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Effect of soil C:N:P stoichiometry on plant-microbe-soil interactions

Ph.D. Thesis

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Annotation

The effect of C:N:P stoichiometry on plant-microbe-soil interactions was studied using glasshouse experiments to maintain constant environmental conditions and manipulate plant and soil characteristics. Plants with different economic strategies were studied on soils with different C:N:P stoichiometry due to C enrichment. This allowed for various plant-soil interactions, which were used to study the strongest links between plants, microbes, and soil. Subsequently, the role of C addition in the plant-exudates-microbe-soil interactions was evaluated, including the correlation of foliar isotopic C and N composition with plant biomass and soil N availability. Finally, plant- soil communication was altered by fertilizing plant leaves without changing soil conditions. In this way, we were able to determine the role of plant C:N stoichiometry in the priming effect of the rhizosphere and detritusphere.

Declaration

I hereby declare that I am the author of this thesis and that I have used only those sources and literature detailed in the list of references.

České Budějovice, 08/03/2022

Julian Cardenas

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List of papers and manuscripts and author's contribution

The thesis is based on the following papers and manuscripts:

I. **Cardenas-Hernandez, J.**, Santa, F., Kaštovská, E., 2021. The exudation of surplus products links plant functional traits and plant-microbial stoichiometry. *Land* 10, 840.
DOI: https://doi.org/10.3390/land10080840. (Journal rank: Q2, IF=3.398 (2020), 2 citations).

Julian Cardenas participated in designing the experiment, sampling, biochemical analyses, was responsible for assembly and statistical evaluation of data, wrote the first version of the manuscript and contributed to its revisions. Contribution: 75%.

II. **Cardenas-Hernandez, J.**, Čapek, P, Kaštovská, E. C addition to the soil alters plant-microbial interactions and it is reflected in foliar $\delta^{15}N$. Manuscript.

Julian Cardenas participated in designing the experiment, sampling, biochemical analyses, was responsible for assembly and statistical evaluation of data, wrote the first version of the manuscript and contributed to its revisions. Contribution: 75%.

III. Kaštovská, E., **Cardenas-Hernandez, J.**, Kuzyakov, Y. 2021. Priming efects in the rhizosphere and root detritusphere of two wet-grassland graminoids. *Plant and Soil. DOI: https://doi.org/10.1007/s11104-021-05191-6* (Journal rank: Q1, IF=4.192 (2020), 2 citations)

Julian Cardenas participated in sampling, biochemical analyses, helped in the statistical analysis and in the discussion of the manuscript. Contribution: 40%.

The agreement of the co-authors to the student's declared share of joint publications

As a senior co-author of Paper I and Manuscript II and the first author of Paper III, I agree with the student's declared share of joint publications.

Eva Kaštovská

CONTENTS

Abreviations

C	carbon
DOC	dissolved organic C
SOM	soil organic matter
N	nitrogen
NH_4^+	ammonium
NO_3^-	nitrate
P	phosphorus
PO_4^{3-}	phosphate
PE	priming effect
RPE	rhizosphere priming effect
PFT	plant functional trait
RS	root-to-shoot ratio
SLA	specific leaf area

1. GENERAL INTRODUCTION

The main players in terrestrial ecosystems, **plants and soil, are intimately linked** (Högberg and Read, 2006; Kuzyakov and Xu, 2013). Plants and soil microorganisms have different nutrient requirements due to their distinctly different functions, metabolism, and biomass composition (Soong et al., 2018). Plants rely on nutrients (mainly nitrogen (N) and phosphorus (P)) from the soil to fix carbon (C). Some of the fixed C is released to the soil by roots in the process called rhizodeposition and fuels microbial activity which in turn accelerates decomposition of soil organic matter (SOM) and mineralization of nutrients (Hunter, 2016). **The "exchange" of C for nutrients between plants and soil microorganisms is one of the most important processes in sustaining the primary production** of terrestrial ecosystems (Bardgett and Wardle, 2010; Gundale and Kardol, 2021; Pugnaire et al., 2019). The net outcome of plant-soil feedback on plant and microbial growth and on rates of SOM decomposition and nutrient recycling depends on both biotic (e.g. plant and microbial functional traits) and abiotic factors (e.g. soil nutrient availability) (Bennett and Klironomos, 2019; Henneron et al., 2020a). The numerous components and factors involved make plant-soil interactions complex and diverse (Bai et al., 2016; Pugnaire et al., 2019; Van der Putten et al., 2013). Synthesizing (generalizing) approaches are needed to uncover and disentangle the complexity of plant-soil interactions. The study of plants and microbes in terms of their functions in the ecosystem (functional analysis) and their elemental ratios (ecological stoichiometry) has proven useful in this regard.

The combination of functional analysis and ecological stoichiometry makes it possible to understand the relationships between plant nutrition and the rates of soil processes that determine ecosystem performance. Each species growing under specific conditions can be characterized by a set of plant functional traits (PFT: e.g. inherent growth rate, specific leaf area, above-to-belowground allocation pattern and others) that allow **the economic strategy of plants** to be classified on a gradient from conservative to acquisitive. Fast-growing (acquisitive) plants dominate in nutrient-rich soils, whereas slow-growing (conservative) plants are adapted to oligotrophic conditions (Henneron et al., 2020b; Ordoñez et al., 2009). Acquisitive plants rapidly take up available nutrients to support their rapid growth and preferentially allocate the resources above ground to improve access to light and maximize photosynthesis. The dominance of acquisitive plants in the ecosystem is related to the fast mineralization of readily decomposable SOM by soil microbes and the rapid release of available nutrients, which translates into rapid plant growth (Carrillo et al., 2017; Henneron et al., 2020b). Under nutrient limitation, plants reduce their growth rate, build the more long-lived, nutrient-poor tissues, and increase the allocation of biomass to roots to

explore a larger soil volume (Güsewell, 2004). The dominance of conservative plants in the ecosystem is associated with slow SOM decomposition and nutrient recycling. Therefore, **plant characteristics reflect well the prevailing processes in the ecosystem** and allow deriving general information about soil conditions (nutrient content, carbon sequestration) (De Deyn et al., 2008; de Vries and Bardgett, 2016). **Plant growth and the rate of soil processes are determined by soil C:N:P stoichiometry**. Nevertheless, the main directions of both frameworks, the trait-based approach and ecological stoichiometry, are independent, which limits the knowledge of the link between elemental stoichiometry and functional traits (Meunier et al., 2017).

Plant-soil interactions are mediated by plant-derived organic compounds that enter the soil both during the growing season (**rhizodeposition, especially root exudation**) and after plant senescence (**plant litter**). Root exudation, the release of simple soluble compounds (mostly primary metabolites), has recently been considered a PFT (Guyonnet et al., 2018) because of its key role in promoting microbial growth and SOM decomposition and thus regulating soil nutrient availability (Bengtson et al., 2012; Carrillo et al., 2017; Drake et al., 2013b). The exudates partly consist of compounds that exceed the actual metabolic needs of the plants (Canarini et al., 2019; Prescott et al., 2020). Therefore, exudates contain not only excess C compounds but also organic and mineral forms of macronutrients, N, and P (Drake et al., 2013b; Edwards et al., 2018). Therefore, exudate flux and its elemental composition should be related to both PFTs and tissue nutrient composition and C:N:P stoichiometry. While the strong positive correlation of exudate flux with plant growth rate and plant biomass is well known (Baptist et al., 2015; Kaštovská et al., 2017), the links between exudate C:N:P stoichiometry and PFTs, as well as the effects of nutrients excreted by roots on microbial processes in the rhizosphere, are unknown. Given the importance, complexity, and difficulty of measuring root exudation, **it is critical to find the PFTs that can predict the quality and quantity of root exudation** for more practical integration of belowground plant processes into C-cycle models and budgets (Gougherty et al., 2018).

Decomposing roots provide another important source of C and nutrients for microorganisms, especially at the end of the growing season when part of the root system gradually dies. The large input of complex root litter, the decomposition of which leads to the release of a large amount of available C, at least in the initial phase, stimulates microbial growth and activity on scale comparable to the exudates released by living roots (Bastian et al., 2009; Mastný et al., 2018; Sokol et al., 2019). The decomposition rate of root litter is controlled by its C:N ratio, which is closely related to plant growth rate and other PFTs. While these relationships are relatively well understood, the effects of microbial activity stimulated by **the root litter inputs on the decomposition and loss of older stabilized SOM have rarely been studied. The role**

of root litter stoichiometry in SOM dynamics and its association with other PFTs remains to be elucidated.

The natural isotopic composition of C and N in leaves ($\delta^{13}C$ and $\delta^{15}N$) is also increasingly used as an indicator of plant metabolism and nutrient cycling in ecosystems. This is because the foliar $\delta^{13}C$ and $\delta^{15}N$ integrate isotopic composition of sources and fractionation during uptake and utilization by plants (Bowling et al., 2008; Cernusak et al., 2013; Dawson et al., 2002; Tcherkez, 2011). Both $\delta^{13}C$ and $\delta^{15}N$ of leaves are affected by nutrient availability (Zhang et al., 2015; Zhang et al., 2017). Therefore, **we included them** in our studies **to investigate their potential as integrators between ecological stoichiometry and PFTs**.

1.1 THE RHIZOSPHERE AND ROOT DETRITUSPHERE – PLANT-GENERATED HOTSPOTS OF MICROBIAL ACTIVITY

Microbial growth and activity in soil are primarily limited by C availability (Soong et al., 2018). The input of plant-derived organic compounds into the soil, either through live or dead roots, alleviates this limitation. It promotes the abundance and activity of microorganisms (Kaštovská and Šantrůčková, 2007; Kuzyakov and Blagodatskaya, 2015) and creates **hotspots of microbial activity** in the soil (Kuzyakov and Blagodatskaya, 2015). The **rhizosphere** is the volume of soil directly influenced by the activity of living roots (Hiltner, 1904). After the roots die, the rhizosphere turns into the **root detritusphere,** which is affected by the decomposition of dead roots (root litter) (Kuzyakov and Blagodatskaya, 2015). Therefore, both have similar spatial distribution through the soil profile.

However, the rhizosphere and detritusphere differ in the decomposability of their major C sources. The release of organic compounds by living roots is referred to as **rhizodeposition** (Jones et al., 2004). It encompasses a variety of processes and forms by which organic compounds are continuously released to the soil, including: Loss of root cap and border cells, death and lysis of root cells (cortex, root hairs, etc.), gaseous losses, secretion of insoluble polymers from living cells near the root cup (mucilage), and leakage of solutes from living cells (**root exudates**) mainly in the zone of cell division (meristematic zone) (Fig. 1) and from root-associated symbionts (e.g. mycorrhiza) (Hinsinger et al., 2003; Jones et al., 2009). In the root detritusphere, the C source consists of dead roots, mostly in particulate, highly polymeric recalcitrant form (Kögel-Knabner, 2017), with low concentrations of labile low molecular-weight organics (LMWO) (Kuzyakov and Blagodatskaya, 2015). **Root litter** can still release significant amounts of labile compounds at an early stage of its decomposition, which primes microbial decomposition of the remaining root tissue and native OM (Bastian

et al., 2009; Mastný et al., 2018). At later stages of decomposition, both the C flush from the decomposing roots and its decomposability decrease (Mastný et al., 2018).

The rhizosphere and detritusphere also differ in the extent of competition for nutrients. In the detritusphere, plant nutrient uptake does not occur and only soil microorganisms compete for available nutrients. In contrast, plant roots play a critical role in the rhizosphere (Kuzyakov and Xu, 2013). They actively take up nutrients, which significantly decreases nutrient availability near the roots. This effect is particularly strong for highly mobile forms of nutrients such as nitrates (Fig. 1). Plant activity makes the rhizosphere a highly competitive environment for nutrients compared to the detritusphere (Bonkowski et al., 2000; Kuzyakov and Xu, 2013). Plant nutrient requirements and the rate of nutrient uptake by roots depend on the **economic strategy of the plant** and are regulated by plant growth rate and tissue N and P concentrations. While slow-growing, conservative plants have lower nutrient requirements, the fast-growing, acquisitive species with greater nutrient requirements cause greater removal of nutrients, especially nitrates, from the rhizosphere (Henneron et al., 2020b). The differences in C flux into the soil between the rhizosphere and detritusphere in combination with the presence or absence of plant-microbe competition for nutrients then influences microbial growth, extracellular enzyme production, and decomposition of SOM in the two environments.

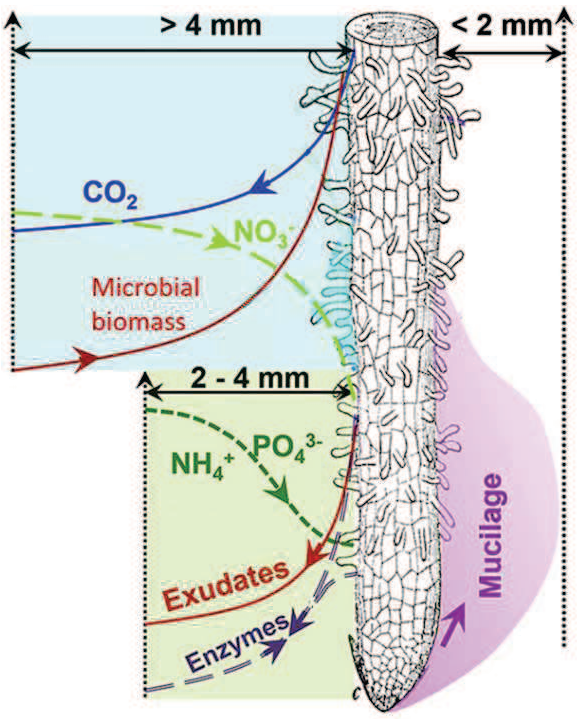

Figure 1. Generalization of rhizosphere extent and gradient types for some of the soil variables focused on in this work: CO_2, root exudates and mucilage, nitrate, ammonium, phosphate, hydrolytic enzyme activities and microbial biomass. Three groups of rhizosphere extents are typical: 0.5–2 mm (right), 2–4 mm (lower left), and >4 mm (upper left). The release of exudates and mucilage is located near the root cap and elongation zone. The gradient of CO_2 concentration is mainly formed by root and microbial respiration. Microbial biomass and activity increases toward the root surface due to intensive use of released exudates. Uptake by roots decreases nutrient concentrations near the roots. The depletion zone is more extensive for nitrates than for ammonium and phosphates, due to a greater adsorption of ammonium and phosphates to soil particles but higher solubility and mobility of nitrates in soil solution. Adapted from Kuzyakov and Razavi (2019).

1.2 ROOT EXUDATION: A TARGETED INVESTMENT OR A RELEASE OF CURRENTLY UNUSED PRODUCTS?

Root exudates are the substances secreted by the roots of living plants. They consist mainly of LMWO compounds that are released freely and passively (Fig. 1), forming a complex mixture of soluble substances such as sugars, amino acids, organic acids (Jones et al., 2009), and even mineral ions (Edwards et al., 2018). The compounds in root exudates are similar to those that are central to cellular metabolism (Jones et al., 2009). This is because these compounds are translocated within the plant from shoots to roots before being exuded (Canarini et al., 2019). The transport of compounds from photosynthesizing organs (source) to roots occurs in the phloem. The flux is driven by concentration gradients determined by source-sink activities (De Schepper et al., 2013). Once in the roots, exudates are released unimpeded and move out of the phloem-pole pericycle by a diffusion gradient. Most of the exudation occurs around the root tip, as this zone consists of non-differentiated cells lacking apoplastic barriers (casparian strip) (Canarini et al., 2019), which allows diffusion of compounds into the soil (Canarini et al., 2019; Jones et al., 2009; Ross-Elliott et al., 2017).

Microbial activities modulate concentration gradients in the rhizosphere (Fig. 1). Microorganisms in the rhizosphere rapidly utilize exudates (Caffaro et al., 2013) and either take up or release available nutrients (Groleau-Renaud et al., 2000; Valentinuzzi et al., 2015). Changes in C and nutrient concentrations in the rhizosphere are sensed by roots and alter plant metabolism, structure, and exudation (Canarini et al., 2019; Eisenhauer et al., 2017). Therefore, microorganisms are an important sink for plant photosynthates (Canarini et al., 2019; Farrar and Jones, 2000; Savage et al., 2016) and their activity regulates root exudation (Phillips et al., 2004; Pii et al., 2015; Vranova et al., 2013).

Like any other resource allocation process, root exudation is often interpreted as a **targeted investment of the plant** to optimize its growth and reproduction in search of limited resources. In the case of exudation, nutrients are the target resource because (larger) plants with increasing N-requirements increase C exudation (Henneron et al., 2020b; Wang et al., 2021; Yin et al., 2018; Zhou et al., 2020). This assumption about directed investment implies that plant growth is limited by fixed C and that the plant is forced to consciously allocate this scarce resource according to current demands. However, the evidences show that plant growth is commonly limited by availability of nutrients, usually N and/or P (Augusto et al., 2017; Harpole et al., 2011; Hartman and Richardson, 2013; Hermans et al., 2006; Jiang et al., 2020; Körner, 2015; Millard et al., 2007). The presence of excess C in plants is supported by many results from fertilization experiments. Plants, which typically face nutrient limitation, must regulate biomass synthesis (which requires C, N, P, S and other micronutrients) more precisely

than photosynthesis, which produces "only" basal C-skeletons. This situation results in **an excess of C compounds** (Fig. 2). This is reflected in an increased concentration of non-structural carbohydrates (Assuero et al., 2004; Kavanová et al., 2006). To prevent/reduce photosynthesis suppression due to accumulation of carbohydrates in leaves (Chen et al., 2005; Jeannette et al., 2000), plants need to get rid of the excess C and exudation is one way to do this (Fig. 2) (Prescott et al., 2020). In summary, under natural conditions, plants often have an excess of C relative to other resources needed for growth, such as nutrients. This has profound implications for both leaf C metabolism and C allocation within the plant, as well as for plant nutrient metabolism, tissue and exudate elemental stoichiometry. The C-surplus viewpoint describes exudation as a consequence of plant metabolism rather that a targeted investment. This facilitates the understanding of nutrient exudation and its prediction in relation to plant traits.

Exudates consist not only of C compounds, but also of nutrients in organic (mostly) and mineral forms (Drake et al., 2013a; Edwards et al., 2018). Therefore, the **conditions of excess (surplus) can be extended to all components of exudates.** The release of nutrients and the C:N:P stoichiometry of exudates should be determined by the most limiting nutrient and the extent of excess of other elements for plant growth under certain conditions. The most limiting nutrient will remain in the plant, while the one currently in "relative" excess will be more exuded, changing the N:P ratio of exudates. Since exudation is associated with plant metabolism, the C:N:P stoichiometry of plants should be reflected in stoichiometry of exudates and thus can be used to study its association with microbial activity. It is of great importance to soil ecology because the nutrients released with exudation alter microbial activity in the rhizosphere. The N in exudates is likely necessary for microorganisms living under C-excessive conditions in the rhizosphere for their extracellular enzyme synthesis and related SOM decomposition (Drake et al., 2013b). Phosphorus is also present in exudates, but its role in rhizosphere ecology is unknown. The relationships between plants, exudates, and soil C:N:P stoichiometry are poorly understood, and important information about the effects of exudation on SOM decomposition dynamics is lacking.

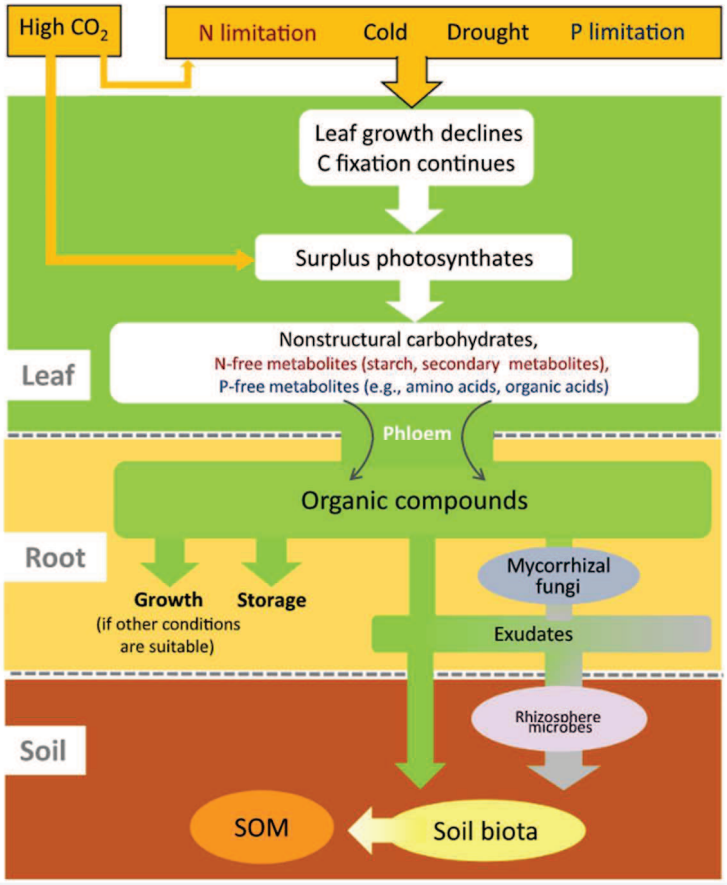

Figure 2. Surplus C Hypothesis. External factors such as insufficient nutrient availability (including low water availability, low temperature) limit growth while C fixation continues (albeit at a reduced rate). Similarly, elevated CO_2 concentration leads to rapid C fixation, while growth is only possible when other resources (nutrients) are available. In such situation, there is a surplus of photosynthates, the accumulation of which in leaves would downregulate photosynthesis. Therefore, they are either converted to non-structural C-rich metabolites that can be accumulated in the biomass (storage and other secondary compounds) or transported through the phloem and further metabolized in the roots. As a result, root growth and C storage usually increase. The remaining C, the excess C, is either exuded directly from the roots (green) or after being metabolized by root symbionts (mycorrhiza) (grey). In the soil, root exudates are rapidly utilized and transformed by rhizosphere microorganisms and organisms of higher trophic levels. The residues and metabolites of soil microorganisms are important precursors of stable SOM (Prescott et al., 2020).

1.3 PRIMING EFFECT – AN IMPACT OF FRESH SUBSTRATE ON SOM DECOMPOSITION

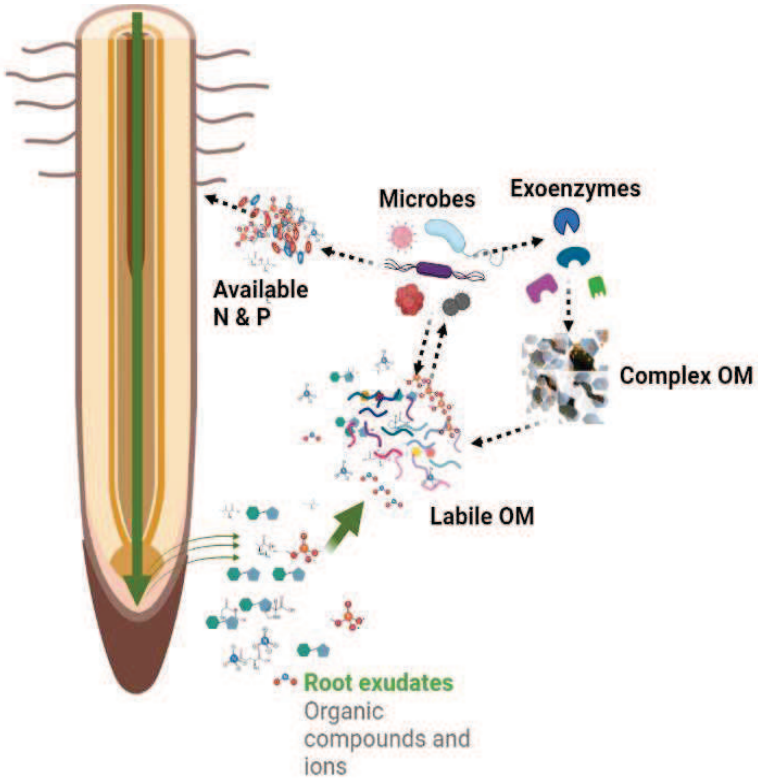

Figure 3. **Microbial nutrient mining hypothesis (rhizosphere priming effect) and the resulting release of nutrients available to plants.** Root exudation, concentrated around the root tip, increases the labile pool of OM in the soil. Microorganisms use these labile forms to trigger the metabolism and initiate growth. Since exudates do not usually meet microbial nutrient requirements (they are rich in C but poor in nutrients), microbes become nutrient limited whenever they (and plants) consume the nutrients available in the soil solution. Therefore, the microorganisms increase the production of exoenzymes that accelerate the decomposition of SOM and the release of organic nutrients. As a result, the microbes can continue to grow and synthesize enzymes (Valadares et al., 2018). Since microbial turnover in the rhizosphere is very rapid (in days), C losses through respiration are very high. Consequently, the activated microorganisms that remain from the ever-growing root tip enter a C shortage. They switch to endogenous reserves and begin to catabolize intracellular compounds and substrates released from dead cells, resulting in a release of mineral forms of N and P. In addition, strong predation pressure in the rhizosphere accelerates the release of mineral nutrients available to plants (Kuzyakov and Xu, 2013).

Root exudates and root detritus contain some labile and more energy rich compounds than older transformed and stabilized SOM. Therefore, their input and utilization stimulates microbial growth and activity (Hinsinger et al., 2009; Jones et al., 2009; Kaštovská et al., 2015; Kuzyakov and Xu, 2013). The activated microbial community soon runs out of nutrients available in the soil solution and the additional nutrient demand often cannot be met by the supply of exudates or root detritus (Kuzyakov and Xu, 2013; Mooshammer et al., 2014). This is because the microbial demand for

nutrients for biomass synthesis is usually higher than what plant inputs provide. That is, the relative nutrient content in plant OM inputs (mean C:N:P of root litter = 4184:43:1) is lower than in microbial biomass (microbial C:N:P = 42:6:1) (Xu et al., 2015). To compensate for this nutrient imbalance, microorganisms increase exoenzyme production above constitutive levels to mine for nutrients in older SOM (Phillips et al., 2011). The stabilized SOM in the rhizosphere is largely of microbial origin and therefore more nutrient rich (mean SOM C:N:P = 168:30:1) compared to the original plant inputs (Cleveland and Liptzin, 2007). Faster decomposition of SOM is associated with release of nutrients in simple organic forms that can be utilized by microorganisms (Henneron et al., 2020b; Koranda et al., 2011; Sun et al., 2017; Valadares et al., 2018; Yin et al., 2018) and later released in plant available forms (Fig. 3).

In general, any change in the mineralization rate of native SOM caused by an input of fresh OM is referred to as a **priming effect (PE)** (Bingeman et al., 1953). The input of C- and energy-rich root exudates usually has a stimulatory effect: it increases the SOM mineralization rate in the process called **positive rhizosphere PE** (RPE). Most studies show a positive RPE of up to 380% (Cheng et al., 2014) in agreement with the model described above and shown in Fig. 3. However, **negative RPE** has also been reported**,** where the SOM decomposition rate (compared to a situation without OM input) was reduced by up to 50% (Zhu et al., 2014). There are several explanations for negative RPE and most of them consider the important regulatory role of nutrient availability for microbes (Fig. 4). First, negative RPE can occur in soils with high nutrient availability. Microorganisms can directly use the nutrients available in the soil solution instead of investing more resources in decomposing recalcitrant SOM (Blagodatskaya et al., 2007; Guenet et al., 2010). In addition, the composition of the microbial community generally shifts towards a faster-growing, more nutrient-rich bacterial-dominated community with relatively lower C requirements and lower production of enzymes that decompose recalcitrant SOM. In a rhizosphere sufficiently supplied with exudates, which are the main source of C and energy for the microorganisms, this leads to a decrease in the decomposition of SOM. This situation is referred to as the preferential substrate utilization hypothesis (Cheng, 1999) (Fig. 4). Second, a negative PE can be observed after the input of fresh, nutrient-rich OM into the soil. This may be plant litter with a low C:N ratio: fresh mulch, fresh crop residues, grain, or manure. The nutrient-rich input is preferentially used by microorganisms, as in the previous example, which temporarily reduce the decomposition of natural SOM (Mastný et al., 2021).

Another scenario that can cause a negative RPE is a situation, in which severe N limitation prevents microorganisms from synthesizing extracellular enzymes-proteins with a very low C:N ratio below 3 (Allison and Vitousek, 2005). P limitation

can also limit enzyme production and thus SOM decomposition (Hill et al., 2014; Kaštovská et al., 2018; Li et al., 2021; Mastný et al., 2018; Sottocornola et al., 2007). Such a situation can occur in the rhizosphere when plant uptake depletes the pool of available nutrients to the point of limiting microbial activity (Dijkstra et al., 2013; Hill et al., 2014; Kaštovská et al., 2018; Li et al., 2021; Mastný et al., 2018; Sottocornola et al., 2007). Negative RPE can be very strong when nutrient availability is already low and limits both plant and microbial growth (competition hypothesis, Cheng, 1999) (Fig. 4). Therefore, under nutrient-limiting conditions, the C:N:P stoichiometry of exudates or other plant OM inputs should determine the extent of PE (Chen et al., 2014; Dijkstra et al., 2013; Fang et al., 2018; Liu et al., 2020).

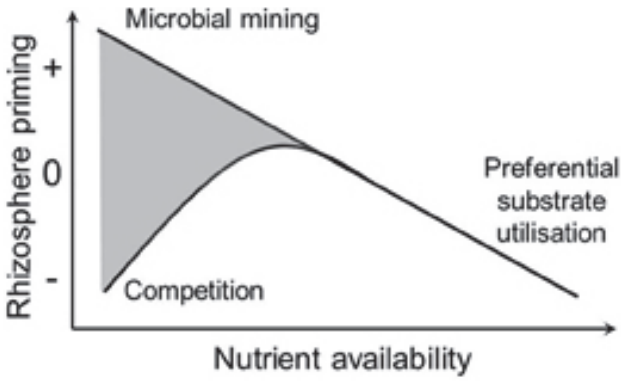

Figure 4. Hypothetical response of RPE to soil nutrient availability. Microbial mining: microbes use rhizodeposition to increase synthesis of extracellular enzymes and mine for nutrients in the SOM, resulting in positive RPE when nutrient availability is low. **Preferential substrate utilization**: microbes switch from decomposing SOM to using rhizodeposition when nutrient availability is high (negative RPE). **Competition**: microbes compete with plants for nutrients, resulting in a negative RPE because microbial growth and decomposition are nutrient limited. Both positive and negative RPE can likely occur when nutrient availability is low (grey area) (Dijkstra et al., 2013).

In summary, the effects of fresh substrate input to the soil on the SOM decomposition are controlled by the composition and C:N:P stoichiometry of the input, the size and the composition of microbial community, and the nutrient availability in the soil itself (the actual stoichiometric imbalance between microbial requirements and available resources). In this sense, the study of root exudates and their relationship to plant and soil conditions should lead to a better understanding of the role of plant and soil conditions in RPE.

Due to the significant influence of fresh substrate input on SOM transformation, PE may play an important role in global biogeochemical cycles (Finzi et al., 2015; Kuzyakov, 2010; Zhu et al., 2014). Recently, the RPE has been intensively studied. However, due to the complex control mechanisms, many aspects of the RPE require more attention (Cheng and Kuzyakov, 2015; Feng and Zhu, 2021; Zhu et al., 2014). In particular, the role of strong nutrient limitation that may occur in the rhizosphere of fast-growing plants due to their intense nutrient uptake needs to be clarified.

The PE, caused by root litter, is poorly studied compared to RPE or leaf litter PE. Given the significant OM input that root litter represents to ecosystems, it is an important gap in soil science knowledge (Sokol et al., 2019). The N content of litter is more critical to its decomposition rate than environmental factors (Parton et al., 2007). The decomposition rate of litter is proportional to its N content. That is, detritus with a lower C:N ratio (rich in N) decomposes faster. However, the effect of root stoichiometry on PE in detritusphere is unknown. Because plant and litter stoichiometry are related, a better understanding of the role of root litter C:N ratio in PE could link its influence on SOM dynamics to plant stoichiometry and other plant traits.

1.4 PFTS AS INDICATORS OF NUTRIENT TRANSFORMATION PROCESSES AND SOM DYNAMICS

Plants possess characteristics or trait values at the tissue-to-organism scale that reflect their evolutionary history and current environmental conditions and determine their performance (plant traits, PT) (Cavender-Bares et al., 2009; Westoby and Wright, 2006). The PTs, including functional traits (PFT), provide clues and insights into influence of plants on their surrounding abiotic and biotic environments (Reich, 2014). PFTs vary with plant-soil feedbacks (Bardgett, 2017; Reich, 2014). However, due to the many factors involved, the suitability of PFTs as indicators of soil conditions is still limited (Reich, 2014). PFTs are those that indirectly influence plant fitness through their effects on growth, reproduction, and survival (Violle et al., 2007). Specific leaf area (SLA), root-to-shoot ratio (RS), and tissue nutrient content are among the most commonly used and best studied PFTs. Recently, root exudation has been considered a PFT because of its recognized effect on soil nutrient availability (Guyonnet et al., 2018) (Fig. 3). However, some aspects of exudation, such as the role of nutrients released by plants into the rhizosphere, are unknown.

SLA, calculated as the ratio of total leaf area to total leaf dry mass (Blackman and Evans, 1973), is one of the best-studied PFTs. It is closely related to whole plant growth, lifespan and ability to capture light (Auger and Shipley, 2013; Cheng et al., 2016; Depauw et al., 2020; Poorter and Bongers, 2006). High SLA values are usually associated with high photosynthetic capacity, rapid metabolism, and short leaf lifespan (Blondeel et al., 2020; Poorter and Bongers, 2006; Reich, 2014). It is well described that SLA increases under light-limiting conditions (Feng and Van Kleunen, 2014; Reich et al., 2003; Rozendaal et al., 2006). However, SLA is also affected by soil properties and show a correlation with soil C:N ratio (Gong and Gao, 2019) and N mineralization (Ordoñez et al., 2009). High SLA is characteristic of ecosystems with fast SOM cycle associated with fast decomposition of leaf litter (Wright et al., 2004).

Because this PFT responds to both light and soil fertility conditions (Liu et al., 2017), it has great potential as an indicator of processes involved in plant-soil interactions.

Roots provide the direct contact of plants with the soil. Therefore, root traits (belowground traits) should be the most informative when considering plant-soil interactions (Bardgett, 2017; Lynch, 2007; Paustian et al., 2016). The root-to-shoot ratio, **RS**, is a powerful indicator of nutrient limitations for plant growth. It is well known that nutrient availability is an important stimulus for root growth. Plants suffering from nutrient limitation primarily increase their root growth to prioritize nutrient uptake over C fixation (White et al., 2016). However, knowledge of belowground PFTs lags behind that of aboveground PFTs (e.g. SLA) (Laliberté, 2017), largely due to the challenges of accurately measuring them. Therefore, characterization of plant responses to soil conditions incorporating both above- and below-ground compartments links are necessary to realize the potential of the functional approach in ecological studies.

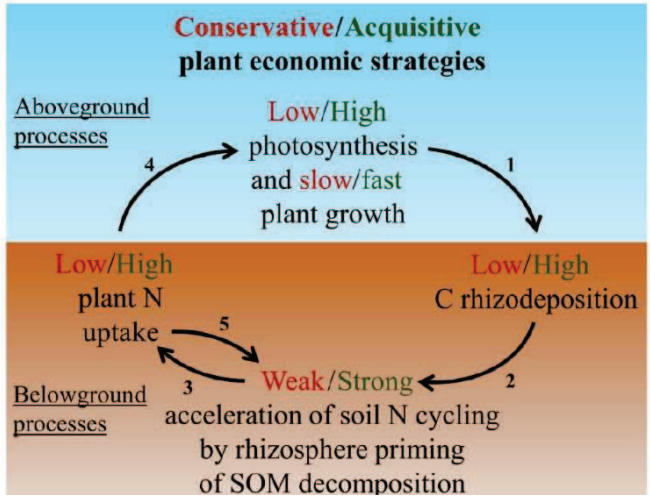

Figure 5. Conceptual model showing how the economic strategies of plant species control soil N cycling via processes in the rhizosphere, which in turn affect N acquisition and plant growth. The arrows represent the flow of causality. The rates of ecosystem processes are indicated in red (left) and green (right) for the resource-conservative and resource-acquisitive strategies, respectively. (1) Acquisitive species are associated with higher photosynthetic C fixation rates than conservative species and release more photosynthate-C into soil through rhizodeposition. Carbohydrates are among the major exudates that provide energy for soil microbes to produce exoenzymes that catalyze SOM decomposition. (2) Therefore, greater rhizodeposition of acquisitive species leads to greater acceleration of soil N cycling through stronger rhizosphere priming effect; this is related to faster gross N mineralization and turnover of mineral and microbial N pools. (3) Faster soil N cycling in the rhizosphere allows acquisitive species to acquire greater amounts of N through root uptake. (4) The absorbed N is then allocated above ground to promote photosynthetic C fixation, sustaining faster growth of acquisitive species. (5) Faster N uptake by acquisitive species further stimulates RPE by imposing more N-limiting growth conditions on soil microbes, resulting in greater microbial mining of N from SOM (Henneron et al., 2020b), unless N limitation is too severe for exoenzyme production.

Among PFTs, tissue nutrient contents are the best studied in terms of their association with soil conditions. Element ratios in biomass of individual plant species reflect changes in environmental conditions according to **biological stoichiometric theory** (Sterner and Elser, 2002). Nutrient fertilization experiments show that plant C:N:P stoichiometry can vary greatly depending on the availability of nutrients in the soil (Ågren et al., 2012; Fornara et al., 2013; Zheng et al., 2012). Variation in soil stoichiometric ratios explains changes in plant biomass and stoichiometry (Güsewell and Koerselman, 2002; Harpole et al., 2011; Olde Venterink, 2011) better than absolute changes in total soil nutrient content (Güsewell, 2005, 2004). Similarly, changes in plant stoichiometry may also reflect nutrient limitation caused by competition with microbes (Eschen et al., 2007; Johnson and Edwards, 1979; Magill and Aber, 2000).

The link among PFTs in nature is summarized in the **plant economics spectrum** (PES). In PES, resource acquisition and processing strategies range from fast-growing **acquisitive** to slow-growing **conservative** species (Reich, 2014). The acquisitive versus conservative strategies have been associated with fast versus slow growth, high versus low tissue nutrient content, high versus low SLA (representing photosynthetic capacity) and low versus high RS (Freschet et al., 2010; Kramer-Walter et al., 2016; Reich, 2014). In addition, fast-growing (acquisitive) plants accelerate biogeochemical C and N cycling more than slow-growing (conservative) plants due to greater exudate flux (Fig. 5). They cause faster gross N mineralization and turnover of mineral and microbial N pools (Cheng et al., 2003; Henneron et al., 2020a, 2020b) via larger RPE (Fig. 5) (Henneron et al., 2020a; Huo et al., 2017). More specifically, it can be concluded that plant photosynthetic activity controls native SOC mineralization through exudation flux and is a major driver of interspecific variation in SOC mineralization. Determining the effects of soil conditions on tissue nutrient ratios and PFTs may lead to the identification of relevant indicators of soil processes. In this way, we expect to help unravel the connections between the above- and belowground compartments, which is key to understanding plant-soil interactions (Bardgett, 2017).

1.5 PLANT ISOTOPIC COMPOSITION AS AN INDICATOR OF SOIL CONDITIONS

In addition to PFTs, other plant traits have the potential to serve as links between above- and belowground processes and the functional and stoichiometric approaches. Plant C and N isotopic signatures ($\boldsymbol{\delta^{13}C}$ and $\boldsymbol{\delta^{15}N}$) are increasingly used as indicators of plant metabolism and nutrient cycling in ecosystems because they account for source isotopic composition and fractionation during plant uptake, transport, and metabolism (Bowling et al., 2008; Cernusak et al., 2013; Dawson et al., 2002; Tcherkez et al.,

2011). Both $\delta^{13}C$ and $\delta^{15}N$ of leaves are affected by nutrient availability (Robinson et al., 2000; Taskos et al., 2020; Zhang et al., 2015; J. Zhang et al., 2017). Leaf $\delta^{13}C$ variations in C3 plants reflect changes in diffusion of CO_2 from the atmosphere to carboxylation sites and during carboxylation itself (Brugnoli and Farquhar, 2000). All of these processes, and thus the $\delta^{13}C$, are affected by water availability in the soil. However, nutrients in the soil also affect the isotopic composition of leaves. Foliar $\delta^{13}C$ increases with increasing N availability (Choi et al., 2005; Livingston et al., 1999; Ripullone et al., 2004). This is because available N stimulates carboxylation efficiency (Ripullone et al., 2004) and increases mesophyll (Choi et al., 2005; Livingston et al., 1999) and stomatal conductance (Flanagan and Farquhar, 2014; Hou et al., 2015; Tang et al., 2019), even when plants are not water-limited.

The $\delta^{15}N$ value is an indicator of the available N in the system. Soils with high N content tend to have more liquid and gaseous losses, i.e., an open N cycle (Hogberg, 1997; Lim et al., 2015). The N that leaves the soil via nitrate leaching and denitrification has higher abundance of the light isotope (^{14}N) (Denk et al., 2017) because ^{14}N reacts faster than the heavier ^{15}N (Möbius, 2013). As a result, the N remaining in the soil has a higher proportion of (is thus enriched in) ^{15}N, which is measured as higher $\delta^{15}N$. In low-N systems, almost all N is recycled and maintained by being exchanged closely between plants and soil microbes and stabilized in SOM while losses are reduced. The N cycle is more closed and there are few opportunities for N to leave the system, resulting in a lower $\delta^{15}N$ of low-N than high-N systems (Stevenson et al., 2010). In summary, $\delta^{15}N$ of system components (soil, plants, microbes) increases with increasing availability of N, N losses, and openness of the cycle.

Consistent with the relationship between N availably and $\delta^{15}N$ at the ecosystem level, many studies report a positive correlation between high $\delta^{15}N$ and N availability to plants (Craine et al., 2015, 2009; Zhang et al., 2017). However, this correlation is not always significant at the species level and changes with the form of N used to fertilize the soil (Xu et al., 2014), suggesting a high sensitivity of $\delta^{15}N$ to changes in soil N cycling and plant metabolism. Plant $\delta^{15}N$ is affected by the uptaken N form and availability in the soil, thus encompassing many of the factors that influence N transformation in the soil (Lim et al., 2015). Foliar $\delta^{15}N$ decreases with increasing soil organic N availability, which is related to increasing N limitation for plants (Gavazov et al., 2016). This is because plants take up N mainly in mineral forms (Biernath et al., 2008; Harrison et al., 2007; Rasmussen et al., 2010; Xu et al., 2008), preferably nitrates (Kuzyakov and Xu, 2013; Liu et al., 2017; Tinker and Nye, 2000). The use of nitrate by plants requires additional steps and investments compared to the use of ammonium. However, it is beneficial in preventing the toxic effect of ammonium, especially when

C-skeletons are not immediately available to fix ammonium for a longer storage. Also, nitrate has additional functions that can benefit the plant under stressful conditions.

Plant N metabolism involves many isotope fractionating steps (Kalcsits and Guy, 2013). One of the best-studied processes affecting plant $\delta^{15}N$ is fractionation during nitrate assimilation by nitrate reductase. As a result of this fractionation, organic products are depleted of ^{15}N and the remaining unconverted nitrate is proportionally enriched by 16‰ (Cui et al., 2019b; Evans, 2001; Karwat et al., 2019; Tcherkez and Hodges, 2008; Yoneyama et al., 2003). Nitrate reduction can occur in both roots and photosynthetic organs (mainly leaves), although it is mainly reduced in leaves in most plants studied (Black et al., 2002; Carelli and Fahl, 2006; Karwat et al., 2019; Scheurwater et al., 2002). According to the Rayleigh model, isotope fractionation occurs only when the reaction is partial. When the entire substrate is completely consumed, there is no isotopic fractionation and the product has the isotopic composition of the substrate (Rayleigh, 1896). This means that two pools with different isotope signals are formed in the cell during nitrate utilization: the ^{15}N depleted organic form and the ^{15}N enriched unconsumed nitrate, which can be stored in vacuoles. This nitrate is the most ^{15}N enriched (up to 55‰) N form in the plant (Cui et al., 2019a). When nitrate is deficient (under severe N limitation), almost the entire nitrate pool is consumed as plants must maximize the use of this limiting nutrient. However, nitrate functions not only as a nutrient but also as a signaling molecule. Cui et al. (2020, 2019a, 2019b) recently found that some of the remaining enriched nitrate in leaves is loaded into the phloem (Wilson et al., 2011) and transported to the roots, likely with a signaling function that regulates the expression of N assimilation genes (Fig. 6).

The model shown in Fig. 6 suggests that the ability of plants to store enriched ^{15}N nitrate can alter the isotopic composition of the whole leaf. This ability is influenced by N availability. Plants living under low-N conditions have limitations in the accumulation of nitrate in leaves because a greater proportion of it is either consumed (converted to organic compounds) or exported. Therefore, its influence on the overall signature of plant tissue decreases, making it more similar to the $\delta^{15}N$ of organic N (depleted in ^{15}N) and that of soil solution N (Canvin and Atkins, 1974; Reed et al., 1983; Tcherkez, 2011). If sufficient N can be taken up, exported and stored (e.g., in vacuoles), the $\delta^{15}N$ of leaf tissue will be higher than that of organic N. These considerations are consistent with general interpretation of **foliar $\delta^{15}N$ as an indicator** of plant N limitation. More specifically, on **N demand**. Leaf $\delta^{15}N$ is negatively correlated with plant biomass (Araus et al., 2013; Raimanová and Haberle, 2010; Robinson et al., 2000; Serret et al., 2018; Yousfi et al., 2012) and leaf C:N ratio (Cui et al., 2020). Larger biomass, N-poorer tissue, or the frequent combination of both conditions indicate increased N demand associated with more negative foliar $\delta^{15}N$. This

is the result of different N distribution between above- and below-ground parts and different concentration of mineral N in plants (Ariz et al., 2015; Tcherkez, 2011).

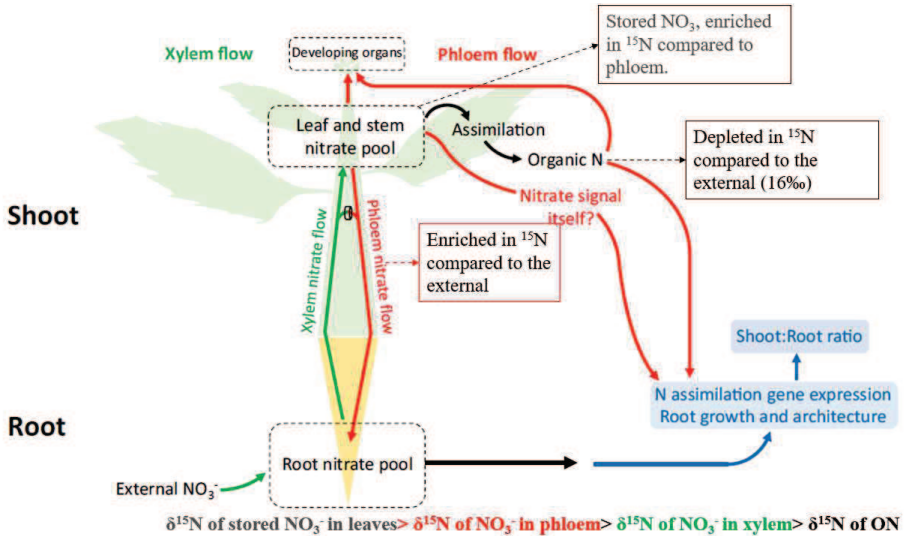

Figure 6. Conceptual model separating N pools in plants according to their $\delta^{15}N$. Nitrate taken up by roots is loaded into the xylem and transported through the plant. The nitrate in the xylem has the same $\delta^{15}N$ as in the soil solution because nitrate uptake does not fractionate N isotopes. In the leaves, the imported nitrate is stored, then converted to organic N (anabolic processes) and the remaining portion is either exported to the phloem or stored in the leaf tissues. Since nitrate reduction discriminates ^{15}N, the organic N products have the most negative $\delta^{15}N$ values, even lower than the nitrate in the xylem. Conversely, the nitrate that remains stored in the leaves and exported to the phloem is enriched in ^{15}N and has the highest $\delta^{15}N$ value of all plant N pools. The export of nitrates to the phloem may have a signaling effect for the root to respond to increased N demand in the leaves: increasing root growth (RS), altering gene expression and root architecture to increase N uptake and utilization. Adapted from (Cui et al., 2021).

The integration of soil conditions and plant metabolism derived from the foliar $\delta^{15}N$ makes it a potential integrator of complex ecological processes. However, the main drivers and particular processes that determine plant $\delta^{15}N$ in nature are still not clear. More knowledge is still needed for practical and precise use of the natural isotopic composition of plants in ecological studies. Nevertheless, the general findings can help to understand how plant-soil interactions are related to foliar $\delta^{15}N$.

2. AIM AND OBJECTIVES

This work addresses an ecologically relevant topic: the interactions among plants, soil microorganisms, and the soil environment under changing nutrient availability. Its main aim was **to understand and describe the mechanisms by which plants relate to soil processes through the flux and C:N:P composition of compounds released by living and dead roots**. The complex plant-microbe-soil interactions were described by **combining two synthesizing scientific approaches: ecological stoichiometry and functional analysis** (i.e., using PFTs). **Identification of reliable and practical PTFs indicators of exudation flux, soil nutrient availability, and SOM decomposition** was expected as a practical outcome of this work.

The specific objectives of this research were defined to:

1. Understand how changing nutrient availability alters plant-microbe-soil interactions over the range from resource-conservative to acquisitive species, and identify key plant and soil drivers of exudation flux and its C:N:P composition.
2. Compare effects of live and dead roots of conservative and acquisitive plants on microbial activities and consequences for decomposition and mineralization of stabilized SOM (priming effects).
3. Evaluate the potential of plant traits - growth rate, SLA, RS, tissue nutrient stoichiometry, and foliar $\delta^{15}N$, as indicators of plant adaptation to changing nutrient availability, exudation, and SOM dynamics.

3. RESULTS AND CONCLUSION

The unifying approach to all objectives was to attempt to link the above- and below-ground components of the system to understand the overall complexity and mechanisms affecting plant-microbe-soil interactions. The studies consisted of greenhouse pot experiments in which temperature, light conditions, and soil moisture were controlled while plant and/or soil properties were manipulated to assess their influence on the soil microbial activity and feedbacks to plants. Each plant-soil system was characterized by a set of plant, exudate, microbial, and soil variables. These extensive sets of measured parameters were analyzed using multivariate methods (network analysis, PCA) that revealed groups of close characteristics and relationships between them.

To address **objective 1**, a set of eight graminoid species typical of temperate grasslands were selected for the pot experiment. The selected species covered a spectrum of plant strategies, from conservative: *Festuca rubra*, *Poa pratensis,* and *Bromus erectus*, through intermediate: *Dactylis glomerata,* and *Lolium perenne,* to acquisitive: *Holcus lanatus, Phleum pretense,* and *Poa trivialis* (de Vries and Bardgett, 2016; Miles et al., 1988). The use of multiple species ensured a gradient in the performance of the plants themselves, as well as in the responses of the entire plant-soil system to changing nutrient conditions. Relative nutrient availability to plants was manipulated by supplementing the soil with C compound (agar as a compound similar to the agar-like mucilage compounds released by roots in rhizodeposition). The C addition enhanced the soil C:N ratio, promoted microbial growth, and reduced relative N availability in the originally nutrient-rich grassland soil. This approach differs from standard fertilizer experiments in which the addition of mineral N forms increases N availability in the system and thus weakens the competition between plant and microbes for N, provides electron donors/acceptors for some microbial processes, and may affect soil pH. To identify the key factors that determine plant-microbe-soil interactions, each system was characterized by more than 40 variables of plants, exudates, soil microbial biomass, soil processes, and soil pools. Network analysis was performed to sort them into functional groups and disentangle the relationships within and between groups of variables.

We demonstrated that plant-microbe-soil interactions were mainly determined by soil N availability, which was well represented by the concentration of water-soluble nitrates. Soil nutrient availability was strongly influenced by plant growth rate, which was well related to plant economic strategies. The faster-growing species formed more biomass and had a greater need for nutrients to build it up than slower-growing plants. Plant growth-induced uptake reduced the availability of mineral N and P in the soil in proportion to plant biomass. As a result, faster- growing plants took up more mineral

nutrients from the soil solution, which led to greater competition for N with microorganisms in the rhizosphere compared with slower-growing plants (**I, II, III**).

The scarcity of available N in the soil forced the large plants to use it more efficiently (**I**). They reduced the N concentration in their tissues and built up the biomass with higher C:N ratio. They further intensified soil exploration by increasing RS. Nitrogen required for root growth came either from reallocated leaf N reserves (which was associated with an increase in SLA) or from increased ammonium uptake from the soil. The exudation flux of fast-growing plants facing low N availability was greater compared to slow-growing plants and relatively richer in C and P compared to N. The altered C:N:P stoichiometry of exudates showed that i) the highly N-limited plants reduced N losses via exudation in agreement with the expectations of ecological stoichiometric theory and that ii) exudation served to release compounds with excess elements from the plants, supporting the plant surplus C hypothesis (**I**). Exudation flux and its C:N:P ratio mediated the coupling of plant and microbial nutrient requirements. Exudate flux correlated with microbial biomass, and the C:P ratio of exudates correlated with the C:P ratio of microbial biomass. These correlations suggest that root exudation is an important source of C, as well as P, for soil microbes. Microorganisms faced with increasing N limitation due to intensive N uptake by plants enhanced N immobilization over N mining from the SOM. This increased competition between plants and microbes for N, led to an increase in C- and P-rich exudation flux, and resulted in tightly closed N recycling between the plant and its associated rhizosphere community (**I**).

The results of the experiment were further exploited. The measured foliar $\delta^{13}C$ & $\delta^{15}N$ showed that plant N metabolism was influenced not only by N availability in the soil but also by the increased concentration of dissolved organic C (DOC) itself. The larger DOC pool came from microbial decomposition of soil added polysaccharide and provided an available source of C that was independent of plant rhizodeposition. Deeper analysis of plant-soil relationships in the C-amended versus non-amended systems served to understand the control mechanisms of foliar N isotopic composition (**II**). This information can contribute to the use of foliar $\delta^{15}N$ as a reliable indicator of plant metabolic limitations related to soil fertility. Individual species produced similar amounts of biomass under both soil conditions, but biomass formed under conditions of high additional DOC in C-amended soil always had a higher C:N ratio. Such lower-N plants were associated with rhizosphere microorganisms with higher biomass C:N ratios, higher specific respiration and increased enzymatic N-mining (**II**). This suggests that after C addition to the soil, plant production was maintained due to increased N use efficiency of both plants and associated soil microorganisms. Foliar $\delta^{13}C$ and $\delta^{15}N$ levels were determined by close relationships between plant growth rate and soil NO_3^- availability. Both $\delta^{13}C$ and $\delta^{15}N$ decreased with increasing plant biomass

(faster growth rate), indicating a significant decrease in NO_3^- availability (due to plant uptake from the soil). For each species, the C-amended soil contained a larger NO_3^- pool than the non-amended one, which may be attributed to the overall lower N uptake by plants and microorganisms, both of which have higher C:N ratios in biomass and thus lower N requirements. This situation was clearly reflected in the foliar $\delta^{15}N$, which was always higher in C-amended than in non-amended soil for the respective species (indicating intraspecific adaptation) and closely correlated with the growth-related variation in NO_3^- availability under both soil conditions (the interspecific variability). Foliar $\delta^{15}N$ was a more robust and sensitive indicator of N availability than tissue C:N, which did not reflect variation in NO_3^- availability under high DOC conditions (**II**). Our results confirm the relationship between $\delta^{15}N$ and plant N demand associated with insufficient N mineralization (supply) relative to plant need (demand) and its importance as an indicator of N limitation in ecosystem production.

In conclusion to objective 1, plant-microbe-soil interactions were mainly determined by soil N availability. Plants facing N limitation were urged to use nitrogen more efficiently when building biomass, reduced its losses via exudation and enhanced RS to obtain more N from the soil. Plant growth rate was the best predictor of exudation flux to soil. The changing C:N:P ratio in exudates reflected well the nutrient limitations of plant metabolism (here the intensity of N limitation) and supported the hypothesis that exudation serves to release excess metabolites. The C:N:P stoichiometry of exudates and soil N availability provided the closest links to microbial stoichiometry and enzymatic N and P mining.

To address **objective 2**, the performance of systems with slower-growing, more resource-conservative *Carex acuta* was compared to a faster-growing, acquisitive *Glyceria maxima.* The focus was on quantifying plant-derived inputs (rhizodeposition, root biomass), their microbial utilization and induced PE in the rhizosphere and detritusphere. Plants were grown for 10 weeks (the rhizosphere period) and continuously labelled with ^{13}C-depleted CO_2, which allowed to partition plant and soil C sources of soil C and CO_2 efflux and thus assessment of PE. Plants received repeated foliar fertilization with urea at concentrations of 0%, 0.3%, and 1% solution, which enabled to manipulate PFTs such as plant growth rate, tissue C:N ratio, and exudation flux without altering the original soils conditions. This allowed us to study the effects of the altered plant activities on soil processes. After the 10 weeks, the shoots were cut off and the roots remained in the soil for 2 years to decompose (the detritusphere period). During both periods, we measured and partitioned the soil CO_2 efflux, and performed two destructive samplings in the rhizosphere period and one at the end of the detritusphere period. There, total and microbial soil C and their isotopic compositions were analyzed to quantify the contribution of plant- and SOM-derived C.

These analyzes were complemented by assessment of plant biomass characteristics and nutrient availability (**III**).

Living roots largely controlled soil processes in the rhizosphere: root-derived C contributed up to 45 % of soil CO_2 efflux, and rhizodeposits were the preferred C source for microorganisms. Rhizodeposition input to soil increased with shoot growth and with decreasing root C:N, partly reflecting root turnover. Microbial utilization of rhizodeposits under plant-microbial competition for N stimulated SOC decomposition. The positive PE increased with plant growth and was enhanced among plants with rapid biomass growth, large input of root-derived C to the soil, high active microbial biomass, and soil phenoloxidase activity. Comparing the activity of the two species, the faster-growing *Glyceria* allocated more C into the soil, induced higher microbial activity and a greater proportion of active microorganisms, and taken up more mineral N than the slower-growing *Carex*. Its rhizosphere PE was 2.5 times stronger than that of *Carex*. Foliar N fertilization further altered the extent of positive PE as a function of the size of the activated microbial community. This suggests that plant-microbial competition for nitrogen and its impact on soil N availability play an important role in controlling SOC mineralization (**III**).

After the shoots were cut off, the root residues began to be decomposed by microorganisms and formed the detritusphere hot spot. The amount of rhizodeposition and active microbial biomass present in the soil at the time the shoot were cut affected root decomposition at the initial stage. Interestingly, decomposition of fresh roots resulted in negative priming, which changed to positive priming after depletion of labile compounds in a few months (1-9 months) and finally levelled off after two years. The negative PEs were due to the fact that plant N uptake was stopped by clipping the shoots and mineral N became more available to the microbes. Therefore, microorganisms shifted their feeding from hard-to-reach SOM to fresh root litter and balanced their N requirements by uptake of available soil N. The lower C:N ratio of *Glyceria* root residues and the larger active microbial community still persisting from the previous rhizosphere stage were associated with fast decomposition and rapid onset of negative PE, but also its their rapid shift to a longer period of small positive PE (within 1-3 months). Positive PE in the detritusphere of the N-poorer *Carex* roots was more intense but did not begin until after 9 months. Thus, the C:N ratio of the decaying roots was crucial for the transformation of more recalcitrant substances from dead roots and for the final contribution of root-derived C to soil C.

Conclusions regarding objective 2 follow. Detritusphere PEs are smaller than rhizosphere PEs but vary more with time as root decomposition progresses. In support of previously published results, the fast-growing, N-acquisitive species were associated with greater rhizodeposition inputs, stronger competition for N with soil microorganisms, and thus with greater SOC losses from the rhizosphere. However, the

faster decomposition of their more decomposable N-rich root litter resulted in a lower proportion of root C remaining in the soil, but was associated with lower SOC losses than the slow-growing N-conservative species.

To achieve **objective 3**, the results of the experiments were summarized to identify the PFTs as reliable predictors of soil nutrient conditions and SOC dynamics (Fig. 7). **Plant growth rate** (represented by plant biomass in our experiments) synthesized the adjustment of all PFTs. Plant growth rate was well suited to predict exudation flux, microbial biomass, and the DOC pool in the soil (**I, II, III**). It was also associated with the degree of nutrient depletion in the rhizosphere, indicating competitive pressure for N between plant and rhizosphere microorganisms (**I, II, III**) (Fig. 7).

The fast growth rate, e.g., resource-acquisitive strategy of species, is associated with high **SLA** and low **RS.** In particular, SLA is often used as a (database) constant for each species. Our results suggest that these two PFTs can be adjusted to support rapid growth under N limitation (**I**). After depletion of nitrate from soil solution by rapid growth, plants increase biomass allocation to roots (higher RS) (White et al., 2016) more than they change SLA (Blondeel et al., 2020). This confirms the importance of root growth in coping with nutrient limitation and the SLA especially under light limitation. However, in response to an external C input (higher soil C:N ratio), fast-growing plants reduced SLA, increased root growth, and decreased rhizosphere ammonium concentration (**I**). This suggests that the magnitude of the response of plants to soil conditions is related to their growth rate. When the plants demand exceeds the supply of nutrients in the soil through mineralization (as shown here by the depletion of mineral nutrients from the soil solution), they are forced to optimize the use of nutrients (higher C:N of tissues), reduce their losses (higher C:N of root exudates), increase the exploration of the soil to access the limited nutrients by promoting root growth (higher RS). Storage in leaf biomass may be reduced to compensate for root growth, but leaf area is maintained to capture light (lower SLA). In this sense, acquisitive species with higher growth rate and higher nutrient requirements showed greater plasticity in their PFTs than conservative species (**I**).

Ecostoichiometry helped to link the nutrient requirements and limitations of plants and microorganisms and predict the C:N:P composition of the exudates. **Tissue C:N ratio** increased with plant growth rate and greater biomass (Fig. 7), indicating increasing plant N limitation and the ability of plants to increase their N use efficiency under N limitation. The tissue C:N ratio served as a good predictor of the C:N ratio of both total SOM and the available pool of organic matter. Under high soil DOC conditions, it functioned better than plant biomass itself, reflecting well the increase in soil C:N ratio, while plant biomass production remained unchanged (**I, II**). There was a close correlation between C:N:P stoichiometry in tissues and exudates (**I**), indicating

that tissue C:N and C:P levels are indicative of exudate stoichiometry. Plant N concentrations, including tissues, root exudates, root biomass (and detritus), decreased with the enhancement of N-limiting soil conditions (**I, II**). This coordination reflected increased N-use efficiency associated with lower N losses to soil and poorer detritus under strong N-limiting conditions. Thus, our results support the notion that exudation is a way to release products in excess, which is determined by the most limiting nutrient (Prescott et al., 2020). The C:N:P stoichiometry of plant exudates were also coordinated at high DOC in the soil solution, which can be directly sensed by plant roots and cause a change in plant N metabolism (Araya et al., 2010; Ma et al., 2018, 2017). Tissue C:N ratio is already known to be a good predictor of the rate of decomposition and sequestration of plant C in soil (also reported in **II**). We extended this knowledge and showed that rapid decomposition of residues with low C:N ratio leads to lower PE on the old stabilized SOM (**II**). Therefore, the rapid SOM dynamics in the systems with dominant acquisitive plants can be mainly attributed to the young SOM.

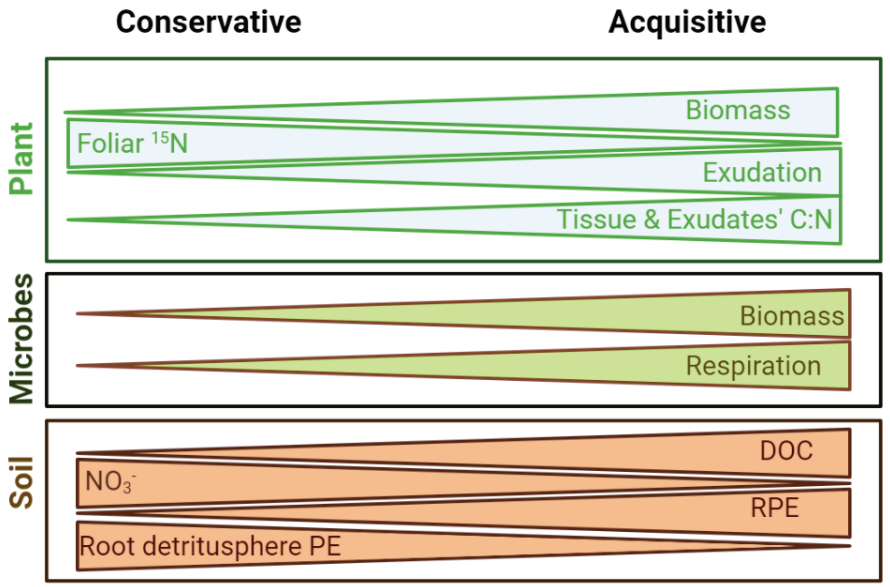

Figure 7. Summary figure of plant, microbial, and soil variables most affected by plant economic strategy. Under the same soil conditions, the acquisitive plants grow faster and exudate more than the conservative species. The acquisitive species have higher nitrogen use efficiency, reflected in higher C:N ratio of tissue and exudates, and also have lower leaf ^{15}N content than the acquisitive species. The greater exudation flux of acquisitive species is associated with higher microbial biomass and activity, reflected in higher respiration. The faster growth and exudation of acquisitive species is related to the depletion of mineral nutrients in the soil, especially NO_3^- (also NH_4^+ and PO_4^{+3}), increased DOC and RPE. The PE of root detritus is higher in conservative species, which is related to the higher availability of N.

We have extended the understanding of a recently used trait – the **foliar δ ^{15}N**. In support of published results, we have shown that isotopic composition is related to plant nutrient requirements (Ariz et al., 2015) and thus to plant C:N stoichiometry (Cui et al., 2020). Foliar δ ^{15}N proven to be more sensitive than tissue C:N ratio as it correlated with nitrate content in soil and exudates (Fig. 7), and changed with soil C treatment, whereas tissue N content and C:N ratio did not.

In summary, our main approach was to link the above- and below-ground components of the system to look for mechanisms that alter plant-microbe-soil interactions and to understand the overall complexity of the system. We have successfully combined the PFT approach commonly used in plant ecology to generalize the role of plants in the system with ecological stoichiometry, which is used to understand the relationships in the overall system in terms of the elemental balances among its components. We focused specifically on factors affecting the exudation flux and its C:N:P composition to complement the very sparse data in this area. At the same time, we have increased our knowledge of the mechanisms that alter foliar□ □^{15}N. With a sufficient understanding of the mechanisms controlling leaf N isotope composition, this trait can serve as a detailed indicator of plant growth limitation in the community.

Our results point to the importance of plant growth for belowground processes. Plant growth, reflecting the adaptations of PFTs, proved to be the most important factor in controlling exudation flux, microbial activity in the rhizosphere, and SOM decomposition. This general conclusion supports the frequently noted coordination between plant nutrient requirements, soil stoichiometry, and microbial SOM mineralization (Henneron et al., 2020b, 2020a). In addition, we have shown that complementarity between C:N:P stoichiometry of plant tissues, exudates, and root litter is important for coordination of plant growth and SOM mineralization. Plant nutrient uptake is also linked to SOM mineralization after plant death, as the stoichiometry of root detritus influences its decomposition rate and also affects the dynamics of stabilized SOM. We have also shown that external C input to the soil uncouples the plant from microbial demand, leading to changes in microbial growth, stoichiometry, respiration, and enzymatic decomposition processes. These changes in soil stoichiometry and microbial activity are reflected in only some of the PFTs, but are sensitively indicated by foliar N isotope composition.

4. RESEARCH PERSPECTIVES

Linking above-to-belowground compartments of the system allows us uncover the mechanisms driving plant-microbe-soil interactions and to understand changes in plant productivity and in ecosystem functioning. This is necessary to make reliable predictions about ecosystem responses to environmental changes. We have shown in pot experiments that PFTs, namely growth rate (biomass), tissue C:N, and foliar $\delta^{15}N$ are accurate indicators of plant N limitation, but also of exudation flux, microbial biomass, their C:N ratio, rhizosphere and detritusphere PEs (thus SOM dynamics) and final sequestration of plant-derived C.

The next steps should be to study the response of microbial communities including structural and functional changes to determine, if there are key functional groups associated with different economic spectra of plants, even in the same soil type, or with changes in soil C:N ratios, or with key soil processes described in this work (N mineralization, nitrification, enzymatic N and P mining, PEs in the rhizosphere and detritusphere).

In addition, it is necessary to validate our results by evaluating the correlation between growth rate, tissue C:N ratio, foliar $\delta^{15}N$ and N availability under field conditions – within an ecosystem, and between low- and high-N grassland ecosystems with more closed and open N cycles, respectively.

Finally, evaluation of these results under prevailing P limitation should lead to clarification of the role of N and P on plant-microbe-soil relationships and of selected PFTs as general indicators of plant nutrient requirements, soil nutrient transformation processes, and SOM dynamics.

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PAPER I

The exudation of surplus products links plant functional traits and plant-microbial stoichiometry

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The Exudation of Surplus Products Links Plant Functional Traits and Plant-Microbial Stoichiometry

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Abstract: The rhizosphere is a hot spot of soil microbial activity and is largely fed by root exudation. The carbon (C) exudation flux, coupled with plant growth, is considered a strategy of plants to facilitate nutrient uptake. C exudation is accompanied by a release of nutrients. Nitrogen (N) and phosphorus (P) co-limit the productivity of the plant-microbial system. Therefore, the C:N:P stoichiometry of exudates should be linked to plant nutrient economies, plant functional traits (PFT) and soil nutrient availability. We aimed to identify the strongest links in C:N:P stoichiometry among all rhizosphere components. A total of eight grass species (from conservative to exploitative) were grown in pots under two different soil C:nutrient conditions for a month. As a result, a wide gradient of plant–microbial–soil interactions were created. A total of 43 variables of plants, exudates, microbial and soil C:N:P stoichiometry, and PFTs were evaluated. The variables were merged into four groups in a network analysis, allowing us to identify the strongest connections among the variables and the biological meaning of these groups. The plant–soil interactions were shaped by soil N availability. Faster-growing plants were associated with lower amounts of mineral N (and P) in the soil solution, inducing a stronger competition for N with microorganisms in the rhizosphere compared to slower-growing plants. The plants responded by enhancing their N use efficiency and root:shoot ratio, and they reduced N losses via exudation. Root growth was supported either by reallocated foliar reserves or by enhanced ammonium uptake, which connected the specific leaf area (SLA) to the mineral N availability in the soil. Rapid plant growth enhanced the exudation flux. The exudates were rich in C and P relative to N compounds and served to release surplus metabolic products. The exudate C:N:P stoichiometry and soil N availability combined to shape the microbial stoichiometry, and N and P mining. In conclusion, the exudate flux and its C:N:P stoichiometry reflected the plant growth rate and nutrient constraints with a high degree of reliability. Furthermore, it mediated the plant–microbial interactions in the rhizosphere.

Keywords: rhizosphere; C:N:P ratios; plant growth; mineral N; soil enzymatic activity; microbial growth

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1. Introduction

The rhizosphere, i.e., the soil directly impacted by the activity of roots [1,2], is one of the most important zones for biogeochemical cycles in terrestrial ecosystems. It closely connects the following two main actors: plants and soil microorganisms. Plant-microbial interactions in the rhizosphere involve multiple components and factors [3]. The root exudation of organic compounds alleviates the C limitation that microbes commonly encounter in bulk soils, and stimulates rhizosphere microbial activity [4–6]. Enhanced microbial activity feeds back to plants through changes in nutrient availability [7] and plant development [8,9]. This complexity represents a major challenge for the understanding of ecological processes.

The application of stoichiometric and functional ecology to soil research has enabled a synthesis of the complexity of soil and plants components, both of which are needed to understand rhizosphere processes. The availability of nutrients determines plant growth as well as the stoichiometric restrictions controlling microbial growth, activity, and organic matter decomposition. It also shapes plant–microbe interactions [10–16]. The rhizosphere is generally a C-surplus and nutrient-limited environment due to the exudation flux of organic compounds and the increased needs of plants and microbes for nutrients. Exudation stimulates microbial growth and the synthesis of exoenzymes that mine nutrients from the soil organic matter (SOM) to fulfil their requirements [17]. Root exudation increases with plant growth rate and nutrient uptake. Consequently, it has been recognised as an important plant strategy for controlling soil nutrient cycling [16,18]. Faster-growing plants are associated with greater microbial activity, faster SOM dynamics, lower N in the microbial biomass, and a different functional community composition than slower-growing plants [15,16,19–23]. Those results are a part of the growing knowledge that link plant functional traits (PFTs) with soil nutrient availability, soil microbial activity-stoichiometry, and SOM decomposition. The current data on PFTs are, however, largely independent of ecological stoichiometry, which limits the capacity of PFT to be a useful indicator [24].

Living roots release not only C compounds but also nutrients in organic and mineral forms, even under nutrient limiting conditions for plants [25]. Such nutrient limiting conditions restrict plant metabolism and generate an excess of some compounds/elements that the plant needs to release. The root exudation flux, thus, partly consists of compounds in excess of the actual plant metabolic requirements [26,27], which should be associated with the variability in both PFT and tissue C:N:P stoichiometry. However, besides the well-established dependence of C exudation fluxes on plant biomass and their stimulation of microbial activity [15,28,29], associations of PFTs with nutrients exuded by roots and their roles in rhizosphere ecology are unknown. The fluxes and stoichiometry of exudates have been suggested as drivers of microbial activity in the rhizosphere [20,30,31], but this is difficult to measure [32]. Identifying PFTs that can predict relevant information about C and nutrient exudation would enable their integration into C cycling models and budgets [33].

In this study, we explored the connections among rhizosphere components, stoichiometry, and PFTs, focusing on the role of exudation stoichiometry in plant–soil interactions. We hypothesised that (H1) faster-growing plants take up more nutrients, which results in greater depletion from the soil solution compared to slower-growing plants. (H2) Plants adapt to decreasing soil nutrient availability by altering the PFT and stoichiometry of tissues and exudates to optimise the uptake and use of nutrients, and (H3) changes in the soil solution and exudation stoichiometry related to plant growth lead to adjustments of the biomass stoichiometry and enzymatic activity of rhizosphere microorganisms. We set up a greenhouse experiment in which we planted eight grass species ranging in nutritional strategy, from conservative to exploitative, in pots with soils adjusted to two different C:nutrient ratios. We changed soil stoichiometry by adding complex C, which intensifies microbial activity and nutrient demands. This is in contrast to most other studies, which have changed soil stoichiometry by adding nutrients (fertiliser) and, thus, alleviating the nutrient limitations of plant production. Diverse plant strategies ensured a gradient in the PFTs and the stoichiometric ratios linked to different soil C:nutrient ratios. We measured and calculated 43 variables defining PFTs, microbial activity, and plant-exudate-microbe-enzyme-soil C:N:P stoichiometry. We performed a network analysis to sort the tightly connected variables into groups with biological (functional) meaning, as demonstrated in previous applications of network analysis [34].

2. Materials and Methods

We used a set of 8 grass species common in European grasslands. The selected species varied along a gradient of increasing specific leaf area (SLA) [35] and ecological strategies [22]; conservative: *Festuca rubra*, *Poa pratensis,* and *Bromus erectus*; intermediate:

Dactylis glomerata and *Lolium perenne*; and exploitative: *Holcus lanatus*, *Phleum pratense*, and *Poa trivialis*.

The soil was classified as cambisol (IUSS Working Group WBR 2015, FAO), collected in an annually mown grassland site (Ceske Budejovice, Czech Republic, 48.9° N, 14.3° E, 450 m a.s.l.). The main soil properties were soil organic C of 12.8 ± 0.3 g kg^{-1}, total N of 1.3 ± 0.03 g kg^{-1}, total P of 0.21 ± 0.04 g kg^{-1}, a C:N ratio of 11.76 ± 0.19 (n = 5) and pH of 5.74 (soil:water mixture, 1:5, w/v); texture loam (55% of sand, 15% of clay). After sieving (2 mm) and removing roots, the soil was split into two parts; one was kept without any treatment, and the other was amended with an agar powder (Fluka agar for microbiology, Millipore Sigma, containing 47% of C) in the final amount of 6.8 g kg^{-1} (3.2 g C kg^{-1}) [36,37]. The addition enhanced the total C of the soil to 16.0 ± 0.1 g kg^{-1} and soil C:N to 14.2 ± 0.6 (n = 5). The polysaccharide addition aimed to ensure a long-term supply of C to the soil microbial community, promoting its growth [36,37] and related N requirements, thus strengthening the plant–microbial competition for the available N. Agar was chosen to represent mucilage polysaccharides rich in galactose, which are secreted from plant roots [38–41], suggesting that agar-like polymers are present in the rhizosphere. In support, agar-degrading bacteria are commonly found in roots and the rhizosphere [42–45]. The soil, either non-amended or amended, was put in pots (8 cm × 8 cm × 13 cm; 800 g) and planted with seeds of one grass species (15 seeds per pot, 4 replications per soil treatment, 5 unplanted pots per non-amended or amended soil as controls). Plants grew from seed in a greenhouse (exposed to natural light, temperature conditions of 25 °C for 18 h of daytime; 16 °C for 6 h of night-time). Soil water content was adjusted to field capacity at the beginning of the experiment. Pots were irrigated with tap water twice per week to keep the soil moisture at the field water capacity, which was checked by weighing the pots. The experiment lasted one month, from May until June. Then, the plant and soil characteristics were evaluated for each pot (soil parameters only in unplanted pots) as follows.

2.1. Exudates

Intact plants were used for measuring exudation flux. The plants were removed from the pot together with the block of soil. The soil was carefully separated from roots by hand, starting from the outside of the block towards the core with the highest root density. About 30 g of the (core) soil that remained attached to the roots (defined here as the rhizosphere soil) was carefully cleaned from the roots by hand and tweezers in an effort to minimise root disturbance. This rhizosphere soil was used for posterior analyses. The complete removal of soil particles and of metabolites lost from potentially damaged roots was completed by gentle washing and pre-soaking of the roots in 500 mL of distilled water [32]. We tried to reduce root damage effects during the whole procedure, but we are aware that it cannot be totally eliminated. Despite the limitations, the described method of exudate sampling is considered to give results closest to real conditions and is widely used (e.g., [25,46]). All plants from the pot were placed in a beaker with roots submerged in 500 mL of redistilled water and left in daylight in the conditioned greenhouse at 25 °C. After 4 h, the plants were removed and the root exudate solution was vacuum-filtered through a 0.2-micrometre express plus PES (polyethersulfone) membrane filter (GPWP14250, Merck Milipore Ltd., Carrigtwohill, Ireland) to remove microbial cells and root debris if present, and immediately analysed. The soluble exuded C and N (C.exu, N.exu) were determined with a TOC-L analyser equipped with the total N measuring unit TNM-L (Shimadzu, Tokyo, Japan). The concentrations of N-NH_4^+ (NH4.exu), N-NO_3^- (NO3.exu) and soluble reactive P in exudates (P.exu) were measured with a Flow Injection Analyzer (FIA Lachat QC8500, Lachat Instruments, Milwaukee, WI, USA). The principal colorimetric methods used in FIA were the ascorbic acid reduction of phosphomolybdic acid for analysis of soluble reactive phosphates, a phenol-hypochlorite assay with the sodium nitroprussite as the catalyst (Berthelot reaction) for ammonium-N measurement, and the diazotisation of sulfanilic acid and subsequent coupling with N-(1-naphthyl)-ethylenediamine in strongly

acid solution for nitrate-N analysis. All exudation characteristics are expressed per pot (total plant biomass) and hour.

2.2. Plant Properties and Functional Traits (PFTs)

After exudation measurements, 10 fresh leaves per pot were immersed in tap water overnight, then wiped, scanned using WinRhizo (Regent Instruments, Québec, QC, Canada), then dried at 70 °C for 48 h and weighed. The remaining plant biomass was separated into shoot and root biomass, also dried at 70 °C for 48 h and weighed. Dry tissues were milled and analysed for C and N contents (C.plant, N.plant) after combustion of approximately 3–4 mg of a sample weighted in the tin capsule at 980 °C in the elemental analyser (vario MICRO cube, Elementar, Germany) and for P content (P.plant) after acid digestion [47] using FIA.

The following set of PFTs were evaluated: the plant biomass (Plant.BMS) calculated as the sum of dry shoot and root biomass per pot; and biomass elemental compositions (C.plant, N.plant, P.plant) and appropriate molar stoichiometric ratios. These are all aspects of growth, which are related to the ecological strategy of a species and the soil nutrient availability in a particular pot system. The specific leaf area (SLA), calculated by dividing the fresh leaf area by the dry mass of scanned leaves (10 leaves), is related to the ability of the canopy to capture light per the mass invested in leaf growth, linked to leaf photosynthetic capacity. The root:shoot ratio (RS), the ratio of root over shoot dry biomass of each pot, is an indicator of resource allocation strategy to capture nutrient/water versus light in a given condition. The exudation flux (per pot; C.exu, N.exu, P.exu, NH4.exu, NO3.exu) shows the release of compounds from the roots, which should partially reflect the metabolic C surplus and imbalance in nutrient availability according to actual plant needs [27]. This is considered to be a component of a species ecological strategy [16]. The specific exudation (spc.C.exu, spc.N.exu, spc.P.exu), calculated by dividing the exudation flux of C, N, and P over the root biomass, then shows the "losses" of the compounds containing the respective elements per root mass.

2.3. Physico-Chemical Properties of Soil and Soil Solution

After harvesting, a part of the rhizosphere soil was air-dried, milled, and analysed for the total C and N contents, as was performed for plant tissues. The total soil P was analysed according to [47]. Part of the soil was frozen at −18 °C and the rest of the fresh soil was stored at 4 °C and analysed for soluble organic C and N (DOC and DON), ammonium-N (NH4.ss) and nitrate-N (NO3.ss) within a week. Fresh soils were extracted with 0.5 M of K_2SO_4 (1:4, *w*/*v*) and analysed as performed for exudates. Extractable P (PO4.ss) was determined in 0.5 M of $NaHCO_3$ (1:15, *w*/*v*, pH = 8.5) and measured with a spectrophotometer at a wavelength of 890 nm (Genesys 10 S, UV–Vis, Thermo Scientific, Waltham, MA, USA) after reacting with molybdenum blue. The pH was determined in a soil:water mixture (1:5, *w*/*v*) using a glass electrode.

2.4. Soil Microbial Biomass and Soil Activities

Microbial C (Cmic), N (Nmic), and P (Pmic) were determined using chloroform fumigation–extraction [48–50]. The extraction before and after the fumigation of the soil with amylene-stabilised chloroform for 24 h was performed with 0.5 M of K_2SO_4 (1:4, *w*/*v*) for C and N, and with 0.5 M of $NaHCO_3$ (1:15, *w*/*v*, pH = 8.5) for P. The Cmic, Nmic, and Pmic were calculated as differences in the concentrations of organic C, total N (measured on TOC-L analyser) and extractable P (measured after the molybdenum blue reaction on a spectrophotometer at a wavelength of 890 nm [50]) in soil extracts before and after fumigation. The values were corrected for extraction efficiencies using $k_EC = 0.41$ [51], $k_EN = 0.45$ [52], and $k_EP = 0.4$ [50]. Total microbial DNA was extracted from 0.25 g of defrosted soil using a PowerSoil Isolation Kit (MO BIO laboratories, Inc. Carlsbad, CA, USA) according to the suggested protocol, using PowerBeat tubes and tube mixer 693TC5. The extracted DNA was quantified using QuantiFluor® dsDNA Dye in a

Quantus Flourometr (Promega, Madison, WI, USA) following the manufacturer's protocol. Cmic, Nmic, and Pmic were considered to be measures of the total microbial biomass and its molar stoichiometric ratios, whereas microbial DNA served as an indicator of microbial growth and the biomass responsive (activated) to soil C inputs from plants and the added substrate.

Respiration of the rhizosphere soil was measured to characterise microbial activity as the cumulative CO_2 production after a 3-day incubation of 10 g of soil in airtight-sealed 100-millilitre flasks at 20 °C, determined using an Agilent 6850 GC system (Agilent Technologies, Santa Clara, CA, USA). Potential activities of five hydrolytic enzymes in the rhizosphere characterised the microbial potential to release C, N, and P from organic substrates. The activity of C-mining (β-glucosidase and cellobiosidase), N-mining (Ala-aminopeptidase and chitinase), and P-mining enzymes (phosphatase) [53] were determined using a microplate fluorometric assay [54]. Defrosted soil samples (1 g) were homogenised in distilled water (100 mL) and sonicated for 4 min; 200 µL of soil suspension was added to 50 µL of methylumbelliferyl solution for β-glucosidase, cellobiosidase, phosphatase, and chitinase (NAG) determination, or to 50 µL of 7-aminomethyl-4-coumarin substrate solution for measurement of Ala-aminopeptidase. From three pre-tested concentrations of each fluorogenic substrate (50, 100, and 300 µM), the one with the highest enzymatic activity was chosen [55,56]. Plates were incubated at 20 °C for 2 h. The fluorescence was measured with an INFINITE F200 microplate reader (TECAN, Crailsheim, Germany) at an excitation wavelength of 365 nm and an emission wavelength of 450 nm.

2.5. Statistical Analyses

A factorial combination of two soil C treatments with eight plant species classified into 3 different ecological strategies (3 conservatives, 2 intermediates, and 3 exploitatives) was performed in order to find a controlled gradient of soil conditions, visible in the distinctive C:N ratio of total soil, as well as a non-controlled gradient of PFTs. After comparing correlation matrices of variables using three different analyses, Pearson, Kendall, and Spearman, the Pearson method was chosen because it showed the best matrix determinant reflected in most links. The Pearson correlation analysis requires numerical variables, for which we had to replace the categorical "plant ecological strategy" with another numerical variable from the measured PFTs. A hierarchical clustering of PFTs (plant biomass, SLA, RS, specific exudation of C, N, and P) using the package *clustofvar* [57] identified plant biomass as the best representative of a plant ecological strategy. The algorithm uses the Spearman correlation when non-continuous variables are present. Figure S1 shows how we chose the PFT. Plant biomass (g per pot) expressed the species-specific growth rate (the increase in biomass of 15 seedlings per pot and month). All three conservative species had low biomass, whereas plants of the intermediate and exploitative categories had more variable biomass, including the largest biomass values (Figure S1). After selecting only countable variables, we applied the Box–Cox transformation to the 43 numerical variables to reduce the effect of skewed distributions [58], and performed a network analysis that included the relationships with correlation coefficients higher than 0.3 or lower than -0.3 ($|r| > 0.3$) and an adjusted p-value < 0.05 (Pearson, London, UK). The variables were then distributed into groups according to the maximal modularity using the *cluster_optimal* function of the library *qgraph*(v. 1.2.6) [34,59]. All the analyses including the additional correlations (Figures 1–4 and the supplementary matrices) were performed in *R* software.

3. Results

3.1. C, N, and P Content and Stoichiometry in All Pools

The C:N:P stoichiometry of plant–microbial–soil pools for all species and both soil C:N treatments (64 experimental units) are summarised in Table 1. The coefficients of variance (in %), which represent the flexibility of the respective pools, show that the characteristics of the total soil were the most stable, whereas those of exudates were the most variable. The N concentration and respective stoichiometric ratios were most variable in the plant biomass

and soil solution, whereas the P concentration and related elemental ratios were most variable in root exudates and microbial biomass. Microbial respiration was, on average, 20.5 μg C g^{-1} (±38%), and the total microbial DNA in soil was 7.5 μg g^{-1} (±18%). Table S1.

Table 1. C, N, P contents and their molar ratios in plant, microbial, and soil pools calculated over all planted treatments (mean and coefficient of variance in %, n = 64).

		Mean						Coefficient of Variance (%)					
POOL	**Unit**	**C**	**N**	**P**	**C:N**	**C:P**	**N:P**	**C**	**N**	**P**	**C:N**	**C:P**	**N:P**
Plant biomass	$mg \cdot g^{-1}$	414.6	19.6	4.0	24.7	266.5	10.8	2.1	31.9	18.3	30.9	21.9	29.2
Root exudates	$\mu g \cdot plant^{-1} \cdot h^{-1}$	793.6	40.2	29.6	22.6	88.0	4.2	61.1	56.3	74.7	31.4	63.7	57.6
Spec. Exudation	$\mu g \cdot g^{-1}$ root$\cdot h^{-1}$	1073.9	59.2	41.6				42.0	46.3	60.1			
Soil solution	$\mu g \cdot g^{-1}$	22.0	2.9	1.1	9.2	53.4	6.0	22.0	26.7	17.9	30.6	30.9	24.5
Soil microbes	$\mu g \cdot g^{-1}$	200.4	29.6	77.9	7.9	6.6	0.8	20.6	28.4	42.6	20.6	40.5	51.4
Enzym. activity	$nM \cdot g^{-1} \cdot h^{-1}$	48.5	16.1	29.5	3.0	1.7	0.6	18.2	15.5	16.7	16.7	12.9	11.8
Total soil	$mg \cdot g^{-1}$	14.2	1.3	0.2	12.9	173.4	13.6	9.4	7.4	19.0	6.5	12.2	13.5

3.2. Variable Clustering and Correlation

3.2.1. Central Group: Close Link to Plant Biomass

The 43 variables characterising interactions in the plant–soil system were sorted into four groups in the network analysis (Figure 1). Details of the correlations (r and p-value) between variables within each group and variables connecting the groups are given in correlation matrices in the supplementary information (Figures S2–S6). Group 1 was in the centre to the network and contained most of the strongest linked variables. It consists of plant biomass (representing plant growth rate) and variables expressing plant C excess and N economy (Figure 1). Faster-growing plants decreased the N content in tissues (N.plant) and enhanced the N use efficiency, which was evidenced by reduced N:P and enhanced C:N ratios of their tissues (Figures S2 and S6). They were also associated with a larger exudation flux of C, P, and organic N, and with higher microbial DNA contents in the rhizosphere soil. The higher C:N (CN.exu) and lower N:P ratio (NP.exu) of exudates correlated with plant C:N:P stoichiometry (Figures S1 and S4). It also reflected the higher N use efficiency and regulation of N losses of the faster-growing plants. This group also includes the variables characterising nutrient availability in the soil (NO3.ss, NH4.ss, PO4.ss). Faster plant growth caused a stronger depletion of mineral nutrients from the soil, which resulted in increased C:N and C:P ratios of the soil solution (CN.ss, CP.ss) (Figure S2).

3.2.2. Upper Group: DOC, Microbial Respiration, and Enzymatic Activity

The second, upper group of variables was connected to the central group. It was strongly influenced by the C:N ratio of total soil. The group consisted mainly of the organic compounds in the soil solution, microbial respiration, and exoenzymatic activity (Figure 1). The correlation matrix showed the following relations among the variables (Figure S3). The soils with higher C:N ratios contained more dissolved organic matter (DOC and DON) than soils of lower C:N ratios. The plants growing in soils with a high DOC concentration, originating from both added soil C and the large exudation flux, released root exudates poor in nitrates (NO3.exu). Under high DOC, the microbial communities had higher respiration (Resp.mic) and invested more into N-mining enzyme activity (AlaAPasa). The increase in N-mining was further accompanied by enhanced phosphatase activity, which resulted in a lower C:N and C:P but a higher N:P of enzymatic activities under a high soil C:N (Figure S3). All these relations indicate a deepening nutrient limitation in plant–soil systems under high DOC concentrations.

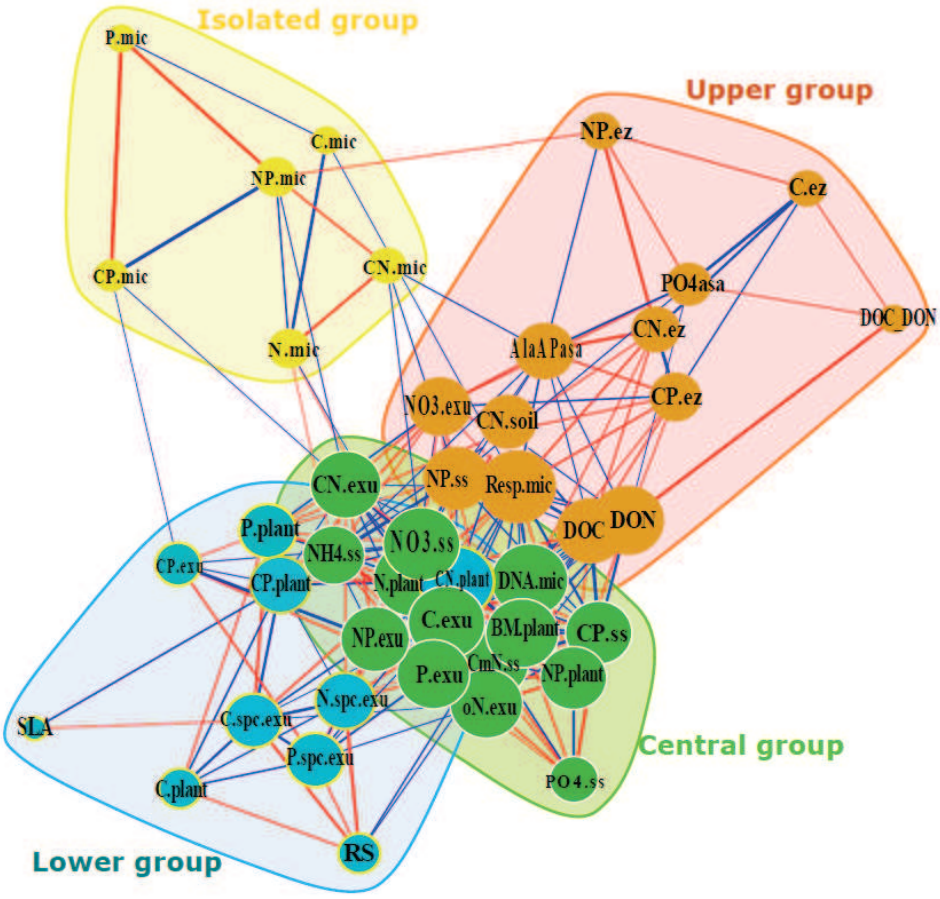

Figure 1. Network of rhizosphere-related variables including soil characteristics, plant functional traits, and indicators of microbial growth and activity. The variables are grouped into 4 groups, which are differently coloured. The size of the circles represents the strength of the variables (number of connections). Lines represent significant correlations between variables ($| r | > 0.3$, $p < 0.05$). Line width is proportional to the absolute value of the correlation coefficient and the colour indicates whether they are positive, blue, or negative, red.

3.2.3. Lower Group: Plant and Exudates Stoichiometry

The third, lower group includes variables that were linked to plant growth less than those in the central group (Figure 1). The strongest variable of the group was the CN.plant, which increased with plant growth and with the soil CN (Figure S6). The plants with enhanced tissue C:N were also poor in P (having a higher biomass C:P). The C.plant and P.plant were negatively correlated and together formed the CP.plant. The plants with increasing CP.plant exuded compounds enriched in P (decreasing CP.exu, Figure S4). Another important PFT in the group was the RS, which was controlled by plant growth after a certain threshold. Small slower-growing plants/species had comparable RSs, but they started to increase, when the plant biomass exceeded 3 g (Figure 2). Faster-growing plants, which invested more in root growth (increasing RS), released less exudates per root-mass (C.spc.exu, N.spc.exu, and P.spc.exu). Decreasing C.spc.exu was associated with

a larger SLA (Figure 1 and Figure S4) and with ammonium availability in the soil solution (Figure S6).

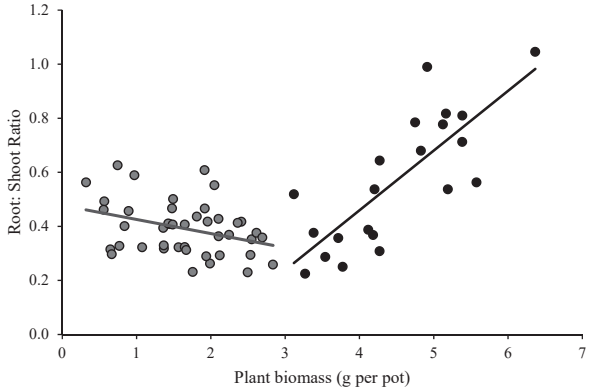

Figure 2. Pearson correlation between plant biomass and root:shoot ratio. The complete data (64 units) had $r = 0.54$, $p = 2.9 \times 10^{-6}$. Small plants with biomass lower than 3 g (42 units) had $r = -0.32$, $p = 0.02$. Large plants with biomass exceeding 3 g (22 units) had $r = 0.79$, $p = 2.1 \times 10^{-5}$.

3.2.4. Isolated Group: Microbial Biomass and Stoichiometry

The fourth, most isolated group includes all the variables of microbial biomass and C:N:P stoichiometry (Figure 1). All microbially bound elements, C, N, and P, were correlated (Figure S5). However, microbial C and P (C.mic, P.mic) were decoupled from non-microbial variables in other groups. Microbial and exudate C:P ratios were positively correlated (Figure 3a), which points to a close link between plant and rhizosphere microorganisms mediated via exudation. Microbial and enzymatic N:P ratios were negatively correlated (Figure 1, Figure S6), and phosphatase activity was linked to the N:P ratio of the soil solution (Figure 3b), in support of the microbial adaptation to the actual nutrient availability by shifts in exoenyzmatic activity. The strongest variables of this group were related to microbial N (N.mic), which connects the microbial biomass and stoichiometry with N.plant, mineral forms of N in the soil solution and enzymatic activity (Figure 4). An increasing microbial N was linked to decreasing N concentrations in the plant (Figure 4a) and decreasing NH_4^+ concentrations in the soil solution (Figure 4b). The lower C:N of the microbial biomass was related to NO_3^- depletion (Figure 4c) and lower Ala-aminopeptidase activity (Figure 4d).

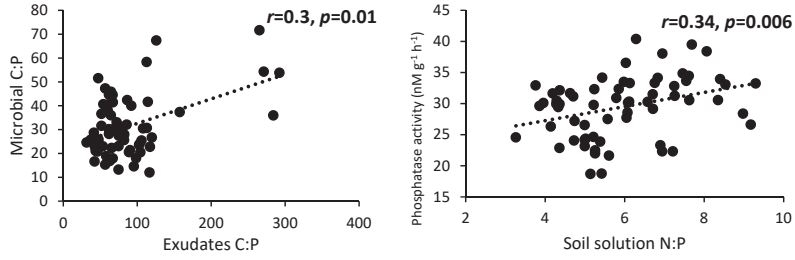

Figure 3. Correlation between C:P ratio of exudates and microbial biomass (**a**) and between soil solution N:P ratio and phosphatase potential activity (**b**).

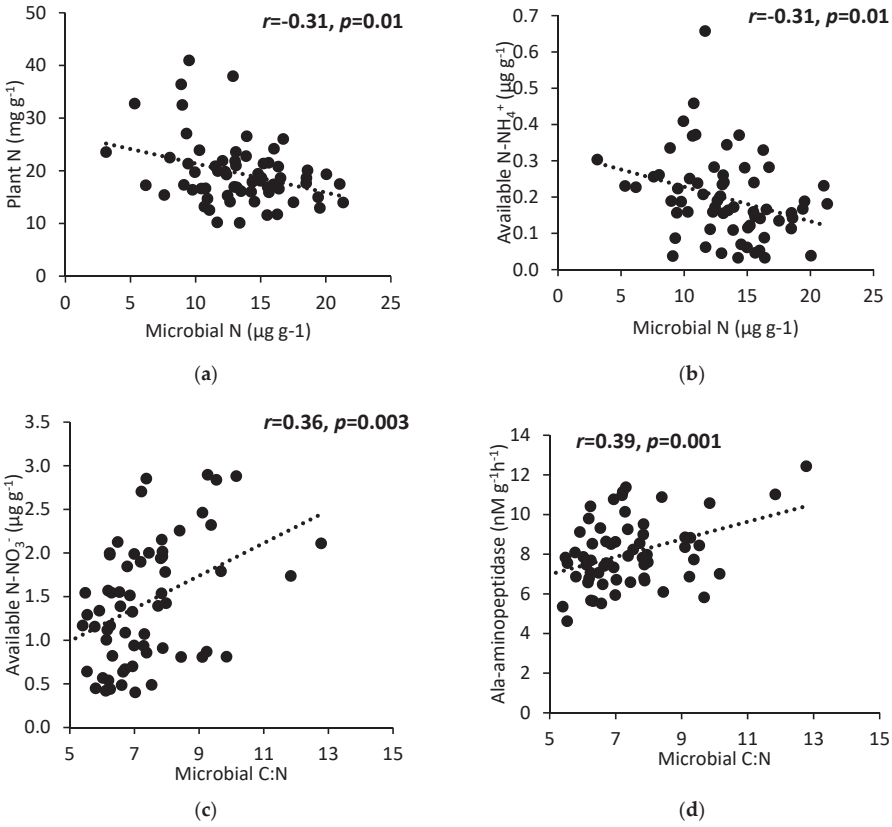

Figure 4. Correlation of microbial N with plant N (**a**) and with N-NH_4^+ amount in soil solution (**b**), and of microbial C:N ratio with N-NO_3^- in soil solution (**c**) and with alanine-aminopeptidase potential activity in soil (**d**).

4. Discussion

4.1. Larger Plants Optimised Their Utilisation of N, the Most Limiting Element

The central group was composed of variables characterising plant growth and related nutrient economics. The size of the plants was proportional to the depletion of mineral nutrients from the soil solution, with the strongest decline for nitrate availability, followed by ammonia and phosphates (Figure 1, central group), in support of H1. The reductions in nitrate and ammonium availability due to plant size are in line with the widespread evidence that plants preferentially take up N in its mineral forms [60–63] and rapidly deplete the highly mobile NO_3^- [64]. Plant biomass was further strongly negatively linked with the N content in tissues and exudates. Larger plants, associated with lower mineral N concentrations in the soil solution, were forced to optimise their N use. They reduced N contents in the tissues (demonstrated by a lowering of N.plant and NP.plant) and in exudates, which had higher C:N and lower N:P ratios. These N savings resulted in the higher efficiency in the N use of the larger plants. The close connections among the C:N stoichiometry of the soil solution, plant biomass, and exudates suggests that the N availability in the plant–soil system played a key role in controlling the plant production and N economy, supporting H2. The soluble phosphate pool was more strongly linked to the plant N:P ratio than to plant biomass (Figure 1, central group, Figure S2), in support

of a stronger control of plant production by N than P availability in our experiment. The change in tissue stoichiometry (shown by both the absolute and relative lowering of the N content) was accompanied by differential biomass allocation. The large plants in which the biomass exceeded a certain threshold (3 g in our case) enhanced their RS ratio (Figure 2). Such a response has been observed in plants under conditions of N deficiency [65,66]. We, thus, suggest that larger (faster-growing) plants induced a strong N limitation in the soils due to their high N demands, supporting H2.

4.2. Root Exudation Served to Get Rid of Surplus Elements

Plant biomass was associated with a larger C exudation flux (Figure 1, central group), which has been previously observed [15,16,28,67,68]. Notably, we report here for the first time that the larger exudation of C compounds was accompanied by a larger release of organic N and total P from the roots. This increased net exudation of nutrients. However, it contradicts the assumption that root exudation serves as a plant investment of C aimed at stimulating nutrient mining from the soil organic matter by microorganisms. Instead, we observed that the exudation of larger plants was mostly composed of products that plants could not immobilise due to their actual N limitation. Larger plants that faced lower soil N availability had an excess of C and P compounds in relation to their needs. Therefore, they released C and P compounds via exudation, which had higher C:N and lower N:P ratios than smaller plants, reflecting the plant tissue stoichiometry (H2) (Figure 1, central group). Additionally, the fast-growing plants also reduced their mass-specific exudation of N but not that of C and P, in accordance with their "N saving" economy and higher N use efficiency compared to the smaller plants. In summary, our results well support the concept that exudation is more a release of surplus products than a targeted investment of plants [27].

4.3. Fast Growth Implied an Adjustment of PFT to Depletion of Mineral N

Fast growing plants, with their large root systems and, thus, high RS, released fewer exudates per root-mass (Figure 1, lowest group). The C that is fixed aboveground in excess to other limiting biogenic nutrients is exported from leaves to prevent damage to photosynthetic structures caused by sugar accumulation [27]. The flow goes belowground, where transported sucrose serves as a signal for root growth. The N required for building root tissues [69] can be obtained either from remobilised shoot reserves, which alters PFT (e.g., SLA, plant N content) or from the soil solution, which affects the concentration of N-forms in the soil solution. Graminoids growing under low N availability increase their phloem load with C and amino acids to support root growth [69–71]. The active remobilisation of leaf reserves may reduce leaf thickness and result in an increase in SLA. Therefore, SLA can be used as an indicator of leaf storage. In our experiment, an increasing SLA was connected to a higher NH_4^+ availability in the soil solution. We, thus, speculate that those species having a higher SLA remobilised their N reserves from leaves to supply root growth, and did not take up extra amounts of NH_4^+ from the soil. In contrast, those plants with a lower SLA kept their reserves in leaves and supplemented their N-needs for root growth via increased NH_4^+ uptake from the soil solution. They were, thus, associated with lower available NH_4^+. This agrees with findings that plants tend to take up more NH_4^+ from the soil when they have high concentrations of carbohydrates in roots to support amino acids' synthesis [72,73]. Though our suggestions are highly speculative and include processes and variables that are rarely linked in the existing literature, it is worth noting them, since these links should be relevant in plant soil interactions. Our pot experiment with species monocultures excluded potential competition between species with different economic traits for aboveground resources (namely light). This explains why SLA was linked to soil conditions but not to plant growth, as has been found in some field experiments [74,75].

4.4. Plant Microbe Links Were Mediated through the Competition for N and Exudation Stoichiometry

The grouping of enzymatic activity and microbial respiration with DOM availability that we observed in the second (upper) group is well supported by the literature [15,16,76–79]. Microbial growth (indicated by microbial DNA amounts) and respiration, though also closely connected, belonged to different groups (Figure 1, central and upper groups). Microbial DNA was tightly linked with variables of plant growth, in support of the key role of easy-available exudation stimulating growth and turnover in rhizospheric microorganisms (Henneron et al., 2020b).

Variables of microbial C:N:P stoichiometry were located in a separate group from the rest of the variables, which could reflect the relatively strict homeostasis of this pool [10,80–82]. The network analysis further revealed that the microbial N and P stoichiometry was influenced by different plant and soil characteristics. Microbial P and related ratios were the most variable within the microbial stoichiometry (Table 1), which agrees with Chen et al. [83]. The variation in P ratios reflects microbial growth activity and the ability of microbes to store it [84,85]. Microbial and exudate C:P ratios were coupled (Figure 3a), suggesting that exuded P was an important source for the P-demanding rhizospheric microbial community. The P exudation and, thus, the P supply of root-associated microbes increased with deepening plant N starvation, occurring proportional to plant size, as discussed above. The enzymatic P-mining from SOM via phosphatase activity was negatively linked to the N:P stoichiometry of the soil solution and of the microbial biomass (Figures 1 and 3b). Microbes, thus, invested in exoenzyme P mining when P availability started to be limited in relation to N, supporting the theory of ecological stoichiometry [53]. We, thus, suggest that microorganisms associated with fast-growing N-limited plants are efficiently supplied by P-rich exudates, while those associated with slower-growing plants facing higher N availability (higher N:P of the soil solution and higher concentrations of mineral N forms) are forced to mine P from SOM more via enhanced phosphatase activity, in support of H3. This agrees with Drake et al. [31], who suggested that root exudate stoichiometry determines microbial activity in the rhizosphere.

Fast-growing plants extracted more N from the soil, which almost certainly resulted from accelerated N mineralisation compared to small plants, as already demonstrated [16]. They further observed that fast-growing plants were accompanied by a soil microbial biomass with a higher C:N ratio than slow-growing species. However, we found the opposite trend, with a depletion of mineral N forms in soil under fast-growing plants, stimulating N immobilisation in microorganisms and resulting in a low C:N microbial biomass (Figure 4). We attribute this contradiction to the difference in soil fertility between the experiments. The soil C and N contents were eight times lower in our case (Table 1), representing a stronger microbial C and N co-limitation, expressed also in a much lower microbial biomass in our experiment (Table 1) [15]. In the short term, microorganisms are superior competitors to plants for organic N and also better competitors for mineral N, especially under very low concentrations [86]. Of the mineral forms, they preferentially consume available NH_4^+ over NO_3^- [86–88], while plants preferentially take up mobile nitrates, which alleviates their competition for N through chemical niche separation [89]. However, we observed a strong depletion of both mineral N forms from the soil solution under fast-growing plants, indicating conditions of strong competition for N and high energy expenses in N incorporation into the microbial biomass [90]. The microbial biomass built under such conditions had a low C:N ratio. It was associated with low alanine aminopeptidase activity (H3) (Figure 4c,d). This supports the suggestion that very low mineral N availability limits microbial investments into the highly N-demanding production of exoenzymes [31,91–94]. In our case, microorganisms reduced only N mining but not the activity of other hydrolytic enzymes. This suggests that the repression of N mining is a coordinated microbial strategy for the efficient N use and competition for N uptake by fast-growing plants under very low N availability, supporting H3. Such conditions push plants to adapt their metabolism and PFTs to increase N use efficiency (CN.plant) and N

uptake (RS) by the optional re-translocation of leaf reserves (SLA) and decreased specific N exudation.

5. Conclusions

The plant growth-related uptake of nutrients from the soil reduced the availability of both mineral N and P in the soil. The nutrient uptake was proportional to the plant biomass. The depletion of nutrients from the soil solution caused by fast plant growth forced large plants to reduce N concentrations in tissues and losses via exudation, and to relocate biomass to intensify soil exploration by increasing SLA and RS. The exudation of plants facing low N availability was relatively richer in C and P against N, which indicates that exudation served to release surplus elements from plants. The exudation C:P ratio correlated with the C:P of the microbial biomass, suggesting that root exudation serves as an important source of C and P for soil microbes. Therefore, exudation mediates the coupling of plant and microbe nutrient requirements. Microorganisms responded to deepening N limitation by enhancing N immobilisation over N mining, which strengthened the plant-microbe competition for N and resulted in tightly closed N recycling. Our data showed that soil nutrient availability and soil solution stoichiometry control plant–microbe interactions.

Our results cast light over the use of plant stoichiometry and PFT as indicators of exudation and microbial stoichiometry. There is a need for similar studies, which would focus on plant–exudates–microbial links under a strong P limitation to complement the knowledge. We propose field studies evaluating these links as the next step for their validation under complex natural rconditions.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/land10080840/s1, **Figure S1**: Dendrogram of correlations among plant category (X.qualy) and biomass (bm.plant), SLA, RS and specific exudation of C, N and P (_.spc.exu) (a). Spearman correlation analysis between plant category and biomass (b), SLA (c), and RS (d), **Figure S2**: Pearson correlation matrix of variables included in the central group. Under the diagonal are writen the correlation coeffients proportional to the colour of each cell, **Figure S3:** Pearson correlation matrix of variables included in the upper group. Under the diagonal are writen the correlation coeffients proportional to the colour of each cell, **Figure S4:** Pearson correlation matrix of variables included in the lower group. Under the diagonal are writen the correlation coeffients proportional to the colour of each cell, **Figure S5:** Pearson correlation matrix of variables included in the microbial biomass stoichiometry group. Under the diagonal are writen the correlation coeffients proportional to the colour of each cell, **Figure S6**: Pearson correlation matrix of variables from different groups. Under the diagonal are writen the correlation coeffients proportional to the colour of each cell, **Supplementary Table S1**: Strenght of the variables by groups in Figure 1. *Strength means the numeber of linkns of each variable.*

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Supplementary Materials:

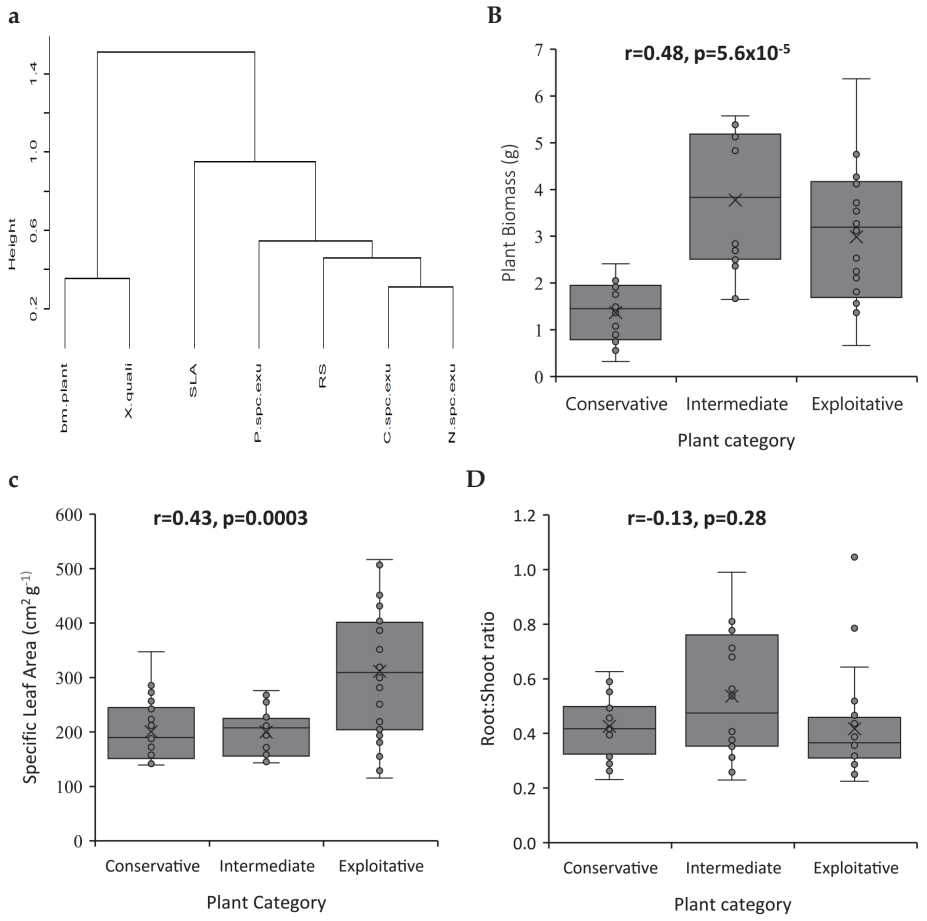

Figure S1. Dendrogram of correlations among plant category (X.qualy) and biomass (bm.plant), SLA, RS and specific exudation of C, N and P (_.spc.exu) (a). Spearman correlation analysis between plant category and biomass (b), SLA (c), and RS (d). Spearman correlation coefficient (r) and p-value (p) are expressed in each graph.

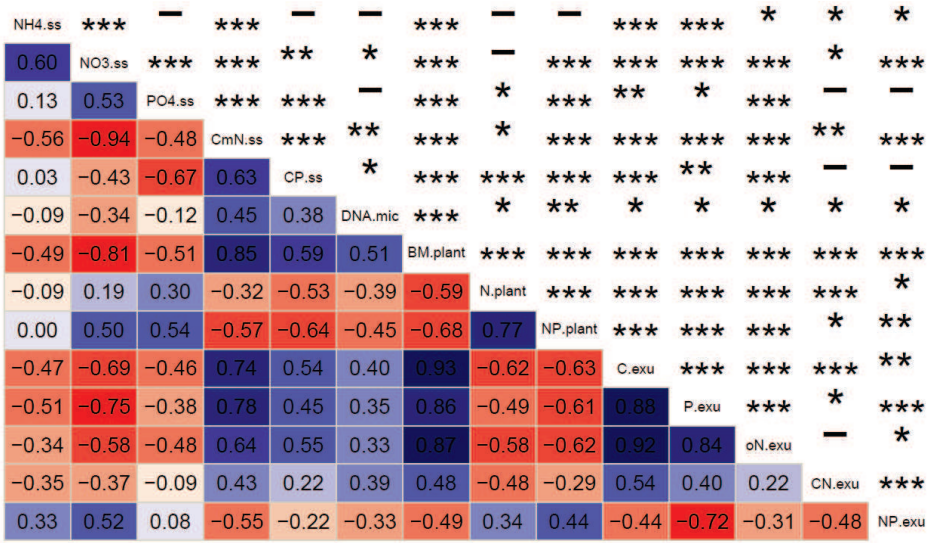

Figure S2. Pearson correlation matrix of variables included in the central group. Under the diagonal are writen the correlation coeffients proportional to the colour of each cell. Over the diagonal the p values, p>0.05;-, 0.05<p>0.001=*, 0.001<p>0.0001=**, p<0.0001=***.

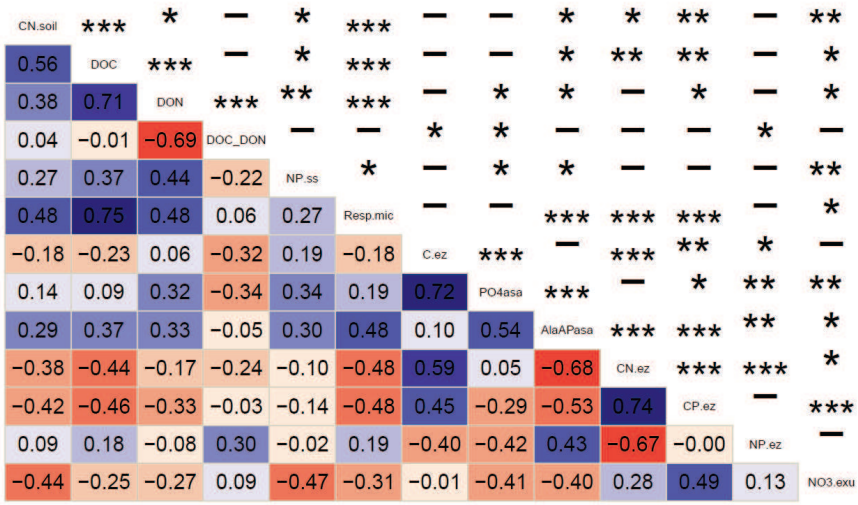

Figure S3. Pearson correlation matrix of variables included in the upper group. Under the diagonal are writen the correlation coeffients proportional to the colour of each cell. Over the diagonal the p values, p>0.05;-, 0.05<p>0.001=*, 0.001<p>0.0001=**, p<0.0001=***.

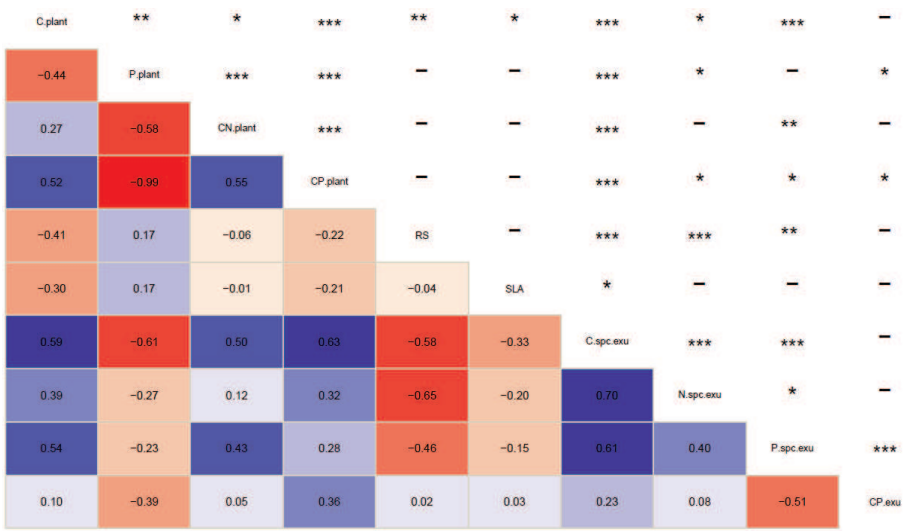

Figure S4. Pearson correlation matrix of variables included in the lower group. Under the diagonal are writen the correlation coeffients proportional to the colour of each cell. Over the diagonal the p values, p>0.05;-, 0.05<p>0.001=*, 0.001<p>0.0001=**, p<0.0001=***.

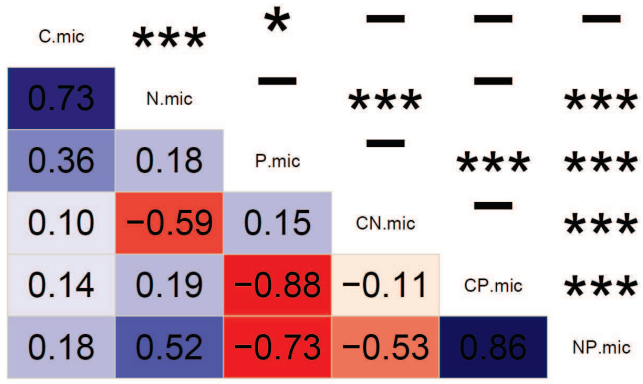

Figure S5. Pearson correlation matrix of variables included in the microbial biomass stoichiometry group. Under the diagonal are writen the correlation coeffients proportional to the colour of each cell. Over the diagonal the p values, p>0.05;-, 0.05<p>0.001=*, 0.001<p>0.0001=**, p<0.0001=***.

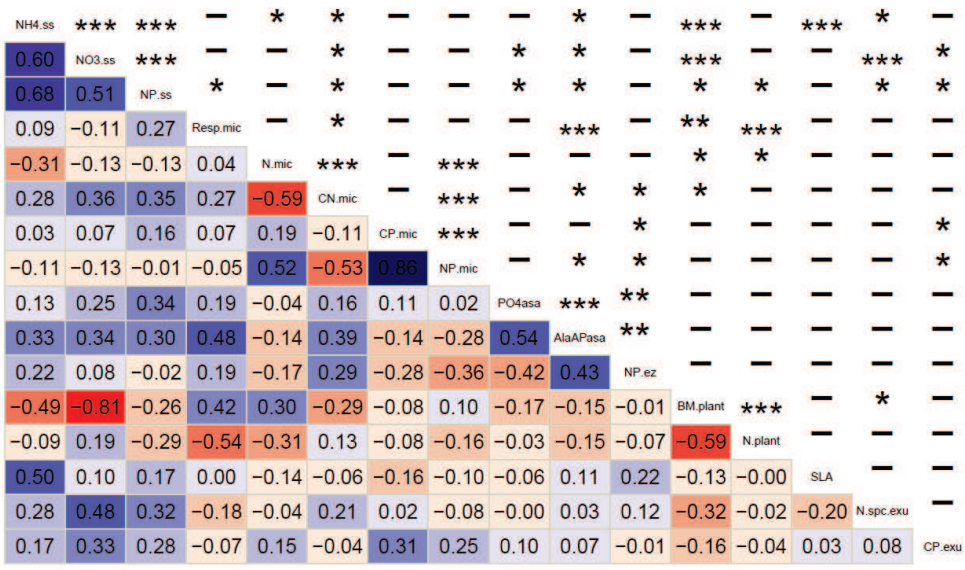

Figure S6. Pearson correlation matrix of variables from different groups. Under the diagonal are writen the correlation coeffients proportional to the colour of each cell. Over the diagonal the p values, p>0.05;-, 0.05<p>0.001=*, 0.001<p>0.0001=**, p<0.0001=***.

Supplementary table ST1. Strenght of the variables by groups in figure 1. *Strength means the numeber of linkns of each variable.*

1. Central		**2. Upper**		**3. Lower**		**4. Microbial CNP**	
Variable	Strength	Variable	Strength	Variable	Strength	Variable	Strength
N.plant	21	Resp.mic	20	CN.plant	19	N.mic	6
DNA.mic	20	DON	18	C.plant	16	NP.mic	6
C.exu	20	DOC	16	CP.plant	12	CN.mic	5
CmN.ss	19	NP.ss	15	N.spc.exu	12	CP.mic	4
BM.plant	18	NO3.exu	13	P.plant	11	C.mic	3
oN.exu	18	AlaAPasa	12	C.spc.exu	11	P.mic	3
P.exu	18	CN.soil	11	P.spc.exu	11		
CN.exu	17	CP.ez	9	CP.exu	7		
NP.exu	16	CN.ez	8	RS	6		
CP.ss	15	PO4asa	8	SLA	2		
NP.plant	15	C.ez	5				
NO3.ss	13	NP.ez	5				
NH4.ss	13	DOC_DON	4				
PO4.ss	8						

PAPER II

Manuscript

C addition to the soil alters plant-microbial interactions and it is reflected in foliar $\delta^{15}N$

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C addition to the soil alters plant-microbial interactions and it is reflected in foliar $\delta^{15}N$

ABSTRACT

The close links and feedbacks between plant and microbial activity regulate both the growth of soil microorganisms and the ecosystems production. Plant-soil interactions are affected by carbon (C) addition to the soil, which relieves the dependence of rhizosphere microbial activity on C-supply via root exudation and induces stronger competition for nitrogen (N) between microorganisms and plants. We hypothesize that the response of the plant-soil system to the labile C addition would be modulated by plant economic strategy, because N requirements and N uptake rate from soil solution increase from conservative to acquisitive plant species. The altered soil C and N availabilities influence plant metabolism and would be reflected in C and N isotopic composition of the leaves (foliar $\delta^{13}C$ & $\delta^{15}N$). To evaluate the role of the C addition in plant-soil interactions, we run a glasshouse experiment. Eight grass species on a range from conservative to acquisitive strategy were grown either in non-amended or C-amended soil conditions. Plant, soil and microbial variables were measured. The C addition enhanced dissolved organic C (DOC), microbial DNA and specific respiration in planted and unplanted soils, and N mining enzymatic activity in planted soils compared to non-amended soils. Plants in C-amended soils had higher tissue C/N ratio but comparable biomass production as in non-amended soils. Foliar $\delta^{13}C$ & $\delta^{15}N$ was primarily linked to plant growth rate and soil N availability: both decreased with plant biomass and with decreasing nitrate availability. Additionally, $\delta^{15}N$ was also altered by C addition: it increased in C-amended soils due to overall lower N uptake by plants and microorganisms both having higher biomass C/N ratios. In result, the plant production was maintained after C addition to the soils due to the increased N use efficiency of both plants and microorganisms. Our results corroborate the link between $\delta^{15}N$ and plants N demand, linked to unbalanced N mineralization (offer) relative to plant requirements (demand), and its importance as an indicator of N limitation for ecosystem production.

Key words: Foliar isotopic composition, Nitrogen use efficiency, Microbial specific respiration, Plant C/N, Microbial C/N, Enzymatic stoichiometry.

INTRODUCTION

Within natural ecosystems, plants are essentially nutrient limited, whereas microbial decomposers in the soil are primarily carbon (C) limited (Soong et al., 2018). Root exudation relieves C limitation of microorganisms in rhizosphere (Kaštovská and Šantrůčková, 2007), promoting their growth in comparison to bulk soil (Blagodatskaya et al., 2014). The microbial growth stimulated by labile C input may increase the enzymatic soil organic matter (SOM) decomposition to gain more C and nutrients, mainly nitrogen (N), to support additional growth (Kuzyakov, 2010). After depletion of easily decomposable SOM, the soil microbial activity slows down (Cotrufo et al., 2015) indicating that it depends not only on the C input but also on its quality. Root exudation is an important part of plant economic strategy, which links the plant growth with rhizosphere microbial activity and ultimately controls SOM decomposition and N cycling (Guyonnet et al., 2018; Henneron et al., 2020a). Fast-growing (acquisitive) plants accelerate the N cycle more than slow-growing (conservative) plants through higher exudate input. It causes faster gross N mineralization and faster turnover of the mineral and microbial N pools (Cheng et al., 2003; Henneron et al., 2020a, 2020b). However, the close coupling between plant and microbial activities, which is conditioned by the supply of root exudates to the C limited soil microorganisms, may be impaired by an input of additional C source to the soil.

According to Kuzyakov and Xu, (2013) the increase in N availability in rhizosphere is based on the coordination between root growth and the alternation of microbial C surplus, stimulated by exudation, with a posterior C starvation. This model implies that changes in C availability in the soil can alter this coordination, leading to unbalances between plant growth and N availability. Thus, the inputs of additional C sources such as fresh litter, atmospheric depositions (Iavorivska et al., 2017; Willey et al., 2000) and dissolved organic C (DOC) released after freezing-thawing and drying-rewetting events (Dong et al., 2021; Melick and Seppelt, 1992; Schmidt et al., 1997; Zhao et al., 2010) could alter plant-soil feedback. Additional C input makes microbial activity partly independent of plant exudation. The extra C supply further enhances microbial growth rate and related nutrient demand, which may rapidly decrease N availability in the rhizosphere (Eschen et al., 2007; Johnson and Edwards, 1979; Magill and Aber, 2000). The additional C input to the soil may thus finally induce N limitation for both plants and microorganisms and decrease plant productivity. The effect of C-addition to the soil on plant activity is species-specific (Eschen et al., 2006). Plants slow down their growth rate and/or enhance the N use efficiency, i.e. reduce N concentration in tissues (Zhang et al., 2020) to adapt to N deficiency (Reich, 2014; Reich et al., 1997; Wright et al., 2004). We expect that the plant response to the additional C-input to the soil and consequent changes in microbial activity will depend on plant economic strategy. Due to the stronger coordination between plant growth and soil processes in fast-growing acquisitive than in slow-growing conservative, we expect a proportional impact of C addition, related to the disruption plant-microbe

interaction reflected in stronger changes in growth and/or metabolism of plant and microbes.

Foliar C and N isotopic signatures ($\delta^{13}C$ and $\delta^{15}N$) are increasingly used as indicators of plant metabolism and nutrient cycling in ecosystems because they integrate isotopic composition of N sources and the fractionation during plant uptake, transport and metabolism (Bowling et al., 2008; Cernusak et al., 2013; Dawson et al., 2002; Tcherkez et al., 2011). Both leaf $\delta^{13}C$ and $\delta^{15}N$ are influenced by nutrient availability (Robinson et al., 2000; Taskos et al., 2020; Zhang et al., 2015, 2017). Variation in leaf $\delta^{13}C$ of C3 plants reflects changes in diffusion of CO_2 from atmosphere to carboxylation sites and during carboxylation itself (Brugnoli and Farquhar, 2000). The foliar $\delta^{13}C$ increases with increasing N availability (Choi et al., 2005; Livingston et al., 1999; Ripullone et al., 2004). Available N stimulates carboxylation efficiency (Ripullone et al., 2004) and increases mesophyll (Choi et al., 2005; Livingston et al., 1999) and stomatal conductance (Flanagan and Farquhar, 2014; Hou et al., 2015; Tang et al., 2019) even when plants are not limited by water. Variation in foliar $\delta^{15}N$ is attributed to changes in soil N cycling, plant N acquisition, transformation, and translocation between and within plant organs and cells. Generally, $\delta^{15}N$ decreases with decreasing N concentrations in tissues (e.g. higher tissue C/N or lower N/P ratios) (Ariz et al., 2015; Cui et al., 2019b; McKee et al., 2002; Tcherkez, 2011; Zhang et al., 2017). Therefore, we suggest that decreasing N availability due to higher immobilization in microbial biomass in C-amended soils will reduce $\delta^{13}C$ and $\delta^{15}N$ of plants in comparison to those grown in non-amended soils.

We aimed to determine an effect of additional C supply to the soil on the plant-soil interactions over the range of plant economic strategies and a potential of foliar $\delta^{13}C$ and $\delta^{15}N$ to indicate plant adaptations to the changed soil conditions. Therefore, we conducted a pot experiment with eight grass species with known economic strategies (based on specific leaf area according to de Vries and Bardgett, (2016)) from conservative to competitive. The species were grown from seeds in the non-amended control grassland soil or in the same soil amended by agar, representing the mucilage-like organic C in the rhizodeposition released to the rhizosphere (Knee et al., 2001; Moody et al., 1988). We hypothesize that (**H1**) the addition of C substrate to the soil will enhance microbial biomass and enzymatic N-mining from SOM to cover microbial metabolic N demands. (**H2**) Plants growing in C-amended soils will reduce production and/or tissue N content compared to those in control soils. This response will be more pronounced in fast-growing plant with larger N demands than in slow-growing conservative plants with inherently lower N demands. (**H3**) Plants growing in C-amended soils will have lower foliar $\delta^{13}C$ and $\delta^{15}N$ in comparison to plants grown in control soil. The leaf ^{13}C and ^{15}N depletion will be proportional to the decrease in plant biomass and/or leaf N content of respective species grown in C-amended compared to control soils. To test the hypotheses, we measured the effect of C addition on soil properties including microbial variables in planted and non-planted soils, and on plant growth, tissue N content and foliar isotopic composition.

METHODS

Experimental design and setting

A soil classified as cambisol (WBR, FAO, 2015) was collected in an annually mown grassland site (České Budějovice, Czech Republic, 48.9°N, 14.3°E, 450 m a.s.l.). The main soil properties were total soil organic C 12.8 ± 0.3 g kg^{-1}, total N 1.3 ± 0.03 g kg^{-1}, total P 0.21 ± 0.04 g kg^{-1}, total soil C/N ratio 11.76 ± 0.19 (n=5) and pH 5.74; texture loam. Soil was sieved (2 mm) and cleaned from roots. The sieved soil was split into two parts; one was kept without any treatment (non-amended) and the other was amended with an agar powder (C-amended). Agar addition enhanced total soil C to 16.0±0.1 g kg^{-1} and soil C/N to 14.2±0.6 (n=5). We used agar because it is a component of rhizodeposition and agar degrading microorganisms are commonly found in rhizosphere (Bacic et al., 1986; Chaboud and Rougier, 1984; Hosoda et al., 2003; Knee et al., 2001; Moody et al., 1988; Sakai et al., 2014; Song et al., 2015, 2014). The polysaccharide addition aimed to ensure a long-term supply of C to the soil microbial community, enhancing microbial biomass and strengthening the plant-microbial competition for the available N.

The soil, either non-amended or amended, was filled into pots (8x8x13 cm; 800 g) and planted with seeds (15 seeds per pot) of single grass species (four replicates per each particular species and soil treatment). Five unplanted pots filled with non-amended or C-amended soil were further used to assess the effect of C-addition itself on the soil properties. We evaluated eight grass species with prescribed economic strategies from conservative to acquisitive ones according to de Vries and Bardgett (2016). Conservative: *Festuca rubra* (Feru)*, Poa pratensis* (Popr) and *Bromus erectus* (Brer), intermediate: *Dactylis glomerata* (Dagl) and *Lolium perenne* (Lope). Acquisitive: *Holcus lanatus* (Hola)*, Phleum pratense pratense* (Phpr) and *Poa trivialis* (Potr). The experiment had a full factorial design of 2×8×4 (2 C-addition treatment × 8 plant species × 4 replicates) plus the non-planted pots distributed completely random.

Plants grew in a greenhouse (exposed to natural light, 25/16 °C for 18/6 h of day/night time). Soil water content was adjusted to field capacity and maintained over the entire experiment by weighing the pots twice per week. One month after germination (May-June), plants and soils were harvested to evaluate the following variables.

Plant and soil harvest

Plants with soil were removed from the pots. The plant-soil blocks were loosened, shaken, and the soil that remained attached to the roots (defined here as the rhizosphere soil) was carefully cleaned from the roots by hand and tweezers (and with a few ml of distilled water from a pipet) to minimize root disturbance. The removal of soil particles was completed by washing the roots with distilled water. Part of the rhizosphere soil of each pot was used right after separation from roots to measure physico-chemical properties, C and N in microbial biomass and respiration. The rest of the soil was frozen at -20 ºC and later used to measure the total microbial DNA and potential enzymatic activity. Intact plants were taken immediately after root cleaning to collect exudates and then dried to measure elemental concentrations and foliar isotopic signatures.

Physico-chemical properties of soil and soil solution

After harvesting, a part of the rhizosphere soil and all leaf and root tissues were air-dried, milled, and analysed for the total C and N contents on an elemental analyzer (vario MICRO cube, Elementar, Germany). The fresh soils were extracted with 0.5M K_2SO_4 (1:4, w/v) and analysed for the concentrations of soluble organic C and total N (DOC and DN), N-NH_4^+ and N-NO_3^-. The DOC and DN were determined with a TOC-L analyzer equipped with the total N measuring unit TNM-L (Shimadzu, Tokyo, Japan). The concentrations of N-NH_4^+ and N-NO_3^- (available N-NO_3^-) were measured with a Flow Injection Analyzer (FIA Lachat QC8500, Lachat Instruments, USA). The mineral N is the sum of N-NH_4^+ and N-NO_3^-. The dissolved organic N (DON) was calculated by subtracting the mineral N from DN.

Soil microbial biomass, specific microbial respiration (qC) and enzymatic activity

C and N in microbial biomass were determined by chloroform fumigation-extraction method (Brookes et al., 1985; Vance et al., 1987). The extraction before and after the fumigation of the soil with amylene-stabilized chloroform for 24 h was done with 0.5M K_2SO_4 (1:4, w/v). The microbial C and N were calculated as the differences in the concentrations of DOC and DN in sulphate extracts, respectively. The values were corrected for extraction efficiencies using k_{EC} = 0.41 (Sparling et al., 2000) and k_{EN} = 0.45 (Chen et al., 2014). We used the same correction values for all soil conditions as recommended by Joergensen et al. (2011). The C/N ratio of microbial biomass was calculated on molar basis.

Respiration rate of the rhizosphere soil was measured as the cumulative CO_2 production after a 3-day incubation of 10 g soil in airtight-sealed 100 ml flasks at 20 °C, recalculated per day. The CO_2 concentration in headspace was determined using an Agilent 6850 GC system (Agilent Technologies, CA, USA). This value was further divided by microbial biomass C to determine the specific respiration quotient (qC, C-CO_2/C-microbial biomass, w/w). Here we use qC as an indicator of C use efficiency (CUE) of microbes in soil (Li et al., 2021; Manzoni et al., 2017).

Total microbial DNA was extracted from 0.25 g of defrosted soil using a PowerSoil Isolation Kit (MO BIO laboratories, Inc. Carlsbad, CA) according to the suggested protocol, using PowerBeat tubes and tube mixer 693TC5. The extracted DNA was quantified using QuantiFluor® dsDNA Dye in a Quantus Flourometr (Promega) following the manufacturer's protocol.

C-mining (β-glucosidase and cellobiosidase) and N-mining (alanine-aminopeptidase and chitinase) potential enzymatic activity (Sinsabaugh et al., 2009) were determined using a microplate fluorometric assay (Marx et al., 2001). Defrosted soil samples (1 g) were homogenized in distilled water (100 mL) and sonicated for 4 min; 200 µL of soil suspension was added to 50 µL of methylumbelliferyl solution for β-glucosidase, cellobiosidase and chitinase determination, or to 50 µL of 7-aminomethyl-4-coumarin substrate solution for measurement of alanine-aminopeptidase. To determine the saturation substrate concentration, three concentrations of each fluorogenic substrate (50, 100, and 300 µM) were tested and the one with the highest decay rate was chosen (Bárta et al., 2014; Burns et al., 2013). Plates were incubated at 20 °C for 2 h. The linearity of substrate decay over the entire

incubation was verified beforehand. The fluorescence was measured using an INFINITE F200 microplate reader (TECAN, Crailsheim, Germany) at an excitation wavelength of 365 nm and an emission wavelength of 450 nm. The C/N ratio of the enzymatic activity was calculated by dividing the sum of the C-mining over the sum of the N-mining potential enzyme activities. The enzymatic C/N ratio was used here as an indicator of the primary requirement by soil microbes; either C or N, as suggested by the ecoenzymatic stoichiometry hypothesis (Sinsabaugh et al. 2009).

Plant biomass, C/N stoichiometry and foliar isotopic composition

After exudation measurements, plant biomass was separated into shoot and root biomass, dried at 70°C for 48 h and weighted. Plant biomass was calculated as the sum of shoots and roots dry mass per pot. Dry tissues were milled and analysed for total C and N contents using a micro elemental analyzer (vario MICRO cube Elementar, Germany). The molar C/N ratio was calculated. C and N natural isotopic composition ($\delta^{13}C$ and $\delta^{15}N$) of leaf biomass was measured with an isotope ratio mass spectrometer (IR-MS Delta X Plus, Finnigan, Germany).

Root exudates

Intact plants with clean roots were used for measuring exudation flux. All plants from the pot were placed in a beaker with roots submerged in 500 ml of redistilled water and left in daylight in the conditioned greenhouse at 25 °C. After 4 h, the plants were removed and the root exudate solution was vacuum-filtered through a 0.2 µm express plus polyethersulfone membrane filter (GPWP14250, Merck Milipore Ltd. Ireland) to remove microbial cells and root debris if present, and immediately analysed. The exuded DOC, DN and $N\text{-}NO_3^-$ were measured as reported for the soil solution above. The $N\text{-}NO_3^-$ in exudates is expressed as the proportion of total N.

Statistical analyses

To evaluate the effect of C addition on soil variables in planted and non-planted pots, a t-test was applied (Fig. 1, S1, S2). A two-way ANOVA was performed to evaluate the effect of C-addition, plant species and their interaction on the plant biomass, foliar C/N and available $N\text{-}NO_3^-$ (Fig. 2). 15 variables including soil, microbial, and plant variables were subjected to principal component analysis (PCA) to explore the main drivers of variation in C-amended and non-amended soils (Fig. 3). We evaluated the linear relationship of most changing variables among PCAs of C-amended and non-amended treatments. The correlation of microbial DNA with microbial N and with qC was analysed separately for each soil treatment taking all data from planted soils (Fig. 4). In planted soils, ANOVAs were performed to compare the slope of the linear model between plant biomass and available $N\text{-}NO_3$ (Fig. S3), between plant $\delta^{13}C$ and $\delta^{15}N$ (Fig. S4), and of each isotopic signature with plant biomass, foliar C/N and N-NO3 availability (Fig. 5). All the analyses were performed in *R* v. 3.6.3 (R Core Team, 2021).

RESULTS

Effect of C addition on DOC, microbial biomass and exoenzymatic activity

Unplanted soils were poorer in microbial DNA and DOC compared to planted soils (Fig. 1a, b). Both unplanted and planted C-amended soils contained more microbial DNA and DOC than non-amended soils, with more pronounced C effect in unplanted ones (Fig. 1a, b). Microbial C and N pools were not affected by C addition (Fig. S1a, b), however C/N ratio of microbes increased with C-addition in planted soil (Fig. S1d). The microbial DNA and DOC were positively correlated across all planted and unplanted soils ($r=0.4$, $p<0.001$). The C/N ratio of potential enzymatic activity was higher in C-amended compared to non-amended unplanted soils (Fig. 1e). There was, however, opposite trend in planted soils (Fig. 1f), where C-addition enhanced Ala-aminopeptidase activity compared to non-amended soils (Fig. S1f). In summary, C-addition increased the investments into C mining in unplanted soils but into N mining, when plants were present.

Effect of C addition on plant biomass and tissue C/N, and NO_3^- availability

Plant biomass was linked to plant economic strategy. Conservative species: Popr, Brer and Feru, generally grew slower and built the lowest dry masses among all species until the end of experiment. The largest production was found for two acquisitive (Phpr and Hola) and one intermediate (Lope) species (Fig. 2a) ($F=85.5$, $p<0.001$). The soil C-addition did not affect plant biomass (Fig. 2a) but increased the tissue C/N ratio of all species ($F=102.6$, $p<0.001$) except Lope and Potr (Fig. 2b) (soil treat*species: $F=4.6$, $p<0.001$). The NO_3^- availability was significantly reduced by the presence of plants in comparison to unplanted soils (Fig. S2). Concentration of available N-NO_3^-, being the preferred N source for grasses, differed among species ($F=57.6$, $p<0.001$, Fig. 2c) and was on average higher in C-amended than non-amended soils ($F=21.3$, $p<0.001$). Therefore, the lower tissue N content (higher leaf C/N ratio) in C-amended than in control soils was not due to a decreased mineral N availability (Fig. 2b, c). In both C-amended and non-amended soils, NO_3^- availability decreased with increasing plant biomass (Fig. 3, S2).

Effect of C-addition on plant-soil interactions

The negative correlation between plant growth and NO_3^- availability in soil formed the main gradient in the multivariate analyses (the PC1) in both non-amended and C-amended systems (Fig. 3), due to the strong negative correlation among N-NO_3^- amount and plant biomass (Fig. S2). The PC1 represented more variability in non-amended (49%) than in C-amended soils (34%). It indicates that the depletion of nitrates due to plant growth affected all other measured variables less in the C-amended than in non-amended soils. Most variables were similarly distributed under both soil conditions. Foliar $\delta^{15}N$ and $\delta^{13}C$ decreased with decreasing NO_3^- availability and with increasing plant biomass under both soil conditions. DON showed opposite trend to nitrates and increased with plant biomass, which suggests that plant activity stimulated DON production (via enhanced Ala-aminopeptidase activity, see Fig. S1f) and plant biomass

production was supplied by the NO_3^- uptake. Faster-growing plants built tissues with higher C/N ratio than slow-growing ones. The C/N ratio of plant tissues was negatively correlated with a proportion of NO_3^- in exudates (exudates NO_3 in Fig. 3) indicating that high tissue N concentration was accompanied by high contribution of NO_3^- to exuded N. All the above-mentioned variables were more closely inter-correlated in the non-amended than in C-amended soils (Fig. 3). The C exudation flux closely correlated with plant biomass under both soil conditions.

The largest contrasts in the distribution of variables between non-amended and C-amended soil were related to microbial variables. Microbial DNA increased with plant biomass in both treatments but correlated with microbial C and N pools only in non-amended soils (Fig. 4a) and with qC only in C-amended soil (Fig. 4b). The variation of microbial C and N along the plant biomass gradient was greater in non-amended than in C-amended soil, which suggests different controlling factors of microbial elemental pools.

Effect of C-addition and NO_3^- availability on foliar isotopic composition

Foliar $\delta^{15}N$ (‰) decreased with increasing plant biomass under both soil conditions (Fig. 3 and 5a). However, small plants were more enriched in ^{15}N and the decrease of foliar $\delta^{15}N$ with increasing plant biomass was steeper in non-amended than C-amended soils ($F=8.2$, $p=0.005$). The negative correlation between foliar $\delta^{15}N$ and plant C/N ratio found in non-amended soils was lost in C-amended soils (Fig. 5c) ($F=9.4$, $p=0.003$). Under both soil conditions, foliar $\delta^{15}N$ increased with increasing N-NO_3^- availability, with slower foliar ^{15}N enrichment in C-amended than in non-amended soils (Fig. 5e) ($F=9.1$, $p=0.003$). The correlations of foliar $\delta^{13}C$ with plant biomass, tissue C/N and available N-NO_3^- were similar to those with $\delta^{15}N$ but none of the links was altered by C-addition to the soil (Fig. 5b, d, f).

DISCUSSION

C-addition stimulated microbial growth, enzymatic N mining and specific respiration in planted soils

We hypothesized in **H1** that the addition of C substrate to the soil will enhance microbial biomass and enzymatic N-mining from SOM to cover microbial metabolic N demands. Our assumption about the support of microbial growth by the additional soil C input was valid in both unplanted and planted soils (Fig. 1a, b). However, the enzymatic response to the added substrate was modified by the presence of plants. While microorganisms in unplanted soils enhanced exoenzymatic C-mining to utilize the added polysaccharide (Fig. 1e), the microorganisms associated with plants preferentially invested into N-mining. The different microbial response showed that plant activity modified original conditions in bare soil and shifted microorganisms from prevailing C limitation to the N shortage.

Planted soils always contained more microbial DNA and larger DOC pool than unplanted soils (Fig. 1a-d). Both, microbial DNA and DOC further increased with

faster plant growth and larger C exudation flux (Fig. 3) due to the direct contribution of root exudates to the DOC pool and consequent promotion of microbial growth (Canarini et al., 2019; Li et al., 2021, 2018; Prescott et al., 2020) and of exoenzymatic activity (Henneron et al., 2020b). At the same time, planted soils were poorer in available mineral N than unplanted soils (Fig. S2) and the N availability further diminished under faster-growing plants due to their more intensive mineral N uptake (Fig. 3). In summary, plants promoted growth of rhizosphere microorganisms via exudation but put them in N shortage by pumping out the available mineral N. The fast plant growth rate and large exudation input make these plant-microbial interactions more intensive (Cardenas et al., 2021; Henneron et al., 2020b).

The C-amendment of the planted soil magnified the microbial N demands in the rhizosphere due to the additional enhancement of microbial DNA in comparison to non-amended soil (Fig. 1b). The C-supplied microorganisms increased activity of Ala-aminopeptidase (Fig. S1e), which lowered the C/N ratio of enzymatic activity compared to non-amended soils (Fig. 1e), in accord with the ecoenzymatic stoichiometry hypothesis (Sinsabaugh et al., 2009, 2008; Waring et al., 2014). In support of our results, Feng and Zhu (2021) recently proposed that the influence of soil stoichiometry on SOM degradation is especially important under strong N limitation conditions, which is the case for our C-amended planted soils. At the same time, microorganisms in C enriched soils enhanced their specific respiration, showing larger energetic demands (Manzoni et al., 2010; Spohn, 2015), which was probably linked to a higher production of exoenzymes (i.e. Ala-aminopeptidase) and faster biomass turnover. The higher investment into N-mining enzymes and higher specific respiration (Fig. 3, 4b) are indications of decreasing C use efficiency (CUE) of microorganisms under N limiting conditions (Li et al., 2021; Manzoni et al., 2017, 2012; Soong et al., 2018). In summary, the C-amendment of planted soils increased DOC, microbial biomass, its specific respiration, and N-mining enzymatic activity, suggesting the magnifying of microbial N limitation in rhizosphere. Those altered rhizosphere conditions significantly influenced N content and metabolism of plant species compared to the non-amended soils.

The combined effect of soil C-amendment and plant growth rate on tissue C/N and soil N availability

The H2 was focused on plant response to the soil C-amendment and its relationship with plant economic strategy. To answer **H2**, C-addition did not alter plant growth rate but increased the tissue C/N ratio (Fig. 2). Differently from the non-amended soil, where the tissue C/N further increased with plant biomass, it was independent of plant growth in C-amended soils. It suggests that the C-addition effect (DOC effect) exceeded that of plant growth rate on the tissue C/N. We expected the increase in tissue C/N ratio to be a response to N limitation, which was observed for rhizosphere microorganisms after the C-addition (Fig. S1d). However, the concentration of nitrates in soil solution, which serves as a preferential N form taken up by grasses and a good indicator of N availability for plants (Biernath et al., 2008; Harrison et al., 2007; Liu et al., 2017; Rasmussen et al., 2010; Tinker and Nye, 2000; Xu et al., 2008), was similar in non-amended and C-amended soils or even increased after C-addition under some

species (Fig. 2b). Therefore, the building of N poorer tissue by plants in C-amended soils cannot result from a shortage in this preferable N source compared to the non-amended soil conditions. It rather implies a direct effect of C, likely in the DOC form, on plant N uptake and net metabolism, which agrees with results from hydroponic solutions or solid media *in vitro.* The studies show that increasing soluble C concentration reduce plant mineral uptake, alter gene expression of C and N metabolism, and ultimately increase C/N in tissues (Araya et al., 2010; Delhon et al., 1996; Goel et al., 2016; Gutiérrez et al., 2007; Hanisch ten Cate C.H., 1981; Lejay et al., 2003, 1999; Ma et al., 2018, 2017; Palenchar et al., 2004; Price et al., 2004; Thum et al., 2004). Therefore, the increased DOC originated from enzymatic cleavage of added complex C substrate to the soil (Fig. 1a, b) could be responsible for the increase in tissue C/N in our case. This effect was significant in small (slow-growing) plants but not in Lope (species with the largest biomass) (Fig. 2c), probably because the strong N limitation of Lope had a definitive effect on N stoichiometry due to the lowest NO_3^- availability, overlapping the effect of DOC. In summary, our results suggest that not only the N availability but also DOC concentration strongly influenced plant traits. However, the effect of DOC should be less evident with increasing N limitation. The high DOC input induced higher N use efficiency in plants and microorganisms (low CUE), while plant productivity was kept similar to control conditions.

Foliar isotopic composition reflects N-NO_3^- depletion better than changes in tissue C/N stoichiometry

Fast plant growth rate producing more biomass resulted in lower leaf $\delta^{13}C$ and $\delta^{15}N$ compared to slow growth (Fig. 6). We attribute it to the intensified depletion of mineral N from the system by larger plants (Fig. 3). The common cause is supported by the correlation between $\delta^{13}C$ and $\delta^{15}N$ (Fig. S3). Plant N limitation can reduce plant stomatal conductance that results in more negative $\delta^{13}C$ (Flanagan and Farquhar, 2014; Hou et al., 2015; Tang et al., 2019). The negative relation of foliar $\delta^{15}N$ with plant biomass has been reported before (Araus et al., 2013; Raimanová and Haberle, 2010; Robinson et al., 2000; Serret et al., 2018; Yousfi et al., 2012). This is attributed to increased N demand of plants with larger biomass, commonly connected with building N poorer tissues (Ariz et al., 2015; Tcherkez, 2011). It agrees with our results on a range of species differing in biomass production and tissue C/N ratio (Fig. 2 and 5). The small, slow-growing conservative plants responded to the soil C-addition distinctly by an increase in tissue C/N (Fig. 2b) and foliar $\delta^{15}N$ (Fig. 5a), while the largest plants did not react et al. The pronounced response of small plants corresponds well with the significant enhancement of NO_3^- concentration in their rhizospheres in the C-amended versus non-amended conditions, which did not occur under the largest plants. We thus suggest that small plants mainly faced an effect of high DOC concentration, which was superimposed by the effect of strong N limitation in case of the largest plants. The changing NO_3^- availability in the soil was well reflected by foliar $\delta^{15}N$ but not by $\delta^{13}C$ (Fig. 5 e,f). From this view, the foliar $\delta^{15}N$ was more sensitive to plant-soil interactions than $\delta^{13}C$ showing its higher potential as indicator of soil nutritional conditions.

In connection to the plant demand, we propose $\delta^{15}N$ of leaves is altered by differential exportation of NO_3^- from leaves to phloem, and its losses via exudation.

NO_3^- remobilization from leaves to roots has a signaling function, e.g. regulating N distribution in shoots and roots (Cui et al., 2020, 2019b, 2019a). Similarly as phloem NO_3^- load correlates with its availability in plant (Cui et al., 2020), we found that NO_3^- exudation positively correlated with N concentration in plants (Fig. 3). Smaller plants with lower N demands had more NO_3^- available in plant-soil system than fast-growing plants, having higher N uptake due to larger biomass and thus lower overall nitrate availability (the main gradient in Fig. 3). The resulting shortage of NO_3^- in plant-soil system resulted in lower phloem NO_3^- load and lower observed NO_3^- exudation (Fig. 3). Wilson et al. (2011) suggest that phloem sap is composed of enriched NO_3^- and depleted organic N. The NO_3^- is the most ^{15}N enriched (up to 55‰) N form in the plant (Cui et al., 2019a), in consequence of the isotopic fractionation during nitrate reduction (~16 ‰)): depleting the products (organic N) and enriching the remnant (not used NO_3^-) (Evans, 2001; Karwat et al., 2019; Tcherkez and Hodges, 2008; Yoneyama et al., 2003). We propose that smaller plants store in leaves more NO_3^- because there is more NO_3^- available, which increases their foliar $\delta^{15}N$. The leaf $\delta^{15}N$ of large plants is more similar to the $\delta^{15}N$ of the organic N. The difference in $\delta^{15}N$ between soil N (4 ‰) and plant N (-11‰) was 15‰ in large plants, similar to the 16‰ fractionation during NO_3^- reduction to organic N (Cui et al., 2019b; Tcherkez and Hodges, 2008; Yoneyama et al., 2003).

The observed tight correlations among foliar $\delta^{13}C$, $\delta^{15}N$ (Fig. S4), plant biomass and soil NO_3^- availability likely arises from the prevailing N limitation of plant production in our experiment. The foliar ^{15}N depletion was tightly related to decreasing NO_3^- availability due to faster plant growth under both soil conditions (Fig. 5e). In comparison to the foliage C/N ratio, which accounts for all N forms in the leaf, the foliar $\delta^{15}N$ is sensitive to the concentration of both mineral N (enriched) and organic N (depleted), giving a more detailed picture of plant growth under N limitation. Since N limitation of plant production is common across grassland ecosystems (LeBauer and Treseder, 2008), our data suggest that foliar $\delta^{15}N$ can be used as a sensitive indicator of plant N limitation within the system. Increasing foliar $\delta^{15}N$ is a sign of a considerable NO_3^- storage in the foliage and thus of relatively lower N limitation of plant growth in comparison to plants with lower leaf $\delta^{15}N$ within an ecosystem. We further suggest that the coupling of foliar $\delta^{15}N$, plant biomass and NO_3^- availability could be lost, when plant productivity will be limited by nutrients other than N. The testing/verification of the hypothesis would represent a big tool in understanding plant-soil interactions.

Conclusion

We tested plant and microbial response to additional soil C-supply, which enhanced soil C/N ratio and stimulated microbial growth. Microbial community formed under C-amended conditions had higher C/N ratio and higher specific respiration. Plants growing in both C-amended and non-amended soils took up NO_3^- from the soil; the NO_3^- depletion increased with plant growth rate. All the species growing under conditions of high additional DOC in the C-amended soil built tissues of higher C/N. The close relations between plant growth rate and soil NO_3^- availability and the response to C-addition changed plant C and N metabolism, which was reflected well

in natural isotopic composition of leaves. Both the $\delta^{13}C$ and $\delta^{15}N$ declined with increasing plant biomass (faster growth rate) and indicated well declining availability of NO_3^-. The foliar $\delta^{15}N$ was more robust and sensitive indicator of N availability for plants than their tissue C/N, which failed in indication of lowering NO_3^- availability under high DOC conditions.

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Figure 1. Effect of C-addition to the soil on microbial DNA (a, b), DOC (c, d) and C/N of enzymatic activity (e, f) in non-planted (a, c & e) and planted soils (b, d & f).

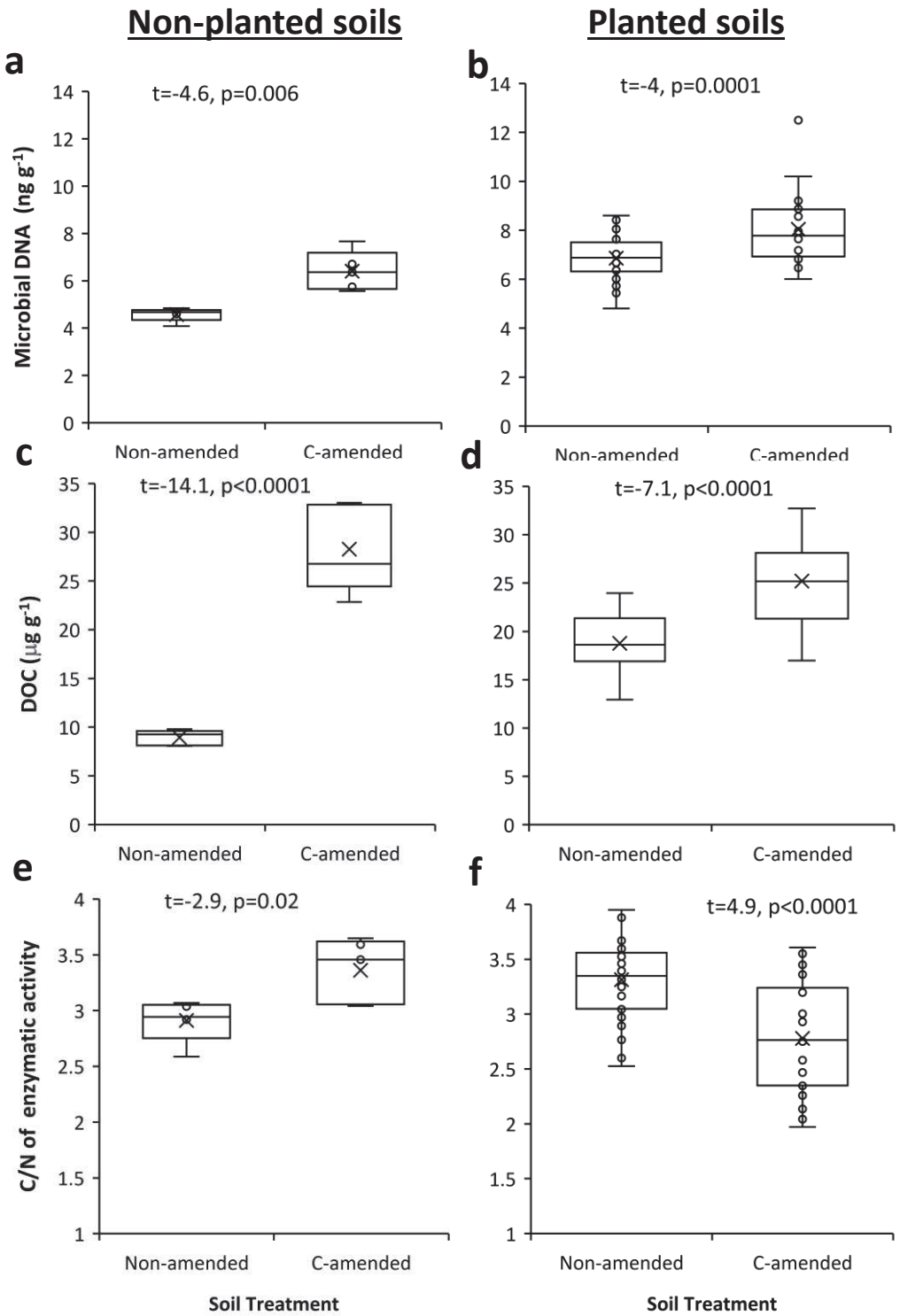


Figure 2. The effect of C-addition to the soil on plant biomass (a), C/N ratio of plant tissues (b) and nitrate availability (c) in the soils of eight plant species from conservative on the left to acquisitive on the right.

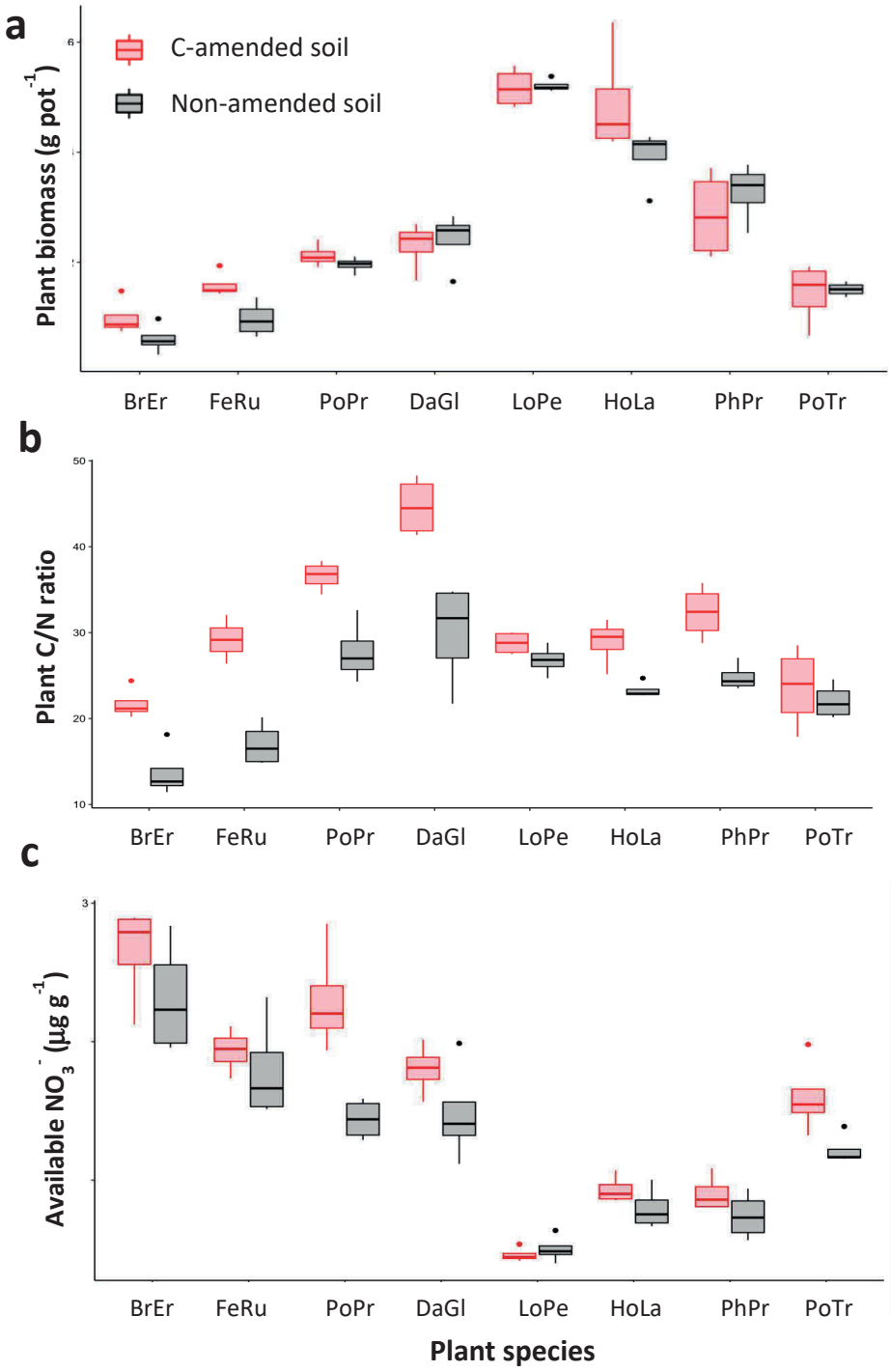


Figure 3. Relations of plant, soil solution and microbial variables in non-amended (a) and C-amended soil (b). contrib= contribution to the principal components (PC).

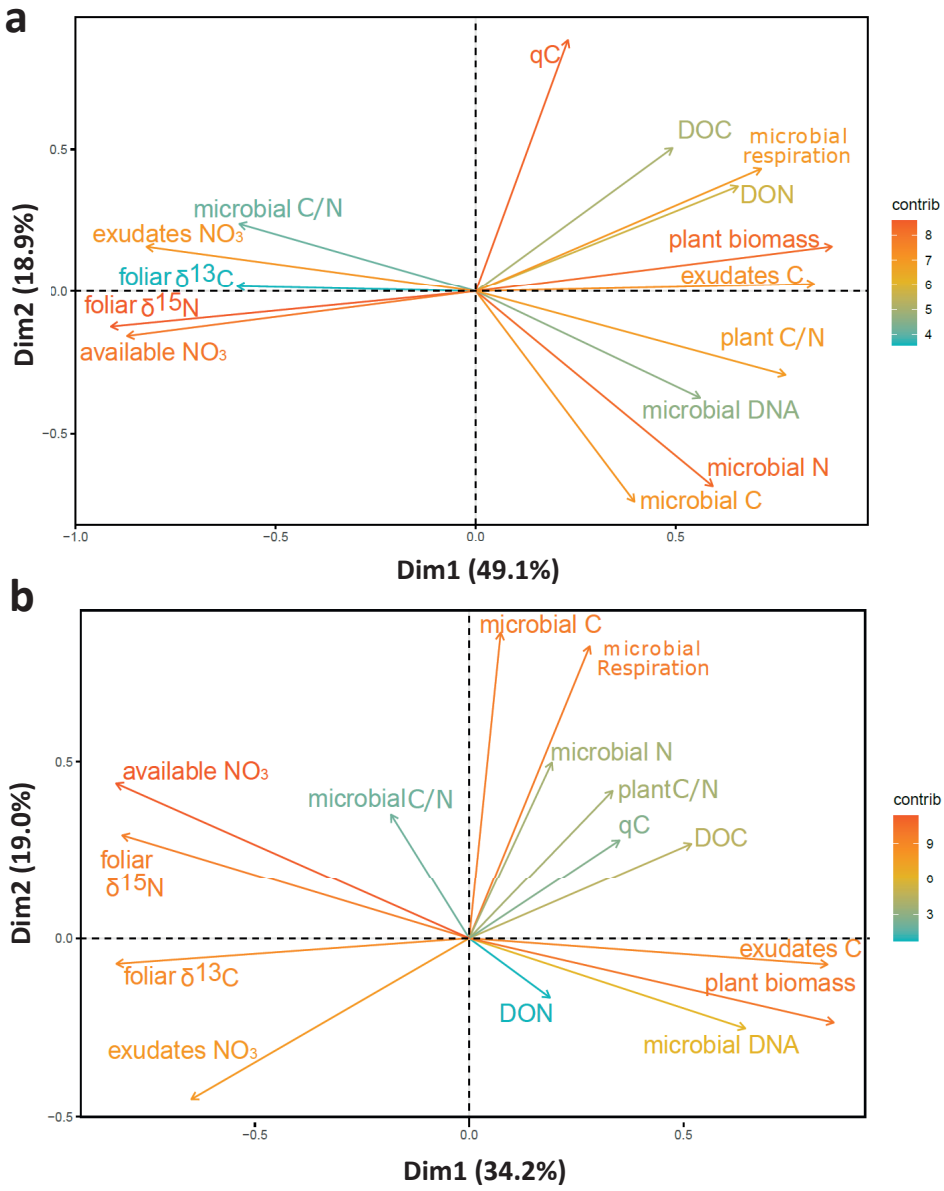


Figure 4. Relationships between microbial DNA and microbial biomass N (a) and specific respiration (b) in non-amended compared with C-amended soils (Pearson-r and significance of the correlations are shown).

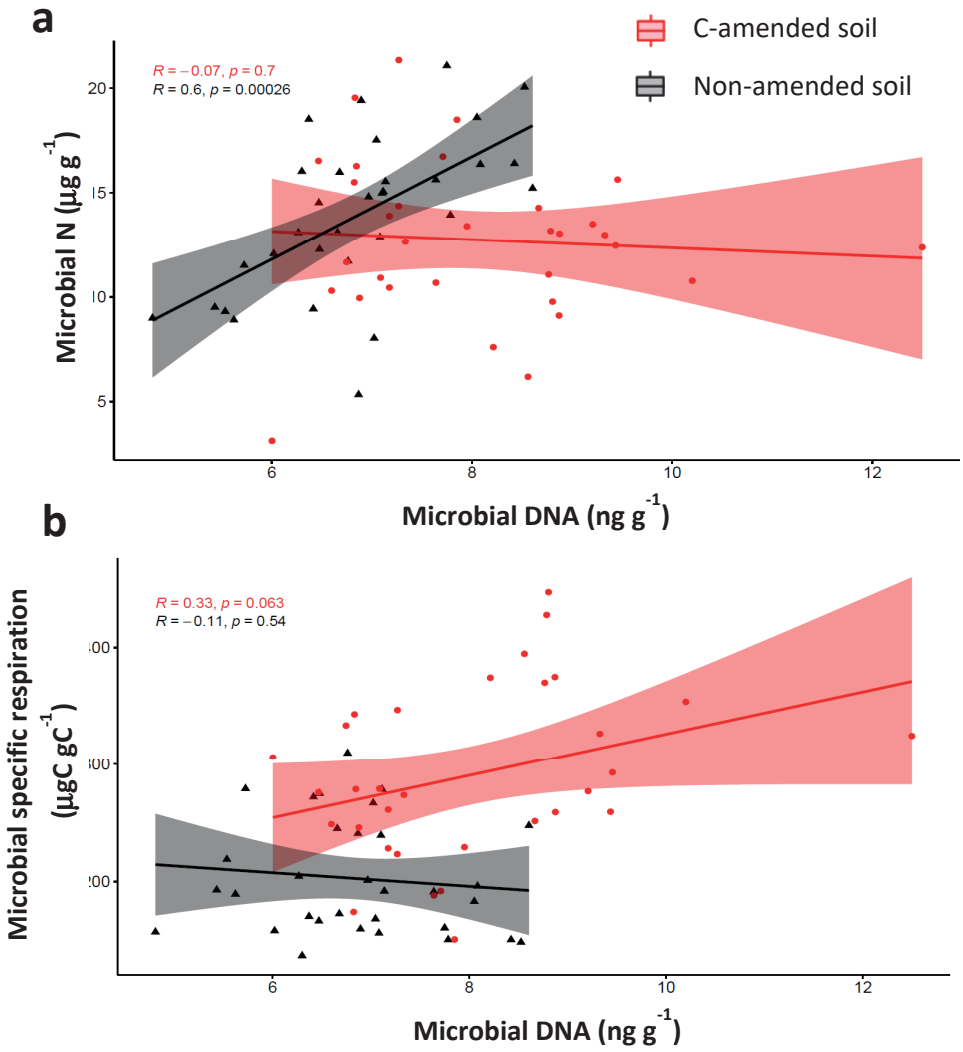


Figure 5. Relationships between foliar $\delta^{15}N$ and $\delta^{13}C$ and plant biomass (a, b), plant C/N ratio (c, d) and available N-NO_3^- (e,f) in non-amended compared with C-amended soils (Pearson-r and significance of the correlations are shown).

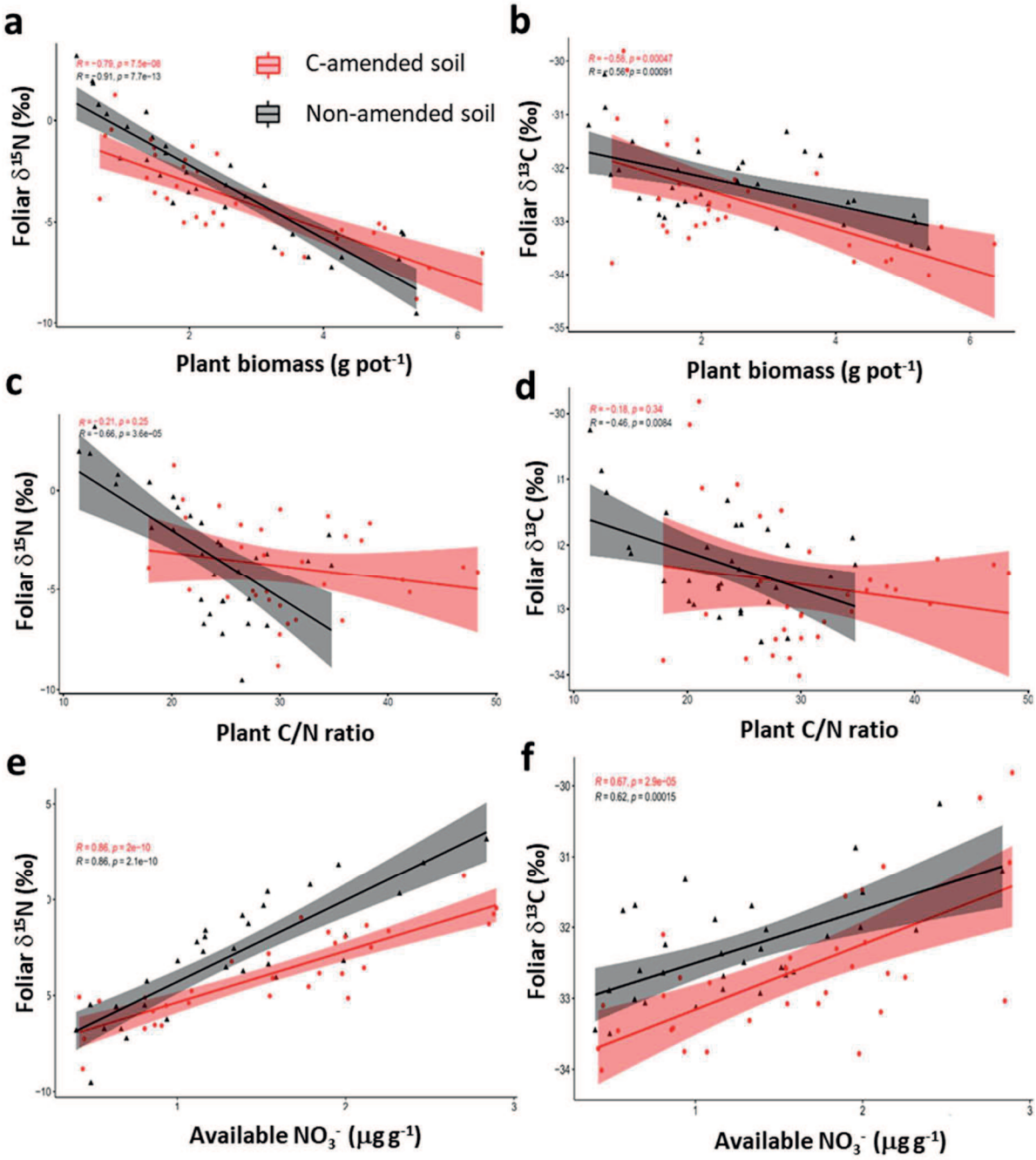


Figure S1. Effect of C addition to the soil on soil microbial N (a,b), microbial C/N ratio (c,d) and soil Ala-aminopeptidase poteantial activity (e,f) in unplanted (a, c & e) and unplanted soils (b, d & f).

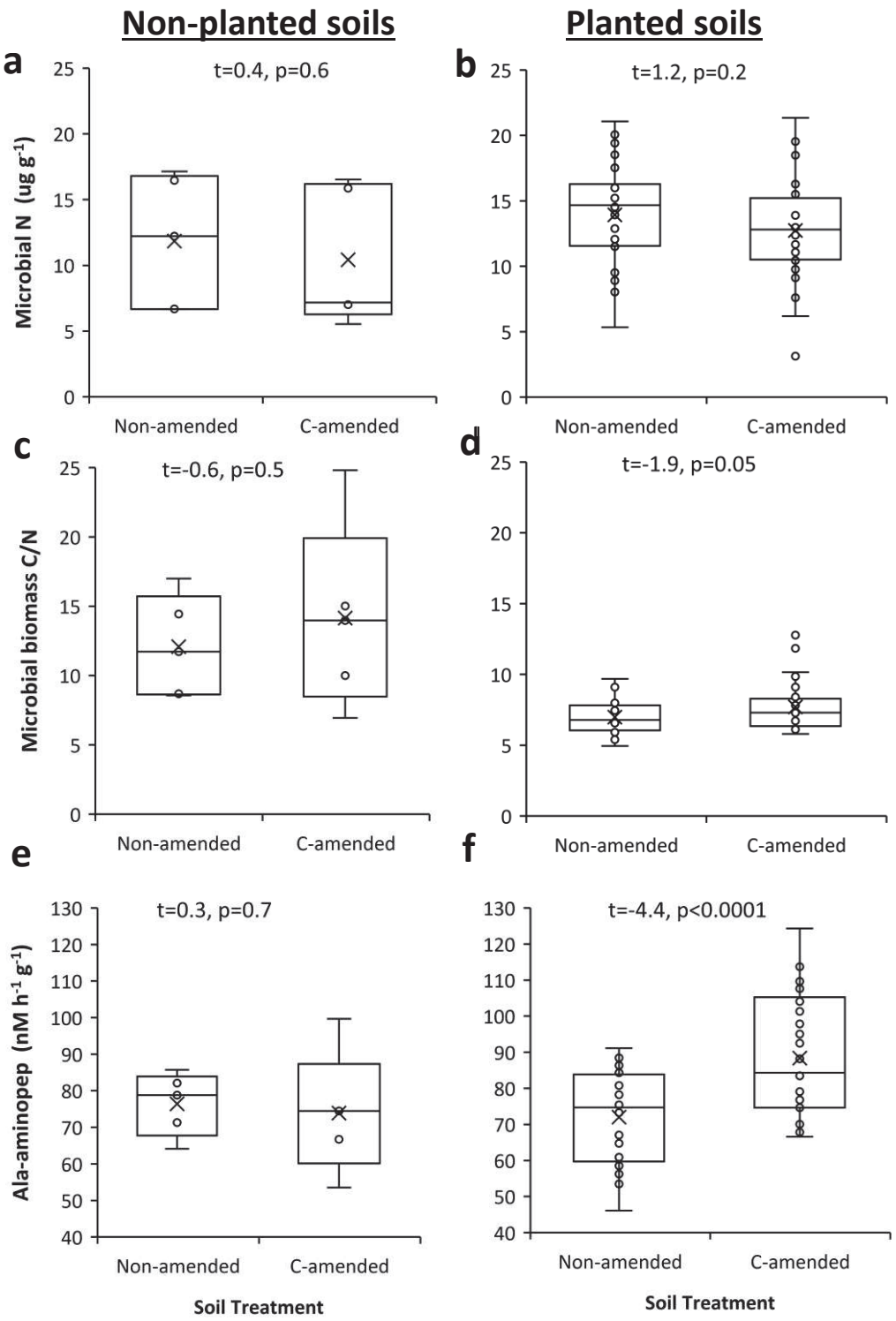


Figure S2. Effect of C addition to the soil on nitrate availability in planted (a) and non-planted soils (b).

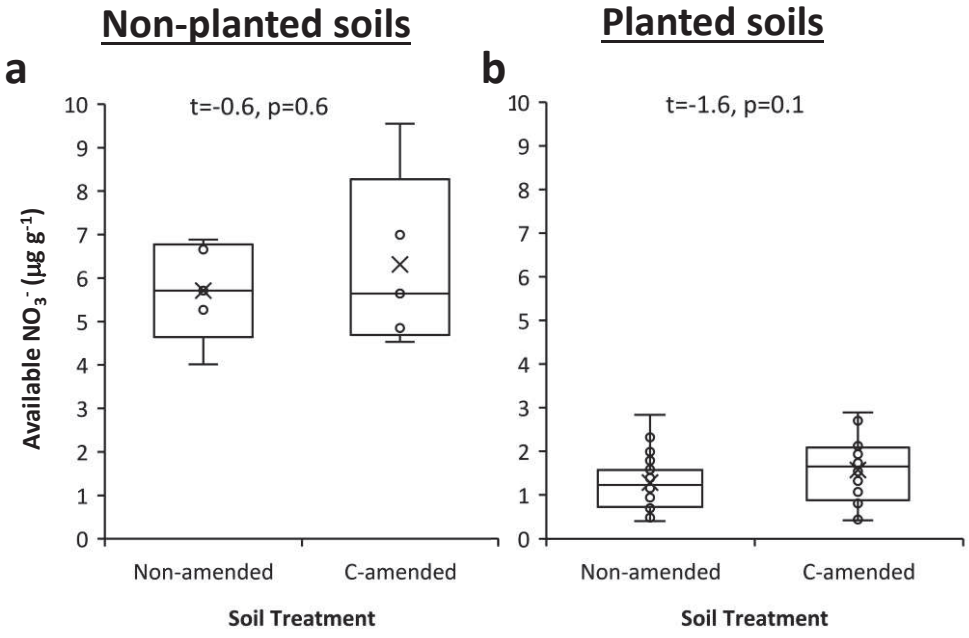


Figure S3. Pearson correlation analysis of nitrate availability and plant biomass in non-amended compared with C-amended soils

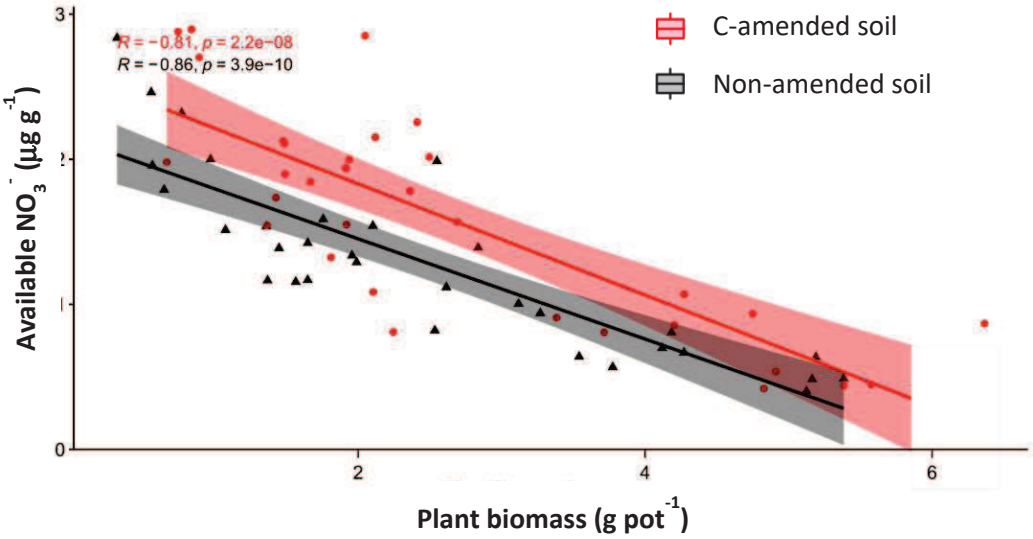
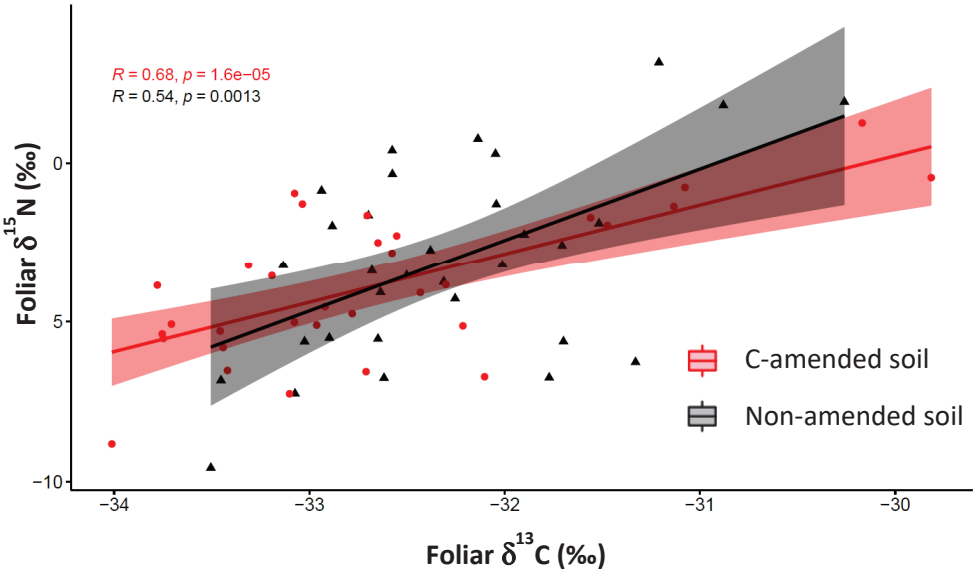


Figure S4 Pearson correlation analysis of foliar $\delta^{13}C$ and $\delta^{15}N$ in non-amended compared with C-amended soils



PAPER III

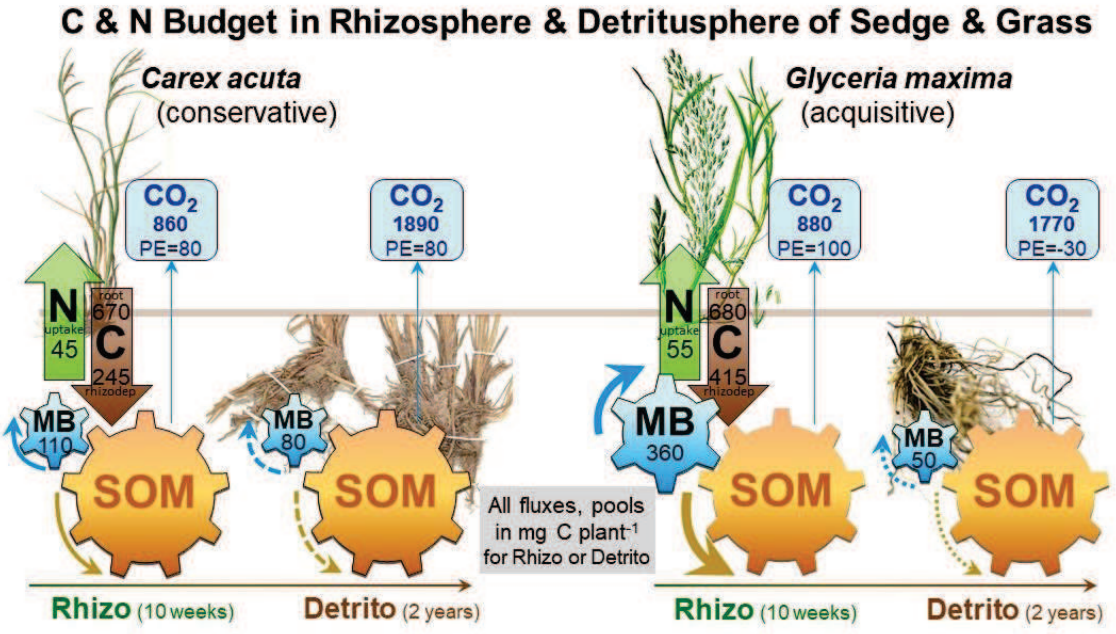
Priming effects in the rhizosphere and root detritusphere of two wet-grassland graminoids

Kaštovská, E., **Cardenas, J.**, Kuzyakov, J.

2021

Plant and Soil

Graphical abstract: A conceptual figure summarizing the differences in microbial transformation of fresh plant input and soil organic matter (SOM) in the rhizosphere and detritusphere of two graminoids in the context of their different functional traits. The lower rhizodeposition flux of the slower-growing conservative Carex acuta results in lower microbial biomass MB), whose activity leads to slower decomposition and mineralization of organic matter (lower rhizosphere priming effect, PE), and releases less available N. The roots of the conservative plant with a higher C/N ratio degrade more slowly, but the lack of N needed for microbial growth results in a stronger detritusphere PE. The opposite is true for the faster growing, acquisitive Glyceria maxima, whose rhizosphere and detritusphere are more dynamic environments where most C fluxes occur due to better decomposability and faster turnover of plant C inputs.



Plant Soil
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REGULAR ARTICLE

Priming effects in the rhizosphere and root detritusphere of two wet-grassland graminoids

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Abstract
Aims The rhizosphere and root detritusphere are hotspots of microbial activity, where root-derived inputs induce intensive priming effects (PE) on soil organic carbon (SOC) decomposition. These conditions for induced PE differ between rhizosphere and detritusphere and are modified by plant traits.
Methods Continuous labelling with ^{13}C-depleted CO_2 allowed for the partitioning of plant and soil C sources of CO_2 efflux and the investigation of the PE in the rhizosphere and detritusphere of slow-growing conservative *Carex acuta* and fast-growing acquisitive *Glyceria maxima.*
Results *Glyceria* allocated more C into the soil, induced higher microbial activity and a larger portion of active microorganisms, and depleted mineral N stronger than *Carex*. Its rhizosphere PE was 2.5 times stronger than that of *Carex*. Root residues (detritusphere) induced negative PE at the early stage of decomposition (1–9 months). The depletion of available organic substances in the detritusphere of more easily decomposable *Glyceria* roots resulted in positive PE after 3 months. The PE in the detritusphere of N-poorer *Carex* roots was more intensive but started after 9 months.
Conclusions The rhizosphere PE was positive and stronger than the detritusphere PE, which switched from initially negative to positive PE after depletion of available substances during few months. More productive species with faster N-uptake and higher belowground C input (here *Glyceria*) induce larger rhizosphere PE than slower-growing species (here

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Carex). The N-rich *Glyceria* roots decompose faster than N-poor roots of *Carex* and, consequently, have a lower impact on SOC dynamics and induced a smaller positive detritusphere PE.

Keywords Rhizosphere processes · Root decomposition · Nitrogen dynamics · Mechanisms of priming effects · Continuous ^{13}C labelling · Plant traits

Introduction

Plants create important hotspots of (micro)biological activity through the flux of fresh photosynthates from the living roots (Nguyen 2003; Jones et al. 2009; Pausch and Kuzyakov 2018) and via the carbon (C) fluxes arising from the decomposition of root litter (Poll et al. 2008; Gargaglione et al. 2019). Microbial processing of plant inputs forms new root-derived SOC (Rasse et al. 2005; Sokol et al. 2019). At the same time, root-derived C influences SOC mineralization: a phenomenon called the priming effect (PE) (Kuzyakov 2002a; Cheng et al. 2014; Shahzad et al. 2015). The PE induced by the root-derived C inputs ranges from a suppression of SOC decomposition by -50 % to its stimulation by +380 % (Cheng et al. 2014), with the positive PE being more frequently reported than the negative PE (Kuzyakov 2002a; Cheng et al. 2014; Shahzad et al. 2015). Considerable attention has been paid to the rhizosphere PE recently. Rhizodeposition and its impact on soil organic matter (SOM) mineralization is recognized as a key component of plant economic strategies (Guyonnet et al. 2018; Henneron et al. 2020a) allowing them to influence SOC dynamics (Han et al. 2020; Henneron et al. 2020a) and regulate nitrogen (N) and phosphorus cycling (Henneron et al. 2020b).

The knowledge about the detritusphere PE connected with the decomposition of dead roots is incomparably smaller, although the root decomposition itself is relatively well understood (Gill and Jackson 2000; Silver and Miya 2001; Freschet et al. 2012; Solly et al. 2014). The detritusphere PE may be of equal importance to SOC dynamics as the rhizosphere PE due to the large inputs of root litter (Rasse et al. 2005; Sokol et al. 2019), followed by a large C flush into the soil in the early phase of decomposition (Poll et al. 2008; Bastian et al. 2009; Mastný et al. 2018). Since the PE importantly affects SOC turnover and balance, greenhouse gas emissions and soil fertility (Fontaine et al. 2004; Finzi et al. 2015), both the rhizosphere and detritusphere PE and their controlling mechanisms need deeper understanding.

The interactive effects of C and N are often used to explain the general phenomenon of the PE. The principle is expressed in the N-mining Hypothesis (Craine et al. 2007; Chen et al. 2014). According to it, the abundant C inputs stimulate microbial growth and demand for N, which can be met by increasing enzymatic decomposition of SOM to access N contained within. The SOC mineralization (and PE) should increase with an increasing C supply and decreasing N availability (Fontaine et al. 2011). In these terms, the rhizosphere and root detritusphere represent complex natural environments, largely different in C inputs and availability of N influenced by the presence/absence of living plants. The rhizosphere constitutes a C-excessive, N-limited, intensely competitive environment (Kuzyakov and Xu 2013). A continual supply by rhizodeposits, predominantly low-molecular weight, energy-rich root exudates (Hinsinger et al. 2009; Jones et al. 2009) accelerates microbial growth and production of exoenzymes that break down SOM (Schimel and Weintraub 2003; Blagodatskaya and Kuzyakov 2008). At the same time, the available N pool is intensely depleted via root uptake. According to the N-mining Hypothesis, the large positive PE should occur in the rhizosphere, through which the N-limited microorganisms cover their nutrient demand. The SOC mineralization grows together with increasing C supply (Cheng et al. 2014; Zhu et al. 2014; Henneron et al. 2020a, b). Therefore, the plant species with inherently fast growth rates, having large plant biomass (Dijkstra et al. 2006; Huo et al. 2017), high mass-based photosynthetic capacity (Kuzyakov and Cheng 2001, 2004) and fast N uptake (Henneron et al. 2020b), are associated with larger exudate release (Guyonnet et al. 2018; Cardenas et al. 2021), soil microbial activity (Vale et al. 2005; Legay et al. 2014) and larger positive rhizosphere PE (Henneron et al. 2020a) than slow growing plants.

The detritusphere is formed around dead roots composed mainly of complex substrates, which decompose without any direct influence from living

plants (e.g. release of exudates and nutrient uptake). The fast early stage of root residue decomposition is accompanied by a large flush of easily decomposable substrates (Poll et al. 2008; Bastian et al. 2009; Mastný et al. 2018). It supports abundant microbial communities (Marschner et al. 2012; Shahzad et al. 2015) and stimulates their enzyme activities to a level comparable to the rhizosphere (Spohn and Kuzyakov 2014) or even higher (Ma et al. 2017). The residues are usually N-poor relative to microbial demands; microorganisms thus immobilize available N from their environment (Mooshammer et al. 2014b) and may promote N mining from SOM. However, there is no competitive pressure for N between plants and microorganisms in the detritusphere, so the induced PE should be less intensive than in the rhizosphere, or even negative. N-richer litter poor in lignin decomposes quicker relative to N-poor and lignified residues (Cornwell et al. 2008; Freschet et al. 2013), leading to the suppressed formation of new root-derived SOC (Henneron et al. 2020a) and fast C turnover (De Deyn et al. 2008; Schmidt et al. 2011). Whether the better decomposable residues induce larger PE due to the larger C flush or lower PE due to higher N content compared to less degradable roots has yet to be found.

Comparing the magnitude and dynamics of rhizosphere and detritusphere PE associated with species differing in functional traits may help to test the general validity of the N-mining Hypothesis, which has recently come into question (Mason-Jones et al. 2018). The forms and complexity of the substrates, metabolic pathways of their utilization (Mason-Jones et al. 2018), composition of microbial community and actual soil conditions (Lloyd et al. 2016) create unique environments. Microorganisms have several other mechanisms to cope with the stoichiometric C/N imbalance between their sources and requirements except the extra N mining from SOM (Mooshammer et al. 2014b). They include compositional changes within the community, changes in enzyme affinities and alterations of C and N use efficiencies (Mooshammer et al. 2014a), which must not result in the positive PE. It points to the fact that the PE is controlled by multiple interacting factors, which are not recognized yet.

We aimed to compare the PE in the rhizosphere and detritusphere induced by living and dead roots, respectively, and link the PE to plant functional traits. We ran a pot experiment with two graminoids differing in carbon economy, e.g. represented by growth rate, rhizodeposition and the root C/N ratio (based on our previous data from Kastovska and Santruckova 2011; Kastovska et al. 2014; Kastovska et al. 2017): slow-growing conservative *Carex acuta* with lower rhizodeposition input and larger root C/N and fast-growing acquisitive *Glyceria maxima* with larger rhizodeposition input and lower root C/N (hereinafter referred to as *Carex* and *Glyceria*). Due to its faster growth rate and the formation of N-richer roots, the actual demand for N is higher for *Glyceria* than *Carex*, thus meaning more vigorous competition for N with rhizosphere microorganisms (Knops et al. 2002; Kastovska and Santruckova 2011). To monitor C released by living and dead decomposing roots, as well as to distinguish it from the SOC, we continuously labelled plants in the $^{13}CO_2$ depleted atmosphere during their 10-week growth. Additionally, both plants were foliar N fertilized to stimulate plant growth, without a direct effect of N fertilizer on soil processes. Using this method, we aimed to extend a range of quantitative and qualitative differences in root-derived C entering the soil and, consequently, in microbial activities in the rhizosphere and root detritusphere.

We hypothesize that (H1) both fluxes from living and decomposing roots will stimulate SOC decomposition. The rhizosphere PE will be more substantial than detritusphere PE and will increase with plant growth. The detritusphere PE will be lower and will occur only during the initial rapid decomposition of fresh root residues. Then it will disappear. We tested H1 by assessing the rhizosphere PE after 4 and 10 weeks of plant growth and the detritusphere PE at 0.5, 1, 3, 9, 16 and 24 months after shoot cutting. We further hypothesize that (H2) *Glyceria* will increase the rhizosphere PE more than *Carex* because of the greater N requirements and a larger rhizodeposition (Kastovska et al. 2014). However, its N-richer root residues will induce lower PE in the detritusphere compared to *Carex*. (H3) The foliar N fertilization will enhance plant growth and, consequently, rhizodeposition flux but will not increase plant N uptake from the soil, which will modulate the rhizosphere PE associated with both species. We tested H2 and H3 by analyzing plant biomass, its nutrient content and soil characteristics: the amount of root-derived C,

microbial biomass, soil CO_2 efflux and their $\delta^{13}C$, using destructive samplings after the 4th and 10th weeks of plant growth and relate the measures to the PE values.

Materials and methods

Two graminoids in the study and their planting

As previously mentioned, our study involved two common graminoid wet meadow species: *Carex* and *Glyceria*. Seedlings of *Glyceria* and *Carex* were sampled by spade from an unfertilized, regularly mown wet grassland located in the Třeboň Basin Biosphere Reserve, Czech Republic, at the beginning of the vegetation season (April 15, 2017). Plants from sampled turfs were separated into individuals, washed with tap water to remove soil particles, and 36 individuals of each species of similar biomass (three leaves, height of 6–7 cm) were chosen. Their shoots and roots were reduced by cutting with scissors to 3 cm before planting.

The selected individuals of *Glyceria* and *Carex* were planted into pots (1 plant per pot, diameter 8 cm, height 15 cm, with a perforated bottom) into a soil/sand mixture (800 g). The soil was sampled from the same location as the plants, sieved (5 mm mesh size) and mixed with sand in a 3:1 ratio, which enabled easier separation of roots during destructive samplings. The soil mixture had the following characteristics: organic C (SOC) of 32.0±2.7 g kg^{-1}, total N of 2.4±0.2 g kg^{-1}, total P of 0.5±0.05 g kg^{-1}, pH 5.43±0.06 (measured in soil:water solution, 1:5). The contents of water soluble nutrients, available to plants were following: ammonium-N of 6.4±1.9 mg kg^{-1}, nitrate-N of 9.1±1.0 mg kg^{-1}, soluble reactive P of 0.13±0.01 mg kg^{-1}.

General experimental design and timetable: The rhizosphere and detritusphere periods

The experiment included 2 species x 3 foliar N treatments x 3 destructive samplings (2 rhizosphere and 1 detritusphere) with 4 replicates, being 72 planted pots in total and 12 additional unplanted control soils (4 replicates x 3 destructive samplings). Plants were grown for 10 weeks (from mid-April to the end of June), which represented here the rhizosphere period. We took two destructive samplings, which were always preceded by the measurement of the soil CO_2 efflux: after 4 weeks in a stage of active plant growth and after 10 weeks of labelling at the time of maximum biomass. After 10 weeks, the remaining pots were treated as follows: we cut the shoots and let roots decompose for 2 years, which represented the detritusphere period here. The pots were incubated at 20 °C in the dark and regularly watered to keep the soil at the field water holding capacity (checked by weighing). During the detritusphere period, we measured the soil CO_2 efflux after 14 days, then 1, 3, 9, 16 and 24 months. The last measurement was followed by

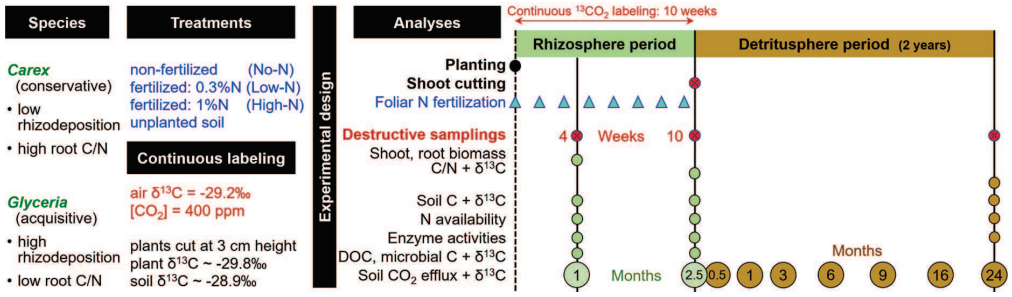

Fig. 1 Design of the experiment with two species, *Carex* and *Glyceria*, which were continuously labelled with ^{13}C-depleted CO_2 for 10 weeks and repeatedly foliar-fertilized with three doses of N (urea solution, Δ). Timing of the treatments and particular analyses done in-between and after (three) destructive pot samplings are marked with o

destructive sampling of the pots. The experimental design is depicted in Fig. 1.

Experimental conditions and set-up for continuous $^{13}CO_2$ plant labelling

The planted pots (36 per species) together with 12 unplanted ones were placed in a plexiglass chamber (100×60×70 cm) with the lower edge immersed in 5 cm of water in a tray (110×65×7 cm); the pots were immersed in the water to a height of 1 cm, maintaining the high moisture preferred by the species. The chamber with 3 metal inflows and 3 outflows was coupled to a facility for continuous $^{13}CO_2$ production. We used ^{13}C-depleted air produced by continual injection of fossil-fuel derived CO_2 ($\delta^{13}C$=-29.20±0.15‰) in CO_2-free air to reach ambient CO_2 concentration of 400 ppm. The CO_2-free air was produced with a compressor injecting ambient air into a molecular sieve (Siliporite® beads from zeolite, 3 Å), which removed the CO_2. The chamber with pots was continuously supplied with re-humidified ^{13}C-depleted air for 10 weeks. The flow was adjusted to renew the chamber volume every 2 min in order to maintain constant CO_2 concentration and its $\delta^{13}C$ (Cros et al. 2019). The chamber was located in an air-conditioned glasshouse (day temperature 22 °C for 16 h, night temperature 10 °C for 8 h). The air flow through the chamber also helped to maintain the inside temperature close to glasshouse conditions. A 10-week continuous labelling of plants using ^{13}C-depleted CO_2 decreased the $\delta^{13}C$ of the shoots and roots' biomass from their original values (-29.88±0.54‰ for shoots and -28.94±0.50‰ for roots, n=8) by *approx.* 11‰ in roots and 12–13‰ in shoots (Supplementary Table 1).

Foliar N fertilization

The plants were repeatedly treated by foliar N fertilization with urea at concentrations 0% (distilled water control), 0.3% and 1% solution at four replicates. The fertilization was done by manual brushing on all the leaves. During brushing, the soil was covered with a foil to prevent any N contamination. Plants were fertilized at the start of the experiment and then repeatedly at 10-d intervals. The foliar fertilization aimed at stimulating plant growth and potentially shifting the biomass C/N ratio while maintaining the same initial conditions of soil N availability. Maintaining this design, any changes which we find in the soil can be ascribed to plant activities. The fertilizer application on leaves was tested prior the experiment, and the doses not damaging leaves were chosen.

Measuring total soil CO_2 efflux for assessing rhizosphere and detritusphere PE

The soil CO_2 efflux from the pots to assess the rhizosphere PE was measured twice: after 4 weeks and 10 weeks of labelling. The detritusphere PE was assessed after shoot cutting: after 14 days then 1, 3, 9, 16 and 24 months. The incubations of pots with living plants were always done in mid-afternoon, allowing plant photosynthesis for most of the daylight, which supplies the rhizosphere with C (Kuzyakov and Gavrichkova 2010). The incubations of pots with decomposing roots were done in the morning. Four pots per species and N fertilization level were measured. In pots with plants, we first packed shoots carefully into a plastic bag which was sealed airtight just above the ground with silica tape and a rubber band. The pots treated in this way were closed in opaque airtight chambers, which were thoroughly ventilated with ambient air for 1 min just before closing; the pots with cut shoots were incubated the same way. Then we sampled ambient air (initial conditions) and the air from the chamber headspace after 40 min of incubation at 22 °C to measure CO_2 concentration and its $\delta^{13}C$ using gas chromatography (TCD detector, Agilent 6850 GC System, USA) and GasBench II connected with IRMS Delta X Plus (both Finnigen, Germany), respectively.

Analyses of plant and soil samples from the destructive samplings

Total C contents, C/N ratio and $\delta^{13}C$ of shoot, root and soil

After measuring the respiration, we cut the shoots and separated roots from the soil (by hand and tweezers) and homogenized the soil. We are aware that homogenization of all soil from the pot could reduce the rhizosphere effect. However, because the soil in the pots was relatively densely rooted already after four weeks of plant growth, the homogenization was chosen as the best approach for quantification of the total

amount of root-derived C. Similarly, the soil from the pots incubated for two years after shoot cutting was homogenized and cleaned from remaining roots, the biomass of which was negligible. All shoot and root materials (when present) and a part of the soil were dried at 60 °C for 48 h, and the shoot and root biomasses were weighed. The plant and soil materials were milled and analyzed for C, N and $\delta^{13}C$ on NC Elemental analyzer (ThermoQuest, Germany) connected to an isotope ratio mass spectrometer (IR-MS Delta X Plus, Finnigan, Germany).

Soil microbial C, dissolved organic C and their $\delta^{13}C$, soil N availability

The content of the soil microbial biomass C (Cmic) was analyzed using the chloroform fumigation-extraction method (Bruulsema and Duxbury 1996) with slight modifications. Briefly, subsamples (10 g of fresh soil) were extracted with 50 mM K_2SO_4 (20 ml) for 30 min either before or after fumigation with amylene-stabilized chloroform (24 h) to obtain the dissolved and chloroform-labile C pools. The extracts were filtered, and the total N and organic C concentrations were analyzed on a TOC-L analyzer equipped with the total N measuring unit TNM-L (Shimadzu, Japan). The C content in the non-fumigated extract represented dissolved organic C (DOC), and microbial C (Cmic) was calculated as a difference between C concentrations in the fumigated and non-fumigated extracts. The freeze-dried extracts were analyzed for $\delta^{13}C$ using an isotope ratio mass spectrometer. The $\delta^{13}C$ values obtained for the soil, DOC and Cmic were used to calculate the portion of SOC-derived and root-derived C in the pools. The extract from non-fumigated soils were further analyzed for the ammonium and nitrate concentrations using FIA (FIA Lachat QC8500, Lachat Instruments, USA). The amounts of dissolved N forms enabled a comparison of the actual N availability between species*fertilization treatments, in particular sampling times.

Activity of hydrolytic and oxidative exoenzymes

Extracellular hydrolases activities were determined by a microplate fluorometric assay. One gram of fresh soil was suspended in 100 ml of distilled water and sonicated for 4 min. Soil suspension (200 µl) was amended by 50 µl of methylumbelliferyl (MUF) substrate solution for β-glucosidase (BG), exocellulase (CEL), phosphatase (PHO) and chitinase (CHIT) determination or by 50 µl of 7-amine-4-methylcoumarin substrate solution for Leucine-aminopeptidase (LEU) determination (Barta et al. 2014). Three concentrations of each substrate were pre-tested (50, 100 and 300 µM) and the concentration with the highest enzymatic activity (substrate-saturated enzyme) was selected for each enzymatic assay. Plates were incubated at 20 °C for 120 min. Fluorescence was quantified at an excitation wavelength of 365 nm and an emission wavelength 450 nm (Infinite F200 Microplate Reader, TECAN, Germany). We calculated the % C-enzymatic activity (BG+CEL), N-enzymatic activity (LEU+CHIT) and P-mining (PHO) (Sinsabaugh et al. 2009), which are used to compare relative investments to C *versus* N and P mining associated with the species. The activities of oxidases were measured to characterize the soil potential for decomposition of phenolic and lignin-like compounds. Activities of peroxidases (PerOX) and phenoloxidases (PhOX) were determined photometrically using L-3,4-dihydroxyphenylalanine substrate and L-DOPA, with and without the addition of H_2O_2, respectively (Barta et al. 2010). The substrate oxidation was immediately measured as well as after incubation for 13 h at 20 °C photometrically as an absorbance at 450 nm. Finally, we calculated a sum of activities of hydrolases and of oxidases.

Isotopic partitioning of C sources in the soil fluxes and pools, and calculation of priming effects

The continuous labelling of plants with ^{13}C-depleted air allowed for the partitioning of the SOC-derived and root-derived sources into the soil C pools. For the soil C (and for DOC and Cmic), we used the following equations (calculated for each pot separately):

$$C_{SOC} = C_{total} \times \frac{\delta^{13}C_{root} - \delta^{13}C_{total}}{\delta^{13}C_{root} - \delta^{13}C_{SOC}} \quad (1)$$

$$C_{root} = C_{total} \times \frac{\delta^{13}C_{SOC} - \delta^{13}C_{total}}{\delta^{13}C_{SOC} - \delta^{13}C_{root}} \quad (2)$$

where C_{SOC} refers to the C from the native SOC and C_{root} to the "new" C released to the soil from roots as rhizodeposition during the rhizosphere phase and from

root decomposition during the detritusphere phase. The C_{total} and $\delta^{13}C_{total}$ are the total soil organic C amounts and their $\delta^{13}C$ measured in the respective planted pots; $\delta^{13}C_{SOC}$ refers to the isotopic composition of natural SOC in control, unplanted soils (-28.91±0.02‰, mean±standard deviation, n=8, samplings in the 4th and 10th week). The mean values of $\delta^{13}C_{total}$ for species x fertilization treatments are in Supplementary Table 2. The $\delta^{13}C_{root}$ refers to the mass-weighted $\delta^{13}C$ of the shoot and root biomass calculated for particular pots (according to Henneron et al. 2020a), which ranged between -37.20‰ and -43.30‰ during the rhizosphere period (Supplementary Table 1). This is based on the assumption that the rhizodeposition is fuelled by recent assimilates-exudates and root lysates (Jones et al. 2009; Kastovska et al. 2017). The equations were analogically used for the DOC partitioning, using measures for DOC rather than for C.

Cmic and its isotopic signature were calculated as follows:

$$Cmic = C_{fumigated} - DOC \quad (3)$$

$$\delta^{13}Cmic = \frac{(C_{fumigated} \times \delta^{13}C_{fumigated}) - (DOC \times \delta^{13}C_{DOC})}{Cmic} \quad (4)$$

where DOC and $\delta^{13}C_{DOC}$ refer to the amounts and isotopic composition of dissolved organic C (C extracted from non-fumigated soil by 0.05 M K_2SO_4), while $C_{fumigated}$ and $\delta^{13}C_{fumigated}$ to the values obtained for the chloroform-fumigated soil. The SOC- and root-derived parts of Cmic were further calculated analogically using modified Eqs. 1 and 2.

When we partitioned sources into the soil respiration, we first corrected the amount and $\delta^{13}C$ of the total soil CO_2 efflux (R_{total}) for background atmospheric CO_2 using Eqs. 5 and 6 and then separated the root-derived (R_{root}) from the SOC-derived CO_2 efflux (R_{SOC}, SOC decomposition) using the following two-source isotope mixing Eqs. 7 and 8:

$$R_{total} = CO_2 inchamber - CO_2 inair \quad (5)$$

$$\delta^{13}C_{Rtotal} = \frac{(CO_2 inchamber \times \delta^{13}CO_2 inchamber) - (CO_2 inair \times \delta^{13}CO_2 inair)}{R_{total}} \quad (6)$$

$$R_{SOC} = R_{total} \times \frac{\delta^{13}C_{root} - \delta^{13}C_{Rtotal}}{\delta^{13}C_{root} - \delta^{13}C_{SOC}} \quad (7)$$

$$R_{root} = R_{total} \times \frac{\delta^{13}C_{SOC} - \delta^{13}C_{Rtotal}}{\delta^{13}C_{SOC} - \delta^{13}C_{root}} \quad (8)$$

where R_{total} and $\delta^{13}C_{Rtotal}$ are the total soil CO_2 efflux from the planted pots and its measured isotopic composition. For $\delta^{13}C_{root}$ value see above. R_{SOC} and $\delta^{13}C_{SOC}$ are the CO_2 flux and its $\delta^{13}C$ from native SOC mineralization from unplanted pots (mean $\delta^{13}C_{SOC}$ -25.69±0.63‰, data in Supplementary Table 5). This value was *approx*. 3.2‰ enriched in comparison of the $\delta^{13}C$ of the natural SOC (-28.91±0.02‰), showing a fractionation during native SOC mineralization, which remained stable during the experiment's duration. The value is in the range published in Santruckova et al. (2000). The actual rhizosphere or detritusphere PE was calculated by subtracting the mean CO_2 efflux from unplanted pots (n=4) from the R_{SOC} calculated for each planted pot at a particular sampling time.

At the end of experiment (after 2 years of root decomposition), root-derived C contribution to the soil C was calculated using Eq. 2, where $\delta^{13}C_{root}$ was represented by the values from the destructive sampling in the 10th week (data in Supplementary Table 1). The values were used to estimate the total amounts of root-derived C mineralized during the detritusphere period (Table 4).

Statistical analyses

All data were checked for normality and homoscedasticity and log transformed, if necessary, to improve the normality of the residuals. We used general linear models (GLM) to assess the species (*Carex* and *Glyceria*) and fertilization (no-N, low-N, high-N) effects and their interactions on plant characteristics (shoot and root biomass, C/N ratios, $\delta^{13}C$), on the amount of root-derived C and its fate in the soil (recovery in DOC, Cmic, soil CO_2 efflux), and on the SOC-derived soil CO_2 efflux and the PE. Time was treated as a random factor (covariate) in the models. A Tukey-HSD post-hoc test followed, where significant effects were found. We calculated a matrix of correlation of measured plant and soil variables with the soil CO_2 fluxes and PE at particular sampling times (using Pearson correlation) to identify significant relations. In the case of identified predictors, we show fitted linear regressions characterized by R^2 and *p* values (shown in figures). Statistical tests were

considered significant at $p<0.05$. All the analyses were done in Statistica 13 (StatSoft, USA).

Results

Differences between species in biomass characteristics and soil properties

Both species markedly increased their root and shoot biomass and their C/N ratios from the 4th to 10th week (Fig. 2) and consistently maintained the following species-specific differences. *Glyceria* had approx. 21 % larger shoot biomass with about a 20 % higher C/N ratio but roots with a lower C/N ratio than *Carex* (the root C/N of 48 and 65 for *Glyceria* and *Carex*, respectively, Fig. 2). The root biomass of both species was comparable. Foliar N fertilization did not affect plant growth but decreased C/N ratio of shoots (by more than 30 % for *Carex* and 10 % for *Glyceria*), while root C/N remained unaffected (Fig. 2).

Soils with *Carex* and *Glyceria* contained similar Cmic and DOC amounts (Table 1) and showed comparable potential activities of hydrolytic and oxidative enzymes (Table 2). DOC and Cmic decreased during plant maturation from the 4th to 10th week (Table 1), while hydrolytic and oxidative enzymatic activities remain stable (Table 2). Mineral N (ammonium and nitrate) strongly decreased during plant growth (Table 1), which reflects an intensification of competition for N between roots and microorganisms. Soils under the *Glyceria* were poorer in nitrate and showed elevated levels of investments into N mining compared to *Carex*, which points to increased N limitation in the plant-microbial system. *Glyceria* was further associated with a lower ratio of hydrolytic to oxidative activities than *Carex*, which indicates decomposition of more complex substances (Table 2). Foliar N fertilization further increased the activities of all hydrolases (BG, CEL, PHO, CHIT) except for the purely N-mining LEU (Supplementary Table 3).

Differences between species in root-derived C in the rhizosphere and its recovery in DOC and Cmic

The amount of root-derived C in the soil increased from *approx.* 225 µg of C g^{-1} to approx. 425 µg of C g^{-1} between the 4th and 10th week; it was higher in *Glyceria* than *Carex* soil ($p<0.001$) and increased with foliar N fertilization ($p<0.001$) (Fig. 3a). The root-derived C recovered in the soil was positively correlated with the shoot biomass increasing from the the 4th to 10th week (Fig. 3d). The final amounts of root-derived C in the soil in the 10th week was negatively correlated to the root C/N ratio of mature plants (Fig. 3e).

The amount of root-derived C in the microbial biomass increased during plant growth (Fig. 3b), while

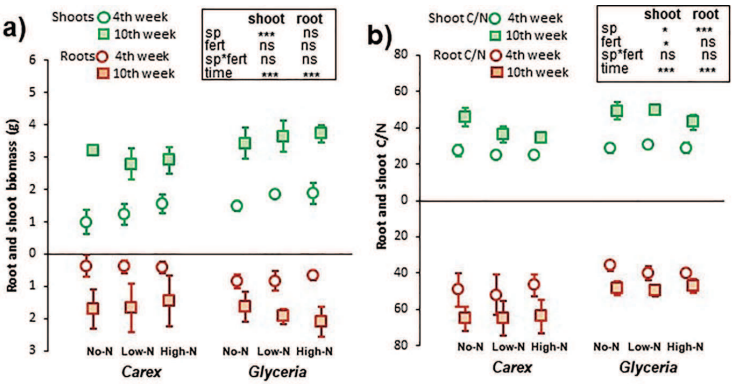

Fig. 2 Comparison of shoot and root biomass (**a**) and their C/N ratios (**b**) of *Carex* and *Glyceria* growing for 4 and 10 weeks and fertilized with increasing N doses. Means and standard deviations (n = 4) as well as results of GLM models assessing the effects of species, fertilization, their interactions, and time are shown. GLM results are presented in the box at the top and denote: sp: species, fert: fertilization, sp*fert: interactions, and time: time effect (4 and 10 weeks)

Table 1 Content of dissolved organic C (DOC), microbial C (Cmic) and of ammonium and nitrate-N in the soils with *Carex* and *Glyceria* subjected to three foliar N fertilization doses found in rhizosphere in the 4th and 10th week

Rhizosphere 4th week		DOC (μg g^{-1})		Cmic (μg g^{-1})		NH_4^+ (μg N g^{-1})		NO_3^- (μg N g^{-1})	
Carex	No-N	213 ± 20		604 ± 32		1.32 ± 0.37		1.63^{b} ± 0.81	
	Low-N	190 ± 19		420 ± 59		1.40 ± 0.16		1.01^{ab} ± 0.15	
	High-N	200 ± 24		549 ± 110		2.32 ± 0.96		1.52^{b} ± 0.63	
Glyceria	No-N	184 ± 21		511 ± 90		1.68 ± 0.32		1.52^{b} ± 0.60	
	Low-N	207 ± 13		499 ± 89		1.34 ± 0.19		0.87^{a} ± 0.08	
	High-N	184 ± 16		481 ± 66		1.39 ± 0.25		0.95^{a} ± 0.14	
Soil		181 ± 21		635 ± 50		6.43 ± 1.89		9.08 ± 0.99	
Rhizosphere 10 week									
Carex	No-N	154 ± 59		464 ± 71		0.88 ± 0.56		1.60^{b} ± 1.14	
	Low-N	143 ± 75		351 ± 78		0.49 ± 0.20		1.01^{b} ± 0.51	
	High-N	161 ± 42		329 ± 42		0.49 ± 0.08		1.15^{b} ± 0.40	
Glyceria	No-N	110 ± 17		342 ± 60		0.44 ± 0.06		0.56^{a} ± 0.09	
	Low-N	134 ± 18		341 ± 93		0.44 ± 0.11		0.49^{a} ± 0.16	
	High-N	101 ± 29		589 ± 98		0.41 ± 0.14		0.38^{a} ± 0.12	
Soil		247 ± 37		714 ± 89		0.81 ± 0.09		16.21 ± 1.31	
GLM	variable	F	*p*	F	*p*	F	*p*	F	*p*
	species	0.01	ns	0.58	ns	1.41	ns	**10.47**	**0.002**
	fertilization	0.86	ns	2.08	ns	1.58	ns	2.59	ns
	sp*fert	2.78	ns	1.55	ns	2.33	ns	0.52	ns
	time	**149.4**	**<0.000**	**12.07**	**0.001**	**85.46**	**<0.001**	**9.21**	**0.004**

Mean ± SD are shown, n = 4. Results of GLM model are shown for species, fertilization, interaction and time (covariate). Post-hoc test of species effect on NO_3^- concentration is marked by different letters in subscript. Data for unplanted soils are also shown for comparison

Table 2 Total activity of hydrolases and oxidases, relative investment into C, N and P-mining and the ratio of hydrolytic/oxidative activity in soil with *Carex* and *Glyceria* subjected to three foliar N fertilization doses in the 4th and 10th week of sampling (rhizosphere) and in 2 years after shoot cutting (detritusphere)

Rhizosphere 4th week		Sum hydrolases (nmol $g^{-1} h^{-1}$)		Sum oxidases (nmol $g^{-1} h^{-1}$)		%C-mining		%N-mining		%P-mining		Hydrolase/oxidase	
Carex	No-N	$722^{b} \pm 181$		2727±101		23.6 ± 3.8		$11.8^{ab} \pm 2.0$		64.5 ± 3.1		$0.26^{b} \pm 0.04$	
	Low-N	$632^{ab} \pm 121$		2575 ± 80		25.0 ± 1.1		$10.2^{a} \pm 2.9$		64.6 ± 2.7		$0.24^{b} \pm 0.01$	
	High-N	$881^{c} \pm 108$		2589 ± 120		25.0 ± 5.4		$10.0^{a} \pm 0.9$		64.9 ± 6.2		$0.34^{c} \pm 0.07$	
Glyceria	No-N	$531^{a} \pm 144$		2772 ± 156		19.5 ± 3.8		$14.2^{b} \pm 2.7$		66.2 ± 3.1		$0.19^{a} \pm 0.04$	
	Low-N	$677^{b} \pm 176$		2754 ± 53		21.5 ± 4.1		$12.3^{ab} \pm 2.6$		66.1 ± 1.5		$0.24^{b} \pm 0.05$	
	High-N	$794^{bc} \pm 171$		2575 ± 194		23.7 ± 4.0		$11.1^{ab} \pm 1.8$		65.1 ± 3.3		$0.30^{c} \pm 0.05$	
Soil		888 ± 72		2257 ± 208		24.1 ± 2.0		10.4 ± 1.0		65.3 ± 1.3		0.39 ± 0.03	
Rhizosphere 10th week													
Carex	No-N	$525^{a} \pm 160$		2608 ± 53		22.2 ± 4.0		$11.2^{a} \pm 1.7$		66.5 ± 2.4		$0.20^{a} \pm 0.04$	
	Low-N	$707^{b} \pm 70$		2430 ± 240		20.1 ± 2.3		$11.2^{a} \pm 0.6$		68.6 ± 1.7		$0.29^{b} \pm 0.03$	
	High-N	$733^{b} \pm 91$		2509 ± 58		24.6 ± 0.3		$10.3^{a} \pm 1.0$		65.0 ± 0.8		$0.29^{b} \pm 0.00$	
Glyceria	No-N	$601^{ab} \pm 120$		2578 ± 116		21.8 ± 2.3		$12.0^{b} \pm 0.6$		66.1 ± 2.1		$0.23^{a} \pm 0.02$	
	Low-N	$616^{ab} \pm 53$		2628 ± 89		21.5 ± 1.1		$10.5^{a} \pm 0.6$		67.8 ± 1.6		$0.23^{a} \pm 0.01$	
	High-N	$770^{b} \pm 95$		2543 ± 299		20.1 ± 2.5		$11.8^{ab} \pm 0.9$		68.0 ± 3.4		$0.30^{b} \pm 0.04$	
Soil		756 ± 176		2382 ± 113		23.2 ± 3.4		9.4 ± 1.9		67.3 ± 1.8		0.31 ± 0.05	
Detritusphere 2 years													
Carex	No-N	497 ± 31		1000 ± 17		13.8 ± 1.0		8.5 ± 0.6		77.5 ± 0.9		0.49 ± 0.04	
	Low-N	508 ± 24		982 ± 19		14.5 ± 0.6		9.7 ± 0.5		75.7 ± 1.0		0.51 ± 0.02	
	High-N	528 ± 45		979 ± 19		14.4 ± 1.1		8.9 ± 0.5		76.6 ± 1.5		0.53 ± 0.04	
Glyceria	No-N	451 ± 26		953 ± 17		14.3 ± 1.4		8.7 ± 1.2		76.9 ± 2.3		0.47 ± 0.05	
	Low-N	441 ± 15		950 ± 27		14.6 ± 1.4		8.9 ± 1.7		76.3 ± 2.5		0.46 ± 0.05	
	High-N	448 ± 34		931 ± 27		17.6 ± 0.5		10.1 ± 0.6		72.2 ± 1.0		0.48 ± 0.02	
Soil		558 ± 45		969 ± 9		13.5 ± 1.7		10.5 ± 0.9		75.8 ± 2.2		0.57 ± 0.07	
GLM	variable	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*
	species	2.15	ns	0.93	ns	0.02	ns	**4.00**	**0.049**	0.01	ns	**4.93**	**0.030**
	fertilization	**7.17**	**0.001**	2.32	ns	1.13	ns	0.96	ns	1.15	ns	**9.48**	**0.001**
	sp*fert	0.02	ns	1.62	ns	0.13	ns	0.63	ns	0.11	ns	0.16	ns

Rhizosphere 4th week	Sum hydrolases (nmol $g^{-1} h^{-1}$)		Sum oxidases (nmol $g^{-1} h^{-1}$)		%C-mining		%N-mining		%P-mining		Hydrolase/oxidase	
time	**20.72**	**0.001**	**1128**	**0.001**	**83.05**	**0.001**	**12.23**	**0.001**	**82.05**	**0.001**	**151.5**	**0.001**

Mean ± sd are shown, n = 4. Results of GLM model are shown for species, fertilization, their interaction and time (treated as covariate)

^{13}C recovery in DOC remained stable (Fig. 3c). The distribution of root-derived C between the two labile pools, however, differed between species ($p<0.001$ for DOC and $p<0.001$ for Cmic). About 40 % of DOC and approx. 30 % of microbial C under *Carex* originated from the roots, whereas *Glyceria* contributed much less to DOC (10 %, Fig. 3c) and much more to Cmic (60 %, Fig. 3b). Foliar N fertilization further shifted a distribution of the root-derived C between the two pools. In the case of *Carex*, fertilization decreased the ^{13}C recovery in Cmic in preference to DOC, but it functioned oppositely in the case of *Glyceria* (Fig. 3b, c) (species*fertilization $p<0.001$ for DOC and $p=0.006$ for Cmic).

Species and time effects on sources of soil CO_2 efflux and induced rhizosphere PE

The total (SOC- and root-derived) CO_2 efflux from the planted soil were approx. 2 times larger compared to unplanted soil for both species (Fig. 4a). The root-derived C, which included root respiration and respiration of root-derived microbial products, formed 20–45 % of the total soil CO_2 efflux, being larger for *Glyceria* than *Carex* (Fig. 4b, Supplementary Table 6). The SOC mineralization increased in all planted treatments compared to the unplanted soil (positive rhizosphere PE, Fig. 4c). The rhizosphere PE increased over time (Fig. 4c, $p<0.001$); the SOC was mineralized 3–15 % faster than in unplanted soils in the 4th week and 9–23 % faster in the 10th week. The PE was influenced by species*fertilization interactions ($p = 0.01$): the PE was larger under unfertilized than in both fertilized *Carex* systems, while it was lower in no-N *Glyceria* systems compared to both fertilized ones in the 10th week (Fig. 4c).

The root and SOC components of soil CO_2 efflux were related (Pearson-r=0.41*) and correlated with shoot and root biomass, the root-derived C in the soil (net rhizodeposition) and soil phenoloxidase activity, all increasing over time from the 4th to 10th week (Table 3). The correlations between shoot biomass and root-C supplied activities, represented by root-derived CO_2 efflux and activated microbial biomass, were preserved in particular samplings, whereas those with SOC-derived respiration and PE were not (data not shown). It suggests that although plant activity importantly

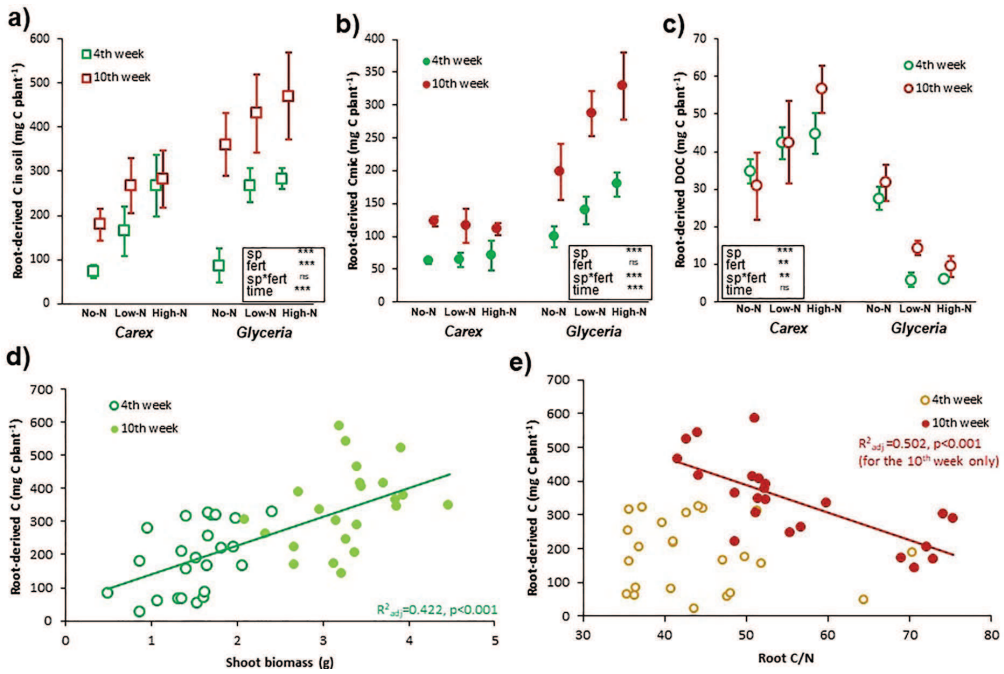

Fig. 3 Effects of growth of *Carex* and *Glyceria* (for 4 and 10 weeks) and N fertilization on recovery of root-derived C (labelled C) in the soil (**a**), microbial biomass (Cmic, **b**), and dissolved organic C (DOC, **c**). Means and standard deviations (n = 4) as well as the results of GLM models assessing the effects of species, fertilization, their interactions, and time are shown. GLM results are presented in the box at the bottom and denote: sp: species, fert: fertilization, sp*fert: interactions, and time: time effect (4 and 10 weeks). Effects of shoot biomass over the whole gradient (**d**) and root C/N ratio from the 10th week of sampling (**e**) on the amount of root-derived C in soil under *Carex* and *Glyceria*

influences the activity of soil microorganisms, its effect on SOC mineralization is modified by additional factors (species*fertilization interaction, Table 3). Specifically, the plant-soil relations differed between species, with closer correlations in *Glyceria* than *Carex* systems (Table 3). The SOC-derived respiration and PE were correlated with the total amount of root-derived C in the soil under *Glyceria* but only with the root-supplied Cmic and DOC under *Carex* (Table 3).

Species and time effects on root- and SOC-derived CO_2 efflux from the detritusphere

Shoot clipping halted rhizodeposition and respiration of roots, and induced root dying and microbial decomposition of residues. The CO_2 efflux thus decreased compared to that from soil with living roots but was still larger than from unplanted soils during the first month. After a month, the soil CO_2 efflux became similar to that from unplanted soils and remained relatively stable during the following two years (Fig. 4a). CO_2 from decomposing roots formed 11–29 % of the total CO_2 in 14 days after shoot cutting and decreased to 9–20 % after one month, with a larger contribution beneath the *Glyceria* than *Carex* during the very early stage of decomposition (Fig. 4b).

The SOC-derived efflux formed a majority of total CO_2 from the detritusphere and was closely related to the PE (Pearson-r=0.99***). The dynamics of the detritusphere PE was influenced by

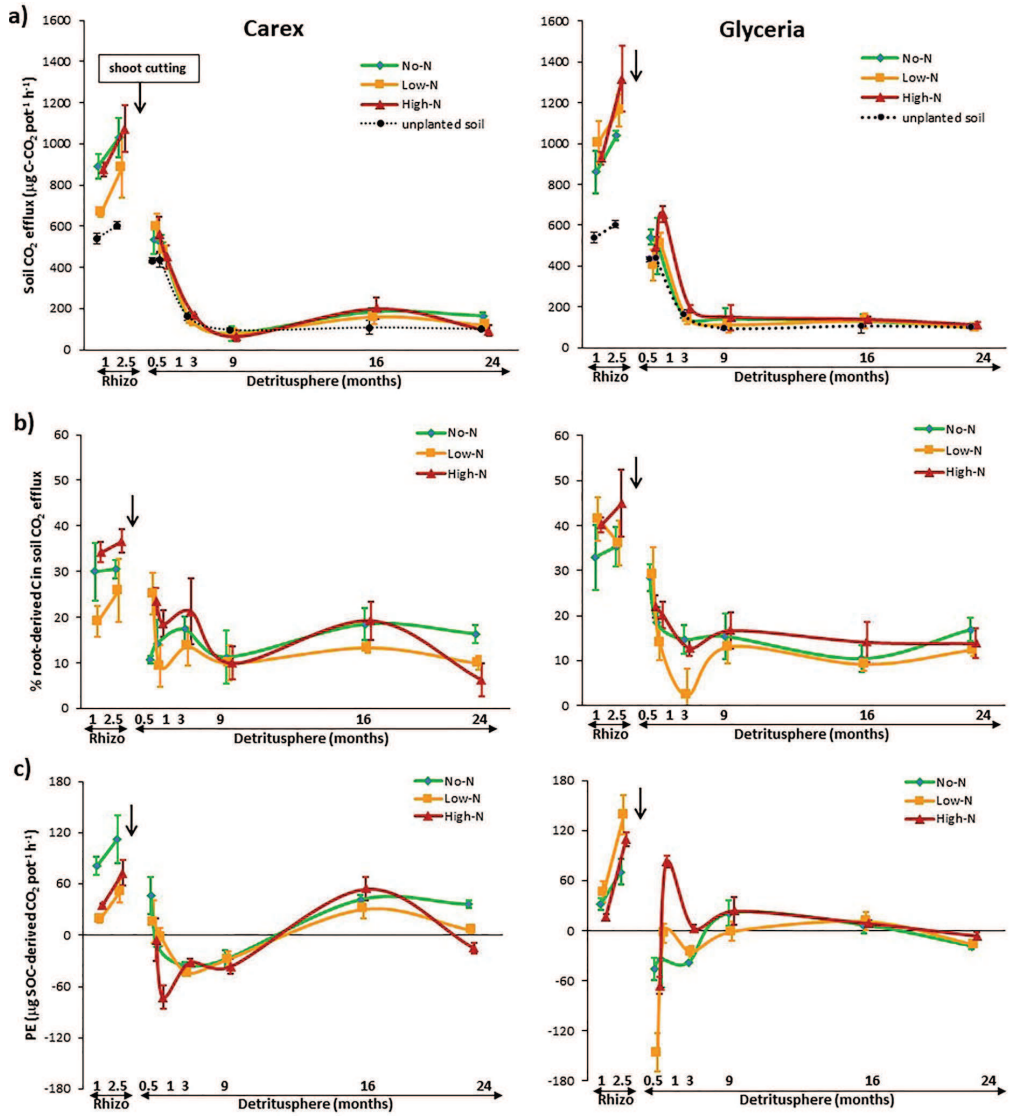

Fig. 4 Total soil CO_2 efflux (**a**), the contribution of the root-derived C to the total CO_2 (**b**), and the absolute priming effect (PE). Measurements were done in unplanted soils and soils with *Carex* and *Glyceria* with foliar fertilization with increasing N doses in the 4th and 10th weeks of their growth (Rhizo) and in 0.5, 1, 3, 9, 16 and 24 months after shoot cutting (Detritusphere). Mean values and standard deviations (n = 4) are shown. The cutting of shoots corresponding to the switch between rhizosphere and detritusphere is marked by vertical arrows

species*fertilization interactions (p=0.019). The shoot cutting and the induced root dying caused a drop in SOC mineralization underneath both plant species (Fig. 4c). Large negative PEs were common in all soils

Table 3 Correlation matrix for soil CO_2 efflux and its parts and related plant and soil microbial characteristics over the two samplings in the rhizosphere period

	Soil CO_2 efflux	Root-derived CO_2 efflux	SOC-derived CO_2 efflux	PE	shoot biomass	root biomass	root-derived C in soil	root-derived Cmic	root-derived DOC	total Cmic	PhOx
Soil CO_2 efflux	---	**0.92*** **	**0.73*** **	**0.65*** **	**0.67** (0.53/0.71)	**0.53** (0.42/0.72)	**0.54** (ns/0.64)	**0.36** (0.46/ns)	**ns** (0.45/ns)	**ns** (0.54/ns)	**0.51** (ns/0.55)
Root-derived CO_2 flux	---	---	**0.41** **	**0.33***	**0.54** (0.42/0.51)	**0.41** (ns/0.57)	**0.45** (ns/0.42)	**0.47** (ns/ns)	**-0.32** (ns/ns)	**ns** (0.49/ns)	**0.37** (ns/ns)
SOC-derived CO_2 flux	---	---	---	**0.93*** **	**0.67** (0.60/0.71)	**0.56** (0.54/0.63)	**0.50** (ns/0.70)	**ns** (0.47/ns)	**ns** (0.45/ns)	**0.34** (0.52/ns)	**0.58** (0.45/0.63)
PE	---	---	---	---	**0.39** (ns/0.49)	**0.32** (ns/0.43)	**0.36** (ns/0.61)	**ns** (0.42/ns)	**ns** (ns/ns)	**ns** (ns/ns)	**0.46** (ns/0.58)
GLM											
species	22.1***	26.5***	0.62	0.24							
fertilization	5.07**	7.80**	0.54	0.40							
sp*fert	8.07***	4.52*	5.51**	4.51**							
time	44.9***	11.8**	72.5***	15.2***							

Pearson-r correlation coefficients significant at $p<0.05$ are shown for both species together (in bold) and for *Carex/Glyceria* separately. The results of GLM model are shown for species, fertilization, interactions and time – the 4th and the 10th week (as a covariate)

with the *Glyceria*-root 14 days after shoot cutting but appeared only for a short period of 1–3 months in the case of no-N and low-N and even shorter with high-N fertilization. Cutting *Carex* shoots caused a slowed decrease of SOC mineralization than that of *Glyceria*, but the subsequent negative PE lasted longer, from the 3rd to the 9th month (Fig. 4c). All soils with *Carex*-roots had negative PE ranging between -20 % and 40 % reduction of SOC mineralization compared to the unplanted control soil.

The period of reduced SOC mineralization switched to the phase of the positive PE in all the soils with roots. This positive PE phase mirrored the period of enhanced contribution of decomposing roots to CO_2 (compare Fig. 4b and c). The positive PE appeared from the 9th to 16th month (from the 1st month for high-N) after clipping the *Glyceria* shoots and between the 16th and 24th month in the case of *Carex* soils. The PE magnitudes for *Carex* soils were larger and resulted in larger SOC losses in this period compared to those from *Glyceria* soils (Fig. 4c). The decomposition of *Glyceria* roots with lower C/N ratio had less impact on SOC dynamics and mineralization in the detritusphere compared to N-poorer *Carex* roots.

Mineralization of root-derived C and its final contribution to soil C

Soils under *Carex* contained approx. 360 mg of root-derived C per plant (forming 1.6–2.0 % of soil C) and soils under *Glyceria* only approx. 220 mg of root-derived C per plant (0.8–1.3 % of soil C) (p = 0.018) (Table 4). These values represented about 50 % and 30 % of all the root-derived C, which were in soils with *Carex* and *Glyceria*, respectively, before the shoots were clipped (Table 4). It showed that the root-derived C inputs from *Glyceria* decomposed and mineralized faster compared those from *Carex*. The amount of root-derived C remaining in the soil after 2 years increased with the root C/N ratio (Pearson-r=0.55**).

Discussion

We first compared the magnitude and dynamics of the PE in the rhizosphere and root detritusphere of two plant species, which differed in traits important for SOC mineralization. Although there is an increasing understanding that rhizosphere processes (Guyonnet et al. 2018; Henneron et al. 2020a, b) and soil C and N dynamics and sequestration (De Deyn et al. 2008, 2009; de Vries and Bardgett 2012; Bardgett et al. 2014) are closely associated with plant economic traits, both rhizosphere and detritusphere PE phenomena have always been studied separately.

Based on the N-mining Hypothesis (Craine et al. 2007; Chen et al. 2014), we hypothesized that (H1) C fluxes from living and decomposing roots will stimulate SOC decomposition with larger PE in the

Table 4 Estimated amounts of root-derived C mineralized and remaining in the soil after 2-year decomposition of roots of *Carex* and *Glyceria*

		Total amount of root-derived C in soil in 10th week before shoot clipping (mg C pot^{-1})		Remaining root C after 2 years (mg C pot^{-1})	The fate of root-derived C present in soil before shoot cut (%)	
		Net rhizodeposition[1]	C in root residues[2]	[3]	remaining	mineralized
Carex	No-N	173	832	468	47	53
	Low-N	274	615	405	46	54
	High-N	289	560	459	54	46
Glyceria	No-N	369	609	362	37	63
	Low-N	438	701	253	22	78
	High-N	442	755	335	28	72

[1]the amount of labelled root-derived C deposited to soil by living roots (measured in soils of destructively sampled pots after shoot clipping in the 10th week of plant growth)

[2]root biomass C measured in soils of destructively sampled pots after shoot clipping in the 10th week of plant growth

[3]the amount of labelled root-derived C remained in soils after 2 years of root decomposition

rhizosphere than in the detritusphere due to continual input of energy-rich and easily available exudates to the severely N-limited rhizosphere. The rhizosphere PE will increase with plant productivity; the detritusphere PE will occur only during the fast early stage of decomposition and will later fade. We further hypothesized that (H2) the larger exudation and greater N requirements of *Glyceria* (Kastovska et al. 2014) would induce larger positive rhizosphere PE compared to *Carex*, while decomposition of N-richer roots of *Glyceria* will be associated with lower positive detritusphere PE in comparison to N-poorer *Carex* roots. (H3) The PE will be further modulated by foliar N fertilization with well utilizable urea-N (Mayer et al. 2003) via reduced plant N demand from soil and consequent changes in plant-microbial interactions. Our results support H1, partly also H2 and reveal complex dynamics of the detritusphere PE. The foliar N fertilization altered rhizosphere PE through enhanced rhizodeposition input and species-specific differences in its utilization by microorganisms, with effects persisting until the detritusphere period (H3). We will first turn our discussion to the results from "the rhizosphere period".

Rhizosphere PE is influenced by plant biomass, rhizodeposition and N depletion from the soil (H1, H2)

Plants stimulated SOC mineralization by 3–23 % compared to the unplanted soil. The induced positive rhizosphere PE increased during plant growth (Fig. 4c), in support of our H1 and findings of others (Dijkstra et al. 2006; Huo et al. 2017). The best predictor of the SOC mineralization and rhizosphere PE (both were tightly linked, Table 3) was the shoot biomass that differed between the faster-growing *Glyceria* and slower-growing *Carex* (Fig. 2a). This corresponds to Henneron et al. (2020a), who identified a strong link between the rhizosphere PE and aboveground net primary productivity, absolute growth rate, biomass allocation pattern and leaf mass-based photosynthetic activity. This is clear evidence that plant photosynthetic activity controls SOC mineralization.

The rhizosphere PE increased with the amount of root-derived C in the soil (net rhizodeposition), with microbial community actively utilizing rhizodeposits and with the root-derived CO_2 efflux from the soil (Table 3). This indicates that plant photosynthesis control over rhizosphere PE takes place through the input of recent assimilates to the soil. It provides energy and stimulates microbial activity and exoenzyme production, supporting the microbial activation hypothesis (Drake et al. 2013). Rhizodeposits (forming only approx. 2 % of the soil C) represent a key source for microorganisms (Hutsch et al. 2002; Butler et al. 2003; Boddy et al. 2007) because they have much better availability compared to most other C pools. The 30–45 % contribution of the root-derived C to the soil CO_2 efflux (roughly half of it possibly originating from microbial respiration) (Kuzyakov 2002b; Baggs 2006; Hopkins et al. 2013) and the 27–60 % portion in the microbial C in the 10th week of plant growth (Fig. 3b) point to high microbial activity and fast biomass turnover close to the living roots (Boddy et al. 2007; Kastovska and Santruckova 2007).

When comparing the two species (H2), *Glyceria* rapidly builds up the aboveground photosynthetic tissues with a lower need for N per unit of fixed C in comparison to *Carex*. A similar link between fast growth and high N use efficiency of acquisitive species has been found in other studies (Cardenas et al. 2021). Oppositely, the *Glyceria* roots were N-richer than those of *Carex*, which is the trait commonly associated with the higher root metabolic activity (Craine et al. 2005; Tjoelker et al. 2005), faster turnover (Sun et al. 2016) and release of lysates to the soil (Kastovska et al. 2017). A combination of the acquisitive properties above- and belowground explains why more productive *Glyceria* was associated with larger rhizodeposition (Kastovska et al. 2017; Guyonnet et al. 2018; Henneron et al. 2020a) and larger microbial biomass utilizing root-derived compounds than *Carex* (Fig. 3a, b). The more active soil microorganisms and roots of the *Glyceria* depleted stronger soil mineral N pool (Table 1). It implied a greater need for the *Glyceria*-associated microbial community to mine N from complex soil organic matter compared to *Carex*-system in accordance with H2. The faster SOC mineralization due to stronger N insufficiency was corroborated by enzymatic activities in support of the N-mining Hypothesis (Craine et al. 2007; Chen et al. 2014; Mooshammer et al. 2014b). Strongly competitive *Glyceria*-system differed from soils with *Carex* by a higher investments into N mining and lower ratio of hydrolytic/oxidative enzyme activity (Table 2) indicating faster degradation of complex soil organic

matter. Accordingly, the activity of phenoloxidase degrading phenolic compounds and other complex substances in soil organic matter (Sinsabaugh 2010) positively correlated with the SOC mineralization and rhizosphere PE (Table 3).

Foliar N fertilization altered the rhizosphere PE through rhizodeposition increase and its microbial utilization (H3)

To test H3, we modulated the interactions of plants and microorganisms in the rhizosphere using foliar N fertilization. While the N addition directly into the soil affects the pH (Zamanian et al. 2018), composition and activity of microbial community and plant-microbial competition for N (Treseder 2008; Liu and Greaver 2010), the leaf N feeding (Mayer et al. 2003) has no direct effects on the soil. Repeated foliar N fertilization changed some plant functional traits: It accelerated the plant growth in the initial phase (Fig. 2a) and increased shoot N content in mature plants of both species (Fig. 2b), thereby increasing the photosynthetic activity of fertilized plants compared to unfertilized ones (Evans 1989). Therefore, fertilized plants released more rhizodeposits (including exudation and root turnover products) into the soil than unfertilized ones during 10 weeks. This root-derived C was recovered either in DOC (*Carex*) or in microbial biomass (*Glyceria*) (Fig. 3) as well as in root-derived CO_2 efflux of both species (Fig. 4b). Larger total root-derived C in the soil under N fertilized plants (Fig. 3a, d) is in line with studies of Henneron et al. (2020a; Baptist et al. (2015); Cardenas et al. (2021)) and others, connecting higher rhizodeposition with faster plant growth and higher photosynthetic activity.

Applying N directly to the leaves of the plants reduced the need to take up N from the soil and the N remained available for microbial needs, corroborated by the unchanged microbial enzymatic N-mining. Activities of all other hydrolases increased under fertilized plants, which resulted in higher ratio of hydrolytic/oxidative enzymatic activities compared to soils under non-fertilized plants (Table 2). These enzymatic shifts reflected microbial preferential use of the large inputs of readily available rhizodeposits against SOC. The consequence for the SOC mineralization was, however, species-specific. The rhizosphere PE decreased under fertilized *Carex* but increased under fertilized *Glyceria* (Fig. 4c), mediated by the response of the microbial community utilizing the root-derived inputs. The "extra" rhizodeposition activated but did not increase the root-derived microbial community under fertilized *Carex* (Fig. 3b). A sufficient root C supply and weaker competition for N with fertilized plants suited microbial C and N demands (Mooshammer et al. 2014b) and lowered SOC decomposition in support of the N-mining Hypothesis (Chen et al. 2014). This was similar to the decrease in the rhizosphere PE after soil amendment with mineral N (Dijkstra et al. 2013; Kirkby et al. 2014; Jiang et al. 2021). In contrast, the "extra" rhizodeposition flux from fertilized *Glyceria* enhanced microbial biomass grown on root-C (Fig. 3b) and SOC mineralization compared to non-fertilized *Glyceria* systems (Fig. 4c). This larger rhizosphere PE could result from both targeted SOM decomposition to cover increased nutrient growth demands and its co-metabolic decomposition. The observed species-specific differences in fertilization effect point to the key role of the size and activity of the soil microbial community utilizing the root-derived substances in driving the rhizosphere PE.

In summary, the activity of living roots of both species stimulated SOC mineralization. The increased rhizosphere PE accompanied the acquisitive properties of *Glyceria.* Its greater shoot biomass and lower root C/N were associated with faster growth, higher root activity, larger input of root-derived C into the soil and activation of larger microbial community and oxidase activity in the rhizosphere. The SOC mineralization and rhizosphere PE decreased under lower plant-microbial competition for N in the soil, evidenced in the rhizosphere of fertilized *Carex*. Foliar N fertilization has proven to be an appropriate approach to shift some functional traits of plants and test their effects on rhizosphere processes, including the rhizosphere PE.

The detritusphere priming by root decomposition under *Carex* and *Glyceria* (H1-H3)

The starting conditions for the detritusphere phase were the following: soils with both species contained similar root biomass but those of *Carex* had a higher root C/N ratio, less rhizodeposits and smaller root-derived microbial biomass than soils with *Glyceria*. There were also additional residual fertilization effects – larger amounts of root-derived C (Fig. 3a)

and higher hydrolytic enzymatic activity (Table 2) in the soil under both species as compared to the non-fertilized systems.

The clipping of shoots interrupted transport of assimilates into the soil and release of rhizodeposits, which was substituted by a concentrated flush of soluble C from the root litter starting to decompose (Mastný et al. 2018). We hypothesized in H1 that this early stage of decomposition will be joined with a positive detritusphere PE (Nottingham et al. 2009; Pascault et al. 2013). Instead, either a negative (or no) detritusphere PE lasted about 3 months in the soil with *Glyceria* roots and more than 9 months in the soil with *Carex* roots (Fig. 4c). The negative PEs are because plant N uptake was stopped by shoot clipping and mineral N became more available to microbes. Microorganisms thus switched their feeding from hardly accessible SOM to fresh root litter and balanced their N requirements through the uptake of available soil N. Such microbial activity supports the preferential substrate utilization hypothesis (Mooshammer et al. 2014a). Our experimental design distinguishes from others regarding the detritusphere PE (for example Nottingham et al. 2009; Pascault et al. 2013; Shahzad et al. 2015) since they commonly add plant residues to the soil. In contrast, our plants grew in and influenced microbial communities via the rhizodeposition for 10 weeks. Subsequent shoot clipping did not disturb the specific soil environment and root decomposition was provided by the "preconditioned" microbial community. Therefore, this approach better reflects natural conditions.

A preferential utilization of fresh root residues led to a depletion of easily decomposable substances and their mineralization and transformation within 3–9 months. The negative PE switched to the phase of stimulated SOC decomposition under both species (Fig. 4c). We speculate that this change was connected with a shift in microbial community composition from groups utilizing soluble C substrates to those decomposing microbially transformed compounds (such as necromass) and those efficiently degrading remaining complex compounds in the root residues. Such microbial succession connected with a decreasing decomposability of root residues can be one of the regulatory factors in the detritusphere PE (Pascault et al. 2013). The positive PE appeared earlier, after 9 months after shoot cutting, in soils with N-richer better decomposable roots of *Glyceria* than in soils with *Carex* roots, where the positive PE started only after 16 months (Fig. 4c). The detritusphere PE dynamics were closely related to the degradability of the root residues, which was higher for acquisitive than conservative species. We additionally observed a persistent fertilization effect in *Glyceria*-soils that influenced SOC mineralization dynamics. The shift from negative to positive PE occurred earlier in soils with the roots of fertilized plants (Fig. 4c), which contained larger rhizodeposition and active microbial biomass than soils under unfertilized *Glyceria* (Fig. 3a, b). Consequently, the easily degradable substances and available nutrients were depleted faster there due to higher microbial activity. However, the positive PE induced in soils with N-richer *Glyceria* roots was smaller than in soils with N-poorer *Carex* roots, in agreement with H2 and in support of the N-mining Hypothesis (Craine et al. 2007; Chen et al. 2014).

To summarize the detritusphere period, the early stage of root decomposition was associated with negative or no PE period lasting for several months. This was a consequence of the preferential decomposition of fresh C-rich root residues, nutrient deficiency of which was balanced by microbial uptake of soil N available without plant N uptake. A depletion of easily decomposable compounds and associated shift in the microbial community resulted in its decreased activity and induced the positive PE, which was larger in relation to N-poorer root residues of *Carex*. Approx. 50 % and 70 % of all root-derived C (root mass and rhizodeposition) of *Carex* and *Glyceria*, respectively, were mineralized during 2 years of root decay. Thus, decomposability of root litter (indicated here by the root C/N) was the main driver of its utilization rate but also of the dynamics of related PE.

Conclusions

Living roots largely controlled soil processes in the rhizosphere: root-derived C contributed up to 45 % to the soil CO_2 efflux, and rhizodeposits were the preferred C source for microorganisms. The rhizodeposition input into the soil increased with shoot growth and with decreasing root C/N, partly reflecting root turnover. Microbial utilization of rhizodeposits under plant-microbial competition for N stimulated SOC decomposition. The positive

PE increased with plant growth and was intensified under plant with fast biomass growth, large input of root-derived C into the soil, high active microbial biomass and activity of phenol-oxidase in the soil. Foliar N fertilization altered the magnitude of positive PE dependent on the size of activated microbial community. It indicated that plant-microbial competition for N and its impact on soil N availability play an important role in controlling SOC mineralization.

After shoots were clipped, the detritusphere was formed. The amounts of rhizodeposition and active microbial biomass present in the soil at the time of shoot cutting influenced the root decomposition at the early stage. The root C/N ratio of decaying roots was crucial for the transformation of more recalcitrant substances from dead roots, during detritusphere ageing and for the final contribution of root-derived C to the soil C. Decomposition of fresh roots induced negative priming, which switched to a positive priming after exhaustion of the labile compounds in several months and finally was levelled out after two years. Lower C/N of *Glyceria* root residues and larger active microbial community persisting from the previous rhizosphere stage were associated with fast decomposition and rapid onset of negative PE, but also its rapid change to a longer period with small positive PE.

From this study on two graminoid species, we conclude that the rhizosphere PEs are larger that detritusphere PEs, which are, however, more variable over time depending on the progressive decomposition of the root litter. The fast-growing N-acquisitive species was associated with larger SOC losses from rhizosphere, whereas decomposition of its better decomposable N-richer root litter was accompanied by lower SOC losses compared to the slow-growing N-conservative species.

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Code availability Not applicable.

Declarations

Conflict of interest/Competing interests We declare no conflicts of interests.

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