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**MYCORRHIZAL COMMUNITIES IN A GLACIER FOREFIELD -
SIZE, COMPOSITION AND FUNCTIONING**

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Front cover

Ectomycorrhizal root tips (vertical panel) and arbuscular mycorrhizal structures (horizontal panel) detected in the roots of *Salix helvetica* (Light microscope; photo M. Welc, 2008-2010)

Back cover

Series of pictures imaging ecosystem developmental gradient from a rock material (upper left corner) to vegetation cover with *Salix helvetica* (bottom right corner), including possible intermediate stages (photo M. Welc, 2007-2011)

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List of abbreviations

AM	Arbuscular mycorrhiza
AMF	Arbuscular mycorrhizal fungi
ANOVA	Analysis of Variance
C	Carbon
DCA	Detrended Correspondence Analysis
DW	Dry weight
ECM	Ectomycorrhiza / ectomycorrhizal fungi
ER	Ericoid mycorrhiza / ericoid mycorrhizal fungi
FAME	Fatty Acid Methyl Esters
GLFA	Glycolipid Fatty Acids
LMWOA	Low Molecular Weight Organic Acids
MHB	Mycorrhizal Helper Bacteria
N	Nitrogen
¹⁵ N	Stable isotope of nitrogen
NLFA	Neutral Lipid Fatty Acids
<i>p</i>	Probability level
P	Phosphorus
³³ P	Radioisotope of phosphorus
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PLFA	Phospholipid Fatty Acids
qPCR	Quantitative Polymerase Chain Reaction
RDA	Redundance Analysis

List of abbreviations

SOM	Soil Organic Matter
TMCS	Trimethylchlorosilane
UTS	Fungi of uncertain trophic status
WCFA	Whole Cell Fatty Acids

Summary

More than 90% of all terrestrial plant species establish mycorrhizal associations supporting their growth, nutrition and resistance against environmental stresses. These associations seem to be particularly important for the mountain vegetation by facilitating plant establishment, development and species succession on recently deglaciated areas. However, the current knowledge about mycorrhizas in mountain ecosystems consists mostly of the information on taxonomic diversity, whereas the link between the composition and functions of mycorrhizae remains poorly understood. The main objective of the research project described in this thesis was to characterize the size, composition (structure and abundance) and functional diversity of the mycorrhizal fungal communities associated with *Salix helvetica* (Swiss willow) in a glacier forefield.

The research included field work in a young alpine ecosystem developed at the Damma glacier forefield, and laboratory experiments, mimicking different stages of ecosystem development. Biochemical (fatty acid profiling, chapter 1; enzymatic activity assays, chapters 2 and 4) and molecular techniques (quantitative PCR (qPCR); chapters 2 to 4) as well as isotope labeling (^{15}N and ^{33}P ; chapters 3 and 4) were the main methods employed in this study.

In order to characterize the composition of ectomycorrhizal (ECM), arbuscular mycorrhizal fungi (AMF) and other fungi (called fungi of uncertain trophic status, UTS) associated with roots and rhizosphere soil of willow, a number of taxon-specific qPCR molecular markers were designed. The composition of mycorrhizal fungi was linked with their functional diversity by measuring the activities of enzymes in the rhizosphere soils (field and lab studies) and from ECM root tips (field). In the experiments, willows were inoculated with indigenous fungi originating from different stages of ecosystem development at the Damma glacier forefield (approximately 16, 60 and 116 years after deglaciation). The effect of mycorrhizal communities on willow growth and nutrition was tested by following the transfer of nutrients from labeled sources to the willow plants. We used two external sources of nutrients: an apatite labeled with ^{33}P and bacterial biomass labeled with ^{15}N and ^{33}P . The transfer of nutrients from these two sources to willow was studied in two distinct rhizobox experiments.

The field study demonstrated that the size of the fungal communities in soils at different developmental stages (i.e., 23 sites spanning 7 through 137 years of soil development) increased and was strongly correlated to soil pH and total organic carbon (C) content. AMF dominated over other fungi (including ECM) at the early stages of soil development, whereas other fungi (including ECM) presented stable co-dominance with soil bacteria along the entire developmental gradient (chapter 1). Concerning the structure of the mycorrhizal fungal communities established with adult willows (roots and rhizosphere soil) colonizing the sites of different developmental stage (i.e., 7 sites spanning 7 through 110 years of soil development), changes in abundance but not composition were observed. The highest abundance of AMF, ECM and UTS was observed in willow bushes at the central part of the glacier forefield. The high activity of chitinase and β -glucosidase observed in the rhizosphere soil of willows suggested possible participation of associated ECM fungi in willow nutrition (chapter 2). The composition of mycorrhizal fungal communities established with willow in the two rhizobox experiments depended on the origin of the inoculum and C content of the growth medium. The abundance of ECM and UTS fungi significantly correlated with the dry weight of willow and N and P uptake (chapter 3). The added sources of nutrients were used by the willow in association with the fungal communities but at different rates. Moreover, release, uptake and transfer of ^{15}N and ^{33}P from labeled bacteria correlated predominantly with the abundance of UTS fungi (chapter 4).

Conclusions

- Mycorrhizal fungal communities are important components of the developing ecosystem. Their size and composition were related to soil pH and total soil organic C and to the structure of plant communities (chapters 1 to 4).
- Enzymatic activity of fungal communities should be considered as one (but not the only) strategy of nutrient acquisition. The link between the composition and functions of mycorrhizal communities (in term of enzymatic activities) was difficult to reveal under field conditions, but it could be better observed in the rhizobox experiments (chapters 2 and 4).
- Mycorrhizal fungal communities contributed to willow growth and nutrition from inorganic and organic nutrient sources. The rate of nutrient release, uptake, and transfer was dependent on the fungal community composition (origin of the fungal inoculum) and the type of external nutrient source (chapters 3 and 4).

Zusammenfassung

Mehr als 90% aller Landpflanzen sind mit Mykorrhizapilzen vergesellschaftet, die Wachstum, Ernährung und Widerstand der Pflanzen gegen Umwelteinflüsse unterstützen. Diese Vergesellschaftungen scheinen besonders wichtig zu sein für die Bergvegetation, weil durch sie Ansiedlung und Entwicklung der Pflanzen und Artenabfolge in kürzlich vom Gletschereis freigegebenen Gebieten erleichtert werden. Der Stand des Wissens über Mykorrhiza in Bergökosystemen besteht jedoch hauptsächlich aus Informationen über taxonomische Diversität, wogegen über die Verbindung zur Artenzusammensetzung und Funktion der Mykorrhizen noch wenig bekannt ist. Das Hauptziel dieses Forschungsprojekts war es, die Grösse, Zusammensetzung (Struktur und Häufigkeit) und funktionale Diversität von Mykorrhizapilzgemeinschaften in Vergesellschaftung mit *Salix helvetica* (Schweizerische Weide) in einem Gletschervorfeld zu charakterisieren.

Die Forschungsarbeiten beinhalteten Feldarbeit in einem jungen alpinen Ökosystem auf dem Dammagletschervorfeld und Laborexperimente, die verschiedene Stadien der Ökosystementwicklung nachahmten. Die Hauptmethoden dieser Arbeit waren biochemische (Fettsäuremuster, Kapitel 1, und Enzymaktivitätsmessungen, Kapitel 2 und 4) und molekulare Techniken (quantitative PCR (qPCR), Kapitel 2 bis 4) und Isotopenmarkierung (^{15}N und ^{33}P , Kapitel 3 und 4).

Um die Zusammensetzung von Ektomykorrhizapilzen (ECM), arbuskulären Mykorrhizapilzen (AMF) und anderen Pilzen (genannt Pilze von unbekanntem trophischem Status, UTS), die mit den Wurzeln und dem Rhizosphärenboden der Weide assoziiert sind, zu charakterisieren, wurden eine Reihe von gattungsspezifischen qPCR molekularen Markern entworfen. Die Zusammensetzung der Mykorrhizapilze wurde mit ihrer funktionellen Diversität verknüpft, indem die Enzymaktivitäten im Rhizosphärenboden (Feld- und Laborstudien) und in ECM-Wurzelspitzen (Feld) gemessen wurden. In den Versuchen wurden Weiden mit einheimischen Pilzen inokuliert, die von verschiedenen Stadien der Ökosystementwicklung auf dem Dammagletschervorfeld (ca. 16, 60 und 116 Jahre nach dem Abschmelzen) stammten. Die Auswirkungen von Mykorrhizagemeinschaften auf Wachstum und Ernährung der Weide wurden anhand des Transfers von Nährstoffen aus markierten Quellen zu den Weidepflanzen untersucht. Zwei externe Nährstoffquellen wurden verwendet: ein mit ^{33}P markierter Apatit und bakterielle Biomasse, die mit ^{15}N und ^{33}P markiert war. Der

Transfer der Nährstoffe aus diesen beiden Quellen wurde in zwei verschiedenen Topfversuchen untersucht.

Die Feldstudie zeigte, dass die Grösse der Pilzgemeinschaften mit dem Alter der Böden verschiedener Entwicklungsstadien (23 Flächen von 7 bis 137 Jahren Bodenentwicklung) zunahm und stark mit dem pH und Gesamtkohlenstoffgehalt (C-Gehalt) des Bodens korreliert war. In frühen Stadien der Bodenentwicklung dominierten AMF über andere Pilze (einschliesslich ECM), wogegen andere Pilze (einschliesslich ECM) eine stabile Kodominanz mit Bodenbakterien entlang des gesamten Gradienten zeigten (Kapitel 1). Bei der Struktur der Mykorrhizapilzgemeinschaften von adulten Weiden (Wurzeln und Rhizosphärenboden) auf verschiedenen Bodenentwicklungsstadien (7 Flächen von 7 bis 110 Jahren Bodenentwicklung) wurden Veränderungen in der Häufigkeit, nicht aber in der Zusammensetzung beobachtet. Die grösste Häufigkeit von AMF, ECM und UTS wurde bei Weidenpflanzen im mittleren Teil des Gletschervorfelds festgestellt. Die hohe Aktivität von Chitinase und β -Glucosidase im Rhizosphärenboden der Weiden wies auf eine Rolle der assoziierten ECM-Pilze bei der Ernährung der Weide hin (Kapitel 2). Die Zusammensetzung der Mykorrhizapilzgemeinschaften, die sich in den zwei Topfversuchen entwickelten, hing von der Herkunft des Inokulums und vom C-Gehalt des Wachstumsmediums ab. Die Häufigkeit von ECM- und UTS-Pilzen war signifikant mit dem Trockengewicht der Weiden sowie N- und P-Aufnahme korreliert (Kapitel 3). Die zugegebenen Nährstoffquellen wurden von den Weiden genutzt, die mit Pilzgemeinschaften vergesellschaftet waren, aber in unterschiedlichen Raten. Darüberhinaus korrelierten Freisetzung, Aufnahme und Transfer von ^{15}N und ^{33}P aus den markierten Bakterien vor allem mit der Häufigkeit von UTS-Pilzen (Kapitel 4).

Schlussfolgerungen

- Mykorrhizapilzgemeinschaften sind wichtige Bestandteile sich entwickelnder Ökosysteme. Ihre Grösse und Zusammensetzung korrelierte mit dem Boden-pH-Wert und dem Gehalt an organischen Kohlenstoff im Boden sowie mit der Struktur der Pflanzengemeinschaften (Kapitel 1-4).
- Enzymaktivitäten von Pilzgemeinschaften sollten als eine (aber nicht als einzige) Strategie zur Nährstoffaneignung betrachtet werden. Der Zusammenhang zwischen Zusammensetzung und Funktion von Mykorrhizagemeinschaften (im Sinne von

Enzymaktivitäten) war unter Feldbedingungen schwierig zu zeigen, konnte in den Laborversuchen aber besser beobachtet werden (Kapitel 2 und 4).

- Mykorrhizapilzgemeinschaften trugen zu Wachstum der Weiden und Aufnahme von Nährstoffen aus anorganischen und organischen Quellen bei. Die Rate der Freisetzung, Aufnahme und Verlagerung der Nährstoffe hing von der Zusammensetzung der Pilzgemeinschaft (Herkunft des Inokulums) und von der Art der externen Nährstoffquelle ab (Kapitel 3 und 4).

General introduction

Mountain ecosystem in ecological studies

Mountains cover approximately 21% of land surface of Earth and sustain globally important habitat for a variety of organisms (Körner, 2003; Nagy and Grabherr, 2009). Mountain ecosystems are regarded as the most fragile and still – compared with lowlands – relatively undisturbed. Soils gradually emerging from underneath of retreating mountain glaciers received scientific attention since at least 40 years (Bernasconi et al., 2011; Lazzaro et al., 2010; Matthews, 1992; Ohtonen et al., 1999; Tscherko et al., 2003). Called chronosequences, they replace temporal with spatial gradients thus forming the basis for numerous studies on the ecosystem development and succession (Huggett, 1998; Walker et al., 2010).

Great contribution to the development of the terrestrial ecosystems is attributed to mycorrhizal fungi (Cázares et al., 2005; Chapin et al., 1994; Helm et al., 1996). They facilitate vegetation establishment and survival, especially on lands where vegetation cover is formed anew as a result of primary succession.

Mycorrhizal associations in the mountains

Roots of more than 90% of extant terrestrial plant species establish mycorrhizas with specialized soil fungi (Smith and Read, 2008). Several types of mycorrhizal associations exist in nature, differing in the fungal and plant species involved and in the morphological features of the association. On the global scale, arbuscular mycorrhiza (AM) and ectomycorrhiza (ECM) are the most common relationships engaging about 260 000 plant species (Finlay, 2008). Enhancing the uptake of nutrients and water, alleviating response to stresses such as drought and cold, mycorrhizas are important for establishment and succession of mountain vegetation (Cripps and Eddington, 2005). Investigations of the last decade focused mainly on the distribution and taxonomic diversity of the mycorrhizas in the mountains (Cázares et al., 2005; Kernaghan and Harper, 2001; Mühlmann et al., 2008; Oehl et al., 2011), whereas their functions remains still poorly understood. Thus, on one side, a great amount of knowledge accumulates about the fungal taxa colonizing different mountain plant species such as *Salix herbacea*, *Polygonum viviparum*, *Kobresia myosuroides* (Graf and Brunner, 1996; Mühlmann et al., 2008; Mühlmann and Peintner, 2008a, 2008b). But on the other side, the link between the diversity and functions of mycorrhizas in the mountain ecosystem remains weak (Pritsch

and Garbaye, 2011; Rineau and Courty, 2011). Another aspect which deserves attention is the impact of environmental factors on mycorrhizal fungal diversity and functions. Again, since the abiotic and biotic factors affect the composition of AM and ECM fungal communities in the field (Bahram et al., 2012; Becklin et al., 2011a; Becklin et al., 2011b; Jansa et al., 2009), there is still lot more of unknowns that discoveries concerning their impact on mycorrhizas functions.

Mycorrhiza and nutrients acquisition strategies

The strategies of nutrient acquisition from different soil resources such as inorganic or organic differ between types of mycorrhizas (e.g., ECM and AM) but also between different fungi forming the same type of the symbiosis (e.g., different ECM taxa). As proposed in the literature, ECM fungi exude variety of exoenzymes which facilitate hydrolysis of complex organic macromolecules (e.g., proteins, nucleic acids, chitin, lipids) deposited in the soil organic matter (SOM) (Finlay, 2008; Finlay et al., 1992). Upon SOM hydrolysis essential nutrients such as nitrogen (N) and phosphorus (P) are released, then captured by ample net of mycorrhizal hyphae and transferred to the host plant. As an alternative strategy, ECM are also able to access nutrients from inorganic recalcitrant forms such as minerals, upon their dissolution with low molecular weight organic acids (LMWOA) (Arocena et al., 2011; Hoffland et al., 2004; Wallander and Wickman, 1999). In case of arbuscular mycorrhizal fungi (AMF), production of exoenzymes and LMWOA have been claimed to be limited (Smith and Read, 2008), but studies confirming utilization of organic and inorganic forms of nutrients have been also published (Hawkins et al., 2000; Hodge and Fitter, 2010). Exact mechanisms behind these phenomena remain speculative for AMF which are rather believed efficient scavengers of inorganic nutrients (e.g., NO_3^- , PO_4^{3-}) released upon microbial degradation of SOM or mineral dissolution than active participants of these processes (Smith and Read, 2008).

Differences between AM and ECM concerning preferential use of inorganic or organic nutrients underlie the hypothesis of their distribution in nature. Following this concept, ECM fungi can colonize both mineral and organic-rich patches in the soil, where they would mainly mobilize the nutrients by mineral dissolution and enzymatic hydrolysis of SOM, respectively. In contrast, AMF will preferentially occupy niches providing nutrients in form of simple inorganic ions. Although such a concept may seem oversimplified, it has been repeatedly observed in studies of spatial distribution of AMF and ECM in the soil profile (vertical aspect)

or different stages of soil development (horizontal aspect) (Lambers et al., 2009; Read, 1984; Rosling et al., 2003).

Mycorrhizal associations of *Salix* spp.

The genus *Salix* is claimed to be the earliest recorded flowering plants in pre-Ice Age period (Newsholme, 1992). The number of *Salix* species in today's flora range from 300 to 500 with representatives in almost every part of the globe (Lautenschlager-Fleury, 1994). Although this genus is believed to originate from the subtropics, it found its ecological optimum rather in moist ecosystems of the temperate and Arctic regions of the northern hemisphere (Newsholme, 1992). Occurring usually as dwarf shrubs, willows are resistant to flooding, freezing but also avalanches and landslides thus can be commonly found in alpine¹ ecosystems. The flora of the Swiss Alps contain approximately 20 species of *Salix* (Lauber et al., 2012). *Salix helvetica* is a dwarf willow, first time described in 1789 by Dominique Villars (Villars, 1789) in the French Alps. Latter studies confirmed this species also in Italy, Switzerland, Austria (Alps), as well as Slovakia and Poland (West Carpatian Mountains). This willow colonizes non-calcareous shallow grounds usually susceptible to periodical flooding (Lauber et al., 2012).

To the best to our knowledge, no study has yet specifically focused on the mycorrhizal associations of *Salix helvetica*. Willow species are known to be able to establish ECM (Linkins and Antibus, 1980; Loree et al., 1989; Miller and Laursen, 1978), AM (Antibus et al., 1980) or form both of these symbioses on the same root system. This phenomenon is called dual mycorrhizal colonization and is known in *Salix* spp. (Dhillion, 1994; van der Heijden, 2001) as well as in poplars and few other plant species (Chilvers et al., 1987; Lodge, 1989; Truszkowska, 1953). Until now, the reasons or factors driving dual colonization have not yet been fully elucidated (Chilvers et al., 1987; Lodge, 1989; McGuire et al., 2008; van der Heijden, 2000). Dual colonization is regarded as an ancient remnant of adaptation to nutrient-poor conditions during evolution. Since AM and ECM were expected to explore different nutrients pools, their co-existence or preferential formation was treated as functional complementarity.

¹ 'Alpine' is defined in both geographic and biological sense, i.e., referring to the European Alps and zone above the climatic high altitude timberline (Körner, 2003) and are used in this meaning in entire doctoral thesis.

Moreover, co-formation of AM and ECM explain willows plasticity in adaptation to different environmental conditions (Lodge, 1989). This feature nowadays is highly appreciated in environment protection (e.g., control of water and wind erosion), phytoremediation (e.g., regeneration of degraded habitats) and production of the timber fuel (i.e., renewable sources energy). Most of these issues are, however, far beyond the scope of the presented doctoral thesis.

General objectives of the doctoral project

The overall objective of the doctoral project was to characterize the size², composition³ (structure and abundance) and functioning⁴ of the mycorrhizal fungal communities at different stages of ecosystem development. More specifically, I aimed at:

- Comparing the size of the different microbial populations (i.e., AMF, other soil fungi including ECM, and bacteria) along a soil developmental gradient (**chapter 1**).
- Characterizing the composition of mycorrhizal fungal communities and their functions in the rhizosphere of willow colonizing the sites of different developmental stage (**chapter 2**).
- Measuring the functions of indigenous fungal communities of young and developed soils in willow growth and nutrient acquisition from external inorganic (**chapter 3**) and organic (**chapter 4**) forms.

This project aimed at providing a more complete and holistic picture of the mountain ecosystem functioning (Fig. 1). Thus, my investigations ranged from the broad scale of the entire ecosystem (**chapter 1**) through the level of the plant community (**chapter 2**) to the very detailed level of the individual plant (**chapters 3 and 4**). Such an arrangement of the research mirrored the construction of the entire doctoral project (Fig. 3) aiming at establishing the link between the mycorrhizal fungal community composition and functioning. The forefield

² 'Size' is defined in the doctoral thesis as estimation of the biomass of microbial populations (Frostegård and Bååth, 1996) in analysed samples.

³ 'Composition' is defined in the doctoral thesis as occurrence of the different fungal taxa in analysed samples.

⁴ 'Functioning' is defined in the doctoral thesis as performance of fungal communities in analysed samples meaning their enzymatic activity or ability of transfer of nutrients to the host plant.

of retreating glacier (detail description **Materials and methods**, **Sampling sites**, chapter 1) and lab experiments (detail description **Materials and methods**, chapter 3) mimicking natural conditions (e.g., use of the substrates collected from the Damma glacier, alpine plant species) gave a unique insight into the functioning of mycorrhiza-plant-soil as an integral system.



Figure 1

Investigation levels of the doctoral project.

Hypotheses

The following hypotheses were tested in this project (Fig. 2):

- H1) The composition (i.e., taxonomic structure) and functioning (i.e., functional diversity) of mycorrhizal fungal communities associated with roots and rhizosphere soils of *Salix helvetica* will increase along an ecosystem developmental gradient at the glacier forefield.
- H2) The communities of mycorrhizal fungi from old soils will affect the growth and nutrition of willows better as compared with young soils.
- H3) The communities of mycorrhizal fungi mobilize nutrients from different sources: 1) in young soils – more readily from recalcitrant inorganic sources (i.e., apatite) through production of LMWOA and protons, 2) in old soils – more readily from organic sources (i.e., bacterial biomass) through production of hydrolytic enzymes.

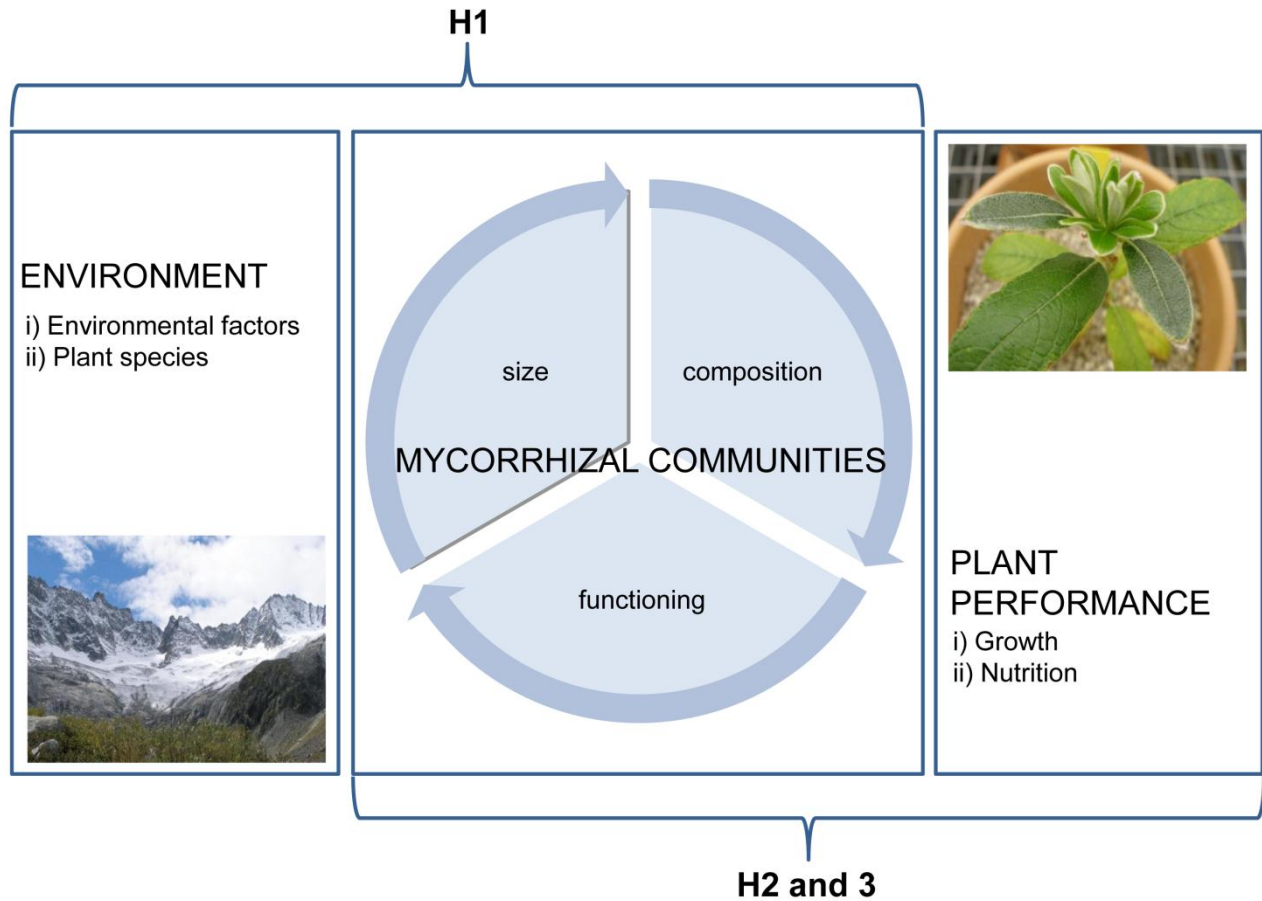


Figure 2

Schematic representation of the hypotheses tested in the project.

Objectives of individual chapters

The **Chapter 1** challenged the hypothesis that bacteria are more abundant in the young soils, whereas the importance of fungi would increase in the later stages of soil development. The results provided important background information for further investigations and - together with other studies - contributed to a better characterization of the processes driving ecosystem succession (Bernasconi et al., 2011).

Chapter 2 addressed whether the type of mycorrhizal association (i.e., AMF, ECM and ericoid mycorrhizal fungi, ER) influenced enzymatic activities in the rhizosphere soils. From a practical point of view, this research substantiated the selection of the willow as a model plant

for our further investigations. This plant presented a wide range of distribution along the soil developmental gradient and was able to form symbioses with ECM and AMF simultaneously.

The last two chapters (**chapters 3 and 4**), aimed at linking the composition of mycorrhizal fungal communities associated with willow with their functions (Fig. 2). A contribution of mycorrhizal fungi to the uptake of N and P by the willows and to the growth of the plants was studied under controlled conditions.

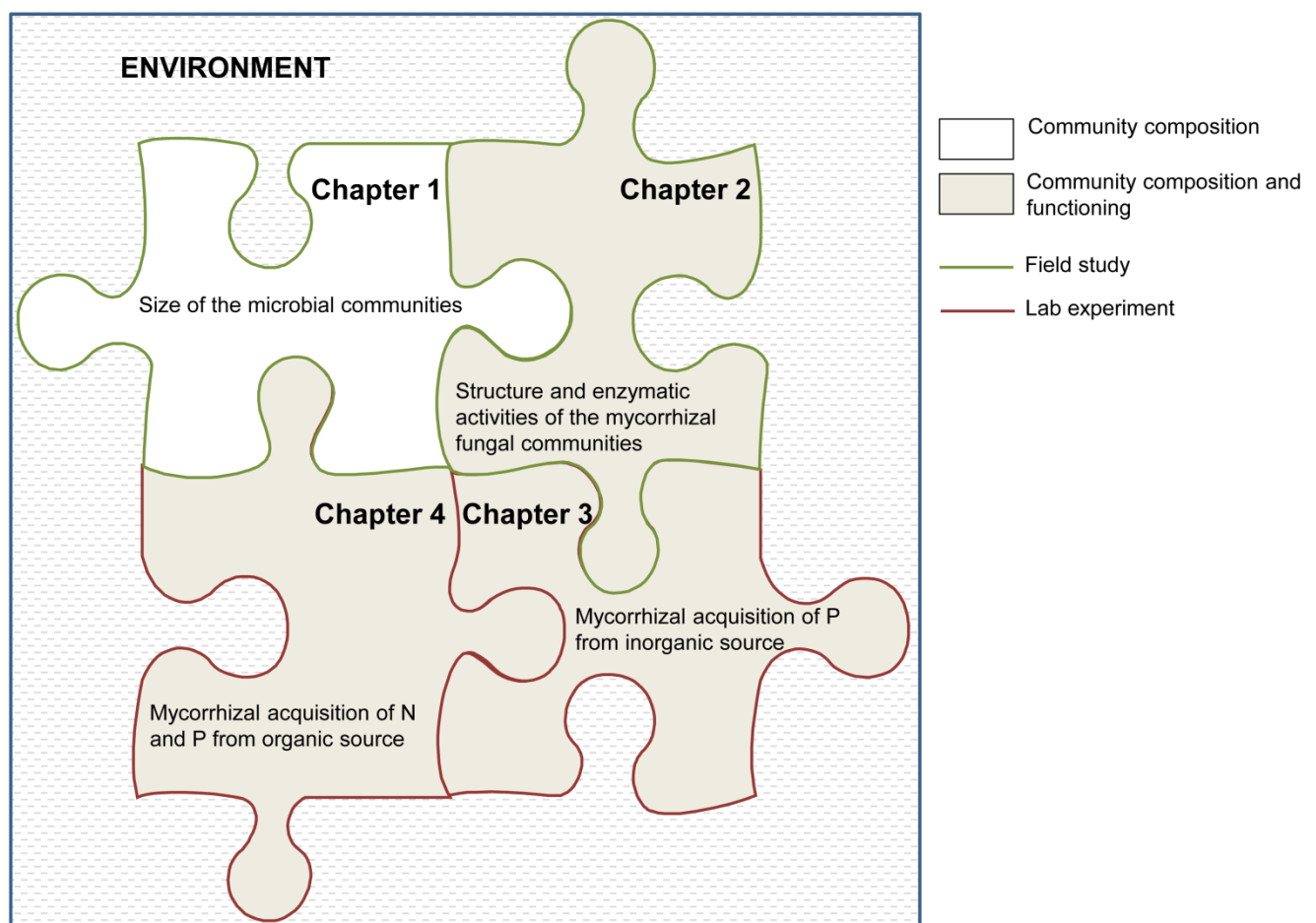


Figure 3

Project construction: each part of the puzzle represents one chapter. The focus (community composition or composition and functions) as well as the character of each study (field study or lab experiment) of each chapter is specified in the legend.

Methodological approaches

Different methods were employed in the doctoral project (Table 1). Fatty acids analyses and enzymatic activity assays were used in chapters 1, and 2 and 4, respectively. Characterization of the fungal community composition (structure and abundance) required the development - specifically for investigated environment - new molecular markers for quantitative PCR (qPCR). These qPCR markers were designed using DNA sequences generated exclusively for the investigated ecosystem. Isotope labeling was employed to track nutrient fluxes between soil and plants as mediated by the mycorrhizal fungi (chapters 3 and 4).

Table 1

Methods employed for characterization of the fungal community size, composition and functions.

Chapters	Methods	
	Size / composition	Functions
Chapter 1	FAME	nd
Chapter 2	qPCR	enzymatic activity assays
Chapter 3	qPCR	isotopic labeling
Chapter 4	qPCR	enzymatic activity assays isotopic labeling

Abbreviations: (FAME) Fatty Acid Methyl Esters; (qPCR) quantitative PCR; (nd) not determined.

Since *Salix helvetica* was selected as a model plant, one of the prerequisites for set-up of the rhizobox experiments was propagation of willows under controlled conditions. Preliminary propagation started in 2008 and this procedure was successfully established in 2010. This allowed to conduct the experiments by the end of 2010 (**chapter 4**) and at the beginning of 2011 (**chapter 3**).

The PhD work was embedded in the BigLink project (<http://www.cces.ethz.ch/projects/clench/BigLink>, 6th April 2012) conducted at the Damma glacier forefield. This project examined the interactions between biosphere and geosphere in order to understand the processes of the ecosystem development and succession. The group of about 30 researchers from different field of expertise (e.g., botany, geology, glaciology, hydrology, microbiology, mineralogy, mycology) have been working in BigLink and other projects directly related to BigLink. The results of these investigations were shared with international scientific community either via multidisciplinary (Bernasconi et al., 2011; Bernasconi et al., 2008) or specialized publications (Dümig et al., 2011; Frey et al., 2010; Göransson et al., 2011; Kiczka et al., 2010; Magnusson et al., 2011; Welc et al., 2012).

Chapter 1: Soil bacterial and fungal communities along a soil chronosequence assessed by fatty acid profiling

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Abstract

Microbial communities are important components of terrestrial ecosystems. The importance of their diversity and functions for natural systems is well recognized. However, a better understanding of successional changes of microbial communities over long time scales is still required. In this work, the size and composition of microbial communities in soils of a deglaciation chronosequence at the Damma glacier forefield were studied by fatty acid profiling. Soil fatty acid concentrations clearly increased with soil age. The abundances of arbuscular mycorrhizal fungi (AMF), bacteria and other soil fungi, however, were more affected by abiotic soil parameters like carbon content and pH than by soil age. Analysis of ratios of the different microbial groups (AMF, fungi, bacteria) along the soil chronosequence indicated that: i) the ratios of AMF to bacteria and AMF to other fungi decreased with soil age; and ii) the ratio of fungi to bacteria remained unchanged along the soil chronosequence. These two pieces of evidence suggest that the evolution of this ecosystem proceeds at an uneven pace over time and that the role of AMF is less important in older, more organic and acidified soils than in mineral soils. In contrast to other studies, no successional replacement of bacteria with fungi in more acidified and organic soil was observed.

Keywords: Fatty Acid Methyl Esters (FAME), glacier forefield, soil chronosequence, microbial community, mycorrhizal fungi, community size, environmental determinants

Introduction

Soil is a fundamental component of terrestrial ecosystems. Soil properties develop over time through the interplay of abiotic and biotic processes (Paul, 2007). Several centuries to millennia may need to pass until rock material is transformed into soil, making the process of soil and ecosystem development impossible to observe within human life time. Thus ecologists commonly study areas with a soil developmental gradient resulting from natural processes (e.g., retreat of glaciers, succession on volcanic ashes and islands of uneven ages), thus replacing temporal with spatial gradients (Huggett, 1998; Walker et al., 2010). Chronosequences established on land gradually emerging from underneath of retreating glaciers have formed the basis for numerous studies on soil formation (Dümig et al., 2011; Mavris et al., 2010; Stevens and Walker, 1970) and biological succession (Chapin et al., 1994; Dobas-Miranda et al., 2008; Garibotti et al., 2011; Grzesiak et al., 2009; Sigler et al., 2002). The development of the ecosystem is strongly interlinked with soil microbial activity. Thus, composition, taxonomic and functional diversity of microbial communities on deglaciated lands have been studied intensively (Cázares et al., 2005; Tscherko et al., 2003). This has mostly been accomplished by nucleic acid-based techniques, which offer a deep resolution of identities, but have shortcomings with respect to quantitiveness. Quantitative aspects of microbial communities are best assessed using biochemical markers such as fatty acids (Kaštovská et al., 2007; Tscherko et al., 2005).

The establishment of microbial communities on newly deglaciated areas is a complex and multifaceted process. The gradient of environmental conditions along the soil chronosequence has been postulated as the main factor governing the structure (composition and diversity) and activity of the microbial communities (Lazzaro et al., 2009; Lazzaro et al., 2010; Noll and Wellinger, 2008). Although differences between recently and earlier deglaciated soils in temperature (lower in the vicinity of ice), rates of weathering and accumulation of soil organic matter (SOM) seem to be obvious, their impact on microbial communities is still poorly understood. Therefore, in this study we attempted to disentangle the effects of different soil properties (i.e., nutrients, soil age and pH) along a natural gradient of soil development on the biomass of the main groups of the microbial community.

The size of a nutrient pool such as phosphorus and nitrogen in the topsoil usually increases with increasing soil age, and this is mostly accompanied by an increase of

microbial and plant biomass with soil age. However, nutrients typically limit plant and microbial growth differentially at various stages of soil development. Moreover, microbial groups differ in nutrient acquisition strategies and trophic requirements, which should be reflected in correlations between the biomass of different soil organisms and the levels of soil nutrients in different soil developmental stages. Soil fungi are active participants but also main beneficiaries of SOM decomposition. Thus, their biomass is expected to correlate with soil age as well as soil carbon (C) content. In case of arbuscular mycorrhizal fungi (AMF) and bacteria obtaining C from other sources (AMF: photoassimilates from the host plant and bacteria: autotrophic C fixation) a weaker correlation with soil age and a dependency on elements other than C can be presumed. Phosphorus (P) could be a potentially important agent, lowering the biomass of AMF community as shown in other environments (Jansa et al., 2009), whereas the availability of nitrogen (N) and other biogenic elements preferentially affects bacterial communities (Madigan and Martinko, 2006).

Acidification is an integral process of soil development which has been found to be important in shaping the structure of microbial communities (Noll and Wellinger, 2008; Rousk et al., 2010). However, to the best of our knowledge, the influence of pH on the biomass of different microbial groups in recently deglaciated areas as it is presented in our work has not been elucidated. The Damma glacier forefield exhibits a gradient of soil pH ranging from 4.4 to 3.5, which is expected to shift the microbial community from dominance of bacteria to that of fungi (Aliasgharzad et al., 2010). Moreover, based on experience from other ecosystems such as forests and an experimental agroecosystem (Coughlan et al., 2000; Wang et al., 1993) a shift in AMF biomass along the soil pH gradient can be hypothesized, i.e., a positive correlation between pH and AMF biomass.

In the present work we aimed to estimate the microbial biomass and to identify the abiotic determinants of the size and coarse diversity of microbial communities in a soil chronosequence along the Damma glacier forefield, distinguishing AMF, other fungi, and bacterial communities. In particular, the dominance of fungi over bacteria in older soils and the successional replacement of AMF with other symbiotic associations (i.e., ericoid and ectomycorrhizal fungi) were tested. To ensure the selection of the lipid fraction with the highest informative power for the investigated ecosystem, different lipid fractions were analyzed separately by fatty acid profiling.

Materials and methods

Sampling sites

This study was conducted at the forefield of the retreating Damma glacier, situated in the Western Alps in the Canton of Uri, Switzerland (N46°38.117', E8°27.677'). The glacier forefield is situated in front of an ice block, which is a remnant of a glacier tongue and currently detached from the main glacier body. The forefield is oriented south-west to north-east and measures approximately 1000 m in length and 600 m in width. Average inclination of the forefield area from the ice block to the bottom of the forefield valley is about 21%. Two main recent advances of the glacier resulted in terminal moraines dated back to 1992 and 1927. Side moraines date back to 1850.

Twenty three experimental sites along the forefield chronosequence were selected in July 2007. These sites are identical to the so called “common sampling design” of the BigLink project (Bernasconi et al., 2011). Of these, 21 sites were distributed along a recent soil chronosequence (Fig. S1) at the valley bottom, thus minimizing effects of the side slopes. The approximate ages of soil (i.e., years after the last deglaciation) at each experimental site were calculated based on historic glaciological records available from the Swiss Glacier Monitoring Network (glaciology.ethz.ch/messnetz/glaciers/damma, accessed 11th March 2012). The soil age at the forefield ranged between 7 and 137 years. The two remaining sites, at a distance of about 500–800 m from the forefield (Fig. S1), were deglaciated much earlier (probably more than 3000 years ago) and served as reference sites. An approximate age of 3000 years was assigned to the reference sites for statistical analyses. Soils at the experimental and reference sites were classified as Hyperskeletal Leptosol and Haplic Cambisol, respectively (Bernasconi et al., 2011).

The vegetation is very patchy in the young part of the chronosequence and dominated by individual herb species. As succession continues, plant cover is increasing up to 100% coverage. The initial dominance by herbs and grasses (*Agrostis gigantea*, *Poa alpina*, *Leucanthemopsis alpina*) is gradually replaced by dominance of woody plants (*Salix helvetica*, *Salix herbacea*) and shrubs (*Rhododendron ferrugineum*, *Vaccinium* sp., *Calluna vulgaris*, *Loiseleuria procumbens*) in the middle and old parts of the chronosequence, respectively. The reference sites are dominated by grasses (*Agrostis gigantea*, *Poa alpina*, *Festuca rubra*).

Soil sampling and processing

Soil samples were collected from the 23 sites between 10th and 12th September 2007 (see Bernasconi et al. 2011 for further details). Soil from each site was collected from three subplots (0.5 m × 0.5 m each) within a 2 m × 2 m quadrant, with separate sampling from two depths (0-5 cm and 5-10 cm). Soil from each depth was passed through an 8 mm sieve directly in the field, homogenized by mixing, preserved in plastic bags, transported to the laboratory and stored at -20°C until analyses. Subsequently, soil samples were freeze-dried, passed through a 2 mm sieve, ground to a fine powder with mortar and pestle at room temperature and weighed directly into 50 ml glass centrifuge tubes for lipid extraction. Fifteen, 10 and 5 g dry soil was processed for young (deglaciated approx. 7–14 years ago), middle-aged (deglaciated approx. 59–79 years ago), and old soils (deglaciated more than 100 years ago), respectively.

Lipid analyses

Lipids were extracted from all samples following the procedure of Frostegård et al. (1991) with slight modifications. Briefly, 15 ml of a chloroform: methanol: citrate buffer (0.15 M, pH 4.0) mixture (1:2:0.8, v:v:v) was added to the samples in the centrifuge tubes. Each sample was spiked with 900 µg of the first internal standard (Fig. S2), nonadecanoic fatty acid (19:0, Sigma-Aldrich, Buchs, Switzerland) dissolved in hexane. Samples were incubated at room temperature for 2 h with occasional stirring. Phase separation was attained by addition of 6.2 ml of chloroform: citrate buffer (1:1, v:v) and centrifugation at 750 g at 16°C for 15 min. The lower organic phase was collected with a 10 ml Hamilton syringe, filtered through syringe-driven hydrophobic filters (Millex-FR, Millipore, Zug, Switzerland), transferred to a new 50 ml glass tube and evaporated to dryness under mild vacuum (water-driven pump). Total lipids in this fraction were redissolved in 200 µl of chloroform and divided in two parts. One half was evaporated to dryness under vacuum and stored at -20°C for analysis of whole cell fatty acids (WCFA). The other half was used for lipid fractionation using pre-packed silica columns (Bond Elut LRC- Si 100 mg, Varian, Darmstadt, Germany). Elution volumes for each fraction were adjusted experimentally to assure complete elution, with 5.2 ml of chloroform, 7.8 ml of acetone and 5.2 ml of methanol eluting neutral lipids (NL), glycolipids (GL), and phospholipids (PL) from the silica column, respectively (Fig. S3). These lipid fractions were

evaporated to dryness under mild vacuum (water-driven pump) for 30-45 min. Each fraction then received 300 µg of heneicosanoic acid (C21:0, Sigma-Aldrich, Buchs, Switzerland) dissolved in hexane as a second internal standard (Fig. S2).

Unfractionated lipids as well as the three lipid fractions were subjected to transmethylation with trimethylchlorosilane (TMCS) that was found superior to the often used mild alkaline methanolysis (MAM) (Fig. S4). Two ml of a TMCS: methanol mixture (1:9, v:v) was added to each sample, followed by incubation at 40°C for 45 min (Poerschmann and Carlson, 2006). The resulting fractions i.e., methyl esters of unfractionated WCFA, neutral lipid fatty acids (NLFA), glycolipid fatty acids (GLFA) and phospholipid fatty acids (PLFA) (Fig. S2), were filtered through syringe-driven hydrophobic filters (Millex-FR) and evaporated to dryness under mild vacuum (water-driven pump) for 20 min. Prior to gas chromatography (GC) analyses, samples were dissolved in hexane (25 to 100 µl per sample, depending on the expected fatty acid concentrations). GC analyses were carried out on a Perkin Elmer Gas Chromatograph 8700, fitted with a capillary column (Supelcowax 10TM, 30 m × 0.53 mm, 2 µm coating, Sigma-Aldrich) and equipped with a splitless capillary injector and flame ionization detector. Helium was the carrier gas with a flow rate of 2 ml min⁻¹. The injector temperature was 260°C and the detector temperature was 300°C. The temperature program was as follows: initial oven temperature 110°C held for 10 min, then ramped to 240°C at a rate of 2°C min⁻¹, followed by an isothermal phase at 240°C for 35 min. Fatty acids were identified by comparing their retention times with those of several commercial standards provided by Sigma-Aldrich, Buchs, Switzerland (Product Number: 18918-1AMP, 47033 and 47015-U). The position of the peak of fatty acid 16:1 ω 5 was determined by comparison of lipid extracts of mycorrhizal vs. non-mycorrhizal roots of *Medicago truncatula*. The identities of the different fatty acids were further confirmed by gas chromatography - mass spectrometry (GC-MS) analysis of selected samples, using Agilent Technologies 6890N Network GC System – Mass Selective Detector 5973, equipped with a capillary column (Supelcowax10TM, 60 m × 0.25 mm, 0.5 µm coating, Sigma-Aldrich). The running conditions for both instruments were identical.

The nomenclature of the fatty acids in this paper follows IUPAC recommendations (IUPAC-IUB, 1977) using ω -reference abbreviations (position of double bond(s) counted from the methyl (ω) carbon in the molecule). The number of C atoms in the molecule is expressed by

the number before the colon; the number after the colon indicates the number of double bonds in the molecule.

Selected PLFA were used as signatures for different soil organisms (Table 1). The sum of 15:0, 16:1 ω 9, 16:1 ω 7, 17:0 and 18:1 ω 7 fatty acids was used as a signature for bacteria (Frostegård et al., 1996), the sum of 16:1 ω 5, 20:4 ω 6 and 20:5 ω 3 as a signature for arbuscular mycorrhizal fungi (Olsson, 1999), and 18:2 ω 6 as a signature for other soil fungi, including ecto- and ericoid mycorrhizal fungi (Frostegård et al., 1996).

In total, 53 fatty acids were detected in the four different fatty acid fractions. In WCFA profiles, 49 fatty acids were present, out of which 34 could be identified (Table 1). The concentration of each individual fatty acid was calculated based on the quantification of the internal standards introduced into each sample (Fig. S2). The peak area was converted into μ g amounts and used for the calculation of fatty acid concentrations per unit of dry soil weight.

Soil properties

Soil samples for chemical analyses were air-dried, sieved through a 2 mm sieve and ground to a fine powder with a laboratory ball mill (Retsch mixer mill 200M, 30 Hz) for 4 min. Total carbon (C_{tot}) and total nitrogen (N_{tot}) were analyzed by dry combustion (NCS Analyzer, FlashEATM 1112 Series, ThermoElectronic Corporation). Total phosphorus (P_{tot}) concentrations were estimated in 300 mg soil aliquots after their incineration (16 h, 550°C), dissolution in 1.0 ml of 65% HNO_3 (2 min, 250°C) and filtration through 0.2 μ m nitrocellulose membranes.

Concentrations of plant available phosphorus (P_{av}), calcium (Ca_{av}), potassium (K_{av}), and magnesium (Mg_{av}) were assessed following the extraction of soil samples with ammonium acetate–EDTA solution (1:10 w:v) (Stünzi, 2006) by shaking at 3.75 Hz at room temperature for 1 h. Before the analyses, extracts were filtered using ashless filter paper (Whatman 40). The concentrations of P_{tot} and P_{av} in the extracts were measured colorimetrically at $\lambda=610$ nm (UV 1601 Spectrophotometer, Shimadzu), using the malachite green method (Ohno and Zibilske, 1991). The concentrations of Ca_{av} , K_{av} and Mg_{av} were determined by Inductively Coupled Plasma – Mass Spectrometry (Agilent 7500 C) in 10-fold diluted extracts. Soil pH

was measured in suspensions of the soil in 0.01 M CaCl₂ (1:2.5, w:v), using a Corning 125 pH-meter (Corning B.V. Life Sciences, Amsterdam, The Netherlands).

Statistical analyses

Fatty acid data were combined with several environmental variables and subjected to multivariate statistics using Canoco 4.5 (ter Braak and Šmilauer, 2002). Multivariate statistical analyses were conducted on centered and standardized data matrices. Detrended Correspondence Analysis (DCA) indicated a short length of the gradient (1.411 and 1.031 for WCFA and PLFA, respectively), which justified employment of Redundancy Analysis (RDA). Statistical significance of the relationship between fatty acid profiles and environmental variables was evaluated using the Monte Carlo permutation test (unrestricted permutation, reduced model, 9999 permutations). Paired-sample comparison tests (t-test, Statgraphic Plus for Windows 3.1), were employed to compare the differences between fatty acid concentrations in the different soil depths.

Table 1

Codes and identities of the different fatty acids detected in the whole cell fatty acid fraction in this study. Also, assignment of signature phospholipid fatty acids to different groups of organisms is given with their respective bibliographic references. Abbreviations: (PLFA) phospholipid fatty acids (methyl esters); (AMF) arbuscular mycorrhizal fungi.

Compound code	Fatty acid identity according to IUPAC (IUPAC-IUB, 1977)	Signature PLFA	Reference
C1	12:0		
C2	unidentified		
C3	unidentified		
C4	14:0		
C5	14:0 12Me		
C6	14:1		
C7	unidentified		
C8	unidentified		

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C9	15:0	Soil bacteria	(Frostegård et al., 1996)
C10	unidentified		
C11	15:0 14Me		
C12	unidentified		
C13	15:0 ethyl		
C14	16:0		
C15	16:1 ω 7	Soil bacteria	(Frostegård et al., 1996)
C16	16:1 ω 5	AMF	(Olsson et al., 1995)
C17	16:0 14Me		
C18	17:0	Soil bacteria	(Frostegård et al., 1996)
C19	unidentified		
C20	unidentified		
C21	unidentified		
C22	unidentified		
C23	17:1		
C24	18:0		
C25	18:1 ω 9		
C26	unidentified		
C27	18:1 ω 7	Soil bacteria	(Frostegård et al., 1996)
C28	18:1 ω 5		
C29	18:2 ω 6	Soil fungi (excluding AMF)	(Frostegård et al., 1996)
C30	18:3 ω 3		
C31	18:3 ω 6		
C32	unidentified		
C33	18:4 ω 3		
C34	20:0		
C35	20:1 ω 11		
C36	20:2		
C37	20:3 ω 6		
C38	20:4 ω 6	AMF	(Graham et al., 1995; Larsen et al., 1998)
C39	unidentified		
C40	20:3 ω 3		
C41	20:5 ω 3	AMF	(Graham et al., 1995; Johansen et al., 1997)
C42	22:0		
C43	22:1 ω 11		
C44	unidentified		
C45	22:1 ω 9		
C46	unidentified		
C47	22:2 ω 6		
C48	23:0		
C49	22:4 ω 6		

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Results

1. Effect of soil age and depth on the total concentration of lipid fractions

The concentration of fatty acids increased significantly with soil age and showed similar trends for both soil layers, both in the WCFA (Fig. 1) and in the different lipid fractions (data not shown). As confirmed by multivariate analysis (Fig. 2), 39.8% of the variability in the WCFA profiles was explained by soil age and depth. Moreover, the multivariate analyses suggest that: i) the concentrations of all WCFAs increased with or remained unaffected by soil age, with none decreasing with soil age, ii) the abundances of only a few WCFAs correlated with soil depth (either positively or negatively), and iii) the influence of soil depth was smaller than that of soil age (Fig. 2). Therefore, the lower layer was excluded from further analyses. Neutral lipid fatty acids (NLFAs) represented about half, and glycolipid fatty acids (GLFAs) and phospholipid fatty acids (PLFAs) about a quarter each of the amount recorded for the WCFAs (Figs. 1, 3 and S5). Since all fractions showed a similar trend and PLFAs presented the strongest correlation with soil age (Fig. 3), only this fraction was analyzed in the further steps.

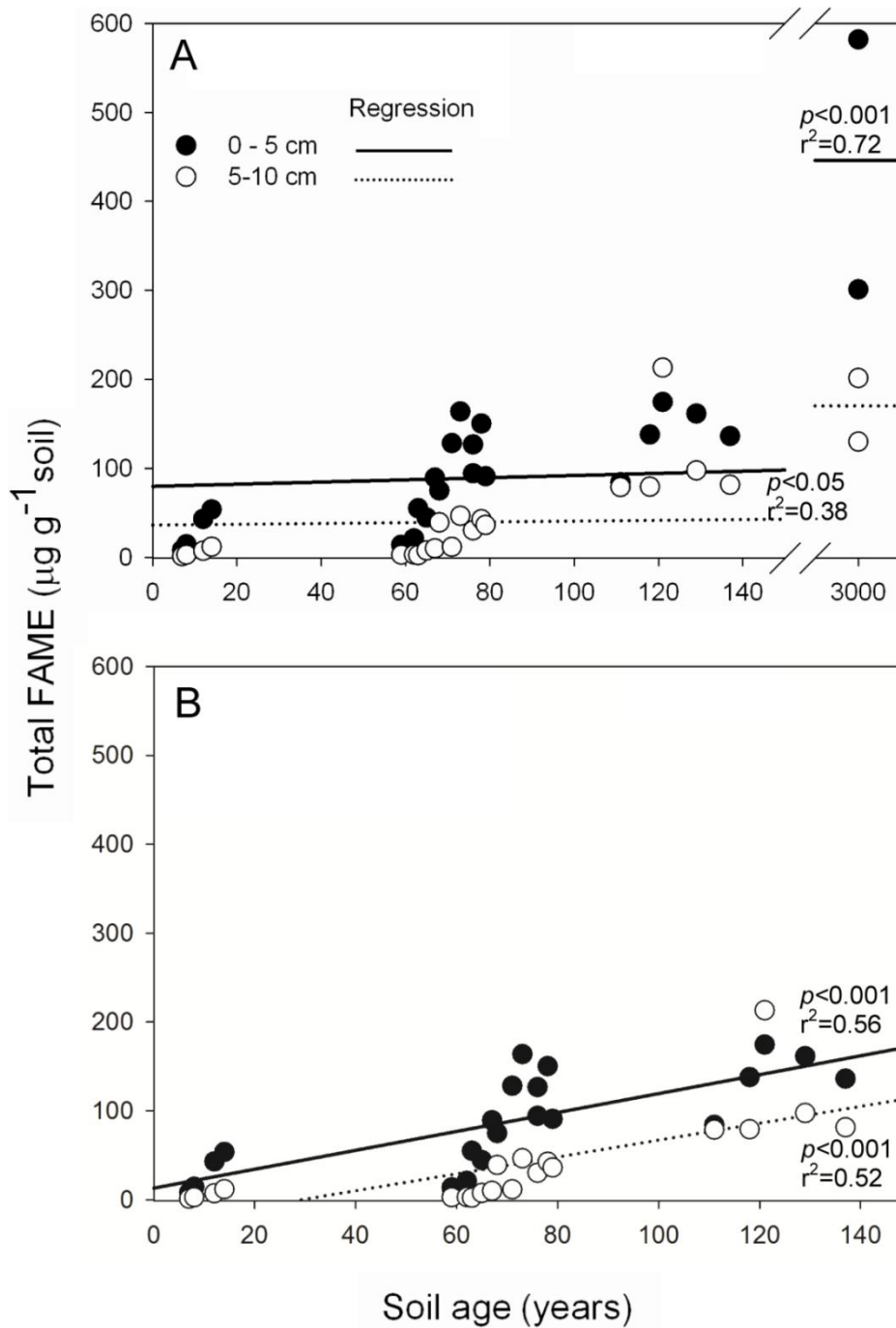


Figure 1

Sums of fatty acid methyl ester (FAME) concentrations detected in the whole cell fatty acid fraction of the individual samples along the soil chronosequence at two soil depths (0-5 cm and 5-10 cm), including (A) or excluding (B) the two reference sites. Statistical significances and r^2 values for the linear regression models are given next to the respective lines.

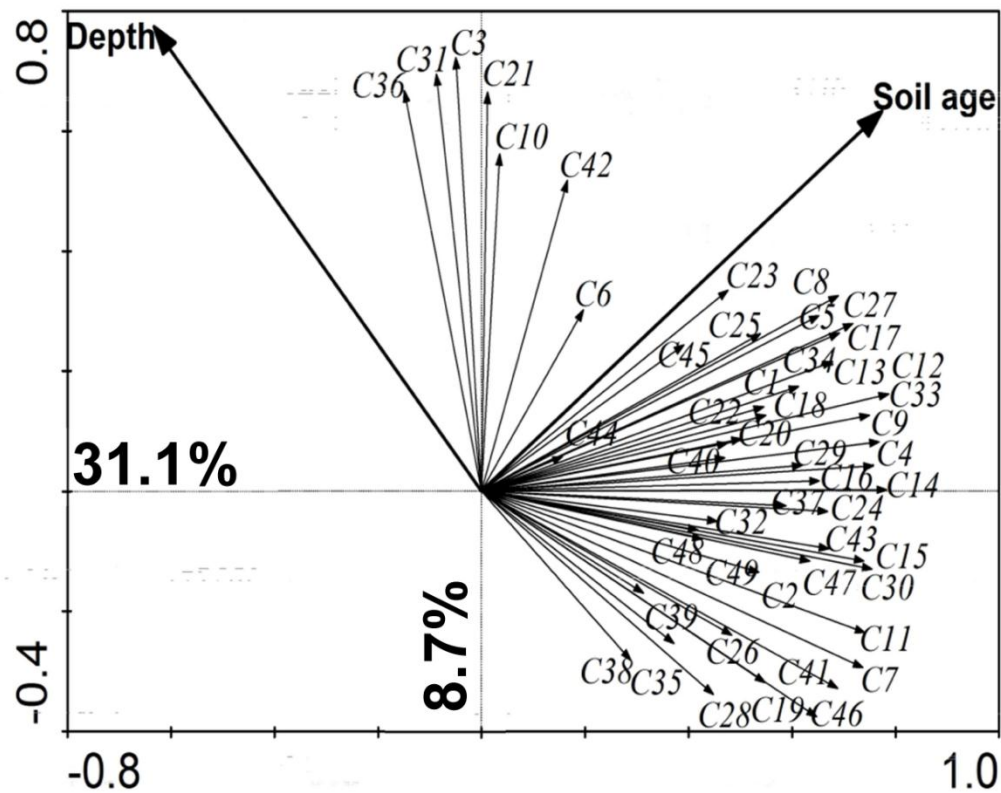


Figure 2

Redundancy analysis ordination diagram showing dependencies between whole cell fatty acid profiles from two soil depths and explanatory variables (soil age and soil depth). Monte Carlo permutation tests carried out for two canonical axes (which were the only axes) confirmed the significance of the relationship between the two datasets ($F=12.9$, $p=0.0001$). Individual compounds were encoded as in Table 1.

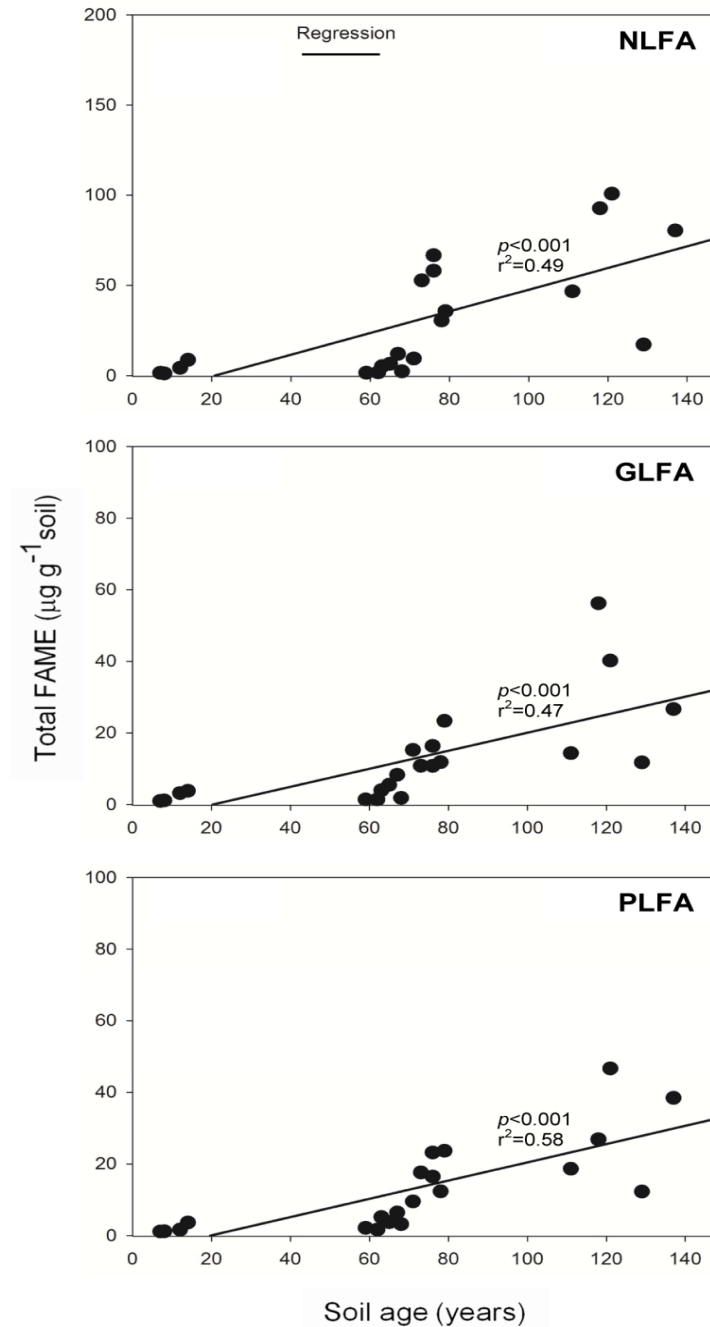


Figure 3

Sums of all fatty acid methyl ester (FAME) concentrations in the different lipid fractions, detected in the individual samples (0-5 cm) along the soil chronosequence upon exclusion of the reference sites. NLFA – neutral lipid fatty acids (methyl esters), GLFA – glycolipid fatty acids (methyl esters), PLFA – phospholipid fatty acids (methyl esters). Statistical significances and r^2 values for the linear regression models are given next to the respective lines.

2. Effect of soil age and soil properties on the concentration of lipid fractions and signature PLFAs

Multivariate analysis relating the signatures PLFA (AMF, fungi, bacteria) to environmental factors (Fig. 4) revealed that they explained 84.6% of the dataset variability. Soil pH and C_{tot} prominently affected the size of the different microbial communities, and signature PLFA were more strongly correlated to C_{tot} than to soil age (Fig. 4). The concentrations of signature PLFAs correlated negatively with soil pH (Figs. 4 and 5). Soil age correlated positively with each of the signature PLFAs, but explained less than 50% of the variability (Fig. 5). The correlation strengthened when C_{tot} and pH were related to signature PLFAs, showing positive and negative correlations, respectively, with more than 50% of the variability explained in all cases. When the ratios of signature PLFAs for AMF, other soil fungi and bacteria were related to soil age (Fig. 6), the following dependencies were revealed: i) the relative abundance of AMF to bacteria decreased with soil age; ii) the relative abundance of other soil fungi to bacteria remained unaffected throughout the chronosequence, and iii) the relative abundance of AMF to other soil fungi decreased with soil age (Fig. 6). Additionally, principal component analysis (PCA) revealed differences in the concentrations of PLFA (total and signatures) along soil developmental gradient (Fig. S6).

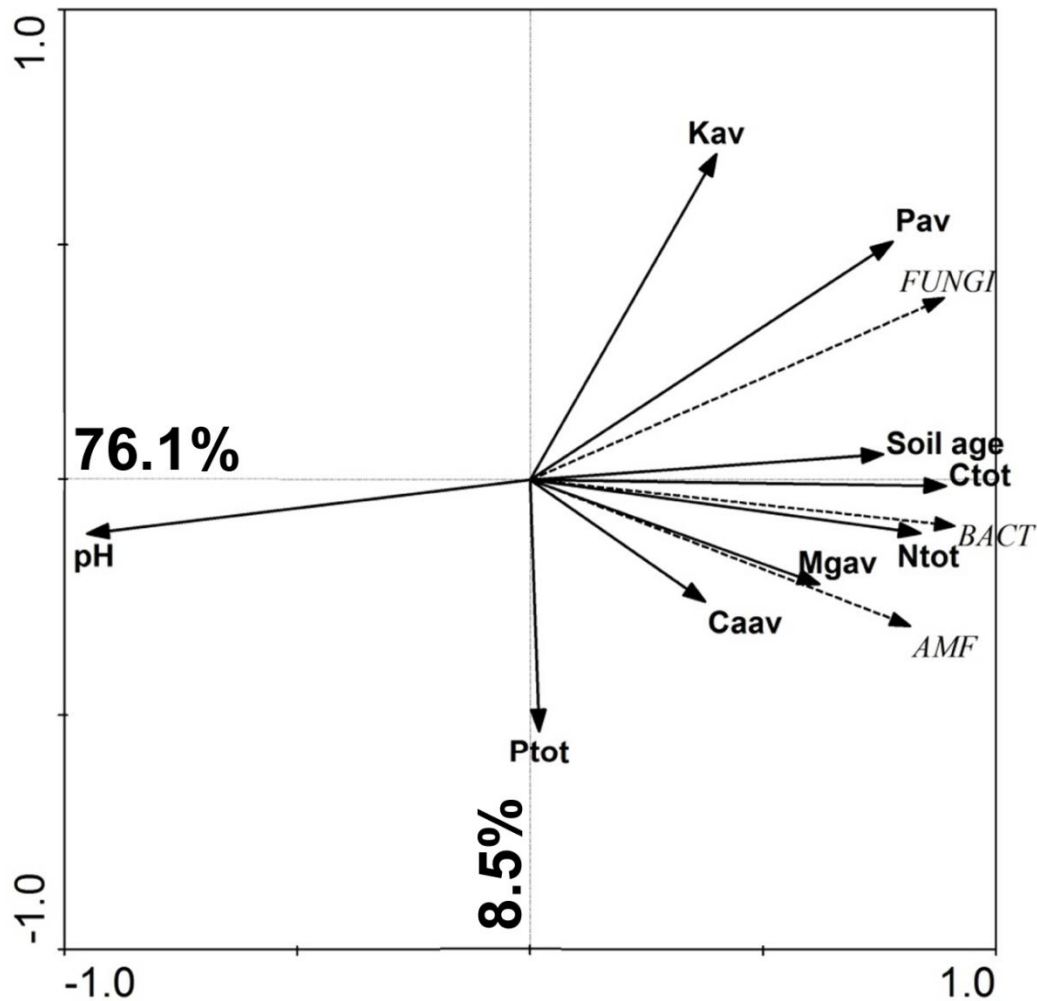


Figure 4

Redundancy analysis ordination diagram showing dependencies between the concentrations of the phospholipid fatty acid signatures in the 0-5 cm soil depth (dashed lines, italics labels) and soil properties (solid lines, bold labels). The signature PLFAs indicative for the main microbial groups, bacteria (BACT), other soil fungi (FUNGI) and arbuscular mycorrhizal fungi (AMF; as in Table 1) are related to soil properties. Monte Carlo permutation tests carried out for all canonical axes confirmed the significance of the relationship between the two datasets ($F=6.9$, $p=0.0003$). Abbreviations: Ptot – total phosphorus concentration, Ntot - total nitrogen concentration, Ctot – total organic carbon concentration, Pav – plant-available P concentration, Kav – plant-available potassium concentration, Mgav – plant-available magnesium concentration, Caav – plant-available calcium concentration.

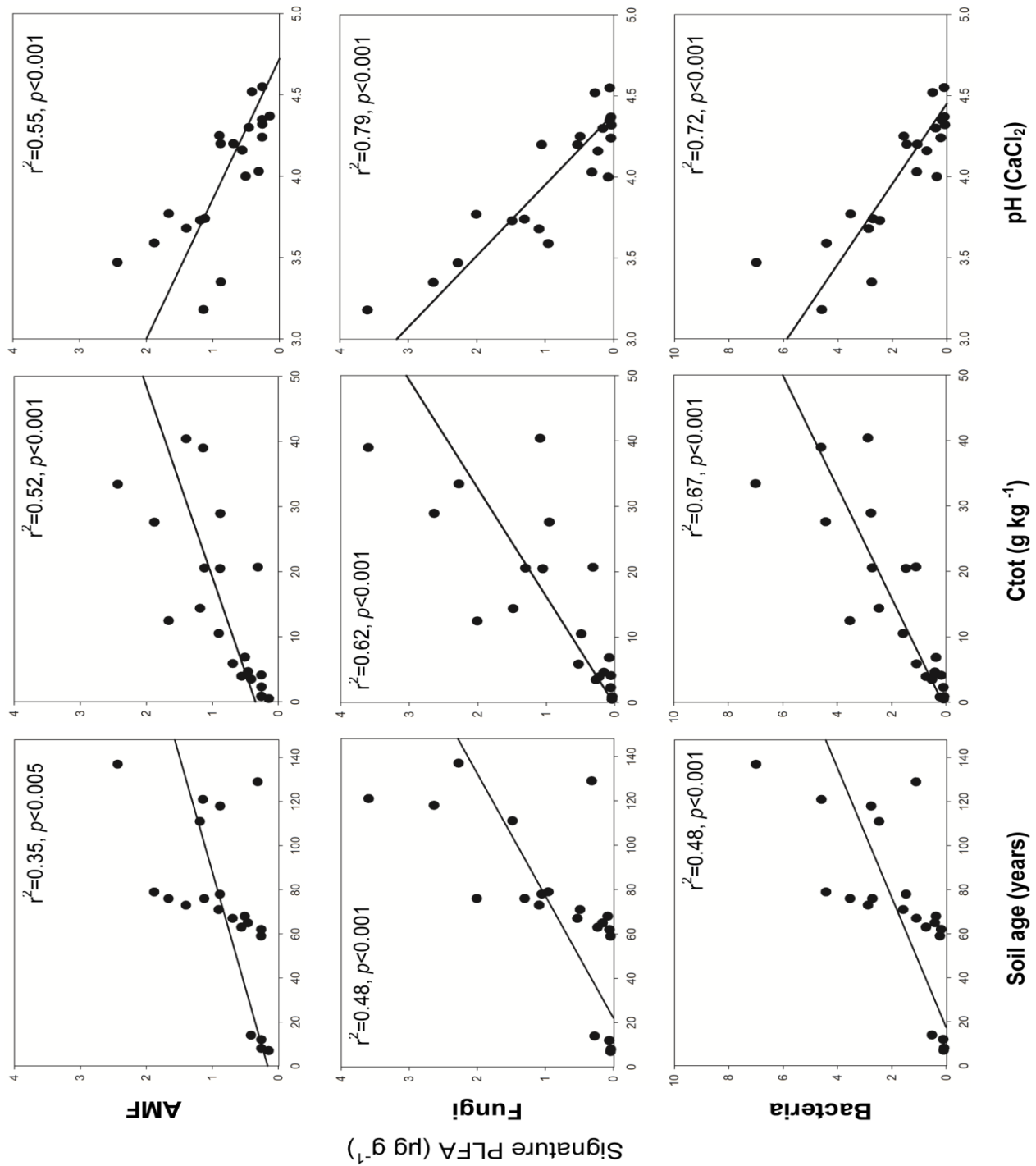


Figure 5

Relationships between the concentrations of signature phospholipid fatty acids (in 0-5 cm soil depth) and soil age (i.e., years after deglaciation), soil organic carbon concentration (Ctot) and soil pH assessed in CaCl₂ (10 mM) soil slurry. Linear regression lines with corresponding r^2 values and levels of statistical significance are shown.

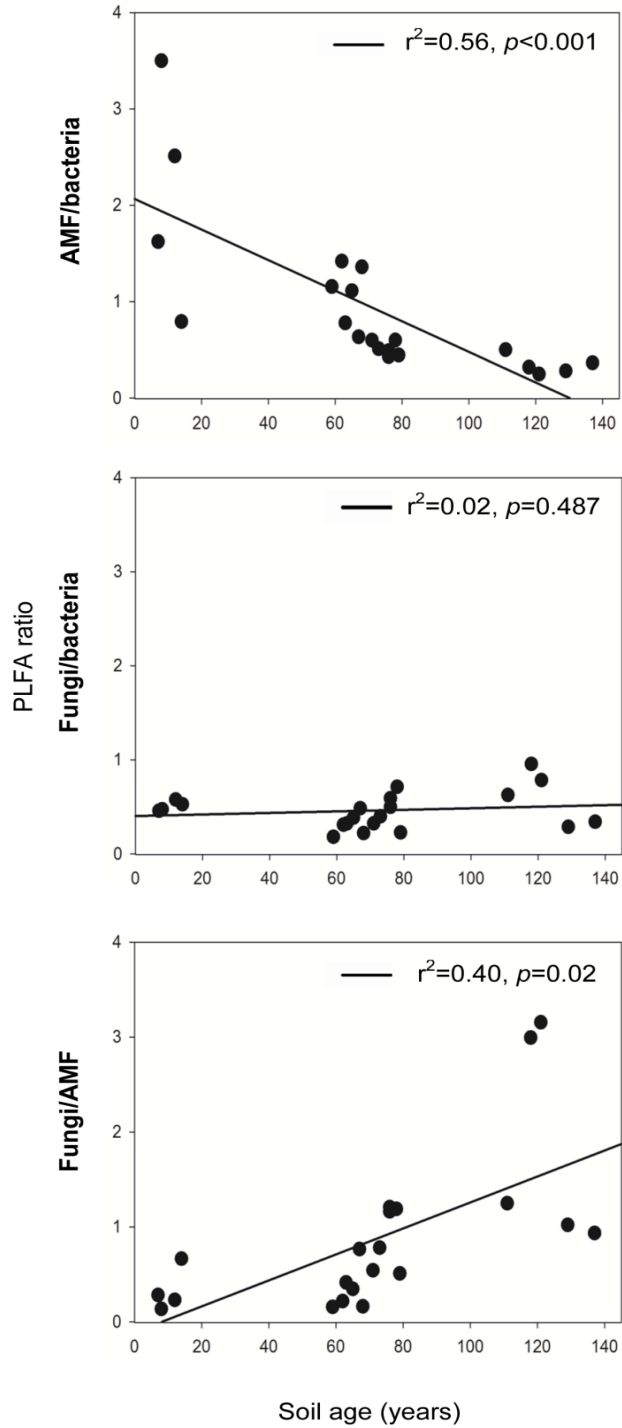


Figure 6

Ratios between the concentrations of signature phospholipid fatty acids for arbuscular mycorrhizal fungi (AMF), other fungi and bacteria, in soils of different ages (i.e., years after deglaciation) in 0-5 cm soil depth. Linear regression lines with corresponding r^2 values and levels of statistical significance are shown.

Discussion

1. Soil age and fatty acid concentrations

Biological activity in recently deglaciated areas was often found to be influenced primarily by soil age (Noll and Wellinger, 2008; Tscherko et al., 2003). However, the importance of soil age is only tentative: soil development can proceed at uneven rates for different areas and periods of time and may strongly depend on local (micro-environmental) conditions (Vidic, 1998). The fact that in this study the concentrations of fatty acids in soils deglaciated approximately 7 and 60 years ago were similar (Figs. 1, 3 and 5, Table S1) is probably a consequence of the re-advancement of the glacier between the 1960s and 1992, which erased (or set back) most of the soil developmental changes on sites close to the advancing glacier tongue. Prolonged cooling of the soils close to the glacier tongue and exposure to rapid erosion upon glacier melting presumably limited plant and microbial development and/or activities and caused the loss of organic C and fine soil particles, thus starting the soil and ecosystem development anew.

2. Soil C and pH and fatty acids concentrations

Although significant correlations were found between WCFA concentrations and soil age (Fig. 1), which corresponds well to previous studies (Noll and Wellinger, 2008; Tscherko et al., 2003), an even better correlation was found between fatty acid concentrations and soil C and pH. Namely, the signature PLFAs, considered to provide relevant insights into the composition of active microbial communities, showed stronger correlations with soil developmental indicators such as soil C content and pH than with the plain soil age (Figs. 4 and 5). Mechanistically, this can also be seen as increased productivity of the (plant-driven) ecosystems thriving on older soils, feeding back on soil microbial communities via mycorrhizal symbioses, root exudation and litter deposition (Bardgett and Shine, 1999; Chapman and Newman, 2010; Esperschütz et al., 2011; Frostegård and Bååth, 1996).

3. Soil depth and fatty acids concentrations

The concentrations of WCFAs (Fig. 1) as well as fatty acids in the other lipid fractions (data not shown) were generally higher in the 0-5 cm than in the 5-10 cm soil layer, consistent with higher activity of soil microbes in the soil layer with higher root proliferation and a greater C

content (Cardon and Whitbeck, 2007). At the Damma glacier forefield - as in other young soils in cold ecosystems - the majority of the root biomass is concentrated in the first 0-5 cm of soil whereas the lower soil layer, poorly weathered and C-deprived, limits root growth.

4. Size of the microbial communities in soils

Fatty acid analyses deliver information about the biomass of microbial communities in soil. Especially by employing PLFA (Figs. 4 and 5), the size of active communities of the main microbial groups can be estimated (Frostegård et al., 2010). However, the fatty acid concentrations should be translated into biomass carefully (Balkwill et al., 1988; Lindahl et al., 1997; Rejendran et al., 1992). In this study, the biomass of soil fungi was deduced from one fatty acid whereas for AMF and bacteria, the sums of three and five fatty acids, respectively, were used. This approach can easily lead to misinterpretation of the results (Ohtonen et al., 1999) and thus will be briefly discussed here. For example, the most common constituent of PLFA in soil fungi, the 18:2 ω 6 (Ratledge and Wilkinson, 1988), accounts on average for 51% of total PLFA in fungi (Dembitsky et al., 1992). In the case of bacteria, only 26% of all PLFAs can be attributed to the signature PLFAs used in our study (Lindahl et al., 1997). For the AMF, the most frequently used signature PLFA, the 16:1 ω 5, was found to make up about 18% of total PLFA in two *Glomus* species (Olsson and Johansen, 2000). These proportions should be considered for the interpretation of the results presented above. For example, the maximum amount of AMF, fungal and bacterial signature PLFAs in the 0-5 cm soil layer in the glacier forefield were about 3, 4, and 8 $\mu\text{g g}^{-1}$ soil, respectively (Fig. 4, upon exclusion of 20:4 ω 6 and 20:5 ω 3 signatures for the AMF). If the conversion factors above are (close to) correct, this would translate into the following values: 16.7, 7.8 and 30.8, for AMF, other fungi and bacteria, respectively. This means that: i) the soil microbial communities were generally dominated by bacteria, ii) the biomass of AMF was intermediate, and iii) the lowest active biomass could be attributed to the other soil fungi.

5. Ratios of PLFAs as indicators of ecosystem development

Acidification of soils due to C accumulation in other ecosystems has been suggested to cause a shift in microbial communities from bacteria- to fungi-dominated (Bardgett, 2000; Ohtonen et al., 1999), but such a trend did not substantiate in our study (Fig. 6). The results

indicated rather a constant proportion of bacteria to fungi along the whole soil chronosequence. Several potential explanations can be proposed. Firstly, dominance of fungi in ecosystems is usually connected with accumulation of organic C and formation of soil macroaggregates (Bardgett et al., 1993; Beare et al., 1992). Accumulation of organic C at the Damma glacier forefield has clearly been observed (Dümig et al., 2011). Macroaggregate formation has not yet been specifically addressed, but appears to be slow (own observations). This is mainly because of a low clay content (Bernasconi et al., 2011) and irregular drying-rewetting cycles (Denef et al., 2002).

Secondly, the fungal community could be stressed by soil disturbances (Drijbera et al., 2000). This is probably a common phenomenon at the Damma glacier forefield (avalanches, soil/rock slides, water erosion by streams, presence of animals and tourists, etc.). Thirdly, another possible explanation is based on the hypothesis that soil microbial communities exhibit a great adaptability to local soil properties, including pH (Fernández-Calviño and Bååth, 2010; Rousk et al., 2010; Wang et al., 1993). Thus, growth of bacteria, even at the latest stages of soil development with low pH, was not greatly limited. Possibly, also the gradient of pH along the chronosequence was not steep enough to generate detectable changes.

According to another hypothesis, a heterogeneous distribution of nutrients in the soil, either in spatially discrete patches or in different forms (i.e., inorganic, organic, easily available vs. recalcitrant), could affect the distribution and size of both AMF and fungal communities in space as well as along the soil developmental gradient. The ratio of AMF to either fungal or bacterial signatures decreased with soil age (Fig. 6). This seems to support the hypothesis that AMF (Glomeromycota) are important companions of plants colonizing soils with low levels of nutrients in organic forms, whereas the ecto- and ericoid mycorrhizal fungi (containing the same PLFA 18:2 ω 6 as saprotrophic soil fungi from the Asco- and Basidiomycota) replace the AMF at later stages of ecosystem development (Finlay, 2008; Read, 1991; Reddell and Malajczuk, 1984; van der Heijden, 2000). It remains unclear whether the decline in the ratio of AMF to bacteria in our study depended upon the nutrient forms available at certain stages of soil succession as observed for other ecosystems (Allison et al., 2005) or whether it was due to other factors (e.g., moisture or biological competition).

6. Limitations of the method and interpretation of the results

The limitations of fatty acid profiling result from the restricted power of: i) individual fatty acids or ii) lipid fraction, as a signature of certain group of microorganisms, and iii) technical constraints related to the analyses. Previous investigations clearly stated that none of the signature fatty acid used in our study can be traced back to a narrow ecological group of (micro)organisms. Thus, the results should always be interpreted with caution: the fatty acids 16:1 ω 5, 20:4 ω 6 and 20:5 ω 3, although used as signatures for AMF here and elsewhere (Graham et al., 1995), have also been found in bacteria, protozoa, some oomycetes and chytridiomycetes (Kaštovská et al., 2007; Lechevalier and Lechevalier, 1988; Olsson et al., 1995). The fatty acids 15:0, 16:1 ω 9, 16:1 ω 7, 17:0 and 18:1 ω 7 used in our study as signatures of bacteria (Frostegård and Bååth, 1996), and fatty acid 18:2 ω 6 as a signature for other soil fungi (Lechevalier and Lechevalier, 1988), have also been detected in other organisms (Lechevalier and Lechevalier, 1988; Olsson, 1999). To resolve the possible origin of individual fatty acids, the NLFA to PLFA ratio can be calculated. It has been suggested that a bacterial origin of a given fatty acid is signaled by a lower NLFA to PLFA ratio, whereas the opposite would be true for fatty acids of fungal origin (Bååth, 2003). In this study, we calculated these ratios (Table S1); confirming the proposed general trend. However, the ratios still varied markedly between the sampling sites, indicating potential shortcomings in the underlying theory or a great dynamic of the fatty acids or of the microbial communities along the soil chronosequence. For example, the NLFA to PLFA ratio calculated for 16:1 ω 5 ranged from 0.77 to 18.42, with two of the values (0.77 and 0.80) clearly lower than the remaining ones. These low values would indicate a bacterial rather than fungal origin of this fatty acid in two out of the 23 sampling sites. However, when the ratio was presented as average for all investigated sites, the value (6.24) suggested this signature being of fungal origin. We suspect that the usefulness of this approach is limited in our study as the system is extremely heterogeneous (i.e., spatial heterogeneity on the centimeter scale caused by local disturbances, as well as heterogeneity introduced by the glacier advancements on the meter scale), especially when compared with other environments for which this approach was developed (Bååth, 2003).

The selection of the adequate lipid fraction certainly needs to be considered when microbial biomass is estimated by FAME profiling. For example, the biomass of AMF should

be estimated with the 16:1 ω 5 fatty acid from the NLFA rather than the PLFA fraction (Bååth, 2003): AMF are able to store neutral lipids, thus a higher content of 16:1 ω 5 is predicted in the NLFA than in the PLFA fraction. In contrast, 16:1 ω 5 originating from bacteria should be found mostly in the PLFA fraction since these lack neutral lipid storage (Madigan and Martinko, 2006). Both of these fractions were investigated separately in our work (Figs. 3, S5); the use of different fatty acid fractions to assess the biomass of different microbial groups in our study has been avoided in order not to jeopardize the comparability of the different data. Furthermore, the property of PLFAs as a signature of active cells (Zelles, 1999) justified the selection of this fatty acid fraction in our investigations.

Some fatty acids (i. e., branched, cyclic, hydrocyclic and methylated) were difficult to detect with our instrumentation due to limited sensitivity of detection, quantification and identification as well as chemical alteration during the entire sample processing (particularly the transmethylation). For example, it is well known that cyclic and hydrocyclic fatty acids tend to decompose or rearrange on some GC columns, that they overlap with other peaks, and that aggressive methylation agents such as TMCS can decompose methylated fatty acids (Christie, 1993).

Conclusions

- In this study, we used fatty acid profiling to assess the size of the main microbial groups in soils representing different soil developmental stages at the Damma glacier forefield.
- We demonstrated that from an array of tested soil properties, soil C content and pH were the most important determinants of the size and composition of soil microbial communities.
- Moreover, the dominance of AMF over bacteria and fungi at the early stages of soil development disappeared with increasing soil age. A stable co-dominance of fungi and bacteria was observed in the different soils across the chronosequence spanning 7 through 137 years of soil development.

Acknowledgments

We would like to thank the members of the BigLink project for their support with soil sampling and processing in autumn 2007. The Competence Center Environment Sustainability (CCES, BigLink project) and Swiss National Science Foundation (SNSF, project number 31003A-125491) are acknowledged for their financial support.

Chapter 1 - supplementary material**Supplementary table****Table S1**

Ratios NLFA to PLFA for signature fatty acids used in this study for arbuscular mycorrhizal fungi (AMF), other fungi and bacteria. Values for each individual soil age plus average over soil ages are given. Fatty acid 16:1 ω 9 was not detected in 0-5 cm soil depth. Double lines indicate the position of the terminal moraines in the chronosequence (Fig. S1); NC- not calculated (the fatty acid not detected in the PLFA fraction).

Soil age	AMF			Bacteria				Fungi
	16:1 ω 5	20:4 ω 6	20:5 ω 3	15:0	16:1 ω 7	17:0	18:1 ω 7	18:2 ω 6
7	0.80	3.62	1.26	2.68	1.15	0	NC	3.99
8	1.47	0.55	1.13	2.07	0.87	NC	2.86	3.57
12	18.42	0.80	0.99	6.23	3.43	NC	2.32	11.98
14	5.76	2.44	4.28	1.33	1.26	NC	0.57	8.36
59	0.77	0.31	0.99	1.39	1.29	NC	NC	4.01
62	2.66	0	1.39	1.04	1.22	NC	7.89	2.50
63	1.73	1.60	1.14	1.78	1.39	NC	1.61	2.45
65	7.96	1.37	1.77	2.07	1.73	NC	3.49	5.54
67	7.15	1.47	0.86	1.96	2.17	NC	1.32	2.46
68	2.97	0.95	0.73	1.08	0.63	NC	0.60	2.28
71	2.14	1.05	2.94	0	0.55	NC	5.05	1.21
73	10.86	3.06	4.79	1.62	2.20	NC	0.41	5.31
76	10.29	4.16	7.15	1.59	2.44	NC	0.62	5.08
76	18.33	2.58	5.21	1.22	2.17	0.40	6.06	2.73
78	3.84	3.03	4.08	0.78	1.33	0.87	2.13	3.32
79	5.50	2.00	3.11	0.50	1.16	0.26	1.21	2.40
111	10.00	3.99	3.49	1.63	1.43	NC	0.50	3.53
118	6.71	9.14	NC	2.13	1.96	2.40	0.91	5.56
121	5.30	NC	NC	0.90	1.18	0.53	0.87	4.61
129	7.62	NC	NC	1.10	1.24	0.56	0.76	3.23
137	2.00	0	10.23	1.34	1.04	0.43	1.16	2.25
3000	5.85	1.48	24.63	2.11	2.46	0.53	1.65	3.52
3000	5.42	4.02	0.60	1.65	1.27	NC	1.91	4.13
AVERAGE	6.24	2.27	4.04	1.66	1.55	0.66	2.09	4.09

Supplementary figures

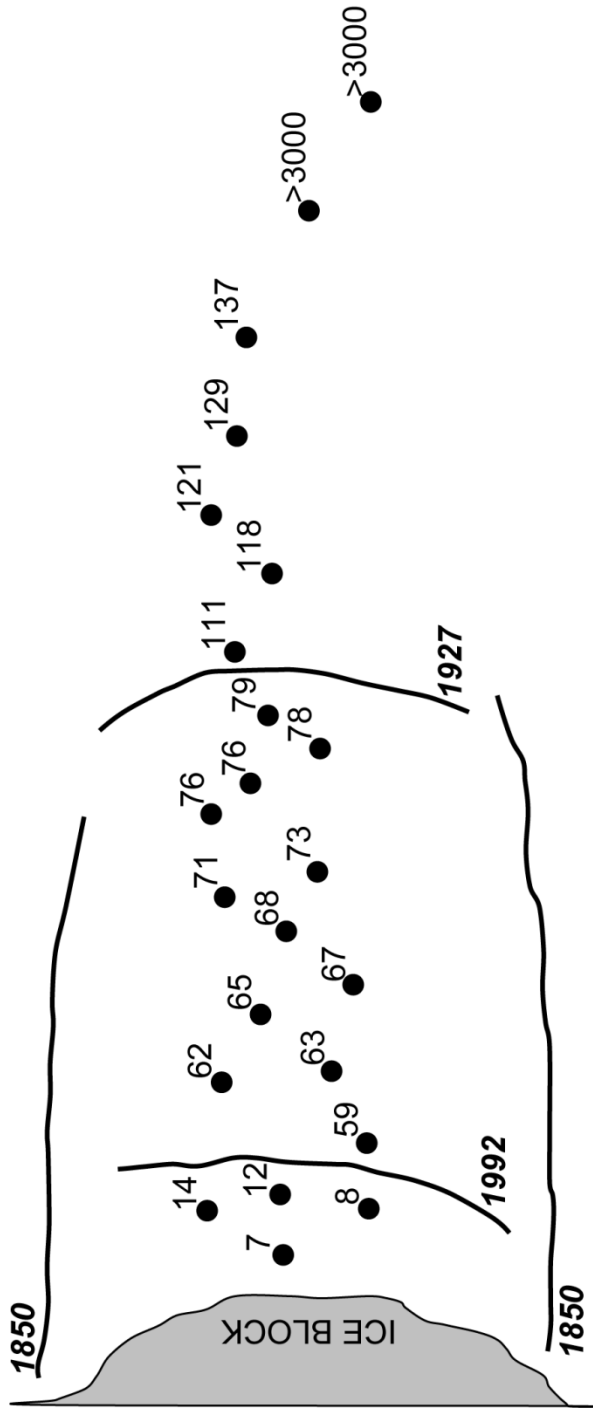


Figure S1

Position of the experimental sites at the Damma glacier forefield. Plain numbers indicate soil age (i.e. number of years since last deglaciation). Lines with numbers in italics represent terminal (years 1992 and 1927) and side (year 1850) moraines. Drawing not exactly to scale.

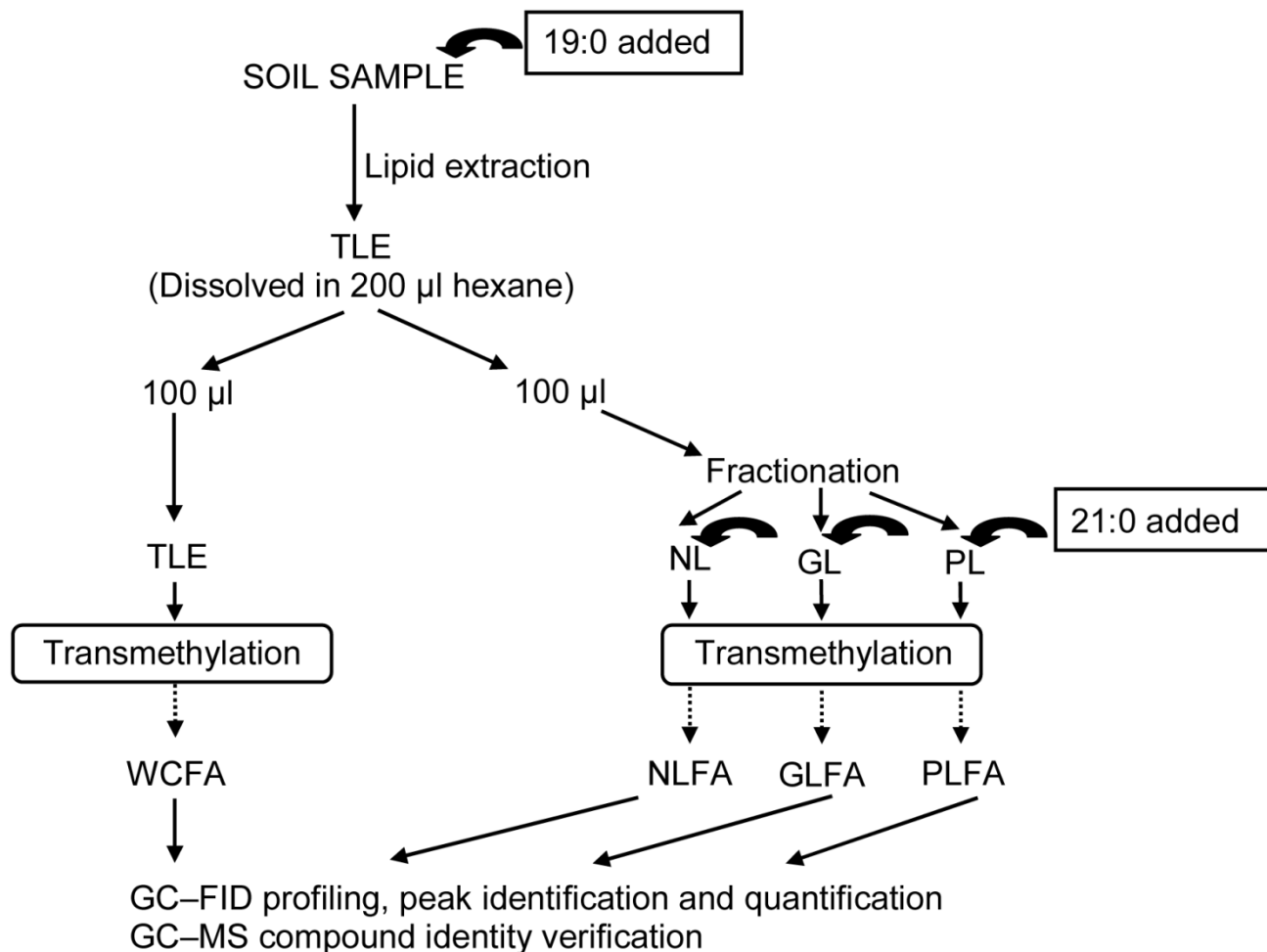


Figure S2

Steps included in extraction and fractionation of soil lipids. Abbreviations: TLE- total lipid extract, WCFA – whole cell fatty acids (methyl esters), NL – neutral lipids, GL– glycolipids, PL – phospholipids (methyl esters), NLFA – neutral lipid fatty acids (methyl esters), GLFA – glycolipid fatty acids (methyl esters), PLFA – phospholipid fatty acids (methyl esters), GC – gas chromatography, FID – flame ionization detection, MS – mass spectrometry. 19:0 – nonadecanoic acid (used as internal standard), 21:0 – heneicosanoic acid (used as internal standard).

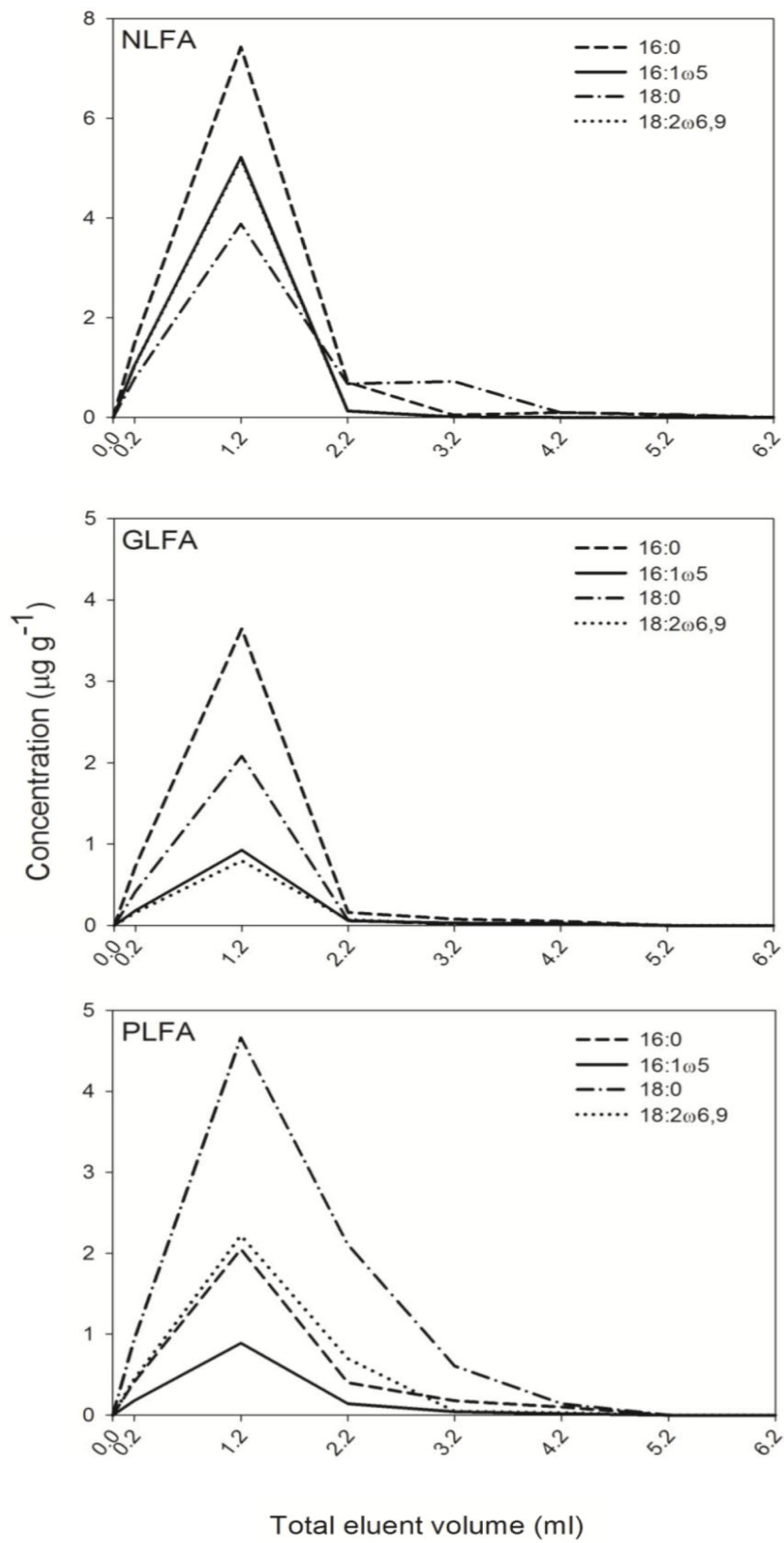


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Figure S3

Elution of different fatty acid fractions such as neutral lipids (NLFA), glycolipids (GLFA) and phospholipids (PLFA) from pre-packed silica columns (Bond Elut LRC - Si 100 mg, Varian, Darmstadt, Germany) with different volumes of eluents. Results presented in the graphs were obtained from soil samples (5–10 cm soil depth) collected at one of the reference site at the Damma glacier forefield (deglaciated more than 3000 years ago). Concentrations of selected fatty acids in different fractions are shown in μg per g dry weight of soil. The void volume of the column (1.3 ml) was estimated by repeated introduction of 100 μl -portions of chloroform to the upper surface of the column until flow-through was observed. The silica column was pre-activated by addition of 1.5 ml of chloroform to the pre-packed column, and then left to dry for 2 min after the last droplet of chloroform left the column. Next, the total lipid extract dissolved in 100 μl of chloroform was loaded on the column and left at room temperature for another 2 min. Thereafter, 100 μl of pure chloroform was applied on the column to facilitate infiltration of the lipid extract into the column. No outflow of eluent was observed at this stage. Addition of another 100 μl of pure chloroform yielded the first sample of the NLFA, which was collected in a 4 ml glass tube. Another 1.0 ml of chloroform was applied on the column and this fraction was collected in a separate 4 ml tube. The last step was repeated five more times to complete elution of the NLFA from the silica column (in total 6.2 ml of eluent was used). Elution of GLFA and PLFA was carried out using a similar scheme with acetone and methanol, respectively. The elution test yielded 7 samples for each of the fatty acid fractions. All samples were evaporated to dryness under mild vacuum (water-driven vacuum pump), transmethylated, and the fatty acid profiles analyzed and quantified using GC-FID (see Materials and methods section for details).

For each 100 mg Si column, 1.5 ml of chloroform, 6.0 ml of acetone and 1.5 ml of methanol were previously suggested as appropriate elution volumes for NLFA, GLFA and PLFA fractions, respectively (Bligh and Dyer, 1959). According to our results, however, these volumes do not guarantee full elution of each fatty acid fraction (especially the first and the last fraction required more eluent than previously thought). In this study, therefore, higher volumes of the eluents were used to wash the column in each of the fractionation steps.

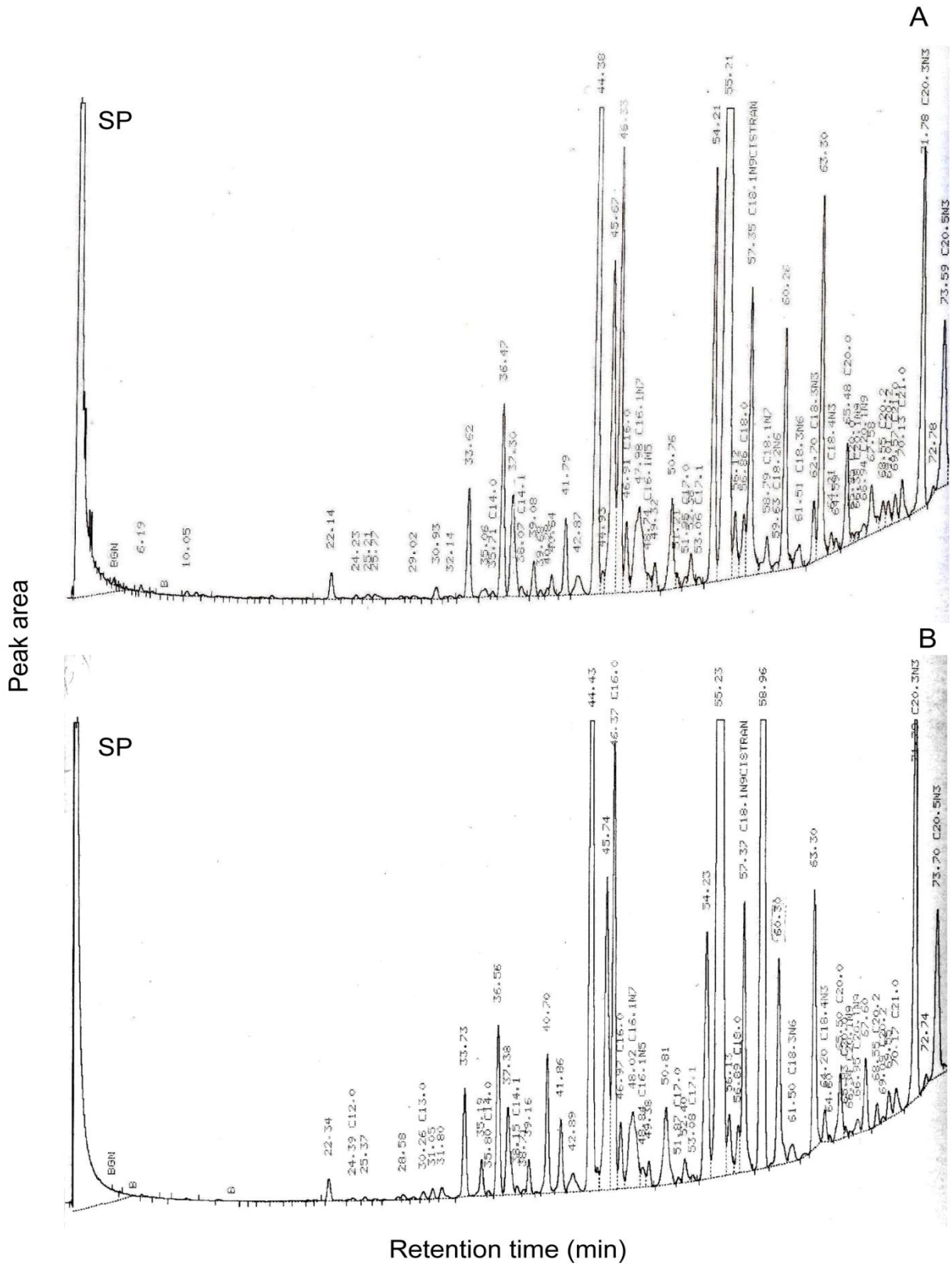


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Figure S4

Fatty acid profiles obtained after two different transmethylation procedures of the total lipid extract of soil deglaciated for more than 3000 years (0-5 cm soil depth). The internal standard was nonadecanoic acid (C19:0, 646-30-0, Sigma-Aldrich, Buchs, Switzerland) introduced directly to the soil before extraction (Fig. S2). The total lipid extract was evaporated to dryness under mild vacuum (water-driven pump), dissolved in 1.0 ml of chloroform and split to two halves, which were again evaporated to dryness under the same conditions. These samples were then subjected to transmethylation, either by mild alkaline methanolysis (MAM, Fig. S4A) or by trimethylchlorosilane (TMCS, Fig. S4B). The graphs represent scanned chromatograms from the gas chromatograph (GC) equipped with a flame ionization detector, without any further modifications. Retention times of peaks (in min) are given. The retention time of the internal standard (C19:0) is approximately 60.3 min. The results were very similar for most fatty acids, with some differences in the region of saturated and moderately unsaturated fatty acids containing 18 carbon atoms (retention times 54 – 60 min). Because of its simplicity, the TMCS procedure was used in this study.

Briefly, in the MAM procedure, fatty acids were dissolved in 1.0 ml of toluene: methanol mixture (1:1, v:v) and vortexed at room temperature for 1 min. Subsequently, 1.0 ml of 2.0 M KOH in methanol (freshly prepared) was added to the sample, which was then incubated at 37°C for 15 min. While the sample cooled down at room temperature for 15 min, the following solutions were added separately to the sample: 2.0 ml of hexane : chloroform (4:1, v:v), 0.3 ml of 1.0 M acetic acid and 2.0 ml of distilled water. The sample was mixed at room temperature for 10 min and then left to stand for another 15 min to allow phase separation. The pH of the lower water phase was 6.0 (checked with commercial indicator strips). The upper organic phase containing the fatty acid methyl esters was transferred into a new tube with a Hamilton syringe, filtered through a syringe-driven hydrophobic fluorophore filter (Millex-FR, Millipore, Zug, Switzerland), and evaporated to dryness under mild vacuum (water-driven pump) for 20 min. The TMCS procedure is described in the Material and method section in the main text. Following the transmethylation (either approach), the samples were re-dissolved in 20 µl of hexane and the volume of 0.5 µl was injected into the GC. Abbreviations: (SP) solvent peak.

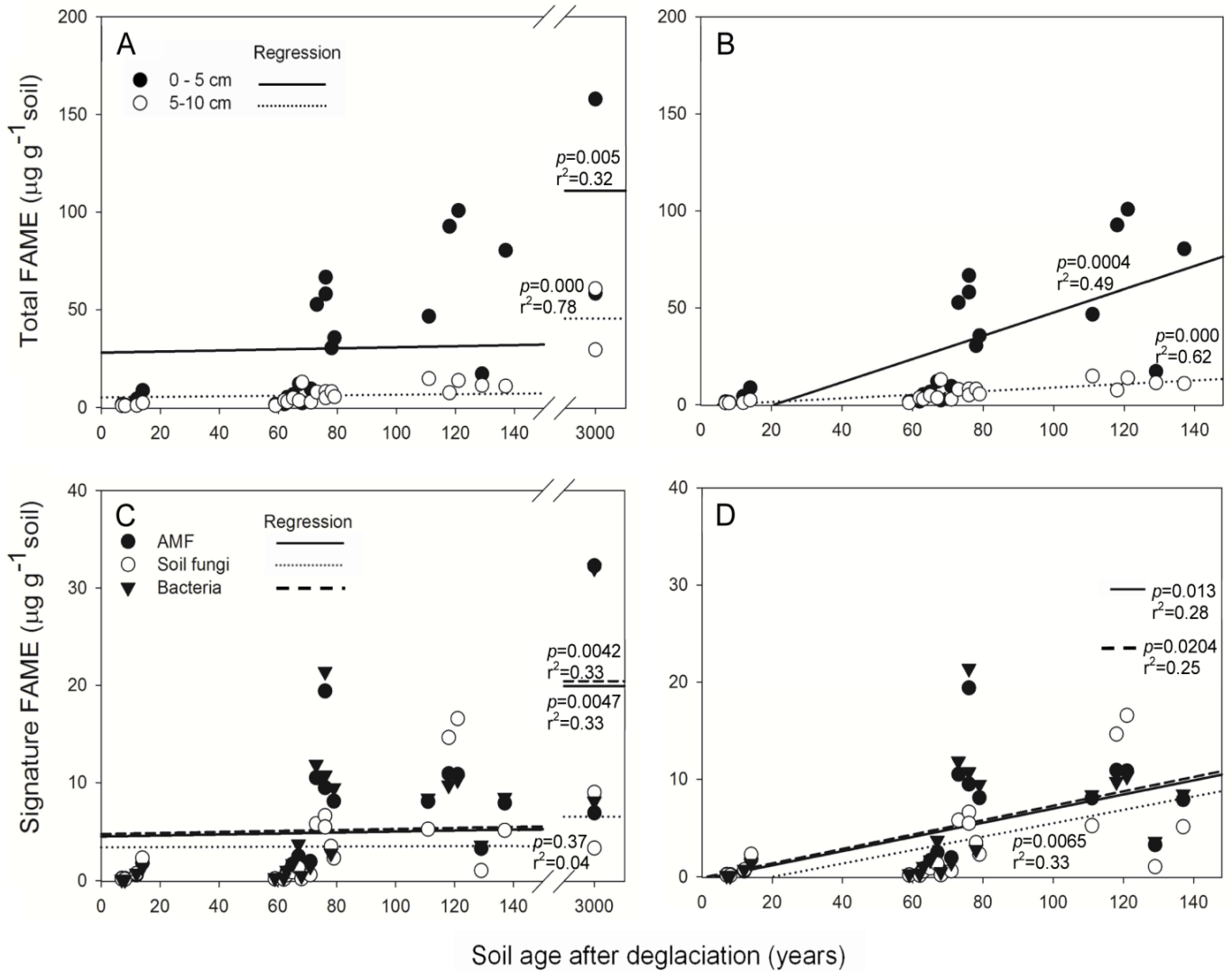


Figure S5

Concentrations of fatty acid methyl esters (FAME) from the NLFA fraction detected in the individual samples along the soil chronosequence: Sum of total NLFAs at two soil depths (0-5 cm and 5-10 cm) when the reference sites were included (A) or excluded (B) from the analyses; signature fatty acids used in this study for arbuscular mycorrhizal fungi (AMF), other fungi and bacteria in upper soil depth (0-5 cm) when reference sites were included (C) or excluded (D) from the analyses. Statistical significances and r² values for the linear regression models are given next to the respective lines.

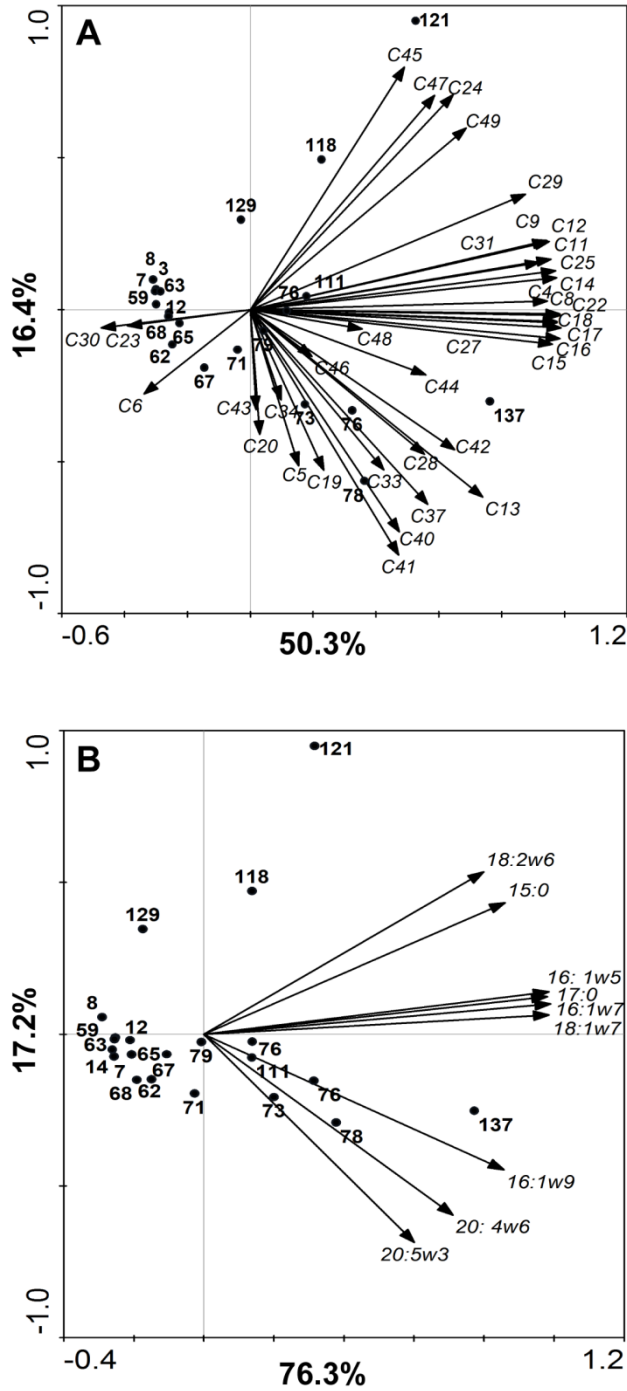


Figure S6

Principal Component Analysis (PCA) diagrams relating concentrations of (A) all fatty acids detected in the phospholipid fatty acid fraction (identities as encoded in Table 1), and (B) signature phospholipid fatty acids used in this study with the age of soil (7-137 years after deglaciation) at the different collection sites (0-5 cm soil depth).

**Chapter 2: Rhizosphere fungal assemblages and soil enzymatic activities
in a young alpine ecosystem**

Abstract

Although fungal diversity in alpine ecosystems has been studied in the past, the link between their abundance, distribution and functions remains poorly understood. In presented study we aimed at characterization of the structure and enzymatic activity of mycorrhizal fungal communities associated with *Salix helvetica* (Swiss willow) colonizing the sites of different developmental stage (i.e., 7 sites spanning 7 through 110 years of soil development). These parameters were also assessed for three other plant species, differing in their mycorrhizal status (i.e., *Agrostis gigantea* and *Leucanthemopsis alpina*, AM plants; *Rhododendron ferrugineum*, ericoid plant) and the bare soil, serving as the control. The hypothesis of this study was that the enzymatic activity assessed in plant rhizosphere will differ with mycorrhizal status of the plants. More precisely, ample enzymatic activity of ectomycorrhizas (ECM) and ericoid fungi (ER) will be mirrored in the rhizosphere of *S. helvetica* and *R. ferrugineum*, respectively, whereas plants colonized by arbuscular mycorrhizal fungi (AMF) will present lower enzymatic activities.

Quantitative PCR with molecular markers targeting several fungal taxa (ECM, AMF, uncertain trophic status fungi, UTS) were combined with enzymatic activity assays in order to link the structure and functions of mycorrhizal fungal communities in the plant rhizospheres.

The results suggest that: 1) plant identity influenced the structure of ECM and AMF communities, whereas the structure of the community of remaining fungi (i.e., UTS) were more influenced by the soil developmental stage, suggesting those were saprotrophs; 2) from all investigated samples, the higher activity of chitinase and α -glucosidase was observed in the rhizosphere of *S. helvetica* possibly due to activity of ECM. In contrast, rhizosphere of *A. gigantea* presented higher activity of phosphatase and β -glucosidase, but this can not be explained only by the activity of AMF but also by bacteria and roots themselves.

Keywords: fungal community, community structure, community functions, mycorrhiza, rhizosphere soil, soil development, soil enzymes, *Salix helvetica*, alpine tundra

Introduction

Numerous studies during the last decade have aimed to disentangle main processes driving ecosystem formation. Land gradually emerging from underneath of retreating glaciers provide natural laboratory for such investigations (Huggett, 1998; Walker et al., 2010). Living organisms - especially plants - contribute substantially to ecosystem development in general and to soil formation in particular (Chapin et al., 1994). Acting as island of available nutrients and carbon (i.e., rhizodeposition, litter fall), their rhizospheres harbor a variety of microorganisms (Hawkes et al., 2007), including fungi.

Communities of fungi, especially mycorrhizal fungi, in alpine ecosystems were mainly influenced by an environmental properties and/or plant identity (Becklin et al., 2011a; Becklin et al., 2011b; Kernaghan and Harper, 2001). In our study, natural gradient of soil physico-chemical properties along a soil developmental gradient at the Damma glacier forefield (N46°38.1170', E8°27.677', Switzerland) was accompanied by gradual increase of plant coverage. This gives a rare opportunity to disentangle the joint impact of these both factors on the fungal community composition and functions.

Up to now, many studies surveyed mycorrhizal associations in a mountain ecosystems (Cázares et al., 2005; Cripps and Eddington, 2005; Gardes and Dahlberg, 1996), characterized their diversity (Becklin et al., 2011a; Gao and Yang, 2010; Mühlmann and Peintner, 2008a, 2008b; Ryberg et al., 2009) and dynamics (Oehl et al., 2011), but insights into their functions are still very limited (Rineau and Courty, 2011).

Research of the last decades confirmed that ectomycorrhizal (ECM) and ericoid (ER) fungi exude various exoenzymes (Read and Perez-Moreno, 2003), which facilitate hydrolysis of complex biomolecules deposited in soil organic matter (SOM). Essential nutrients released upon enzymatic hydrolysis can be further uptaken by an ample net of mycorrhizal hyphae and transferred to the host plant. The second strategy of nutrient acquisition, described mainly for ECM, employs low molecular weight organic acids (LMWOA). These acids effectively enhance dissolution of recalcitrant nutrient sources such as minerals (Hoffland et al., 2004). In case of AMF neither enzymatic activity not exudation of LMWOA was confirmed unambiguously (Smith and Read, 2008; Hodge and Fitter, 2010).

These differences in enzymatic activity of different mycorrhizal types encouraged us to investigate wheater different mycorrhizal status of the plant will reflect enzymatic activities in

their rhizospheres. We hypothesized that: i) the rhizosphere soils of *S. helvetica* and *R. ferrugineum*, associated with ECM and ER fungi, respectively, will present higher enzymatic activity of all enzymes as compare to AMF which exhibit scarce enzymatic activity; ii) the enzymatic activities will change along soil developmental gradient, being the highest in the soils where ecological conditions seem to be optimal for a given plant species, i.e., soils located at the middle and old part of the development gradient for *S. helvetica* and *R. ferrugineum*, respectively.

These hypotheses aimed to reconcile two concepts existing in the current knowledge. From one side, the origin of enzymes in soil could not be clearly tracked back to any specific group of organisms (Tabatabai and Dick, 2002). It means that enzymes which were exuded by bacteria, fungi, plants, etc. will constitute in fact one total pool of given enzyme in soil. From other side, the differences in enzymes exudation strategies was confirmed for ECM fungi (Pritsch and Garbaye, 2011) thus are expected to mirror enzymatic activity in the rhizosphere of ECM plant. Based on these two arguments, we attempted to bridge the composition (structure) of fungal communities with their functioning.

Materials and methods

The study area and the sampling sites

The study area was the forefield of the Damma glacier. It is situated in Central Swiss Alps (Canton of Uri, N46°38.1170', E8°27.677') and was described in detail elsewhere (Bernasconi et al., 2011). In July 2009, eight sites at different soil developmental stages (i. e., years after last deglaciation) were selected for these investigations. Approximate ages of soils on these sites ranged from 7 to 110 years (Fig. S1, Table S1). These ages were calculated based on the historical glaciological records available from The Swiss Glacier Monitoring Network (<http://glaciology.ethz.ch/messnetz/glaciers/damma>, accessed 29th March 2012).

Selected sites were distant from the streams, terrain depressions or elevations and spots where fine rock material had been accumulated by temporary streams.

Soil and roots sampling

At each experimental site, four different alpine plants, representing different mycorrhizal types, were selected: *Salix helvetica* (dual mycorrhizal plant, with scarce arbuscular mycorrhiza (AM) and predominant ectomycorrhiza (ECM)), *Leucanthemopsis alpina* and *Agrostis gigantea* (AM) and *Rhododendron ferrugineum* – (ER). Individual plants were uprooted and soil adhering to the root system (approximately 200 g) was gently shaken-off. These soils, called further rhizosphere soils (=rhizospheres), were preserved in a plastic bags (separately for each plant) until the analyses. At the same time, bare soil (i.e., not covered by vegetation) was collected from each experimental site and served as a reference for the rhizosphere soils. Upon arrival to the lab, soils were stored at +4°C and processed within next 12h.

Samples preparation and processing

Shoots and roots were processed separately: soil still adhering to the roots was shaken-off, pooled with the respective sample of soil from the fields, homogenized by mixing, passed through a 2 mm sieve and aliquoted (approximately 5 g each subsample). These subsamples were either frozen (-80°C) or freeze-dried (one subsample from each soil). Frozen samples were used for enzymatic activity assays, whereas nutrient analyses and the DNA extraction was conducted on the freeze-dried samples prior their grinding (4 min, 98 Hz) to a fine powder.

Root samples were gently cleaned in distilled water, observed under dissecting microscope, where gravels, organic debris and roots of other plants were manually eliminated. Morphotyping (ECM, Agerer (1987-2002) (Fig. S2, Table S2) and the presence of extraradical spores (AMF) was conducted. Only a fine roots (≤ 0.5 mm diameter) were separated from the samples, cut into 1-cm long segments and gently homogenized by mixing. These roots (approximately 0.2 g of roots) were either stained with 0.05% trypan blue, to visualize the mycorrhizal fungi (Kormanik and McGraw, 1982) (Table S3), or freeze-dried. From each freeze-dried sample, a portion of approximately 0.8 g was subjected to bead-beating (2×30 sec, 46 Hz) to obtain a fine powder for further analysis of the total carbon (C_{tot}) and nitrogen (N_{tot}) content (see **Soil nutrient analyses** for details) and the DNA extraction (see **DNA extraction from the root and soil samples** for details).

DNA extraction from the root and soil samples

Genomic DNA was extracted from the root and soil samples with Nucleo Spin Plant II[®] Kit and Nucleo Spin Soil[®] Kit (Macherey-Nagel, GmbH & Co. KG, Germany), respectively, according to manufacturer's recommendations. Prior to the DNA extraction, each sample was spiked with 20×10^9 copies of the APA 9 plasmid (vector pUC19 with insert of a cassava mosaic virus; GenBank accession number AJ427910 (von Felten et al., 2010)), serving as an internal standard in order to control the efficiency of the DNA extraction and any inhibition of PCR reaction.

qPCR markers and cycling conditions

Taxon-specific qPCR molecular markers (Table S4), targeting ECM, AMF and UTS from investigated ecosystem, were designed with AlleleID[®] software (PREMIER Biosoft International, USA) and synthesized by Microsynth AG, Balgach, Switzerland (see ***Design of the qPCR markers***, supplementary materials). Each taxon-specific qPCR marker was subjected to cross-specificity tests to confirm amplification of the target fungus only (see ***Cross-specificity test for qPCR markers***, supplementary materials). Single qPCR reaction contained 0.18 μ l of each primer (25 μ M), 0.04 μ l of the hydrolysis probe (25 μ M), 1.80 μ l of the Roche Master Mix (Roche Diagnostics, Rotkreuz, Switzerland; Cat. No. 04736536001), and 4.55 μ l Light Cycler water (Roche Diagnostics, Rotkreuz, Switzerland). All qPCR reactions were run on Light Cycler 2.00 (Roche Diagnostics, Rotkreuz, Switzerland) according to the qPCR reaction cycling conditions optimized individually for each qPCR molecular marker (Table S5).

Soil enzymatic activity assays

All chemicals used for enzymatic activity assays had high chemical quality (Premium quality level for substrates and standards, or >99% purity for buffers; Sigma-Aldrich, Buchs, Switzerland). Used glass labware was sonicated (3 \times 10 min, 35 kHz) and autoclaved to assure chemical purity. All used consumables (i.e., microplates, pipette tips) were sterilized prior to use. Two buffers: MES (2-(N-morpholino)ethanesulfonic acid, Buchs, Switzerland) and modified universal buffer (Stemmer, 2004) were applied for all enzymatic activity assays and they were used sterile (filter sterilization or autoclaving for MES and modified universal

buffer, respectively). Buffer pH was experimentally adjusted prior the measurement (Table S6) and monitored before and after the assay (Electrode Mettler Toledo 403-M8-S7/120).

Stocks of the substrates and standards were prepared on sterile distilled water and protected from light with an aluminum foil. Working solutions of the substrates and standards were stored at +4°C not longer than one week. Fluorogenic substrates were used to measure activities of the following enzymes: acid phosphatase, chitinase, protease, α -glucosidase, β -glucosidase, xylosidase, and sulfatase (Table S6). Depending on the enzyme, 4-Methylumbelliferone (4-MUF) or 7-Amino-4-Methylcoumarin (AMC) were used as a standards (Table S6). Standard curves were prepared for each enzymatic activity assay and each soil separately. Following concentrations of the standards were achieved on the microplate: 0, 100, 200, 500, 800, 1200 and 1500 pmol of MUF or AMC per well (Stemmer, 2002).

Enzymatic activities were assessed in the soil slurries. Approximately 1 g of fresh soil was suspended in 40 mL of sterile distilled water, sonicated (2 min, 35 kHz) and incubated at room temperature on horizontal lab shaker (1.5h, 2.3 Hz). After short sedimentation (5 min, room temperature), soil slurries were distributed into a black 96-well microplates (Greiner Bio-One, Cat. no. 655900, Germany) containing relevant buffer, substrate and standard (Fig. S3, Table S6). Each sample had respective blank, in which substrate for measured enzyme was absent. The fluorescence of all used solutions (buffers, distilled water, standards and substrates) was continuously monitored for all assays to exclude their accidental contamination.

Prepared microplates were incubated at 37°C for the following times: 0, 30, 60, 90, 120 and 180 minutes. Enzymatic activities were quantified using a Biotek FLx800 microplate fluorometer (excitation 360 nm, emission 460 nm) and fluorescence of measured samples was converted to pmol per well based on the standard curves, generated for 4-MUF or AMC, respectively (Table S6).

Soil nutrient analyses

The concentrations of total C (C_{tot}) and total N (N_{tot}) were analyzed in the samples of 100 mg, 50 mg, and 25 mg, for young (deglaciated 7 to 12 years ago), middle-age (deglaciated 60 to 70 years ago) and old (deglaciated 78 and 110 years ago) soils, respectively, by dry combustion (NCS Analyzer, FlashEATM 1112 Series, Thermoelectronic Corporation). The

concentration of total phosphorus (P_{tot}) was estimated in 100 mg-soil aliquots. The samples, weighted into ceramic crucibles, were incinerated for 16 h at 550°C and then the ashes were dissolved in 1.0 ml of 65% HNO_3 at 250°C for 2 min. The extracts were filtered through 0.2 μm syringe-driven hydrophilic filters (Minisart, Sartorius-Stedim, Switzerland).

The concentrations of plant-available soil elements, such as phosphorus (P_{av}), calcium (Ca_{av}), potassium (K_{av}), and magnesium (Mg_{av}) were assessed in ammonium acetate–EDTA extracts (Stünzi, 2006), after shaking at 3.75 Hz at room temperature for 1 h. The soil: extractant ratio was 1:10 (w:v). Before the analyses, extracts were filtered through 0.2 μm syringe-driven hydrophilic filters (Minisart, Sartorius-Stedim, Switzerland). The concentrations of P_{tot} and all plant-available nutrients were estimated by Inductively Coupled Plasma – Mass Spectrometry (ICP-MS, Agilent 7500 C) on 10-fold diluted extracts. Soil pH was measured in suspension of the soil in water (1:2.5, w:v), using the Corning 125 pH-meter (Corning B.V. Life Sciences, Amsterdam, The Netherlands).

Statistical analyses

The average and differences of enzymatic activity were calculated in Excel (Microsoft Excel 2010). Significance of difference from zero (mean signed rank test), paired samples comparison (t-test) and simple regressions were performed with Statgraphic®Plus for Windows 3.1. The abundance of fungi (ECM, AMF, UTS) in roots and soils were combined with the results of enzymatic activities of soils and several soil properties (Table 1) and subjected to multivariate statistics using Canoco 4.5 (ter Braak and Šmilauer, 2002). Multivariate statistical analyses were conducted on a centralized and standardized datasets. Selection of Redundancy Analysis (RDA) was justified by a short length of the gradient (2.038 and 1.379 for first axis, for Figures 2 and 5, respectively) in Detrended Correspondence Analysis (DCA). The RDA ordination diagram presented on Figure 2 shows the dependencies between soil properties and plant species (handled as explanatory variables) and enzymatic activities (handled as explained variables). The RDA ordination diagram presented on Figure 5 shows the dependencies between soil properties and plant species (handled as explanatory variables) and the abundance of different fungi in soil (fungus name S) and roots (fungus name R). The statistical significance of the relationships

presented in Figure 2 and 5 was evaluated using the Monte Carlo permutation test (unrestricted permutation, reduced model, 9999 permutations).

Table 1

Selected chemical properties of the rhizosphere soils collected for four alpine plants (*Salix helvetica*, *Agrostis gigantea*, *Leucanthemopsis alpina* and *Rhododendron ferrugineum*) and a bare soil collected at each of the experimental site (with the exception of the sites deglaciated 7 and 8 years ago). The values are averages of three technical replicates \pm SE.

Soil age (years)	pH (H ₂ O)	C _{tot}	N _{tot}	P _{tot}	P _{av}	K _{av}	Mg _{av}	Ca _{av}
		(g ⁻¹ kg)		(mg ⁻¹ kg)				
7	5.4 (\pm 0.16)	0.7 (\pm 0.1)	0.03 (\pm 0.001)	293.4 (\pm 46.8)	18.6 (\pm 2.4)	45.7 (\pm 6.1)	10.3 (\pm 0.9)	123.1 (\pm 8.8)
8	5.4 (\pm 0.09)	0.8 (\pm 0.3)	0.05 (\pm 0.01)	228.0 (\pm 25.5)	21.5 (\pm 1.6)	67.2 (\pm 5.3)	12.6 (\pm 2.0)	126.2 (\pm 20.5)
12	5.2 (\pm 0.04)	7.4 (\pm 2.0)	0.4 (\pm 0.07)	552.6 (\pm 37.8)	55.0 (\pm 11.7)	221.6 (\pm 36.9)	77.5 (\pm 8.7)	443.6 (\pm 47.1)
60	5.2 (\pm 0.05)	4.1 (\pm 1.7)	0.2 (\pm 0.09)	349.8 (\pm 29.0)	39.5 (\pm 9.6)	82.6 (\pm 21.2)	28.8 (\pm 4.9)	202.7 (\pm 23.9)
65	5.1 (\pm 0.02)	5.8 (\pm 1.3)	0.4 (\pm 0.08)	472.8 (\pm 43.8)	44.5 (\pm 9.4)	185.4 (\pm 62.8)	76.8 (\pm 23.7)	430.6 (\pm 80.4)
70	5.0 (\pm 0.11)	3.6 (\pm 0.7)	0.2 (\pm 0.05)	379.9 (\pm 10.8)	37.2 (\pm 4.0)	90.9 (\pm 11.7)	35.2 (\pm 2.6)	201.2 (\pm 16.6)
78	5.0 (\pm 0.08)	8.5 (\pm 1.6)	0.4 (\pm 0.08)	254.1 (\pm 11.2)	29.4 (\pm 4.5)	105.6 (\pm 19.4)	36.6 (\pm 4.7)	257.2 (\pm 42.2)
110	4.7 (\pm 0.05)	17.5 (\pm 4.9)	0.9 (\pm 0.28)	262.2 (\pm 10.0)	45.3 (\pm 4.5)	143.0 (\pm 23.0)	45.4 (\pm 8.7)	245.8 (\pm 34.9)

Abbreviations: (C_{tot}) concentrations of the total carbon and (N_{tot}) concentration of the total nitrogen measured by dry combustion; (P_{tot}) concentrations of the total phosphorus, and plant-available elements such as (P_{av}) phosphorus, potassium (K_{av}), magnesium (Mg_{av}) and calcium (Ca_{av}) estimated by Inductively Coupled Plasma – Mass Spectrometry.

Results

1. *Fungal communities in roots and rhizospheres*

1.1. *The abundance of ECM: roots vs. soil of individual plant species along the soil developmental gradient*

ECM fungi associated only with *S. helvetica* (Fig. 1A-B). Distribution of ECM fungi along soil developmental gradient assessed by qPCR, but not microscopy (Fig. S4 B), presented following pattern: the lowest abundance was recorded in young (7-12 years after deglaciation) sites. Thereafter, it increased markedly in middle-age sites (peak of abundance at the site deglaciated 65 years ago), after which it tended to decrease towards the oldest site (110 years after deglaciation). Among the dominant ECM species associated with roots and soils of *S. helvetica*, *Russula emetica* and *Laccaria pumila* were found. In contrast, *Inocybe lacera* commonly occurred in the rhizosphere soil (Fig. 1 A-B).

1.2. *The abundance of AMF: roots vs. soil of individual plant species along the soil developmental gradient*

AMF were detected in roots of *S. helvetica*, *L. alpina* and *A. gigantea* (Fig. 1A). *S. helvetica* presented very low colonization with AMF (Figs. 1A, S4 A), however these fungi were present in the rhizosphere of willow, especially at the site deglaciated 65 years ago (Fig. 1A-B). *L. alpina*, was colonized scarcely by AMF, and no clear trend in AMF distribution along soil developmental gradient was found (Fig. 1A-B). Low abundance of AMF in the roots contradicted microscopic observations, which suggested high colonization density for this plant (Fig. S4 A). *A. gigantea* presented the highest abundance of AMF (roots and soils), with clear peak of abundance at site deglaciated 65 years ago (Fig. 1A-B). High colonization density for this plant was also confirmed by microscopy (Fig. S4 A). *G. sinuosum* and *G. mosseae* were detected in all investigated samples (Fig. 1A-B).

1.3. *The abundance of UTS : roots vs. soil of individual plants along the soil developmental gradient*

The UTS were detected in all investigated roots (Fig. 1A) and soils (Fig. 1B) and presented variable abundance and distribution patterns.

Uncultured *Mortierella* spp. and *M. bicolor*, abundant in *S. helvetica* roots, were present already at young sites, and decreased the abundance in the older soils (Fig. 1A).

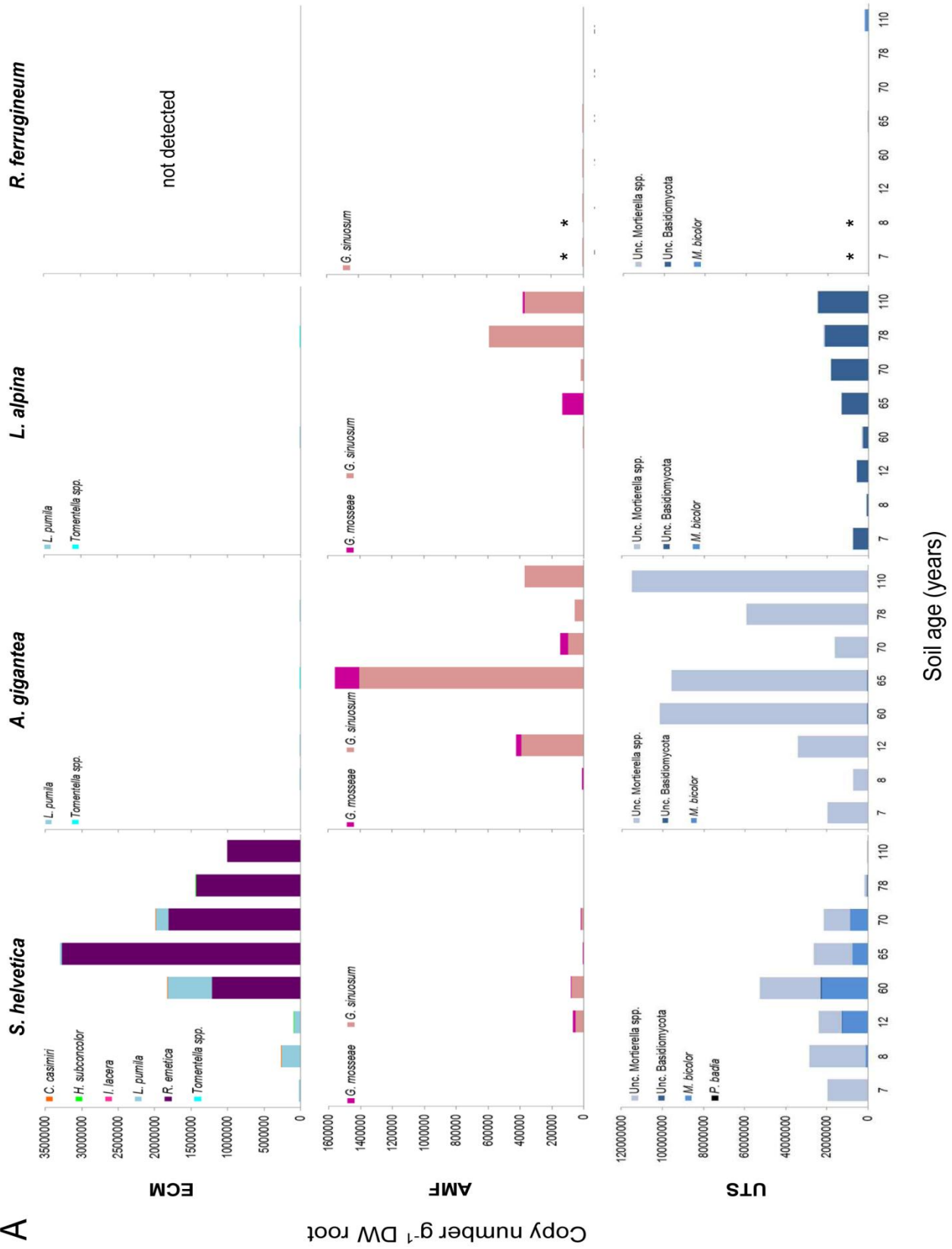
M. bicolor and Uncultured *Mortierella* spp. was detected in rhizosphere, whereas Uncultured Basidiomycota and *M. bicolor* dominated on willow roots (Fig. 1A-B).

In the roots and rhizosphere of *L. alpina* (Fig. 1A-B), Uncultured Basidiomycota was frequent, and its abundance show not clear trend as soil development proceeded, for both roots and soils.

Roots of *A. gigantea* were amply colonized by Uncultured *Mortierella* spp. (Fig. 1A), whereas Uncultured Basidiomycota constituted the majority of fungal communities in the rhizosphere (Fig. 1B). The UTS were not detected in roots (Fig. 1A) but in the rhizosphere (Fig. 1B) of *R. ferrugineum*.

A

Figure caption on the page 58



B

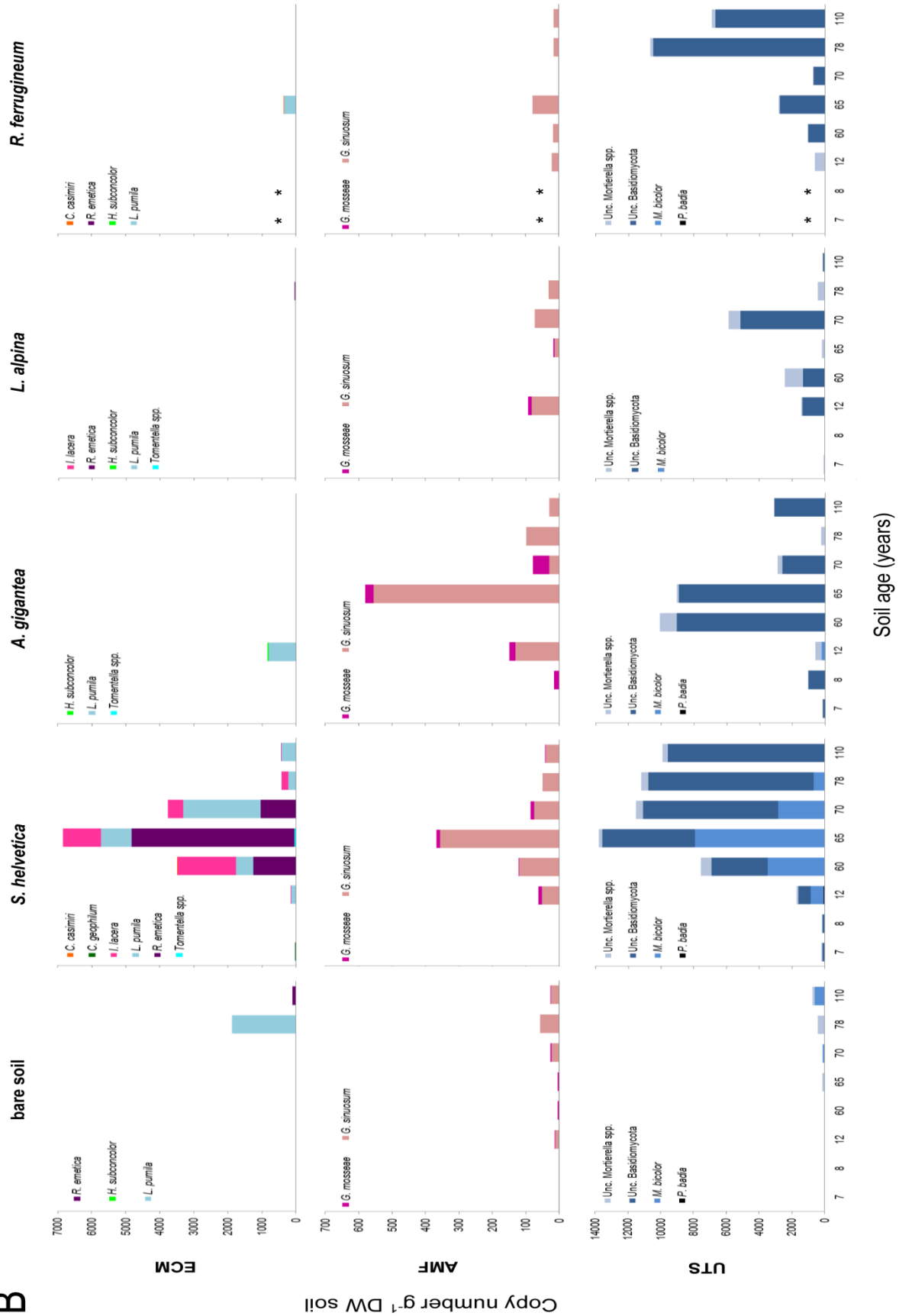


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Figure 1

The abundance of the different fungi (ectomycorrhizal, ECM; arbuscular mycorrhizal, AM; and fungi of uncertain trophic status, UTS) associated with roots (A) and in the rhizosphere soils (B) of four alpine plant species (*Salix helvetica*, *Agrostis gigantea*, *Leucanthemopsis alpina* and *Rhododendron ferrugineum*) and bare soil along soil developmental gradient assessed with taxon-specific molecular markers. Fungal identities - (*C. geophilum*) *Cenococcum geophilum*; (*C. casimiri*) *Cortinarius casimiri*; (*H. subconcolor*) *Hebeloma subconcolor*; (*I. lacera*) *Inocybe lacera*; (*L. pumila*) *Laccaria pumila*; (*R. emetica*) *Russula emetica*; (*G. mosseae*) *Glomus mosseae*; (*G. sinuosum*) *Glomus sinuosum*; (*M. bicolor*) *Meliniomyces bicolor*; (*P. badia*) *Peziza badia*. Asterisks indicate the absence of *R. ferrugineum* at the site deglaciated 7 and 8 years ago.

2. The influence of soil origin and soil properties on the abundance of root- and soil-associated fungal communities

In general, soil pH decreased and total soil C and N increased along soil developmental gradient (Table 1). These parameters were included in the multivariate statistic. Multivariate analysis relating soil origin (rhizosphere or bare soil) and soil properties (Table 1) to abundance of fungal taxa in roots and rhizosphere soils (Fig. 2) confirmed that these parameters explained a large part (58.5%) of the dataset variability. Plants strongly affected the fungal taxa associated with their roots and rhizospheres. For example, the abundance of *Tomentella* spp., *R. emetica* (roots and soils), *M. bicolor* and *G. sinuosum* (soil only) associated with *S. helvetica* correlated positively with K_{av} and Mg_{av} ($p \leq 0.0016$, $r^2 \leq 0.74$) (Fig. 2). In contrast, the abundance of Uncultured Basidiomycota correlated positively with C_{tot} but not N_{tot} or soil age (for *S. helvetica*, *A. gigantea* and *L. alpina*, $p \leq 0.04$, $r^2 \leq 0.52$, data not shown). Interestingly, the abundances of ECM, AMF and UTS (presented as a sum abundances of all fungi for individual group) were related to soil age and C_{tot} , significant correlations were found only between UTS, bare soil and rhizosphere of *S. helvetica* and roots of *L. alpina* roots ($p \leq 0.04$, $r^2 \leq 0.52$, data not shown). Soil pH did not affected the fungal communities significantly (Fig. 2). Interestingly, the correlations between abundances in roots and in soils assessed for the same fungal taxon were predominantly statistically insignificant (Fig. 2, Table S8).

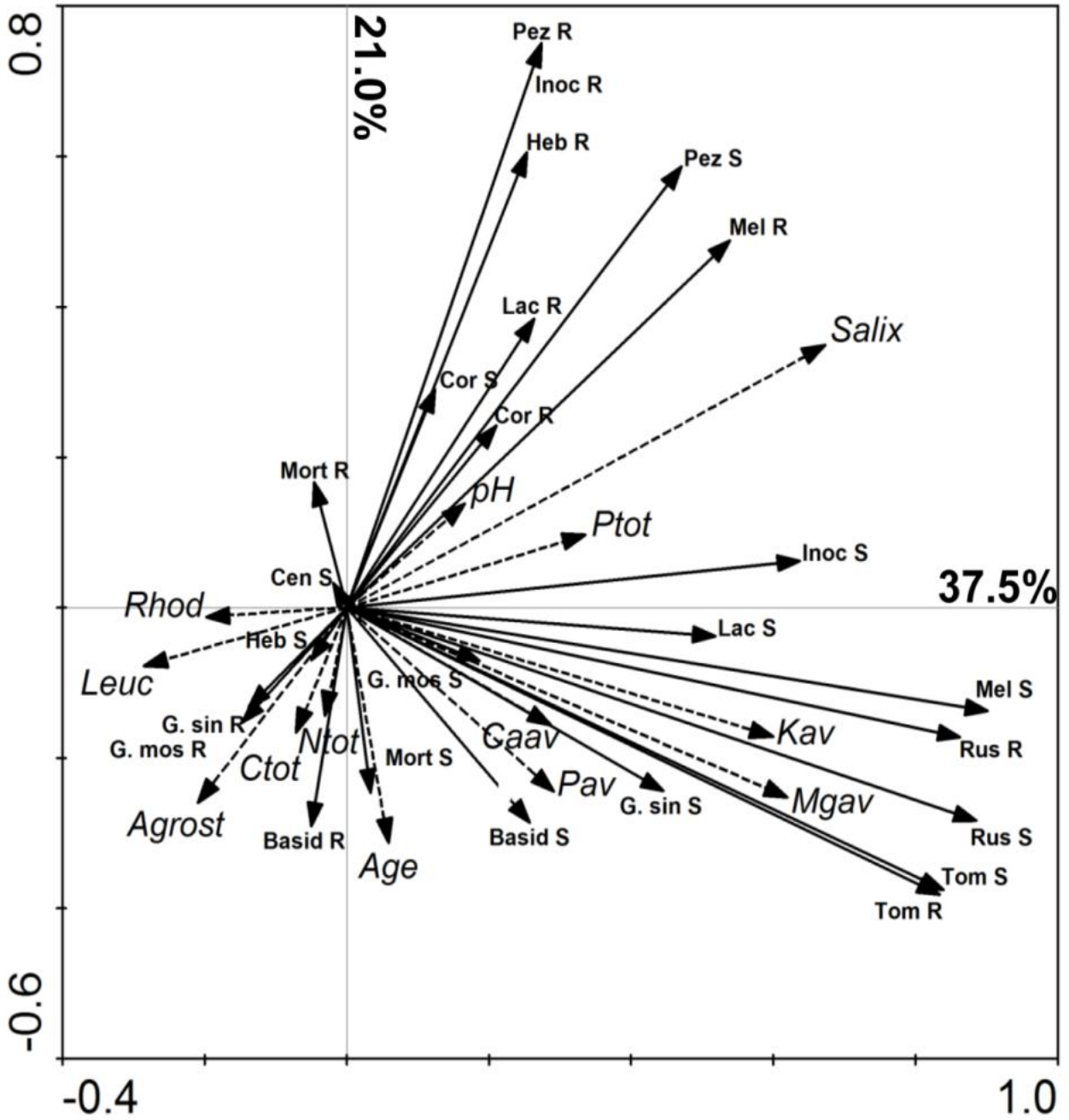


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Figure 2

Redundancy analysis ordination diagram showing dependencies between soil origin (rhizosphere soils, with bare soil excluded) and soil properties (dataset 1, dashed lines, italic labels), and abundance of fungi detected in the root and rhizosphere soils (dataset 2, solid lines, bold regular labels). Monte Carlo permutation test carried out for all four canonical axes confirmed the significance of the relationship between investigated datasets ($F=2.122$, $p=0.0015$). Abbreviations: plant identity – (Salix) *Salix helvetica*; (Agrost) *Agrostis gigantea*; (Leuc) *Leucanthemopsis alpina*; (Rhod) *Rhododendron ferrugineum*; (soil) bare soil; soil properties – (age) soil age after deglaciation (years); (C_{tot}) total organic carbon concentration; (N_{tot}) total nitrogen concentration; (P_{tot}) total phosphorus concentration; (P_{av}) plant-available phosphorus concentration; (K_{av}) plant-available potassium concentration; (Mg_{av}) plant-available magnesium concentration; (Ca_{av}) plant-available calcium concentration; fungi abbreviated as **fungus name R** or **fungus name S**, were detected in roots or soils, respectively; fungal identities - (Cen) *Cenococcum geophilum*; (Cor) *Cortinarius casimiri*; (Heb) *Hebeloma subconcolor*; (Inoc) *Inocybe lacera*; (Lac) *Laccaria pumila*; (Rus) *Russula emetica*; (Tom) *Tomentella* spp.; (G. mos) *Glomus mosseae*; (G. sin) *Glomus sinuosum*; (Mel) *Meliniomyces bicolor*; (Pez) *Peziza badia*; (Basid) Uncultured Basidiomycota; (Mort) Uncultured *Mortierella* spp.

3. Factors influencing enzymatic activities of soils

3.1. The influence of soil age on the soil enzymatic activity

The activity of enzymes in all soils was either unaffected by (chitinase, protease, α -glucosidase and sulfatase) or increased (phosphatase, β -glucosidase and xylosidase, $p \leq 0.03$, $r^2 \geq 0.56$, data not shown) with soil age (Fig. 3). Enzymatic activity in the rhizosphere soil of *L. alpina* tended to increase ($p=0.0081$, $r^2=0.71$, data not shown) with soil age, whereas other investigated rhizospheres did not follow similar pattern (Figs. 3 and S5).

3.2. The influence of plant species on the soil enzymatic activity – general trends

Bare soil exhibited significantly lower enzymatic activity compared to soils with a vegetation cover, irrespective of the soil development stage (Figs. 4 and S5). Enzymatic activities in the rhizosphere soils varied markedly between investigated plant species (Fig. 4). This suggests that plant roots and root-associated microbial communities specifically influence the enzymatic activities. For example, enzymatic activity of chitinase and α -glucosidase was found significantly higher in the rhizosphere of *S. helvetica* than other plants. In contrast, phosphatase and β -glucosidase presented significantly higher activities in the rhizosphere of *A. gigantea* (Fig. 4).

3.3. The influence of plant species and soil properties on the soil enzymatic activity

Multivariate analysis relating soil origin (rhizosphere or bare soil) and soil properties (Table 1) to enzymatic activities revealed that these parameters explained 64.5% of the dataset variability (Fig. 5). Above all investigated parameters, plant species prominently affected enzymatic activities of soils. This trend was observed for *S. helvetica*, *R. ferrugineum* and *A. gigantea*, whereas *L. alpina* seemed to influence activity of soil enzymes to a lesser extent. Following relations between plant species and enzymatic activity could be noted: *S. helvetica* correlated positively with activity of chitinase ($p=0.0058$, $r^2=0.74$) and α -glucosidase ($p=0.0028$, $r^2=0.22$), *R. ferrugineum* correlated positively with sulfatase ($p=0.0000$, $r^2=0.37$) and protease ($p=0.0080$, $r^2=0.18$), whereas *A. gigantea* with β -glucosidase ($p=0.038$, $r^2=0.11$). The vector of *L. alpina* (Fig. 5) was short thus no significant correlation was found between this plant species and enzymatic activity for none of the enzyme in this dataset.

Neither soil age, nor C_{tot} or N_{tot} , exhibited significant impact on the activity for assayed enzymes (Fig. 5). On the contrary, soil pH correlated significantly with enzyme activities in the soils ($p \leq 0.05$), except α -glucosidase and sulfatase. Especially in rhizosphere of *S. helvetica*, soil pH could be considered as a factor influencing activity of β -glucosidase ($p=0.0058$, $r^2=0.74$) and chitinase ($p=0.0058$, $r^2=0.74$).

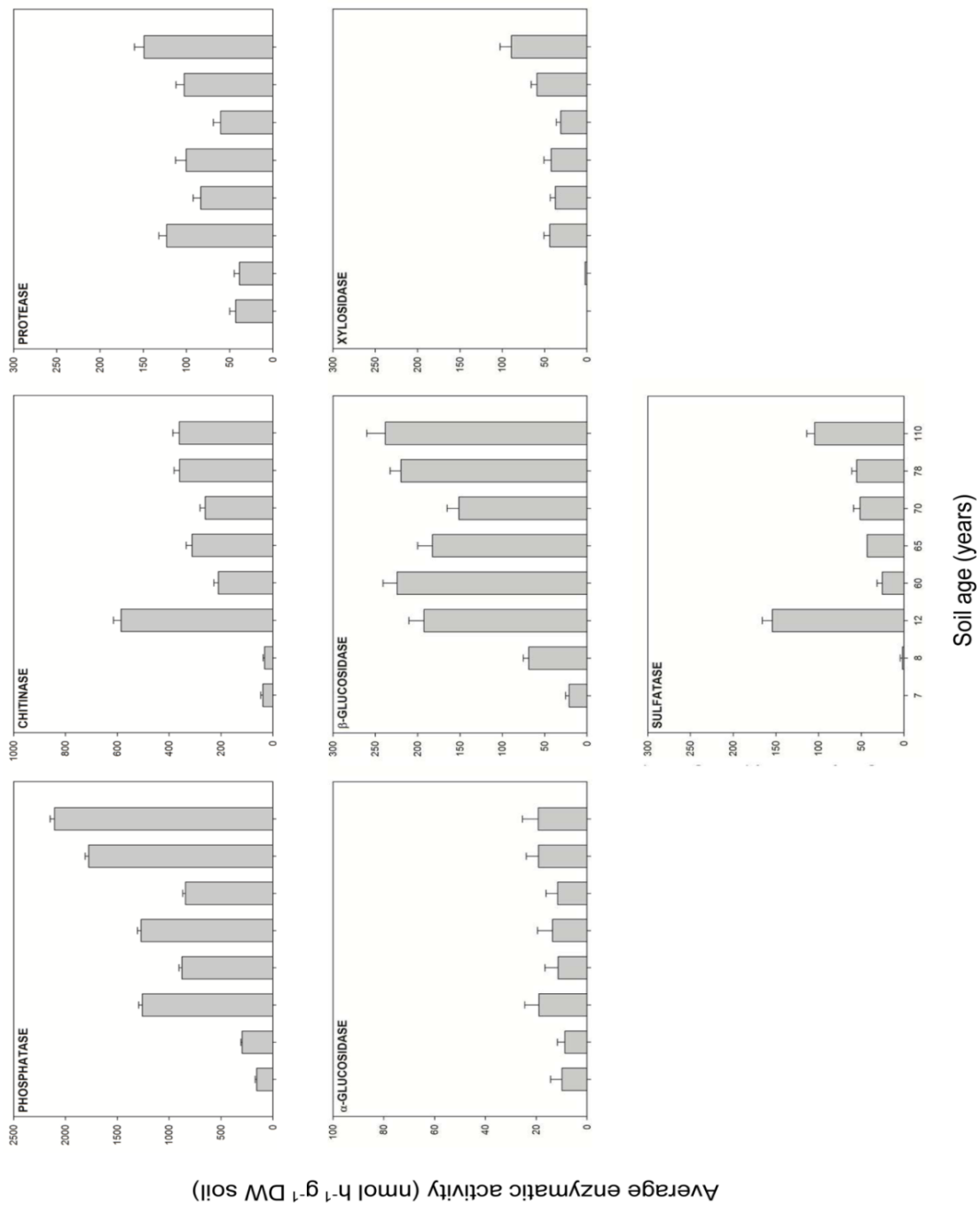


Figure 3

Enzymatic activities of soils along developmental gradient. The values are averages \pm SE of the enzymatic activities measured in the rhizosphere soils of four alpine plant species (*S. helvetica*, *A. gigantea*, *L. alpina* and *R. ferrugineum*) and bare soil at each sampling site, with exception of sites deglaciated 7 and 8 years ago, where *R. ferrugineum* was absent.

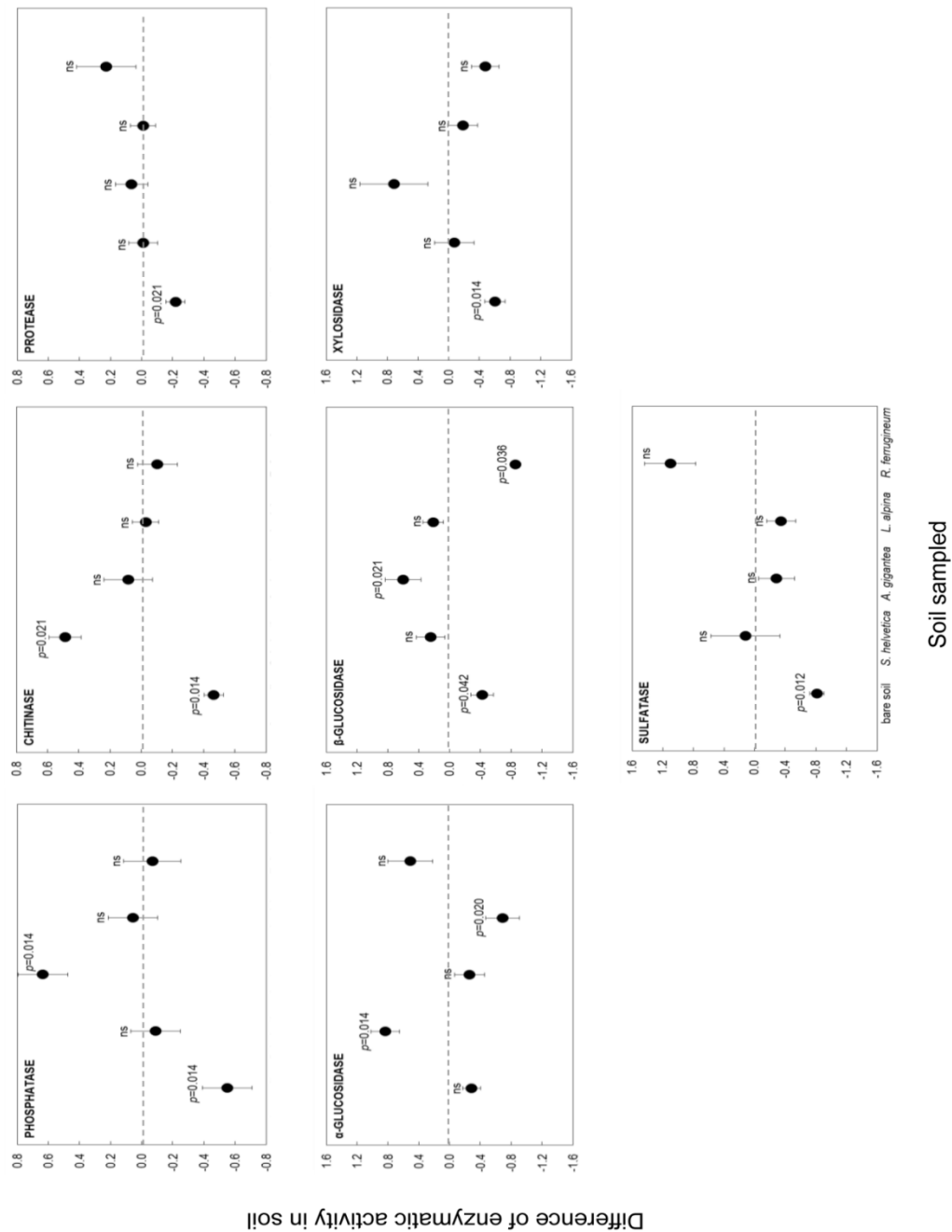


Figure 4

The effect of plant species on the enzymatic activities in the rhizosphere soil. The values were standardized to the average enzymatic activity per sampling site by the following calculation: $\frac{\text{enzymatic activity of individual soil}}{\text{average enzymatic activity per sampling site}} - 1$. Standardization eliminated influence of soil age and entitles plant species to be main factor affecting enzymatic activity of assayed soils. Significance of differences of the values from zero is shown according to mean signed rank test; (ns) not significant $p \geq 0.05$.

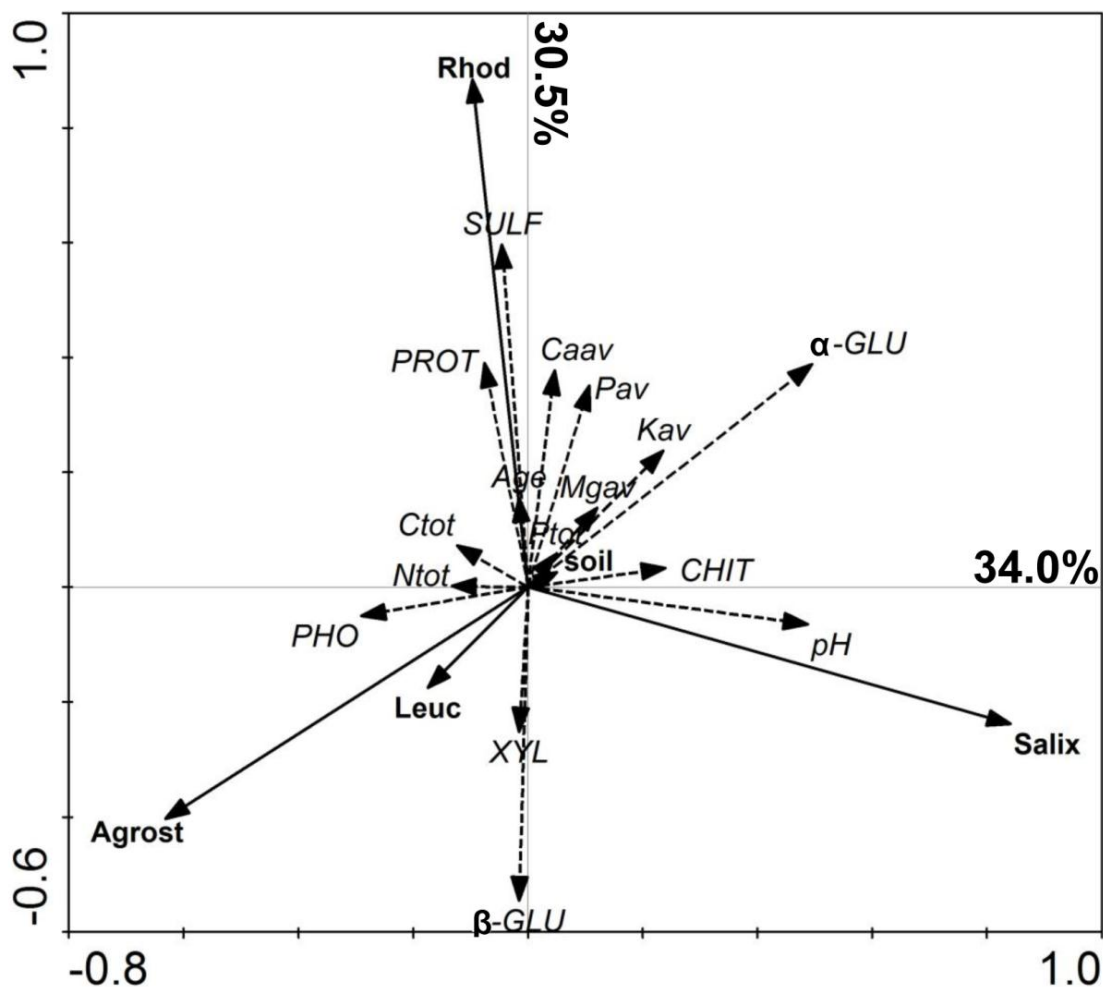


Figure 5

Redundancy analysis ordination diagram showing dependencies between soil origin (rhizosphere or bare soil, dataset 1, solid lines, bold regular labels), soil properties and soil enzymatic activities (dashed lines, italic labels). Monte Carlo permutation test carried out for all four canonical axes confirmed the significance of the relationship between investigated datasets ($F=2.731$, $p=0.0001$). Abbreviations: plant identity – (Salix) *Salix helvetica*; (Agrost) *Agrostis gigantea*; (Leuc) *Leucanthemopsis alpina*; (Rhod) *Rhododendron ferrugineum*; (soil) bare soil; soil properties – (age) soil age after deglaciation (years); (C_{tot}) total organic carbon concentration; (N_{tot}) total nitrogen concentration; (P_{tot}) total phosphorus concentration; (P_{av}) plant-available phosphorus concentration; (K_{av}) plant-available potassium concentration; (Mg_{av}) plant-available magnesium concentration; (Ca_{av}) plant-available calcium concentration; enzymes – (PHO) phosphatase; (CHIT) chitinase; (PROT) protease; (α -GLU) α -glucosidase; (β -GLU) β -glucosidase; (XYL) xylosidase; (SULF) sulfatase.

Discussion

1. The impact of plant species on the structure of mycorrhizal fungal community.

Our investigations indicated that plants specifically affected the community of mycorrhizal fungi associated with their roots and rhizospheres (Fig. 1A-B). Plant species, depending on their mycorrhizal preferences, harbored predominantly certain mycorrhizal fungi (i. e., *S. helvetica* – ECM, *A. gigantea* – AMF) (Fig. 1A-B). Both, AM and ECM fungi exhibit symbiotic life-style (Baldrian, 2009; Smith and Read, 2008) and there is strong dependency between plant species and mycorrhizal fungi. This has been repeatedly shown in a variety of ecosystems (Kummel and Lostroh, 2011; Mummey et al., 2005). For example, the study from Pennsylvania Mountain confirmed that the identity of the herbaceous host plant (*Taraxacum officinale*, *Taraxacum ceratophorum* and *Polemonium viscosum*) strongly affected the community of symbiotic AMF, erasing other (subordinated) habitat effects such as plant community type (open meadow or willow understory) (Becklin et al., 2011a).

Our study show that composition of fungal assemblages colonizing roots usually differed from those in the rhizosphere soils (Fig. 1A-B). Differences in the relative abundance of mycorrhizal fungi between roots and soil can be explained by very intensive colonization of the roots. In contrast, the web of mycorrhizal hyphae penetrating rhizosphere soil, although dense and expanded, usually constitute smaller biomass than root-associated fungal structures (Smith and Read, 2008).

Pattern in assemblage of the fungal communities in soil could be affected by interactions between members of the microbial community (Bending et al., 2006) or associated plant (Becklin et al., 2011b; Mummey et al., 2005). Moreover, rhizosphere soil can act as a refuge for fungi (Fig. 1B), which are unable to colonize particular plant species, but originate from neighboring plants or were transported and deposited there by a soil-dwelling fauna.

2. The impact of soil development on the community structure of other fungi

The abundance of UTS (“endophytic”, *sensu lato*¹) seem to be modulated by soil properties rather than the plant species (Fig. 2). Soil age (to a lesser extent also C_{tot}) correlated well

¹ 'Endophytes' are defined here as group of non-mycorrhizal fungi associated with plant roots. Morphologically, these fungi do not form only the structures inside the roots (e.g., sclerotia, sclerotium-like structures, hyphae) but can develop hyphae or mantle-like structures outside the roots (Peterson et al., 2008).

with the total abundance of these fungi (Fig. 1A-B, Fig. 2), especially in bare soil and rhizosphere of *S. helvetica* ($p \leq 0.01$, $r^2 \leq 0.68$, data not shown). This correlation was mainly driven by Uncultured Basidiomycota and *Mortierella* spp.; neither *Meliniomyces bicolor* nor *Peziza badia* contributed significantly to this pattern ($p \geq 0.1$, data not shown) suggesting that another factor/s such as vegetation or soil properties underlie their distribution and abundance (Kohout et al., 2011; Tedersoo et al., 2009). Correlation with soil development (Fig. 2) could potentially indicate saprotrophic preferences of these fungi (Brunner et al., 2011; Thormann, 2006). Their predominance in rhizospheres (especially Uncultured Basidiomycota), as compared with bare soil, and their absence or low abundance in young soils (Fig. 1B) seem to support this hypothesis. Further, it needs to be emphasized that the abundance and diversity of saprotrophic fungi can be greatly imprinted by spatial and temporal heterogeneity between and within investigated sites, difficult- to- track local disturbances (e.g., continuity of SOM layer, sediments deposition by ephemeral streams) and differences (e.g., SOM composition, manure deposition by grazing animals). These effects could be more important for the distribution of the saprotrophs than for the mycorrhizal fungi.

3. The impact of soil developmental stage on soil enzymatic activity

No uniform pattern was found for activities of measured soil enzymes along developmental gradient (Fig. 3). When effect of individual plant species was erased by averaging of the values from each sampling site, only phosphatase, β -glucosidase and xylosidase seemed to increase with soil age ($p \leq 0.03$, $r^2 \geq 0.56$) (Fig. 3). Increase in enzymatic activities with soil development was commonly found in other alpine ecosystems (Tscherko et al., 2003), however these investigations focuses mainly on the soil in general sense, not on the rhizospheres of a particular plant species (Miniaci et al., 2007). General increase along soil development can be then attributed to the succession of microbial communities and plant cover. Accumulation of SOM stimulate microbial growth and activity (Göransson et al., 2011), what in turn increase the pool of enzymes in soil.

4. Soil enzymatic activity as impacted by the association plant-fungi

Plant species influenced the soil enzymatic activity (Fig. 4) and different patterns of this activity were be observed (Fig. S5). Evidences exist that ECM in natural ecosystems are

characterized by higher, compare with other mycorrhizal fungi, enzymatic activities of phosphatase (Grierson and Comerford, 2000) and chitinase (Kluber et al., 2011). However, these enzymes had been argued to have both fungal, bacterial or plant origin. None of the studies, to the best to our knowledge, tried to resolve this contentious issue. We could, however, potentially assume, that enzymatic activity of given enzyme (e.g., chitinase) in soil will increase with increasing fungal biomass. In work of Baldrian et al. (2010) a significant correlation between enzymatic activity in litter and organic horizon of *Quercus petraea* forest soil, and concentration of fungal biomass indicators (i.e., 18:2 ω 6,9 phospholipid fatty acids and ergosterol) was found (Baldrian et al., 2010). If our assumption will be true, these results could suggest (predominantly) fungal origin of some enzymes in investigated soils. Unfortunately, until now, the separation of different groups of fungi/microbes and plants as contributors to enzymatic activity still present shortcomings (Baldrian, 2009 and references therein).

Despite all these uncertainties, the impact of fungal communities associated with plant roots on the enzymatic activity detected in soils, was observed (Fig. S6 A). Surprisingly, similar trend was not confirmed when soil enzymatic activity were correlated with the abundance of fungal communities associated with soil, not plant roots (Fig. S6 B). Technical limitations such as detection limits of qPCR reactions, soil heterogeneity, or sampling procedure (Taylor, 2002) could possibly be found among explanations for observed discrepancy. Additionally to that, we are aware that our investigations covered only a part (and probably only a minority) of fungal diversity: the qPCR markers were developed only for a handful of fungal taxa. Further, spatial and/or seasonal aspects were omitted in this study. In face of all the constrains presented above, studies disentangling enzymatic activity of individual ECM root tips are nowadays of a great meaning (Pritsch et al., 2011). Although still not free from discussions (i. e., activity of plant root and associated microbes, bias of ECM tips selection, representativity of the selection, etc.), this approach revealed the diversity of ECM enzymatic activities for various environments, host plants and conditions (Courty et al., 2005; Pritsch and Garbaye, 2011; Rineau and Garbaye, 2009). To track the origin of enzymes in rhizosphere soils in our study, enzymatic activities of *S. helvetica*-associated ECM root tips were inspected (Fig. S8, Table S7). However, high heterogeneity of investigated system and technical limitations (i. e. selection and vitality of ECM tips, age and physiological state of

plants) overruled the impact of fungal species itself contributing to inconsistency in observed patterns (Fi. S7, supplementary materials).

Conclusions

- The composition (structure) of the fungal communities varied for the different alpine plants. The composition of mycorrhizal fungi (ECM and AMF) was strongly affected by the plant species, whereas soil development influenced the community of other soil fungi (i.e., UTS), suggesting those to be saprotrophs.
- Root-associated fungal (microbial) communities were important factors influencing the activity of enzymes in the rhizosphere soil.
- Enzymatic activity of chitinase and α -glucosidase was significantly higher in the rhizosphere of *S. helvetica* than other plants, what can be due to the activity of ECM.
- Our understanding of fungal communities in alpine ecosystems profoundly improved over last decade. Nevertheless, shift from the taxonomic diversity toward diversity of functions is highly required in current ecological studies. These will certainly contribute to deeper understanding of subtle dependencies between plants and mycorrhizal fungi associated with them under natural conditions, and thus expand our knowledge about ecosystem succession, functioning and sustainability.

Acknowledgements

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Chapter 2 - supplementary material

Supplementary Materials and methods

Design of the qPCR markers

Quantitative PCR (qPCR) molecular markers were designed specifically for the fungal taxa occurring in the studied environment (Damma glacier, Central Swiss Alps, N46°38.117', E8°27.677'). Particularly, the design targeted fungi associated with roots of *Salix helvetica* (dual mycorrhizal plant, with scarce arbuscular mycorrhiza (AM) and predominant ectomycorrhiza (ECM)) and *Leucanthemopsis alpina* and *Agrostis gigantea* (both AM plants). Morphotyping of ECM associated with *S. helvetica* (Agerer, 1987-2002) yielded detailed description and photographic documentation of discerned ECM morphotypes (Fig. S2, Table S3). Representatives of each morphotype were excised from the roots, two to eight ECM tips of the same morphotype were placed in 2.0 ml Eppendorf tube and frozen. For *L. alpina* and *A. gigantea*, samples of approximately 0.6 mg of the fine roots (≤ 5 mm diameter) were placed in 2.0 mL Eppendorf tubes and frozen. Further, all samples were freeze-dried, homogenized by beat-beating (2x20 sec, 46 Hz) and subjected to the DNA extraction (DNeasy Plant Mini Kit, Qiagen, Switzerland). Five μ l of all DNA extracts (undiluted or diluted 1:5, 1:10 or 1:20, depending on the coloration of the extract) were combined with 20 μ l of the PCR mix, which consisted of 2.5 μ l of 10x CoralLoad buffer (15 mM), 5.0 μ l of 5x Q-solution, 2.0 μ l of MgCl₂ (25 mM), 0.5 μ l of the mix of dNTPs (10 mM each nucleotide), 1.0 μ l of each primer (25 mM), 0.1 μ l of Taq DNA polymerase and 7.9 μ l molecular-grade PCR water. The following primer combinations were used to amplify ITS region of ECM fungal rDNA: ITS1/ITS4, ITS1F/ITS4, ITS1/ITS4B, ITS1F/LR15, ITS1/LR15, ITSF/ITS4B, ITS1/NL4, ITS1F/NL4 (Gardes and Bruns, 1993; Mühlmann et al., 2008; Mühlmann and Peintner, 2008a; White et al., 1990). To amplify AM fungi from *L. alpina* and *A. gigantea*, nested PCR reaction was performed with primers targeting LSU of the rDNA: ITS3/NDL22 in the first, and LR1/FLR2 in the second step of nested PCR amplification (van Tuinen et al., 1998a). All PCR reactions were performed with a Biometra GmbH thermocycler (Göttingen, Germany). The following conditions were selected for amplification of the DNA from ECM fungi: 94°C for 5 min followed by 38 cycles of denaturation at 94°C for 15 s, annealing at 50°C for 75 s and extension at 72°C for 90 s. Final extension was at 72°C for 6 min. The following conditions

were selected for amplification of the DNA from AMF: 94°C for 3 min followed by 38 cycles of denaturation at 94°C for 20 s, annealing at 58°C for 70 s and extension at 72°C for 1 min. Final extension was at 72°C for 5 min.

Agarose gel electrophoresis with ethidium bromide as a DNA staining agent substantiated selection of only positively amplified samples for the post-PCR purification (QIAquick PCR Purification Kit, Qiagen, Switzerland). Purified DNA fragments were cloned into blue-script vector (pGEM®-T Easy Vector System, A1360, Promega, Switzerland) according to the manufacturer protocol. MiniPrep technique was used to isolate the plasmid DNA carrying inserted DNA of interest (Sambrook et al., 1989). Plasmid inserts were sequenced by Microsynth AG (Balgach, Switzerland), using M13 primers. Each obtained sequence was individually edited and aligned with sequences available from the electronic database (www.ncbi.nlm.nih.gov/BLAST/, 09.09.2011). The sequences representing good quality (i. e., no biased identity of any nucleotides, reads longer than 600 bp) were deposited in National Center of Biotechnology Information (NCBI) Blast nucleotide collection (accession numbers: under the procedure), and served as the templates for the design of the qPCR molecular markers. The sequences of each fungal group were separately aligned with sequences deposited in the database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to confirm their identity. Optimal design conditions required by the software (i. e., optimal kinetics, length of the primers/probes, 0 to 2 mismatches allowed) were highly respected during the design of the molecular markers.

Twenty-three, seven and thirteen different molecular markers were designed to detect ECM, AMF and UTS, respectively. Sequences of those molecular markers were individually blasted to the database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>, 28th April 2009), what revealed that only 18 markers for ECM and 7 for AMF and UTS, respectively, exhibited high specificity toward targeted fungus. These markers were synthesized by Microsynth AG, Balgach, Switzerland and tested further.

qPCR reaction performed with these 32 markers at low stringency conditions (40 cycles, denaturation at 95°C for 10 s, annealing at 52°C for 30 s, amplification at 72°C for 5 s) and plasmidic DNA (of target fungus and non-target fungi) as amplification template substantiated final selection of 13 the most specific markers (7 for ECM, 2 for AMF and 4 UTS,

respectively) for further tests (see **Cross-specificity test for qPCR molecular markers** for details) and finally- for routine use.

Cross-specificity test for qPCR molecular markers

Designed qPCR markers were subjected to three-step validation procedure, similar to that outlined in recent publication (Thonar et al., 2012):

- i) qPCR reaction was performed at low stringency conditions (40 cycles, denaturation at 95°C for 10 s, annealing at 52°C for 30 s, amplification at 72°C for 5 s) with plasmidic (of both target fungus and non-target fungi and plants) and genomic (extracted from individual root tips/root samples and plant roots and leaves) DNA available for *S. helvetica*, *L. alpina* and *A. gigantea*;
- ii) qPCR reaction was performed with plasmidic DNA (of both target fungus and non-target fungi) at optimized cycling conditions adjusted for each qPCR molecular marker individually (Table S5);
- iii) qPCR reaction was performed with genomic DNA (extracted from individual root tips/root samples) available for *S. helvetica*, *L. alpina* and *A. gigantea* at optimized cycling conditions (Table S5). These programs of optimized cycling conditions were implemented for routine analysis on the root and soil DNA extracts.

The detection limit

The detection limit for each qPCR molecular marker was defined. First, the concentration of plasmid DNA, carrying fragments of ITS or LSU (Table S3) regions of each fungus, was measured by PicoGreen (Invitrogen, Switzerland). Measured fluorescence was converted to concentration unit (μg of the DNA in μl) based on the standard curve established with the standard plasmid DNA. Taking for account the length of pGEM[®]-T Easy Vector (3018 bp, A1360, Promega, Switzerland), length of target DNA insert (length of the sequence) and additional nucleotides (two adenosine nucleotides added during the PCR reaction), length of entire plasmid was calculated. Number of copies of the template DNA in one microliter of solution (undiluted plasmid sample) was calculated based on Avogadro's constant ($A_v = 6.022 \times 10^{23}$). This sample was further diluted to obtain gradient of the template concentration and was run with respective molecular marker under optimal conditions (Table

S5). The threshold cycle (C_t) values obtained from this analysis were plotted against log-transformed template concentration and from equation of negative linear correlation detection limit of qPCR reaction was calculated and expressed as threshold number of copies of template DNA per unit volume. The detection limits and efficiency of qPCR reaction were also calculated (Lee et al., 2006; Vaerman et al., 2004).

Supplementary tables

Table S1

Soil age (after deglaciation) and geographical location of the sampling sites.

Soil age (years after last deglaciation)	Coordinate	Elevation (m)
7	N 46°38.160' E 8°27.550'	2052
8	N 46°38.196' E 8°27.591'	2046
12	N 46°38.226' E 8°27.623'	2037
60	N 46°38.233' E 8°27.718'	2037
65	N 46°38.281' E 8°27.735'	2024
70	N 46°38.314' E 8°27.786'	2012
78	N 46°38.364' E 8°27.826'	1990
110	N 46°38.412' E 8°27.909'	1966

Table S2

Morphotypes of ectomycorrhizal fungi associated with *S. helvetica* – identity based on sequencing information, showing the most similar hits from the GenBank database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>, 28th April 2009), and their morphology description (DEEMY, www.deemy.de, 14th March 2009).

ECM identity	Accession number	% of identity	Mycorrhizal system	Axis form	Mantle color	Mantle surface	Emanating hyphae
<i>Cenococcum geophilum</i>	DQ474290.1	95	monopodial	short or long	black	grainy	black, straight
<i>Cortinarius casimiri</i>	FJ717538.1	99	monopodial	long	whitish, pale creamy	smooth	white, woolly
<i>Hebeloma subconcolor</i>	AY320393.1	98	monopodial	long	creamy-silverish, shiny	grainy	creamy, long, undulated
<i>Inocybe lacera</i>	HQ604445.1	99	monopodial	short	gray	grainy	no
<i>Laccaria pumila</i>	GU234161.1	99	monopodial	long	gray-brownish	smooth	creamy, short, woolly
<i>Meliniomyces bicolor</i>	AY394885.1	97	monopodial	short, thick	gray-brown	grainy	no
<i>Peziza badia</i>	DQ384574.1	94	monopodial	long	end of the tip creamy, changing to gray toward the base	grainy	gray, long, undulated
<i>Phialocephala fortinii</i>	AY394921.1	98	monopodial	long, very thin	gray	grainy	no
<i>Russula emetica</i>	AY228350.1	98	monopodial	short or long	brown-reddish	smooth	no
<i>Tomentella</i> spp.	FJ581421.1	97	monopodial	long, thick	creamy, brownish while senescent	smooth to grainy while senescent	no

Photographic representation of these morphotypes is presented on Figure S2.

Table S3

Methodological details of the four-step non-vital staining procedure adjusted for the roots of investigated plant species. The concentrations and the type of used chemicals as well as duration and temperature for each staining step are detailed. Mycorrhizal status and plant form is indicated in the bracket following the plant name.

Staining step Plant	Maceration	Bleaching	Acidification	Staining solution LGW +
<i>Salix helvetica</i> (ECM+AMF shrub)	10% KOH 4h, 90°C	1% H ₂ O ₂ + 10% NH ₄ OH 2h, 25°C	1% HCl 30 min, 25°C	0.10% TB + 0.05% MB 1h, 90°C
<i>Agrostis gigantea</i> (AMF grass)	10% KOH 20 min, 90°C	NONE	1% HCl 20 min, 25°C	0.05% TB + 0.05% MB 20 min, 90°C
<i>Leucanthemopsis alpina</i> (AMF herb)	10% KOH 20 min, 90°C	1% H ₂ O ₂ + 10% NH ₄ OH 25 min, 25°C	1% HCl 20 min, 25°C	0.05% TB + 0.05% MB 20 min, 90°C
<i>Rhododendron ferrugineum</i> (ER shrub)	5% KOH + 5% NaOH 8h, 90°C	1% H ₂ O ₂ + 10% NH ₄ OH 3h, 25°C	1% HCl 12h, 25°C	0.03% CB 4h, 90°C

Abbreviations: (AM) arbuscular mycorrhiza; (ECM) ectomycorrhiza; (ER) ericoid mycorrhiza; (LGW) staining solution containing lactic acid: glycerol: water (14:1:1, v:v:v); (TB) Trypan Blue; (MB) Methylene Blue; (CB) Chlorazol Black.

Table S4

Taxon-specific qPCR molecular markers developed for quantification of the root-associated fungi. Species for genera names according the sequences database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>, 29th April 2009). Characterization of each fungus (trophic status and origin of fungus) is provided with detailed description of the designed qPCR molecular markers.

Fungus identity	Fungus trophic status	Origin of the fungus	% of identity (accession number)	Target region	Sequence (5'-3')		Amplicon length (base pairs)
					Primers: forward and reverse	TaqMan probe (FAM- fluorescein; BHQ1- black hole quencher chromophore)	
<i>Cenococcum geophilum</i>	ECM	<i>S. helvetica</i>	95 (DQ474290.1)	ITS	CCGTAGAACAAGTCTTGC TCCATGTCGTCTGTTAC	FAM- AATGATTCCCTCCGGCAGTGTG- BHQ1	251
<i>Cortinarius casimiri</i>	ECM	<i>S. helvetica</i>	99 (FJ17538.1)	ITS	GTCCTCGGACAAATTCATC GCGGATAACACTTCTCTAG	FAM- ATTACAACTCGGACAGCCATAAGAC- BHQ1	196
<i>Hebeloma subconcolor</i>	ECM	<i>S. helvetica</i>	98 (AY320393.1)	ITS	CCTCCCATCCTCATCTCTCC GCAAAGAAAGCCAGCTAAGTG	FAM- AGTCCTCACGACCGAGTTGCCT- BHQ1	106
<i>Inocybe lacera</i>	ECM	<i>S. helvetica</i>	99 (HQ604445.1)	ITS	GTAGGCCTGGAATCTTCTG AGGTAAACATAGACCTGGAAAG	FAM- TGAAGCACAAACAGTCCCTCAACTATA CAA- BHQ1	94
<i>Laccaria pumila</i>	ECM	<i>S. helvetica</i>	99 (GU234161.1)	ITS	GAGCAACTCGGATTTTAGG GATGACATTCTTTAAACATACATTTGG	FAM- AGCTTTCCTGTCAATTTCCAAGACTAT GTTT- BHQ1	105
<i>Meliniomyces bicolor</i>	UTS	<i>S. helvetica</i>	97 (AY394885.1)	ITS	GGAAGGATCATTAAAGAATC TACTCAGACATCACTAAAC	FAM- TATTTCCAAACCCTGTATATAC- BHQ1	162
<i>Peziza badia</i>	UTS	<i>S. helvetica</i>	94 (DQ384574.1)	ITS	TTGATCTTGGAAATGATTTGGAGAG GTTAAGGCAAGCACAAATCATACC	FAM- ATGTAGACGCCCTCCAGAAAATTCAT AG- BHQ1	138
<i>Russula emetica</i>	ECM	<i>S. helvetica</i>	98 (AY228350.1)	ITS	TTGGCGTGATAAGTTGTTTC TTGAGGTCAAGGTTTTGTTTC	FAM- ACACCTGCTTTCTAAACCCTCTCAT- BHQ1	143
<i>Tomentella</i> spp.	ECM	<i>S. helvetica</i>	97 (FJ581421.1)	ITS	TGCTGGTCTCAAACAAGG CGTAGAACCCACAGCAGGTG	FAM- CCCAAGCCATAGGAACATACAGAGG- BHQ1	116
<i>Glomus sinuosum</i>	AMF	<i>A. gigantea</i>	99 (AJ634628.1)	LSU	CGATAGCGAACAAGTACC CGTCACATCTACCAAGAGG	FAM- AACACTTCAATACGAGACCCAAAGC- BHQ1	297
<i>Glomus mosseae</i>	AMF	<i>A. gigantea</i>	90 (FR17196.1)	LSU	TCTGCGTGTGATTTTAGC GACTCTTAGAAAAACGATCG	FAM- AAAGCATCTTGTATCTAACCAGACG- BHQ1	104
Uncultured Basidiomycota	UTS	<i>L. alpina</i>	99 (DQ341907.1)	LSU	GAACAGAGCGTCATAGAGG TATTTAGCTTTAGATGGAAATTTACC	FAM- AACAACTCGACTCGCTGAGAGCT- BHQ1	134
Uncultured Mortierella spp.	UTS	<i>L. alpina</i>	91 (HQ680438.1)	LSU	GTTGTTTGGCGGTACAATGC CATTACGTC AACATCTCTAAGC	FAM- CGCCTTGCTTCTCCATACTCGCT- BHQ1	119

Table S5

Optimized qPCR cycling conditions for each taxon-specific qPCR molecular marker.

Fungus identity	qPCR cycling reaction			Number of cycles
	Denaturation (°C / s)	Annealing (°C / s)	Amplification (°C / s)	
<i>Cenococcum geophilum</i>	95 / 10	60 / 10	72 / 1	50
<i>Cortinarius casimiri</i>		56 / 10	72 / 1	
<i>Hebeloma subconcolor</i>		60 / 10	72 / 1	
<i>Inocybe lacera</i>		60 / 10	72 / 1	
<i>Laccaria pumila</i>		60 / 10	72 / 1	
<i>Meliniomyces bicolor</i>		48 / 10	72 / 1	
<i>Peziza badia</i>		58 / 5	72 / 1	
<i>Russula emetica</i>		58 / 10	72 / 1	
<i>Tomentella</i> spp.		58 / 10	72 / 1	
<i>Glomus sinuosum</i>		58 / 10	72 / 5	
<i>Glomus mosseae</i>		58 / 10	72 / 1	
Uncultured <i>Basidiomycota</i>		58 / 10	72 / 1	
Uncultured <i>Mortierella</i> spp.		55 / 20	72 / 5	

Table S6

Characterization of the enzymes assayed for the soils. Systematic names and enzyme commission (EC) numbers according to International Union of Biochemistry and Molecular Biology. Functions of enzymes according to BRENDA database (<http://www.brenda-enzymes.org/>, 8th May 2009). Methodological details of the assays are included.

Common name of enzyme	Systematic name / EC number	Function	Buffer type / pH	Standard type / concentration	Substrate type / concentration	Incubation time / temperature
Phosphatase	Phosphate-monoester phosphohydrolase (acid optimum) EC 3.1.3.2	Release of phosphate from organic compounds via random hydrolysis of phosphoric monoester bonds under acidic conditions	0.1 M MES / pH=5.0	4-MUF / 10 μ M	4-methylumbelliferyl phosphate / 1 mM	0-180 min / 37°C
Chitinase	(1 \rightarrow 4)-2-acetamido-2-deoxy- β -D-glucan glycanohydrolase EC 3.2.1.14	Random hydrolysis of N-acetyl- β -D-glucosamine (1 \rightarrow 4)- β -linkages in chitin and chitin-derivates	0.1 M MES / pH=5.5	4-MUF / 10 μ M	4-methylumbelliferyl N-acetyl- β -D-glucosaminide / 1 mM	0-180 min / 37°C
Protease	Leucyl aminopeptidase EC 3.4.11.1	Hydrolysis of terminal Leu and other amino acids (except Arg or Lys) from N-terminus of peptides	Modified universal buffer / pH=7.0	AMC / 10 μ M	L-leucine-7-amido-4-methylcoumarin hydrate / 1 mM	0-180 min / 37°C
α -glucosidase	α -D-glucoside glucohydrolase EC 3.2.1.20	Hydrolysis of terminal, non-reducing (1 \rightarrow 4)-linked α -D-glucose residues with release of α -D-glucose	0.1 M MES / pH=5.0	4-MUF / 10 μ M	4-methylumbelliferyl α -D-glucopyranoside / 1 mM	0-180 min / 37°C
β -glucosidase	α -D-glucoside glucohydrolase EC 3.2.1.21	Hydrolysis of terminal, non-reducing β -D-glucosyl residues with release of β -D-glucose	0.1 M MES / pH=5.5	4-MUF / 10 μ M	4-methylumbelliferyl β -D-glucopyranoside / 1 mM	0-180 min / 37°C
Xylosidase	1,4- β -D-xylian xylohydrolase EC 3.2.1.37	Hydrolysis of (1 \rightarrow 4)- β -D-xylans, to remove successive D-xylose residues from the non-reducing termini	0.1 M MES / pH=5.0	4-MUF / 10 μ M	4-methylumbelliferyl β -D-xylopyranoside / 1 mM	0-180 min / 37°C
Sulfatase	Aryl-sulfate sulfohydrolase EC 3.1.6.1	Releasing of sulfate from phenol sulfate compounds	0.1 M MES / pH=5.0	4-MUF / 10 μ M	4-methylumbelliferyl sulphate potassium salt / 1 mM	0-180 min / 37°C

Abbreviations: (MES) 2-(N-morpholino)ethanesulfonic acid; (4-MUF) 4-Methylumbelliferone; (AMC) 7-Amino-4-Methylcoumarin.

Table S7

Characterization of the enzymes assayed for the ectomycorrhizal root tips. Systematic names and enzyme commission (EC) numbers according to International Union of Biochemistry and Molecular Biology. Functions of enzymes according to BRENDA database (<http://www.brenda-enzymes.org/>, 8th May 2009). Methodological details of the assays are included.

Common name of enzyme	Systematic name / EC number	Function	Buffer type / pH / concentration	Standard type / concentration	Substrate type / concentration	Incubation time / temperature
Phosphatase	Phosphate-monoester phosphohydrolase (acid optimum) EC 3.1.3.2	Release of phosphate from organic compounds via random hydrolysis of phosphoric monoester bonds under acidic conditions	Tris-maleic acid / pH=4.5 / 75 mM	4-MUF / 10 µM	4-methylumbelliferyl phosphate / 2.4 mM	10 min / 37°C
Chitinase	(1→4)-2-acetamido-2-deoxy-β-D-glucan glycanohydrolase EC 3.2.1.14	Random hydrolysis of N-acetyl-β-D-glucosamine (1→4)-β-linkages in chitin and chitin-derivates	Tris-maleic acid / pH=4.5 / 75 mM	4-MUF / 10 µM	4-methylumbelliferyl N-acetyl-β-D-glucosaminide / 1.5 mM	20 min / 37°C
Protease	Leucyl aminopeptidase EC 3.4.11.1	Hydrolysis of terminal Leu and other amino acids (except Arg or Lys) from N-terminus of peptides	Tris-maleic acid / pH=6.5 / 75 mM	AMC / 10 µM	L-leucine-7-amido-4-methylcoumarin hydrate / 1.2 mM	60 min / 37°C
β-glucosidase	α-D-glucoside glucohydrolase EC 3.2.1.21	Hydrolysis of terminal, non-reducing β-D-glucosyl residues with release of β-D-glucose	Tris-maleic acid / pH=4.5 / 75 mM	4-MUF / 10 µM	4-methylumbelliferyl β-D-glucopyranoside / 1.5 mM	40 min / 37°C
β- glucuronidase	β-1,4-glucuronidase EC 3.2.1.31	Hydrolysis of complex carbohydrates by removing glucuronic acid residues from non-reducing termini	Tris-maleic acid / pH=4.5 / 75 mM	4-MUF / 10 µM	4-Methylumbelliferyl-β-D-glucuronide / 1.5 mM	60 min / 37°C
Cellobiohydrolase	Cellulose 1,4-β-cellobiosidase (non-reducing end) EC 3.2.1.91	Hydrolysis of (1→4)-β-D-glucosidic linkages in cellulose/ cellotetraose, releasing cellobiose from the non-reducing termini	Tris-maleic acid / pH=6.5 / 75 mM	4-MUF / 10 µM	4-Methylumbelliferyl-cellobiohydrofuran / 1.2 mM	40 min / 37°C
Xylosidase	4-β-D-xylan xylohydrolase EC 3.2.1.37	Hydrolysis of (1→4)-β-D-xylans, to remove successive D-xylose residues from the non-reducing termini	Tris-maleic acid / pH=4.5 / 75 mM	4-MUF / 10 µM	4-methylumbelliferyl β-D-xylopyranoside / 1.5 mM	60 min / 37°C

Abbreviations: (MES) 2-(N-morpholino)ethanesulfonic acid; (4-MUF) 4-Methylumbelliferone; (AMC) 7-Amino-4-Methylcoumarin.

Table S8

The significance of the interactions between the abundance of fungi in root and rhizosphere tested for each investigated plant species separately (considering the different sampling sites as experimental replicates). Significance levels in the table: (ns) $p \geq 0.05$; (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$.

Plant identity	S. <i>helvetica</i>	A. <i>gigantea</i>	L. <i>alpina</i>	R. <i>ferrugineum</i>	S. <i>helvetica</i>	A. <i>gigantea</i>	L. <i>alpina</i>	R. <i>ferrugineum</i>
Fungus identity	Paired samples comparison (t-test)				Simple regression			
<i>Cenococcum geophilum</i>	-	-	-	-	-	-	-	-
<i>Cortinarius casimiri</i>	ns	-	-	-	-	-	-	-
<i>Hebeloma subconcolor</i>	ns	ns	-	-	-	-	-	-
<i>Inocybe lacera</i>	ns	-	ns	-	ns	ns	ns	ns
<i>Laccaria pumila</i>	ns	ns	ns	ns	ns	*** $r^2=0.99$	ns	-
<i>Meliniomyces bicolor</i>	ns	ns	ns	ns	ns	ns	ns	ns
<i>Peziza badia</i>	ns	ns	ns	ns	ns	ns	-	ns
<i>Russula emetica</i>	**	-	ns	ns	*** $r^2=0.74$	-	-	-
<i>Tomentella</i> spp.	ns	ns	ns	ns	*** $r^2=0.99$	ns	-	-
<i>Glomus sinuosum</i>	ns	ns	ns	**	ns	*** $r^2=0.93$	ns	ns
<i>Glomus mosseae</i>	ns	ns	ns	-	ns	ns	ns	-
Uncultured <i>Basidiomycota</i>	***	***	***	ns	* $r^2=0.52$	ns	ns	** $r^2=0.81$
Uncultured <i>Mortierella</i> spp.	ns	**	ns	*	ns	ns	** $r^2=0.67$	ns

Abbreviations: (S. *helvetica*) *Salix helvetica*; (A. *gigantea*) *Agrostis gigantea*; (L. *alpina*) *Leucanthemopsis alpina*; (R. *ferrugineum*) *Rhododendron ferrugineum*; (ns) not significant; (-) test impossible to perform due to lack of data.

Supplementary figures

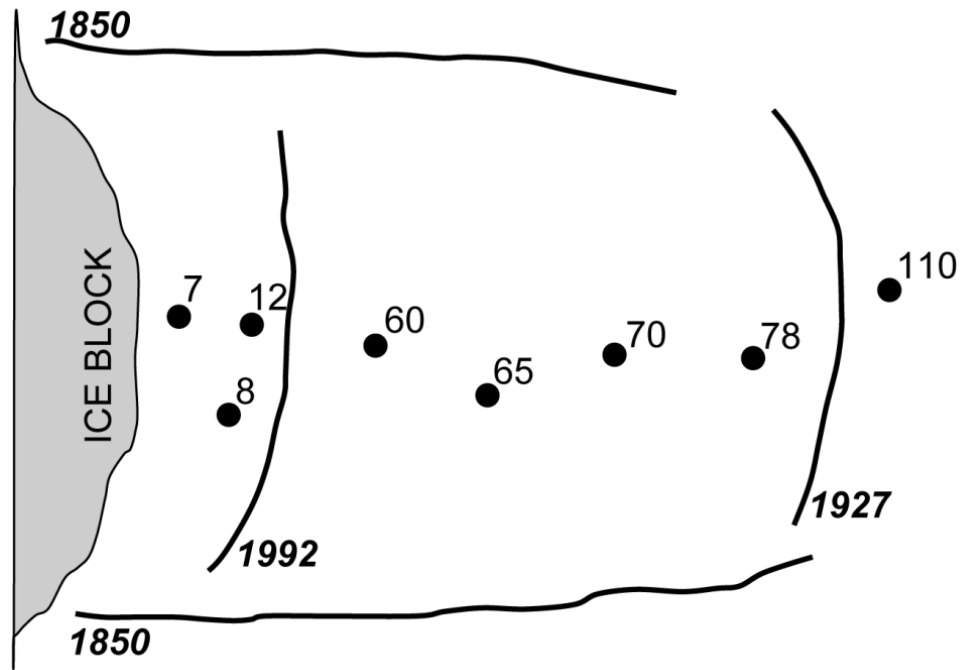


Figure S1

Location of the sampling sites at the Damma glacier forefield. Plain numbers indicate soil age (years after deglaciation). The lines inscribed with italic numbers represent terminal (years 1992 and 1927) and side (year 1850) moraines. Drawing not exactly to scale.

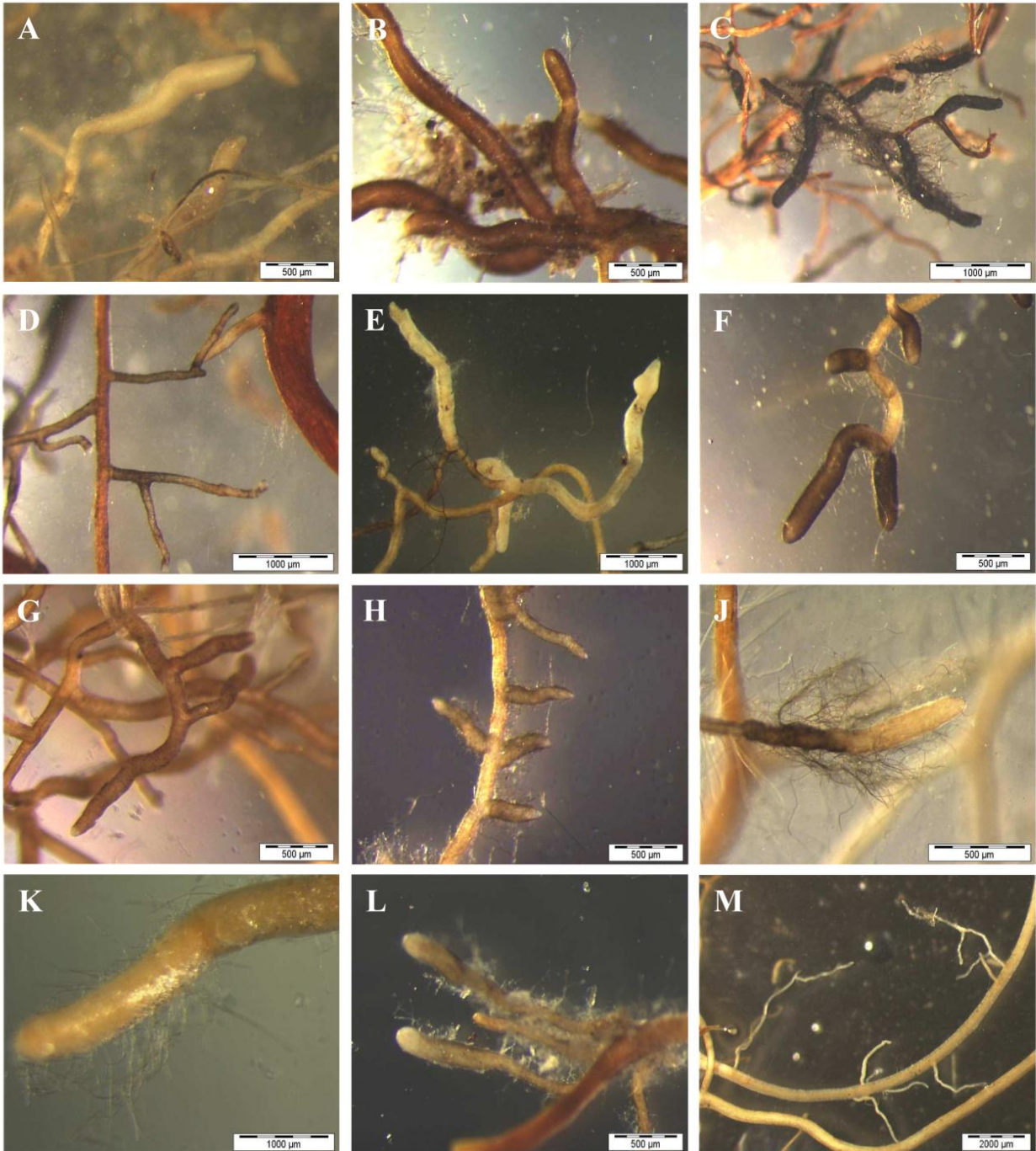


Figure S2

The morphotypes of ectomycorrhizal fungi associated with fine roots of *Salix helvetica*. (A-B) *Tomentella* spp.; (C) *Cenococcum geophilum*; (D) *Phialocephala fortinii*; (E) *Cortinarius casimiri*; (F) *Meliniomyces bicolor*; (G) *Russula emetica*; (H) *Inocybe lacera*; (J) *Peziza badia*; (K) *Hebeloma subconcolor*; (L) *Laccaria pumila*; (M) non-ectomycorrhizal fine roots. For detailed description of the morphotypes see Table S3.

		STANDARDS												Concentration (picomol/well)
		Soil 1	Soil 2	Soil 3	Soil 4	Soil 5	Soil 6	Soil 1	Soil 2	Soil 3	Soil 4	Soil 5	Soil 6	
		1	2	3	4	5	6	7	8	9	10	11	12	
SAMPLES	A													0
	Buffer 50 µl													100
	Soil suspension 50 µl													200
	Substrate 100 µl													500
BLANKS	E													800
	Buffer 50 µl													1000
	Soil suspension 50 µl													1200
	Water 100 µl													1500
	F													
	G													
	H													

Figure S3

Schematic representation of a microplate design used for all soil enzymatic activity assays. Six different soils were analysed on one microplate; wells with samples, respective blanks and standards are indicated.

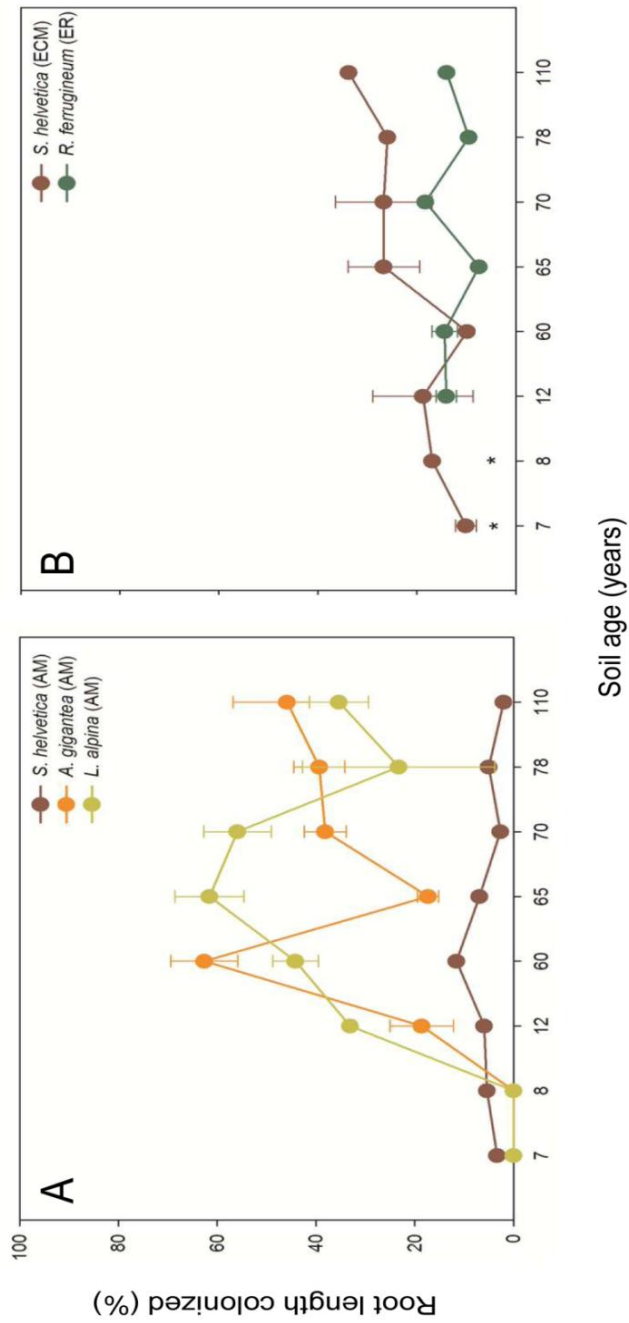
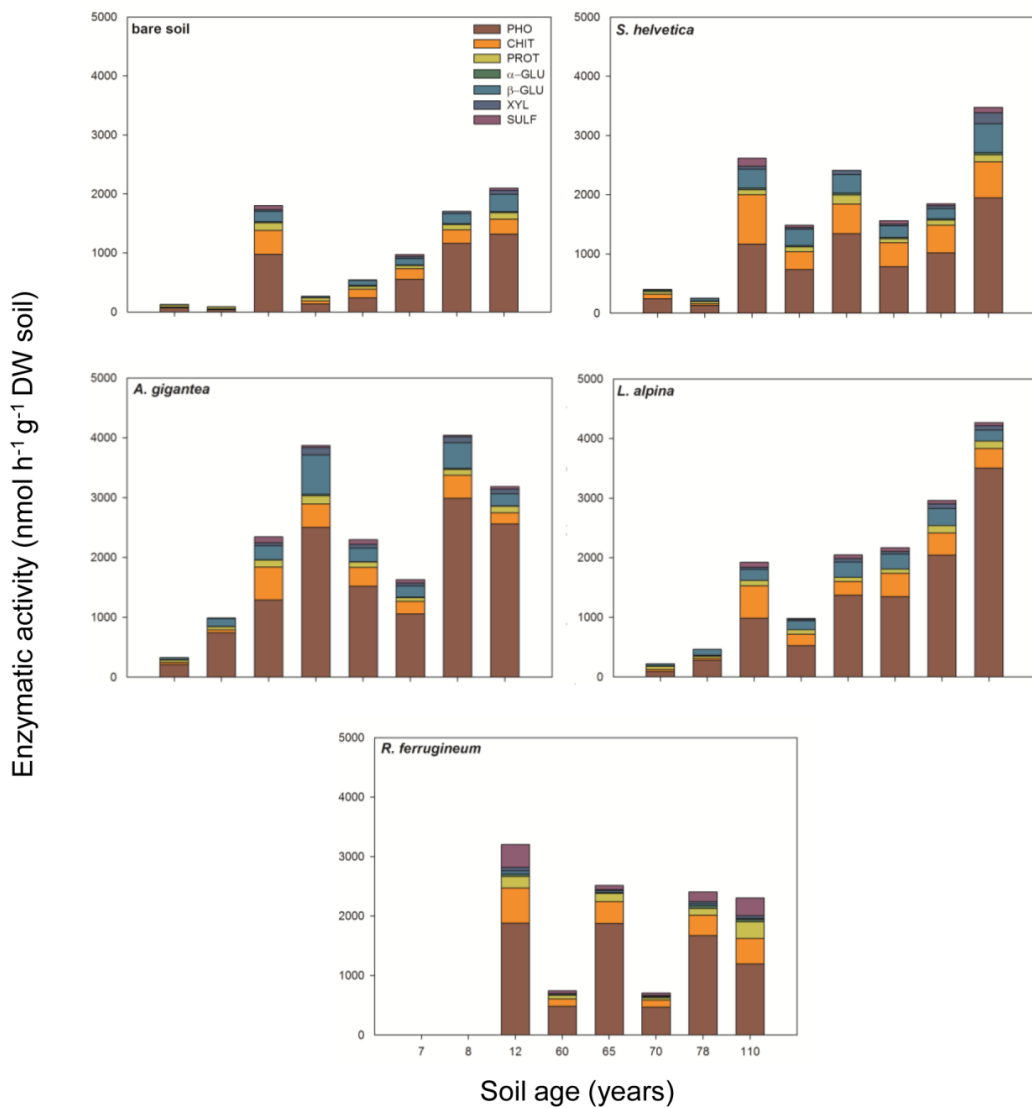


Figure S4

Microscopically estimated percentage of the root length colonized by arbuscular mycorrhizal (A) or ectomycorrhizal and ericoid (B) fungi performed for investigated plant species distributed along soil development gradient. *Salix helvetica* was a dual-mycorrhizal colonized plant (A-B); assessment of colonization by ectomycorrhizal fungi required no staining. The values represent the mean of calculations from multiple microscopic slides (n=2 through 5, ±SE). Abbreviations: plant identity – (*S. helvetica*), *Salix helvetica*; (*A. gigantea*), *Agrostis gigantea*; (*L. alpina*), *Leucanthemopsis alpina*; (*R. ferrugineum*), *Rhododendron ferrugineum*; mycorrhizal status – (AM) arbuscular mycorrhiza; (ECM) ectomycorrhiza; (ER) ericoid mycorrhiza. Details of non-vital staining procedure facilitating visualization of the mycorrhizal structures in the roots are presented in Table S2.

**Figure S5**

Enzymatic activity of bare and rhizosphere soils investigated in this study. Stacked bars show activity of the different enzymes per sampling site. The values are means (n=3, technical replicates), however for graph clarity, standard errors not indicated on the graph. Abbreviations: plant identity – (*S. helvetica*), *Salix helvetica*; (*A. gigantea*), *Agrostis gigantea*; (*L. alpina*), *Leucanthemopsis alpina*; (*R. ferrugineum*), *Rhododendron ferrugineum*; enzymes – (PHO) phosphatase; (CHIT) chitinase; (PROT) protease; (α-GLU) α- glucosidase; (β-GLU) β-glucosidase; (XYL) xylosidase; (SULF) sulfatase.

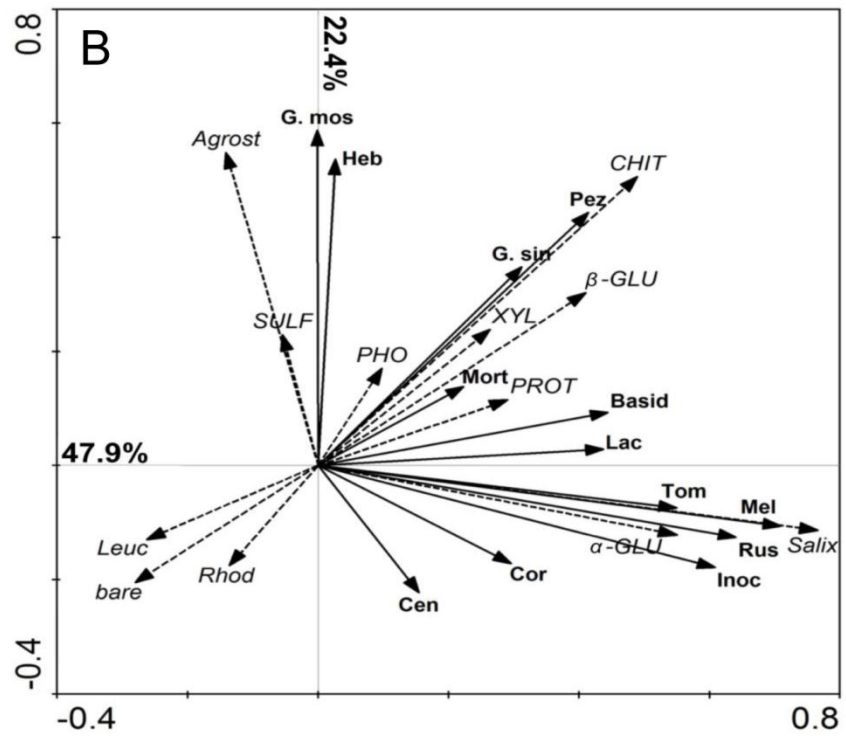
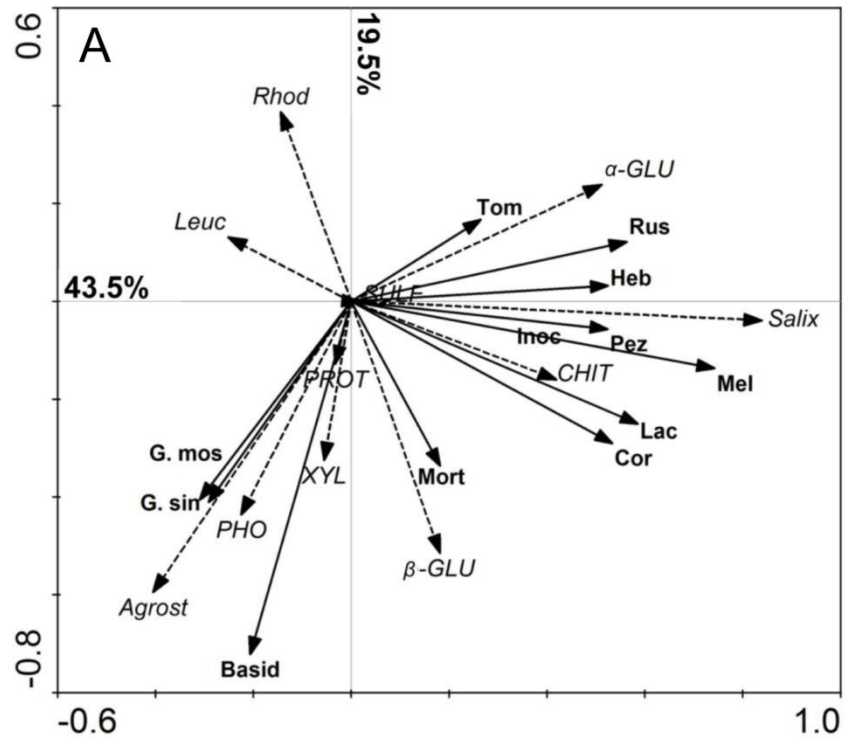


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Figure S6

Redundancy analysis ordination diagrams showing dependencies between plant identity (dataset 1, solid lines, bold regular labels) and soil enzymatic activity and abundance of fungi (dataset 2, dashed lines, italic labels) detected in the roots (A) and rhizosphere soils (B). Monte Carlo permutation test carried out for all four canonical axes confirmed the significance of the relationship between investigated datasets in graph A ($F=1.870$, $p=0.0133$), but not in graph B ($F=1.309$, $p=0.1600$). Abbreviations: plant identity – (Salix), *Salix helvetica*; (Agrost), *Agrostis gigantea*; (Leuc), *Leucanthemopsis alpina*; (Rhod), *Rhododendron ferrugineum*; (soil), bare soil; enzymes – (PHO) phosphatase; (CHIT) chitinase; (PROT) protease; (α -GLU) α -glucosidase; (β -GLU) β -glucosidase; (XYL) xylosidase; (SULF) sulfatase; fungus identity – (Cen) *Cenococcum geophilum*; (Cor) *Cortinarius casimiri*; (Heb) *Hebeloma subconcolor*; (Inoc) *Inocybe lacera*; (Lac) *Laccaria pumila*; (Rus) *Russula emetica*; (Tom) *Tomentella* spp.; (G. mos) *Glomus mosseae*; (G. sin) *Glomus sinuosum*; (Mel) *Meliniomyces bicolor*; (Pez) *Peziza badia*; (Basid) Uncultured Basidiomycota; (Mort) Uncultured *Mortierella* spp.

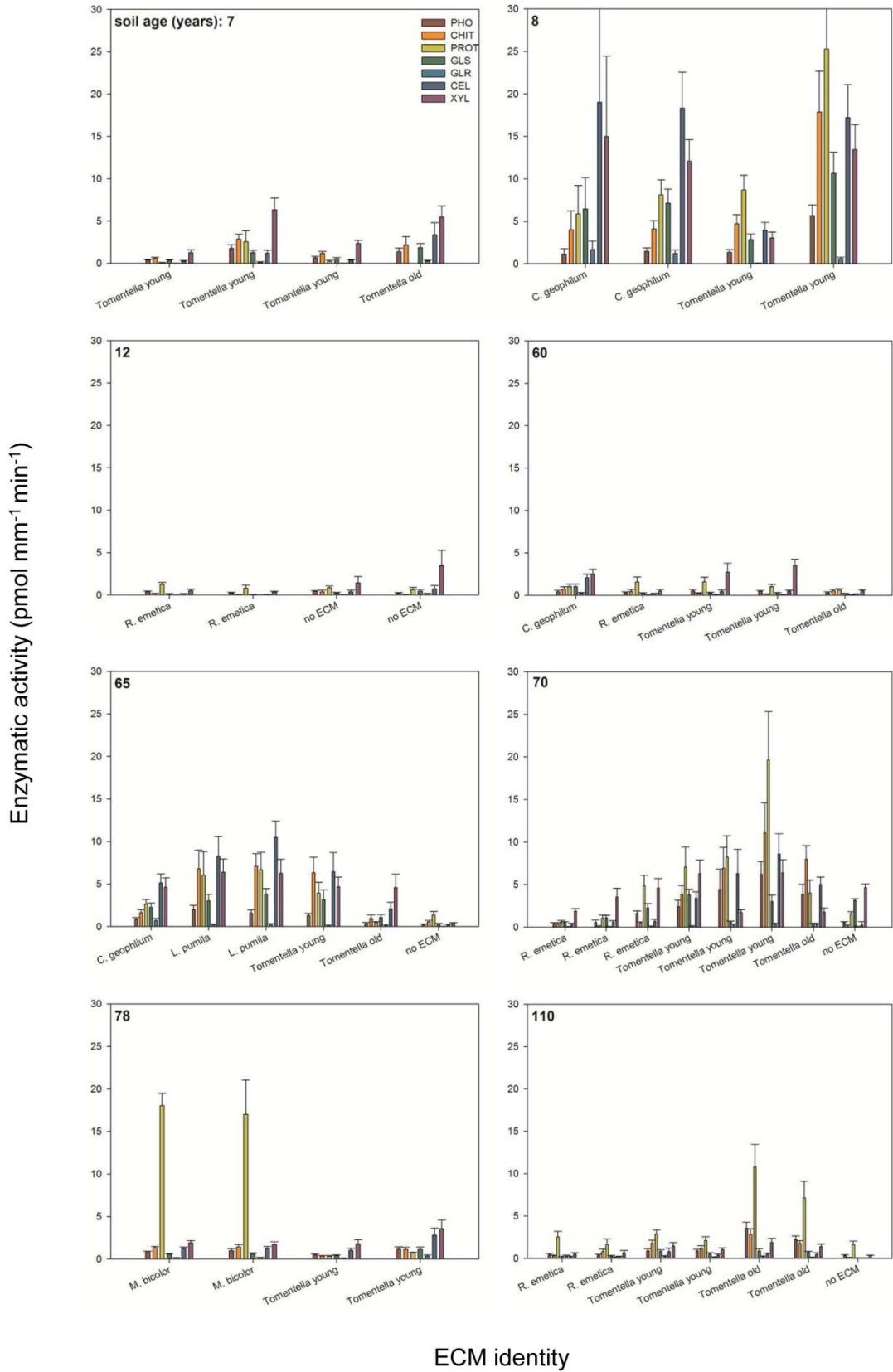


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Figure S7

Enzymatic activity of individual ectomycorrhizal root tips. The values are means (n=7, +SE). Ectomycorrhizal root tips excised from freshly harvested *Salix helvetica* plants were subjected to the enzymatic activity measurements within 3 h after excizing. Plants of *S. helvetica* from sites (in the close vicinity of sites selected for study presented in this work, see Table S1) and distributed along soil development gradient (bold numbers in the upper left corner of the graph represent soil age) were excavated carefully with intact root system in late summer 2009, transferred to a plastic pot (20 cm in diameter; 20 cm deep) and grown in the growth chamber (Photoperiod 16h; Photon Flux Density $175.5 \mu\text{mol m}^{-2} \text{s}^{-1}$, day/night: temperature (°C) 20/18, humidity 70%) for 3 months. Thereafter, each plant was harvested separately, roots prepared and subjected to enzymatic activity assays following optimized protocol (Pritsch et al., 2011). Abbreviations: enzymes – (PHO) phosphatase; (CHIT) chitinase; (PROT) protease; (GLS) β -glucosidase; (GLR) β -glucuronidase; (CEL) cellobiohydrolase; (XYL) xylosidase; fungus identity – (*C. geophilum*) *Cenococcum geophilum*; (*L. pumila*) *Laccaria pumila*; (*R. emetica*) *Russula emetica*; (*M. bicolor*) *Meliniomyces bicolor*; (no ECM) non-ectomycorrhizal root tips.

**Chapter 3: Fungal inoculum and growth medium determinantes the
composition of willow-associated fungal communities in a rhizobox
experiment**

Abstract

Mycorrhizal fungi are involved in the uptake of essential nutrients such as nitrogen (N) and phosphorus (P) from the soil, and their further transfer to the host plant. However, the environmental factors which affect the structure and function of fungal communities for the host plant are still poorly understood. In this study, we inoculated alpine willow species, *Salix helvetica* with indigenous mycorrhizal fungi in a rhizobox experiment. These communities of indigenous fungi (originating from the soil deglaciated 16 and 116 years ago) were introduced to the growth media of two different carbon (C) content (low C and high C). The experiment aimed to imitate different stages of ecosystem development. We hypothesized that: i) the fungal inoculum and the growth medium will affect the composition and the abundance of the mycorrhizal fungal communities associated both with the rhizosphere soil and roots of willows, ii) the differences in fungal communities will mirror growth and content of N, P and ^{33}P of the willows, and iii) mycorrhizal fungi will be able to dissolve ^{33}P -labeled apatite and transfer ^{33}P to the willows. Our experiment demonstrated that: i) both the fungal inoculum and the growth medium influenced the composition and abundance of the fungal communities associated with willow. The communities established from old inoculum (sampled from the site deglaciated approximately 116 years ago) and combined with high C growth medium presented the highest abundance of ectomycorrhizal and arbuscular mycorrhizal fungi; ii) the differences in the fungal community composition translated into the differences in uptake of N but not P by the willows; iii) in spite of large differences in fungal community compositions, no large difference either in willow P content nor ^{33}P transfer from ^{33}P -labeled apatite (upon its dissolution), was observed. The results of this experiment suggest that the composition of the fungal communities was strongly affected by the content of C in the growth medium. However, the direct influence of the composition of fungal communities on the plant growth and nutrition remains unclear.

Keywords: fungal community, community assemblage, environmental factors, dissolution, ectomycorrhiza, *Salix helvetica*, rhizobox, qPCR, ^{33}P -labeled apatite

Introduction

Abiotic and biotic factors were reported to influence the composition of arbuscular mycorrhizal (AM) and ectomycorrhizal (ECM) fungal communities. Arbuscular mycorrhizal fungi (AMF) were found affected by soil texture, total P concentration, pH and the identity of the host plant (Bever et al., 1996; Dumbrell et al., 2010; Jansa et al., 2009; Mathimaran et al., 2005). In case of ECM, altitude, soil pH, concentration of the total C and P, C:N ratio and identity of the host plant were postulated to be the most important factors shaping ECM community composition (Bahram et al., 2012; Fujimura and Egger, 2012; Navarro-Ródenas et al., 2012).

Impact of AM and ECM associations on the plant growth parameters and nutrition had been investigated since at least a century (Frank, 1885). Over this time, mycorrhizal fungi were repeatedly confirmed to enhance the transfer of nitrogen (N) and phosphorus (P) from the soil to the host plant (Smith and Read, 2008), which often resulted into a better plant growth.

Several mechanisms of nutrients uptake were described for AMF and ECM. For example, AMF were claimed to capture N and P as a simple organic (e.g., amino acids) or inorganic (e.g., ions) forms (Bücking and Shachar-Hill, 2005; Hawkins et al., 2000), most likely released upon the hydrolysis of soil organic matter (SOM) by other soil microorganisms. ECM fungi can access nutrients stored in SOM and in minerals by releasing in their extracellular medium, respectively, hydrolytic enzymes or low molecular weight organic acids (LMWOA) and protons (Casarin et al., 2004; Finlay, 2008; Wallander and Wickman, 1999). These mechanisms, however, can be regulated internally, by the fungal identity (Pritsch and Garbaye, 2011) or externally, by the environmental conditions (e.g., ash amendment (Mahmood et al., 2003)). For example, some ECM fungi hydrolyze SOM more readily (e.g., proteins, *Cenococcum* sp., *Amanita* spp., *Paxillus* spp.) (Abuzinadah and Read, 1986), whereas other were found to efficiently enhance mineral dissolution¹ (e.g., *Suillus* spp.) (Landeweert et al., 2001; Wallander and Wickman, 1999; Wallander et al., 1997b). The dependence of fungal functioning from the fungal identity and environmental conditions is a complex problem invoking numerous questions. For example, if the communities of

¹ 'Dissolution' is defined here as a breakdown of a mineral crystals (i.e., ³³P-labeled apatite) into individual ions (i.e., ³³P) (Banfield et al., 1999). Dissolution conducted in the experimental system was termed as dissolution *in vitro*, whereas dissolution in plant-mycorrhiza system was termed as biodissolution (see ***Dissolution of ³³P-labeled apatite in vitro*** and ***Biodissolution of ³³P-labeled apatite*** in the results section).

mycorrhizal fungi present adaptation to dramatically changed environmental conditions, what will happen when the fungal community will be transplanted from one ecosystem to another of contrasting properties (e.g., low vs. high nutrients concentration)? Logically, it can be assumed that fungal communities exposed to similar conditions as in their native soil, will be more fit than those exposed to contrasting conditions? Second, how or if the adaptation to a new conditions will affect the composition of the mycorrhizal fungal community? Finally, will this adaptation affect the growth or nutrition of the host plant? Those issues remain still poorly understood.

In order to answer some of these questions, we designed a rhizobox experiment with *Salix helvetica* (Swiss willow) (Lauber et al., 2012) inoculated with two unsterilized soils collected at the glacier forefield (from the sites deglaciated approximately 16 and 116 years ago, for young and old fungal inoculum, respectively). These soils were further combined with two growth media, mimicking two different environments (low and high carbon content). We aimed to disentangle, whether the composition of mycorrhizal fungal communities was more affected by the source of the fungal inoculum than the environment. Second, we aimed to interlink the composition of the mycorrhizal fungal communities with willow nutrition. Thus, we measured the uptake of N and P by the willows from unlabeled nutrient source. Additionally, using isotopic labeling, we aimed to assess biodissolution of ^{33}P -labeled apatite by mycorrhizal fungal communities. We hypothesized that: i) the fungal inoculum and the growth medium will affect the composition and abundance of mycorrhizal fungal communities associated with willows; in turn, some fungi from a specific habitats will prefer a growth medium with properties similar to their native soil; ii) the differences in fungal communities will mirror growth and content of N and P of the willows, especially when the communities will be best adapted to the environmental conditions; iii) mycorrhizal communities will be able to dissolve ^{33}P -labeled apatite and transfer ^{33}P to the host plant.

Materials and methods

Origin and preparation of the substrates and fungal inocula used in the experiment

All substrates used in this study (i.e., *Salix helvetica* propagation procedure and the experiment) consisted of the sand and the soil organic layer collected from the vicinity of the Damma glacier forefield (sand: N46°39.032' E8°30.968'; soil organic layer: N46° 38.455' E8°28.707'). According to FAO (FAO, 2006), sand was defined as a very coarse.

Sand was sieved at 2 mm, acid-washed (1% HCl) and sterilized by heating (150°C, 48h). The soil organic layer was sieved at 5 mm and sterilized by γ -irradiation through applying a dose of 25-75kGy with ^{60}Co source (LEONI Studer Hard AG, Däniken, Switzerland). Selected chemical properties (Table 1) were measured for the sand and soil organic layer. The soil organic layer was mixed with the sand in the ratio 1:100 (low C growth medium) and 1:2.5 (high C growth medium) to mimic young (approximately 8 to 12 years after deglaciation) and old (approximately 111 to 117 years after deglaciation) soil development stage.

The fungal inocula were collected in the vicinity of *Salix helvetica* plants at the Damma glacier forefield in late June 2010. Two collection points, differing in age after deglaciation were selected: i) young, deglaciated approximately 12 years ago (N 46°38.208' E 8°27.368', elevation 2051 m) and ii) old, deglaciated approximately 116 years ago (N 46°38.399' E 8°27.912', elevation 1962 m). The fungal inocula were collected to plastic bags, sieved at 5 mm upon the arrival to the laboratory and stored at +4°C for five months. The inocula were subjected to nutrient and molecular analyses (Table 1, Fig. S1).

Experiment duration and conditions

All preparation steps preceding the experiment and the experiment itself lasted altogether 4.5 month (Fig. 1). The willow propagation was conducted over 1.5 month (see ***Vegetative propagation of Salix helvetica*** for details). Then two willow seedlings were transplanted and established in each rhizobox for 2 months (Figs. 1 and 2) in the presence of indigeneous fungal inocula. After this time, ^{33}P -labeled apatite was added to the nutrient compartment of the rhizobox (Fig. 2). Plants were harvested 30 days after introduction of the ^{33}P -labeled apatite to the nutrient compartment (Fig. 1).

The experiment was conducted in the green house under following conditions: temperature (°C) 18/16 (day/night, respectively), relative aerial humidity 80%, 16h photoperiod, natural and incandescent light of $170.7 \mu\text{mol photon m}^{-2} \text{s}^{-1}$. Rhizoboxes were watered daily with

approximately 60 mL of distilled water, avoiding water leakage. Water was poured to all rhizobox compartments to assure comparable development of the microbial communities in all the compartments.

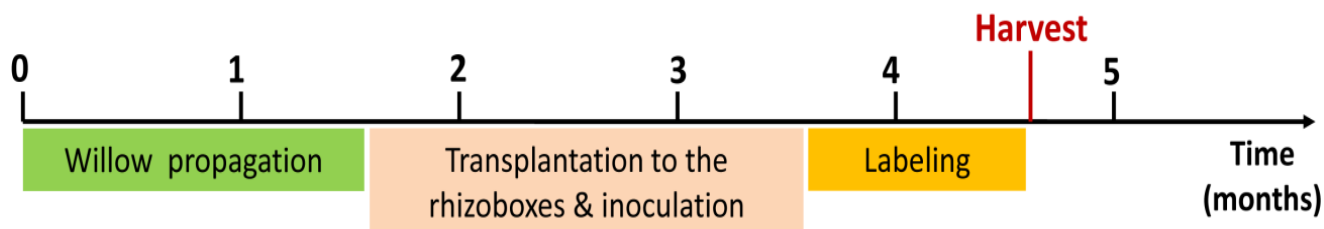


Figure 1

The scheme outlining main steps in experiment preparation and set-up.

Table 1

Selected chemical properties of the substrates (i.e., sand and soil organic layer) and fungal inocula used in the experiment. The values are single measurements (C_{tot} , N_{tot} and P_{tot} for sand and soil organic layer) or averages of three analytical measurement (\pm SE).

Substrate	pH (H ₂ O)	Analytical replicates	C_{tot}	N_{tot}	P_{tot}
			(g kg ⁻¹)		(mg kg ⁻¹)
Sand	6.2 (\pm 0.01)	n=1	0.007	nd	37.0
Soil organic layer	4.3 (\pm 0.04)		106.8	0.5	367.3
Fungal inoculum young	5.7 (\pm 0.09)	n=3	0.3 (\pm 0.15)	0.03 (\pm 0.004)	212.4 (\pm 1.13)
Fungal inoculum old	4.9 (\pm 0.09)		5.0 (\pm 0.12)	0.4 (\pm 0.01)	277.3 (\pm 2.07)

(nd) not detected (value below the detection limit).

Soil pH was measured in suspensions of the soil in 0.01 M CaCl₂ (1:2.5, w:v), using a Corning 125 pH-meter (Corning B.V. Life Sciences, Amsterdam, The Netherlands). Methods employed for the element concentration measurement described in detail in **Chemical analyses of plant and soil material** section.

The rhizobox construction

The experiment was conducted in 16 rhizoboxes, each of them made of three compartments: a plant, a buffer, and a nutrient compartment (Fig. 2). The plant compartment was filled with total amount of approximately 450 g of the growth media. This consisted of about 420 g of either low or high C growth medium, and approximately 30 g of the fungal inoculum (either young or old). The fungal inoculum was deposited in the vicinity of the roots (see **Inoculation procedure** for details). A nylon mesh (30 μm mesh size, Sefar AG, Heiden, Switzerland), penetrable for mycorrhizal fungal hyphae but acting as a barrier for the roots, was installed to separate the plant from the buffer compartment. The buffer compartment contained approximately 300 g of either low or high C growth medium, and was separated from the nutrient compartment by a polyamide mesh (PA 6, 1 mm mesh size, Sefar AG, Heiden, Switzerland). The nutrient compartment was filled with approximately 300 g of low C growth medium for all treatments and received 0.5 mL of ^{33}P -labeled apatite suspension (see **Labeling procedure** for details).

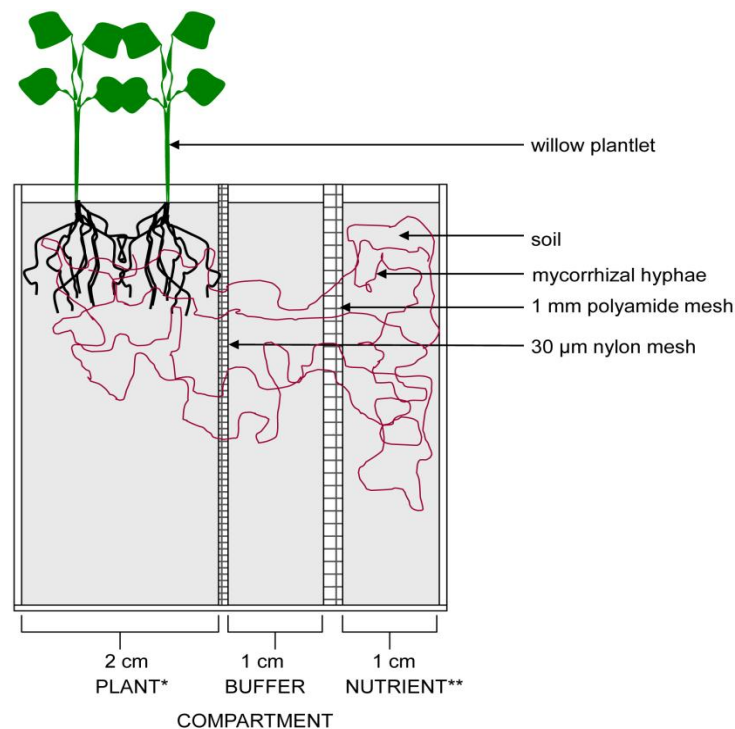


Figure 2

Schematic representation of the individual rhizobox.

(*) fungal inoculum introduced to the plant compartment; (**) nutrient compartment received ^{33}P -labeled apatite as a source of P for mycorrhizal willows.

Experimental design

The experiment had four treatments: two growth media with either low or high C content (introduced to the plant and buffer compartment), were combined with either young or old fungal inoculum (introduced to the plant compartment) (Fig. 3). Each treatment had four replicates.

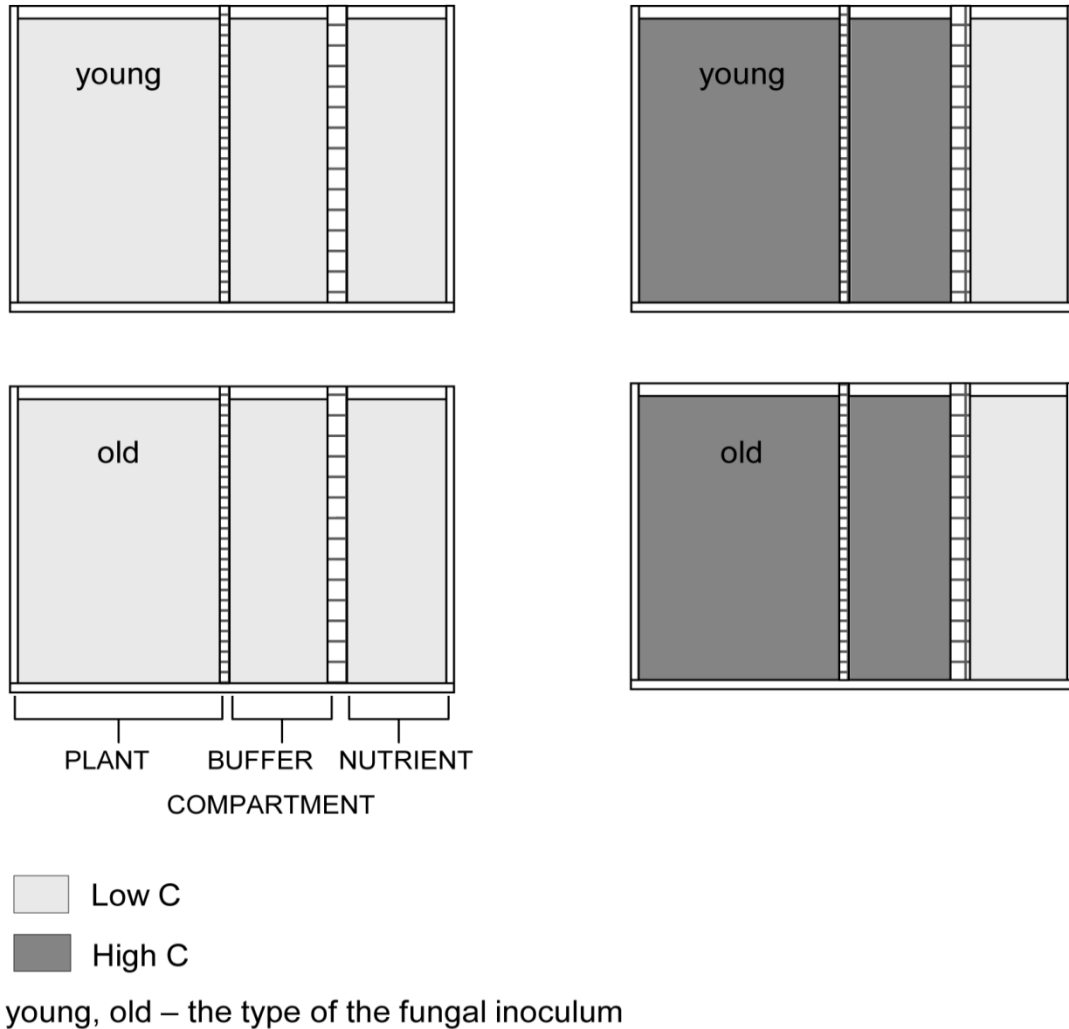


Figure 3

Schematic representation of the experimental treatments. Light and deep gray color represents growth media of low or high C content, respectively. Labels: young and old represent the type of the fungal inoculum (see **Origin and preparation of the substrates and fungal inocula used in the experiment** for details).

Vegetative propagation of *Salix helvetica*

Salix helvetica was propagated vegetatively from stem cuttings. The stems were collected from the field in September 2010 (N46°38.347' E8°27.801, elevation 1982 m), leaves were peeled off the stem (Fig. 4A) and stored at +4°C in the dark. All cuttings were prepared only from main axis of each stem. They were approximately 5 to 6 cm long, had some 0.3 to 0.5 cm in diameter and a fresh weight of about 1.0 to 1.5 g. Each cutting had at least two healthy buds. Under the flow cabinet, cuttings were surface sterilized by dipping them (in batch of 10) in 50 mL of freshly prepared 6% H₂O₂ (10 min, room temperature). Thereafter, the cuttings were washed (in batch of 10) in 50 mL of sterile distilled water (10 min, room temperature). Each cutting was then aseptically transferred to a 50 mL Falcon tube (SPL Lifesciences, Gyeonggi-do, Korea) prefilled with 25 mL of 0.8% water agar (GibcoBRL, 10695-039). Each tube was tightly closed with Parafilm and transferred to a growth chamber for three weeks under the conditions: day/night temperature (°C) 22/20, relative aerial humidity 60%, 16h photoperiod, incandescent light of 175.5 μmol photon m⁻² s⁻¹. After this time, the plantlets which developed roots and leaves (Fig. 4B) were transferred to 45 g of sterile growth medium with low C content (see ***Origin and preparation of the substrates and fungal inocula used in the experiment*** for details). Each plantlet² was watered daily with 4 mL of sterile distilled water. During the first 4 days after transfer to the growth medium, the plantlets were kept under transparent gas-permeable chamber (Fig. 4C) to minimize evapotranspiration and assure adaptation of the plantlets to a new conditions. After three weeks, healthy plantlets were selected and transferred to the rhizobox experiment.

² 'Plantlet' is defined in entire doctoral thesis as a young plant produced vegetatively from a stem cutting (Hartmann et al., 2010).

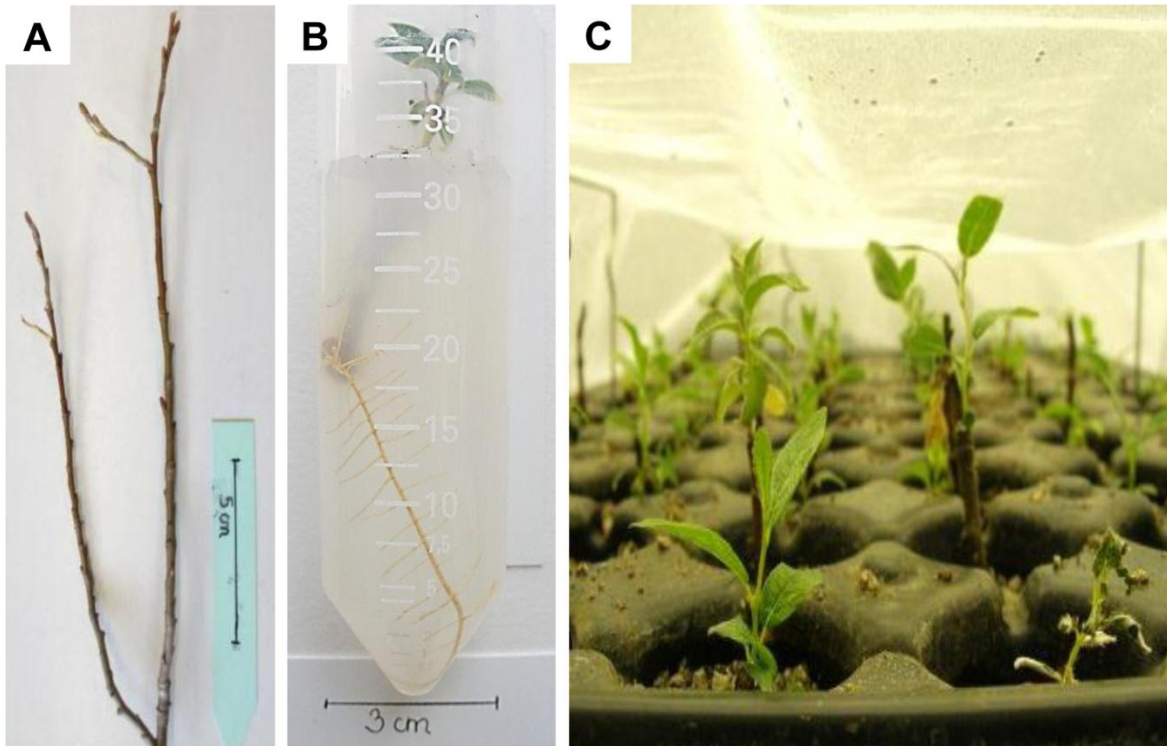


Figure 4

Vegetative propagation of *Salix helvetica* – procedural outlines. (A) One- to-three year-old branch of *S. helvetica* with well-developed buds; (B) Three-week old cutting of *S. helvetica* in 0.8% sterile water agar; (C) Seven-week-old plantlets of *S. helvetica* grown in a transparent chamber in a sterilized low C growth medium.

Inoculation procedure

Inoculation was performed as follows: approximately 300 g of the growth media – either low or high C content – was introduced and distributed evenly in the plant compartment (Fig. 2). Next, the compartment received 30 g of the fungal inoculum (either young or old) which was distributed in a 0.5 cm thick layer. The roots of two plantlets of *S. helvetica* introduced in the plant compartment had direct contact with the fungal inoculum. After planting, 120 g of the appropriate growth media (either low or high C, depend on the treatment, Fig. 3) was added to stabilize the plantlets.

Hydrothermal synthesis of the ³³P-labeled apatite

The hydrothermal synthesis was carried out in a golden (Au) pipe, 7×45 mm (Fig. 5A). This was closed on one side using electric arch welding equipment, filled with 155 mg of amorphous Ca₃(PO₄)₂. Then, 144 μL of water with carrier free ³³P (6 mCi, Hartmann Analytic, Braunschweig, Germany) was added and overlaid with 536 mg of Ca₃(PO₄)₂. The pipe was then closed by welding and the tightness of the capsule (Fig. 5A) was tested in an acetone bath. The capsule was then subjected to pressure of 10.13 MPa in a heated pressure chamber, which was then slowly brought to 650°C. Thereafter, the pressure was increased to 172.25 MPa over next 30 min and kept for the 9 following days. After turning off the instrument, the pressure was slowly released and the temperature decreased to 50°C, and the capsule was then carefully removed from the heating block. The capsule was opened and subjected to heating at 110°C for 1.5 h. After that ³³P-labeled apatite was transferred to a porcelain mortar (Fig. 5B) and finely ground. The powder was transferred to 50 mL Falcon tube, washed 3 times in 40 mL distilled water and centrifuged for 15 min at 1700 g. ³³P activity in each wash and in ³³P-labeled apatite suspension (data not shown) was measured by liquid scintillation counting (see ***Liquid scintillation counting***, supplementary materials). The washed ³³P-labeled apatite powder was finally transferred to 2.0 mL Eppendorf tube and dried at 50°C in a vacuum centrifuge for 5 h at 20°C. Microscopic observations (Fig. S2), XRD analyses (Fig. S3) and dissolution experiments in inorganic (HCl and H₂SO₄) and organic (citric, oxalic and acetic) acids (Fig.10) were performed to characterize the apatite.

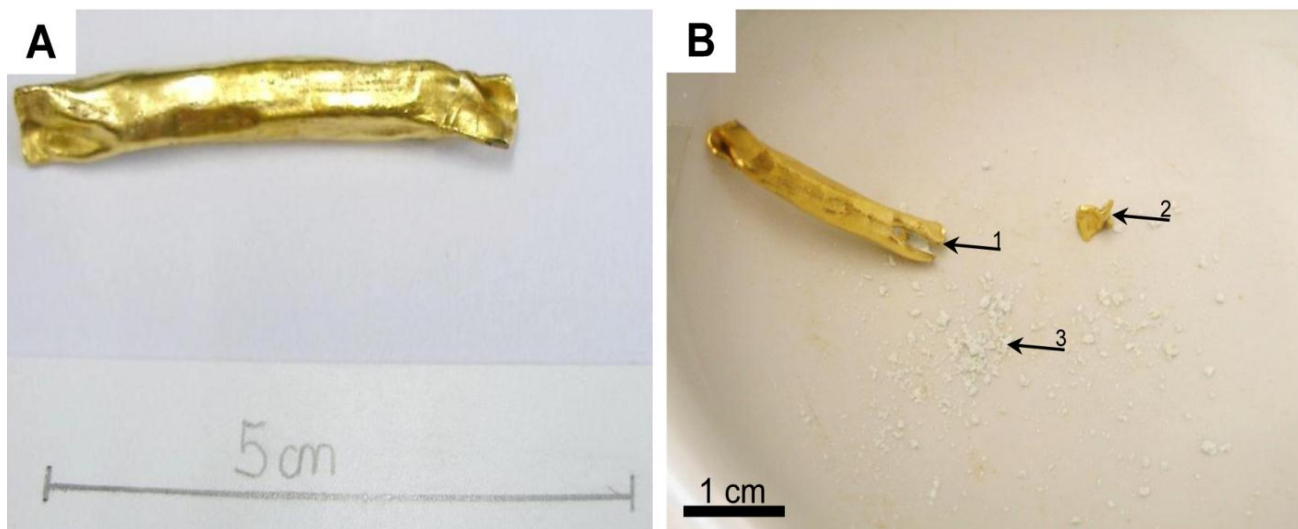


Figure 5

Golden capsule used for hydrothermal synthesis of ^{33}P -labeled apatite; (A) the capsule filled with the amorphous $\text{Ca}_3(\text{PO}_4)_2$ and carrier free ^{33}P before hydrothermal synthesis of the apatite; (B) the capsule after hydrothermal synthesis containing ^{33}P -labeled apatite; (1) the end of the capsule opened with a pincers, (2) cut end of the capsule, (3) ^{33}P -labeled apatite removed from the capsule.

Dissolution of ^{33}P -labeled apatite in vitro

The dissolution experiments in HCl , H_2SO_4 (inorganic acids), citric, oxalic and acetic acids (organic acids) were conducted as follow. Approximately 5 mg of washed ^{33}P -labeled apatite was weighted into 50 mL Falcon tube, combined with 40 mL of different dissolution promoters (water, 1M inorganic and organic acids), and incubated at room temperature upon shaking (2.3 Hz). The solution was sampled at regular intervals: 1, 3, 6, 24 and 48 h after application of the dissolution promoters. Five mL subsample was taken from each tube with a sterile syringe and passed through a 20 μm syringe-driven filter. ^{33}P activity of the solution was assessed by liquid scintillation counting using 1 mL of the filtrate from each sampling event and 5 mL of scintillation cocktail (UltimaGoldTM, PerkinElmer, Schwerzenbach, Switzerland). The scintillation counting rate of a channel having energy 0 to 220 keV was measured over 3 min (see ***Liquid scintillation counting***, supplementary materials). Initial pH of each dissolution promoter as well as pH of the filtrates after 48 h incubation (Fig. S4) was

monitored with Mettler Toledo SevenMulti pH-meter equipped with Metler Toledo inLab[®]Routine Pro electrode.

Labeling procedure

Five hundred mg of ³³P-labeled apatite was transferred to 50 mL Falcon tube, combined with 42 mL of distilled water and mixed vigorously by hand. Then half a mL of the suspension was introduced to the nutrient compartment of each rhizobox, which was prefilled with the layer of 200 g of the low C growth medium and over layered with another 100 g of the same growth medium (see ***The rhizobox construction*** for details). In order to monitor homogeneity of the ³³P in the suspension, aliquots of 100, 200, 250, 300, 400, and 600 µl of the solution were sampled during the procedure and

³³P activity was measured by liquid scintillation counting (data not shown).

Harvest and processing

Roots and shoots were processed as follows: roots were washed under tap water and dried between layers of paper. The leaves were separated from the wood (the wood of cutting was excluded for the analyzes). All samples were placed in the paper bags, dried at 65°C for 48h, weighed and subjected to bead-beating (2×30 sec, 46 Hz) to obtain a fine powder for further measurements of ³³P activity, contents of total elements such as phosphorus (P_{tot}), carbon (C_{tot}) and nitrogen (N_{tot}) in plant tissues and for the DNA extraction from the roots (see ***DNA extraction from the root and soil samples*** for details).

Soil from each compartment of the rhizobox was homogenized by mixing and representative samples of approximately 5 g had been freeze-dried, ground to a fine powder (4 min, 98 Hz) and subjected to the DNA extraction.

Chemical analyses of plant and soil material

Plant samples (leaves, wood and roots) were subjected to N_{tot} analyses by the dry combustion (NCS Analyzer, FlashEATM 1112 Series, Thermoelectronic Corporation). P_{tot} was extracted from plant samples after incineration (16 h, 550°C), dissolution of the ashes (1.0 ml of 65% HNO₃, 2 min, 250°C) and filtration of the extracts (0.2 µm nitrocellulose syringe-driven membranes). ³³P activity was measured in the extracts by liquid scintillation

counting (see **Liquid scintillation counting**, supplementary materials), while P_{tot} was measured by Inductively Coupled Plasma – Mass Spectrometry (Agilent 7500 C).

P_{tot} , plant-available (P_{av}) and water-extractable (P_{water}) P and ^{33}P activity in plant-available and water-extractable fractions ($^{33}\text{P}_{\text{av}}$, $^{33}\text{P}_{\text{water}}$) was measured in the growth media (from nutrient, buffer and plant compartment of the rhizobox). P_{tot} was extracted and measured as previously in the plant material. Plant-available P was extracted with ammonium acetate–EDTA solution with soil: extractant ratio 1:10 (w:v)(Stünzi, 2006). Water-extractable P was extracted with distilled water with soil : water ratio 1:1.6 (w:v).

Both extracts were filtered (0.2 μm nitrocellulose syringe-driven membranes) and P was measured by Inductively Coupled Plasma – Mass Spectrometry (Agilent 7500 C). ^{33}P activity in plant-available and water-extractable fraction was assessed by liquid scintillation counting (see **Liquid scintillation counting**, supplementary materials).

Microscopic observation of the ^{33}P -apatite

Small sample (<1 mg) of ground and washed ^{33}P -labeled apatite (see **Hydrothermal synthesis of the ^{33}P labeled apatite** for details) was placed in a drop of distilled water on a microscope slide. This was observed under visible and polarized light (Olympus AX70 microscope equipped with Olympus XC10 camera and assisted by analySIS pro software; Olympus Software Imagine Solutions[®]) in order to assess ^{33}P -labeled apatite crystallinity (Fig. S2). The amorphous $\text{Ca}_3(\text{PO}_4)_2$ was observed under identical conditions and served as a control (Fig. S2).

DNA extraction from the root and soil samples

Genomic DNA was extracted from the root samples and soils from the nutrient and plant compartments (Fig. 2) with Nucleo Spin Plant II[®] Kit (Macherey-Nagel, Oensingen, Switzerland) and MOBIO Power Soil[®] Kit (MOBIO, Carlsbad, USA), respectively, according to manufacturer's recommendations. In order to control the DNA extraction efficiency and potential inhibition of the qPCR reaction, each sample was spiked prior to the DNA extraction with internal DNA standard. This spike consisted of 20×10^9 copies of the APA 9 plasmid carrying a fragment of a cassava mosaic virus, GenBank accession number AJ42791(von Felten et al., 2010).

qPCR markers and qPCR cycling conditions

The abundance of ECM (i.e., *Cenococcum geophilum*, *Cortinarius casimiri*, *Hebeloma subconcolor*, *Inocybe lacera*, *Laccaria pumila*, *Russula emetica*, and *Tomentella* spp.), AMF (i.e., *Glomus sinuosum*, and *Glomus mosseae*) and fungi of uncertain trophic status (UTS, i.e., *Meliniomyces bicolor*, *Peziza badia*, Uncultured *Basidiomycota* and Uncultured *Mortierella* spp.) were assessed in roots and soils with taxon-specific qPCR markers (see **Primer design**, chapter 2, supplement). Individual qPCR reaction contained 0.18 µl of forward and reverse primer (25 µM), 0.04 µl of the hydrolysis probe (25 µM), 1.80 µl of the Roche Master Mix (Roche Diagnostics, Rotkreuz, Switzerland; Cat. No. 04736536001), and 4.55 µl Light Cycler water (Roche Diagnostics, Rotkreuz, Switzerland). All qPCR reactions were run on LightCycler 2.0 (Roche Diagnostics, Rotkreuz, Switzerland) according to the qPCR reaction cycling conditions presented previously (see **Primer design**, chapter 2, supplement).

Statistical analyses

Microsoft Excel 2010 was used to calculate the averages and standard errors of the data, whereas the graphs were prepared in SigmaPlot 11 (Systat Software Inc., San Jose, USA). The analysis of variance (two-way ANOVA) for the differences in plant and soil parameters (Table 2) in different treatments was tested with StatGraphics®Plus (Statistical Graphic Corporation).

The data matrix containing the abundance of the different fungi (in both the soils and roots), several soil properties and plant growth parameters were subjected to multivariate statistics using Canoco 4.5 (ter Braak and Šmilauer, 2002). Multivariate statistical analyses were conducted on a centralized and standardized dataset. Selection of Redundancy Analysis (RDA) was justified by a short length of the gradient (2.029, total inertia 0.917) in Detrended Correspondence Analysis (DCA). The statistical significance of the relationship between the treatments, fungal abundances, soil properties and plant growth parameters was evaluated using Monte Carlo permutation test (unrestricted permutation, reduced model, 9999 permutations).

Results

Until destructive sampling, all willows were well-developing and healthy with no visible signs of growth limitation.

1. Fungal communities as affected by fungal inocula in combination with different growth media

Fungal communities established in the experiment differed from those detected in the native fungal inocula (Fig. S1). Fungal communities differed also between the treatments containing young or old fungal inoculum and were significantly modulated by the type of the growth medium (low or high C) (Fig. 6). In general, the abundances of fungi from three investigated groups (ECM, AMF, UTS) were lower when any of the growth medium was combined with young fungal inoculum (except UTS in the soils and roots, Fig. 6). Moreover, root samples presented higher abundance of all investigated fungal groups as compared to the soil samples (Fig. 6).

1.1. The abundance of ECM

Inocybe lacera was the dominant ECM taxon in both the soil and roots. In the root samples, some other ECM were recorded but in generally much lower abundances than *I. lacera* (Fig. 6).

1.2. The abundance of AMF

No AMF were detected in the soil samples, whereas their presence was clearly confirmed in the willow roots (Fig. 6). *Glomus sinuosum* was detected in roots from all established treatments. *Glomus mosseae* presented lower abundance than *Glomus sinuosum* and was not present in the treatments with high content of C in the growth medium (Fig. 6).

1.3. The abundance of UTS

Peziza badia dominated in the soils, especially in the treatments where old fungal inoculum was applied (Fig. 6). In contrast, roots were abundantly colonized with Uncultured Basidiomycota especially in the treatments with low content of C in the growth medium (Fig. 6). Moreover, *Meliniomyces bicolor* was present where old inoculum was applied for both growth media (Fig. 6).

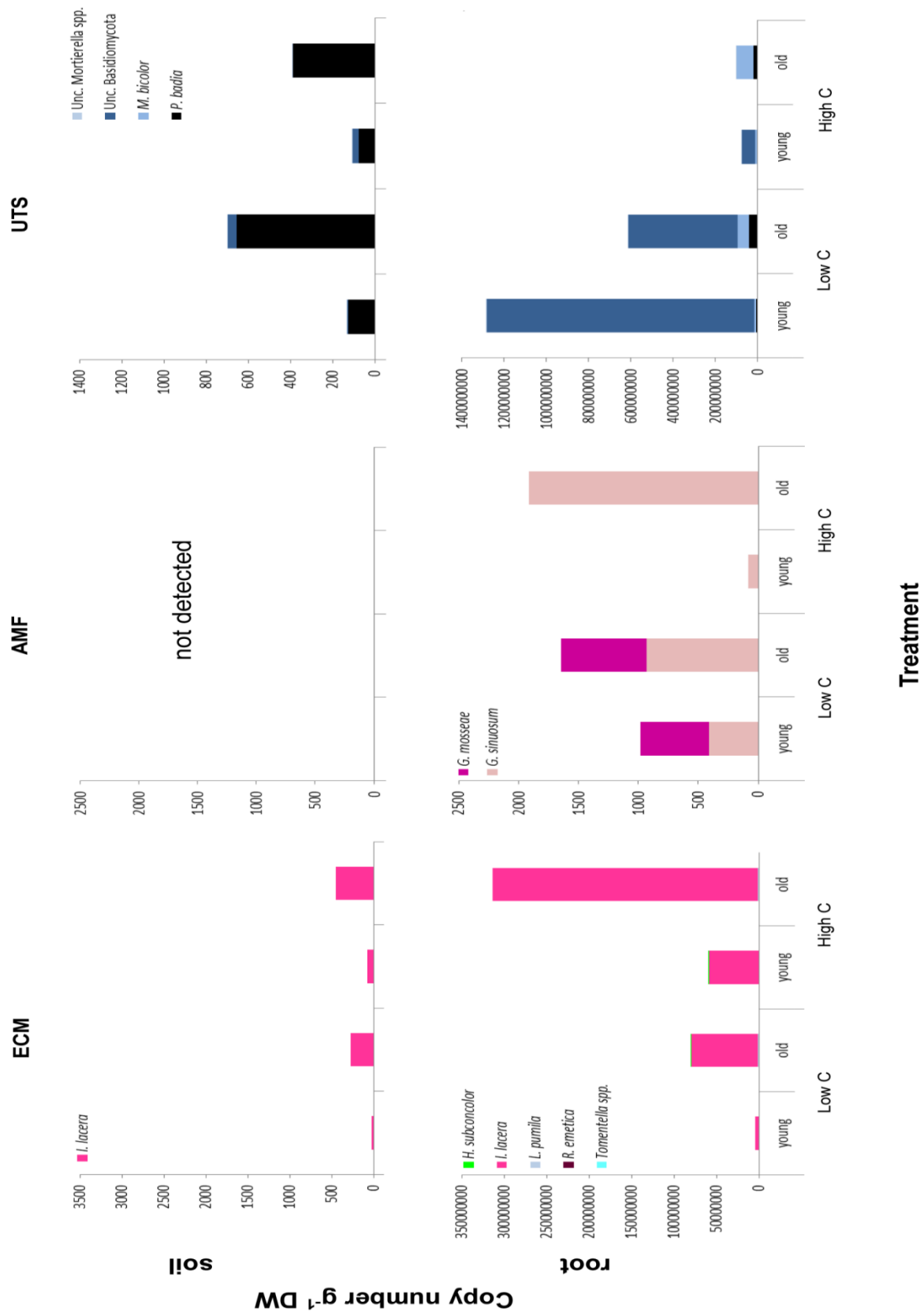


Figure caption on the following page

Figure 6

The abundance of different fungi (ectomycorrhizal, ECM; arbuscular mycorrhizal, AMF; and fungi of uncertain trophic status, UTS) in soils and roots of *Salix helvetica* grown in the rhizobox system, assessed with taxon-specific qPCR molecular markers. The results are presented for four different treatments. The values are means (n=4), for clarity of the graph, the SE bars were not included. Fungal identities – (*H. subconcolor*) *Hebeloma subconcolor*; (*I. lacera*) *Inocybe lacera*; (*L. pumila*) *Laccaria pumila*; (*R. emetica*) *Russula emetica*; (*G. mosseae*) *Glomus mosseae*; (*G. sinuosum*) *Glomus sinuosum*; (*M. bicolor*) *Meliniomyces bicolor*; (*P. badia*) *Peziza badia*; (*Unc. Basidiomycota*) Uncultured Basidiomycota; (*Unc. Mortierella spp.*) Uncultured *Mortierella* spp. Note the different scales for soils and roots.

2. Impact of the fungal inoculum and growth media on the fungal communities and willow growth parameters

Both factors, fungal inoculum and growth medium shaped the communities of indigenous soil fungi (Table 2). The type of fungal inoculum significantly affected the abundance of entire fungal communities as well as of the individual fungal taxa (Tables 2 and 3, Fig. 7). The abundance of ECM in roots was also affected by the fungal inoculum while UTS in roots were greatly affected by the growth medium (Table 2, Fig. 7).

The fungal inoculum influenced the abundances of *Inocybe lacera* (ECM) and *Peziza badia* (UTS), both for the soils and the roots. The abundances of *Meliniomyces bicolor* and uncultured Basidiomycota were significantly dependent from the applied fungal inoculum, but only in the root samples. In contrast, *Glomus mosseae* was the only taxon that was strongly affected by the type of the growth medium (Table 3, Fig. 7).

Willow growth remained unaffected by the growth medium or fungal inoculum (Table 2). The content of P, but only in the leaves, was influenced by the growth medium at the low significance level. In contrast, growth medium significantly influenced the plant N content, N content in the wood and in the leaves. The only parameter significantly affected by the fungal inoculum was N content in the plant leaves (Table 2). In spite of large differences in fungal communities established in this experiment, no large differences in ³³P transfer from ³³P-labeled apatite to willows was observed (Table 2, Fig. 9).

Table 2

P values of main factors (**G**rowth medium and **I**noculum) and their interaction (**G×I**) obtained from two-way analyses of variance (ANOVA) for different soil and plant parameters (n=4). Significance levels in ANOVA table: (nd) not detected; (ns) not significant $p \geq 0.05$; (*) $p < 0.05$; (**) $p < 0.01$ (***) $p < 0.001$. Abbreviation: (DW) dry weight.

Parameters	Main factors		Interaction
	Growth medium	Inoculum	G×I
ECM abundance (copy number g ⁻¹ DW soil)	ns	**	ns
AMF abundance (copy number g ⁻¹ DW soil)	nd		
UTS abundance (copy number g ⁻¹ DW soil)	ns	**	ns
ECM abundance (copy number g ⁻¹ DW root)	*	**	ns
AMF abundance (copy number g ⁻¹ DW root)	ns	*	ns
UTS abundance (copy number g ⁻¹ DW root)	***	ns	ns
Plant dry weight (g)	ns	ns	ns
Leaves dry weight (g)	*	ns	ns
Wood dry weight (g)	ns	ns	ns
Root dry weight (g)	ns	ns	ns
Root: shoot dry weight	ns	ns	ns
Plant P content (mg plant ⁻¹)	ns	ns	ns
Leaves P content (mg plant ⁻¹)	*	ns	ns
Wood P content (mg plant ⁻¹)	ns	ns	ns
Root P content (mg plant ⁻¹)	ns	ns	ns
P content root: shoot	ns	ns	ns
Plant N content (mg plant ⁻¹)	*	ns	ns
Leaves N content (mg plant ⁻¹)	***	**	ns
Wood N content (mg plant ⁻¹)	**	ns	ns
Root N content	ns	ns	ns
N content root: shoot	ns	ns	ns
Plant ³³ P content (Bq plant ⁻¹)	ns	ns	ns
³³ P content root: shoot	ns	ns	ns

Table 3

P values of main factors (**G**rowth medium and **I**noculum) and their interaction (**G×I**) obtained from two-way analyses of variance (ANOVA) for different fungi detected in soil and root samples (n=4). Significance levels in ANOVA table: (nd) not detected; (ns) not significant $p \geq 0.05$; (*) $p < 0.05$; (**) $p < 0.01$ (***) $p < 0.001$. Abbreviation: (DW) dry weight.

Fungal identity	Unit	Main factors		Interaction
		Growth medium	Inoculum	G×I
<i>H. subconcolor</i>	copy number g ⁻¹ DW soil	nd		
<i>I. lacera</i>		ns	**	ns
<i>L. pumila</i>		ns	ns	ns
<i>R. emetica</i>		nd		
<i>Tomentella</i> spp.		nd		
<i>G. mosseae</i>		nd		
<i>G. sinuosum</i>		nd		
<i>M. bicolor</i>		ns	ns	ns
<i>P. badia</i>		ns	**	ns
Unc. <i>Basidiomycota</i>		ns	ns	ns
Unc. <i>Mortierella</i> spp.		ns	ns	ns
<i>H. subconcolor</i>		copy number g ⁻¹ DW root	ns	ns
<i>I. lacera</i>	*		**	ns
<i>L. pumila</i>	ns		ns	ns
<i>R. emetica</i>	ns		ns	ns
<i>Tomentella</i> spp.	ns		ns	ns
<i>G. mosseae</i>	***		ns	ns
<i>G. sinuosum</i>	ns		ns	ns
<i>M. bicolor</i>	ns		***	ns
<i>P. badia</i>	ns		**	ns
Unc. <i>Basidiomycota</i>	ns		***	ns
Unc. <i>Mortierella</i> spp.	ns		ns	ns

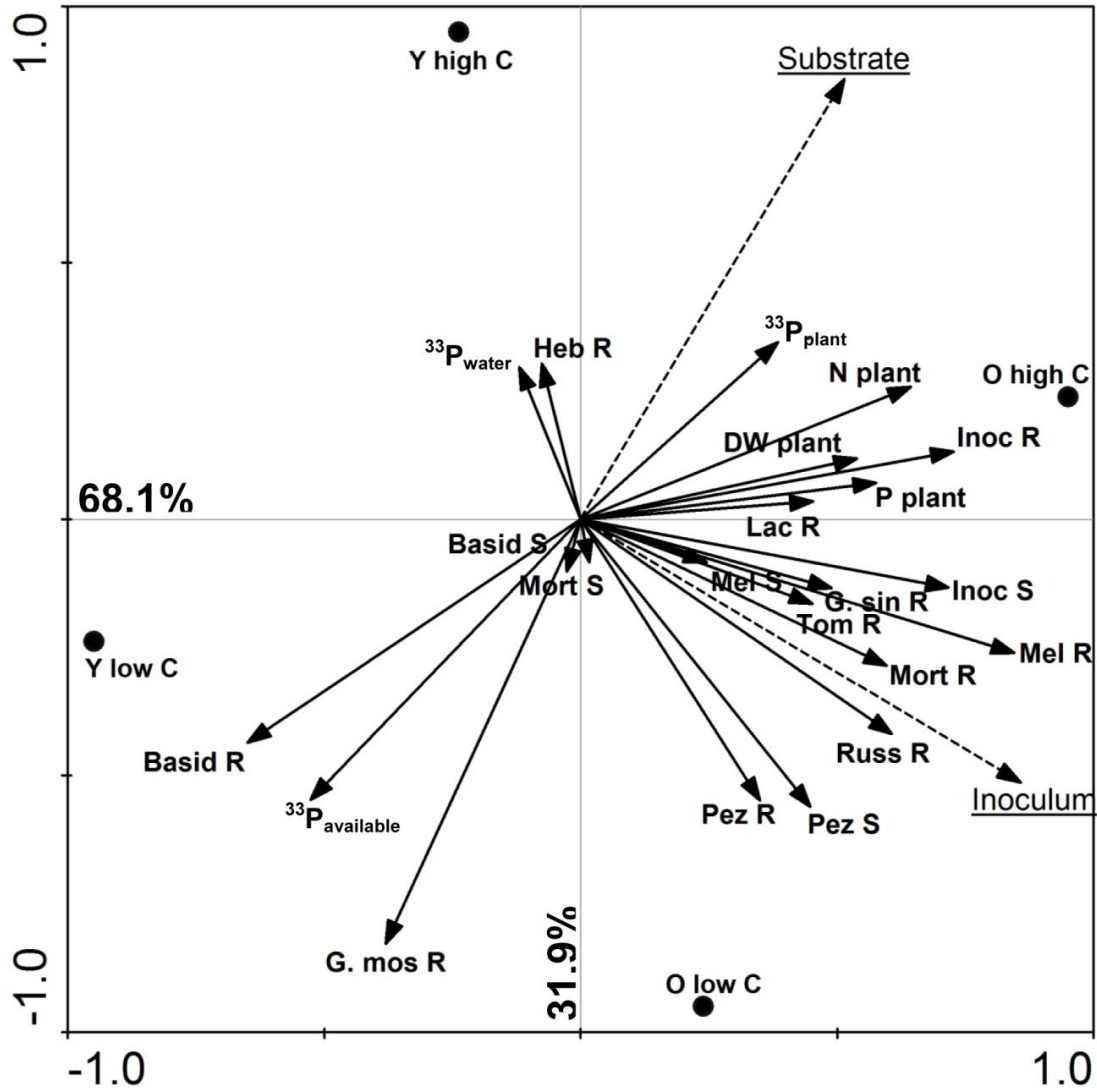


Figure 7

Redundancy analysis ordination diagram presenting dependencies between experimental treatments (fungal inoculum and the type of the growth medium, handled as explanatory variables) and a range of explained variables: ^{33}P activity soil, plant growth parameters (plant dry weight, ^{33}P , N and P content in plant), and the abundance of different fungi in soil (fungus name S) and roots (fungus name R). Monte Carlo permutation test carried out for two canonical axes confirmed the significance of the relationship between investigated datasets ($F=3.838$, $p=0.0001$). Abbreviations: treatments – (Y low C) treatment combining young fungal inoculum with low C growth medium; (O low C) treatment combining old fungal inoculum with low C growth medium; (Y high C) treatment combining young fungal inoculum with high C growth medium; (O high C) treatment combining old fungal inoculum with high C

growth medium; soil properties – ($^{33}\text{P}_{\text{water}}$) activity of ^{33}P in water-extractable phosphorus fraction of labeled soil; ($^{33}\text{P}_{\text{available}}$) activity of ^{33}P in plant-available phosphorus fraction of labeled soil; plant growth parameters – (DW plant) plant dry weight; (^{33}P plant) content of ^{33}P in plant; (N plant) content of nitrogen in plant; (P plant) content of phosphorus in plant; fungal identities – (Heb) *Hebeloma subconcolor*; (Inoc) *Inocybe lacera*; (Lac) *Laccaria pumila*; (Russ) *Russula emetica*; (Tom) *Tomentella* spp.; (G. mos) *Glomus mosseae*; (G. sin) *Glomus sinuosum*; (Mel) *Meliniomyces bicolor*; (Pez) *Peziza badia*; (Basid) Uncultured Basidiomycota; (Mort) Uncultured *Mortierella* spp.

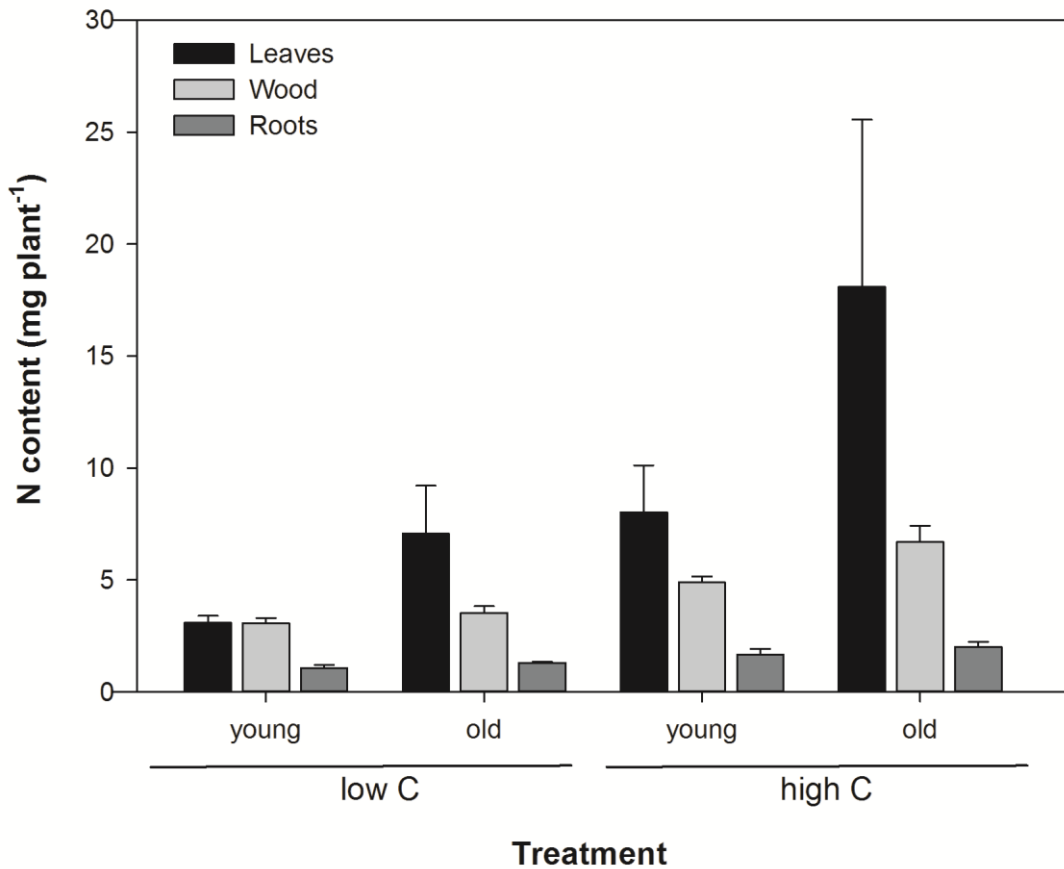


Figure 8

The content of N in the willow dry weight specified for individual organs. Young and old refer to the type of the fungal inoculum. The growth media of contrasting C content are denoted as low and high C. The values are means (n=4 ±SE).

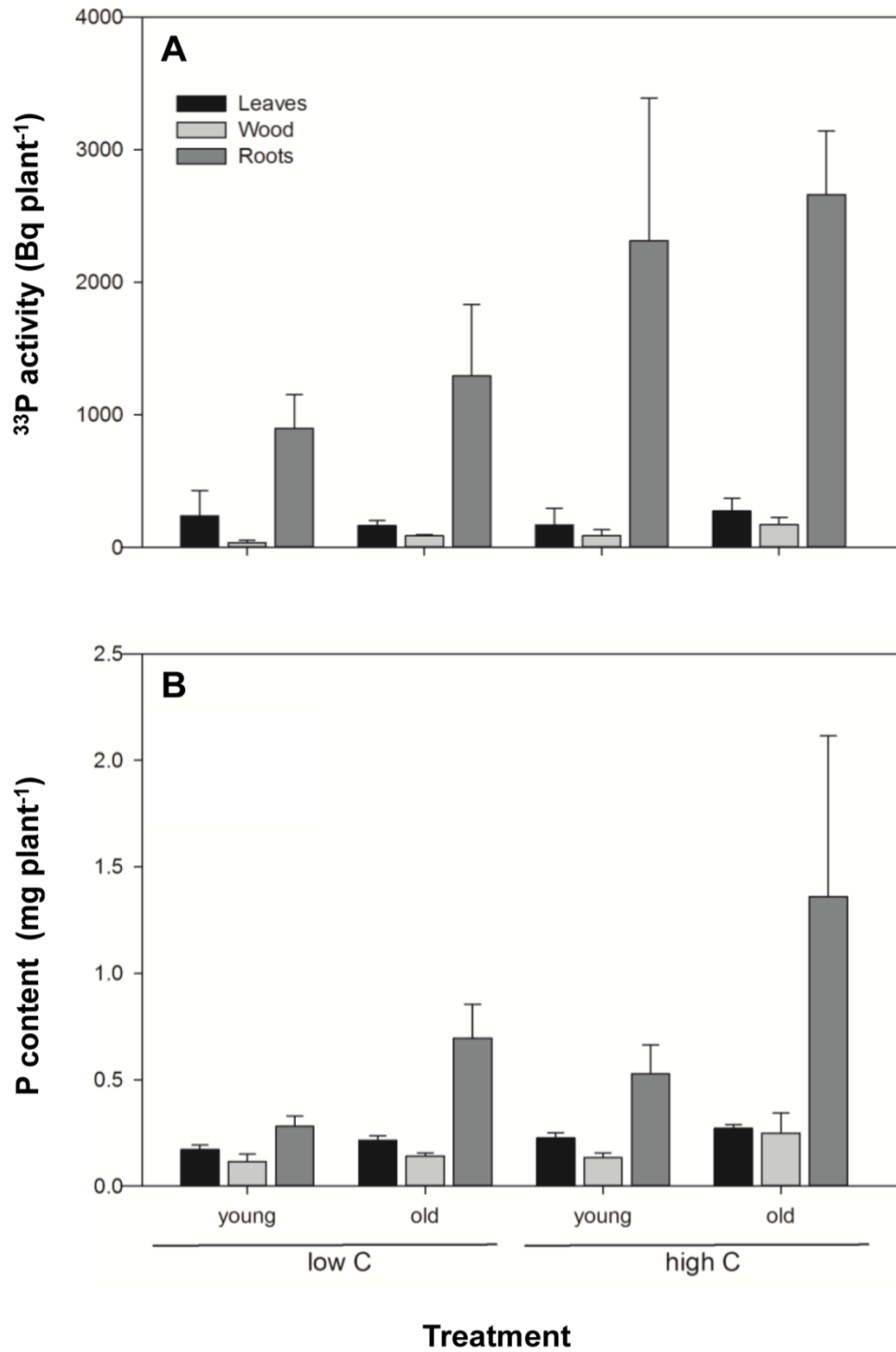


Figure 9

³³P activity (A) and content of P (B) in the willow dry weight specified for individual organs. Young and old refer to the type of the fungal inoculum. The growth media of contrasting C content are denoted as low and high C. The values are means (n=4 ±SE).

Biodissolution of ^{33}P -labeled apatite

The rate of the transfer of ^{33}P from the ^{33}P -labeled apatite to the willows was very low. Over the period of 30 days fungal communities established in the experiment transferred to the willows approximately 0.09% (average for all treatments) of initially applied ^{33}P activity (^{33}P -labeled apatite introduced to the nutrient compartment of each rhizobox) (Fig. 10). This transfer was not affected by the fungal inoculum but was significantly higher in high C growth medium as compare with low C growth medium (one-way ANOVA, $p=0.019$, $F=7.02$). Low rate of ^{33}P transfer to the plant was partially caused by the low solubility³ of ^{33}P -labeled apatite (Fig. 11). ^{33}P was detected in water extracts of soil in approximately 0.02% (average for all treatments, data not shown). The ratio between ^{33}P transferred to the plant and ^{33}P in water-extractable soil P fraction of the nutrient compartment was higher for the treatments where high C growth medium was applied (Fig. 12). This indicates that relatively greater portion of the ^{33}P was transferred to the plants growing in these treatments, leaving less ^{33}P in the water-extractable P pool.

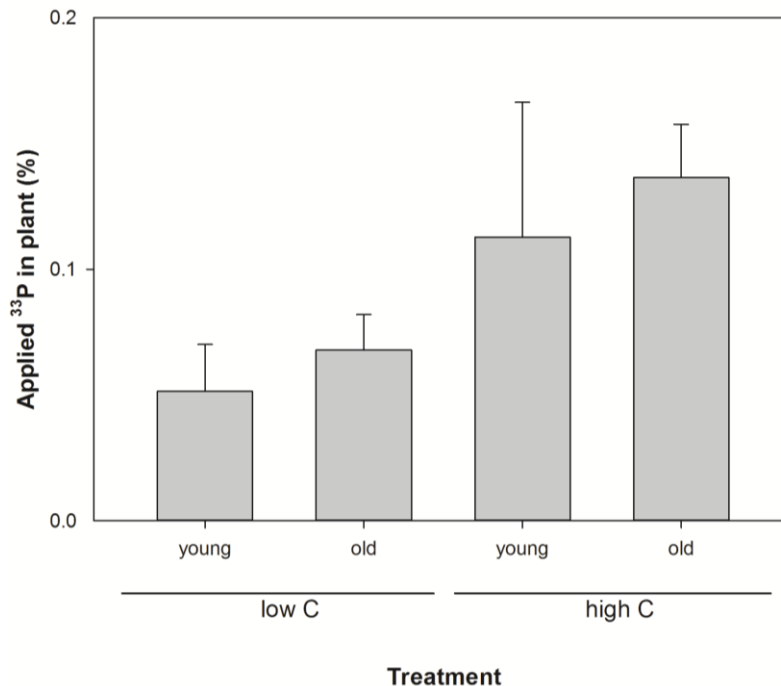


Figure 10

^{33}P activity in plant dry matter as percentage of ^{33}P initially introduced to the nutrient compartment. The values are means ($n=4 \pm \text{SE}$).

³ 'Solubility' is defined here as the ability of a mineral (i.e., ^{33}P -labeled apatite) to be dissolved in mycorrhiza-plant system.

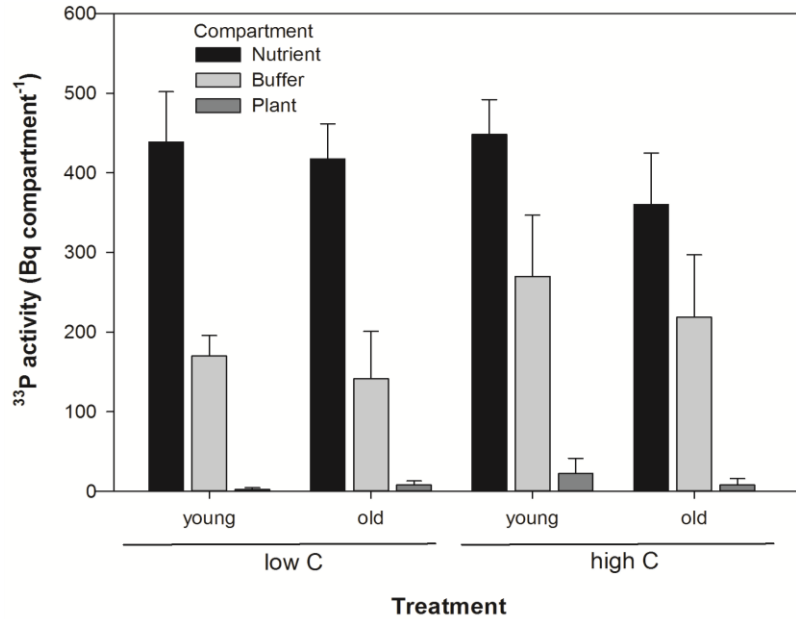


Figure 11

^{33}P activity detected in water-extractable fraction soil P pool. Soil from all treatments was analysed in different compartments of the rhizobox (i.e., nutrient, buffer and plant). The values are means ($n=4$) \pm SE.

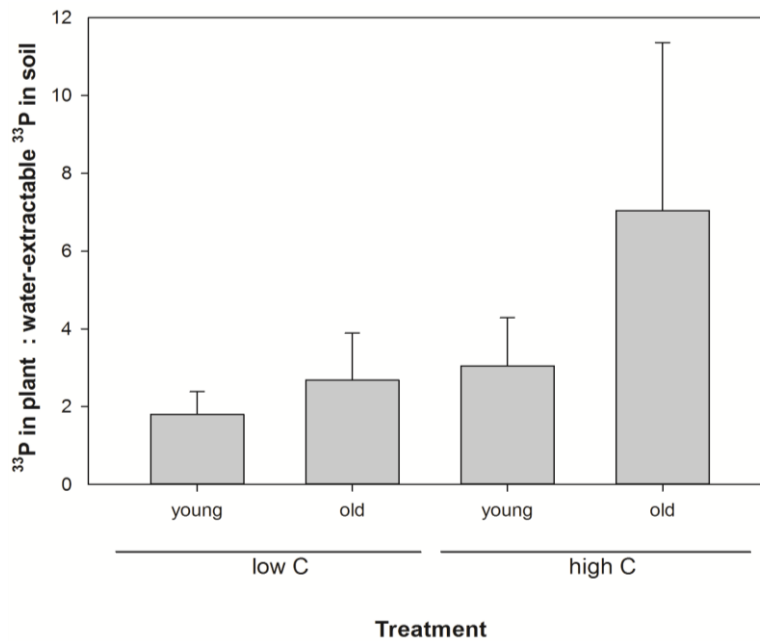
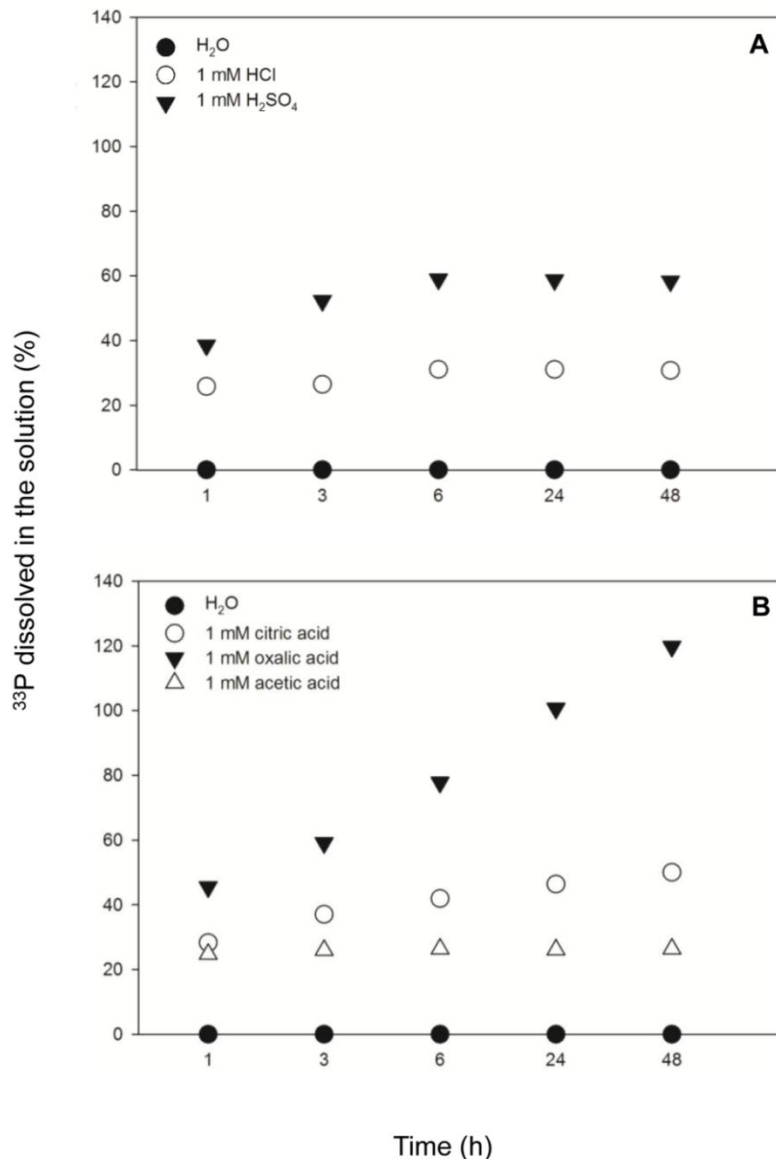


Figure 12

The ratio between ^{33}P transferred to the plant organs and ^{33}P in water-extractable P fraction in the soil from the nutrient compartment. The values are means ($n=4$) \pm SE).

Dissolution of ^{33}P -labeled apatite in vitro

Inorganic (Fig. 13A) and organic acids (Fig. 13B) favored the dissolution of ^{33}P -labeled apatite in vitro. After 48 h of incubation of ^{33}P -labeled apatite in the presence of 1 mM H_2SO_4 and 1 mM HCl, 58% and 30% of apatite was dissolved, respectively (Fig. 13A). Comparable results were obtained for organic acids: oxalic (50%) and acetic acid (26%), whereas the dissolution of ^{33}P -labeled apatite by citric acid was virtually 100% (Fig. 13B). Negligible fraction of ^{33}P (i.e., 0.002%) was dissolved in pure water over this period of time.

**Figure 13**

Percentage of ^{33}P dissolved in the inorganic (A) and organic (B) acids used as dissolution promoters. The points represent single values.

Discussion

In order to understand the development and functioning of the mycorrhizal associations at the glacier forefield, we inoculated alpine willow with indigenous mycorrhizal fungi. The communities of these fungi were exposed to two growth media differed in C content as is the case in the different stages of ecosystem development. Although in this experiment the growth media are describing as low or K (data not shown).

1. Impact of environmental factors on the fungal communities composition

The composition and abundance of fungal communities established in the experiment (Fig. 6) significantly differed from those detected in the fungal inocula (Fig. S1). This could be expected as a result of environmental filtering and adjustment of the fungal communities to the created experimental conditions (see ***Fungal communities under the experimental and field conditions*** for details). First, fungal inocula were collected from the field, thus there were complete fungal communities, containing propagules of fungi not only specific for *S. helvetica* but also establishing mycorrhiza with other plants. Because of this, only fungi able to establish mycorrhizal associations with *S. helvetica* in experimental system were expected to establish. Second, mycorrhizal fungal community could be affected by the willow age. The inocula were collected in the close vicinity of well-developed (approximately 12 to 60 years old) *S. helvetica* individuals thus the compositions of mycorrhizal fungi was certainly different from those established in the presence of young, only approximately four-month old willows (Fig. 6) Third, our experimental conditions (e.g., uniform watering, stable temperature, low UV radiation as compared with the field conditions) could also impact the fungal communities' composition and abundance.

Incoybe lacera dominated soil and root-associated willow ECM communities, whereas other ECM taxa presented very low abundances (Fig. 6). Moreover, higher content of C in the growth media (possibly also other elements) favored *I. lacera* suggesting this fungus to occur rather at the later stages of soil development. This observation seems to correlate with the presence of the fruitbodies of *I. lacera* at the later stages of the ecosystem succession at the Damma glacier forefield (approximately 65 to 110 years after deglaciation, personal observation). This observation, however, contradict earlier reports classifying *I. lacera* as an early-succession fungus (Jumpponen et al., 2012; Nara et al., 2003) which readily colonize plant roots and outcompete other ECM taxa (Fox, 1986).

AMF were detected in the willow roots but not in the soil samples (Fig. 6). Interestingly, AMF were always more abundant in the presence of the old fungal inoculum (for both low and high C growth medium). However, higher content of C in the growth medium seemed to suppress the growth of *Glomus mosseae* (Fig. 6). This can indicate that: i) this fungus was suppressed not by the high C content itself but by e.g., P introduced with the growth medium as well; ii) failed to establish due to high competition with another (AMF) fungus/fungi. Since retarded development of AMF at high P supply is well documented (Jansa et al., 2009; Jasper et al., 1979), interspecies competition between AMF fungi is still poorly understood (Thonar, 2009) thus can be only speculated in our investigations.

The abundance of UTS differ for soil and roots (Fig. 6). *Peziza badia* dominated in soil, especially when old fungal inoculum was applied. This observation somehow contradict earlier research where fruitbodies (and mycorrhizas) of *Peziza badia* were found at the early stages (a stand of birches planted 6 years ago) of ecosystem development (Last et al., 1983).

Uncultured Basidiomycota was abundant colonizer of the willow roots (Fig. 6), but ecological importance of this fungus remain unknown. It can be only speculated that this fungus probably thrive on C and can only dominate the treatments in which competition with another (micro)organisms is low. The presence of *Meliniomyces bicolor* in the treatments with old fungal inoculum stays in agreement with other reports. This fungus mainly associates with ericaceous plants (they occurred at the location from which old fungal inoculum was sampled) this fungus can also form ECM with trees, possibly also willows (Grelet et al., 2009; Kohout et al., 2011; Vohník et al., 2007).

2. Impact of fungal communities on the transfer of nutrients

This experiment did not include nonmycorrhizal controls so any judgements about absolute levels of nutrient transfer by the mycorrhizal communities to the plants remain speculative. No consistent differences were observed that fungal communities better adapted to the different growth media (i.e., young inoculum-low C growth medium and old inoculum-high C growth medium) would provide much larger nutrient benefits than the others, as it was hypothesized. Moreover, there is a number of factors potentially influencing mycorrhizal functioning, such as host plant (i.e., identity but also physiological state and age) (Becklin et al., 2011; Fujimura and Egger, 2012), interactions within the fungal community (e.g,

competition, suppression or facilitation) as well as experimental conditions which sometimes deviate markedly from the natural ones (e.g., regular watering vs. fluctuations in water availability in the field, lower UV radiation than in the field, etc.). Moreover, we still do not have sufficient knowledge about the interaction between nutrients in the natural systems and their direct effect on the mycorrhizal colonization and the plant nutrition. Sylvia and Neal (1990) studying interaction between AMF, N and P revealed that root colonization was not affected by P addition when plants were N-deficient but when N was delivered in sufficient amount, it feedback the impact of P on root colonization rate (Sylvia and Neal, 1990). Thus, plant N stress modulated the resistance of the roots to colonization by AMF. Up to now, none of the studies considered such a situation of the ECM fungi or suggested impact of N-P interactions on the plant nutrition.

Since *I. lacera* was the dominant ECM taxon, this transfer can be (most likely) predominantly attributed to this fungus. However, the literature reports that *I. lacera* develops rather scarce extraradical mycelium thus was classified as short-distance exploration ECM type (Agerer, 1995, 2001; Cripps and Miller, 1995). It is, however, impossible to present any exact distance (on mm or cm scale), which will define short-distance expression more precisely. In our experiment *I. lacera* penetrated the soil (Figs. 2 and 6) away from the plant compartment (where fungal inoculum was applied) of (at least) 2 cm. The mechanism which facilitate uptake of nutrients by this ECM fungus remain unknown. Based on our observations, we speculate that *I. lacera* was able: i) to hydrolyze SOM (introduced here with the growth medium) as the significant relation of the abundance of this fungus with N in plants was found (Table 2, Fig. 7), ii) to solubilize recalcitrant inorganic P nutrient source such as ^{33}P -labeled apatite. In the latter case, however, no significant relation between fungus abundance and ^{33}P in plant was observed (data not shown).

Also, in our experiment plant growth remained unaffected either by the fungal inoculum or the growth medium (Table 2). Similar observation was reported for *Pinus sylvestris* associated with ECM fungi, which did not improve the growth of seedlings cultivated with apatite as a P source (Wallander et al., 1997b). A few explanations can be given for such an observation in our experiment. First, carbon which could be allocated to the biomass was invested into establishment of the mycorrhizal associations (Hobbie, 2006). Second, ^{33}P released from dissolved apatite was not only captured by mycorrhizal fungi but possibly by other, more competitive soil microbes. Third, the solubilization of ^{33}P -labeled apatite in experimental

system was very slow: only 0.09% (in average) of initially applied ^{33}P was transferred to the plants. In the study of Wallander (2000) the rate of dissolution of apatite was also low: 0.3-0.9% of apatite added to the pots was solubilized over 210 days. Despite this fact, seedlings of *Pinus sylvestris* grew significantly better in the presence of apatite as a P source than the control plants (Wallander, 2000). These results suggest that: i) the duration of the experiment presented in this chapter was too short to observe relevant effects, ii) established mycorrhizal associations release LMWOA in low concentrations, what retarded the solubilization rates (Figs.12 and 13), iii) established mycorrhizal associations were not well able to dissolve the ^{33}P -labeled apatite, and/or iv) the rate of dissolution was sufficient to cover the nutritional requirements of the (slowly-growing) willow.

Participation of AMF in the transfer of N and P to the willows seem to be negligible as they were absent in the soil. However, the presence and function of other AMF than *G. mossae* and *G. sinuosum* should be considered. It is difficult to elaborate the ecological importance of other soil fungi such as *Peziza badia* or of the Uncultured Basidiomycota, which were abundant in our experiment. There is, however, clear evidence that the fungi which were not classified as mycorrhizal, can participate directly (e.g., as endophytes) or indirectly in the transfer of nutrients to the plant (Jumpponen, 2001; Lindahl et al., 1999).

3. Fungal communities under the experimental and field conditions

The abundance and the composition of the fungal communities developed in presented experiment differed from those established with *Salix helvetica* under the natural conditions (Fig. 6; Fig. 1A-B chapter 2). In contrast to the rhizosphere soil from the field, AMF were not detected under the experimental conditions. Colonization of willow roots from the natural environment by AMF was approximately 50-fold higher than in the rhizobox experiment (Fig. 6; Fig. 1A-B chapter 2).

The abundance of ECM in the soils was comparable with their abundance in the rhizobox experiment but only in case of young and old part of the chronosequence (i.e., 7 to 12 and 78 to 110 years after deglaciation) (Fig. 6; Fig. 1A-B chapter 2). The abundance of ECM fungi in the rhizospheres from the middle part of the chronosequence was approximately 9-fold higher than for remaining sites. Additionally to that, the differences in ECM community composition were observed between experimental and field study (Fig. 6; Fig. 1B chapter 2). *Inocybe lacera* dominated ECM communities established in the soil under the experimental

conditions, whereas these associated with the willows' rhizospheres in the field changed with soil age and favored other ECM taxa. At the youngest site (i.e., 7 years after deglaciation) *Cenococcum geophilum* was found as a dominant species. Interestingly, this ECM had never been detected under the experimental conditions (Fig. 6). Willow rhizospheres at the sites deglaciated approximately 8 and 12 years ago were dominated by *Laccaria pumila* and *Inocybe lacera* whereas middle part of the chronosequence (approximately 60 to 70 years after deglaciation) favored mainly *Russula emetica*, *Laccaria pumila* and *Inocybe lacera* in the ECM community. At the oldest part of the chronosequence, similarly to this deglaciated 8 and 12 years ago, *Laccaria pumila* and *Inocybe lacera* occurred as main constituents of the ECM community. The abundance of ECM in the rhizobox experiment was approximately 10-fold higher compared with the field communities. Furthermore, root-associated ECM communities were dominated by either *Inocybe lacera*, or *Russula emetica* and *Laccaria pumila*, under the experiment and field conditions, respectively (Fig. 6; Fig. 1A chapter 2). Field conditions favored the growth and development of the UTS in the rhizosphere soils (Fig. 6; Fig. 1B chapter 2). The abundance of these fungi was approximately 10-fold lower in the experiment than in the field study. Concerning the composition, *Peziza badia* and Uncultured Basidiomycota were the dominant taxa under the experimental and field conditions, respectively. These fungi, moreover, were approximately 40-fold more abundant on the roots from the experiment than these sampled from the field (Fig. 6; Fig. 1A chapter 2). Uncultured Basidiomycota dominated the fungal community in the rhizobox experiment, whereas field conditions favored mostly Uncultured *Mortierella* spp. and *Meliniomyces bicolor* in the root-associated fungal community.

Conclusions

- In this experiment, we examined mycorrhizal associations with alpine willow. Communities of indigenous fungi (originated from the soil deglaciated 16 and 116 years ago, for young and old fungal inoculum, respectively) were combined with the different growth media (low and high C content) that imitated different stages of ecosystem development.

- We demonstrated that the composition and the abundance of the fungal communities associated with willow (roots and rhizosphere soil) were affected by the origin of fungal inoculum as well as by the growth medium.
- The differences in the fungal community composition translates to the differences in content of N in the willows.
- In spite of large differences in fungal community compositions, no significant difference in willow P nutrition and ^{33}P transfer to the willows, upon dissolution of ^{33}P -labeled apatite, was observed.

Acknowledgements

I greatly acknowledge Prof. Peter Ulmer (Institute of Geochemistry and Petrology, ETH Zurich) for his help with hydrothermal synthesis of ^{33}P -labeled apatite; Irena Jansova-Vacinova for preparation of all samples for the nutrient analyzes; Fabio Grasso is thanked for an excellent technical support upon harvest and qPCR analyses and Thomas Flura for performing of the ICP and CN analyses. The authors acknowledge The Swiss National Science Foundation (SNSF, project number 31003A-125491) for the financial support.

Chapter 3 - supplementary material

Supplementary text

XRD analyses

The analyses were performed at the Institute for Mineralogy and Petrology, ETH Zurich. The sample of ^{33}P -labeled apatite was dispersed on a silicon disk and analyzed with Powder X-Ray Diffractometer (Bruker, AXS D8 Advance), equipped with a Lynxeye superspeed detector.

Liquid scintillation counting

The ^{33}P activity was determined by liquid scintillation counting (Wallac, Perkin Elmer, Waltham, USA) in the plants (i.e., leaves, wood and roots), soils (i.e., nutrient, buffer and plant compartment of the rhizobox) and ^{33}P -labeled apatite suspension and washes resulting from the washing procedure (see ***Hydrothermal synthesis of the ^{33}P -labeled apatite*** for details). Two, 3 and 1 mL of plant extracts, extracts of plant-available and water-extractable elements of soil, and ^{33}P -labeled apatite suspension and washes were combined with 10, 15 and 5 mL of scintillation cocktail, respectively (UltimaGoldTMAB or UltimaGoldTM, PerkinElmer, Schwerzenbach, Switzerland). Scintillation counting rate of a channel having energy 0 to 220 keV was measured over 30 and 3 min, for plant and soil extracts, and ^{33}P -labeled apatite and washes, respectively.

Supplementary figures

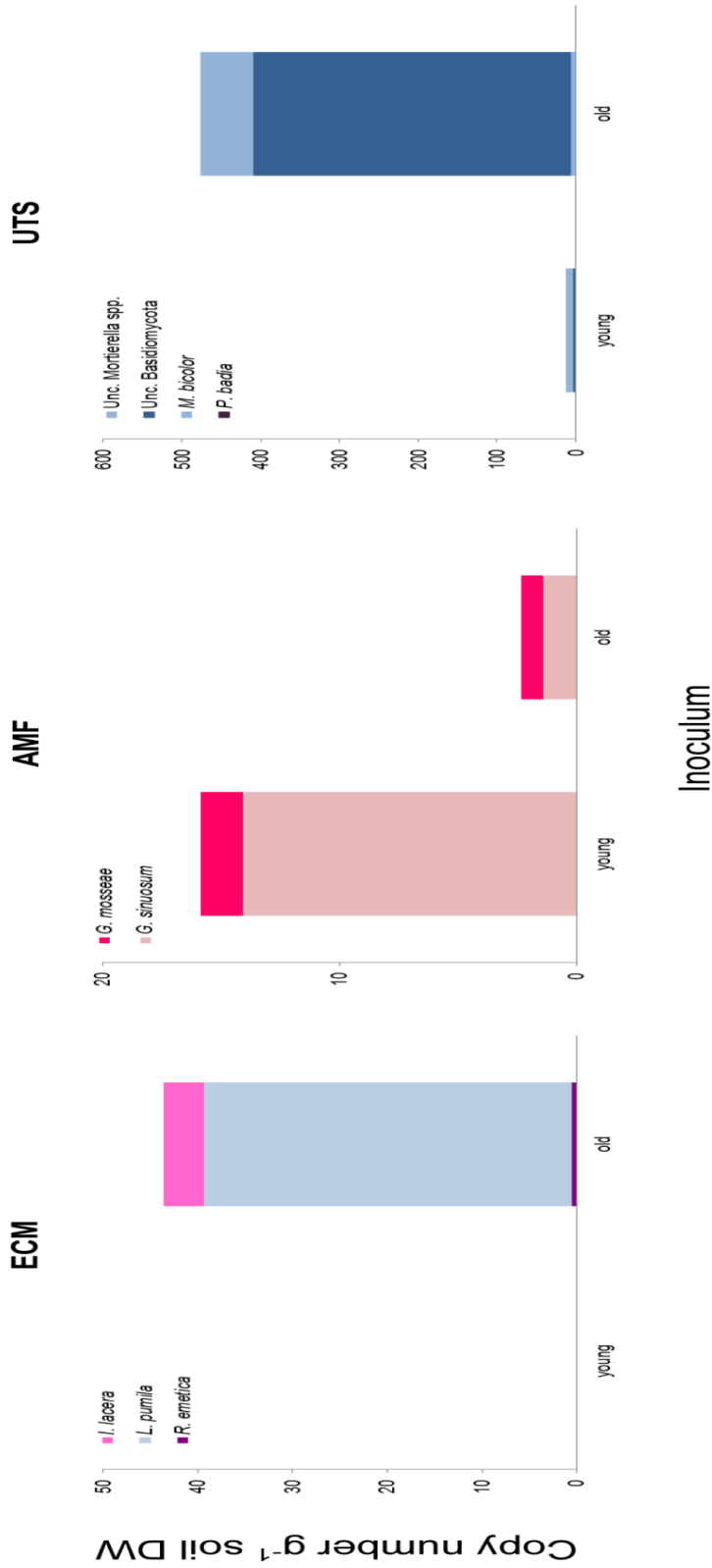
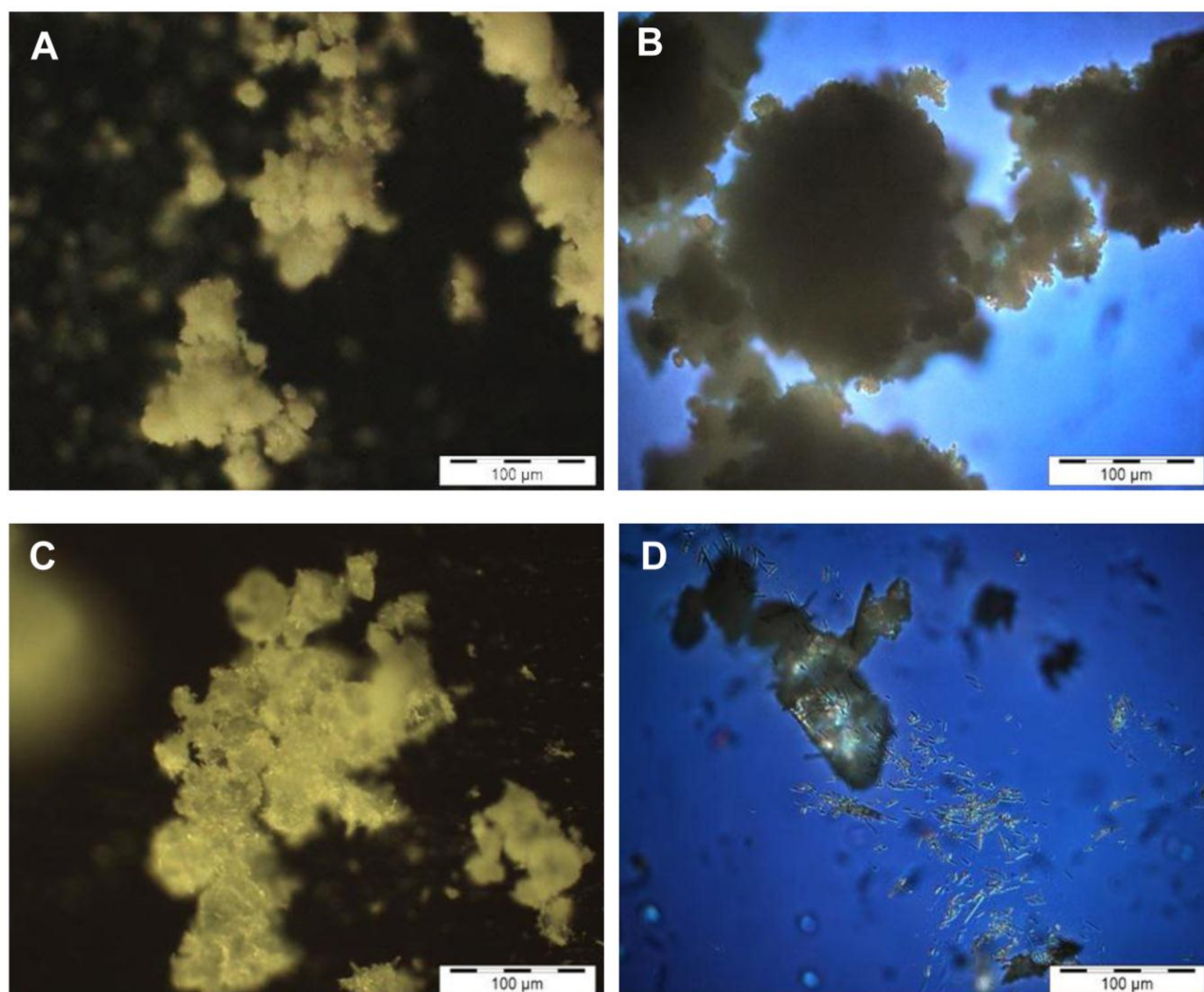


Figure S1

The abundance of different fungi (ectomycorrhizal, ECM; arbuscular mycorrhizal, AMF; and fungi of uncertain trophic status, UTS) in soils serving as fungal inocula (young and old) for rhizobox experiments, assessed with taxon-specific molecular markers. The values are means of analytical replicates (n=5). For clarity of the graph, SE bars not included. Fungal identities – (*I. lacera*) *Inocybe lacera*; (*L. pumila*) *Laccaria pumila*; (*R. emetica*) *Russula emetica*; (*G. mosseae*) *Glomus mosseae*; (*G. sinuosum*) *Glomus sinuosum*; (*M. bicolor*) *Meliniomyces bicolor*; (*P. badia*) *Peziza badia*; (*Unc. Basidiomycota*) Uncultured Basidiomycota; (*Unc. Mortierella* spp.) Uncultured *Mortierella* spp. Note the different scales for soils and roots.

**Figure S2**

$\text{Ca}_3(\text{PO}_4)_2$ used as a product for ^{33}P -labeled apatite hydrothermal synthesis and ^{33}P -labeled apatite observed under the light and polarization microscope; amorphous $\text{Ca}_3(\text{PO}_4)_2$ under the (A) light and (B) polarization microscope; ^{33}P -apatite after hydrothermal synthesis under light (C) and (D) polarization microscope. All microscopic slides were prepared from <1 mg apatite powder distributed in a drop of distilled water and observed under the microscope (Olympus AX70), equipped with Olympus XC10 camera and assisted by analySIS pro software (Olympus Software Imagine Solutions[®]).

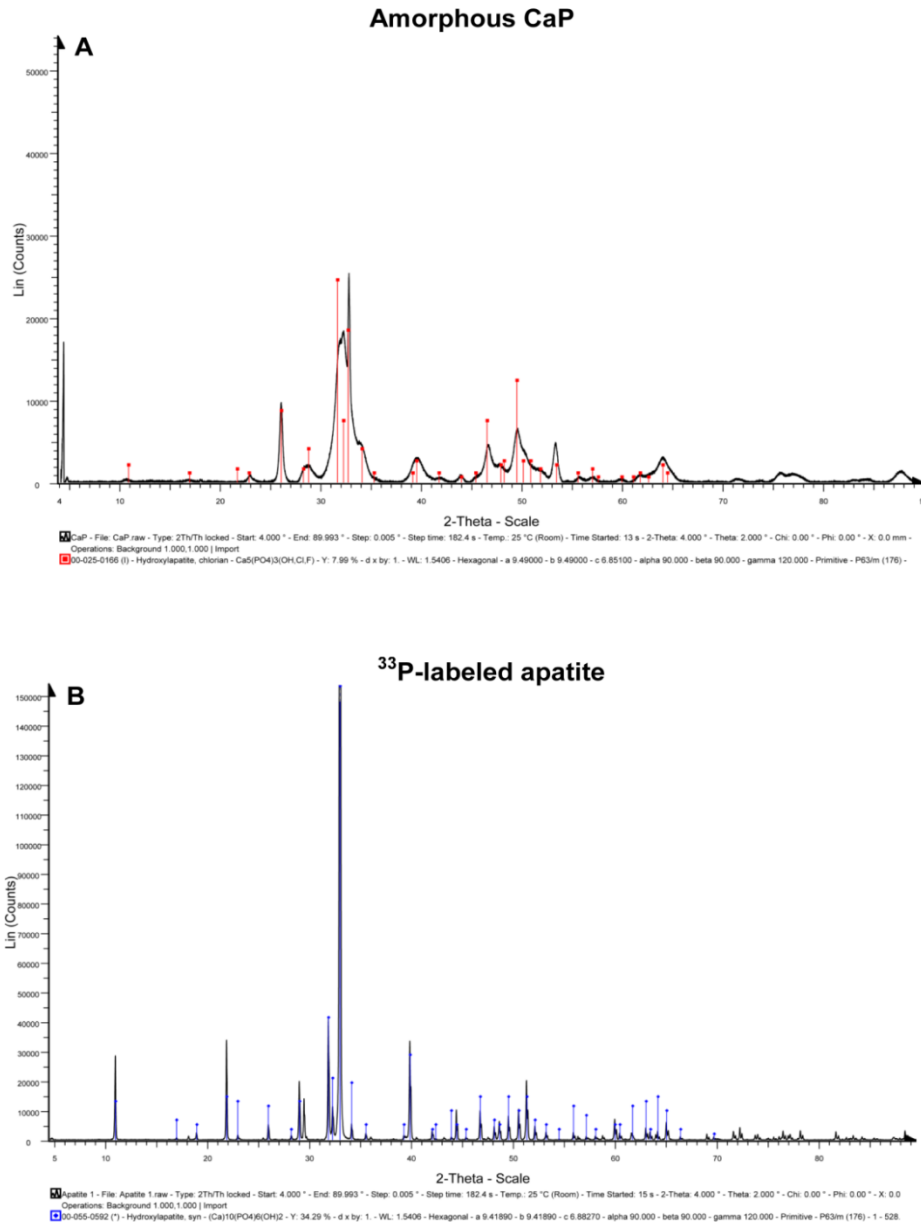
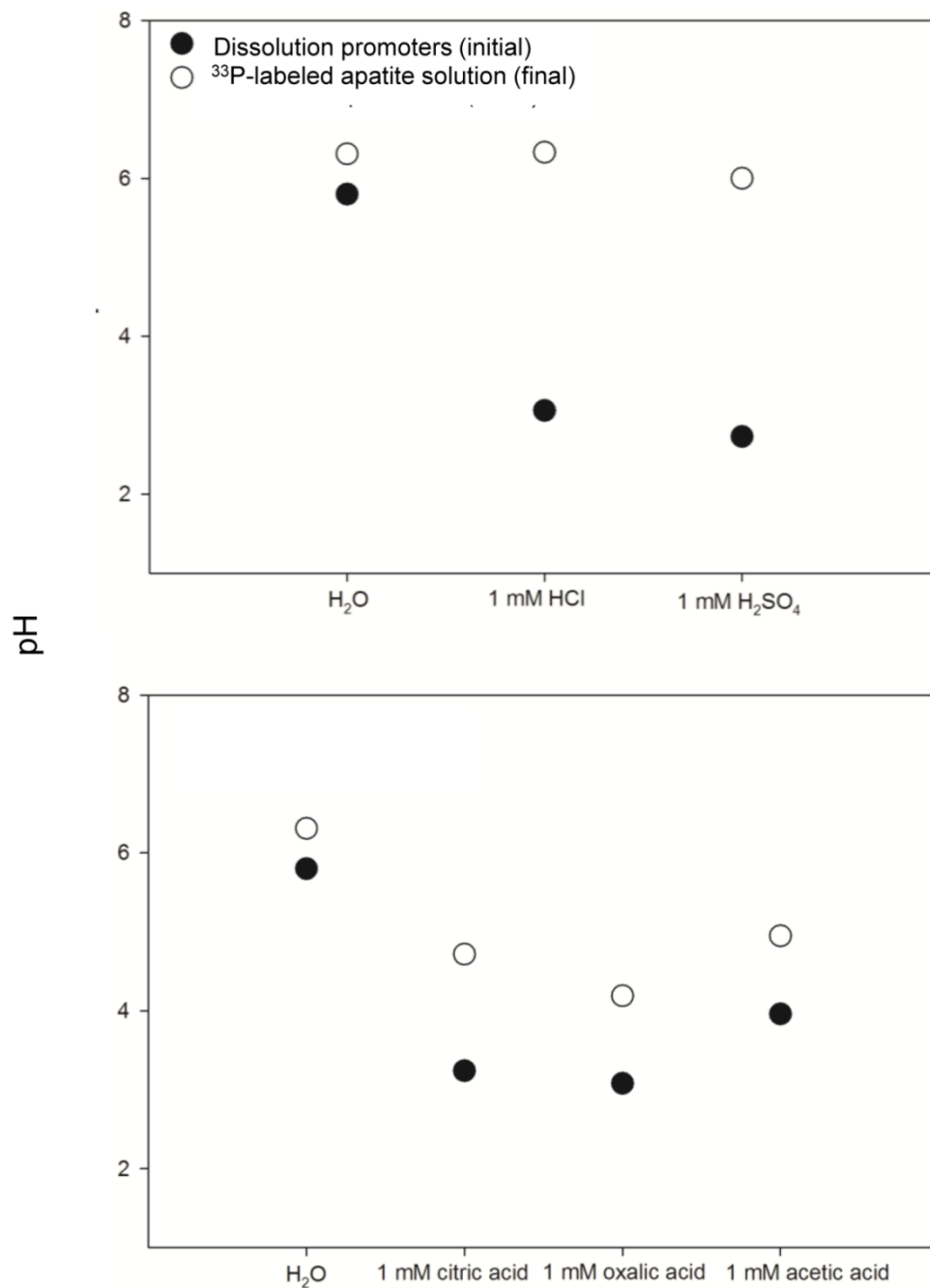


Figure S3

X-Ray diffraction analysis spectra revealing the structure of (A) amorphous $\text{Ca}_3(\text{PO}_4)_2$ and (B) ^{33}P -labeled apatite. Red and blue dots, for $\text{Ca}_3(\text{PO}_4)_2$ and ^{33}P -labeled apatite, respectively, denotes results of a structure of reference sample. The diffraction spectra consists of a record of photon intensity according to linear scale (Lin(count)) versus detector angle (2-theta scale; correspond to the angular position of the detector that rotates around the sample). Since $\text{Ca}_3(\text{PO}_4)_2$ occurs in the form of aggregates, the peaks are broader; ^{33}P -labeled apatite occur in the form of small crystals thus the peaks are thicker.

**Figure S4**

pH measured at the initial phase and at the end of the ³³P-labeled apatite dissolution in vitro. Dissolution promoters (initial) on the graph denotes the pH of each solution prepared in the sterile conditions before the dissolution experiment. ³³P-labeled apatite solution (final) on the graph denotes the pH of each solution after 48 h incubation. Each symbol represents single value.

**Chapter 4: Functional diversity of fungal communities in transfer of N and
P from organic source to willow plants**

Abstract

Mycorrhizal fungi are important deliverers of N and P which are exchanged with the host plant for carbohydrates. These fungi greatly support the establishment of plants, especially in developing ecosystems. Lab works conducted with single mycorrhizal strains proved that different mycorrhizal fungi can have different effects on plant growth. However, the causal relationship between the presence of specific mycorrhizal fungus/i in the fungal communities and the growth and nutrition of plants have been scarcely studied. In the present rhizobox experiment, we aimed to assess the impact of mycorrhizal communities on the growth and nutrition of *Salix helvetica*. Willow was inoculated with three fungal inocula taken from alpine soils (deglaciated 16, 65 and 116 years ago) and applied unsterilized or sterilized. Moreover, these fungal inocula were combined with two growth media: low and high carbon (C) and content. Such a set-up aimed to mimic young (i.e., deglaciated 8 to 12 years ago, referring to low C growth medium) or old (i.e., deglaciated 111 to 117 years ago, referring to high C growth medium) stage of soil development. In order to track the effectiveness of the fungal communities in uptake and transfer of nutrients to the willows, we applied ^{15}N - ^{33}P -labeled bacterial biomass as a source of labile organic N and P. After 4 weeks of experiment, willow dry weight, content of N, ^{15}N , P and ^{33}P in willows was measured. Using quantitative PCR molecular markers, we assessed also the abundance of ectomycorrhizal (ECM), arbuscular mycorrhizal (AMF) and fungi of uncertain trophic status (UTS) in soils, in order to disentangle participation of these fungi in the nutrient transfer to the willows. Our results show that: i) willow-associated mycorrhizal communities were able to access, uptake and transfer ^{15}N and ^{33}P to the willows; ii) the dry weight, content of N, ^{15}N , P, and ^{33}P in willows was higher in the presence of unsterilized compared to the sterilized fungal inocula; iii) willows inoculated with the old fungal inoculum in the presence of high C growth medium presented the highest dry weight and content of nutrients (N, ^{15}N , P, and ^{33}P); iii) content of ^{15}N and ^{33}P were correlated with the abundance of selected UTS, less strong - ECM. We demonstrated that different fungal inocula (i.e., different ages, sterilized vs. unsterilized) translated into different dry weight and different content of N, ^{15}N , P and ^{33}P in the willows grown in a model system. Possible function of UTS in willow nutrition was confirmed.

Keywords: willow growth, willow nutrition, fungal inoculum, qPCR, ^{15}N - ^{33}P -labeled bacteria, enzymes, alpine ecosystem, *Salix helvetica*

Introduction

Mycorrhizal fungi constitute an important component of the ecosystems developed on the land exposed by the melting alpine glaciers (Cázares et al., 2005; Jumpponen et al., 2012; Mühlmann and Peintner, 2008a; Trowbridge and Jumpponen, 2004). These fungi enhance the uptake of nutrients such as nitrogen (N) and phosphorus (P) from the soil and their subsequent transfer to the host plant (Smith and Read, 2008). Thus, formation of the mycorrhizal associations by the plants facilitate their spreading, colonization of recently deglaciated areas, and establishment of the plant communities (Chapin et al., 1994; Lambers et al., 2009).

Uptake of nutrients by mycorrhizal fungi, their further transfer to the plants as well as needs for plant-derived C can be influenced by different factors (Dickie et al., 2002; Mozafar et al., 2000; Sylvia and Neal, 1990; Thonar, 2009). Among them, fungal identity and fungal developmental stage can be mentioned as endogenous factors. In addition, environmental factors such as composition of existing vegetation, soil disturbances and fertilization greatly influence the uptake of nutrients by mycorrhizas.

As suggested by numerous studies, mycorrhizal fungi differ also in their mechanisms of uptake and preferential use of different forms of nutrients (e.g., inorganic vs. organic, easily available vs. recalcitrant)(Lambers et al., 2009). For example, arbuscular mycorrhizal fungi (AMF) mainly support acquisition of P by plants from inorganic pool in the soil beyond the depletion zone around the roots (Thonar, 2009), whereas their importance in recycling organic nutrients remains equivocal (Hawkins et al., 2000) or denied (Smith and Read, 2008). In his experiments, Joner et al. (1994; 1995) confirmed that production of extracellular phosphatases by AMF is very scarce thus their role in uptake of P upon soil organic matter (SOM) decomposition is negligible (Joner and Jakobsen, 1994, 1995; Joner et al., 1995). Low uptake of non isotopically exchangeable P by AMF supported this observation and suggested that these fungi rather efficiently gather P dissolved in the soil solution than participate in SOM hydrolysis (Morel and Plenchette, 1994).

In contrast, ectomycorrhizas (ECM) are known to efficiently hydrolyze SOM and recover N and P from it (Finlay et al., 1992; Lambers et al., 2009). Exudation of hydrolytic and oxidative enzymes enables ECM, ericoid (ER) but also saprotrophic fungi to acquire N and P

upon SOM upon decomposition (Baldrian, 2009; Finlay, 2005; Jansa et al., 2011; Lundley and Robinson, 2008; Read and Perez-Moreno, 2003; Unestam, 1966).

The use of organic N and P forms were intensively studied for axenically cultivated ECM (Chalot and Brun, 1998; Turner, 2008), but only a few presented similar abilities in the ECM-plant system (Wallander, 2002; Wallander et al., 1997). Plants colonized with ECM had been presented to efficiently access N and P as amino acids, or mobilized from bovine serum albumin, chitin or phytate (Baxter and Dighton, 2005; Chalot and Brun, 1998; Finlay et al., 1992; Lipson et al., 1999). However, in soil, mineralization of many biomolecules take place simultaneously thus ECM plants can access organic N and P from various organic forms degraded by microorganisms. The dead microbial biomass constitute an important pool of nutrients, which is supposed to be mineralized and recycled more dynamically than plant debris or humus (Jenkinson and Parry, 1989; Paul, 2007).

In the present green house study, we assessed the effectiveness of mycorrhizal fungi in uptake of N and P and dry weight production of *Salix helvetica* (Swiss willow) (Lauber et al., 2012). This alpine willow was inoculated with three fungal inocula taken from alpine soils (i.e., deglaciated 16, 65 and 116 years ago), that had been sterilized or not. Moreover, willows were grown in a low and a high C growth media, mimicking young or old stage of soil development, respectively (i.e., deglaciated 8 to 12 years ago, or 111 to 117 years ago, respectively). In order to track the effectiveness of the fungal communities in uptake and transfer of nutrients to the willows, we applied ^{15}N - ^{33}P -labeled bacterial biomass as a source of fast-cycling labile organic N and P. We hypothesized that: i) mycorrhizal fungi will access and transfer ^{15}N and ^{33}P to the willows directly affecting their growth and nutrition; ii) mycorrhizal fungal communities established from mid inoculum (approximately 65 years after deglaciation) will affect the growth and nutrition of willows better than remaining inocula as the mid inoculum was collected from a part of the glacier forefield amply colonized by willow bushes; iii) ECM able to release hydrolytic enzymes will be the most efficient in ^{15}N and ^{33}P uptake from freshly added fast-cycling pool such as ^{15}N - ^{33}P -labeled bacterial biomass.

Materials and methods

Origin and preparation of the substrates and fungal inocula used in the experiment

The origin and preparation of the growth media used in this experiment was identical with previously used (***Origin and preparation of the substrates and fungal inocula used in the experiment***, chapter 3). The fungal inocula (i.e., young and old) were applied as previously described, and an additional inoculum, collected from the middle part of the glacier forefield (mid, collected from the spot deglaciated approximately 65 years ago, N46°38.262' E8°27.724', elevation 2028 m) was applied. All three fungal inocula were used either unsterilized (as in Chapter 3) or sterilized by γ -irradiation through applying a dose of 25-75kGy with a ^{60}Co source (LEONI Studer Hard AG, Däniken, Switzerland). Selected chemical properties of the fungal inocula have been presented in chapter 3 (Table 1, chapter 3), whereas the mid inoculum presented the following properties: $\text{pH}_{(\text{H}_2\text{O})} = 4,8 (\pm 0.06)$; $\text{C}_{\text{tot}} = 1.3 (\pm 0.15) \text{ g kg}^{-1}$; $\text{N}_{\text{tot}} = 0.09 (\pm 0.01) \text{ g kg}^{-1}$; $\text{P}_{\text{tot}} = 294 (\pm 1.42) \text{ mg kg}^{-1}$; $\text{P}_{\text{av}} = 2.7 (\pm 0.75) \text{ mg kg}^{-1}$ (analytical replicates $n=3 \pm \text{SE}$).

Experiment duration and conditions

The experimental conditions maintained during this experiment were similar to those presented in the chapter 3 (see ***Experiment duration and conditions***, Chapter 3). ^{15}N - ^{33}P -labeled bacteria were introduced to the nutrient compartment when mycorrhizal associations were established on the willow roots (Fig. 1, chapter 3). Destructive harvest was performed 30 days after introduction of the ^{15}N - ^{33}P -labeled bacteria to the experimental system (Fig. 1, chapter 3).

The rhizobox construction

We used the same rhizoboxes as in the preceding experiment (see ***The rhizobox construction***, Chapter 3). In total, 60 individual rhizoboxes were established. Experimental units (sets of 10 rhizoboxes) were randomized once a week to assure homogeneous conditions for willow growth.

Experimental design

The experiment had twelve treatments: two growth media with either a low or a high C content introduced to the plant and buffer compartment, were combined with either unsterilized or sterilized fungal inocula: young, mid and old, introduced to the plant compartment (Fig. 1). Each treatment had five replicates.

Vegetative propagation of *Salix helvetica*

Vegetative propagation of *Salix helvetica* followed the procedure previously established (***Vegetative propagation of *Salix helvetica****, chapter 3). The stems for vegetative propagation were collected from mature willow plants in September 2010 from three field locations (N46°38.355' E8°27.811'; N46°38.348' E8°27.805' and N46°38.365' E8°27.817'). Three different populations of cuttings were produced from the starting material. To assure genetic uniformity of the willows selected for the experiment, only one population of seedlings (the largest and the best developed) was used.

Inoculation procedure

Inoculation procedure was performed as previously (***Inoculation procedure***, chapter 3).

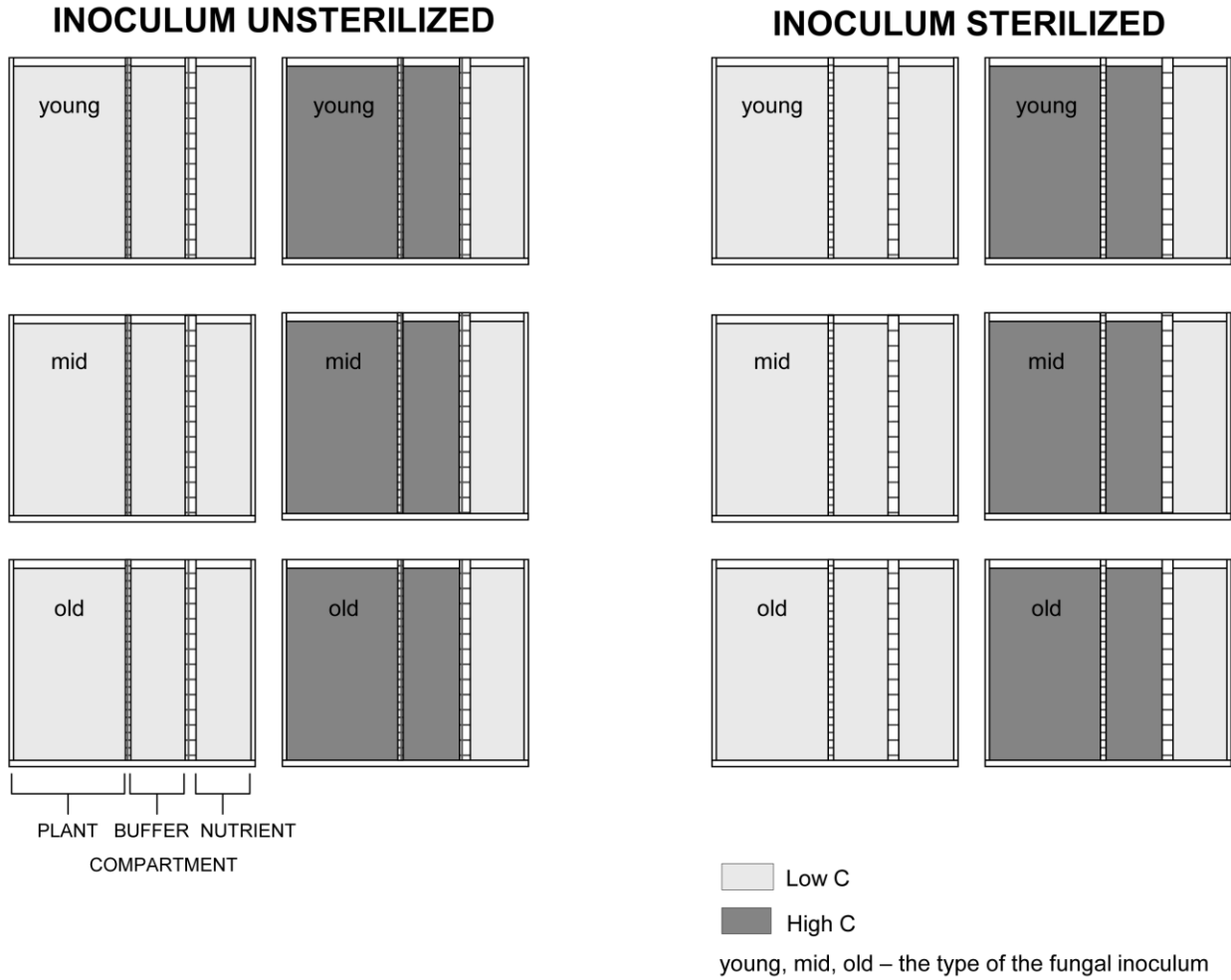


Figure 1

Schematic representation of the experimental treatments. Light and deep gray color represents growth medium of low and high C content, respectively. Labels: young, mid and old fungal inocula collected from three locations at the Damma glacier forefield differed in age after last deglaciation. The fungal inoculum was applied either as unsterilized or sterilized.

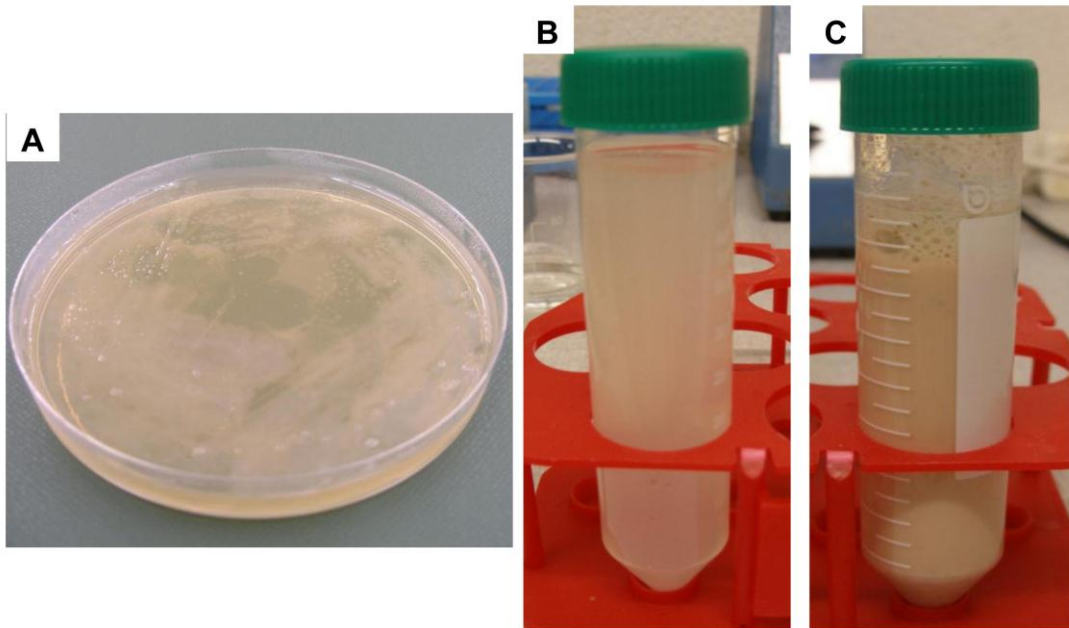
Preparation of the ¹⁵N-³³P-labeled bacteria

Strain of *Arthrobacter* sp. isolated from a fine granitic sand collected at the front of the Damma glacier (database accession no. GU213306) (Frey et al., 2010), preserved in glycerol at -80°C served as a stock culture for propagation (Fig. 4A-B). Prior to labeling, the growth of *Arthrobacter* sp. was tested on a few mineral broths (Fig. S1) in case to optimize bacterial

biomass production. This test led to the selection of a slightly modified M9 medium (Sambrook et al., 1989). The original M9 medium was supplemented with (g L^{-1}): glucose (10) and microelements ($\mu\text{g L}^{-1}$): H_3BO_3 (15), $\text{MnSO}_4 \times 7\text{H}_2\text{O}$ (0.8), $\text{ZnSO}_4 \times 7\text{H}_2\text{O}$ (6), $\text{CuSO}_4 \times 7\text{H}_2\text{O}$ (1), $(\text{NH}_4)\text{Mo}_7\text{O}_{24} \times 4\text{H}_2\text{O}$ (0.2), $\text{C}_6\text{H}_5\text{FeO}_7$ (0.12) while vitamins were omitted. The pH of the medium was 7.0. Double-labeled ammonium nitrate ($^{15}\text{NH}_4^{15}\text{NO}_3$, Catalog no. 366528, 98+% atom labeled, ISOTEC, Switzerland) was the only nitrogen source. $\text{H}_3^{33}\text{PO}_4$ (6mCi, Catalog no. FF-1H, Hartmann Analytic, Germany) was introduced with a solution of KH_2PO_4 (3.0 g/L) to the medium as phosphorus source. M9 medium (2.0 L) was prepared and autoclaved (121°C , 20 min). Two mL of $^{15}\text{NH}_4^{15}\text{NO}_3$ was dissolved in sterile distilled water and 1.5 mL of $\text{H}_3^{33}\text{PO}_4$ (activity 19909569 Bq, December 9th 2010) was introduced to the medium under sterile conditions. Next, the medium was inoculated with 2.0 mL of concentrated *Arthrobacter* sp. culture which was cultivated overnight on Luria-Bertani (LB) medium (Sambrook et al., 1989). Inoculated medium was mixed gently and distributed (approximately 62 mL) to 32 Erlenmeyer flasks (polycarbonate, cat. no. 215-2222), which were incubated at 30°C for 88 h at shaking mode 2.2 Hz. After that, the content of each flask was transferred to 50 mL Falcon tubes and subjected to repetitive centrifugation-washing to obtain a concentrated ^{15}N - ^{33}P -labeled bacterial biomass. Centrifugation-washing cycles were performed at 20°C and 1500 g with following volumes of 1% NaCl and following duration: 1st washing: 20 mL/10 min, 2nd 10 mL/15 min, 3rd 15 mL/15 min and 4th 30 mL/15 min. Volumes of the supernatant from each centrifugation step was recorded and ^{33}P activity assessed by liquid scintillation counting (data not shown). The procedure ended with 40 mL of concentrated ^{15}N - ^{33}P -labeled bacterial biomass (Fig. 2C) which was introduced to the nutrient compartment of each rhizobox (see **Labeling procedure** for details). In the bacterial biomass ^{15}N was only form of nitrogen (N from bacterial turnover negligible) thus was measured as total N by dry combustion (NCS Analyzer, FlashEATM 1112 Series ThermoElectronic Corporation). Total concentration of P and ^{33}P was assessed in the bacterial biomass by Inductively Coupled Plasma Mass Spectrometry and liquid scintillation counting, respectively (**Liquid scintillation counting**, chapter 3 supplement). Bacterial biomass presented following properties: N_{tot} concentration= $5.64 (\pm 1.42) \text{ g kg}^{-1}$; P_{tot} = $28.60 (\pm 0.81) \text{ mg kg}^{-1}$; ^{33}P activity= $73662.75 (\pm 9428.86) \text{ Bq mL}^{-1}$ (December 14th 2010) (analytical replicates $n=3 \pm \text{SE}$).

Introduction of the labeled bacterial biomass into the soil

The labeled bacterial biomass was introduced to the nutrient compartment. This compartment contained only low C growth medium for all treatments to assure comparable development of bacteria. Forty mL of ^{15}N - ^{33}P -labeled bacterial culture in tightly closed 50 mL Falcon tube were mixed end-over-end for 5 minute at room temperature. Half mL of the bacteria suspension was added to approximately 30 g of the growth medium placed in 50 mL Falcon tube and shaken vigorously to assure homogeneous distribution of the labeled bacteria in the low C growth medium. This procedure was repeated 60 times to add the bacterial biomass to each rhizobox. The bacterial suspension was always end-over-end mixed for 1 minute at room temperature before taking the next volume aliquot. This labeled growth medium was introduced to the nutrient compartment prefilled with 140 g of the low C growth medium, distributed in an approximately 0.5 cm thick layer and then covered with another 100 g of the low C growth medium.

**Figure 2**

Arthrobacter sp. strain used for ^{15}N - ^{33}P -labeled biomass production; (A) one-week-old culture (solid Pp medium, see caption of Fig. S1 for detail description of the medium); (B) 24 h culture (liquid Pp medium; see caption of Fig. S1 for detail description of the medium); (C) concentrated three-day-old culture of ^{15}N - ^{33}P -labeled *Arthrobacter* sp.

Harvest and processing

Roots and shoots were processed and analyzed according the procedure established in the previous experiment (***Harvest and processing***, chapter 3).

Chemical analyses of plant and soil material

Plant and soil samples were processed as previously described (***Chemical analyses of plant and soil material***, chapter 3). Measurement of the stable isotope of ^{15}N were performed by stable-isotope mass-spectrometry at Paul Scherrer Institut (PSI), Villigen, Switzerland (Delta-S Finnigan MAT, Bremen, Germany). ^{33}P activity was measured by liquid scintillation counting (***Liquid scintillation counting***, chapter 3 supplement). Two mL of plant extracts were combined with 10 mL of scintillation cocktail (UltimaGoldTM, PerkinElmer, Schwerzenbach, Switzerland). The scintillation counting rate of a channel having energy 0 to 220 keV was measured over 60 min.

DNA extraction from the root and soil samples

DNA extraction was performed by using the same DNA extraction kits appropriate for plant roots and soils, as used previously (***DNA extraction from the root and soil samples***, chapter 3).

qPCR markers and qPCR cycling conditions

The abundance of ECM, AMF and UTS were assessed in root and soil samples with taxon-specific qPCR markers as described previously (***qPCR markers and qPCR cycling conditions***, chapter 3).

Statistical analyses

The averages and standard errors calculated in Excel (Microsoft Excel 2010) were used to generate the graphs with software SigmaPlot 12 (Systat Software Inc., San Jose, USA). The analysis of variance (three-way ANOVA) for the differences in plant and soil parameters in different treatments was tested with StatGraphics[®]Plus (Statistical Graphic Corporation). The data matrix combining experimental factors (fungal inoculum, growth medium and sterilization), the abundance of the different fungi (in both the the soils and roots), enzymatic

activities of soils, and the amount of ^{15}N and ^{33}P in the plants were subjected to multivariate statistics using Canoco 4.5 (ter Braak and Šmilauer, 2002). Multivariate statistical analyses were conducted on a centralized and standardized dataset. Selection of Redundancy Analysis (RDA) was justified by a short length of the gradient (1.843, total inertia 0.658) in Detrended Correspondence Analysis (DCA). The statistical significance of the relationship between the treatments, fungal abundances, soil properties and plant growth parameters was evaluated using Monte Carlo permutation test (unrestricted permutation, reduced model, 9999 permutations).

Results

All willows remained healthy until the harvest of the experiment.

1. Plant growth parameters as affected by the fungal inoculum and growth medium

Depend on the treatments, 4.1 to 8.5-fold increase of the dry weight of willows was observed (Fig. 3), as compared with their initial biomass. The dry weight of the willows was mostly affected by the fungal inoculum and an interaction between fungal inoculum and sterilization (Table 1). The dry weight was greatly promoted by the old fungal inoculum, whereas mid and young fungal inocula impact this parameter to lesser extent (Fig. 3).

Compare with initial, content of N increased in willows at the harvest of 1.2 to 2.9-folds (Fig. 4A). Content of N was mostly affected by the fungal inoculum and growth medium (Table 1). The highest N content as observed in the presence of the old fungal inoculum and the high C growth medium (Fig. 4A).

Content of total P in willows, compared with initial, increased 1.5 to 5.3-folds depend on the treatment (Fig. 4B). Total content of P in the willows was greatly affected by the fungal inoculum and sterilization, to the lesser extent by the growth medium (Table 1). The content of P as dependent from the applied fungal inoculum increased in the order: old > young > mid (Fig. 4B).

Acquisition of ^{15}N and ^{33}P from the labeled bacteria by the willows was affected by the inoculum sterilization, with the unsterilized treatments yielding higher isotope transfer to the

willow than the sterilized treatments (Table 1, Fig. 5). Stronger contrast between unsterilized and sterilized treatments were noted in the presence of the young fungal inoculum (Fig. 5).

Partitioning of the biomass between roots and shoots (Fig. 6A) as well as content of ^{15}N (Fig. 6B) and ^{33}P (Fig. 6C) in roots and shoots was greatly affected by the growth medium, to lesser extent – by the fungal inoculum (Table 1). In all these cases, willows growing in the low C growth medium tended to invest more into the roots.

Table 1

P values of main factors (**G**rowth medium, **S**terilization and **I**noculum) and interactions between them (**G×S**, **G×I**, **S×I** and **G×S×I**) obtained from three-way analyses of variance (ANOVA) for different plant and soil parameters (n=5). Significance levels in ANOVA table: (ns) ($p>0.05$), * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$). Abbreviations: (DW) dry weight; (ECM) ectomycorrhizal fungi, (AMF) arbuscular mycorrhizal fungi; (UTS) fungi of uncertain trophic status. The abundance is presented as a sum of the abundances of individual fungi in three separated groups of fungi: ECM, AMF and UTS.

Parameters	Main factors			Interaction			
	Growth medium	Sterilization	Inoculum	G×S	G×I	S×I	G×S×I
Plant dry weight (g)	ns	ns	***	ns	ns	*	ns
Root: shoot ratio dry weight	***	ns	ns	ns	ns	ns	ns
P content (mg plant ⁻¹)	**	***	***	**	ns	*	ns
P content root: shoot	***	*	ns	ns	*	ns	ns
N content (mg plant ⁻¹)	**	ns	***	ns	ns	ns	ns
N content root: shoot	***	**	ns	ns	*	ns	*
Plant ³³ P content (Bq plant ⁻¹)	ns	***	*	*	ns	*	ns
³³ P content root: shoot	***	ns	ns	ns	ns	ns	ns
Plant ¹⁵ N content (mg plant ⁻¹)	ns	***	*	ns	ns	ns	ns
¹⁵ N content root: shoot	***	ns	ns	ns	ns	ns	ns
ECM abundance (copy number g ⁻¹ DW soil)	***	ns	*	**	*	ns	ns
AMF abundance (copy number g ⁻¹ DW soil)	ns	ns	***	ns	ns	ns	ns
UTS abundance (copy number g ⁻¹ DW soil)	ns	***	ns	ns	ns	**	ns
Applied ¹⁵ N in plant (%)	**	ns	***	ns	ns	*	*
Applied ³³ P in plant (%)	ns	***	**	***	**	ns	ns

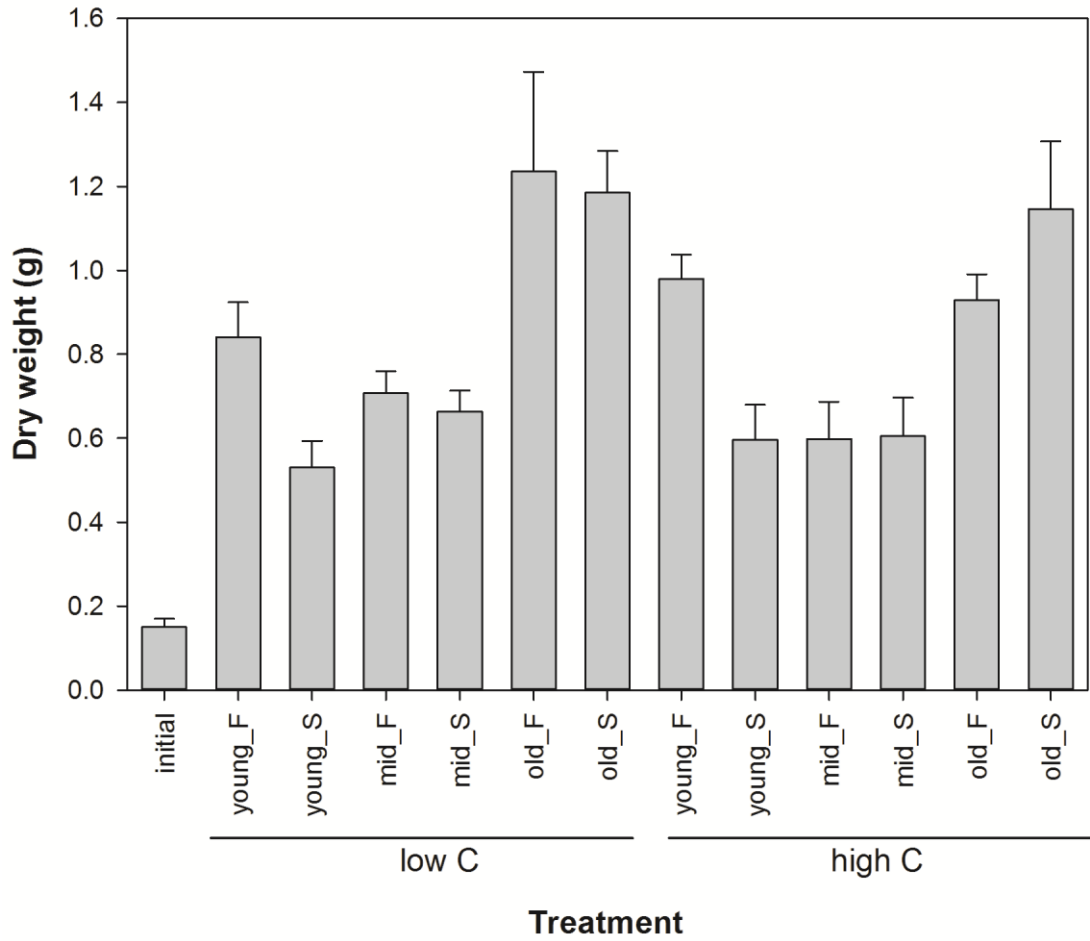


Figure 3

The dry weight of the willows (shoot and roots) at the beginning of the experiment (initial) and in all treatments. Young, mid and old refer to the type of the fungal inoculum applied as unsterilized (F) or sterilized (S). The growth media of contrasting C content are denoted as low and high C. The values are means (n=5 ±SE).

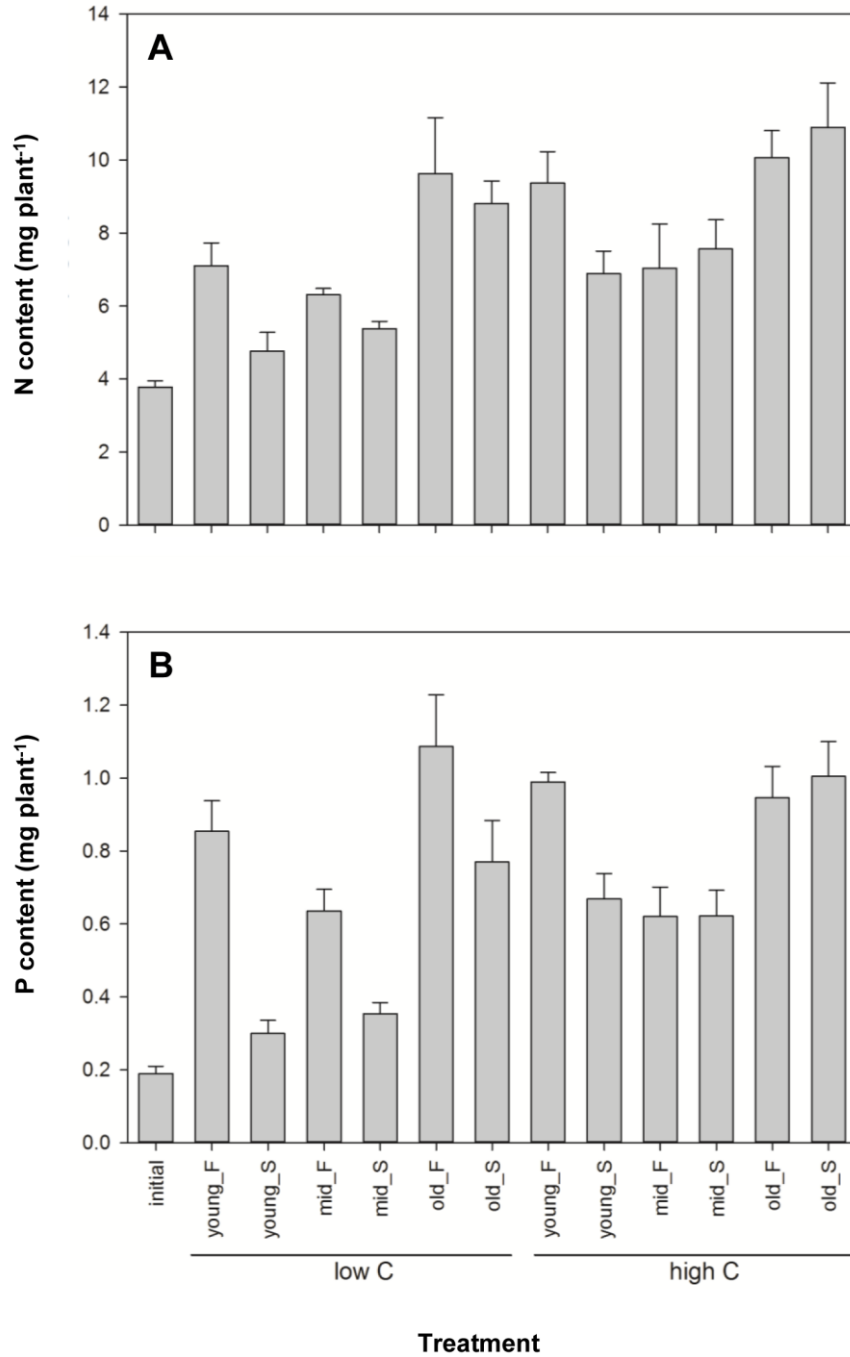


Figure 4

The content of N (A) and P (B) in the willows dry weight (shoots and roots combined) at the beginning of the experiment (initial) and in all treatments. Young, mid and old refer to the type of the fungal inoculum applied as unsterilized (F) or sterilized (S). The growth media of contrasting C content are denoted as low and high C. The values are means ($n=5 \pm SE$).

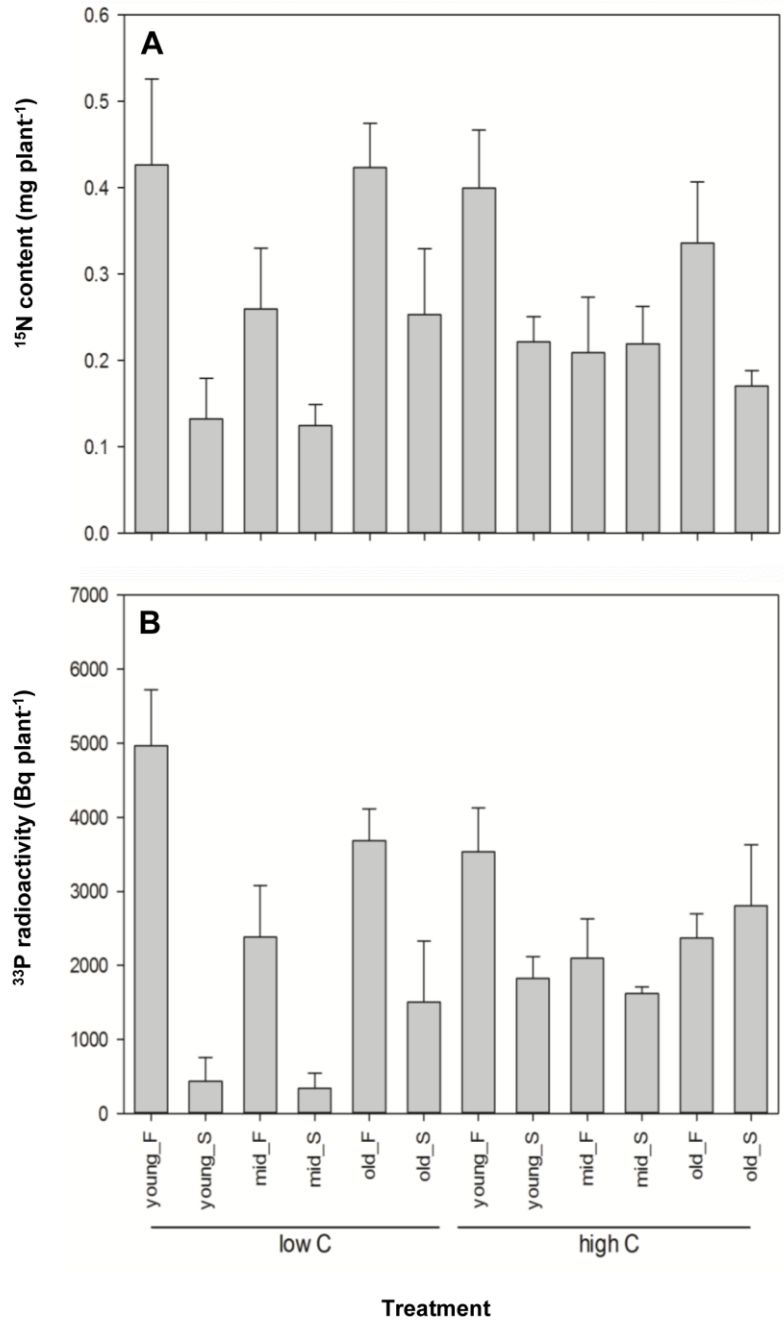


Figure 5

Content of ^{15}N (A) and activity of ^{33}P (B) in the willows (shoots and roots combined) in all treatments. Initial ^{15}N and ^{33}P negligible or not detected, respectively. Young, mid and old refer to the type of the fungal inoculum applied as unsterilized (F) or sterilized (S). The growth media of contrasting C content are denoted as low and high C. The values are means ($n=5 \pm \text{SE}$).

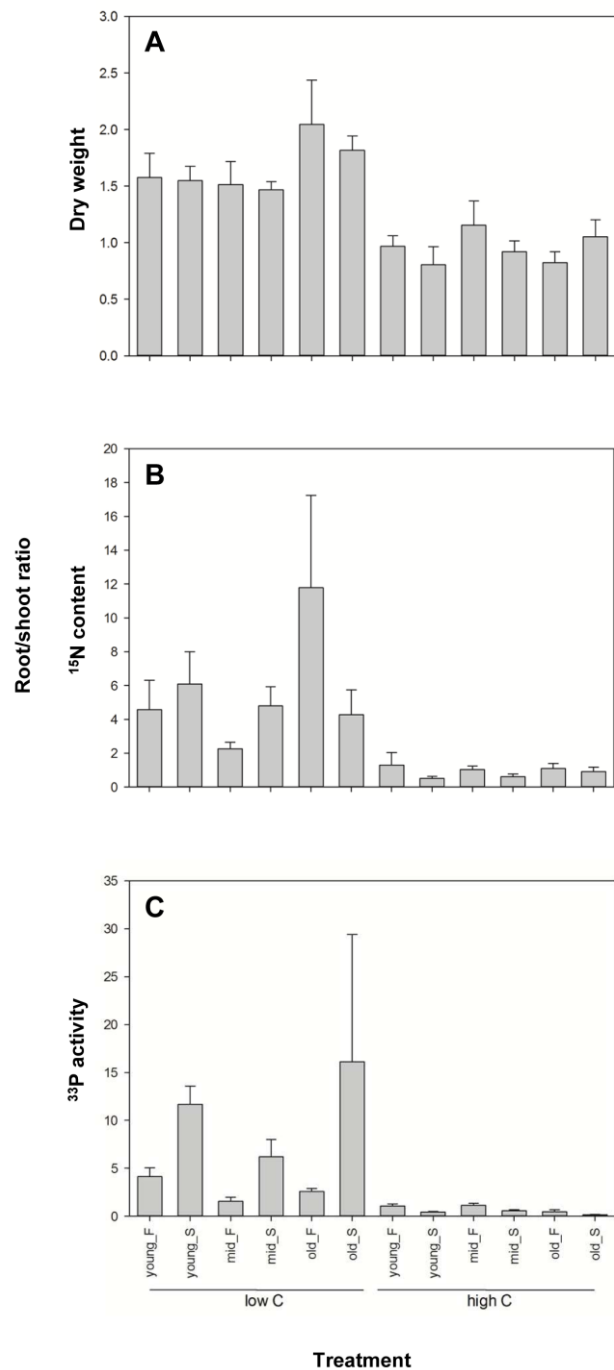


Figure 6

Root/shoot ratio of the willow dry weight (A), content of ¹⁵N (B) activity of ³³P (C). Young, mid and old refer to the type of the fungal inoculum applied as unsterilized (F) or sterilized (S). The growth media of contrasting C content are denoted as low and high C. The values are means (n=5 ±SE).

2. Soil fungal communities as affected by different fungal inocula and the growth media

The composition of AMF, ECM and UTS was assessed only in the soils from the nutrient compartment as their presence in there can be directly linked with the transfer of ^{15}N and ^{33}P to the willow (Fig. 7). AMF were not detected in qPCR analyses.

2.1. The abundance of ECM

The growth media and the interaction between growth media and fungal inoculum sterilization were the main factors influencing the abundance of ECM in the soil (Table 1). Sterilization effectively reduced the abundance of ECM fungi in the treatments conducted with the low C growth medium (Fig. 7). *Inocybe lacera* was a dominant ECM taxon in all treatments.

2.2. The abundance of UTS

The abundance of UTS was strongly affected by the sterilization of the fungal inoculum and by an interaction between sterilization and fungal inoculum (Table 1). Although no significant impact of the fungal inoculum on this group of fungi was found, the abundance of these fungi tended to increase when old fungal inoculum was applied (Table 1, Fig. 7). The dominant fungal taxon in all soil samples was Uncultured Basidiomycota.

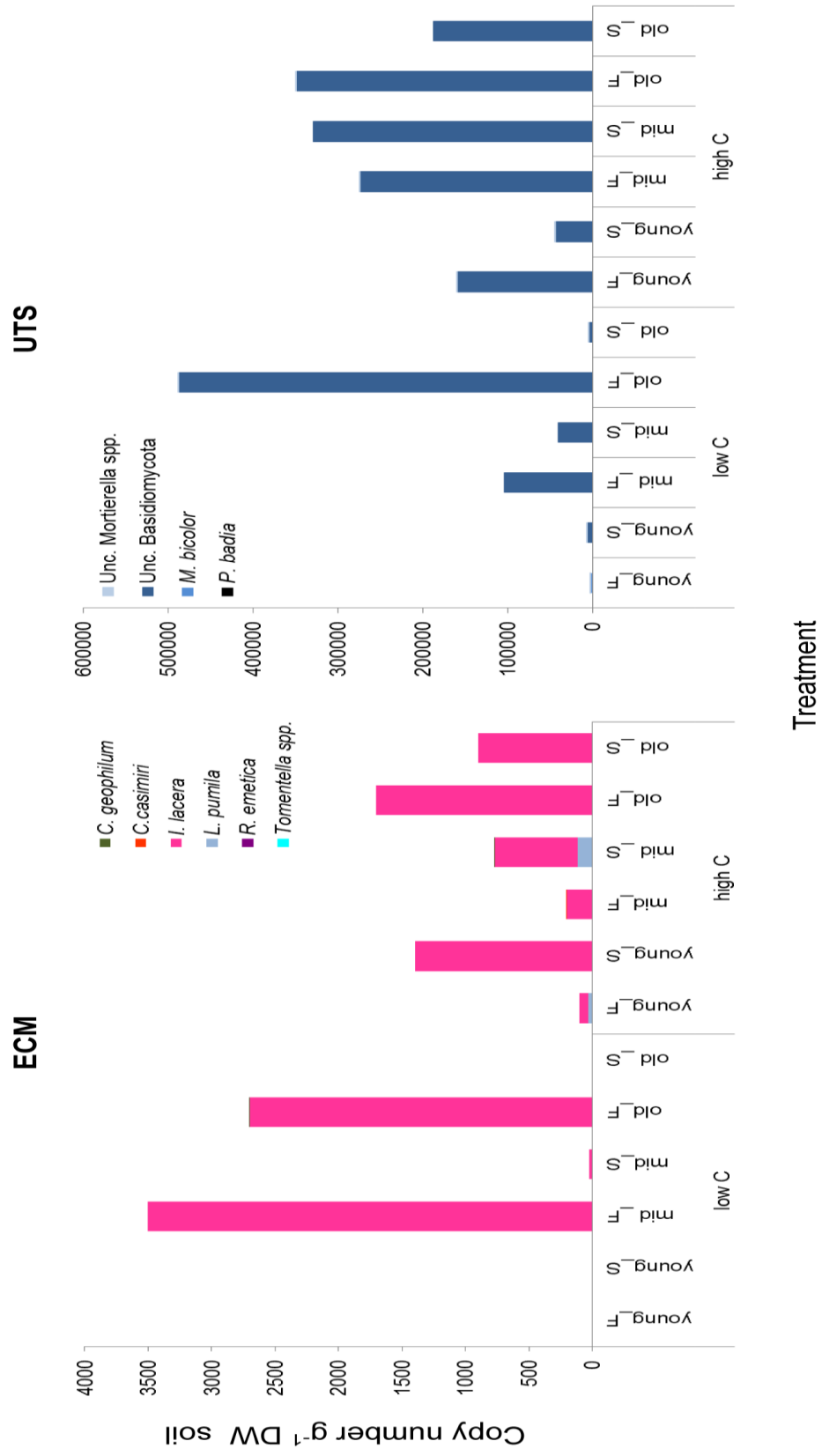


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Figure 7

The abundance of different fungi (ectomycorrhizal, ECM and uncertain trophic status, UTS) in soils from nutrient compartment of the rhizobox assessed with taxon-specific qPCR molecular markers. The results are presented for twelve different treatments. The values are means (n=5), the SE bars were not included on the graph for the sake of clarity. Fungal identities – (*C. geophilum*) *Cenococcum geophilum*; (*C. casimiri*) *Cortinarius casimiri*; (*I. lacera*) *Inocybe lacera*; (*L. pumila*) *Laccaria pumila*; (*R. emetica*) *Russula emetica*; (*M. bicolor*) *Meliniomyces bicolor*; (*P. badia*) *Peziza badia*; (*Unc. Basidiomycota*) Uncultured Basidiomycota; (*Unc. Mortierella spp.*) Uncultured *Mortierella* spp. AMF were not detected in the soil samples. Abbreviation: (DW) dry weight.

3. The transfer of ^{15}N and ^{33}P to the willows as affected by the fungal inoculum and the growth medium

Mycorrhizas formed on the roots of willows accessed and transferred ^{15}N and ^{33}P from the labeled bacteria to the plant (Fig. 5). In average, 9.4% and 14.8% of initially applied ^{15}N and ^{33}P , respectively, was transferred to the willows (Fig. 8A-B) over one month of the experiment duration. Amount of ^{15}N in willow (expressed as % of ^{15}N applied) was strongly affected by the fungal inoculum and growth medium, but also interactions between inoculum sterilization and fungal inoculum, as well as fungal inoculum, sterilization and growth medium (Table 1). Amount of ^{33}P in willow (expressed as % of ^{33}P applied) was strongly affected by inoculum sterilization and interaction between inoculum sterilization and growth medium and to lesser extend – by the inoculum itself or interaction inoculum - growth medium (Table 1).

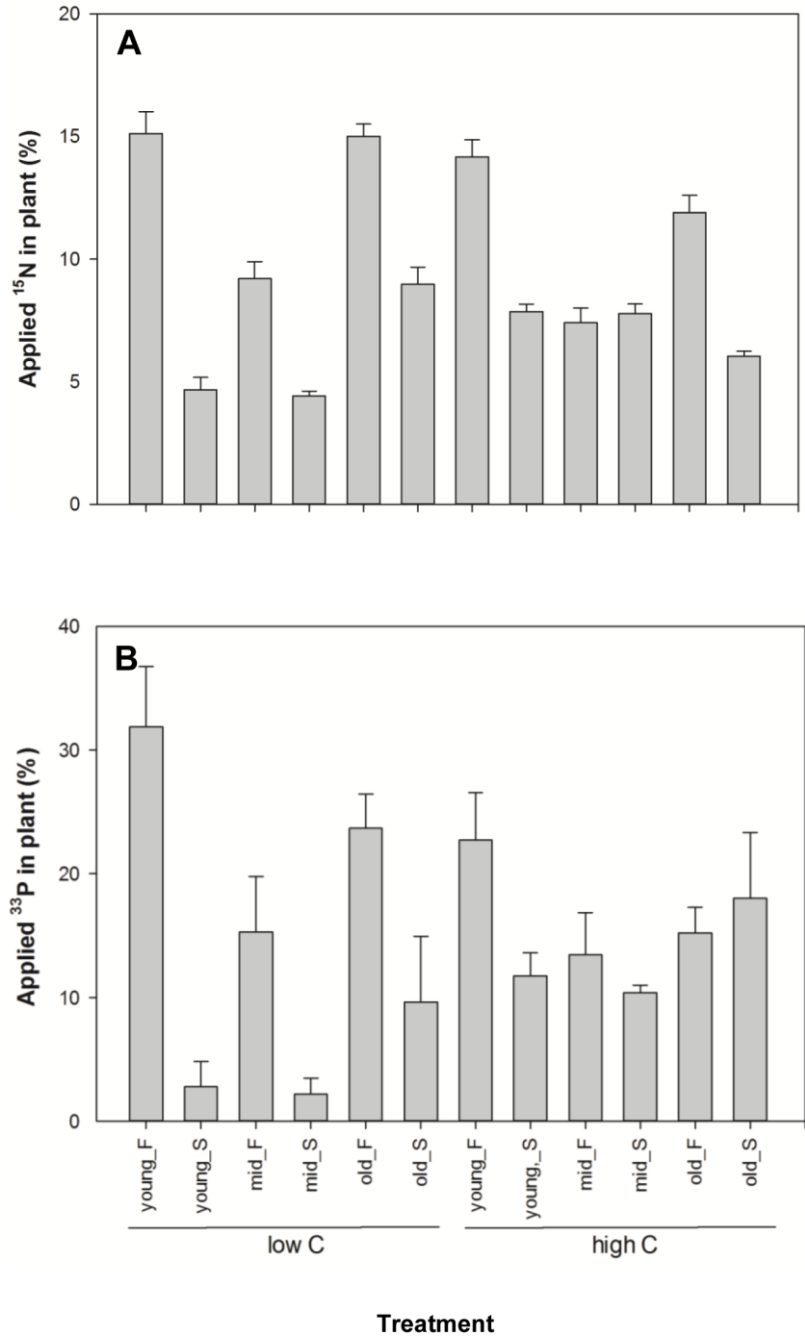


Figure 8

The percentage of ¹⁵N (A) and ³³P (B) applied to the nutrient compartment and detected in the willows (shoot and roots combined). Young, mid and old state for the type of the fungal inoculum applied as unsterilized (F) or sterilized (S). The growth media of contrasting C content are denoted as low and high C. The values are means (n=5 ±SE).

4. Relation between soil enzymatic activities and fungal community composition

Multivariate analysis relating the experimental factors to the abundances of different fungi in soil and roots, to enzymatic activities of soils, and the content of ¹⁵N and activity ³³P in the willows (Fig. 9) revealed that: i) the growth medium and fungal inoculum affected the enzymatic activity of chitinase and protease, respectively, whereas enzymatic activity of phosphatase remains unaffected by these factors; ii) the activity of protease correlated with the amount of ¹⁵N and ³³P isotopes in the willows, however these correlations vary in strength; iii) the enzymatic activity of protease, to lesser extent, chitinase, correlated with the abundances of selected fungal taxa such as Uncultured Basidiomycota (soil and roots), *Tomentella* spp. (soil) and *Peziza badia* (soil and roots). Enzymatic activity of phosphatase did not present any significant correlation with none of the fungal taxa (Fig. 9).

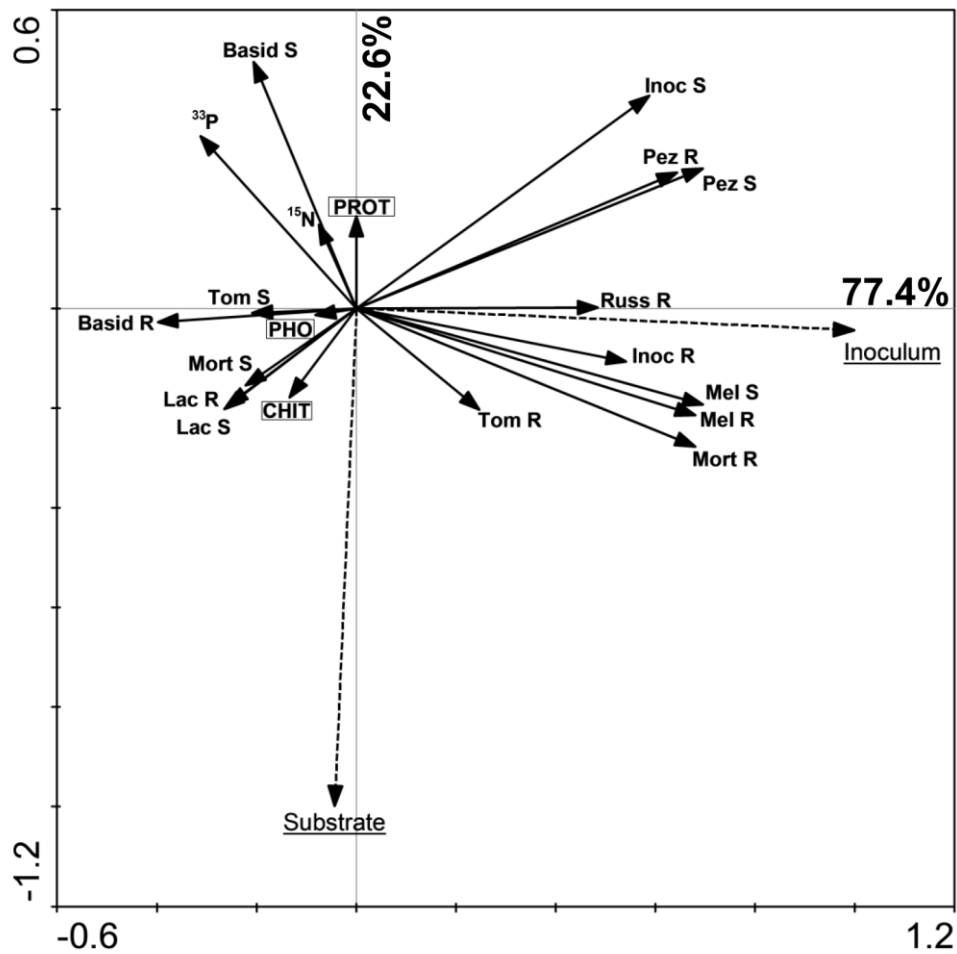


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Figure 9

Redundancy analysis ordination diagram showing dependencies between the experimental factors (i.e., fungal inoculum, growth medium and sterilization, handled as explanatory variables) and a range of explained variables: the abundances of different fungi in soil (fungus name S) and roots (fungus name R), enzymatic activities of soils, the content of ^{15}N and activity ^{33}P in the dry plant weight. Monte Carlo permutation test carried out for two canonical axes confirmed the significance of the relationship between investigated datasets ($F=4.389$, $p=0.0001$). Abbreviations: fungal identities – (Inoc) *Inocybe lacera*; (Lac) *Laccaria pumila*; (Russ) *Russula emetica*; (Tom) *Tomentella* spp.; (Mel) *Meliniomyces bicolor*; (Pez) *Peziza badia*; (Basid) Uncultured Basidiomycota; (Mort) Uncultured *Mortierella* spp.; enzymatic activities – (CHIT) chitinase; (PHO) phosphatase; (PROT) protease; plant parameters – (^{15}N) content of ^{15}N in the plant dry weight; (^{33}P) activity of ^{33}P in the plant dry weight.

Discussion

1. Impact of fungal communities on the willow growth and nutrition

Willow biomass was strongly affected by the fungal inoculum (Table 1). Since mid inoculum was collected from the glacier forefield amply colonized by willows, this fungal inoculum was expected to influence the biomass and nutrition of willows to the strongest. However, the highest biomass accumulation was noted for the treatments where old fungal inoculum was applied. This suggests that: i) our experimental conditions had favored the old inoculum, or ii) fungal communities sampled from the middle part of the glacier forefield could be less adapted to the young plants used in our experiments (approximately four months old). Apparently less willow-specific old fungal inoculum (i.e., willows on the site from which old inoculum was collected consist only approximately 20 to 30% of vegetation cover, personal observation) provided fungal communities better colonizing the roots of the young willows. It is, however, difficult to state that fungi will provide better benefits to the host plant under the

optimal (environmental) conditions. Moreover, enhanced growth and nutrition of the willows is a result of the higher abundance of fungi in the presence of old inoculum rather than their actual functioning. This, however, will only be true when the correlation between fungal abundance and functionality can be proved.

The evidences exist, that different mycorrhizal fungi differ in the effectiveness of the nutrients transfer to the plants (Jansa et al., 2008; Pritsch and Garbaye, 2011; Thonar, 2009). Because in the natural systems environmental conditions fluctuate in space and time, it is impossible to determine the conditions optimal for all fungi contemporarily. Furthermore, fungal identity, developmental stage of mycorrhiza, interaction of fungus with the host plant as well as another components of the microbial community should be expected as indigenous factors influencing mycorrhizal functioning.

Fungi associated with the willows were able to uptake ^{15}N and ^{33}P from labeled bacteria and to transfer them to the willows (Fig. 5). Sterilization of the fungal inoculum the uptake of ^{15}N and ^{33}P by willows (Table 1). This clearly indicates the role of fungi in the transfer of these nutrients to the willows. When grown in the low C growth medium, willows tended to accumulate more biomass and more ^{15}N and ^{33}P in the roots than in the shoots (Fig. 6A-C). Better growth of roots and higher content of ^{15}N and ^{33}P in the roots suggests that ^{15}N and ^{33}P are sequestered either in the root tissues or in the fungal structures. Other possible explanation is that higher accumulation of ^{15}N and ^{33}P in the roots can be only superficial and driven by the high root biomass rather than sequestration in strict sense. The latitude, nutrient and water availability as well as tree age were noted as important factors influencing root: shoot ratio under the field conditions (Gebauer et al., 1996; Gower et al., 1994; King et al., 2007). None of the studies, to the best of our knowledge, aimed exclusively to assess the impact of mycorrhizal symbioses on root: shoot ratio in laboratory experiments yet. Last but not the least, enhanced development of the root system was treated as an indicator of the environmental stresses. While plant need to explore soil in search of water or nutrients, more biomass is invested into roots (Marschner, 2012). Growth medium of high C provided not only more C but also N, P and other elements. This can explain smaller root system which anyway seemed to gather sufficient amount of nutrients (and water) to cover willow's nutritional requirements.

2. Effect of the fungal communities on the transfer of ^{15}N and ^{33}P to the willows

The transfer of ^{15}N and ^{33}P to the plant was probably done by UTS. Their abundance (Uncultured Basidiomycota and *P. badia*, root and soil) clearly correlated with the amount of ^{15}N and ^{33}P taken up by the willows (Fig. 9). Interestingly, out of several ECM fungi, only the abundance of *Tomentella* spp. (roots) correlated with the transfer of ^{15}N by the willows (Fig. 9). These observations contradict the common paradigm of active participation of ECM fungi in the uptake of nutrients from organic sources (Finlay, 2008; Pritsch and Garbaye, 2011). Several possible explanations of obtained results can be proposed here. In other studies fungal abundance (e.g., as hyphal length density) correlated with the uptake of ^{33}P by the plants (Jansa et al., 2005). But our understanding of the link between fungal abundance and functioning is still poorly understood. How far these two parameters affect each other and if the correlation between them exist, remains unclear.

Additionally to that, we need to accept that molecular techniques used nowadays in ecological studies have a limited power in identification of fungi from natural environments. Furthermore, the bigger problem occurs when function of each taxon is of concern. We can not completely exclude the possibility, that Uncultured Basidiomycota will not be in fact another ECM. Additionally, qPCR molecular markers employed in our study allowed us to detect probably minority of the fungal taxa. Thus, it is possible that other, undetected ECM fungus/i participated in the transfer of ^{15}N and ^{33}P to the willows.

Second, although participation of Uncultured Basidiomycota in the transfer of nutrients to the willows can not be completely negated, mechanism behind this transfer is difficult to explain. Evidences exist that mycorrhizas interact with saprotrophic fungi in transfer of nutrients to the host plant. In the microcosm experiment, Lindahl et al. (1999) demonstrated bidirectional transfer of ^{32}P between mycorrhizal and saprotrophic fungi (Lindahl et al., 1999). ^{32}P accessed by ECM from wood-decay fungus *Hypholoma fasciculare* was subsequently translocated to the pine seedlings. The transfer of ^{32}P in the opposite direction - from the labelled mycorrhizal mycelium to the saprotroph - although one (*Paxillus involutus*) to two (*Suillus variegatus*) orders of magnitude lower, was also observed (Lindahl et al., 1999).

Enzymatic activity of fungi can be proposed as a main mechanisms of acquisition of ^{15}N and ^{33}P from ^{15}N - ^{33}P -labeled bacteria (Fig. 9). Protease seem to be responsible for release of ^{15}N from bacterial proteins. Chitinase present relatively high activity but its impact

on the release of ^{15}N was not expected as bacteria do not synthesize this polymer (Madigan and Martinko, 2006). Impact of phosphatase on release of ^{33}P is negligible. Thus, either P was hydrolyzed from other organic compounds by other enzymes (e.g., nucleic acids by nucleases).

Conclusions

- In this study, mycorrhizal associations were established with *Salix helvetica* from inocula of indigenous fungi. The communities of indigenous fungi (originating from soils deglaciated 16, 65 and 116 years ago, for young, mid and old fungal inoculum, respectively) were applied either unsterilized or sterilized (serving as a control) and were combined with growth media of low and high organic C content. Such a set-up aimed to model young and old stage of ecosystem development (i.e., deglaciated 8 to 12 and 111 to 117 years ago).
- In order to track the impact of fungal communities on the willow growth and nutrition, we applied ^{15}N - ^{33}P -labelled bacteria as a source of N and P.
- We demonstrated that different fungal inocula (i.e., different ages, sterilized vs unsterilized) translated into different growth and content of N, ^{15}N , P and ^{33}P in willows. Additionally, we demonstrated the ability of UTS to transfer the nutrients to the willows. This fungi were suggested to exude protease to efficiently hydrolyze ^{15}N - ^{33}P -labeled bacterial biomass.

Acknowledgments

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Chapter 4 - supplementary material
Supplementary figures

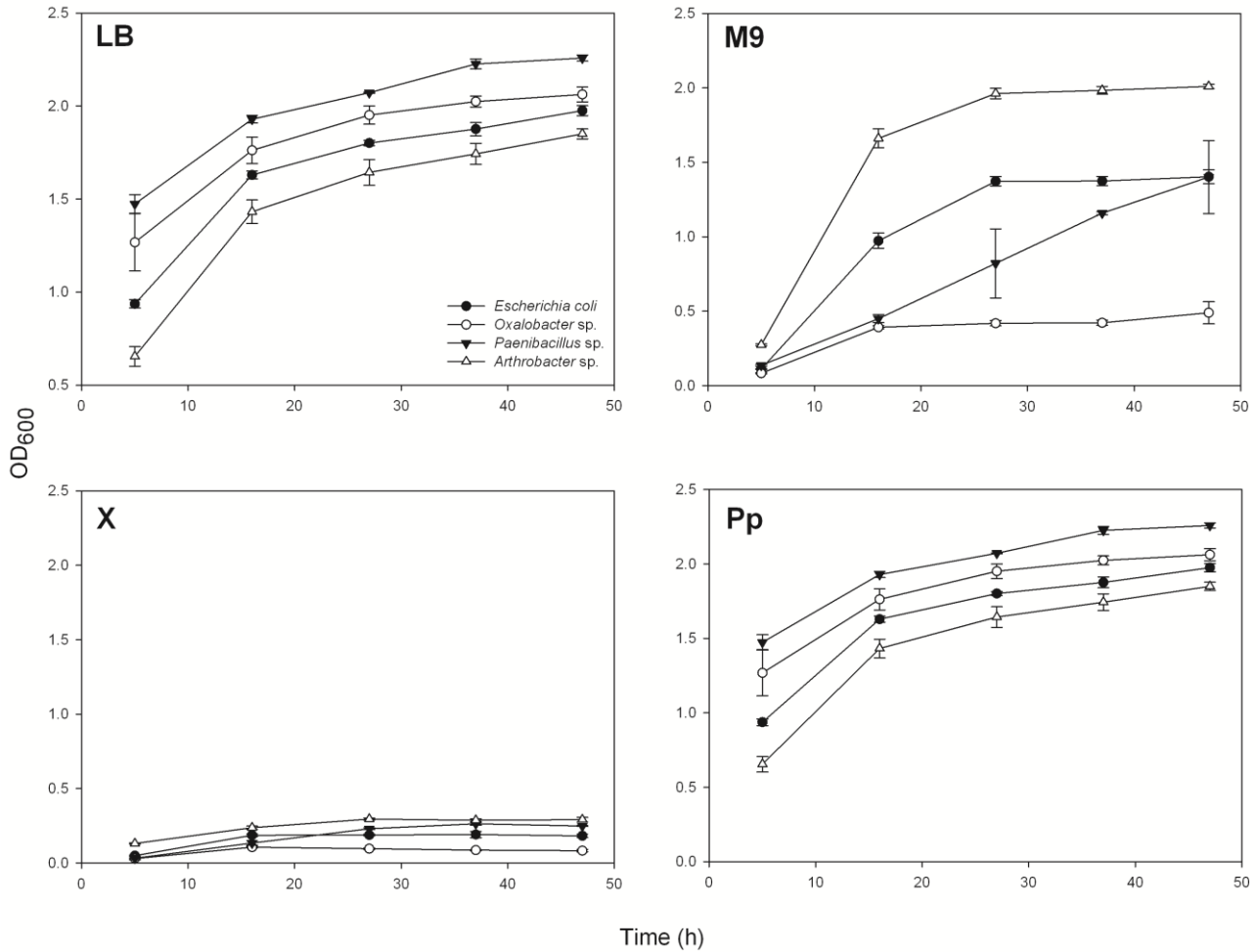


Figure S1

Test of bacteria cultivation on four different liquid media: LB (Luria Bertani), M9, X, and Pp (Pachlewski-Pachlewska) medium. Values are means (n=4) of the optical density (OD=600 nm, UV 1601 Spectrophotometer, Shimadzu, 1.5 mL cuvette) measured for bacterial cultures at regular time intervals (5, 16, 27, 37 and 47 hours) after media inoculation. Bacteria identities – *Escherichia coli* (JM109 phenotype, pGEM[®]-T Easy Vector System, Cat. no. A1380, Promega, Switzerland), *Oxalobacter* sp., *Paenibacillus* sp., and *Arthrobacter* sp. courtesy of Dr Beat Frey, Swiss Federal Institute WSL, Switzerland) (Frey et al., 2010).

Chapter 4

Bacterial cultures were incubated at 30°C upon shaking at 2.3 Hz. Composition of the media: (LB) Luria Bertani medium, ingredients (g/L): Bacto-tryptone (15.0), Bacto-yeast extract (7.5) and NaCl (15.0), pH=7.0 (Sambrook et al., 1989); M9 medium (see **Preparation of ¹⁵N-³³P-labeled bacteria**); (X) medium for *E. coli* (Ihssen and Egli, 2004), modified, ingredients (g/L): (NH₄)₂SO₄ (0.81), KCl (0.08), NaH₂PO₄ × H₂O (0.48), glucose (10.00), (μg L⁻¹): H₃BO₃ (15), MnSO₄ × 7H₂O (0.8), ZnSO₄ × 7H₂O (6), CuSO₄ × 7H₂O (1), (NH₄)₆Mo₇O₂₄ × 4H₂O (0.2), and FeC₆H₅O₇ (0.12), pH=7.0; (Pp) Pachlewski-Pachlewska medium (Pachlewski and Pachlewska, 1974), ingredients (g/L): NH₄NO₃ (0.22), KH₂PO₄ (0.50), MgSO₄ × 7H₂O (0.50), CaCl₂ (5.00), glucose (10.00), (μg L⁻¹): MnSO₄ × 7H₂O (0.5), ZnSO₄ × 7H₂O (2.5), CuSO₄ × 5H₂O (2.5) and FeC₆H₅O₇ (0.12), pH=7.0.

General discussion, conclusions and outlook

General discussion

The overall objective of the project was to characterize the size, composition and functioning of the mycorrhizal fungal communities at different stages of ecosystem development. We did not restrict ourselves to a description of each of these features, but - in order to understand the role of mycorrhizal communities in the ecosystem development and functioning - we also attempted to link them. Since the individual results were discussed separately in each chapter, here I would like to provide more synthetic view and discuss the results in a broader context.

The **size** is an important parameter describing the microbial community (including mycorrhizal fungi). It is also used as an indicator of soil quality, soil health and/or restoration success (Lauer et al., 2011; McKinley et al., 2005; Moeskops et al., 2012; Nielsen and Winding, 2002). Numerous investigations assessed the size of bacterial and saprotrophic fungal communities while fewer studies exist for mycorrhizal fungi (Bååth, 2003; Olsson, 1999; Olsson et al., 1995). Biomarkers such as PLFA, ergosterol, amino sugars or muramic acid are commonly used to assess the size of microbial communities or certain higher-ranking taxa (Glaser et al., 2004; Klamer and Bååth, 2004). Despite some clear linkages to overall soil microbial activity, the size of microbial communities offers only a very crude proxy for functional importance of microbial communities in soil (Frostegård et al., 2010).

Many studies describe the **composition** of microbial communities in soils, water bodies' sediments, and other environments (Dees and Ghiorse, 2001; Garbeva et al., 2004; Smit et al., 2001; Smit et al., 1997; Torsvik et al., 1996). With respect to mycorrhizal fungal communities, a number of studies addressed mycorrhizal abundance and diversity in different ecosystems or different stages of ecosystem development (Blaalid et al., 2011; Dhillon, 1994; Mühlmann and Peintner, 2008a; Tedersoo et al., 2007). But any list, no matter how detailed and whether including the quantitative information or not, of mycorrhizal taxa identified in a given ecosystem, is still rather uninformative in relation to the ecosystem functions of these microbes. This is mostly because we cannot clearly link identity of microbes with their ecosystem function, with exception of a few well defined groups such as symbiotically living diazotrophs or lignin degraders. Additionally to that, numerous taxa still

remain unidentified (e.g., Uncultured *Mortierella* spp. and Uncultured strain of Basidiomycota in our study), which limits the exact and functionally relevant characterization of the microbial community.

Finally, investigations of the **functions** of mycorrhizal communities present a complex task. It can involve studies under controlled conditions, with individual fungal isolates or their communities (Nygren and Rosling, 2009; Thonar, 2009), molecular studies on expression of functional genes of the fungi and/or the associated plants (Tatry et al., 2009), or use of isotopes, either embedded in specific substrates (e.g., ^{14}C in lignin (Šnajdr et al., 2010) or involving system compartmentalization (root and hyphal compartments, Facelli et al., 2010; Jansa et al., 2005; Pearson and Jakobsen, 1993; Smith et al., 2003). There is still limited number of papers describing such efforts and this thesis presents one of the larger study carried so far in this direction.

The combination of information on the **size, composition** and **functioning** of mycorrhizal communities, in the natural ecosystem is indispensable in order to understand microbial contribution to ecosystem functioning, development and sustainability (Bell et al., 2009; Brockett et al., 2012; Godbold, 2005; Waldrop et al., 2000) (Fig. 1). However, some precautions should be kept in mind in order to link the **size, composition** and **functioning** of fungal communities. Namely, fungal communities are greatly interconnected in a number of ways with both **abiotic** and **biotic** components of the ecosystem. This is especially important since the ecosystem is spatially and temporarily heterogeneous and these linkages are potentially changing in both space and time.

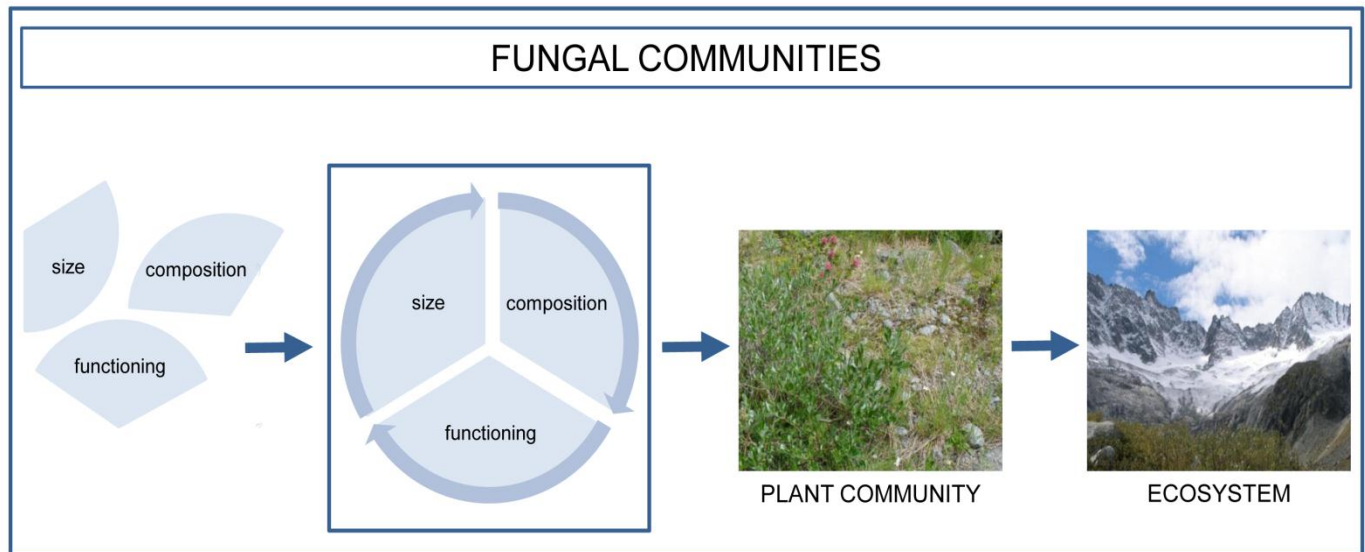


Figure 1

Link between the size, composition and functioning of fungal communities in an ecosystem context.

Evidence exists that **abiotic** conditions greatly influence the size and composition of soil fungal communities (Fig. 2), however the impact on their functions still remains poorly understood. We confirmed here that the size of arbuscular mycorrhizal (AMF), ectomycorrhizal (ECM) but also bacterial communities were strongly affected by the soil pH (chapter 1). These observations stay with agreement with previously published data (Coughlan et al., 2000; Gryndler et al., 2006).

Biotic soil properties can influence the size, composition and functioning of mycorrhizal fungal communities (Fig. 2). We also demonstrated that the abundance of non-mycorrhizal fungi (termed as uncertain trophic status fungi, UTS) was stimulated by the higher content of C in the growth medium, which should be regarded as a biotic factor. Such a trend confirms saprotrophic preferences of this group of fungi. Increase of the biomass of saprotrophic fungi following the increase in soil organic matter accumulation was also observed in primary succession at the glacier forefields (Bardgett, 2000; Ohtonen et al., 1999). Based on the rhizobox experiments performed in the framework of this doctoral project we demonstrated

that biotic conditions strongly affected the function of willow-associated fungal communities (chapter 4). Fungal communities developed in the presence of high C growth medium (higher content of C but also N, P and other nutrients) were more effective in the transfer of ^{15}N and ^{33}P to the willows. Thus it was hypothesized that mycorrhizas enhanced the transfer of nutrients mostly in the high C substrate, because the fungal communities developing under such conditions would be best adapted to the particular environmental conditions (Fig. 2). Likewise, because of large substrate pools of C (and the organic nutrients), the contrast between mycorrhizal and non-mycorrhizal treatments should be most prominent under high C soil conditions. Indeed, mycorrhizas were shown to adapt to the environment and this, in turn, to amplify their infectivity and to enhance certain fungal-mediated ecosystem functions. This somehow resembles earlier results by Henkel et al. (1989), where four isolates of AMF from arid plant community were tested for their infectivity. AMF presented higher infectivity in the soil from which they originated, whereas they were less infective in soils of the properties contrasting with their original soil. Highly infective AMF were then claimed to be more adapted to experimental conditions (Henkel et al., 1989).

The roots and rhizosphere of the host plant induce numerous changes in the associated fungal communities (chapter 2), as recently observed in high Arctic, alpine and other ecosystems (Becklin et al., 2011a; Fujimura and Egger, 2012; Sýkorová et al., 2007; Tran and Cavagnaro, 2010; Vandenkoornhuysen et al., 2003). Plant roots and rhizosphere act as discrete and very selective niches shaping the community of mycorrhizas and other microbes. We could observe, for example, that the abundances of AMF, ECM and UTS fungi in unvegetated soil were lower, as compared with rhizosphere soils (chapter 2). This can be interpreted as evidence for dependency of all these fungal groups on plant-derived C, either exchanged symbiotically with mycorrhizal fungi (Leake et al., 2001), or originating from exudates and root debris (Vasiliauskas et al., 2007). Moreover, it is well recognized that mycorrhizal fungi can differ in their compatibility¹ toward a host plant (Smith and Read, 2008). For example, ECM fungus, *Suillus grevillei* establishes association almost exclusively with *Larix* spp., whereas *Russula ochroleuca* can be hosted by a number of coniferous and deciduous tree species such as *Abies*, *Larix*, *Picea*, *Pinus*, *Betula*, *Carpinus*, *Fagus*, and

¹ 'Compatibility' is defined here as the range of potential mycorrhizal symbionts that are available for a host plant to establish mycorrhizal association (Smith and Read, 2008).

Quercus (Agerer, 1987-2002). Alpine willows (i.e., *S. herbacea* and *S. polaris*) were found to be colonized by a range of ECM from which none was restricted to the willows only. ECM such as *Cenococcum geophilum*, *Sebacina* spp., *Cortinarius* spp., *Entoloma* spp., *Laccaria* spp., *Lactarius* spp. and *Tomentella* spp. occurred frequently on the roots as well as fruit bodies in understory of willow bushes (Graf and Brunner, 1996; Mühlmann and Peintner, 2008a; Ryberg et al., 2010; Schmidt et al., 2011). Similar ECM taxa were also detected for *Salix helvetica* in our research.

An important original finding in the presented project was that the composition of the root-associated fungal communities reflected the enzymatic activity of the rhizosphere soil (chapter 2). Higher activity of chitinase and β -glucosidase detected in the rhizosphere soil of willow suggest exudation of these enzymes by the associated ECM. In work of Pritsch and Garbaye (2011) different ECM fungi were found to exude different enzymes (Pritsch and Garbaye, 2011). For example, *Cenococcum geophilum* exuded more phenoloxidase than other ECM, whereas *Russula ochroleuca* presented higher activity of phosphatase and chitinase (Pritsch and Garbaye, 2011). The examination of enzymatic activities of ECM tips conducted in our project did not present such a clear picture, probably due to the temporal heterogeneity of the investigated system. We could, however, observe some tendencies in the enzymes exudation profiles (chapter 2, Fig. S7). While the higher activity of cellulase and xylosidase was observed for *Cenococcum geophilum*, protease was exuded in relatively high amounts by *Tomentella* spp. and *Russula emetica*. Notable amount of protease was released by *Meliniomyces bicolor* typically associated with ericoid plants. These observations invited some speculations on the role of different ECM fungi in determining the exudation of hydrolytic enzymes by the ectomycorrhizal root tips (Fig. 3). This in turn, would contribute to exploration of different nutrient sources by different ECM taxa, which may be important for functional partitioning in the rhizosphere. In evolutionary view, such partitioning would stabilize spatial and temporal coexistence of the different ECM because the competition for the same nutrient resources by the ECM taxa will be weakened. Additionally, having access to the different nutrient sources provided by the ECM, plants are more plastic and protected from nutrient deficiency when one of the nutrient sources become scarce or exhausted. Such a phenomenon was previously observed for AMF. According to Koide's theory of functional complementarity, plants colonized by multiple AMF will receive more nutrients

mobilized from the different soil pools than plants colonized by a single AMF (Jansa et al., 2008; Koide, 2000; Smith et al., 2000). Based on enzymatic activity assays we can speculate that functional complementarity can also be true for the ECM (Fig. 3), although clear evidence for that is still to be gathered.

Concerning ECM compatibility between different willow species colonizing the same ecosystem, Ryberg et al. (2011) proved that *S. herbacea* and *S. polaris* hosted similar ECM communities and they both presented similar frequencies of colonization on both host species. Interestingly, some ECM taxa such as *Inocybe egenula*, *Russula cf. emetica*, and *Tomentella* sp. were found in meadow ECM communities whereas *Sistotrema cf. alboluteum* and *Tomentella cf. terrestris* were only found in the dry and mesic heath communities (Ryberg et al., 2010). In our research we did not specifically measure water availability at each experimental site. We speculate, however, that ECM community composition was driven more by the presence of the host willow rather than the soil properties, although the willow was colonized by different communities at different sites according to their soil age. The roots of willows colonizing sites deglaciated only recently (approximately 7 to 12 years ago) were mainly colonized by *Laccaria pumila* (chapter 2). When the coverage of willow canopy increased (sites deglaciated approximately 60 to 70 years ago), *Russula emetica* became a dominant ECM taxon. Broad ability of willows in hosting diverse ECM can be regarded as a broad ecological plasticity of this plant species. Compatibility of willows with only a narrow group of ECM would limit their survival in a harsh alpine ecosystems where the distribution, survival and germination of ECM propagules can be limited (Gardes and Dahlberg, 1996). In order to support their growth and development, willows are able to establish ECM with variety of ECM fungi (or even other fungi, including endophytes, Trowbridge and Jumpponen, 2004) which can also be shared with other plant species. For example, *S. helvetica* in our research was colonized with *Meliniomyces bicolor* regarded as the common ericaceous symbiont (Kohout et al., 2011).

Willows can also impact the size, composition and functioning of mycorrhizal fungal communities indirectly, through the litter fall. As proved by Becklin et al. (2011), alpine willows suppressed the AMF colonization of neighbouring herbaceous plants (Becklin et al., 2011b). This suppression was seemingly caused by the allopathic effect of willow- and ECM-derived secondary compounds on the AMF. Moreover, the negative effect on AMF was

postulated to be a result of the high colonization of willows by competitive ECM which restricted availability of nutrients for the AMF. Whether litter accumulation under the canopy of *S. helvetica* in our ecosystem (chapter 2) affects the colonization of AMF, remains unclear. Herbaceous plants investigated in our project were sampled away from *S. helvetica* thus the impact of the litter on their AMF colonization was probably not very strong (chapter 2).

Increasing attention is recently turned to the impact of the interactions **mycorrhiza-mycorrhiza** and **mycorrhiza-other microbes** in shaping the size, composition and functioning of the fungal communities (Bending et al., 2006; Linderman, 1988) (Fig. 2). Although our study did not focused exclusively on any of these issues, we presumed that massive abundance of *Inocybe lacera* in pot experiments was likely to be explain by: i) competition; *I. lacera* was not only ECM introduced to in these experiments from fungal inocula (chapter 3) and its high abundance could be possibly explained by high competitiveness of this ECM taxon in the rhizoboxes, or ii) facilitation; we could speculate that certain strains of bacteria, introduced to the pots with fungal inoculum, acted as mycorrhizal helper bacteria (MHB) (Aspray et al., 2006; Bending et al., 2002; Duponnois and Plenchette, 2003) promoting formation of ECM by the *I. lacera*.

Last but not the least; other non-mycorrhizal fungi frequently assist mycorrhizas in the natural systems (Hijri et al., 2002; Tedersoo et al., 2009a; Weiß et al., 2011). Although their exact role for the plant remains largely unknown, we could speculate that these fungi interact with the other microbes in the ecosystem. In our experiment, for example, Uncultured Basidiomycota, (i.e. a fungus of unknown identity and ecological function) seemed to contribute to the uptake of nutrients by the willows (chapter 4), although exact mechanism behind this uptake remains speculative. However, as revealed by Lindahl et al. (1999), bidirectional transfer of ^{32}P existed between mycorrhizal and saprotrophic fungi. ^{32}P accessed by ECM from wood-decay fungus *Hypholoma fasciculare* was subsequently translocated to the mycorrhizal pine seedlings. The transfer of ^{32}P in the opposite direction - from the labelled mycorrhizal mycelium to the saprotroph - was also observed (Lindahl et al., 1999). Such an interaction not only prove functional interaction between ECM and other fungi but can be regarded as an additional factor affecting ECM community size and structure.

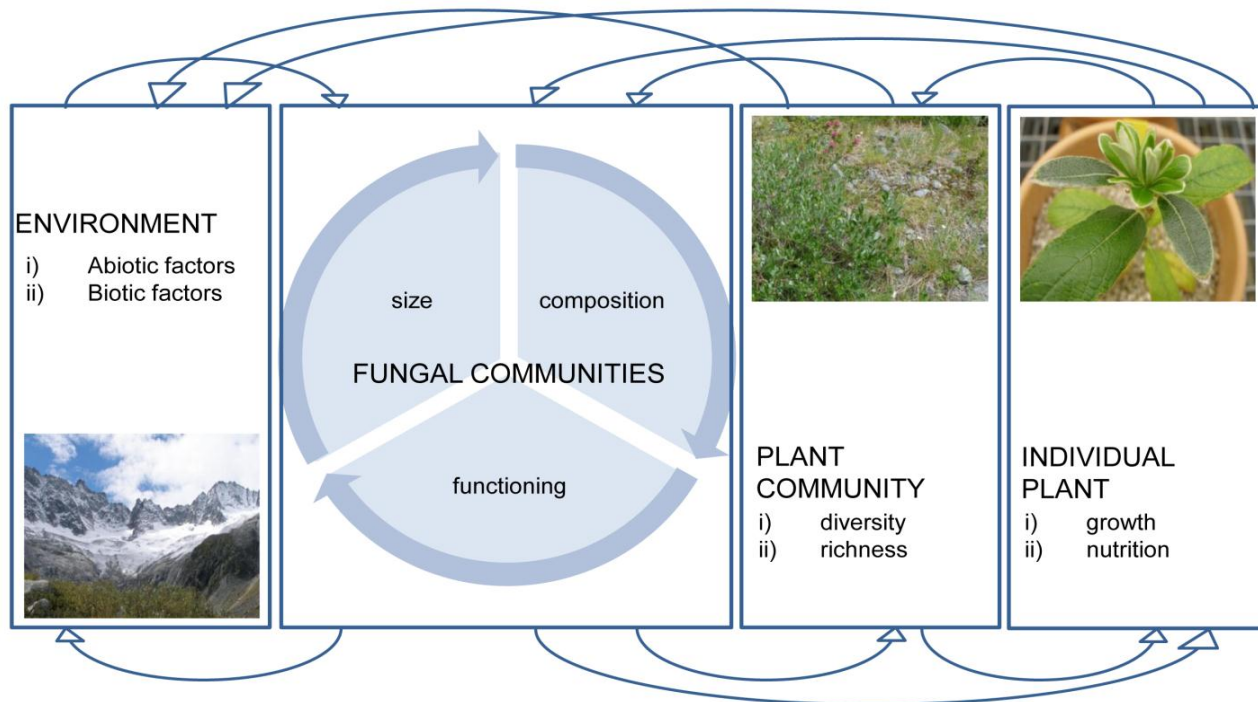


Figure 2

Schematic representation of the influences and feedbacks between the fungal community (size, composition and functioning), plants (plant community and individual plant) and the environment.

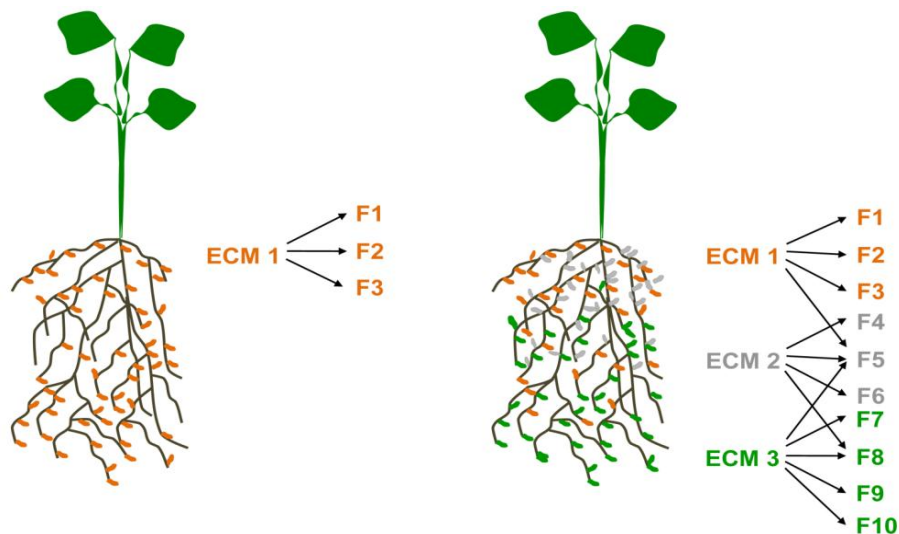


Figure 3

Schematic representation of functional complementarity of ECM. Different colors of root tips indicate different ECM taxa and are encoded as ECM 1 to 3. Numbers F1 to F10 refer to different ecosystem functions.

Conclusions

In our research we attempted to link the size, composition and functioning of the mycorrhizal fungal communities at different stages of ecosystem development. However, this task was difficult to achieve due to the heterogeneity of the investigated ecosystem itself as well as limited power of the used methods. We are fully aware that qPCR approach (chapters 2 to 4), although giving reliable insights into fungal abundance, covered only a part of the fungal community rather than the diversity in the broader sense. We recognized that this heterogeneity affected also enzymatic activities of soils and ECM root tips (chapters 2 and 4). Additionally to that, enzymes released to the rhizosphere soils could not be assigned to individual mycorrhizal type (Tabatabai and Dick, 2002). This problem, however, will probably still persist in ecological studies until we will not be able to assign the enzymes *in situ* to a single cell or organism. It is also worth to mention that enzymatic activity assays performed for ECM root tips seem to be also biased. For example, we recognized that even ECM morphotypes of the same taxa varied markedly in enzymatic activity profiles (chapter 2). Coming to the end, we also need to state that the pot experiments, even though conducted under the conditions highly relevant to the natural situation (e.g., soils and plant species from natural ecosystem, temperature and aerial humidity in the growth chamber similar to the field), cannot be always treated as a proxy of the field situations (chapters 2 to 4, e.g., the abundance of the ECM).

Outlook

Further research should be conducted predominantly in the field whereas lab experiments should only be complementary to them. From a practical point of view, appropriate technical tools to link the size, composition and functioning of the fungal communities are currently available. Enormous efforts have been invested in developing the devices and establish technical methods (e.g., molecular and biochemical) giving deep insights into the black-box of microbial communities. Thus, the future scientific efforts could well be directed now to:

- i) Understand not only **WHO** is in the ecosystem but **WHAT is it doing there** in the broader view, not restricted only to enzymatic activity assays, functional genomics or other proxies, but also to measurement of the realized fluxes of water and nutrients, tolerance to pests and environmental stresses etc.

- ii) Understand what is the meaning of the size and the composition of mycorrhizal fungal community for the functioning. Is functional complementarity theory valid for the ECM and/or other microbes associated with them? How does the size of the mycorrhizal fungal community affect the functions? What is the dominant factor shaping the composition of mycorrhizal fungal communities (e.g., environment, host plant, interactions mycorrhiza-mycorrhiza or mycorrhiza- other soil microbes)?
- iii) Establish a reliable method to assign enzymatic activities to individual mycorrhizal fungi in situ. This could be achieved by functional genomics tools, either on the level of nucleic acids or protein sequences.

Collection of more complex data sets on the size, composition and functioning of the mycorrhizal fungal communities could help us to model the fluctuations, changes and succession of mycorrhizas in natural ecosystems. These will give a base for predictions on the behavior of soils and plants on the local as well as global scale, with respect to major changes in the environment such as global warming. Additionally to that, revealing of the mycorrhizas' role in ecosystems and their functional contribution to plant nutrition, growth, and health could also be used in sustainable agriculture, forestry or ecosystem regeneration practices, especially if mycorrhizal inoculation or in-situ management is implemented. This appears important as adaptations of immense scale are awaiting the humanity in the years and decades to come, especially in regard to the climate change, feeding increasing mankind population and use of renewable sources of energy.

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Appendix

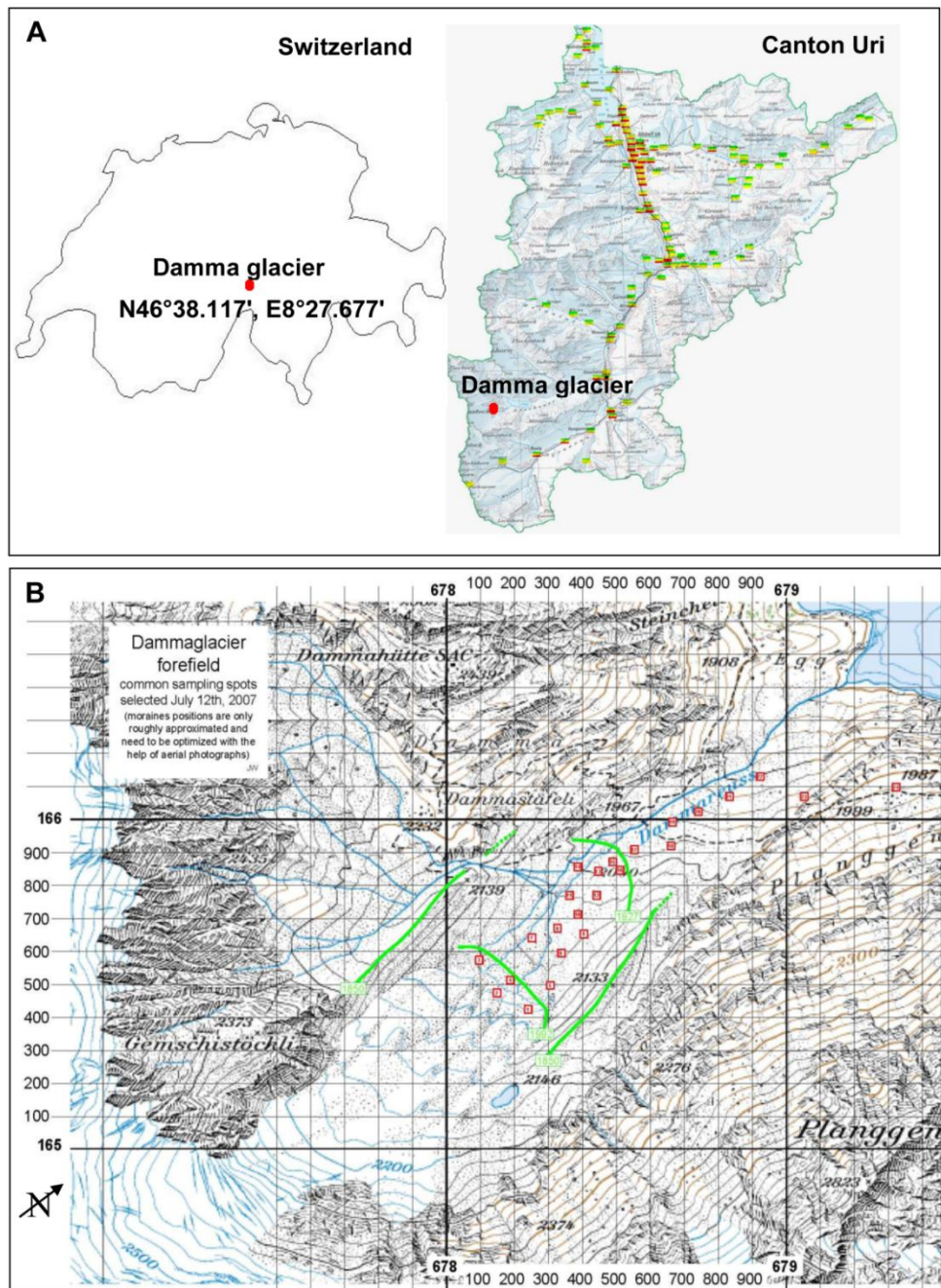


Figure 1

Location of the Damma glacier forefield; (A) Damma glacier forefield located at the map of Switzerland (left panel) and Canton of Uri (right panel) (www.geography.about.com, 22nd April 2009); (B) Detail working map with marked moraines and experimental sites (By courtesy of Dr. Jan Widerhold).

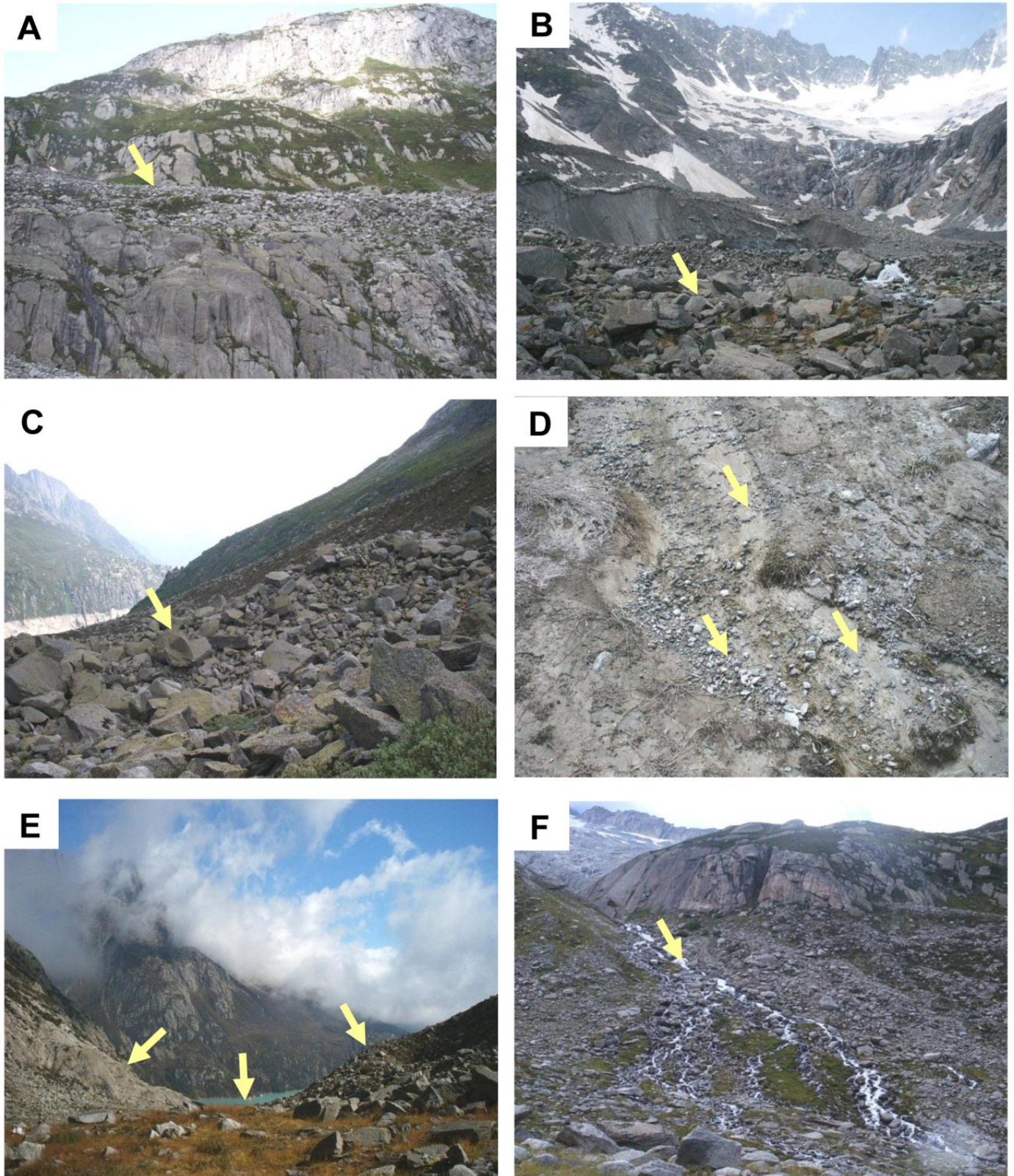


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Figure 2

Postglacial landforms on the Damma glacier forefield (arrows indicate specified object); (A) Side moraine from 1850 on western valley slope (25.06.2008); (B) Bottom moraine in the middle of the picture (on the second plain, on the top right, cirque, and middle left, the ice block) (25.06.2008); (C) Terminal moraine from 1927 (on the second plain, middle left, the lake Göschenerlap) (25.06.2008); (D) Accumulation of silt and small regolith (>5 cm diameter) particles transported with snowmelt streams (25.06.2008); (E) U-shape valley bottom (on the second plain, down middle, the lake Göschenerlap) (07.09.2007); (F) Side stream net running from Genschistöckli and Dammahütte direction (22.07.2008).

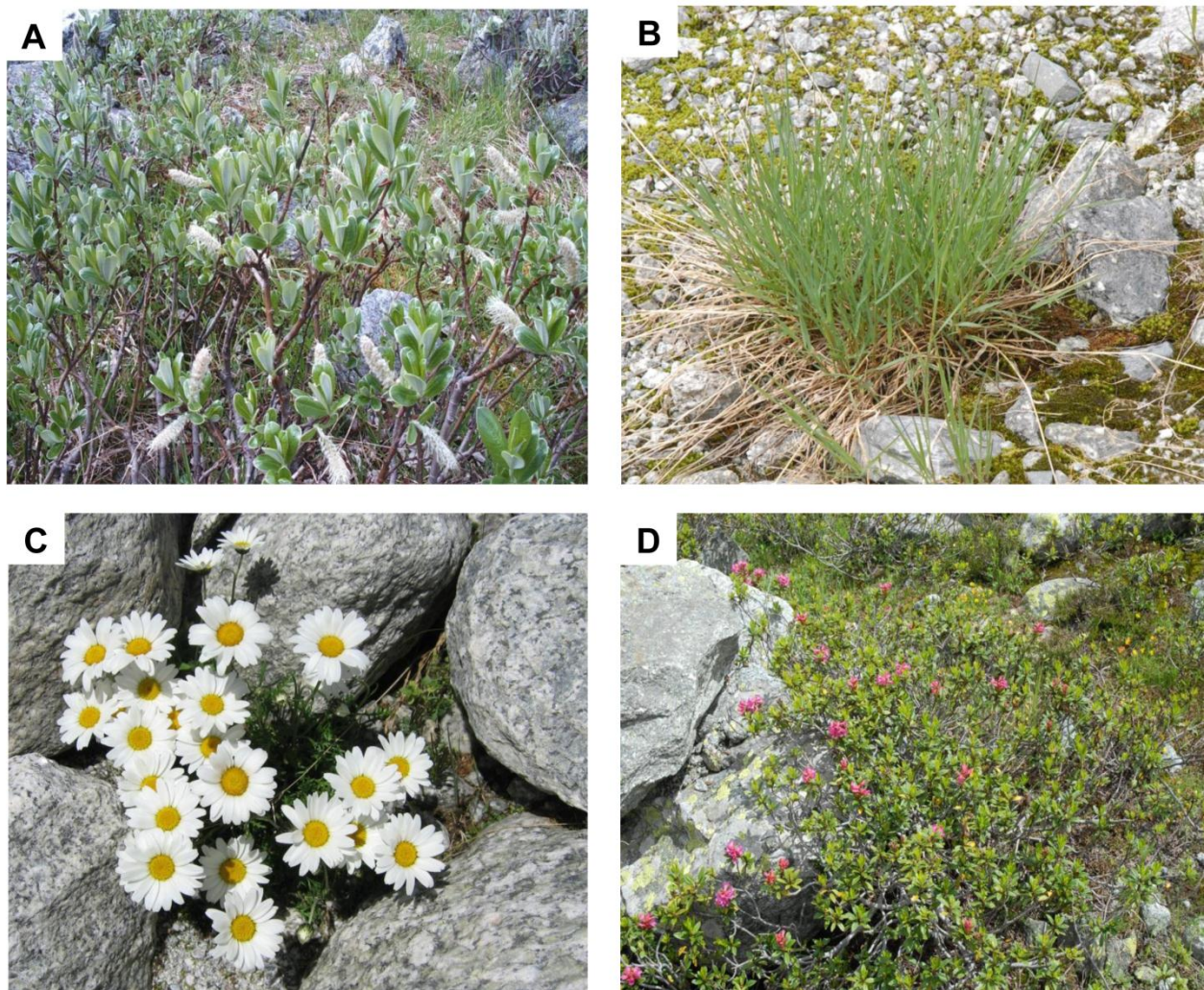


Figure 3

Plant species collected from the Damma glacier forefield; (A) *Salix helvetica*; (B) *Agrostis gigantea*, (C) *Leucanthemopsis alpina*; (D) *Rhododendron ferrugineum*.



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Figure 4

Vegetation cover at the sampling sites along soil developmental gradient. (A) individual plant of *Salix helvetica* (site deglaciated 7 years ago); site is characterized by single plants and scarce discontinuous layer of mosses and lichens; *Leucanthemopsis alpina* and *Agrostis gigantea* as individual plants only; *Rhododendron ferrugineum* not present. (B) well-developed *S. helvetica* (site deglaciated 8 years ago); plant cover become continuous, dominated by grasses; mosses and lichens frequent; *L.alpina* and *A. gigantea* as individual plants only; *R. ferrugineum* not present; (C) *S. helvetica* harboured under the granitic boulder (site deglaciated 12 years ago); plant community dominated by grasses and herbs; layer of mosses and lichens scarce; *A. gigantea* frequent, *L. alpina* as individual plants, *R. ferrugineum* very rare; (D) Mature, well established *S. helvetica* (site deglaciated 60 years ago); grasses and herbs form continuous cover; mosses and lichens scarce, *A. gigantea* and *L.alpina* frequent, *R. ferrugineum* rare but well established; (E) Mature, well established bush of *S. helvetica* surrounded by grass-dominated cover (site deglaciated 65 years ago); mosses and lichens scarce; *A. gigantea* very frequent, individual plants of *L. alpina* and *R. ferrugineum* well developed; (F) *S. helvetica* represented rarely over *R. ferrugineum* (site deglaciated 70 years ago); *A. gigantea* and *L. alpina* between dense *S. helvetica* bush only; mosses and lichens layer very scarce; (G) *S. helvetica* dominated by *R. ferrugineum* (site deglaciated 78 years ago); *A. gigantea* and *L. alpina* represented rarely; mosses and lichens well established; (H) Mature bush of *R. ferrugineum* (site deglaciated 110 years ago); *S. helvetica*, *A. gignatea* and *L. alpina* rare; mosses and lichens occupy available spots between *R. ferrugineum* plants.

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