; Baldrian & Valášková, 2008). It can therefore be assumed that under ECM mostly N and P targeting enzymes are increased while under NMRF also C targeting enzymes are expressed. This can have pronounced effects on soil C and nutrient cycling but has not yet been tested experimentally .

Another difference between the two fungal groups relates to their spatial distribution in soil. While ECM mainly colonize fine roots and spread their extraradical hyphae into the rhizosphere and behind (hyphosphere) (Wu et al., 2002; Teramoto et al., 2012), NMRF are also abundant in the bulk soil (Aguilar-Trigueros et al., 2014). Traditional destructive sampling techniques do not enable considering the spatial heterogeneity of nutrients and enzymes in the soil (Pinton et al., 2001). However, imaging techniques can be used to gain detailed data on the spatial distribution of plant-derived C and of soil enzyme activities. For example, ^{14}C imaging has been used to study ^{14}C allocation in the plant-soil system, and to assess the radial patterns of root exudates (Wu et al., 2002; Pausch & Kuzyakov, 2011; Banfield et al., 2017; Holz et al., 2018). Soil zymography enables the visualization the radial gradients of enzyme activities (Spohn & Kuzyakov, 2014; Razavi et al., 2019). Here, we combine these techniques (^{14}C imaging and soil zymography) to quantify the spatial distribution of recently assimilated C in soil and of exoenzyme activities depending on two major functional fungal groups in soils, the NMRF and the ECM.
We hypothesized that 1) a higher amount of photosynthetic C is allocated to the ECM hyphosphere as compared to NMRF, due to the direct support of ECM by the host plant; 2) ECM colonization, due to the alleviation in C limitation, increases activities of N- and P-related exoenzymes in soil, while NMRF predominantly increase C-degrading enzyme activities; 3) ECM hyphae will extend the spatial distribution of photosynthetic C in soil, accompanied by an increase in the spatial distribution of nutrient-mobilizing enzymes; 4) the competition between ECM and NMRF will reduce plant C allocation to the soil and overall soil enzyme activities.

2. Materials and methods

2.1 Seed germination and plant growth

Seeds of Norway spruce (*Picea abies* L.) were sterilized in 30% H_2O_2 for 20 min and rinsed with pure H_2O for 10 min afterwards. Seeds were germinated on plant agar in Petri dishes (in total 320 seeds distributed to 40 Petri dishes) in a cooling room at 4 °C for 4 days. After that, the seeds were kept in a dark room at approximately 21 °C for 10 days. About one third of the germinated seeds were placed in tubes with agar for another 7 days before planting them on soil. Afterwards, 80 plants were transferred into 50 mL pots with a substrate mixture which consisted of 50% sterilized peat and 50% perlite / vermiculate mix (1:1). During the growth in pots, plants were watered twice a week with approximately 50 mL H_2O per pot and fertilized twice with 10 mL of 1% NPK fertilizer solution (4:1:5; Primaster Universaldünger, Stuttgart, Germany).

Given that spruce (*Picea abies*) occurs as a host of the basidiomycetes *Amanita muscaria* (Sauter and Hager, 1989) and *Hebeloma crustuliniforme* (Meyer, 1989). And *Cenococcum geophilum* is one of the most commonly encountered soil fungi forming ectomycorrhizal (ECM) associations with gymnosperms in diverse habitats throughout northern temperate regions

(Trappe, 1964). The fungal species used in this study are listed in Table 1. The species were isolated by the Department of Forest Botany and Tree Physiology (Georg-August University of Göttingen, Germany). For a period of two years, all fungi were grown in dual pot cultures under similar culture conditions. During that time, mycorrhizal fungi were subcultured at three-month intervals in liquid medium to keep the cultures clean and viable.

Three inoculation treatments were established. Tree seedlings were inoculated with a mixture of three ECM fungal species (ECM), a mixture of three non-mycorrhizal rhizosphere fungal species (NMRF), and a mixture of all ECM and NMRF fungal species (MIX), respectively, as listed in Table 1. Twenty remaining plants were taken as non-inoculated control (Control). During growth in the pot, inoculation was performed three times per each treatment by 10 adding mL of a hyphae solution. The hyphae solution was prepared by adding fungal tissue from liquid medium into H_2O and macerating it with a mixer at 8000 rpm (Ultra-Turrax T25, Janke & Kunkel, IKA Labortechnik).

Table. 1 Fungal types used in the experiment. ECM: ectomycorrhizal fungi, NMRF: non-mycorrhizal rhizosphere fungi.

Fungal type	Taxa		
ECM	*Amanitamuscaria*	*Hebeloma crustuliniforme*	*Cenococcum geophilum*
NMRF	*Trichoderma asperellum*	*Trichoderma viride*	*Cryptococcus terricola*

2.2 Experiment setup

After a growth period of three months at room temperature, 60 plants (15 trees per treatment) were transferred to rhizoboxes (20 × 20 × 3 cm). The rhizoboxes were divided into two compartments, a root and a hyphae compartment, which will be referred to as rhizohyphosphere and hyphosphere in the following. The compartments were disassociated by a nylon mesh (20 µm, Taoyuan, China). The nylon mesh allowed the penetration of hyphae but prevented the passage of roots. The rhizohyphosphere was used to grow host plants that were expected to become colonized by the ectomycorrhizal fungi (in the treatments with ECM inoculation). Extraradical mycorrhizal hyphae were expected to develop from the rhizohyphosphere towards the hyphosphere. The rhizoboxes were filled with 1 kg of a sterilized 1:1:1:1 w/w mixture of nutrient-poor soil, fine sand, coarse sand and perlite. The boxes were transferred to a climate chamber with 14 and 10 h of day and night time, respectively. At day time seedlings were received artificial light with 800 µmol m^{-2} s^{-1} photosynthetic active radiation (Zhou et al., 2020). Deionized water was added to all rhizoboxes every 3 days to keep soil water content at 20%. During the time of growth (three months) in the climate chamber, the hyphosphere was watered once a week with 5 mL of a factor 10 diluted nutrient solution prepared from the previously used nutrition stock solution. In addition, the seedlings were re-inoculated twice a week with 10 mL per rhizobox of each corresponding hyphae solution (5 mL in hyphosphere and 5 mL in rhizohyphosphere). The hyphae solution was added until the mycorrhization of the roots was confirmed under the microscope (Leica M205, Leica

Mikrosysteme Vertrieb GmbH, Germany). Totally hyphae solution was added 4 times to the rhizobox. During the last three months of incubation, bacteria were inoculated in all the rhizoboxes to avoid the contamination of bacteria in air and growth chamber, which was obtained from the original soil solution and grown in the medium with 5% fungicide.

2.3 ^{14}C pulse labeling

After six months growth, $^{14}CO_2$ labeling was conducted in a transparent plastic chamber. The labeling procedure followed Pausch and Kuzyakov (2011). Briefly, 11.8 MBq of ^{14}C as $Na_2{}^{14}CO_3$ (Hartmann Analytic, Brunswich, Germany) was injected into chamber from a connecting glass vial. $^{14}CO_2$ was formed by adding sulfuric acid (5 mL, 1 M) to the labeled $Na_2{}^{14}CO_3$, and then pumped into the chamber with 10 h of circulation. A fan inside the chamber was used to support the even distribution of the $^{14}CO_2$. At the end of labeling, NaOH solution (20 mL, 1 M) was used to capture the remaining $^{14}CO_2$ inside the chamber for half hour. The ^{14}C content of NaOH was determined after adding 3 ml of scintillation cocktail (CarlRoth GmbH + Co. KG, Karlsruhe, Germany) with the liquid scintillation counter Tricarb^{TM} B3180 TR/SL (LSC, PerkinElmer Inc., Waltham, MA, U.S.A.). This measurement showed that approximately 60% of the added ^{14}C was taken up by seedlings after 10 h labeling.

2.4 Measurements and calculations

2.4.1 ^{14}C imaging and quantification

One hour after labeling, one ^{14}C image was taken per rhizobix. To get a good signal, the imaging plates were attached to the rhizobox (rhizohyphosphere and hyphosphere) for 17 h in darkness. ^{14}C imaging information was obtained by using a FLA 5100 scanner (Fujifilm) with setting spatial resolution of 100 µm.

The ^{14}C images were converted to ^{14}C activities by a linear regression which describes the relationship between pixel-wise photo-stimulated luminescence (PSL) and known imaging activities of ^{14}C (Banfield et al., 2017). Briefly, 0.5 g fresh soil sampled from the experiment and added to a sterile 4 ml plastics tube. Next, 100 µL of dissolved ^{14}Cactivities (ranging from 0 to 15 Bq μL^{-1}) was added dropwise to the plastic tube to adjust soil moisture to 30%, and then transferred to a 24-well microtiter plate. To measure its ^{14}C activity, the plates were attached to the imaging plate after smoothed the soil surface. Aliquots of ^{14}C-glucose were dissolved in 3 ml of scintillation cocktail and determined with the LSC. The ^{14}C activity were normalized to the microtiter plate.areas and a linear regression was fitted to the PSL values.

2.4.3 Zymography

Zymography was applied to visualize four hydrolytic enzymes (Razavi et al., 2016). The four enzymes (β-glucosidase, BG; acid phosphatase, ACP; chitinase, NAG; and leucine-aminopeptidase, LAP) were chosen to reflect key enzymes in the soil C, P and N cycle, respectively. Polyamide membrane (20 × 20 cm, pore size of 0.45 mm, Tao yuan, China) were saturated with 4-methylumbelliferyl (MUF) and 7-amido-4-methylcoumarin (AMC) based substrate to visualize the specific enzymes.

4-methylumbelliferyl-β-D-glucoside, 4-methylumbelliferyl-phosphate, 4-methylumbelliferyl-N-acetyl-a-D-glucosaminide, and L-leucine-7-amido-4-methylcoumarin hydrochloride were used to detected the β-glucosidase, acid phosphatase, chitinase, and leucine-aminopeptidase, respectively. Each substrate was separately dissolved in 10 mM MES and TRIZMA buffer for MUF and AMC substrate, respectively. The saturated membranes were attached to the rhizobox for 60 min. Then carefully lifted off the membrane to the rhizobox, and gently removed any attached soil particles with tweezers (Razavi et al., 2016). Enzyme detection sequences followed as: chitinase, β-glucosidase, acid phosphatase, leucine-aminopeptidase activity, with 1 h interval after each zymography. The membranes were placed under ultraviolet (UV) light with 355 nm wavelength) in darkness, and zymogram was taken by camera (EOS 5D, Canon). During zymography, the camera and the UV light was fixed, the distance between membranes and camera was kept same. The grey scale values transferred to the enzyme activities was calibrated using membranes (2 × 2 cm) saturated with a range of concentrations of corresponding products, i.e. MUF and AMC (0. 0.01, 0.2, 0.5, 1, 2, 4, 6, 10 mM). These membranes were imaged under same UV light and environment as samples.

The zymograms were transferred into a 16-bit gray scale by ImageJ (Schneider et al., 2012). The light variations and camera noise were corrected by the images taken in darkness (Razavi et al., 2016). The average grey value of the blank membrane (only with buffer) was viewed as background, and then subtracted from all samples (Zhang et al., 2019). The calibration line obtained for each enzyme was used to convert gray values of each zymography pixel into enzyme activities (Liu et al., 2017).

2.4.4 Plant and soil sampling

After zymography, seedlings were pulled out from the rhizobox and soil samples were carefully collected directly from the rhizohyphosphere and hyphosphere. Seedlings (shoots and roots) and soil (rhizohyphosphere and hyphosphere) were oven-dried (105 °C, 5 days) and then weighed. Afterwards, the dry plant biomass and soil were homogenized with a ball mill before further analysis. Soil microbial biomass C (MBC) and dissolved organic C (DOC) in rhizohyphosphere and hyphosphere soil was extracted with K_2SO_4 (32 mL, 0.05 M) according to Vance et al., (1987) and Zhou et al. (2020). The ^{14}C activity of fumigated and non-fumigated extracts was determined by mixing 1 mL of the K_2SO_4 solution with 4 mL scintillation cocktail and analyzed with a LSC. The ^{14}C-MBC was determined as the difference between fumigated and non-fumigated K_2SO_4-extractable ^{14}C (Zang et al., 2020).

The ^{14}C amounts of plant and soil materials were measured by oxidization in an Oxymat OX500 Biological Oxidiser (RJ Harvey Instrument Corp., Hillsdale, USA), with $^{14}CO_2$ collected in an Oxosol scintillator (C400, Zinsser). Afterwards, the ^{14}C activity was determined by LSC with automated quench correction.

2.5 Calculations

The total ^{14}C recovery in the plant-soil system was the sum of ^{14}C amount in plants, rhizohyphosphere soil and hyphosphere soil:

Total ^{14}C recovered = ^{14}C-shoot + ^{14}C-root + ^{14}C-rhizohyphosphere +

^{14}C-hyphosphere (1)

The ^{14}C incorporation (as proportion of total ^{14}C recovered) of shoots, roots, rhizohyphosphere soil, hyphosphere soil, MBC, and DOC were calculated as follows (Zang et al., 2020):

$$^{14}C_X\ incorporation = \frac{^{14}C_X}{Total\ ^{14}C\ recovered} \times 100\% \quad (2)$$

where X is the corresponding pool, such as shoots, roots, rhizohyphosphere soil, hyphosphere soil, MBC, and DOC.

To quantify the gradients of ^{14}C and enzyme activities, corrected ^{14}C images and zymograms were converted to 16-bit grey values and imported to ImageJ as mentioned above. Root segmentation was easy to perform because of the remarkably contrasting color between soil and roots. We selected 4 single root segments of each tree seedling that showed minimal overlap with other roots. Then, we drew a line in an angle of about 90° to the root and moved the line 10 times for each of the 4 roots with about 1 cm distance. The gray values on this line were extracted and were converted to ^{14}C and enzyme activity based on the corresponding calibration curves. Generally, 10 analyses along one main root were performed. Then 40 different lines were chosen from each zymograme randomly, and the average converted ^{14}C and enzyme activity of these replicates was plotted against distance from root (Zhang et al., 2019). To quantify the radial pattern of ^{14}C and enzyme activity, a logistic curve (four-parameter) was used to ^{14}C and enzyme activity as a function of distance from the root surface (Razavi et al., 2016). We defined the rhizosphere extension (i.e. ^{14}C and enzyme activities) as the radial gradient from the root up to activities of at least 20% higher than the constant level of the regression curve (Fig. 4 and Fig. S3) (Zhang et al., 2019).

The ^{14}C and specific enzyme activities in the rhizohyphosphere and hyphosphere soil for each treatment were calculated as the average of sum of pixel-wise ^{14}C and enzyme activities in the rhizohyphosphere and hyphosphere soil after subtraction of their background, respectively. The area with distinguished intensity color contrast with surrounding area were considered as ^{14}C and enzyme hotspots (Duan et al., 2019).^{14}C and enzyme activities exceeding 20% of mean corresponding activity of the rhizohyphosphere soil were defined as ^{14}C and enzyme hotspots, respectively (Liu et al., 2017). The hotspots areas were expressed as the percentage of total surface area of the rhizohyphosphere. In addition, to avoid effects of different photosynthesis and root biomass between the treatments on ^{14}C allocation into the rhizohyphosphere, the specific ^{14}C activity was calculated as follows:

Specific ^{14}C activity = ^{14}C activity / ^{14}C-root (3)

where ^{14}C activity in the rhizohyphosphere soil is measured by imaging, and ^{14}C-root is the amount of ^{14}C in root biomass measured by LSC.

Enzyme ratios (BG / (NAG + LAP)) and (BG / ACP) represent the relative C or nutrient (N and P) limitations (Sinsabaugh et al., 2008).

2.6 Statistical analysis

All data are presented as the means of four replicates ± SE (n = 4).

Shapiro-Wilk's test and Levene test were used to check the normality and homogeneity. The log_{10} transformed to used to conform the assumption of normality before further analysis if the data were non-normally distributed. A one-way ANOVA (with Tukey test) was used to identify differences between treatments by SPSS version 19.0 (SPSS Inc., USA).

3. Results

3.1 Plant biomass

Ectomycorrhizae (ECM) colonization caused a 50% reduction in root biomass compared to uninoculated (Control) or plants inoculated with non-mycorrhizal rhizosphere fungi (NMRF) ($p < 0.05$, Table. 2). When co-inoculated with ECM and NMRF (MIX), root biomass was less reduced than for plants inoculated with only ECM, but was still 20% lower than that for the control (Table 2). Plants with solely NMRF inoculation showed no changes in root biomass. Contrary to the plant biomass pattern, ECM colonization increased the root-to-shoot ratio up to 2.8, which was the highest among the treatments ($p < 0.05$, Table. 2).

Table. 2 Shoot biomass, root biomass, and the ratio of root to shoot of European spruce (*Picea abies* L.) without inoculation (Control), inoculated with non-mycorrhizal rhizosphere fungi (NMRF), inoculated with ectomycorrhizal fungi (ECM), and inoculated with ECM and NMRF (MIX). Values are averages (±SE) of four replicates. Letters show significant differences ($p < 0.05$).

	Shoot biomass (g)	Root biomass (g)	Root: Shoot
Control	0.26±0.02a	0.54±0.04a	2.038±0.118b
NMRF	0.22±0.01ab	0.52±0.06a	2.180±0.215b
ECM	0.13±0.01b	0.33±0.01b	2.704±0.154a
MIX	0.18±0.02ab	0.41±0.01ab	2.356±0.221ab

3.2 Assimilated ^{14}C allocation in the plant-soil system

Regardless of the inoculation treatment, plant roots were the main sink for assimilated CO_2, incorporating more than 70% of total ^{14}C recovered in the plant-soil system (Fig. 1b). The ^{14}C recovery in soil (Fig. 1c) and microbial biomass (Fig. S1c) showed a similar pattern with lowest ^{14}C allocation to belowground pools for Control and NMRF inoculation, intermediate allocation for MIX, and highest ^{14}C allocation for ECM. The ^{14}C released into the rhizohyphosphere with ECM inoculation was 2-3 times higher compared to Control ($p < 0.05$, Fig. 1c), whereas ^{14}C allocation to the rhizohyphosphere inoculated with NMRF was only around 2.3%. In soils with co-existence of ECM and NMRF, ^{14}C allocation to the rhizohyphosphere was lower than that for soil with ECM inoculation, but it was on average 90% higher compared to plants without inoculation or inoculated with NMRF (Fig. 1c). Moreover, ECM inoculation increased the ^{14}C allocation to the hyphosphere compared to Control ($p < 0.05$); however, there was no difference in ^{14}C allocation to the hyphosphere between Control and NMRF inoculation ($p > 0.05$, Fig. 1d). ^{14}C incorporation into the soil microbial biomass and into the DOC pool represented < 1% of the total ^{14}C recovery (Fig. S1). Similarly to the ^{14}C allocation to soil, ECM inoculation increased ^{14}C allocation to microbial biomass in both the rhizohyphosphere and hyphosphere soil compared to Control ($p < 0.05$), whereas NMRF inoculation did not increase

^{14}C-MBC relative to Control (p > 0.05). Importantly, the ^{14}C allocation to DOC was highest for soils inoculated with both types of fungi (Fig. S1).

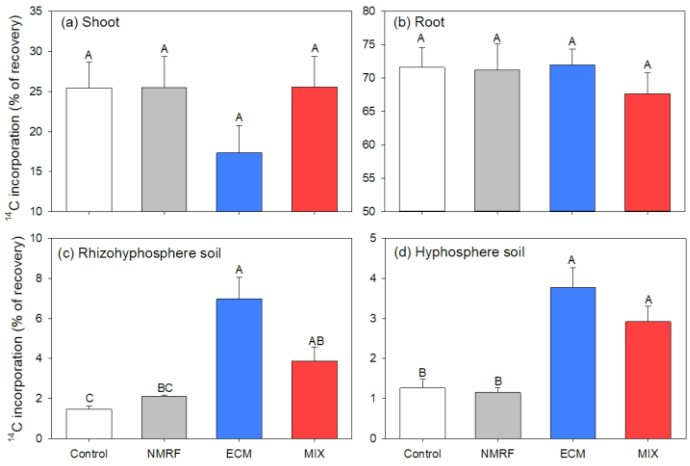

Fig. 1 The allocation of photosynthetically-fixed C, as a percentage of the total ^{14}C recovered, to shoots (a), roots (b), rhizohyphosphere soil (c), and hyphosphere soil (d) of European spruce (*Picea abies* L.) without inoculation (Control), inoculated with non-mycorrhizal rhizosphere fungi (NMRF), inoculated with ectomycorrhizal fungi (ECM), and inoculated with ECM and NMRF (MIX). Values are averages (±SE) of four replicates. Note the difference in y-axis scale and that some do not start from 0.

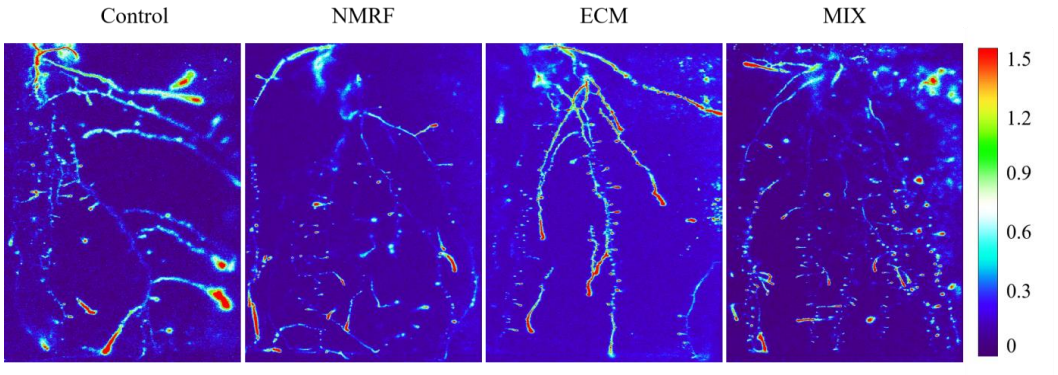

Fig. 2 ^{14}C phosphor images of the root system of European spruce (*Picea abies*L.) without inoculation (control), inoculated with non-mycorrhizal rhizosphere fungi (NMRF), inoculated with ectomycorrhizal fungi (ECM), and inoculated with ECM and NMRF (MIX) in the rhizohyphosphere soil (20 × 10 cm). Side color scale is proportional to the ^{14}C activities (Bq mm^{-2}).

3.3 Spatial distribution of plant-derived ^{14}C

The plant C allocation and spatial distribution was visualized by ^{14}C imaging, with the red colors indicating high ^{14}C activity (Fig. 2). ^{14}C activities in rhizohyphosphere inoculated with ECM or NMRF (4.3-4.8 Bq mm^{-2}) were much higher than in Control (p < 0.05, Fig. 3a). Compared with Control, the ^{14}C specific activity in rhizohyphosphere was 90% and 200% higher for plants inoculated with

NMRF and ECM, respectively ($p < 0.05$, Fig. 3c). When co-inoculated with ECM and NMRF, the specific ^{14}C activity in the rhizohyphosphere was 0.88 Bq mm^{-2} g^{-1} root, decreased by 35% compared to ECM inoculation ($p < 0.05$, Fig. 3c). In the hyphosphere, activity with ECM inoculation was 0.25 Bq mm^{-2}, 8-20 times higher compared to Control or NMRF inoculation ($p < 0.05$, Fig. 3b). This demonstrates that extraradical mycelium penetrated from the root to the hyphosphere. In soils with both types of fungi, ^{14}C activity in the hyphosphere (0.12 Bq mm^{-2}) decreased 2-fold compared to ECM inoculation ($p < 0.05$, Fig. 3b). Interestingly, the ^{14}C activity hotspot area in the rhizohyphosphere was much larger under plants with fungi inoculation (0.17-0.23%) than under Control plants (0.08%, Fig. 3d).

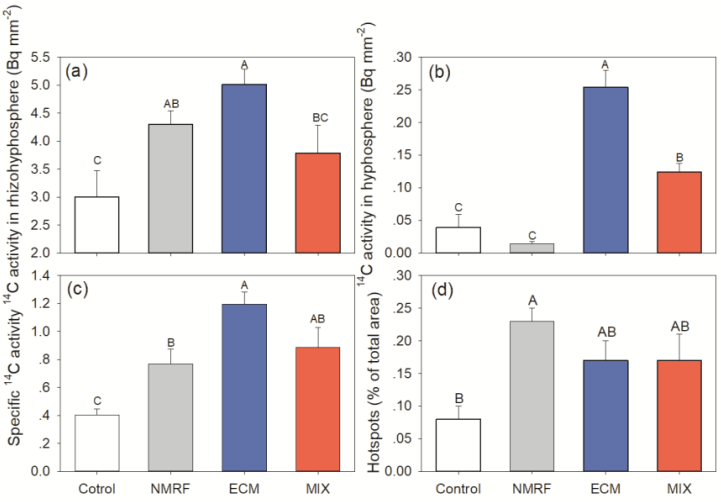

Fig. 3 ^{14}C activity in the rhizohyphosphere soil (a), hyphosphere soil (b), specific ^{14}C activity (c), and percentage of hotspots in rhizohyphosphere soil (b) of European spruce (*Picea abies* L.) without inoculation (Control), inoculated with non-mycorrhizal rhizosphere fungi (NMRF), inoculated with ectomycorrhizal fungi (ECM), and inoculated with ECM and NMRF (MIX). To avoid effects of photosynthesis and root biomass on ^{14}C allocation into the rhizohyphosphere, the ^{14}C activity in the rhizohyphosphere from imaging were normalized by the ^{14}C activity in the root biomass. Values are averages (±SE) of four replicates. Letters show significant differences ($p < 0.05$).

Radial distributions of ^{14}C activities along the main roots as a function of distance from the root surface are given in Fig. 4. The inoculated fungi affected the ^{14}C activity in the rhizohyphosphere. The activity at the root surface was on average 0.8 Bq mm^{-2} for plants inoculated with ECM (ECM and MIX), which was 2-2.5 times higher than that without ECM (NMRF and Control, Fig. 4). Besides higher ^{14}C activity, plants with ECM inoculation transport plant-derived ^{14}C components to a slightly broader rhizosphere area (2.0 mm away from root surface) compared with plants without ECM inoculation (1.6 mm, Fig. 4).

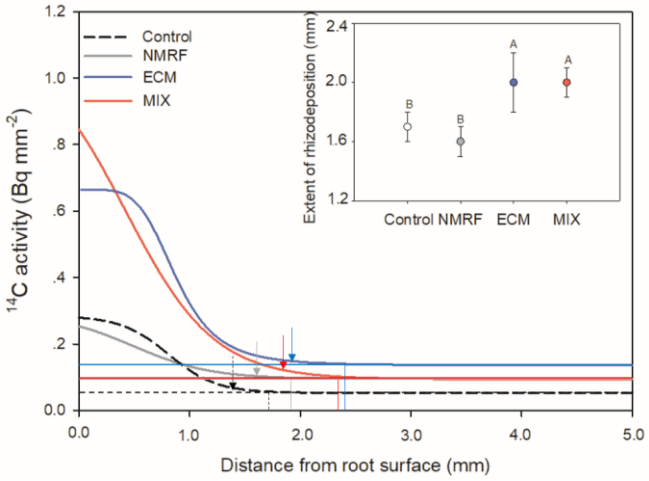

Fig. 4 Vertical ^{14}C gradients as a function of distance from the root surface of European spruce (*Picea abies* L.) without inoculation (control), inoculated with non-mycorrhizal rhizosphere fungi (NMRF), inoculated with ectomycorrhizal fungi (ECM), and inoculated with ECM and NMRF (MIX). Values are averages (±SE) of four replicates. Letters show significant differences (p < 0.05).

3.4 Spatial distributions of enzyme activity

Zymography was used to visualize and evaluate four classes of enzyme activities involved in C, N and P cycles in the rhizosphere of plants inoculated with various fungal groups (Fig. S2). Chitinase and leucine-aminopeptidase activities were 57 and 49% higher in soil with ECM compared to soil with NMRF-inoculation, respectively (p < 0.05, Fig. 5a). In contrast, β-glucosidase in the soil with NMRF inoculation was 20% and 59% higher compared with MIX and ECM inoculation, respectively. The hotspots area of chitinase and leucine-aminopeptidase were broader in rhizohyphosphere with ECM (ECM, MIX) versus NMRF or Control (Fig. 5b).

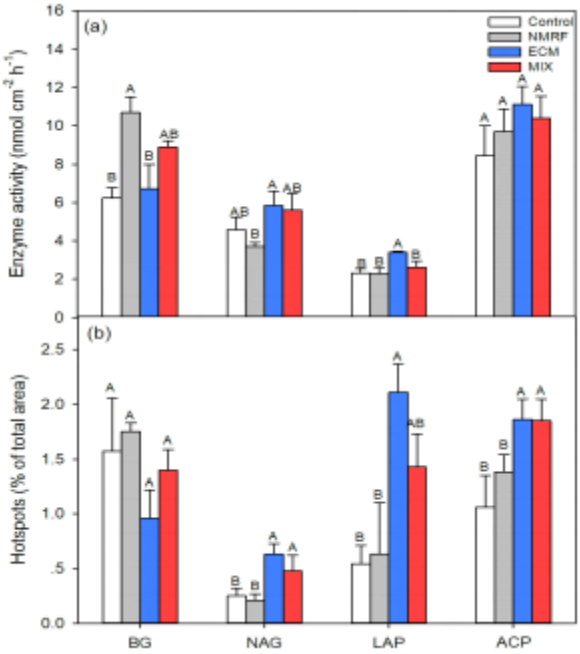

Fig. 5 Enzyme activity (a) and hotspot area (b) of β-glucosidase (BG), chitinase (NAG), leucine-aminopeptidase (LAP), and acid phosphatase (ACP) in rhizohyphosphere soil of European spruce (*Picea abies* L.) without inoculation (Control), inoculated with non-mycorrhizal rhizosphere fungi (NMRF), inoculated with ectomycorrhizal fungi (ECM), and inoculated with ECM and NMRF (MIX). Values are averages (±SE) of four replicates. Letters show significant differences ($p < 0.05$).

The C/N acquisition ratio was about 150% lower in soils with ECM compared to NMRF and Control ($p < 0.05$, Fig. 6). There was no significant difference in C/N ratio between ECM and MIX treatments. Similarly, the C/P acquisition ratio was 30-94% smaller for ECM than that for any other treatments ($p < 0.05$, Fig. 6).

The rhizosphere extent of enzyme activities involved in the N and P cycle, e.g., chitinase and acid phosphatase, was broader for ECM (2.9-3.4 mm) than for NMRF treatment (1.6-1.9 mm) (Fig. S3a, c, d; Fig. S4). In contrast, the rhizosphere extent of β-glucosidase for ECM was 36% smaller than under NMRF inoculation ($p < 0.05$, Fig. S4).

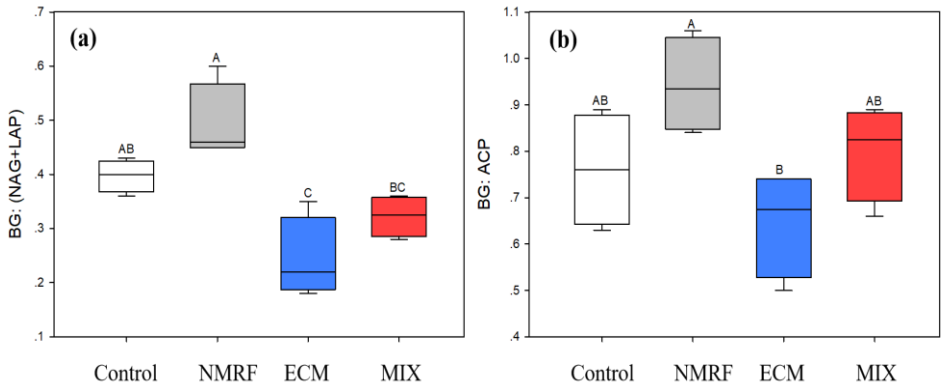

Fig. 6 Enzyme C (β-glucosidase, BG) to N (the sum of chitinase and leucine-aminopeptidase) (a), and C to P (acid phosphatase, ACP) acquisition ratio (b) in rhizohyphosphere soil of European spruce (*Picea abies* L.) without inoculation (Control), inoculated with non-mycorrhizal rhizosphere fungi (NMRF), inoculated with ectomycorrhizal fungi (ECM), and inoculated with ECM and NMRF (MIX). Values are averages (±SE) of four replicates. Letters show significant differences ($p < 0.05$).

4. Discussion

4.1 Ectomycorrhizal and non-mycorrhizal rhizosphere fungi modify photosynthetic carbon allocation belowground

The reduced shoot and root biomass of plants with ECM colonization compared to the other treatments was probably caused by high C investment into extraradical mycelium, a strong sink for C allocation belowground (Conjeaud et al. 1996; Wu et al., 2002). This could help to explain the higher ^{14}C activity in the hyphosphere soil

of ECM compared to other inoculations (Fig. 1d, 3b). As the ^{14}C incorporation into roots was similar in ECM inoculation and in Control soil (Fig. 1b), we conclude that the extra photosynthetic C is used for exudation of low molecular weight exudates or for production and secretion of exoenzymes (Qu et al., 2004). As a consequence, more ^{14}C remained in the soil under ECM inoculation versus Control (Fig. 1c). In this study, the tissue of the ECM hyphae themselves may have contributed to the high ^{14}C activity in the hyphosphere (Fig. 3b) because after destructive sampling the soil sample also contains fungal structures.

Similar to ECM, NMRF inoculation increased plant C allocation belowground relative to that in the Control, as indicated by the higher specific ^{14}C activities in the rhizohyphosphere with NMRF (Fig. 3c). This agrees with earlier studies that NMRF incorporate recently fixed plant C and are therefore major consumers of rhizodeposits (Ballhausen & Bore, 2016; Pausch et al., 2016). On the one hand, NMRF could obtain root-derived C via antagonistic interactions, caused by host defense due to the ability of NMRF to disrupt living plant cell walls (Aguilar-Trigueros et al., 2014; Voříšková et al., 2014). In particular, Hoeksema and Kummel (2003) suggested that plants could promote the mortality of root tips colonized by less beneficial fungi (i.e. *Trichoderma*). Note, two of the NMRF taxa we inoculated - *Trichoderma asperellum* and *Trichoderma viride*, have root-penetrating abilities, which may enable them to feed on assimilated C before released by roots (Harman et al., 2004; Field et al., 2015). Hence, part of the ^{14}C allocated to NMRF may be independent of rhizodeposition or may be directly consumed during the process of rhizodeposition.

The radial rhizosphere extension of ^{14}C-rhizodeposits ranged from 1.4-2.1 mm, which is comparable to previous studies (Kuzyakov et al., 2003; Hafner et al., 2014; Holz et al., 2018). In the rhizohyphosphere soil, plants with ECM inoculation showed a much broader extent of ^{14}C than those with NMRF inoculation, but released the rhizodeposits within a smaller hotspot area (Fig. 3c,d). Hotspots of root-derived C were highly concentrated in small areas around the root tips for ECM-inoculated tree seedlings (Fig. 2), where the ECM often form a hyphal sheath covering the root tips (Teromoto et al., 2012). However, mycorrhizal hyphae can displace plant C beyond the root zone (Smith & Read, 2008), leading to a wider spread and even distribution of C and, hence, to a smaller ^{14}C hotspot area in soil inoculated with ECM (Fig. 3d). Furthermore, the ECM extraradical mycelium secrets ^{14}C photosynthates not only through radial paths from the colonized ECM root tips but also lateral ones in hyphal anastomosis, then ^{14}C photosynthates is transformed freely in every direction through sites of hyphal branching (Teromoto et al., 2016). As a consequence, the rhizosphere extent defined by rhizodeposition was broader for plants inoculated with ECM compared to others (Fig. 4). Overall, both ECM and NMRF increased the belowground C sink strength and modified the C allocation pattern in plant-soil systems.

4.2 Plant photosynthetic C drives rhizosphere enzyme activities

Enzyme activities and their spatial extension in the rhizosphere were strongly affected by the type of fungi present (Fig. 5, S4). ECM are supplied with C by their

host and are generally rather nutrient- than C-limited (Smith & Read, 2010). Accordingly, the increase in the radial extension of photosynthetic C around roots is of particular importance because it expands the soil volume in which these root exudates can interact with microorganisms (Holz et al., 2018). The use of these exudates by microorganisms as C and energy resources implies an increase in the soil volume for nutrient uptake. We therefore observed such a wider rhizosphere extension of chitinase and acid phosphatase for soils inoculated with ECM (Fig. S4). This was also supported by a positive correlation between the extent of rhizodeposition and rhizosphere extension of acid phosphatase ($p < 0.05$, Fig. S5). Besides in exchange nutrients with their hosts for C, the higher C content in the extraradical mycelium of ECM (Trudell & Edmonds, 2004; Trocha et al., 2016) also suggests a higher nutrient demand of ECM compared to NMRF. Accordingly, more N-degrading enzymes were exuded by the extraradical mycelium to hydrolyse SOM to acquire nutrients for the growth of extraradical mycelium. This is indicated by the lower ratio of enzyme activities of C- to N-cycle related enzymes (C:N) and C- to P-cycle related enzymes (C:P) in ECM versus NMRF or MIX treatments (Fig. 6a, b).

Unlike ECM, NMRF not only increased belowground C inputs (Fig. 3c) but also invested more of this C resource in β-glucosidase, as indicated by a higher activity of β-glucosidase activity in the rhizohyphosphere than in any other treatment (Fig. 5a). This could be explained by the fact that the direct exudation by plants may be insufficient to meet the growth demand of the entire hyphal network of NMRF (Hobbie & Horton, 2007) because of the highest hyphal density in soil (Söderström, 1979). Therefore, more β-glucosidase (and presumably also other C-cycle involved enzymes) were released to mineralize native SOM and dead root tissue to acquire energy and C for their hyphal growth. Such an explanation, however, requires further studies to investigate the link between hyphae density, microbial community composition, C-degrading enzyme-related gene expression, and the molecular composition of root exudates as well as their consumption.

The target enzymes released by ECM and NMRF were therefore different due to the acquisition of their most limited or required elements - either for their own tissue (Allison & Vitousek, 2005) or for maintaining their symbiotic relationship (Read & Rerez-Moreno, 2003).

4.3 Competition between NMRF *and ECM affects plant C allocation*

Consistent with our fourth hypothesis, joint NMRF and ECM inoculation reduced C allocation belowground compared to ECM inoculation alone. This points to a competition of ECM and NMRF for the same nutrients ((Lindahl et al., 2001; Bödeker et al., 2016; Clemmensen et al., 2016). In our study, ECM growth is likely suppressed due to the competition with NMRF for soil nutrients. During the competitive processes, ECM may produce less or lose parts of their hyphal biomass (mostly ^{14}C-enriched root tips). As a consequence,^{14}C activities were lower in the hyphosphere soil of MIX compared with ECM treatment (Fig. 3b). However, even though competition exists, ECM remain the winner of this battle, as indicated by a higher ^{14}C activity in the hyphosphere for MIX than for NMRF inoculation (Fig. 1d, 3b). This favorable position of ECM reflects the fact that they gain high proportions

of their C in an available form directly from plants, while NMRF only access the limited C resources from SOM, which has to be mobilized by "costly" enzyme production (Fernandez & Kennedy, 2016). Moreover, ECM can also capture nutrients released from SOM decomposition by saprotrophic fungi (Lindahl et al., 1999; Cairney & Meharg, 2002). This enables them to profit from their energy and C investment into enzyme production. Finally, under certain conditions, ECM were found to release antibiotics to limit NMRF activities (Bödeker et al., 2016) - a process we were unable to detect in this experiment. Nonetheless, this range of processes probably helped ECM to outcompete NMRF when living in competition for C and nutrients. Such an explanation about the competition between ECM and NMRF, however, requires further studies to investigate the links between fungal community composition and ^{14}C activity as well as enzyme activity in the soil inoculated with both fungal species.

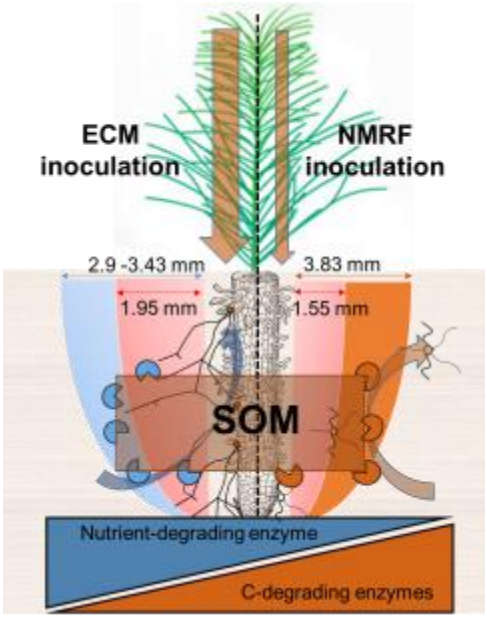

Fig. 7 Graphical abstract of C allocation belowground and specific enzyme production from plants inoculated with ectomycorrhizal (ECM) and non-mycorrhizal rhizosphere fungi (NMRF). Orange arrow indicates C allocated from above- to belowground pools, arrow width indicates C flow strength. Blue arrow: nutrient flow from soil organic matter decomposition by specific enzymes (e.g., C- and N-degrading enzymes).

Conclusions

Both ectomycorrhizal (ECM) and non-mycorrhizal rhizosphere fungi inoculation (NMRF) induced a higher C allocation belowground, due to the direct linkage between the host and extraradical mycelium for ECM, and the indirect uptake of exudates by NMRF. Higher N-acquiring enzyme activities were observed in the rhizohyphosphere soil with ECM inoculation compared with the control and the NMRF inoculation. This suggests that such changes may be regulated by the supply of photosynthates from the host plant. In contrast, NMRF produced 59% higher β-glucosidase compared to plants inoculated with ECM, presumably because NMRF

consumed plant-derived C efficiently whereas ECM received plant C directly from host plants. The ECM growth decreased when co-inoculated with NMRF, which was accompanied by a reduced ^{14}C movement from the rhizohyphosphere to hyphosphere soil. Nonetheless, similar rhizosdeposit allocations into the hyphosphere for ECM and MIX inoculations indicate that ECM outcompeted NMRF, which could slow down SOM decomposition and lead to a potential C storage in forest soils. Overall, *Picea abies* colonized with ECM and NMRF both induced an increased root exudation and promoted enhanced enzyme activities, but ECM focused on nutrient mobilization whereas NMRF presence stimulates enzymes of the C cycle. This suggests that shifts in the fungal community composition of mycorrhizal plants could induce changes in the quality and quantity of rhizodeposition, thereby potentially affecting both soil C and soil nutrient cycles.

Acknowledgments

We thank the China Scholarship Council (CSC) for funding to Jie Zhou (NO.201606850093) and Bin Song in Germany. This study was financially supported by the German Research Foundation (DFG) within the project PA 2377/2-1, GU 1309/5-1 and DI 2136/6, as well as NSFC-CGIAR Joint Research Programme 31861143002. The work was supported by the Government Program of Competitive Growth of Kazan Federal University and with the support of the "RUDN University program 5-100". The pulse ^{14}C labeling was conducted together with Gabriele Lehmann and Rainer Schulz of the Laboratory for Radioisotopes (LARI), Göttingen. The authors would like to thank Immo Shan for his help to incubate plants before labeling. The authors also would like to thank Callum C. Banfield and Bernd Kopka for their laboratory assistance with ^{14}C labeling and imaging.

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Supplementary

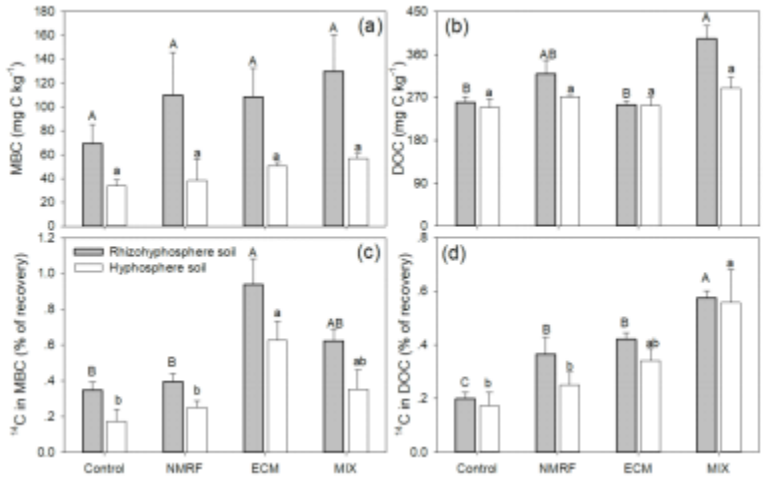

Fig. S1 Microbial biomass C (MBC, a), dissolved organic C (DOC, b), and ^{14}C incorporated into MBC (c) and DOC (d) in the soil planted with European spruce (*Picea abies* L.).

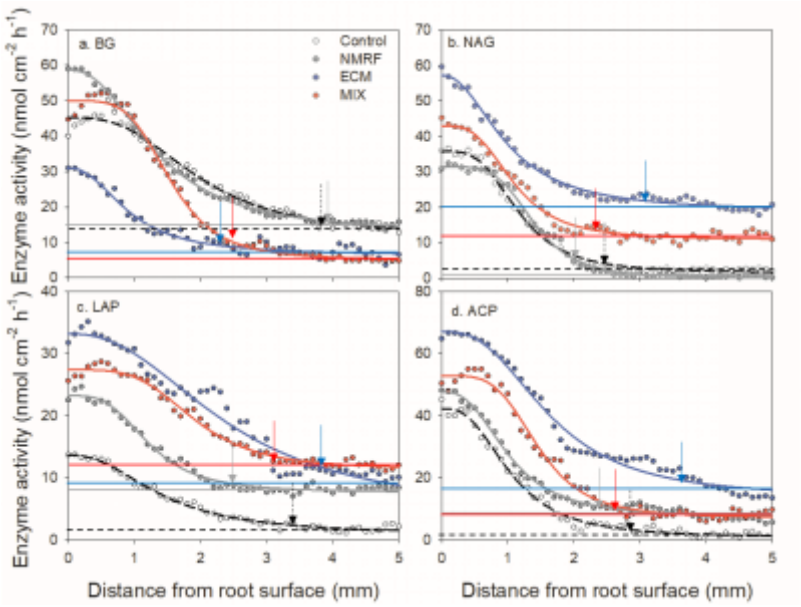

Fig. S2 Profiles of the enzyme activity distribution as a function of the distance from the root surface towards the surrounding soil.

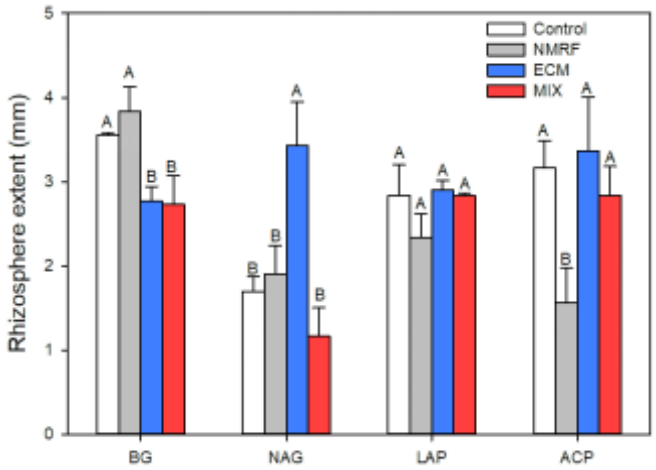

Fig. S3 Rhizosphere extent of β-glucosidase (BG) (a), chitinase (NAG) (b), leucine-aminopeptidase (LAP) (c), and acid phosphatase (ACP) (d)).

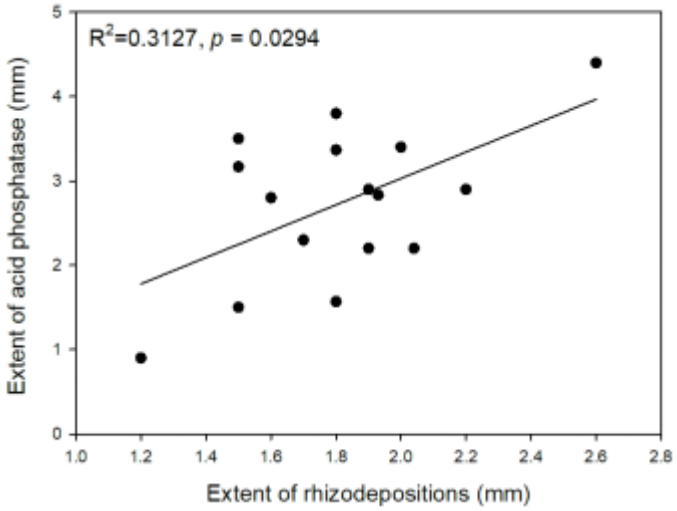

Fig. S4 A correlation between extent of rhizodeposition and the extent of acid phosphatase of European spruce (*Picea abies* L.).

Study 4 Restricted power: is microbial pool still able to maintain the stability of soil organic matter under warming exceeding 2 degrees?

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Status: In preparing

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Abstract

The Paris Climate Agreement is pursuing efforts to limit the increase in global temperature to below 2 °C above the pre-industrial level. Therefore, there is an urgent need to know whether microbial self-regulation is able to maintain the stability of soil organic carbon (SOC) under warming approaching and exceeding 2 degrees. Here, we tested the self-regulatory mechanisms mediating soil C and N cycling through microbial and enzymatic functional traits by long-term warming field study and combined the results with extended literature review. The SOC and total nitrogen (TN) remained stable at warming below 2 °C, while stronger warming (by 2-4 °C) did not affect SOC but it increased the TN content. Possible explanation of increased TN was linked to contrasting response of kinetic parameters of enzymatic and microbial growth functional traits. Warming induced faster microbial growth and turnover (indicated by 71% higher microbial specific growth rates, by 18h and 3h shorter lag- and generation time, respectively). In contrast, warming reduced catalytic efficiency and slowed down the enzymes-mediated turnover of oligosaccharides and polypeptide-like compounds (indicated by 1-1.5 times decreased affinity to substrate of β–glucosidase and leucine aminopeptidase). As fast-growing microorganisms are not able to maintain high level of population for a long time, the total microbial biomass decreased under warming by 32%. Lower enzymatic efficiency and slower turnover of organic residues under warming thus may cause accumulation of microbial necromass. Such a self-regulation by tiny but powerful microbial controller should be included in the models to improve the prediction of soil C and N feedbacks to climate warming.

Keywords: Carbon cycling, Enzyme kinetics, Microbial growth rate, Warming magnitude

1. Introduction

Global temperatures are predicted to rise by 1.0 to 4.8 °C at the end of twenty-first century (IPCC, 2013; O'Neill et al., 2017), with high risk to accelerate soil organic carbon (SOC) mineralization (Emmett et al., 2004; Davidson & Jassens, 2006; Alvarez et al., 2018). Given that soils store vast amounts of carbon (C), any small reduction in SOC would result in strong positive feedback on atmospheric CO_2 and exacerbate climate change (Heimann & Reichstein, 2008). The majority of field experiments investigating the effects of climate warming on soil C cycling, however, have either exceeded the predicted temperature increase (> 4 ℃) or were based on relatively short-term warming (< 5 years) (Allison et al., 2008; Bradford et al., 2008; Melillo et al., 2011; Rousk et al., 2012; Contosta et al., 2015). Therefore, studies with more realistic future temperature regimes conducted over longer periods are required for accurate prediction of the response of soil C and nitrogen (N) cycling to climate change. Specifically, the Paris Climate Agreement aims to limit global mean atmosphere warming to no more than 2 ℃ above pre-industrial levels. There is increasing evidence that climate change will be faster than projected, indicating that soils face accelerating damage (Xu et al., 2018). Thus, it is important to examine microbial functional traits under the 2 ℃ target and to evaluate what will happen if the soils bear greater warming.

Warming-induced acceleration of SOC mineralization is the result of microbial growth on heterogeneous organic substrates and is strongly dependent on the size and functional traits of the active microbial fraction (Schlesinger & Andrews, 2000; Chen et al., 2014). However, the predictions on whether microorganisms will grow faster or slower under climate warming remain controversial. As an immediate response, warming may accelerate the turnover of the fast-growing microbial population (Blagodatskaya et al., 2010), whilst the microbial growth rate may reduce due to the rapid exhaustion of available organic C under long-term warming (Bradford et al., 2008). Therefore, the question ‘How will long-term warming (i.e. < 2 °C and > 2 °C warming for > 5 years) affect soil microbial physiology and in turn, how will this feedback on C cycling?’ still remains to be satisfactorily answered.

Temperature strongly affects microbially-mediated enzymatic processes (Conant et al., 2011; Weedon et al., 2013) by altering the availability of energy and nutrients in soil (Allison, 2006; Sinsabaugh et al., 2009). Enhanced inputs of plant C with warming (Bai et al., 2010; Yin et al., 2013), could induce greater enzyme production due to the increased nutrient demand to meet microbial stoichiometric requirements (Conant et al., 2011). This enhanced enzyme production subsequently causes an increase in the maximal reaction rate (V_{max}), which can be counterbalanced by the reduced substrate affinity of the enzyme (increased K_m) with increasing temperature (Blagodatskaya et al., 2016). Shifts in the enzyme’s intrinsic properties (K_m) could then, in turn, lead to changes in SOC decomposition, C storage, and other biological processes

(Sinsabaugh et al., 2002; Waldrop et al., 2004). It is not clear, however, to what extent the affinity of exo-enzymes towards substrates will be altered under < 2 °C and > 2 °C warming in the long term.

Theoretical models have suggested that the stoichiometric imbalance between microorganisms and organic compounds in soil is the major cause for C or N limitation of SOC decomposition (Manzoni et al., 2008; Kaiser et al., 2014). Warming could stimulate plant nutrient uptake and root exudation, resulting in decreased soil nutrient availability but in increased C availability (Zak et al., 2003; Bird et al., 2011; Lin et al., 2018). These changes in C and N pools with warming could result in a microbial stoichiometric imbalance, thereby inducing the specific release of enzymes to liberate growth-limiting elements from soil (Mooshammer et al., 2014b). However, it remains highly uncertain whether microorganisms maintain their stoichiometric ratios in soil and subsequently influence SOC decomposition under different warming regimes. This knowledge gap limits our ability to build mechanistic mathematical models that can accurately simulate how soil ecosystems will respond to climate warming.

Therefore, we aimed to: 1) estimate the changes in microbial functioning and enzyme kinetics after 8 years of warming; 2) link the stoichiometric ratios of soil and microorganisms; and 3) quantify the changes of soil microbial and enzymatic process, as well as their consequences for C and N pools depending on the magnitude of warming (< 2 °C and > 2 °C). We hypothesized that climate warming would stimulate microbial growth rate, as well as enzyme activities, reduce soil C and N pools, and thereby shrinking soil C sequestration due to plant-derived labile C input under warming. We also predicted that this positive response of soil C and N pools would depend on the magnitude of warming.

2. Materials and methods

2.1. Site description and sampling

This study was conducted on an on-going (since August 2010) long-term warming experiment, located in the northern part of Göttingen, Lower Saxony, Germany (51°33′29.28″N, 9°55′59.46″E) in which soil temperature has been manipulated using heating cables. The mean annual temperature and precipitation were 9.5 °C and 712 mm, respectively (Siebold & von Tiedemann, 2012). The soil is classified as a silt loam textured Haplic Luvisol under an arable cropping regime. The winter oilseed rape cultivar Falcon (NPZ, Hohenlieth, Germany) and the breeding line SEM 05–500256 (SW Seed, Sweden) were sown by hand in a split plot design in August, and harvested in November (Siebold & von Tiedemann, 2012). The three heating regimes included: (1) ambient soil temperature, (2) ambient +1.6 °C, and (3) ambient +3.2 °C. The experimental site consisted of 12 plots (2 m × 2.5 m each) arranged in two rows. Heating cables were buried at a depth of 10 cm in each plot, also in the

control plots, to ensure equivalent physical conditions. Detailed information about the set-up of the warming plots and experimental sites can be found in Siebold & von Tiedemann (2012) and Lukas et al. (2018).

Soil samples were collected from the upper 10 cm of ambient, +1.6 °C and +3.2 °C plots in October 2018. In each of the four field replicates, five sub-samples were pooled to form a mixed soil sample. Once collected, samples were hand-mixed and any visible roots and stones manually removed. Soil samples were stored in gas-permeable plastic bags at 10 °C (approximate field temperature during sampling) until the start of further laboratory experiments (within 5 days of collection).

2.2. Soil basic and microbial properties analyses

Total organic C and N were determined on oven-dried, ground soil using a 2100 TOC/TIC analyzer (Analytik Jena, Jena, Germany). Soil pH was measured in a 1:2.5 (w/v) distilled water extract using a standard calomel electrode (Hanna Instruments Ltd., Leighton Buzzard, UK).

Soil microbial biomass C (MBC) and N (MBN) were determined on fresh soil samples using the chloroform fumigation-extraction procedure of Vance et al. (1987) with minor modifications. After destructive sampling, the soil was carefully mixed and a 5 g sub-sample directly extracted for 1 h using 20 ml of 0.05 M K_2SO_4. Another 5 g of soil was fumigated with chloroform for 24 h and extracted in the same manner. Total C and N concentration in extracts were measured using a 2100 TOC/TIC analyzer (Analytik Jena, Germany). MBC and MBN were calculated by 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 (Wu et al., 1990). The extracted C and N contents from non-fumigated soil samples were considered as dissolved organic C (DOC) and dissolved organic N (DON), respectively (Jones & Willett, 2006). Soil mineral N (NO_3^- + NH_4^+) was measured colorimetrically on the non-fumigated soil extracts using the method of Mulvaney et al. (1996).

The C:N imbalance between resources and microorganisms (both the total form $Soil_{C:N}$ / $MB_{C:N}$) and labile form ($DO_{C:N}$ / $MB_{C:N}$) were calculated as the ratio of the resource C:N ratios ($Soil_{C:N}$ and $DO_{C:N}$) normalized to $MB_{C:N}$ (Chen et al., 2018). Higher C:N imbalance correspond to lower N availability relative to C availability and could therefore be used as a proxy of microbial N limitation (Wild et al., 2012; Mooshammer et al., 2014a). The ratio of DOC to DON was presumed a better representative of the available microbial resource stoichiometry than the bulk soil C:N ratio because the dissolved C and N are more easily available for microbial communities (Wild et al., 2012; Chen et al., 2018).

2.3. Kinetics of substrate-induced growth respiration

The substrate-induced growth respiration (SIGR) approach (Panikov, 1995) was used

to distinguish between the total and active biomass fractions, as well as to determine microbial specific growth rate and lag-time before growth (Blagodatsky et al., 2014). It has to be noted that although C substrate addition is required for the SIGR approach, all kinetic parameters analyzed by SIGR represent the intrinsic features of the dominant microbial populations before substrate addition.

Field-moist soil (1 g) was amended with a mixture containing 10 mg g^{-1} glucose, 1.9 mg g^{-1} $(NH_4)_2SO_4$, 2.25 mg g^{-1} K_2HPO_4, and 3.8 mg g^{-1} $MgSO_4.7H_2O$. Soil samples were then placed in a Rapid Automated Bacterial Impedance Technique bioanalyser (RABIT; Microbiology International Ltd, Frederick, MD), for measuring CO_2 emission at their corresponding field temperature (10 °C as ambient temperature, +1.6 °C, and +3.2 °C). Firstly, we incubated four soil samples at each temperature for 2 days at 45% water holding capacity (WHC) to reduce the impact of the initial disturbance from handling. To measure substrate-induced respiration, a mixture of glucose and nutrients was then added and the samples incubated for a further five days at 75% WHC. The measurement of CO_2 release was based on the conductivity changes in the RABIT system. The average value of CO_2 emission in the 4 h preceding substrate addition was used as a measure of basal respiration (BR). The metabolic quotient (qCO_2) was determined by the ratio of basal respiration to MBC (Anderson & Domsch, 1995). To confirm the adaptation of microbial communities to warming, we also determined microbial growth parameters in soils from all treatments at the same temperature.

The kinetics of microbial growth was estimated by fitting the parameters of Eqn (1) to the measured dynamics of CO_2 evolution (Panikov, 1995):

$$CO_2(t)=A+ B*\exp(\mu \times t) \quad (1)$$

where A is the initial respiration rate uncoupled from ATP generation, B is the initial rate of the growing fraction of total respiration coupled with ATP generation and cell growth, μ is the maximal specific growth rate of soil microorganisms, and t is time. The parameters of Eqn (1) were optimized using a least-square minimization routine. Four replicate respiration curves were used for each treatment. We omitted the first 3 h of measurements from the analysis to exclude the transient effects of glucose addition (Wutzler et al., 2012).

The parameters of microbial growth were calculated from the optimized parameters of the fitted respiration curve Eqn (1). The duration of the lag period (T_{lag}) was calculated using the equation:

$$T_{lag} = \ln (A/B) / \mu \quad (2)$$

The total microbial biomass (TMB) and growing microbial biomass (GMB) before substrate addition were calculated using Eqns (3) and (4), respectively.

$$TMB = B / (r_0 \times Q) \quad (3)$$

$$GMB = TMB \times r_0 \quad (4)$$

where, r_0 is the physiological state index of the microbial biomass before substrate

addition and was calculated as follows:

$r_0 = (B \times (1-\lambda)) / (A + B \times (1- \lambda))$ (5)

where λ is a basic stoichiometric constant, which has an accepted value of 0.9 (Panikov, 1995). *Q* is the total specific respiration activity:

$Q = \mu / (\lambda \times \Upsilon_{CO2})$ (6)

The theory of microbial growth kinetics has been presented previously (Panikov, 1995). Note that in Eqn (6), Υ_{CO2} is the microbial biomass yield per unit of glucose-C and was assumed to be constant throughout the monitoring period with a mean value of 0.6 (Petersen et al., 2005).

In addition, the kinetic approach allowed the assessment of generation time (T_g) of both actively growing and total microbial population consuming glucose. The estimation of for actively growing biomass is based on specific growth rates:

$T_g = \ln (2) / \mu$ (7)

Microbial maximal specific growth rate μ, derived from Eqn (1) was used as an intrinsic property of the microbial population for the estimation of the prevailing growth strategy of the soil microbial community. According to the definitions (Pianka, 1970; Andrews & Harris, 1986), higher μ values reflect a relative domination or shift towards fast-growing *r*-strategists, while lower μ values indicate a relative domination or shift towards slow-growing *K*-strategists.

2.4. Enzyme kinetics

Activity of exo-enzymes: β-1,4-glucosidase (BG) (EC 2.2.1.21), cellobiohydrolase (CBH) (EC 3.2.1.91), xylanase (XYL) (EC 3.2.2.27), β-1,4-N-acetylglucosaminidase (NAG) (EC 3.2.1.52), and leucine aminopeptidase (LAP) (EC 3.4.11.1) were determined using fluorogenic methylumbelliferone (MUF)-based artificial substrates (Marx et al., 2001; Sinsabaugh & Shah, 2012). Briefly, 0.5 g soil (dry weight equivalent) was suspended in 50 mL sterile water by shaking for 30 min, and dispersing with an ultrasonic disaggregator for 2 min using low-energy sonication (50 J s^{-1}). 50 µL of the soil suspension was pipetted into 96-well black pure Grade® microplates (Brand GmbH, Wertheim, Germany), while stirring the soil suspension to ensure uniformity. Afterwards, 50 µL of buffer and 100 µL of the corresponding substrates at concentrations of 2, 5, 10, 20, 50, 100 and 200 µmol substrate g^{-1} soil were added. After substrate addition, the microplates were measured fluorometrically (excitation wavelength 360 nm; emission 450 nm) at 0, 30, 60, and 120 min with an automated fluorometric plate-reader (Victor3 1420 050 Multi-label Counter; PerkinElmer, Waltham, MA, USA). It should be noted that after each fluorescence measurement the microplates were promptly returned to the corresponding climate chambers, so that the measurement time did not exceed 2 min (Razavi et al., 2015).

To describe key enzyme kinetics parameters, we used the Michaelis-Menten kinetic equation which describes how enzyme rate (V) alters with increasing substrate

concentration [S] (Marx et al., 2001):

$V = (V_{max} \times [S]) / (K_m + [S])$ (8)

where V_{max} is the maximal rate of enzyme activity and K_m (Michaelis constant) is the substrate concentration at which $½V_{max}$ occurs.

The substrate turnover time was calcuated according to the following equation: $T = (K_m + S) / V_{max}$ (Panikov et al., 1992), where S is the substrate concentration. The substrate turnover time was calculated at substrate concentration for the situations corresponding of the lack and excess of substrate, as $S = K_m / 10$ and $S = K_m * 10$, respectively.

The catalytic efficiency of enzymes (K_a) was determined as $K_a = V_{max} / K_m$ (Hoang et al., 2016). The K_a characterizes the enzyme catalytic proporties and is used as an indicator to reflect the functional changes of microbial communities (Ticher et al., 2015).

2.5. Data collection

A synthesis of soil microbial responses to temperature was performed on published data using *ISI* Web of Science (http://apps.webofknowledge.com/) and Google Scholar (http://scholar.google.com/) for the period 1990-2019. The search key words were combined with 'warming', 'elevated temperature', 'soil carbon stock', 'microbial biomass', and 'enzyme activity'. The criteria were applied to select appropriate studies as follows: (1) We restricted the data collection to studies where the magnitude of warming was ≤ 4 °C; (2) vegetation, soil parameter, and climate were similar between control and warming treatments; (3) samples size and standard deviations (or standard errors) were reported; (4) warming magnitude and methods were clearly described. Therefore, studies solely focused on the effects of warming under vegetation clipping, precipitation, drought, season, and fertilization regime were excluded. In total, 48 published papers were based on 31 study sites among five ecosystem types (e.g., tundra, shrubland, grassland, forest, or cropland). The data presented in graphs were extracted by digitizing the figures using G3DATA software (http://www. frantz.fi/software/g3data.php). When some critical information was not reported in the published paper, we tried to obtain the information by contact the corresponding author.

2.6. Statistical analysis

The experiment was carried out with four field replicates for each parameter. The value presented in the figures is given as means ± standard error (mean ± SE). Prior to analysis of variance (ANOVA), the data were tested for normality (Shapiro-Wilk) and homogeneity of variance (Levene-test). Any data that were non-normal were either square root or $\log_{10}$-transformed to conform to the assumption of normality before further statistical analysis. One-way ANOVA followed by the Tukey HSD ($p < 0.05$)

was used to assess the effect of temperature on all parameters. All the statistical analyses were performed using SPSS version 22.0 for Windows (SPSS Inc. Chicago).

3. Results

3.1. Stoichiometry of soil organic pools in response to long-term warming

The size of the soil dissolved organic C (DOC) pool was not altered under +1.6 °C. However, +3.2 °C warming increased DOC by 20%, and doubled the DON pool, resulting in a decreased DOC:DON ratio compared to the ambient control ($p < 0.05$, Fig. 1). Soil microbial biomass carbon (MBC) and nitrogen (MBN) were reduced by 32% and 37% under the +1.6 °C, respectively, whilst remained stable with further warming (+3.2 °C; Fig. 1b, e). The C:N ratio of the microbial biomass was similar in all treatments, ranging from 5.3 to 5.9 (Fig. 1h). Although 8 years warming increased total nitrogen (TN) by 26% (+3.2 °C) ($p < 0.05$), the soil organic carbon (SOC) content was not changed (Fig. 1c, f). Therefore, warming decreased the soil C-to-N ratio from 19 to 16 (Fig. 1i). Correspondingly, microbial N limitation decreased, indicated by the decreased C:N imbalance between labile resources and microorganisms (decreased $DO_{C:N}$ / $MB_{C:N}$) in soil with +3.2 °C ($p < 0.05$, Fig. S3d). To prove such a remarkable stoichiometric imbalance revealed by the case study, we analyzed the relevant data about soil response to climate warming available from the literature.

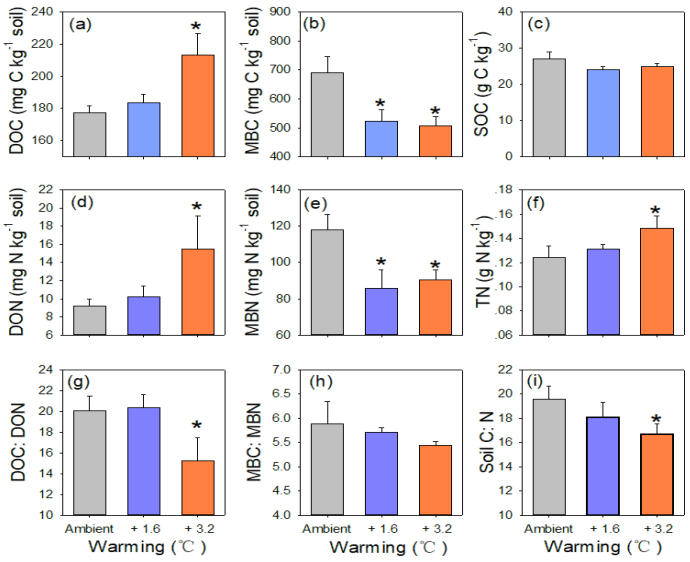

Fig. 1 Dissolved organic carbon (DOC) (a), microbial biomass carbon (MBC) (b), soil organic carbon (SOC) (c), dissolved organic nitrogen (DON) (d), microbial biomass nitrogen (MBN) (e), total nitrogen (TN) (f), and DOC:DON ratio (g), MBC:MBN ratio (h), soil C:N ratio (i) after 8 years warming (ambient, ambient +1.6 °C, and ambient +3.2 °C). Values are the average (± SE) of four replicates. Note the difference in y-axis scale and that some do not start from 0. Asterisks indicate significant differences between warmed and ambient soils.

3.2 Warming alters soil processes (literature review)

Similar to our case study, a synthesis of the literature revealed that a small rise in temperature (< 2 °C above ambient) either has no effect or slightly increased soil C and N related pools (e.g., TN and DON; Fig. 2b). Remarkably, warming (< 2 °C) decreased soil microbial biomass and enzyme activities (xylanase and cellobiohydrolase) by 3.5-4.8% and 8.4-16.5%, respectively. However, higher increases in temperature (2-4 °C) accelerated almost all soil processes, including soil respiration, and specific enzyme activity (chitinase and leucine aminopeptidase). Although 2-4 °C of soil warming increased the content of available N, DOC, and microbial biomass, as well as TN, it did not alter soil SOC pool, consequently reducing the soil C:N stoichiometric ratios. Taking together, both our case study and literature review suggested the restrictions of soil self-regulatory mechanisms. Thus, the 2 °C temperature increase can be generally considered as a threshold for the sustainability of the soil ecosystem. To reveal the drivers of N accumulation in soil, we determined how microbial and enzymatic functional traits were affected by warming.

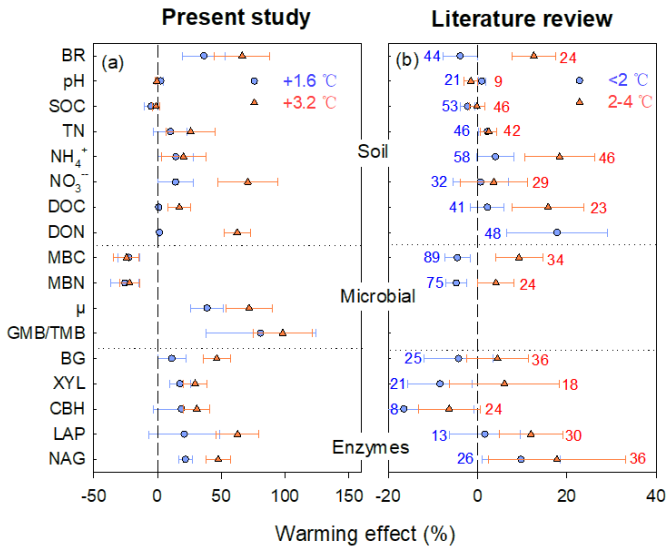

Fig. 2 Effect of warming on soil parameters (SOC, TN, IN, DOC, DON), basal respiration (BS), microbial parameters (MBC, MBN, μ, GMB/TMB), and enzyme activities (BG, XYL, CBH, LAP, NAG) based on our study (panel a) or from a review of the literature (panel b). The left panel shows the changes in parameter value with soil temperature increases of 1.6 and 3.2 °C after 8 years of field warming. The right panel shows the changes in parameter value with soil temperature increases of ≤ 2 °C or 2-4 °C based on 48 observations from the literature. The details for data selection can be found in the text. All value is expressed as percentage change relative to the control (ambient temperature). The vertical dotted black line means control (ambient). The sample size for each variable is shown next to the point.

3.3 Microbial growth kinetics in response to long-term warming

Sight warming (+1.6 °C) did not change maximum specific growth rate (μ_m) of the microbial community, whereas a higher warming (+3.2 °C) increased the μ_m by up to

71% (Fig. 3c). To prove whether such an increase was just a common physiological response to temperature or whether it represents a functional change in the prevailing population, the μ_m was determined at the same temperature (25 °C) for all treatments. Remarkably, the μ_m values in the ambient and +3.2 °C treatments were also significantly different when determined at the same temperature (Fig. S2). +3.2 °C decreased the total microbial biomass (TMB) but increased the proportion of the actively growing microbial biomass within the TMB by 1.4-fold compared to ambient ($p < 0.05$, Fig. 3d). Consequently, the lag-period and generation time of active microbial biomass were, respectively, shortened by 53% and 34% under +3.2 °C warming versus ambient soil, (Fig. 3e, f). The basal respiration (BR) was increased by 58% ($p < 0.05$, Fig. 3b) and the qCO_2 doubled (Fig. S3a) with +3.2 °C warming.

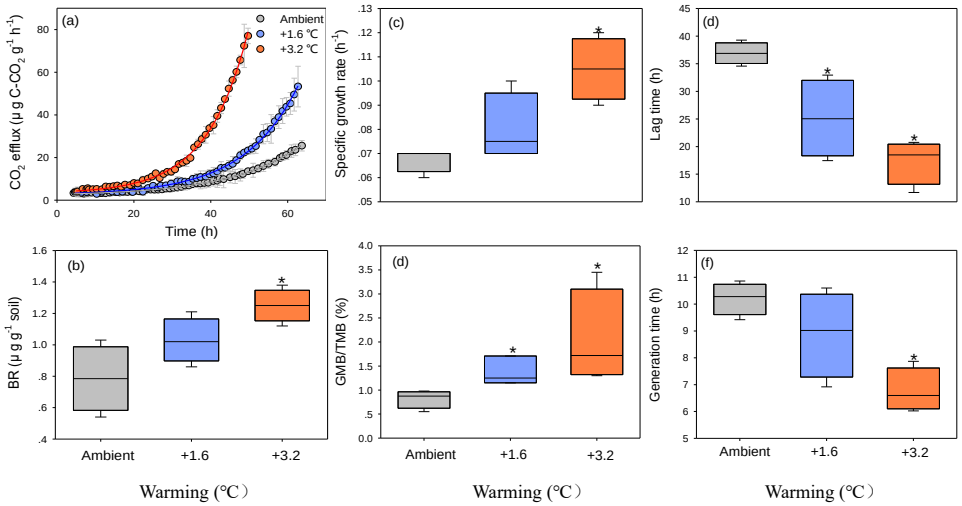

Fig. 3 CO_2 efflux after nutrient addition to soil (a), basal respiration (BR) (b), specific growth rate (μ) (c), the fraction of growing microbial biomass to total microbial biomass (GMB/TMB) (d), and their lag time (e), as well as generation time (f) at soil with long-term warming (ambient, ambient +1.6 °C, and ambient +3.2 °C). Values are the average (± SE) of four replicates. Asterisks indicate significant differences between warmed and ambient soils.

3.4 Enzyme activities and kinetics in response to long-term warming

The activity of the three exo-enzymes β-glucosidcase, chitinase, and leucine aminopeptidase, responded positively to the +3.2 °C increase in temperature. The +3.2 °C warming increased the V_{max} of β-glucosidcase and leucine aminopeptidase, respectively by 35% and 28% versus those at ambient temperature. Furthermore, the increased K_m value indicated that enzyme systems were altered already by +1.6 °C for β-glucosidase and by +3.2 °C of warming magnitude for leucine aminopeptidase ($p < 0.05$, Fig. 4b) towards retarded reaction rate under substrate limitation. This, correspondingly, slowed down the β-glucosidase-driven turnover of oligosaccharides at +1.6 °C warming, whereas the turnover time of peptides (driven by leucine aminopeptidase) changed under +3.2 °C warming ($p < 0.05$, Fig. S5). Similarly, the catalytic efficiency (V_{max} / K_m) for β-glucosidase decreased by 47% under +1.6 °C, while the leucine aminopeptidase responded only to +3.2 °C warming by 50% decrease in catalytic efficiency compared with ambient temperature ($p < 0.05$). In

contrast to β-glucosidcase and leucine aminopeptidase, the V_{max} and K_m of the exoenzymes, which decompose relatively recalcitrant polymeric holocelluloses of plant origin (xylanase and cellobiohydrolase), were not changed with warming (Fig. 4; S5).

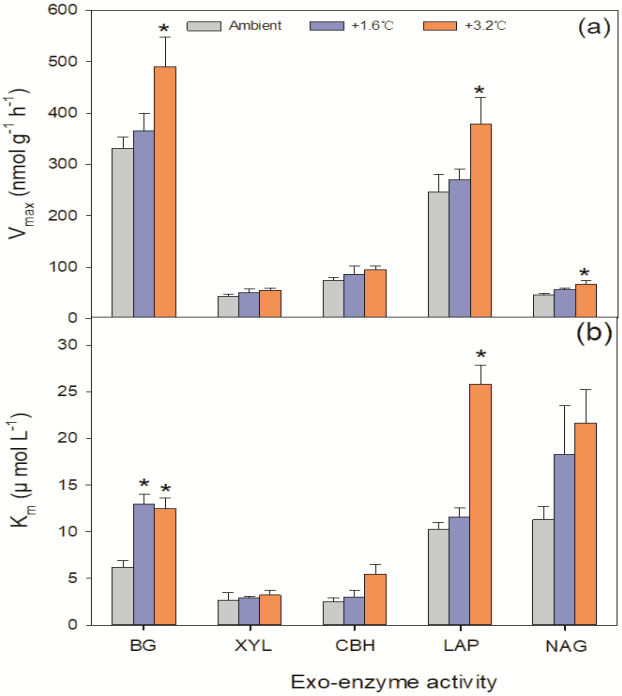

Fig. 4 Potential maximum enzyme activity (V_{max}) and substrate affinity (K_m) of β-glucosidase (BG), xylanase (XYL), cellobiohydrolase (CBH), leucine aminopeptidase (LAP) and chitinase (NAG) in soil exposed to three contrasting long-term temperature regimes (ambient, ambient +1.6 °C, and ambient +3.2 °C). Values are the average (± SE) of four replicates. Asterisks indicate significant differences between warmed and ambient soils.

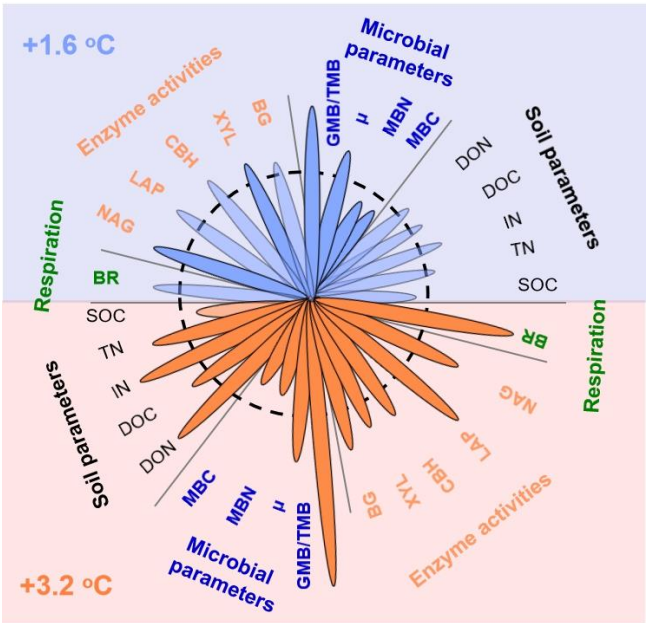

Fig. 5 Effect of long-term warming (+1.6 °C and +3.2 °C during 8 years) on basic soil properties (SOC, TN, IN, DOC, DON), microbial parameters (MBC, MBN, μ, GMB/TMB), and enzyme activities (BG, XYL, CBH, LAP, NAG), as well as basal respiration (BR). Dotted circle means

control (ambient temperature). Blue and red petals mean +1.6 °C and +3.2 °C above ambient temperature. The length of the petals shows the magnitude of the effect of warming relative to the ambient temperature response, i.e. more and less than dotted circle mean increase and decrease, respectively. Shaded petals indicate no significant difference (p > 0.05) between warming and ambient temperature.

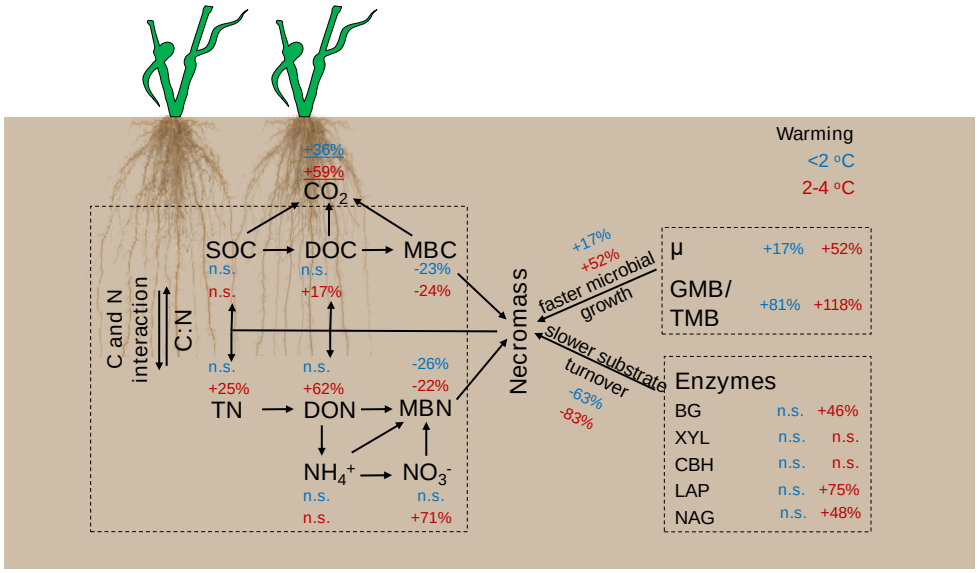

Fig. 6 The fundamental microbial mechanistic framework with soil temperature increases of ≤ 2 °C or 2-4 °C.

4. Discussion

4.1 Is warming exceeding 2 degrees affects soil C and N pools?

Our results are not always in agreement with expectations that warming would induce strong changes in all soil processes (e.g., SOC decomposition, microbial biomass) (Lu et al., 2013; Romero-Olivares et al., 2017). Based on the case study and literature review, we found that the changes in soil C and N pools strongly depends on warming magnitudes. For the first time, we revealed that the active fraction of microbial community was more sensitive to warming compared to total biomass (Fig. 2a). Even a small but long-term temperature increase (<2 °C) was sufficient to increase growing microbial fraction and to shorten lag-time before growth (Fig. 2a; 3). This confirmed that climate warming affects microbial physiology and accelerates microbial metabolism (Walker et al., 2018). Although microbial activity and turnover of labile C were altered by temperature increases below 2 °C (Fig. S5), most stable C and N pools (e.g. dissolved organic and soil organic matter pools) were not affected. This was consistent with the idea that soil microbial response to climate warming is short-lived and leaves little legacy (Cruz-Martínez et al., 2009). However, higher warming magnitude (2-4 °C), which is still in the IPCC prediction for the next 80 years, was sufficient to fundamentally alter almost all soil, microbial pools, and enzyme-related processes significantly (except SOC pool). This could be attributed to the direct changes in microbial growth and activity, but also be related to indirect effects through decreased soil moisture by warming or shifts in microbial community structure (Schindlbacher et al., 2011, 2012; Siebold & Tiedemann, 2013). These

changes will further influence enzyme production and pool size of major soil C and nutrient contents over the long term (Liu et al., 2009; Allison et al., 2010), and thus alter soil biogeochemical processes. When the magnitude of climate warming is not considered, the changes in soil C and N cycling at a global scale maybe underestimated.

There is a strong concern that the goals to eliminate CO_2 emission and safely keep the world from warming above 2 °C can be met according to the Climate action and support trends (UNFCCC, 2019). Consequently, if this target of < 2.0 °C in global warming cannot be met, microorganisms will mobilize N from soil organic matter stronger due to the stoichiometric imbalance between soil and microbial biomass, and stimulating plant growth. This, in turn, may increase a fresh labile C input from the plant and maintaining soil C storage (Luo & Zhou, 2006; Liu et al., 2020). However, given that microorganisms increased soil respiration by 21-59% with exceeding 2.0 °C (Fig. 2a, b), positive feedback to atmospheric CO_2 might be triggered. On the global scale, therefore, such a strong alteration of soil, microbial, and enzyme-related processes caused by warming exceeding 2.0 °C would be large enough to change the predicted temperature effect on the soil C storage in the future.

4.2 Self-regulatory mechanism of soil organic carbon stability under warming

Remarkably, the SOC remained stable even with higher warming magnitude (2-4 °C) in long term, as proved by both case study (Fig. 1c, 2a) and literature review (Fig. 2b). This implies that soil microbial pool still able to maintain the stability of SOC under warming exceeding 2 °C through self-regulation. The stable SOC was attributed to the faster microbial growth and necromass formation, which was counterbalanced by the slower enzyme-mediated substrate turnover. A faster microbial specific growth rate (μ_m) under stronger warming (2-4 °C, Fig. 3c) was attributed to the stimulation of fast-growing microorganisms with *r*-strategy. The increased rhizodeposits as indicated by higher plant biomass with warming in this site (Siebold & Tiedemann, 2013) and more frequent winter freeze/thaw cycles in the warmed soil due to less snow cover (Schimel & Clein, 1996) may stimulate *r*-strategy by marked labile substrates input (Groffman et al., 2001). This explanation was supported by the increased fraction of DOC with 2-4 °C warming (Fig. 1), accompanied by an essential decrease in microbial biomass, implying that necromass (dead microbial biomass) was a possible source of the higher labile organic matter with higher warming magnitude (Miltner et al., 2012). This further favored fast-growing microorganisms, and consequently accelerated microbial turnover, as supported by the increased active fraction of the microbial biomass, and by increased basal respiration (Frey et al., 2008; Hagerty et al., 2014). However, fast-growing microorganisms with *r*-strategy are very sensitive to any limitation (e.g., energy and resource availability), and commonly shift to dormancy or even die when substrate becomes limiting (Salazar-Villegas et al., 2016; Shahbaz et al., 2017). Therefore, the preferential strategy of fast-growing microorganisms is a quick switch from dormancy to activity with available C input. After consumption of labile C, the *r*-strategists reduce their biomass rather than mine available organic compounds from recalcitrant SOC pools. As a result of such

self-regulation, the SOC pool remained unaffected while the DOC was released from lysed fast-growing microbial cells.

The slight warming magnitude (<2 °C) only increased the K_m of β-glucosidase by 1.3 times compared to ambient (Fig. 4b), indicating slow down C turnover and counter-balancing acceleration of chemical reactions by warming. As a result, the real production of glucose-like compounds did not increase, as indicated by stable DOC and SOC pools with slight warming magnitude (<2 °C, Fig. 1). However, higher warming (2-4 °C) further increased growing microbial biomass fraction and shifted the microbial community towards fast-growing microorganisms, which was sufficient to stimulate C-degrading as well as N-degrading enzymes. On the other hand, the 2-4 °C warming changed enzyme systems towards lower affinity to the substrate, i.e. slower glucose or amino-N production, as indicated by higher K_m (Fig. 4b), but lower turnover time and catalytic efficiency of β-glucosidase and leucine aminopeptidase (Fig. S5). Thus, it caused a slower decomposition of organic residues either of plant or microbial origin (i.e., necromass) compared with faster necromass production due to the faster turnover of microorganisms. Specifically, the generation time of microbial population was 3 h faster but the turnover time of peptides (cleaved by leucine aminopeptidase) was 7 h slower at higher warming (2-4 °C) compared with ambient soil (Fig. 3f), resulting in increased total N.

Higher warming (2-4 °C) decreased soil C:N ratio due to increased N along with un-altered C content as mentioned above. The decreased soil C:N ratio might be related to the altered SOM quality with long-term warming (Conant et al., 2008) due to the fast turnover of labile C pools and consequently, greater contribution of microbial necromass (relatively low C:N) to soil organic matter. Moreover, the ambient C:N of soil (~19) was close to the theoretic threshold (20-25 i.e., the C:N value above which the N will be immobilized and below which N will be mobilized by microorganisms (Mooshammer et al., 2014a). The possible consequences of decreased soil C:N to a value of 16 by 2-4 °C warming, is net N mobilization (release) improving N availability for plant growth (Manzoni et al., 2012; Kaiser et al., 2014). Such an N release was further confirmed by the increased DON amount under higher warming, which was mainly contributed from NO_3^- (Fig. 1d, h; Fig. S1c). This also corroborates with the relatively stable microbial C:N ratios with warming (~5.5), which means microorganisms did not immobilize more N even when it was available. Thus, microorganisms were not N limited in the soil with higher warming magnitude. It was further supported by the decreased C/N imbalance between labile resources (dissolved organic pools) and the microbial biomass with warming (Fig. S3d). Therefore, the stoichiometric imbalance between microbial decomposers and their labile resources (dissolved organic pool) may result in enhanced N releases from necromass under the higher magnitude of warming.

5.Conclusions

By combining the long-term field warming study and literature review, we pointed out that a low magnitude of temperature increase (< 2 °C) only altered microbial traits (i.e. microbial biomass), but neither enzyme functioning nor soil basic properties.

However, a higher warming (2-4 °C) was sufficient to change almost all soil, microbial pools, and enzyme-related processes in the long-term. Microorganisms grow and turnover faster under higher warming, and enzyme systems shift towards lower affinity to the substrate, i.e. slower glucose or amino-N production. This was indicated by higher K_m, lower turnover time and catalytic efficiency of β-glucosidase and leucine aminopeptidase. Thus, it caused a slower decomposition of organic residues either of plant or of microbial origin (i.e., necromass). Therefore, N content increased in dissolved organic and soil organic matter pools under higher warming magnitude, thus causing a decreased stoichiometric imbalance between the microbial biomass and their labile resource availability, consequently driving soil C decomposition. In conclusion, this study presents strong evidence showing microbial self-regulatory mechanism mitigating global temperature increase and maintaining the stability of soil C storage as i) faster microbial growth and necromass formation, which is counterbalanced by ii) slower enzyme-mediated substrate turnover, which depends on the magnitude of future climate warming. Consequently, the microbial pool still able to maintain the stability of SOC under warming exceeding 2 degrees but may increase total N due to the self-regulation mechanism proposed above.

Acknowledgments

This study was financially supported by the by Russian Science Foundation project 19-77-30012 and the “RUDN University program 5-100”. Jie Zhou was supported by the China Scholarship Council (CSC). The authors would like to thank Andreas von Tiedemann for access to the long-term warming field and thank Karin Schmidt for her laboratory assistance.

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Supplementary

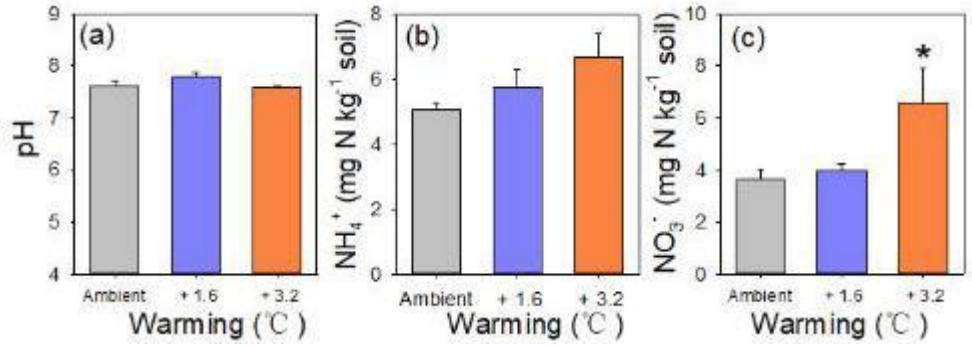

Fig. S1 pH (a) NH_4^+ (b), and NO_3^- content (c) in soil exposed to long-term warming (ambient, + 1.6 ℃, and + 3.2 ℃). Values are average (± SE) of four replicates. Asterisks indicate significant differences between warmed and ambient soils ($p < 0.05$).

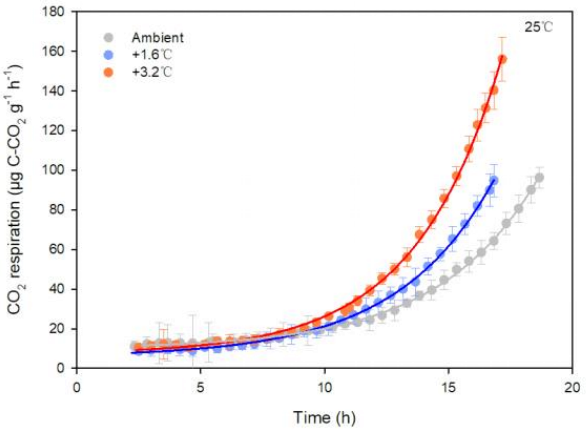

Fig. S2 CO_2 efflux after nutrient addition in soil maintained under ambient, +1.6 ℃, and +3.2 ℃ conditions, but here were determined at the same temperature (25 ℃). Values are average (± SE) of four replicates.

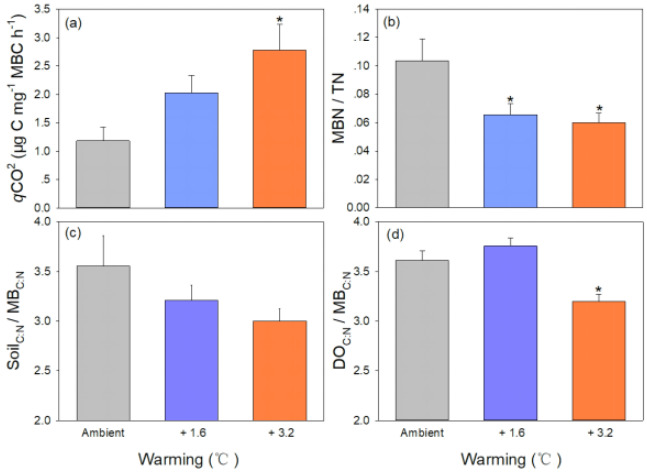

Fig. S3 qCO_2 (BS: MBC) (a), and MBN:TN (b), $Soil_{C:N}$ / $MB_{C:N}$ (the ratio of C: N between soil organic and microbial biomass pools) (c), as well as $DO_{C:N}$ / $MB_{C:N}$ (the ratio of C: N between dissolved organic and microbial biomass pools) (d) at soil with long-term warming (ambient,

+1.6 °C, and +3.2 °C). Values are the average (± SE) of four replicates. Asterisks indicate significant differences ($p < 0.05$) between warmed and ambient soils.

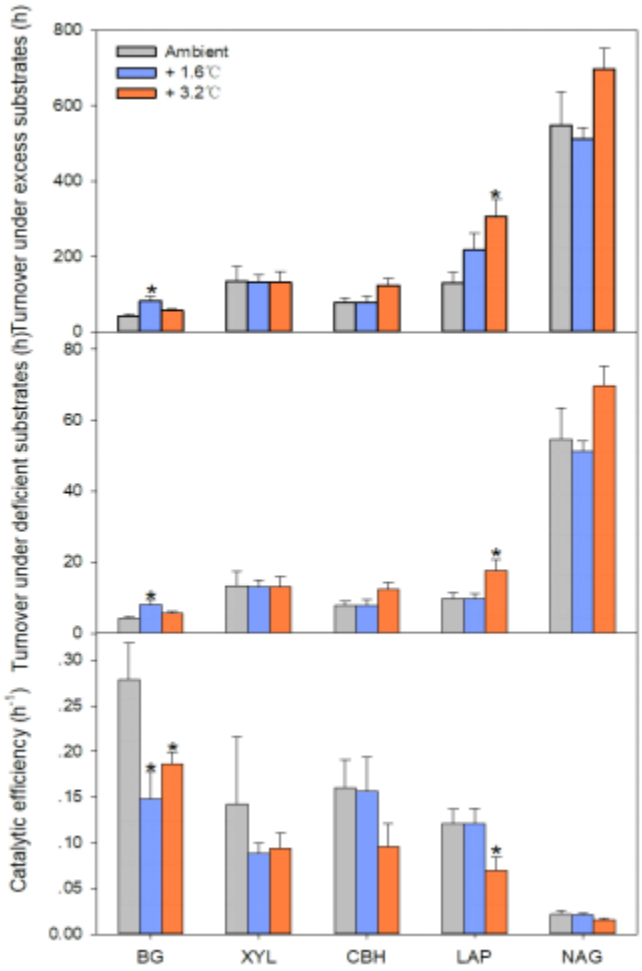

Fig. S5 The turnover time at excess of substrate and lack of substrate, and the catalytic efficiency (ratio of V_{max}/K_m) of β-glucosidase (BG), cellobiohydrolase (CBH), xylanase (XYL), leucine aminopeptidase (LAP), and chitinase (NAG) in soil exposed to ambient, + 1.6 °C, and + 3.2 °C temperature regimes. Values are average (± SE) of four replicates. Asterisks indicate significant differences between warmed and ambient soils ($p < 0.05$).

Study 5 Response of microbial growth and enzyme kinetics to climate change in montane grassland

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Status: In preparing

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Abstract

Mountain grassland systems are of great ecologically importance and are particularly sensitive to global warming. Changes in soil microbial properties are one of the main drivers controlling the soil carbon (C) cycling in response to climate change. Knowledge of the extent to which the functional properties of soil microorganisms vary with climate warming in mountain grassland remains unclear. Here, we analyzed microbial properties in soils from a translocation experiment along an elevation gradient in the Alps. Our aim was to assess the effect of climate change on the microbial growth and enzyme kinetics by substrate-induced growth respiration (SIGR) coupled with kinetics of enzymes involved in C, N, and P cycling. Intact soil columns were translocated downslope to three sites at decreasing elevation. These sites spanned 7 °C increase in temperature (from 13, 15, 17 to 20 °C).

Translocation from colder regions at higher elevation to warmer sites at lower elevation decreased the specific growth rate (μ) of the microbial community, indicating an alteration in their ecological strategy, i.e. a shift towards slow-growing *K*-strategists. This could be attributed to the decreased available C substrate. Further, the increase of enzyme activities with warming, accompanied by the increase of catalytic efficiency, implied the microbial production of less efficient enzymes in the lower versus higher elevation soils. Simultaneously, substrate turnover time of C-degrading enzymes was lower in the soil at lower elevations, implying a stronger and faster C turnover in warmer compared to colder soils due to C rather nutrient limitation. However, K_m of N-related enzymes showed a gradual increase with climate warming, whereas the K_m of C- and P-degrading enzymes increase strongly with translocation from E0 (13 °C) to E1 (15 °C) and then remained unaffected at higher temperatures, i.e. at lower elevations. This could be explained by the expression of iso-enzymes with less flexibility. We conclude that climate warming increases microbial activities, and thus induces a positive soil C-climate feedback in the future warmed world.

Keywords: Climate warming, Enzyme kinetics, Microbial growth kinetics, *r* and *K* strategy, Tranalocation

1. Introduction

Montane grasslands comprise 20-25 % of terrestrial landscape and store 28-37% of the global terrestrial soil carbon (C) stocks, as well as one of the most sensitive ecosystems to global environmental changes and anthropogenic activities (Scurlock and Hall, 1998; Chou et al., 2008). Given the quantitative importance, any change in soil C dynamics could result in a positive climate feedback. Indeed, climate change has led to increasing surface temperatures by roughly 5.8 ℃ by 2100 and changes in precipitation regime (IPCC, 2013). However, the climate-change associated abiotic and biotic factors altering soil processes for Montane grasslands remain largely unclear despite their consideration importance to greenhouse gas emissions, soil fertility, plant nutrition (Makkonen et al., 2012; Althuizen et al., 2018). Therefore, better understanding of how soil organic matter (SOM) respond to climate change in montane grasslands has raised considerable interests.

SOM mineralization is the result of microbial growth on heterogeneous organic substrates and is strongly dependent on the size and functional properties of the active microbial fraction (Schlesinger and Andrews, 2000; Chen et al., 2014). Therefore, measurements of the size and composition of the microbial biomass alongside changes in growth rate and functioning are needed to understand how climate warming affects soil C cycling (Strickland and Rousk, 2010; Reinsch et al., 2013). The kinetic approach based on substrate-induced growth respiration (SIGR) represents a suitable tool to estimate microbial growth parameters (Blagodatsky et al., 2000; Zhou et al., 2020). It is commonly assumed that fast-growing microorganisms with r-strategy benefit by utilizing easily available C substrates, whereas slow-growing K-strategists have an advantage in utilizing more recalcitrant organic substrates (Fontaine et al., 2003). However, the predictions on which microbial group will prevail under climate warming remains controversial. One view is that the increased input of easily available C substrates may accelerate the turnover of the fast-growing microbial population (Blagodatskaya et al., 2010). In contrast, the microbial growth rate may reduce due to the rapid exhaustion of available organic C under long-term warming (Bradford et al., 2008). Therefore, the extent to which the microbial physiological statues changed with climate warming remains an open question.

The break down of soil organic polymers (e.g. dead plant and microbial necromass) is catalyzed by microbially produced extracellular enzymes (Nannipieri et al., 2003). Therefore, the catalytic properties of exo-enzymes determine the direction and magnitude of soil C and nutrients cycling (Sinsabaugh, 2009; Burns et al., 2013). Due to enzyme production by soil microorganisms is regulated by their demand and by substrate availability, which in turn is affected by several factors, such as temperature and soil water content (Burns et al. 2013). Enhanced plant C input with warming (Bai et al., 2010; Yin et al., 2013), could induce greater enzyme reaction rate (V_{max}) due to the increased nutrient demand to meet microbial stoichiometric requirements (Conant et al., 2011). Conditions of low soil water availability, such as caused by warming-induced soil water shortage (Butschoen et al. 2011), however, could in addition lead to diffusion limitations of enzymes and substrates (Allison, 2005). Thus lead to reduced substrate affinity of enzyme (increased K_m) with increasing temperature, which could potentially counterbalance the positive feedback between the V_{max} of hydrolytic enzymes, and thus counteract loss of SOM under warming (Davidson and Janssens, 2006; Davidson et al., 2006; Blagodatskaya et al., 2016). Climate warming responses of soil enzyme kinetics thus demand more attention so that the fate of soil C cycling can be determined under future climate change.

Translocation experiments along elevation gradients have become increasingly important

in understanding the fate of C and its underlying microbial dynamics under climate change (Sundqvist et al., 2013; Looby et al., 2018). Translocation studies have shown that soil respiration (Zimmermann et al., 2009) and litter decomposition (Scowcrofy et al., 2000; Salinas et al. 2011) may increase with changing climate conditions. But, more detailed information is needed on how soil microbial functional traits (i.e. microbial growth rate and enzyme activity) altered with climate warming. Here, microbial growth and enzyme kinetics were determined after one-year translocation in mountainous grassland ecosystems. We coupled soil microbial growth parameters estimated by substrate-induced growth respiration (SIGR) with the kinetic parameters (i.e. V_{max} and K_m) of enzymes involved in C, N, and P cycling to reveal the microbial regulatory mechanisms under climate warming. We aimed to estimate the microbial functioning and enzyme kinetics change with climate warming *in situ*.

2. Materials and method

2.1 Soil site and experimental setup

This study was conducted on four grassland sites along an elevational gradient ranging from 350 m to 1300 m a.s.l. in the European Alps. Four representative grassland sites were selected for downslope translocation of intact plant-soil monoliths. These sites are Esterberg (EB, 1300 m a.s.l.), Graswang (GW, 900 m a.s.l.), Fendt (FE, 550 m a.s.l.), and Bayreuth (BT, 350 m a.s.l.), ranging from pre-alpine to colline ecosystems. For a description of the geographic and climate characteristics see Fig. 1.

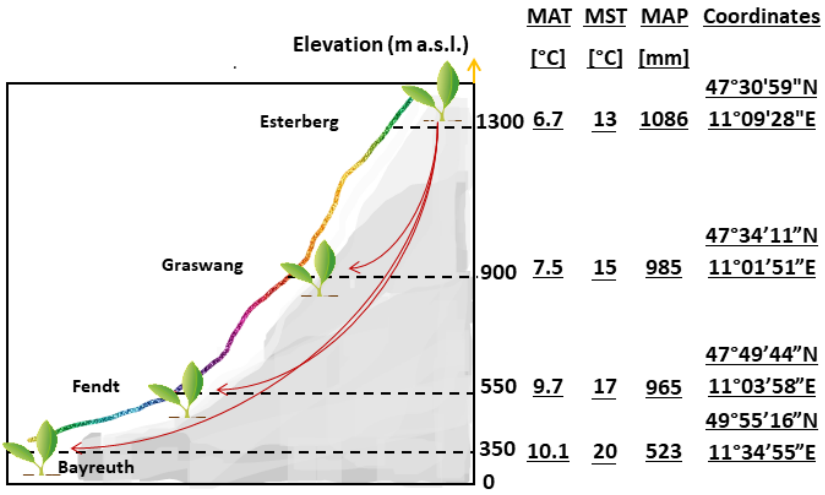

Fig. 1 Geographic and climatic characteristics of the research sites along the elevational gradient in the European Alps. MAT, MST and MAP are mean annual temperature and mean summer temperature and mean annual precipitation, respectively.

In the summer 2016, the intact plant-soil community monoliths were extracted from the pre-alpine grassland in Esterberg by inserting in a 30 cm diameter PVC tube, the bottoms of which were left open to allow for water flow. After excavation of the monoliths at their respective origin site, they were translocated to recipient sites and dug into the extant vegetation with the rim of the monolith level to its surrounding. Monoliths were translocated within site as a control and downslope to each site with a lower elevation than the original site to simulate possible future climate scenarios (Fig. 1). Here, the translocation of control monoliths within original site allowed us to exclude for treatment effects because of the extraction and installation of the PVC tubes. In addition to the treatment of translocation downslope, a randomly selected sub-section of each origin type (Bayreuth, Fendt, Graswang,

Esterberg) received irrigation treatment at the Bayreuth (Recipient) site in 2018, in order to compensate a substantial lower precipitation than that in other sites in Alps. The irrigation amount was calculated as the difference between the amount of mean summer precipitation received at Alps and that at Bayreuth, and then was added to the monoliths twice a week.

The soil in "Esterberg" study site is classified as Rendzic Phaeozem (IUSS Working Group WRB, 2006). The bulk density of soil is 0.5 g cm^{-3} from 0-10 cm depth with a clay texture (43% silt, 5% sand, 52 % clay). A detailed description of soil characters is provided in Table 1. The plant association are Cynosuretum cristate, and the dominant species are *Anthoxanthum odoratum*, *Cynosurus cristatus*, *Elymus repens*, *Festuca pratensis*, *Festuca rubra*, *Lolium perenne*, *Trifolium pratense.*

2.2 Measurement

2.2.1 Plant and soil sampling

During peak growing season of 2018, the aboveground biomass of each monolith was harvested 3 cm above ground level and then divided into three plant functional groups (graminoids, forbs, and legumes), dried at 60 °C for 48 hours and weighed. The *in-situ* ingrowth cores were used for estimating the root production rate. Before the peak growing season (in may 2018), two ingrowth cores (2 mm mesh, 10 cm length by 3 cm diameter) were filled with 2 mm sieved soil from corresponding origin soil and installed into randomly selected monoliths (n = 9) at each site. Towards the end of peak growing season in July 2018, the ingrowth cores were carefully removed from monoliths and brought back to the laboratory stored around 5 °C. In the lab, the wet soil with the roots were removed from ingrowth cores and weighed. Then, root and soil samples were separated by sieving through a 2-mm sieve and stored at 4 °C prior to analyses. Then, root samples were washed over a 5 µm sieve and dried at 60 °C for 48 hours and weighed.

2.2.2 Meteorological and soil parameters

Since the spring 2017, sensors for soil moisture (EcH2O 5-TM, Decagon Devices Inc., USA) and for soil temperature were installed horizontal at 5 cm depth together with data loggers (EcH2O Em50, Decagon Devices Inc., USA) from each origin and each recipient site. Data were recorded at 15 min intervals and were gathered to daily mean values. Hourly air temperature and precipitation was monitored by a Frankenberger psychrometer (Theodor Friedrichs & Co, Schenefeld, Germany) plus a OTT Pluvio (OTT HydroMet GmbH, Kempten, Germany) in Bayreuth, and by the KIT Campus Alpin in Fendt and Graswang with the Weather Transmitter WXT520 (Vaisla, Helsinki, Finland), while in Esterberg the Atmos 41 (MeterGroup AG, Munich, Germany) was used. Soil C and N content were measured using a C/N Elemental Analyzer (Vario EL Cube, Elementary Analysis Systems GmbH, Hanau, Germany). For determining dissolved organic carbon (DOC) and dissolved nitrogen (DN), 10 g of fresh soil was extracted with 20 ml of 0.05 M K_2SO_4 after shaking for 60 min on a reciprocating shaker (Laboratory shaker, GFL 3016) and the filtrates were measured for total extractable C and N with a multi C/N analyzer (multi C/N analyzer 2100S, Analytik, Jena). The soil pH was measured with a calibrated pH meter (FiveEasy F20, Mettler Toledo GmbH, Gießen, Germany) in 10 g soil with 25 mL distilled water.

2.2.3 Kinetic parameters of substrate-induced growth respiration

The substrate-induced growth respiration (SIGR) approach was used to distinguish total and active biomass fractions, microbial specific growth rate, and lag-time before growth (Panikov, 1995; Blagodatsky et al., 2000). It has to be noted that although substrate addition is required for the SIGR approach, all kinetic parameters analyzed by SIGR represent the intrinsic features of the dominant microbial populations before substrate addition (Blagodatsky et al., 2000).

Field-moist soil (1 g) was amended with a mixture containing 10 mg g^{-1} glucose, 1.9 mg g^{-1} $(NH_4)_2SO_4$, 2.25 mg g^{-1} K_2HPO_4, and 3.8 mg g^{-1} $MgSO_4.7H_2O$. Soil samples were then placed in a Rapid Automated Bacterial Impedance Technique bioanalyser (RABIT; Microbiology International Ltd, Frederick, MD), for measuring CO_2 emission at their corresponding field temperature (13, 15, 17 and 20 °C in 1300, 900, 550, 350 m elevation, respectively). Firstly, we incubated four soil samples at each temperature for 2 days at 45% water holding capacity (WHC) to reduce the impact of the initial disturbance from handling. To measure substrate-induced respiration, a mixture of glucose and nutrients was then added and the samples incubated for a further five days at 75% WHC. The measurement of CO_2 release was based on the conductivity changes in the RABIT system.

Microbial respiration in glucose amended soil (Substrate Induced Growth Response, SIGR) was used to calculate the following kinetic parameters: the specific growth rate of microorganisms (μ), the microbial biomass capable for immediate growth on glucose (GMB), physiological state index of microbial biomass before substrate addition (r_0), the total microbial biomass (TMB) responding by respiration to glucose addition, and the lag period (Tlag). This method was suggested by Panikov (1995) and the experimental procedure, calculations, fitting, and statistics are presented in Wutzler et al. (2012).

2.2.4 Enzyme activity

Activity of extracellular enzymes: β-1,4-glucosidase (BG) (EC 2.2.1.21), cellobiohydrolase (CELLO) (EC 3.2.1.91), xylanase (XYL) (EC 3.2.2.27), β-1,4-N-acetylglucosaminidase (NAG) (EC 3.2.1.52), leucine aminopeptidase (LAP) (EC 3.4.11.1) and acid phosphatase (ACP) (EC 3.1.3.2) were determined by the 4-methylumbelliferyl (MUF)-based and 7-amido-4-methylcoumarin (AMC)-based artificial substrates (Marx et al., 2001). Briefly, 0.5 g soil was mixed with 50 ml sterile water and then shaking for 30 min. After 2 min low-energy sonication (40 J s^{-1}) by ultrasonic disaggregation, 50 µl of the soil suspension, 50 µl of corresponding buffer (MES or TRIZMA) and 100 µl of the corresponding substrates at concentrations of 2, 5, 10, 20, 50, 100 and 200 µmol l^{-1} were pipetted into 96-well black microplates (Brand® plates pureGrade, Sigma-Aldrich, Germany). The microplates were determined by an automated fluorometric plate-reader (Victor3 1420-050 Multi-label Counter, PerkinElmer, USA).

To calculate key parameters describing the enzyme kinetics, we fitted a Michaelis-Menten equation to the experimental data (Marx et al., 2001):

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

where V is the enzymatically mediated rate of reaction, V_{max} is the maximal rate of reaction, K_m (Michaelis constant) is the substrate concentration at ½V_{max} and S is substrate concentration. The substrate turnover time (T_t) was based on: T_t (hours) = (K_m + S) / V_{max}, where S is the substrate concentration. The substrate turnover time was calculated at substrate concentration for the situations corresponding to the lack and excess of substrate, as S = K_m /10 and S = 10* K_m, respectively. The catalytic efficiency of enzymes (K_a) was calculated by the ratio of V_{max} and K_m (Hoang et al., 2016). Enzyme activity ratios were determined to examine the relative allocation to labile C versus nutrient acquisition, e.g. V_{max} ratio of BG and sum of LAP and NAG, V_{max} ratio of BG and ACP (Sinsabaugh and Follsatd, 2012).

2.3 Statistical analysis

The experiment was carried out with three replicates of each treatment. The values presented in the tables and figures are given as means ± standard error (mean ± SE). Prior to analysis of variance (ANOVA), the data were tested for normality (Shapiro-Wilk, $p > 0.05$) and homogeneity of variance (Levene-test, $p > 0.05$). One-way ANOVA followed by the Tukey HSD test at a probability level of $p < 0.05$ was used to define temperature ranges with

significantly different specific growth rate (μ), growing microbial biomass (GMB), lag time (T_{lag}), potential enzyme activity (V_{max}), substrate affinity (K_m) and substrate turnover time (T_t), as well as catalytic efficiency (K_a). All statistical analyses were performed using SPSS version 19.0 (SPSS Inc., USA).

3 Results

3.1 Plant and Soil chemical properties

With decreased elevation, soil pH remained unchanged (Table 1). Root and shoot biomass were 8.09 mg g^{-1} soil and 36.88 g in soil at the highest elevation (E0), which was significantly higher than those in soil at the lower elevation (E3) ($p < 0.05$, Table. 1). Furthermore, dissolved organic carbon (DOC) and nitrogen (DON) were 42% and 26% higher in soil under higher elevation (E0) compared to lower elevation soils (E3) ($p < 0.05$, Table 1).

Table. 1 Soil pH, dissolved organic carbon (DOC), dissolved organic nitrogen (DON), and root and shoot biomass in soil under four levels of elevations (E0 with 13°C, E1 with 15°C, E2 with 17°C, E3 with 20°C). Values are means (±SE) of three replicates. Different letter indicate significant difference at a level of $P < 0.05$.

	pH (H_2O)	DOC (mg C kg^{-1})	DN (mg N kg^{-1})	Root biomass (mg g^{-1} soil)	Shoot biomass (g)
E0 with 13 °C	6.83±0.23a	234.77±19.20a	52.66±9.27a	8.09±1.30a	36.88±1.47b
E1 with 15°C	7.12±0.04a	250.39±23.86a	28.48±8.32b	6.82±1.17a	53.57±3.93a
E2 with 17°C	6.99±0.05a	168.52±17.60b	40.01±6.35ab	4.48±0.91ab	48.24±3.08a
E3 with 20°C	7.05±0.02a	136.20±22.97b	39.07±4.53b	2.72±0.46b	25.47±3.89c

3.2 Microbial growth kinetics

The glucose-induced respiration rates increased earlier and responded more intensively in soil at the lowest elevation (E3), i.e. under warmer temperatures (20 °C). Patterns of the exponential CO_2 evolution rate were similar in soils at higher elevations (under 13, 15 and 17 °C). As an inherent property of microorganisms, the specific growth rate (μ) decreased by 22% when comparing E0 with E3, i.e. with increasing temperature ($p < 0.05$, Fig. 2). Total (TMB) and growing microbial biomass (GMB) were significantly higher in soil at the lowest elevation (E3) compared with that at highest elevation ($p < 0.05$, Fig. 3a,b). Further, microorganisms in warmer soil under lower elevation required significantly less time to start exponential growth after glucose amendment compared to that in soils under colder conditions at higher elevation (8 and 27 h in soil at E3 and E0, respectively) ($p < 0.05$, Fig. 3c). Similarly, the generation of microbial populations was around 5 h in soil under E3, 2 h slower than that in the soil under E0 ($p < 0.05$, Fig. 3d).

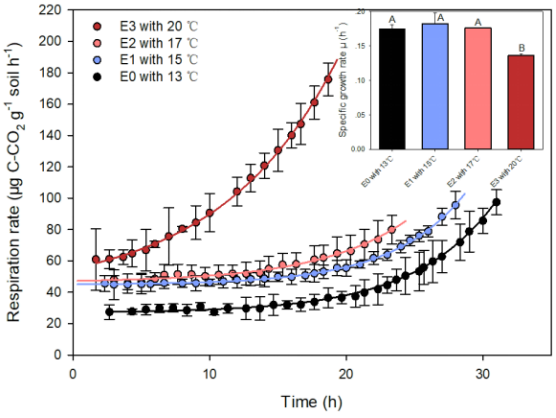

Fig. 2 Substrate-induced respiratory responses of microbial community and their corresponding specific growth rate (μ: insert figures) after a mixture of glucose and nutrient solutions in soil under four levels of elevations (E0 with 13°C, E1 with 15°C, E2 with 17°C, E3 with 20°C). Experimental data are shown as symbols and model simulation (Equation 1) as curves. Values are means (±SE) of three replicates. Different letter indicate significant difference at a level of $P < 0.05$.

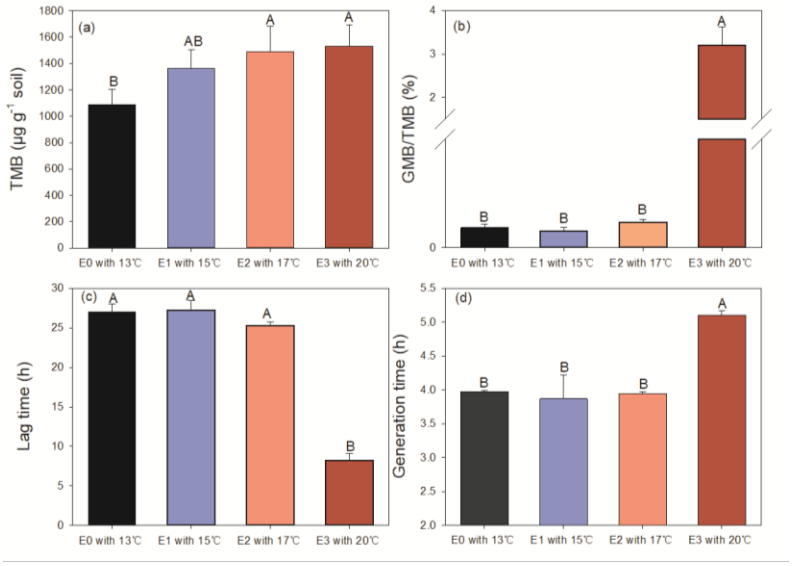

Fig. 3 Growing microbial biomass (a) and its fraction of total biomass (b), lag-time and generation time of actively microbial community consuming substrate in soil under four levels of elevations (E0 with 13°C, E1 with 15°C, E2 with 17°C, E3 with 20°C). Values are means (±SE) of three replicates. Different letter indicate significant difference at a level of $P < 0.05$.

3.3 Enzyme kinetics

The potential enzyme activity (V_{max}) increased with warming from E0 to E3 (i.e. from 13 to 20°C) by 1.2-12.5 times (Fig. 4a). The temperature response pattern of K_m was enzyme specific. NAG and LAP showed a general increase of K_m values from E0 (13°C) to E3 (20°C) (Fig. 4b). For BG, XYL, CELLO, and ACP, however, after the initial increase in K_m from E0 (13°C) to E1 (15°C), the values did not change significantly between E1, E2, and E3.

The turnover time of XYL, NAG, and LAP showed no difference between lower and higher elevations sites, whereas it was shorter at lower elevation with warmer temperature compared to higher elevation for BG and CELLO (Fig. 5). No changes in the catalytic efficiency (K_a, V_{max}/K_m) was detected for XYL, CELLO, and NAG. However, the K_a for BG was 3-folds larger at lower elevation than that athigher elevation ($p < 0.05$, Fig. 5c). The C/N and C/P acquisition ratios were about 3 times higher at warmest temperatures (E3) compared with coldest temperatures (E0) ($p < 0.05$, Fig. 6).

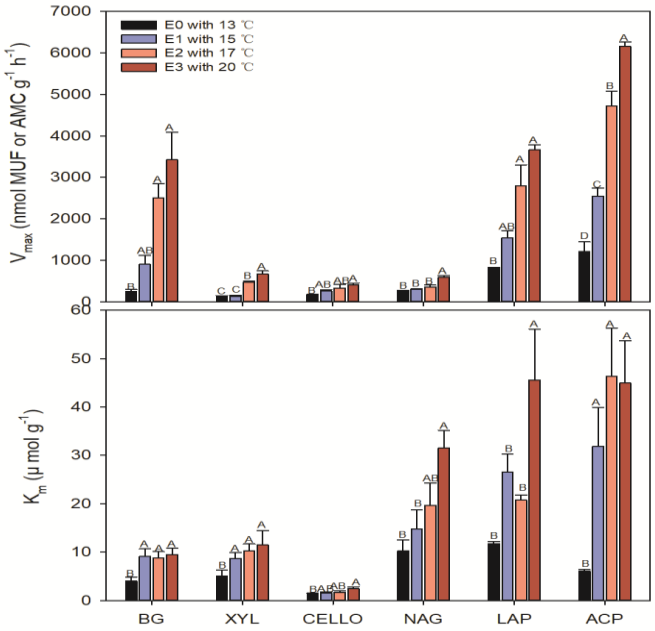

Fig. 4 Enzyme activity (V_{max}) and substrate affinity (K_m) of β-glucosidase (BG), xylanase (XYL), cellobiohydrolase (Cello), chitinase (NAG), leucine aminopeptidase (LAP) and acid phosphatase (ACP) in soil under four levels of elevations (E0 with 13°C, E1 with 15°C, E2 with 17°C, E3 with 20°C). Values are means (±SE) of three replicates. Different letter indicate significant difference at a level of $P < 0.05$.

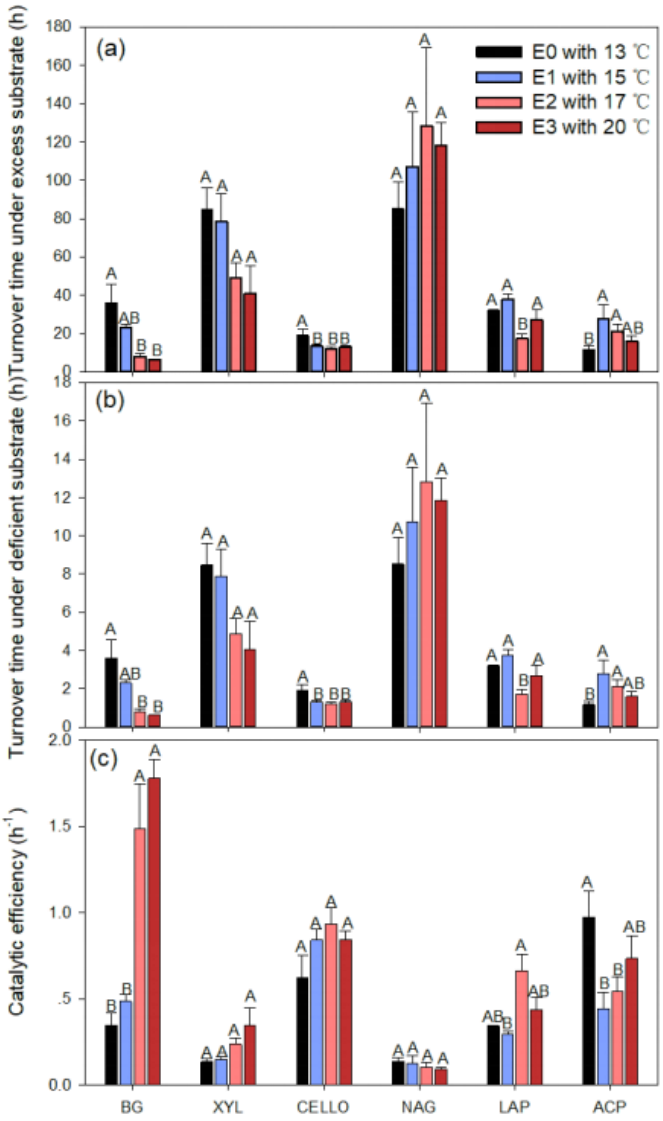

Fig. 5 The turnover time at excess of substrate (a) and lack of substrate, as well as catalytic efficiency of enzymes (V_{max}/K_m) in soil under four levels of elevations (E0 with 13°C, E1 with 15°C, E2 with 17°C, E3 with 20°C). Values are means (±SE) of three replicates. Different letter indicate significant difference at a level of $P < 0.05$.

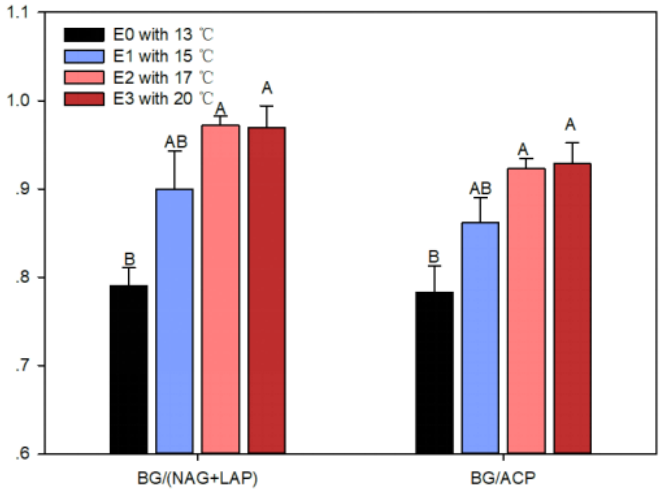

Fig. 6 Enzymatic ratio of labile C versus nutrient (N and P) related enzymes ((BG/(NAG+LAP), BG/ACP)

in soil under four levels of elevations (E0 with 13°C, E1 with 15°C, E2 with 17°C, E3 with 20°C). Values are means (±SE) of three replicates. Different letter indicate significant difference at a level of $P < 0.05$.

4. Discussion

4.1 Soil microbial growth kinetics

A lower microbial specific growth rate (μ) with translocation (decreased elevation) (Fig. 2) was attributed to the stimulation of slow-growing microorganisms. Due to decreased root and shoot biomass with climate warming (Table. 1), less rhizodeposition was released into soil with lower elevation, thus induced a strong competition for easily degradable C sources which favors the *K*-selected microorganisms (Blagodatskaya et al., 2014). On the other hand, the changes in plant community composition could contribute to the shift in microbial community. Because Berauer et al. (2019, unpublished data) found that the species richness of plant community at this site decreased after one-year translocation from higher to lower elevation, which was positively related with plant productivity (Tilmen et al., 1996; 2001). As a consequence, this would result in a reduced supply of C substrate for roots and microorganisms, and thus induce a microbial shift towards to *K*-strategists. Under lower C availability as indicated by lower DOC, active but starving microorganisms, the K-strategists contribute to microbial growth (Chen et al., 2014), as a result the μ values decreases as compared with soil under higher elevation (Fig. 2). Further, the catalytic efficiency of BG increased from elevation 1300 m to 350 m, again indicating a shift to *K*-selected microorganisms (Fig. 5c).

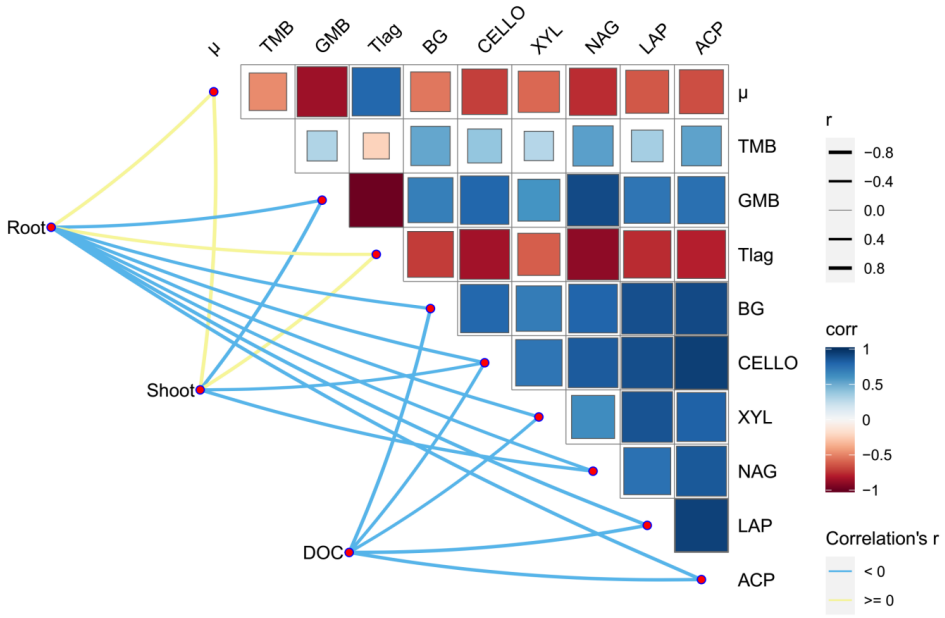

Fig. 7 Relationships between plant and soil properties and microbial functional traits in soil under four levels of elevations (E0 with 13°C, E1 with 15°C, E2 with 17°C, E3 with 20°C). Pairwise correlations of soil properties were presented as Spearman's correlation coefficients. μ, specific growth rate; TMB, total microbial biomass; GMB, growing microbial biomass; T_{lag}, lag time; BG, V_{max} of β-glucosidase; XYL, V_{max} of xylanase; CELLO, V_{max} of cellobiohydrolase; NAG, V_{max} of chitinase; LAP, V_{max} of leucine aminopeptidase; ACP, V_{max} of acid phosphatase.

Although irrigation was applied in the lower elevation soil (E3), it is also likely that reduced soil moisture may played a role in the microbial community shift, perhaps in combination with temperature changes in the field site (Fig. 1). It has been proposed that the

effective *K*-strategist are more dominated by fungi (Fontaine and Barot, 2005; de Graaff et al., 2010; Reinsch et al., 2013). Climate warming has the potential to decrease soil moisture (Fig. 1), which could limit positive effects of warming on microbial activity (Reinsch et al., 2013). For example, drought stress likely facilitates increased survival of fungi, because soil fungi rely on more aerobic conditions and are more tolerant of drought due to their filamentous nature (Zhang et al., 2005). Aerobic filamentous fungi have variable hyphal networks that can relocate water and nutrient resource by cytoplasm translocation (Klein and Paschke, 2004). Therefore, under lower elevation with relative warmer and drier soil, the *K*-strategist overwhelmed r-strategists (with lower μ values).

Besides, GMB increased with climate warming (Fig. 3b), suggesting a quick shift of dormant microorganisms to an active physiological state, which corresponded to the increase in BG activity (Fig. 4a, 7). The microbial community in the lower elevation also has a shorter lag-period (Fig. 3c), and was ready for immediate growth on available substrate compared to the microbial community in soil under higher elevation. This could be explained by the activity state of microbial biomass is responsible of the duration of T_{lag} (Blagodatskaya et al., 2014), which was supported by the positive correlation between T_{lag} and the fraction of GMB (Fig. 7).

4.2 Soil enzymatic kinetics

For all the tested enzymes, we found that activities of C, N and P-degrading enzymes increased with warmer temperatures (Fig. 4a), as a result of enhanced growing microbial biomass (Fig. 4a). Growing microorganisms produce larger amounts of enzymes and thereby enhance the enzyme activity (Blagodatskaya et al., 2016; Tian et al., 2019). Shifts in microbial community composition from *r*- to *K*-microorganisms from high to low elevation ecosystems could have contributed to the changes in enzyme activity, since fungi can be equipped with a larger set of enzymes than bacteria (de Boer et al., 2005; McGuire et al., 2010; Berlemont, 2017). Alternatively, the decreased soil water content could also limit enzyme and substrate diffusion (Allison, 2005), and soil microorganisms may compensate by increasing enzyme production (Bell et al., 2010). Therefore, higher soil temperature may stimulate soil microbial investment in enzyme production.

As microorganisms regulate the production of hydrolytic enzymes in response to environmental resource availability (Allison and Vitousek, 2005), and warming decreased plant C supply as mentioned above, it is possible that microorganisms allocate more resources to the acquisition of C (Sinsabaugh and Follstad, 2012). Hence, these findings indicate that warming-induced decreased C availability stimulated C-degrading enzymes and increased microbial C limitation. There was a clear stoichiometry shift to higher investment in C acquisition in soils with lower elevation. This was indicated by the higher ratio of enzyme activities of labile C- to N-degrading enzymes (BG/(NAG+LAP)) and C- to P-degrading enzymes (BG/ACP) in soil under lower elevation (Fig. 6a, b). In line with the enzymatic ratios, the content of DOC and DN decreased with climate warming, which further suggests that translocation could decrease C availability and stimulate labile C-acquired enzyme activity, which reflects the aggravation of C limitation in lower elevation. On the contrary, soil microorganisms were limited by nutrients in soil under higher elevation, especially in highly diverse plant communities, due to nutrient competition between free-living soil microorganisms and plants (Kuzyakov and Xu, 2013). Alternatively, the higher mobilization of nutrients in soil under higher elevation caused by higher water availability, reduces the need for nutrient mining compared to soil under lower elevation (Zuccarini et al., 2020).

Furthermore, a shorter substrate turnover time and higher K_a of BG in soil at lower elevation (E3, 20 °C) compared with higher elevation (E0, 13 °C) was observed (Fig. 5c), which suggests that the microbial community was more limited by C rather than nutrient in the warmer and drier soils at lower elevation.

K_m increased with warming for all enzyme groups (Fig. 4b), which is in agreement with others (German et al., 2012; Stone et al., 2012). Higher K_m at lower elevation confirmed the expression of low efficient enzymes (Razavi et al., 2016). This means that microorganisms in warmer and drier environments maintained a slow growth rate but maximized substrate use efficiency (Fierer et al., 2007). These accelerated rates are supported by shorter substrates turnover time at warmer environment (Fig. 5a). However, K_m of N-degrading enzymes (NAG and LAP) increased gradually from higher to lower elevation, whereas the K_m of C- and P-degrading enzymes remained nearly constant after sharp increase from 350 m (E3) to 550 m (E2) elevation (Fig. 4b). Microbial communities are often known to produce different isoenzymes that could differ in their intrinsic K_m values (Somero, 1978; Bradford, 2013). The presence of different enzyme isoforms is the plausible reason for having different patterns under climate warming as isozymes are known to conserve their unique K_m values (Somero, 2004). Extracellular enzyme systems therefore adapted to the altered substrate supply resulted in a change of K_a and in a corresponding shift in the functional structure of the microbial community. Thus, a lower K_a indicated the dominance of *r*-strategists at high elevations (E0, 13 °C) as compared with low elevations (E3, 20 °C) , where the *K*-strategists relatively dominated (Loeppmann et al., 2016). In other words, it also supports our first results that *K*-strategists overwhelm *r*-strategists under warming field because of the C limitation, caused by the higher microbial and enzyme activities.

Overall, microbial community shifts towards slow-growing microorganisms after two-year translocation from higher to lower elevations in montane grasslands. This could be attributed to the decreased available C substrate as well as reduced soil water content. Further, the increase of enzyme activities with warming, accompanied by the increase of catalytic efficiency, implied microbial production of less efficient enzymes in the lower versus higher elevation soils. Simultaneously, substrate turnover time of C-degrading enzymes was lower in the soil at lower elevations, implies a stronger and faster C turnover in warmer and drier soil compared to colder soils due to C rather nutrient limitation, which is mainly induced by higher fraction of growing microbial biomass. This has direct consequences for C and nutrient cycling. Warming can therefore lead to proportionally high soil C and N losses when increased N mineralization rates at warmer temperatures are not compensated by rapid plant N uptake and plant-derived C inputs to the soil due to lower root biomass production of less diverse plant communities.

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Study 6 The soil memory: Long-term field warming controls short-term temperature responses of soil microbial functions

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Abstract

Short-term acceleration of soil organic carbon decomposition with warming conflicts with the thermal adaptation observed in long-term studies. The adaption and stabilization mechanisms of microbial functions in response to long-term warming, however, remains poorly understood. Here, we explored these mechanisms using eight-years warming field sites (ambient, +1.6 °C, +3.2 °C) and a short-term constant temperature incubation (from 5 to 25 °C with 5 °C intervals) under microbial steady-state and activated mode. We found that eight-year field warming facilitated the consumption of labile organics due to faster microbial growth and turnover. Two times reduction in native available substrates suppressed total microbial biomass and especially, the fraction of growing microorganisms up to 3 times. Consequently, the microbial growth and enzyme activities acclimated to warming over eight-years responded up to 40% weaker to further short-term temperature increase. Temperature sensitivity of enzyme affinities to substrate did not respond on warming history indicating acclimated microbial communities and enzyme systems and thus caused a soil memory to long-term warming. Under microbial activation induced labile substrates input, however, the higher enzyme activity and temperature sensitivity of Vmax in historically warmed versus ambient soil demonstrated reduced microbial memory effect due to thermophilic nature of activated microorganisms. Thus, we found experimental evidence that microbial memory to warming is driven by reduced amount of native substrate and is strongly dependent on microbial physiological state, which can be quickly altered by the available substrate supply, and therefore constitutes fundamental processes to improve model prediction of C dynamic in response to climate change.

Keywords: Soil enzymes; Temperature sensitivity; Substrate availability; Microbial activation

1. Introduction

Elevated temperature is projected to activate microorganisms and accelerate their turnover, thus promoting terrestrial carbon (C) cycle with potential feedbacks to future climate change (Davidson and Janssens, 2006; Bardgett et al., 2008; Zang et al., 2020). As exoenzymes produced and released by microorganisms are the engine of soil organic matter (SOM) decomposition and labile organic compounds production (Koch et al., 2007; Wallenstein et al., 2009), shifts in enzyme activities change C and nutrient cycling (Sinsabaugh et al., 2002; Wen et al., 2019b). A recent microbial-enzyme model predicts that decreased microbial biomass would promote soil C accumulation with warming, irrespective of accelerated microbial turnover and enzyme activity (Hagerty et al., 2014). The ability of new generation models that incorporate microbial dynamics and functionality, to predict soil C response to warming needs empirical verification considering short- and long-term duration of warming. Additionally, the responses of enzyme kinetics to changes in temperature may feed these new models with empiric data to better predict the vulnerability of soil C stocks and nutrient cycling in a future warmed world. Thus, assessing the impact of global warming on biogeochemical cycles in soils requires a detailed process-based understanding of the interaction of microbial communities and their enzyme systems.

Soil microorganisms can modify their physiology and functionality to compensate for the changes in temperature, and this acclimation can confer resilience to climate change (Davidson and Janssens, 2006; Allison and Martiny, 2008). In long term, this may result in i) the expression of isozymes with different kinetic properties, and ii) changes in the relative abundances of microorganisms expressing enzyme systems with altered kinetics and efficiency (Bradford, 2013; Blagodatskaya et al., 2016). Theoretically, environmental variability (i.e. temperature, moisture, C and nutrient availability) induces stress that results either in thermal adaptation or in the evolution of physiological flexibility (Bradford et al., 2008; Wallenstein et al. 2009; Stone et al. 2012). For example, cold-adapted enzymes tend to be more responsive to increasing temperature than warm-adapted enzymes (Koch et al., 2007; Dong and Somero, 2009). This is primarily due to differences in protein structure that cause cold-adapted proteins to lose functionality more readily as temperatures increase (Hochachka and Somero, 2002). In contrast, this response may vary among enzyme classes (Nottingham et al., 2016). In short-term, however, the sudden temperature changes might affect the kinetic properties of existing enzymes at the physiological level (Razavi et al., 2016; Liu et al., 2017), thereby stimulating the respiratory consumption of C by soil microorganisms (Frey et al., 2013; Bölscher et al., 2017). Therefore, an improved mechanistic understanding of temperature sensitivity of enzyme kinetics is still required to resolve the uncertainty surrounding ecosystem responses to climate warming.

In strong interactions with temperature, the substrate supply is a key factor regulating microbial growth, enzyme production (Allison et al., 2009; Wallenstein et al., 2009; Karhu et al., 2014) and catalytic efficiency of enzymes (Loeppmann et al., 2016). Thus, any enzymatic response of growing microbial populations to temperature can only be identified in excess of substrates (Giardina and Ryan, 2000). Due to the

faster depletion of soil C pools (especially of the easily accessible, hydrolyzable C pools), substrate limitation of hydrolytic enzyme activity is common in warmed soils (Knorr et al., 2005; Conant et al., 2011). Limited substrate input imposes a tradeoff by either reducing the energy available for growth or expression of enzymes (Angilletta et al., 2003). However, there are no studies to date that directly related thedecomposition of soil organics to native amount of corresponding substrate in long-term warming system at field scale. At sufficient supply of energy (i.e. C and nutrients), microorganisms may shift from a steady-state to exponential growth accelerating metabolic reactions and extracellular enzymatic activity (Stolpovsky et al., 2011; Wang et al., 2014). Ignoring microbial activation by increasing C input due to increased net primary productivity under climate warming (Cox et al., 2000; Heimann and Reichstein, 2008; Yin et al., 2013), could result in underestimation of SOM decomposition as well as in the missing feedbacks between the climate and nutrient turnover (Blagodatsky et al., 2000; Barnard et al., 2015; Salazar-Villegas et al., 2016).

Thus, we hypothesized that (1) under long-term warming, faster microbial turnover reduces labile organic compounds and therefore, restricts the energy available for exoenzymes production and their temperature sensitivity, thus, retarding decomposition of SOM. This could work as a self-regulatory acclimation mechanism. (2) This self-regulation can be destroyed by the input of available substrate, (e.g., as a result of increased rhizodeposition under elevated CO2 and warming) altering microbial physiological state, accelerating enzyme activity and concequently, reducing soil C stock. There is a lack of experimental proof of the resilience of this self-regulation under warming considering microbial growth, enzyme activity, and substrate availability. To test this hypotheses, we determined kinetic parameters (V_{max} and K_m) of β-glucosidase, chitinase, leucine aminopeptidase, and acid phosphomonoesterase (involved in C, N, and P cycling) at two physiological modes: i) steady-state (non-activated microbial community) and ii) active growth induced by a mixture of glucose and nutrients addition at temperature gradients in soils from a long-term (8 years) field experiment with soil warming. For the first time, we applied a kinetic approach to estimate the amount of substrate natively present in soil for the set of tested enzymes. Microbial growth parameters, i.e. the specific growth rate (μ) and the portion of growing microbial biomass (GMB/TMB) were determined by substrate-induced growth respiratory response (SIGR).We aimed to study (1) How does long-term warming affect the temperature responses of potential enzyme activities ? (2) Whether or not soil microbial memory occurs persistently when microorganisms were activated by the labile C input with future climate warming?

2. Materials and methods

2.1 Site description and sampling

The soil samples were sampled from an on-going (since August 2010) long-term warming experiment located in the northern part of Göttingen, Lower Saxony, Germany (51°33′29.28″N, 9°55′59.46″E, WGS84), in which soil temperature has

been manipulated using heating cables for 8 years. The annual mean temperature and precipitation were 9.5 °C and 712 mm, respectively (Siebold and von Tiedemann, 2012). The soil at the site is classified as a silt loam textured Haplic Luvisol under an arable cropping regime. The winter oilseed rape cultivar Falcon (NPZ, Hohenlieth, Germany) and the breeding line SEM 05–500256 (SW Seed, Sweden) were sown by hand in a split-plot design in August, and harvested in November every year (Siebold and von Tiedemann, 2012). The three heating regimes included: (1) ambient soil temperature (with an average of 10 °C), (2) ambient +1.6 °C, and (3) ambient +3.2 °C. These were chosen to reflect warming scenarios by the year 2050 and 2100, respectively (Werner and Gerstengarbe, 2007). The experimental site consisted of 12 plots (2 m × 2.5 m each) arranged in two rows. Heating cables were buried at a depth of 10 cm in each plot, also in the control plots, to ensure equivalent physical conditions. Detailed information about the set-up of the warming plots and experimental sites can be found in Siebold and von Tiedemann (2012).

Soil samples were collected from the upper 10 cm of ambient, +1.6 °C and +3.2 °C plots in October 2018. In each of the four field replicates, five sub-samples were pooled to form a mixed soil sample. Once collected, samples were hand-mixed and any visible roots and stones manually removed. Soil samples were stored in gas-permeable plastic bags at 10 °C (approximate field temperature during sampling) until the start of further laboratory experiments (within 5 days of collection). Basic soil properties are shown in Table.1.

Table. 1 Basic properties of the sampling site. Letters mean significant difference between ambient and historical warming in the field ($p < 0.05$).

	pH	SOC (%)	TN (%)	MBC (mg kg^{-1})	MBN (mg kg^{-1})
Ambient	7.61±0.10a	2.701±0.18a	0.119±0.01b	691.2±55a	118.0±7a
+1.6 °C	7.78±0.09a	2.398±0.09a	0.131±0.01a	523.2±38b	85.9±10b
+3.2 °C	7.57±0.04a	2.500±0.08a	0.154±0.01a	506.9±31b	90.6±5b

2.2 Kinetics of substrate-induced growth respiration

The kinetics of substrate-induced growth response (SIGR) in the soil was analyzed according to Blagodatsky et al. (2000). The SIGR approach is based on microbial physiology and, thus, enables distinguishing total and growing biomass fractions along with parameters of microbial growth (Panikov, 1995). It has to be noted that although substrate addition is required by the SIGR approach, all kinetic parameters (e.g., specific growth rate, active and total microbial biomass) analyzed by SIGR represent the proportion of the soil microbial community at the time of sampling, i.e. before substrate addition. For this, one gram of fresh soil was amended with a mixture substrate containing 10 mg g^{-1} glucose, 1.9 mg g^{-1} $(NH_4)_2SO_4$, 2.25 mg g^{-1} K_2HPO_4. Substrate concentrations sufficient for the unlimited exponential growth of microorganisms were estimated in preliminary experiments in which increasing amounts of glucose were added. The quantity of mineral salts was selected

so that any substrate-induced change in soil pH was < 0.1.

For each treatment (ambient, +1.6 °C, and + 3.2 °C, 8 replicates), 1 g soil samples were then incubated in a Rapid Automated Bacterial Impedance Technique bioanalyser (RABIT; Microbiology International Ltd, Frederick, MD, USA), at 5, 10, 15, 20 and 25 °C. First, we incubated the soil samples for 2 days at 45% WHC to stabilize the system. After stabilization CO2 production was monitored every 20 minutes to determine the soil basal respiration (without any substrate addition). Then, glucose and mineral nutrients were added to the four replicates of each treatment. Soil water content was raised up to 75% WHC. Thereafter, the samples were incubated in the RABIT system additional 160, 90, 62, 35 and 24 h at 5, 10, 15, 20 and 25 °C, respectively. Microbial growth respiration in the glucose amended soil was used to model the specific growth rates of microorganisms (μ), the growing microbial biomass (GMB) (Panikov, 1995; Wutzler et al., 2012).

2.3 Enzyme kinetics

Enzyme activities were determined in the soil with and without substrate addition using the method described by Marx et al. (2001) and Zhou et al., (2020). Fluorogenic methylumbelliferone (MU)-based artificial substrates were used to estimate the activities of β-1, 4-glucosidase (EC 2.2.1.21), 4-N-acetylglucosaminidase (EC 3.2.1.52), leucine aminopeptidase (EC 3.4.11.1) and acid phosphomonoesterase (EC 3.1.3.2) (Sinsabaugh and Follstad, 2012). Briefly, 1 g soil (dry weight equivalent) was suspended in 50 mL sterile water by shaking for 30 min, and dispersing with an ultrasonic disaggregator for 2 min using low-energy sonication (50 Js^{-1}). 50 mL of the soil suspension was then pipetted into 96-well black microplates (Puregrade, Germany), while stirring the soil suspension to ensure uniformity. Afterwards, 50 µL of buffer and 100 µL of the corresponding substrates at concentrations of 2, 5, 10, 20, 50, 100 and 200 µmol substrate g^{-1} soil were added. Following substrate addition, the microplates were measured fluorometrically (excitation wavelength 360 nm; emission 450 nm) at 0, 30, 60, and 120 min with an automated fluorometric plate-reader (Victor3 1420 050 Multi-label Counter, PerkinElmer, USA). It should be noted that each fluorescence measurement did not exceed 2 minutes (Razavi et al., 2015) after which microplates were promptly returned to the climate chambers at the corresponding incubation temperature.

To calculate the enzyme kinetics parameters, we used the Michaelis-Menten equation for enzyme kinetics and adapted measured enzyme activities, V, with increasing substrate concentrations [S] where (Marx et al. 2001):

$$V=(V_{max}\times[S])/(K_m+[S]) \quad (1)$$

where V_{max} is the maximal enzyme activity; K_m (Michaelis-Menten constant) is the substrate concentration at which V_{max} is half.

The native available substrate (S_n) for specific enzymes was evaluated based on Monod kinetics (Panikov et al., 1992; Blagodatskaya et al., 2009), considering that the parameter S in Eq. 1 is a sum of naturally existing and of added fluorogenically labelled substrate (S_{add}), i.e.

$S = S_{add} + S_n$;

$V=(V_{max}\times([S_{add}+S_n])) / (K_m + [S_{add}+S_n])$ (2)

We used the routine Q10 function to examine temperature sensitivity and to express the temperature response of each enzyme kinetic parameter (i.e. Vmax and Km) separately:

$Q_{10}=(R(T+10\ °C))/R(T)$ (3)

where R is the rate of a process or a kinetic parameter value and T is the temperature (Razavi et al. 2016; Zang et al., 2020).

The classical Arrhenius equation was used to estimate enzyme denaturation on apparent activation energy that contributed to enzymes reaction rate (Razavi et al., 2015; Liu et al., 2017) :

$k = A \exp(-Ea/RT)$ (4)

Where k is the reaction rate constant; A is the frequency of molecular collisions; Ea is the required activation energy in Joules per mole; R is the gas constant (8.314 J mol^{-1} K^{-1}) and T is the temperature in Kelvin.

Noted, the soil added with and without labile substrate (a mixture of glucose and nutrients) means microbial activation and steady-state mode in our case. The activation effect on enzyme activities and system under different incubation temperatures and historical warming were quantified as effect size:

Activation effect = (EA - ES)/ES (5)

where EA and ES are the kinetic parameters of enzyme activity (i.e. V_{max} or K_m) under microbial activation and steady-state mode, respectively. Activation effect above zero indicates that the activation had a positive effect on V_{max} and K_m.

2.4 Statistical analysis

The experiment was carried out with four replicates for each parameter. The value presented in the figures and tables are given as means ± standard error (mean ± SE). Both Vmax and Km parameters were approximated by the Michaelis-Menten equation (1) with the non-linear regression routine of SigmaPlot (version 12.5; Systat Software, Inc., San Jose, Ca, USA). Fitting was performed for four replicates separately. The R2 values of non-linear regression were greater than 0.9; the p values among all the non-linear fitting in this study were less than 0.05. Prior to the analysis of variance (ANOVA), the data were tested for normality (Shapiro-Wilk, $p > 0.05$) and homogeneity of variance (Levene-test, $p > 0.05$). The effects of incubation temperature and historical warming treatment (ambient, +1.6 ℃, +3.2 ℃) on: specific growth rate (μ); growing microbial biomass (GMB); total microbial biomass (TMB); the ratio of GMB and TMB (GMB/TMB); enzyme activities (V_{max}); and substrate affinity (K_m) were analyzed using a two-way ANOVA, with “temperature” and “warming” as factors, and the different parameters as response variables. The effect of historical warming and microbial activation on the enzyme activity temperature sensitivities (Q_{10}-V_{max}) and substrate affinity (Q_{10}-K_m) at different incubation temperature ranges were also examined using a two-way ANOVA. One-way ANOVA was used to assess the effect of historical warming on V_{max} and on the activation effect of V_{max} at each incubation temperature, as well as onthe activation energy (Ea) of enzymes under steady-state and activation mode. All statistical analyses were

performed using SPSS software (version 19.0; SPSS Inc., Chicago, IL, USA), using Tukey HSD analyses to distinguish significant differences ($p < 0.05$).

3.Results

3.1 Kinetics of substrate induced growth respiration and enzyme under steady-state

The specific growth rate (μ) of soil microorganisms substantially increased with incubation temperature irrespective of historical warming (Fig. 1a), reflecting faster microbial turnover with higher temperatures. The total microbial biomass (TMB) decreased with increasing incubation temperature. However, the growing microbial biomass (GMB) increased at temperatures below 15 °C, but it decreased with further temperature increase (Fig. 1b,c). Consequently, the portion of GMB in TMB generally increased from 5 to 15 °C and then (up to 25 °C) it kept relatively stable or even decreased (Fig. 1d).

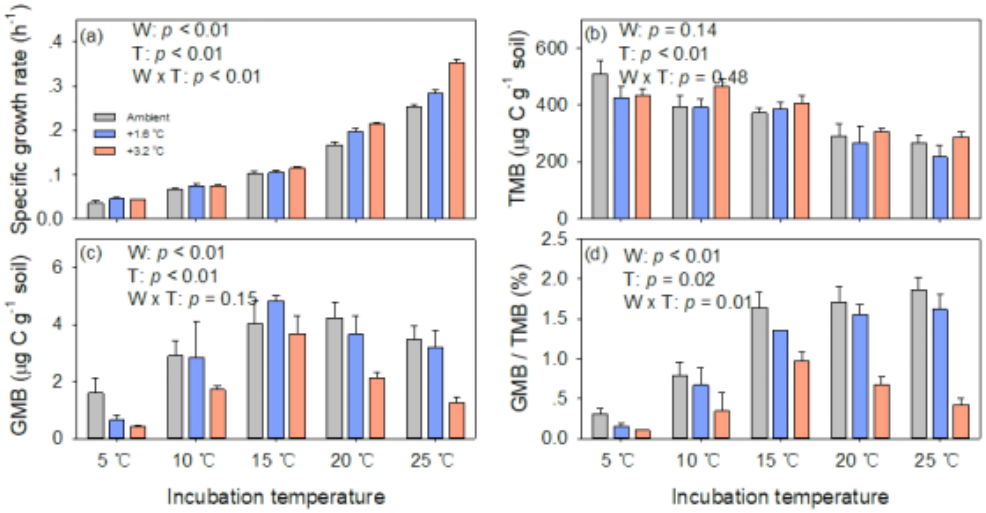

Fig. 1 Specific growth rate (μ) (a), total microbial biomass (TMB) (b), growing microbial biomass (GMB) (c), and the portion of GMB to TMB (d) for soil incubated at temperatures increasing from 5 to 25 °C in 5 °C increments under steady-state mode. Soil was sampled after 8 years of field warming (at: ambient; +1.6 °C; and + 3.2 °C). Values are the average (± SE) of four replicates.

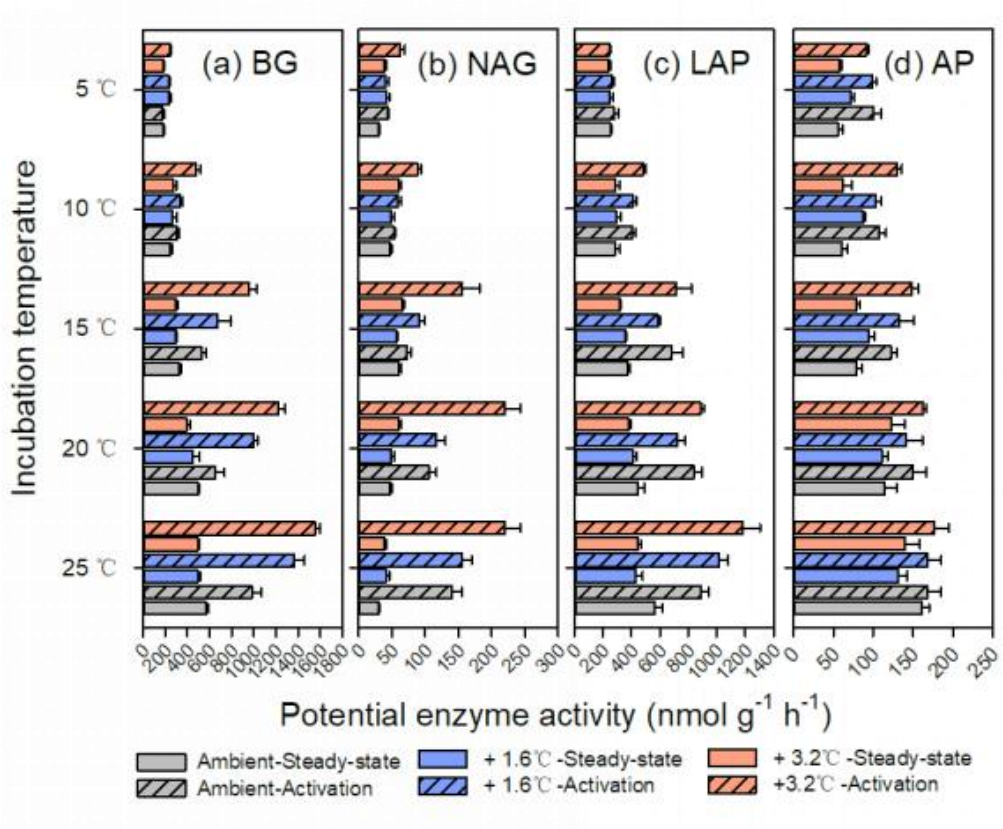

Fig. 2 Potential enzyme activities (V_{max}) of β-glucosidase (a, e), chitinase (b, f), leucine aminopeptidase (c, g), acid phosphomonoesterase (d, h) for soil incubated with temperature increasing from 5 to 25 °C at 5 °C increment under steady-state and microbial activation modes. The soil was sampled after 8 years of field warming (ambient, +1.6 °C, and +3.2 °C). Values are average (± SE) of four replicates. Asterisk indicates significant difference between ambient and historically warmed soil sites at different incubation temperature ($p < 0.05$).

The potential activity (V_{max}) of four hydrolytic enzymes responded positively to increased incubation temperature, regardless of substrate addition and historical soil warming ($p < 0.05$, Fig. 2). The V_{max} of β-glucosidase and leucine aminopeptidase was 11-27% higher in the ambient than in the historically warmed soils ($p < 0.05$, Fig. 2a, c), when incubated at higher temperatures (i.e. 15-25 °C). The K_m remained stable under the different long-term warming regimes (Fig. S1). Under steady-state mode, the native available substrate (S_n) for β-glucosidase, chitinase, leucine aminopeptidase was 49-96%, 34-40%, and 12-74% higher in ambient compared with historically warmed soils ($p < 0.05$, Fig. 3a), respectively. However, the native available substrate for acid phosphomonoesterase was not significantly altered by long-term warming.

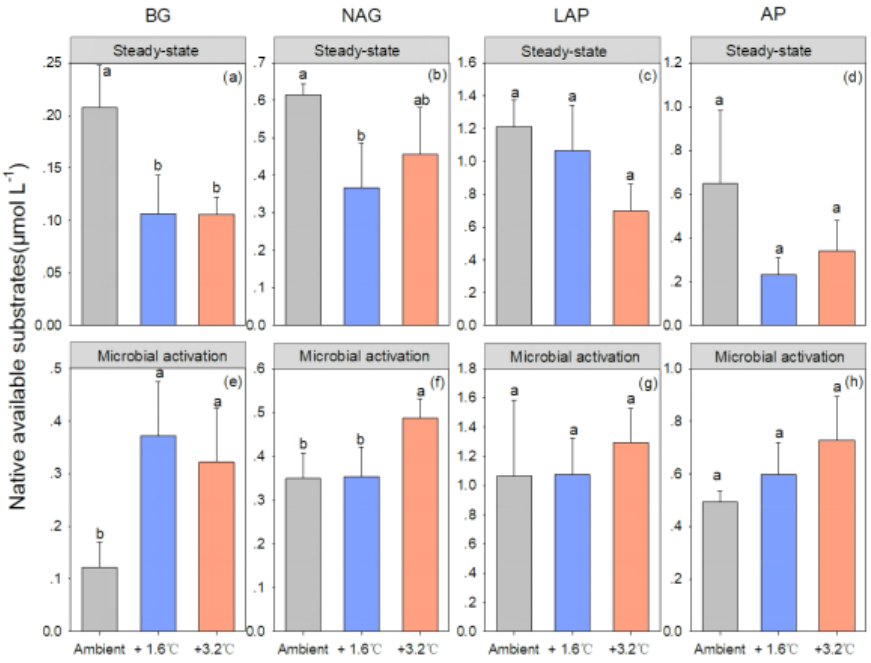

Fig. 3 Native available substrate for β-glucosidase, chitinase, leucine aminopeptidase, acid phosphomonoesterase for soil incubated with 15 °C under steady-state and microbial activation modes. The soil was sampled after 8 years of field warming (ambient, +1.6 °C, and +3.2 °C). Values are average (± SE) of four replicates. Letter indicates significant difference between ambient and historically warmed soil sites ($p < 0.05$). H

3.2 Enzyme kinetics under microbial activation

Under microbial activation mode, β-glucosidase and chitinase activities increased by 23-110% and 37-148% in historically warmed as compared with ambient soil ($p < 0.05$, Fig. 2), respectively. All enzyme affinities under activation mode increased with increasing incubation temperature, except for leucine aminopeptidase (Fig. S1). Microbial activation reduced the Sn pool in ambient, but partly increased that in historically warmed soils (Fig. 3). Therefore, the differences were either smoothed or showed the opposite trend under activation compared to steady-state mode (Fig. 3b).

Activation had a greater effect on V_{max} in the historically warmed soils compared with soil under the ambient temperature (except acid phosphomonoesterase) (Fig. 4). The activation effect on V_{max} increased with temperature from 5 to 15 °C, above which it remained stable. On the contrary, the activation effect on K_m of β-glucosidase, chitinase and acid phosphomonoesterase decreased from 200% to 0% with increased incubation temperature (Fig. 5). The activation did not alter the K_m of leucine aminopeptidase at lower incubation temperature, and thus showed an increased pattern.

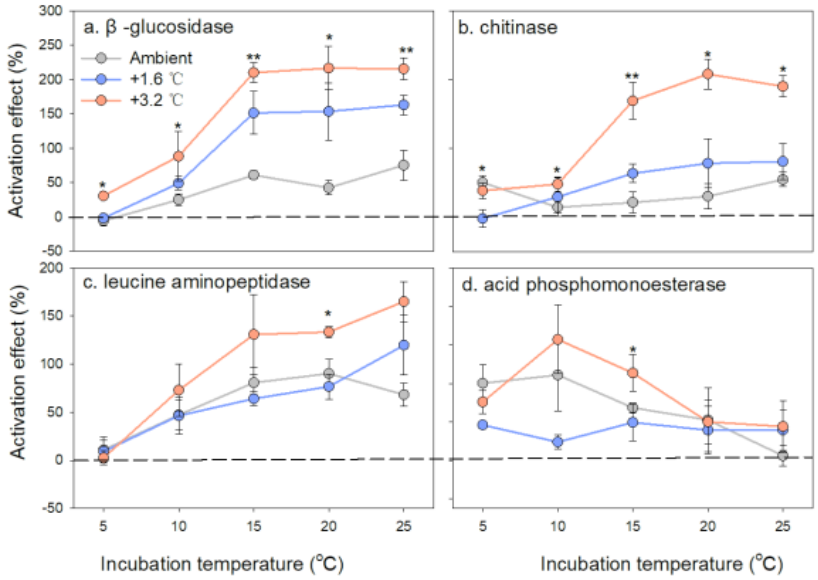

Fig. 4 Activation effect on potential enzyme activities (V_{max}) of β-glucosidase (a), chitinase (b), leucine aminopeptidase (c), acid phosphomonoesterase (d) for soil incubated with temperatures increasing from 5 to 25 °C at 5 °C increments. Soil was sampled after 8 years of field warming (at: ambient; +1.6 °C; and +3.2 °C). Values are the average (± SE) of four replicates. Asterisk indicates significant difference between ambient and historically warmed soil sites at different incubation temperature ($p < 0.05$).

3.3 Temperature sensitivity of enzymes under steady-state and microbial activation

Under steady-state mode, the temperature response of V_{max} differed across enzymes, corresponding to Q_{10}-V_{max} values up to 2.11 (Fig. 6), with the activation energy (E_a) values ranged from 22.9 to 50.5 kJ mol-1 (Fig. S2). Ea was always lower in the historically warmed compared with ambient soil under steady-state mode. The temperature sensitivity of K_m was less than that of V_{max}, and Q_{10}-K_m varied between 0.30 - 2.01 (Fig. S3). The Q_{10}-V_{max} for enzymes involved in C and N cycling (e.g., β-glucosidase, chitinase, and leucine aminopeptidase) were 9-27% lower in historically warmed than in ambient soil, only at lower incubation temperatures ($p < 0.05$, Fig. 6). However, acid phosphomonoesterase had much higher Q_{10}-V_{max} (between 1.2-1.6 irrespective of historical soil warming and incubation temperature).

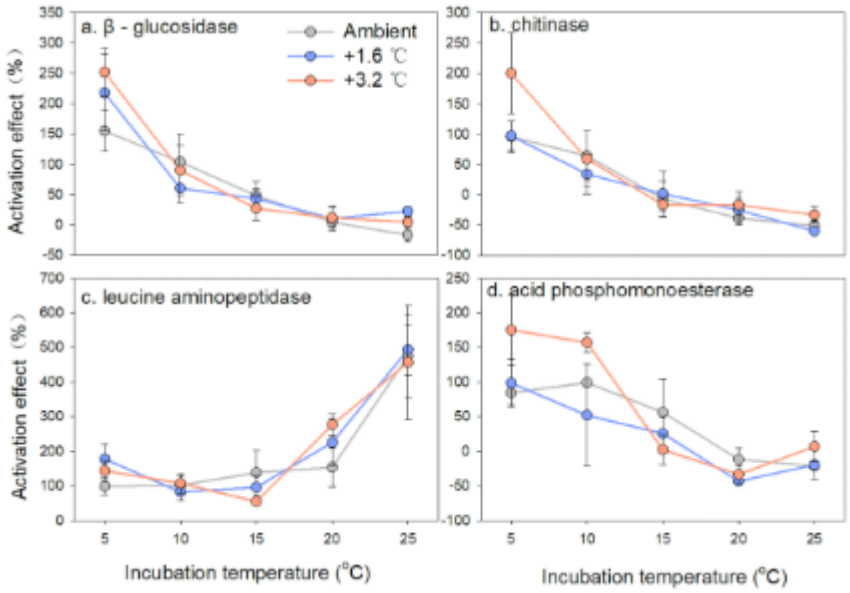

Fig. 5 Activation effect on substrate affinity (K_m) of β-glucosidase (a), chitinase (b), leucine aminopeptidase (c), and acid phosphomonoesterase (d) for soil incubated with temperatures increasing from 5 to 25 °C at 5 °C increments. Soil was sampled after 8 years of field warming (at: ambient; +1.6 °C; and +3.2 °C). Values are the average (± SE) of four replicates.

Under activation mode, the Q_{10}-V_{max} of enzymes ranged from 1.2 to 4.0, two-folds higher than in soils under steady-state mode. This was demonstrated especially under low incubation temperatures (Fig. 6). In contrast to steady-state, the Q_{10}-V_{max} of all enzymes (except acid phosphomonoesterase) was higher in the historically warmed soil than in soil with ambient soil temperatures. The Q_{10}-K_m decreased after microbial activation, but was not affected by long-term soil warming (Fig. S3).

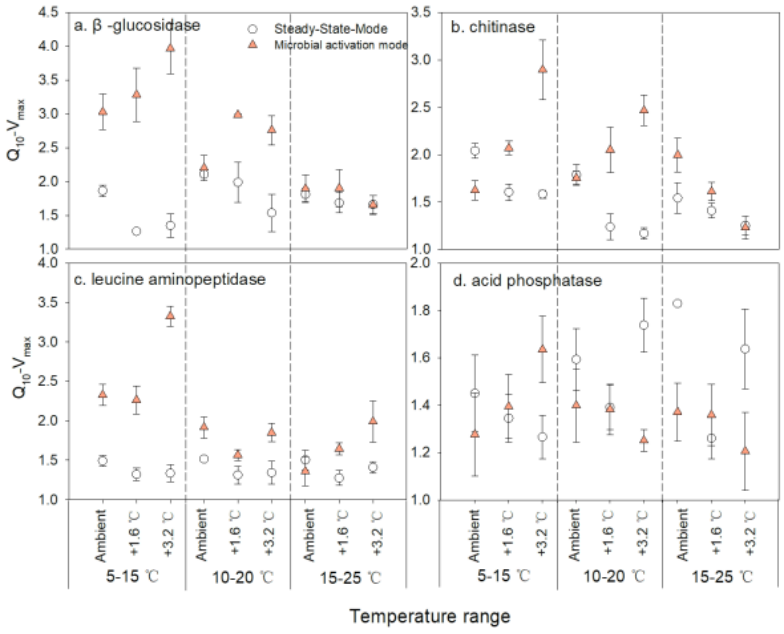

Fig. 6 Temperature sensitivity of potential enzyme activities (Q_{10}-V_{max}) of β-glucosidase (a), chitinase (b), leucine aminopeptidase (c), and acid phosphomonoesterase (d) for soil incubated with temperature increasing from 5 to 25 °C at 5 °C increments. Soil was sampled after 8 years of field warming (at: ambient; +1.6 °C; and +3.2 °C). Values are the average (± SE) of four replicates.

4. Discussion

4.1 Effects of temperature changes on soil enzyme activities under steady-state mode

We observed a weaker increase in enzyme activities (V_{max}) with increasing incubation temperature in samples from historical field warming versus ambient soil, which partly supported our hypothesis that long-term warming reduces the temperature sensitivity of enzymes. In the historically warmed soils, enzymes performance was more efficient as indicated by the lower activation energy (Fig. S2), and therefore, microorganisms can reduce the *de-novo* synthesis of enzymes while achieving the same functions (Koch et al., 2007; Machmuller et al., 2016). However, such a decline in V_{max} of tested enzymes involved in C and N cycling in historically warmed compared with ambient soils was only observed under high incubation temperatures, which suggests that a reduction in enzyme synthesis is a trait of warm-adapted enzymes.

Further, the temperature sensitivity (Q_{10}-V_{max}) of β-glucosidase, chitinase, and leucine aminopeptidase decreased with historical warming (Fig. 6), which supported our first hypothesis that enzymes in the historically warmed soil ambient soil would show lower Q_{10} than in ambient soils, because of the microbial memory effect (thermal acclimation) (Bradford et al., 2008; Tucker et al., 2013; Walker et al., 2018). Microbial memory effect in historically warmed soil could result from limitations by labile organic substrates (Davidson and Janssens, 2006; Frey et al., 2008). Eight years of accelerated C cycling substantially depleted the easily available C pool in the historically warmed soils. This was further proved by the lower content of native substrates for β-glucosidase, chitinase, and leucine aminopeptidase, as well as by lower MBC/SOC ratio in the historically warmed compared with ambient soils (Fig. 3; Table 1). The low amount of available substrates precludes slow microbial metabolic activity, and thus many soil microorganisms shifted to dormancy (Lennon and Jones, 2011), as indicated by a lower proportion of growing in total biomass (i.e., GMB/TMB, Fig. 1d). Given that active microorganisms are more sensitive to the fluctuating environment (Kussell and Leibler, 2005; Blagodatskaya and Kuzyakov, 2013), and therefore showed a higher temperature sensitivity in the ambient soils compared with dormant microorganisms under long-term warming. Furthermore, dormant microorganisms are able to slowly recycle their own cell components and to down-regulate enzyme-expressing gens (Joergensen and Wichern, 2018). As a consequence, they decreased microbial investment in the production of hydrolytic

enzymes, and hence decreased the response of enzyme activity to temperature.

Alternatively, this trend could be related to isoenzyme production by microorganisms (Wallenstein, 2011), which occurs as an acclimation response of the enzyme itself or the enzyme-producing microbial community, as supported by the inapparnet changes of K_m of β-glucosidase and leucine aminopeptidase (Fig. S1). These expressed isoenzymes catalyze the same reaction but differ in protein structure, enabling them to act more effectively in the ambient soil (Nottingham et al., 2016; Razavi et al., 2016). Decreased carbon use efficiency in historically warmed soils could at least partly explain the decline in growing microbial biomass (Tucker et al., 2013; Wen e al., 2019a), thus restricts the Q_{10}-V_{max} in the historically warmed soil. Further, lower total microbial biomass supported the possibility of greater microbial dieback under higher incubation temperatures (Sihi et al., 2017), thus providing via microbial necromass readily available substrate for decomposition and microbial use (Dippold and Kuzyakov, 2016). This C and nutrient source provides the fast-growing microorganisms (Fig. 1a) with their growth substrate (Bore et al., 2017). In contrast to the enzymes involved in C and N cycling, there was an absence of significant trend of Q_{10}-V_{max} for acid phosphomonoesterase (Fig. 6). This was probably an indication of no limitation for P, and thus no any reaction of P cycling enzyme systems even with climate warming.

4.2 Activation altered the temperature sensitivity of soil enzymes

After activating microorganism, we observed an increase both all the tested enzyme activities with incubation temperature (Fig. 2) and temperature sensitivity of enzyme activities (Q_{10}-V_{max}) (Fig. 6). This indicated the interactive effect of substrate and temperature on the catalytic reaction, induced by microorganisms originally adapted to historical warming. Similarly, microbial activation with glucose significantly increased the Q_{10} values of respiration (Zhu et al., 2011). Furthermore, substrate availability might also shift enzyme expression towards lower affinity (Steinweg et al., 2008), where the trade-off is an increase in maximum catalytic rates as supported by the higher K_m (i.e., lower affinity) for all enzymes under microbial activation compared with steady-state modes (Fig. S1). Such a shift in enzyme expression favored fast-growing microorganisms and higher respiration rates under future warming.

Altered C availability in soil are well known to affect microbial physiological status (Cleveland et al., 2007; Fierer et al., 2007). Stronger limitation by the labile native substrate in the historically warmed soil reduced metabolic activity (Fig. 3a), and thus caused a memory effect in response to further short-term increasing temperature. In contrast, the surplus of glucose increased native labile substrates (Fig. 3b), induced microbial growth, as well as increased both enzyme activity and catalytic efficiency (Fig. 2, S2), as a consequence eliminated the microbial memory effect in

the historically warmed soils. Thus, a sudden increase in C availability can induce a short-term fluctuation in community composition shifting the domination from slow-growing oligotrophic microorganisms (favored by low substrate availability) to temporal dominance of copiotrophic microorganisms (Cleveland et al., 2007). Shifts towards more active microorganisms at warmer temperatures combined with increasing labile C input (e.g., activation mode) from enhanced vegetation productivity at higher mineralization rates can thus result in higher temperature sensitivity (Melillo et al., 2002; Hartley et al., 2008). Therefore, the Q_{10}-V_{max} was higher in the historically warmed compared with ambient soils after substrate addition, and substrate input accelerated enzymes-mediated C and N mobilization in the soil under climate warming.

Under the C limitation, microorganisms acclimated to 8- years soil warming with annual fluctuate soil temperature of 5-15 °C, could slowly maintain their metabolism at temperatures below 15 °C (Marchant et al., 2008). At warmer environment (above 15 °C), however, meso/thermophilic microorganisms grow faster and rapidly increase their number during a short time. Therefore, the mesophic (and even thermophilic) nature of acclimated microorganism expressing the enzymes beneficial in warmer environment in long-term warmed soils, could serve as an adaptation strategy to adverse environments (e.g., C limitation) enabling to outcompete or partially replace the mesophiles under favorable conditions i.e., temperature increase and additional labile substrate (Portillo et al., 2012). As a consequence, the input of labile substrate stimulated microbial growth and increased Q_{10}-V_{max} in historically warmed compared to ambient soils. However, the uncommon high temperatures (e.g., above 15 °C which never reached up in the field) not only activate dormant microorganisms (Birgander et al., 2013), but also increase in short-term microbial mortality by thermal denaturation, especially in the ambient soils dominated with psychrophilic microorganisms (Joergensen et al., 1990), as indicated by the stable activation effect of V_{max} above 15 °C, as well as the weaker activation effect in the ambient compared with historically warmed soils (Fig. 4). Furthermore, this could be explained by the limited binding between enzymes and soil organics due to the increased absorption rate between organic-mineral surface with elevated incubation temperature (Nannipieri et al., 1996; Wallenstein et al., 2011). Therefore, the apparent increase of Q_{10}-V_{max} after microbial activation was only observed at low incubation temperatures, and no difference in Q_{10}-V_{max} as well as Q_{10}-K_m between historical warmed and ambient soil was observed at higher incubation temperatures.

5.Conclusions

Overall, this study provided new empirical confirmation that even slight warming (which is highly relevant to current climate change predictions) in long-term reduced the amount of native labile substrates in soil, leading to a soil microbial

memory effect. The temperature sensitivity (Q_{10}) of enzymes involved in C and N cycling under the steady-state mode was higher in ambient than in historically warmed soils. This strongly suggests that microorganisms in the historically warmed soils shifted to dormancy due to the substrate limitation, as indicated by the lower native available substrates for β-glucosidase, chitinase, and leucine aminopeptidase compared with that in ambient soils. Substrate depletion in the historically warmed soils further induced a microbial thermal compensation, as indicated by an inapparent sensitivity of enzyme systems (K_m). Input of labile substrate, however, remarkably activated dormant microorganisms in the historically warmed soils, stimulated enzyme activity, and thus counterbalanced the soil microbial memory effect resulting in higher Q_{10}-V_{max} as compared to ambient soil. Our results identified that the transition between microbial physiological states owing to changes in substrate (C or nutrient) availability is the most likely explanation for the observed variations in temperature sensitivity of enzyme activities. Thus, we showed a strong interaction of C input and climate warming, both suggested to be substantially altered by global change. Therefore, accelerated soil C cycling due to climate warming might induce a self-enhancing feedback reaction resulting in the depletion of the easily available C pool in soils. Shifts in SOM quantity then vice verse will imply shifts in microbial community composition and nutrient cycling functions much more severe as a single altered factor such as temperature alone can induce.

Acknowledgement

We thank the China Scholarship Council (CSC) for funding to Jie Zhou in Germany. This study was financially supported by the Robert Bosch Foundation (Junior Professorship 2017 of Michaela Dippold). The authors would like to thank the permission from Andreas von Tiedemann (Plant Pathology and Crop Protection, Department of Crop Sciences, Georg August University of Göttingen, Göttingen, Germany) for soil sampling from the long-term warming field (northern of Göttingen). The publication was prepared with the support of the "RUDN University program 5-100". Huadong Zang also thanks for the PostDoc position supported by the Department of Crop Sciences, the Faculty of Agricultural Sciences, University of Goetthing.

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Supplementary

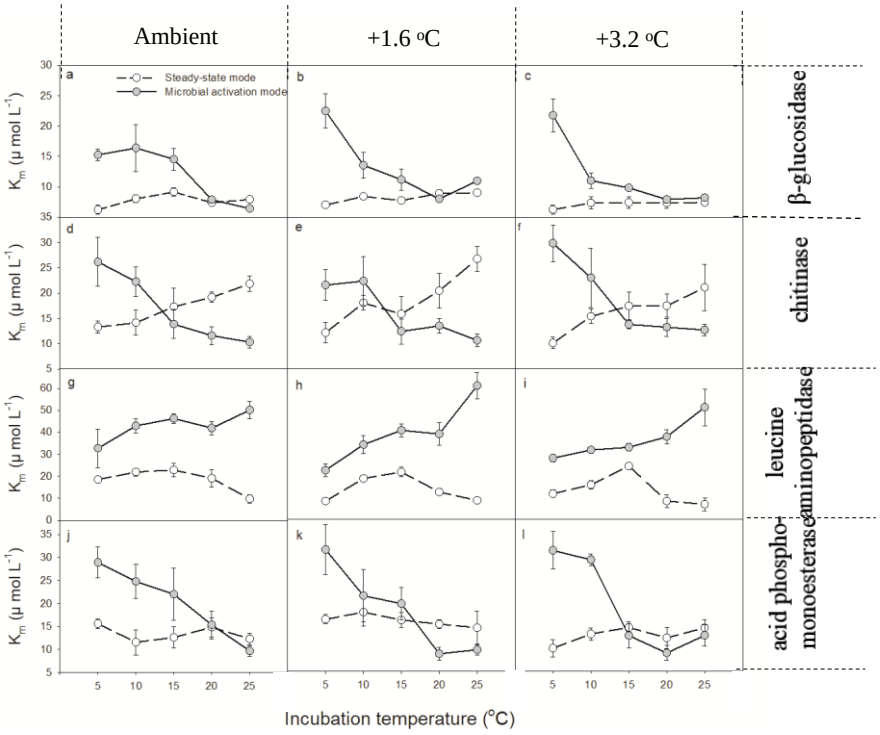

Fig. S1 Substrate affinity (K_m) of β-glucosidase, chitinase, leucine aminopeptidase, and acid phosphomonoesterase for soil incubated with temperature increasing from 5 to 25 °C at 5 °C increment under steady-state and microbial activation modes.

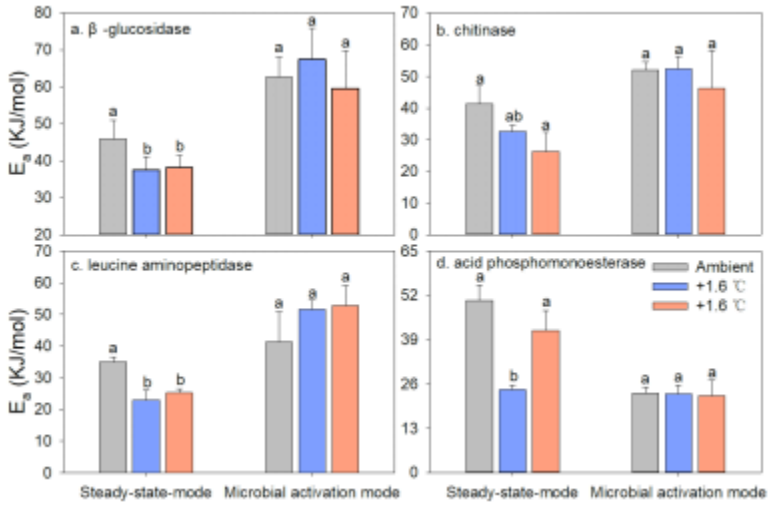

Fig. S2 The activation energy (E_a) of β-glucosidase (a), chitinase (b), leucine aminopeptidase (c), and acid phosphomonoesterase (d) for soil under steady-state and microbial activation modes.

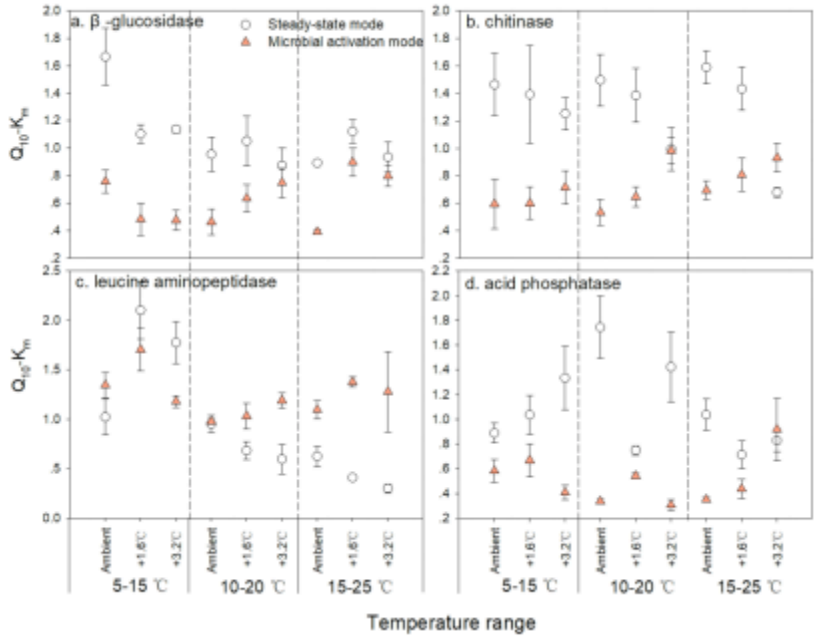

Fig. S3 Temperature sensitivity of enzyme substrate affinity (Q_{10}-K_m) of β-glucosidase (a), chitinase (b), leucine aminopeptidase (c), and acid phosphomonoesterase (d) for soil incubated with temperature increasing from 5 to 25 °C at 5 °C increment under steady-state and microbial activation modes.

Declaration

I hereby declare, to the best of my knowledge and belief, that this thesis contains no material previously published or written by another person, except where due reference has been made in the text of the thesis. This thesis contains no material which has been accepted or definitely rejected for the award of any other doctoral degree at any university.

Erklärung

Hiermit erkläre ich, die vorliegende Arbeit selbst verfasst, keine ander en als die angegebenen Quellen und Hilfsmittel benutzt sowie alle wörtlich und si nngemäß übernommenen Stellen in der Arbeit gekennzeichnet zu haben. Ferne erkläre ich, dass ich nicht anderweitig mit oder ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich einer Doktorprüfung zu unterziehen.

Goettingen, 30-03-2021

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Place/Date Jie Zhou