**Root-induced fungal growth triggers macroaggregation in forest subsoils**

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**Abstract**

Subsoils are typically characterised by low concentrations of organic carbon (OC). However, due to their large volume, they contain more than half of the global soil OC stocks. This discrepancy suggests that subsoils might sequester further carbon (C), thus acting as potential sinks for atmospheric C. Plant roots and associated rhizodeposits are a major source of OC input to subsoils. However, if and how increased OC inputs via plant roots to subsoils affect mechanisms involved in soil C sequestration remains unclear.

Here, we set up a pot experiment to investigate the effect of tree roots and associated rhizosphere development on soil aggregation and C allocation in topsoils vs. subsoils. We planted European beech (*Fagus sylvatica L.*) seedlings in top- and subsoil material collected from three forest sites of different parent material. Over a growth period of five months, the seedlings developed a dense root system transforming the whole soil volume into root-affected (i.e. rhizosphere) soil. Soil samples from rooting treatments and unplanted controls were analysed for OC and total nitrogen (TN) concentrations, microbial community composition, enzyme activities and extracellular polymeric substance (EPS) concentrations. Additionally, we measured OC and TN distribution in four water-stable aggregate size classes.

The findings of our study reveal that the effects of rooting vary amongst soil depths and are also influenced by the soil characteristics. We found that roots and the associated rhizosphere development increased the amount of macroaggregates in the two finest-textured subsoils. For the most C-poor and fine-textured subsoil, this translated into a 15 % increase in bulk OC concentration, indicating considerable potential for C sequestration in subsoils by enhanced macroaggregation. Across subsoils, rooting strongly enhanced microbial abundance and was especially correlated with fungal abundance and a shift in the fungal-to-bacterial- ratio. The strong fungal growth was likely causative for the enhanced macroaggretaion in these subsoils. In topsoils, however, rooting treatment decreased macroaggregate abundance, potentially due to disruption of pre-existing aggregates as indicated by the concomitant increased in microaggregates.

Our study supports the growing awareness that OC dynamics may be governed by different mechanisms in top- and subsoils, respectively. It shows that the enhanced addition of OM via plant roots to subsoils boosts fungal growth and thereby increases macroaggregate formation which potentially facilitates C sequestration by occlusion.

Keywords:

soil organic carbon, soil fungi, rhizosphere, water-stable aggregates, aggregate fractionation, carbon storage

# Introduction

Subsoils, i.e. soil material underlying the upper soil horizons rich in organic matter, are generally characterised by rather low organic carbon (OC) concentrations. However, they contain more than 50 % of the global soil organic carbon (SOC) stocks because they make up the majority of the soil volume in most intermediate and well-developed soils around the world ([Jobbágy and Jackson, 2000](#_ENREF_33)). This discrepancy between low concentrations and high stocks of SOC implies a potential for further carbon (C) sequestration in subsoils given additional input of organic matter (OM) ([Rumpel et al., 2012](#_ENREF_72)). However, comparative studies of the mechanisms and processes controlling SOC storage in top- and subsoils are still scarce ([Salomé et al., 2010](#_ENREF_74); [Rumpel and Kögel-Knabner, 2011](#_ENREF_73)). This lack of mechanistic insight also hampers the representation of subsoils in soil C models as described by [Salomé et al. (2010)](#_ENREF_74): “In essence, subsoil is treated merely as a ‘less concentrated’ topsoil. The corollary of this assumption is that both compartments will respond in the same way to changes in conditions or external perturbations, the only difference being the intensity of the response.”

Yet, evidence is growing that SOC dynamics are controlled by different mechanisms and processes in top- vs. subsoils. Several studies used the addition of labile C to examine how increased C input affected SOC turnover in different soil depths, but results were mixed: While some observe a higher priming effect in subsoils as compared to topsoils ([Karhu et al., 2016](#_ENREF_35); [Shahzad et al., 2019](#_ENREF_79)), others report a lower ([de Graaff et al., 2014](#_ENREF_15)) or even absent ([Salomé et al., 2010](#_ENREF_74); [Wordell-Dietrich et al., 2017](#_ENREF_95)) priming effect in subsoil horizons. [Jia et al. (2019)](#_ENREF_32) found that alpine grassland subsoil responded differently to warming than the corresponding topsoil and [Matteodo et al. (2018)](#_ENREF_51) reported that the thermal stability of OM is controlled by different factors in top- and subsoil. Amongst other factors, abundance and composition of microbial communities might explain part of these differences, as both have been shown to vary significantly with soil depth ([Fierer et al., 2003](#_ENREF_22); [Uksa et al., 2015](#_ENREF_89)). While their number has been growing recently, still relatively few studies explicitly investigated differences in OC storage mechanisms between soil depths: [Vormstein et al. (2020)](#_ENREF_93) observed that with increasing depth, more OC was stabilized in aggregates or by mineral association. This corroborates with findings from [Poirier et al. (2020)](#_ENREF_64) who found that SOC added via crop residues was better retained in the subsoil as compared to the topsoil by both occlusion within aggregates and organo-mineral association. Moreover, a positive response of macroaggregation to elevated CO2 ([Keidel et al., 2018](#_ENREF_37)) and the addition of labile OM ([Baumert et al., 2018](#_ENREF_6)) was observed exclusively in subsoils.

Besides dissolved organic matter (DOM) plant roots are the most important sources of subsoil OM ([Rasse et al., 2005](#_ENREF_68); [Rumpel et al., 2012](#_ENREF_72); [Angst et al., 2016](#_ENREF_3)). There is strong evidence that root-derived OM fosters soil C sequestration more strongly than aboveground residues ([Rasse et al., 2005](#_ENREF_68); [Kätterer et al., 2011](#_ENREF_36); [Sokol and Bradford, 2018](#_ENREF_85)). Root activity triggers a range of biological, chemical, and physical processes in the surrounding soil transforming it into the so-called rhizosphere ([Hinsinger et al., 2009](#_ENREF_31); [Jones et al., 2009](#_ENREF_34)). The release of organic compounds rich in easily accessible OC (i.e. rhizodeposits), for example, generally stimulates the abundance and activity of soil microorganisms, while at the same time altering the composition of microbial communities around plant roots in topsoils ([Singh et al., 2004](#_ENREF_81); [Brant et al., 2006](#_ENREF_9); [Paterson et al., 2007](#_ENREF_59); [Bakker et al., 2013](#_ENREF_4); [Philippot et al., 2013](#_ENREF_60); [Vidal et al., 2018](#_ENREF_91)). Although these parameters are less commonly studied in subsoils, the same pattern can be found there ([Nicolitch et al., 2017](#_ENREF_56); [Shahzad et al., 2018](#_ENREF_80)). For one of the sites that was investigated in the present study, a previous experiment showed that the supply of artificial root exudates can boost fungal growth in the subsoil ([Baumert et al., 2018](#_ENREF_6)). Rhizosphere development also provokes structural changes by altering the aggregation pattern of soil particles ([Hinsinger et al., 2009](#_ENREF_31)). Aggregation leads to the occlusion, i.e. spatial protection from degradation, of OM and therefore can contribute to C sequestration in soils ([von Lützow et al., 2006](#_ENREF_92); [Dungait et al., 2012](#_ENREF_20)). Besides physical mechanisms and cementation by inorganic agents, organic binding and glueing agents play an important role in the formation and stabilisation of soil aggregates ([Amézketa, 1999](#_ENREF_1)). Aggregation is a dynamic process: Formation, stability and destruction of aggregates are regulated by multiple interactions between vegetation, soil organisms, OM, minerals, and type as well as concentration of cations ([Six et al., 2004](#_ENREF_82)). In the rhizosphere, biogenic aggregates can be formed and stabilised by both roots and rhizodeposits themselves, as well as by their associated microorganisms. Plant roots and fungal hyphae thereby act as binding agents, meaning they enmesh and tie together soil particles or smaller aggregates and OM to form macroaggregates (> 250 µm) ([Degens, 1997](#_ENREF_17); [Lehmann et al., 2017](#_ENREF_43)). In addition to polysaccharides exuded by plant roots, extracellular polymeric substances (EPS) of microbial origin that are composed of polysaccharides, proteins, lipids, and nucleic acids are considered as important glueing agents for microaggregates ([Puget et al., 1999](#_ENREF_65); [Verchot et al., 2011](#_ENREF_90); [Nichols and Halvorson, 2013](#_ENREF_55)). An increase in EPS-producing bacteria with rhizosphere development can be expected as these bacteria have recently been shown to thrive in other C-rich environments such as biological soil crusts or reclamation sites ([Cania et al., 2020](#_ENREF_12); [Vuko et al., 2020](#_ENREF_94)).

Deep SOC storage has long been considered to be less affected by biological processes, simply because subsoils are generally less biologically active than OM-rich topsoils ([Lorenz and Lal, 2005](#_ENREF_46); [von Lützow et al., 2006](#_ENREF_92)). However, due to the commonly low SOC saturation and less developed aggregate structure in subsoils, even small inputs of OM can activate the subsoil microbial community and cause all the more pronounced effects ([Poirier et al., 2014](#_ENREF_63); [Baumert et al., 2018](#_ENREF_6)). [Poirier et al. (2014)](#_ENREF_63) suggested that the physical structure of SOC-poor soils, such as subsoils, can be improved rapidly via OM input. Long-term transfer of OM by deep-rooting plants such as trees might thus strongly affect aggregation in subsoils ([Blanco-Canqui and Lal, 2004](#_ENREF_8)). This might especially hold true under ongoing global change as rhizodeposition can increase due to rising atmospheric CO2 concentrations ([Phillips et al., 2009](#_ENREF_62); [Keidel et al., 2018](#_ENREF_37)). Nevertheless, biogenic aggregate formation in subsoil horizons is still insufficiently studied ([Sanaullah et al., 2010](#_ENREF_75); [Baumert et al., 2018](#_ENREF_6); [Keidel et al., 2018](#_ENREF_37); [Torres-Sallan et al., 2018](#_ENREF_88)).

In the present study we aim to determine the effects of rhizosphere development on water-stable aggregates and the soil microbial community in forest subsoils. We therefore established a pot experiment with *Fagus sylvatica* seedlings grown in top- and subsoil material from three forest sites, that led to very densely rooted soil material of which we can assume that all of it has been influenced by the roots and thus transformed into rhizosphere soil. Unplanted pots served as unrooted controls. After plant growth and rhizosphere development for approx. five months, we analysed top- and subsoil samples for microbial community composition by extracting phospholipid fatty acids (PLFA) and microbial activity by measuring potential enzyme activities. Furthermore, EPS were extracted and analysed for their protein and saccharide content. We determined aggregate distribution by wet sieving and analysed OC and total nitrogen (N) for each size class.

We hypothesised that rooting would lead to the formation of water-stable macroaggregates in top- as well as in subsoils, whereby the effects would be larger in OM-poor subsoils. Furthermore, we assumed a root-induced increase in microbial activity and abundance, accompanied by an increase in extracellular polymeric substances (EPS). We expect microbial community composition to shift towards a higher abundance of fungi in the root-affected soils.

# Material and Methods

## Study Sites, Soil Sampling, and Substrate Characteristics

Soil samples were taken in April 2017 at three forest sites located in the state of Lower Saxony in central Germany. All study sites were dominated by mature (> 80 years) even aged European beech (*Fagus sylvatica L.*) stands, and were characterized by similar climatic conditions, but the soils developed from different parent materials: loess (LO), red sandstone (RS), and pleistocene sands (PS) ([Kirfel et al., 2019](#_ENREF_39)). Detailed information regarding the sites is given in Table 1. At each of the sites, three soil profiles (P1-3) were dug and soil material was taken from the uppermost mineral soil horizon (A-horizon) and one subsoil horizon (B-horizon) (subsequently referred to as “topsoil” and “subsoil”, respectively) (Table 1). Roots, litter, and stones were removed both manually and by sieving to < 2 mm. In order to create a homogeneous substrate for planting, equal soil masses of the samples from the three profiles were combined to create one composite substrate per site and soil horizon (n=6). The substrates were stored at 4° C until the start of the pot experiment.

All composite substrates were analysed prior to the start of the pot experiment for general substrate properties (Table 2). Soil texture differed substantially between the three sites. The soil that developed on LO material contained up to 70 % silt and only a minor percentage of sand (topsoil: silt loam, subsoil: silty clay loam). In contrast, soils that developed from red RS and PS material were both characterised by higher sand contents and classified as sandy loam. However, the RS material was dominated by fine sand (60 %), while the PS substrate contained approx. 40 % middle and coarse sand particles (Table 2). All sites were characterized by an acidic soil pH, with PS being most acidic. At the PS site, pH differed between the top- and subsoil with a higher pH in the subsoil (Table 2).

The OC and N concentrations of the topsoils ranged between 28 to 39 mg C g-1 and 1.2 to 2.79 mg N g-1 and increased in the order PS < RS < LO. In the subsoils, the OC concentrations increased in the exact opposite order and ranged there between 2.1 and 6.8 mg g-1, being highest at the PS site. Unlike the topsoil, there was no parallel gradient for N concentrations which varied much less (between 0.27 and 0.35 mg N g-1 in the order PS < LO < RS). C/N ratios decreased with depth for the LO and RS sites but were in the same range for both top- and subsoil at the PS site. In both top- and subsoil, C/N ratios increased in the order LO < RS < PS.

## Experimental Setup and Sample Preparation

Our approach to obtain rhizosphere soil differs from the most common technique which can be summarised as digging out individual roots, shaking them, removing the adhering soil and defining it as rhizosphere soil ([Luster et al., 2008](#_ENREF_48); [Barillot et al., 2012](#_ENREF_5)). However, this technique is associated with several uncertainties, such as the duration and strength of shaking, the soil type and moisture conditions at sampling time and the removal of this rhizosphere soil from the roots that often involves scratched-off root cells or the destruction of soil aggregates. Furthermore, in the less strongly rooted subsoil huge volumes of soil would have to be searched for roots to obtain enough sample material for all analyses. To avoid such issues, we decided to use a different approach and establish a pot experiment where densely rooted soil material from planted pots was compared with unrooted soil from unplanted control pots (Figure 1). Since it was shown in literature that rhizosphere effects can be detected over several cm distance ([Miniaci et al., 2007](#_ENREF_52)), will refer to the rooted soil material as “rhizosphere soil” and to the rooting treatment as “rhizosphere development” in this publication.

Stratified (pre-treatment to ensure germination) European beech (*Fagus sylvatica* L.) seeds were purchased from the Bavarian State Forestry (BaySF, Regensburg, Germany). The seeds were soaked in water at 4° C for 48 hours. Subsequently, the nut wall was carefully ruptured by hand to facilitate germination. The seeds were left for germination in a mixture of field fresh topsoil material from all three sites covered with litter. This ensured equal germination conditions for all seedlings and allowed for the colonization with native mycorrhiza strains. After 3 weeks, the seedlings had developed 2-20 mm long radicles. At this stage of development (mid May 2017), the seedlings were potted into 60-cell potting trays (145 cm3 per cell) filled with the different experimental substrates (topsoil or subsoil material from the three sites ≙ 6 substrates) (Figure 1A). For each site, two plant trays were established with a random arrangement of 25 beech seedlings (15 samples to be pooled to five replicates plus additional 10 seedlings as a reserve) and five unplanted control samples per soil depth. One week after potting, part of the seedlings had to be replanted because of drought damage, especially in the PS soils. To avoid reiterating drought damage, the individual pot cells were watered manually to ensure optimum water supply and root development of the individual seedlings. During this time, the unplanted cells were watered with equal amounts of water as the planted ones of the respective substrate. Afterwards, all trays (including planted and unplanted cells) were watered by saturating them from below every couple of days. The trays were located under a greenhouse roof for the first four months after potting (until mid-September 2017, Figure 1B). Then, the trays were transferred to a closed greenhouse and artificial light was installed upon them to prolong the vegetation period. Throughout the cultivation period, the plant trays were moved on a regular basis to reduce edge effects.

The experiment was harvested 143-145 days after potting in mid-October. Therefore, the plant trays were cut in individual cells from which the soil sample was carefully removed. On some samples a biological soil crust had developed during the experiment, which is why the uppermost 5 mm were removed. The densely rooted soil (Figure 1C) was carefully removed by shaking and manually applying slight pressure (Figures 1D, 1E). Root fragments were sorted out of the rhizosphere soil manually. The beech seedlings in the planted samples were then cut above the soil surface and separated into leaves, buds and stem. Roots were washed upon a 2 mm sieve with deionized water to remove the soil that still adhered to them. This rhizoplane material was not analysed in the present study because we wanted to avoid effects of scratched-off root cells within the rhizosphere samples. All plant parts were freeze-dried. The unplanted control samples were sampled directly. Three rhizosphere soil samples were pooled to one composite sample in order to level effects of genetic variation amongst beech seedlings (Figure 1F). Aliquots of all soil samples were air-dried and frozen for further analysis.

## Aggregate Fractionation

Samples were fractionated into different water-stable aggregate size classes by wet sieving in deionized water as described in Baumert et al. (2018). Briefly, rewetted soil was placed on a sieve tower within a beaker with deionized water and the sieve tower was moved up and down at 30 cycles per minute for 5 min. The smallest fraction that remained in the beaker after sieving was filtered at 0.45 µm. All fractions were washed off the sieves or the filter, respectively, with deionized water and freeze-dried. This procedure yielded four size fractions (>250 µm, 250-53 µm, 53-20 µm, and <20 µm) and a dissolved organic matter (DOM) fraction.

We deliberately did not perform a sand correction for the aggregate size classes as some authors performed it for the > 250 µm (sand) fraction ([Christensen, 1986](#_ENREF_14); [Six et al., 2000](#_ENREF_84)). We argue that not only the largest size fraction but also the smaller ones can contain non-aggregated, solitary primary particles. These, however, cannot be corrected for the respective content of primary particles without performing disaggregation and measuring texture of the respective size class. Since this was not feasible with the given amount of soil material, we decided to treat all size classes the same way and not to correct them. Furthermore, we argue that aggregation may already start with some clay particles that attach to a sand grain and thus, that most particles in well-developed soils are present in an aggregated and not a solitary state ([Felde et al., 2020](#_ENREF_21)). Finally, any root-induced increase or decrease in mass yielded for a specific size class must logically reflect a corresponding change in aggregation since well-mixed, homogeneous substrate was used and a change in texture due to rooting treatment can be excluded.

## Carbon and Nitrogen Analysis and pH Measurement

Carbon and nitrogen (N) concentrations of the rooted and unrooted soil samples and the aggregate size fractions were analyzed by dry combustion using an elemental analyzer (EuroEA, Eurovector, Milan, Italy). Soil samples and all fractions > 53 µm were finely ground in a ball mill prior to analysis. All measurements were performed in duplicates. Since the soils did not contain carbonates, total C concentrations are taken as OC concentrations. The filtrate < 0.45 µm from the aggregate fractionation (section 2.5) was analyzed for dissolved OC (DOC) and dissolved total nitrogen (dNt) on a total carbon analyzer coupled to a total bound nitrogen (TNb) module (DIMATOC 2000 and DIMA-N, Dimatec Analysentechnik GmbH, Essen, Germany). Freeze-dried roots and leaves were finely ground and their C and N concentrations determined using an elemental analyser (Vario MAX cube, Elementar Analysensysteme GmbH, Langenselbold, Germany).

The soil pH was measured in deionised water (1:2.5, m/m) with a pH electrode.

## Elemental Analysis of Plant Material

Freeze-dried plant material was finely ground and weighted into digestion tubes where 10 ml concentrated nitric acid was added. After 16 h, the tubes were digested at 180 °C for 20 minutes using the microwave pressure method (Mars Xpress, CEM GmbH, Kamp-Lintfort, Germany). Subsequently, the samples were filtered through acid-washed filter paper no. 640 (Sartorius AG, Göttingen, Germany) and element concentrations were determined by ICP-OES (Spectro Analytical Instruments GmbH, Kleve, Germany).

## Analysis of Potential Enzyme Activities

We investigated the potential activities of several extracellular hydrolytic enzymes involved in the C, N, or P cycling in soils. Those were analysed according to [Marx et al. (2001)](#_ENREF_50) using methylumbelliferyl (MUF)- and amido-4-methylcoumarin (AMC)-labelled substrates. The enzymes β-glucosidase, β-xylosidase and β-cellobiosidase that are involved in C cycling were determined using 4-MUF-β-D-Glucoside, 4-MUF-β-D- Xylopyranoside and 4-MUF-β-D-cellobioside, respectively. N-acetyl-β-glucosaminidase (chitinase) involved in both the C and the N cycle was determined using 4-MUF-N-Acetyl-β-D-Glucosaminide. The enzymes solely associated with N cycling were N-cycling arginine-aminopeptidase and tyrosine-aminopeptidase and were determined with L-Arginine-aminopeptidase-7-AMC and L-tyrosine-7-AMC, respectively. Acid phosphatase which is involved in P cycling was determined by using 4-MUF phosphate disodium salt. Enzyme activities were calculated from the slope of substrate utilization during 180 min and expressed as nmol g-1 h-1. Detailed information about the enzyme activity calculation can be found in [Heitkötter et al. (2017)](#_ENREF_30).

## Analysis of Extracellular Polymeric Substances

Extracellular polymeric substances were extracted from 2.5 g (dry weight equivalent) frozen soil samples based on the cation exchange resin (CER) method of [Redmile-Gordon et al. (2014)](#_ENREF_69). In brief, soluble (microbial) products were first extracted from the soils by cooled CaCl2 (0.01M, 1:10 soil:CaCl2). Next, EPS were extracted using the cation exchange resin (AMBERLITE ®, strongly acidic Na ion exchange resin, Sigma-Aldrich) in phosphate buffered saline (PBS) (1:10 soil:PBS). To account for different OC contents of the soil, 11 and 1.9 g resin were used for top- and subsoil samples, respectively. Extracts were immediately frozen at -20° C and thawed at 4° C upon further analysis. Before analysis, extracts were filtered through a 0.45 µm syringe filter (PET-45/25 Chromafil, Macherey-Nagel, Düren, Germany). From these EPS extracts, total saccharide content was determined by the phenol-sulphuric acid method of [Dubois et al. (1956)](#_ENREF_19), using D(+)-Glucose as a standard. To quantify the protein content in the EPS extracts, a modification of the Lowry assay ([Lowry et al., 1951](#_ENREF_47)) was used that corrects for polyphenolic compounds as described by [Frølund et al. (1995)](#_ENREF_26). Bovine serum albumin was used as a protein standard for quantification.

## Phospholipid Fatty Acid Analysis

Phospholipid fatty acids (PLFA) were extracted based on the method by [Frostegård et al. (1991)](#_ENREF_27) with modifications by [Kramer et al. (2013)](#_ENREF_40). Briefly, lipids were extracted from freeze-dried aliquots of the samples with Bligh & Dyer solution (methanol, chloroform, citrate buffer (pH=4); 2:1:0.8; v/v/v). The PLFA fraction was separated on silica columns by solid phase extraction (0.5 g SiOH, Chromabond ®, Macherey-Nagel, Düren, Germany) and subsequently subjected to alkaline methanolysis which converted them into fatty acid methyl esters (FAME). The extracts were measured in isooctane with a Trace 1300 gas chromatograph with flame ionization detection (GC-FID; Thermo Fisher Scientific, Waltham, USA), using a ZB-5HT fused silica capillary column (60 m, 0.25 I.D., 0.25 µm film thickness; Phenomenex LTD, Aschaffenburg, Germany). PLFA quantification was based on nonadecanoid acid methyl ester (19:0) as an internal standard. Concentrations were normalized to the average long-term results of a parallel extracted standard soil. Specific biomarker PLFAs were selected from the entirety of recorded FAMEs for further evaluation. This was done based on the unambiguous identification of their microbial origin via mass spectrometry. The selected PLFAs were categorized as indicative for gram positive bacteria (i15:0, a15:0, i16:0, i17:0), gram negative bacteria (cy17:0, cy19:0), unspecified bacteria (15:0, 16:1n7, 17:0), fungi (18:2n6, 18:1n9) and unspecified microbes (14:0, 16:0, 18:1n9t, 18:0, 20:0). The sum of fungal PLFAs relative to that of bacterial ones was used as fungal-to-bacterial-ratio (F:B). The sum of all the above mentioned PLFAs is further referred to as total microbial PLFA content and is indicative for the microbial biomass in soils ([Frostegård et al., 1991](#_ENREF_27)).

## Statistical Analysis

All statistical analyses were done with R v4.0.2 ([R Development Core Team, 2020](#_ENREF_66)). We used two-way analysis of variance (ANOVA) to test for effects of rooting treatment, site and their interaction on chemical and microbial soil variables separately for samples from top- and subsoil, respectively. Therefore, we built linear models for each response variable specifying rooting treatment, site and their interaction as fixed effects (*stats* v4.0.2) ([R Development Core Team, 2020](#_ENREF_66)) followed by calculation of ANOVA tables using type-III sum-of-squares (*car* v3.0-8) ([Fox and Weisberg, 2018](#_ENREF_24)). Model residuals were checked visually by diagnostic plots (qq, residuals vs. fitted, histogram of scaled residuals). In case assumptions were violated, analyses were re-run on ln-transformed data, which generally stabilized variances and improved normality of residuals. Plant variables including biomass and chemistry of leaves and roots were available only for rooted treatments. We used two-way ANOVAs as described above to test for the effects of site, soil depth and their interaction on plant variables. Post-hoc comparisons were based on estimated marginal means and followed by Dunnett’s tests (*emmeans* v1.4.8) ([Lenth et al., 2020](#_ENREF_45)). Significance was generally accepted at α=0.05, while p-values 0.05<p<0.1 are indicated in figures and referred to as “marginally significant” or “trend” in the text. We employed principle component analysis (PCA) to ordinate samples in reduced space based on scaled loading variables (*FactoMineR* v2.3) ([Lê et al., 2008](#_ENREF_42)). Loading variables included PLFA markers for total microbes, fungi, gram-positive bacteria, gram-negative bacteria, the PLFA-based F:B ratio, summed C-cycling enzymes, summed N-cycling enzymes, chitinase, acid phosphatase as well as EPS saccharides and EPS proteins.

# Results

## Plant Parameters

Seedlings of *F. sylvatica* developed significantly more root biomass when grown in topsoil material, both across and within the three investigated sites. Compared to those seedlings that were grown in topsoil material, seedlings grown in subsoils developed 36, 22, and 22 % less root biomass for LO, RS, and PS site, respectively. Additionally, seedlings grown on LO material also had 44% less shoot and 39% less total biomass in subsoil vs. topsoil samples. The root/shoot ratio was > 1 in all soils and did not differ between soil depths (Table 3). Amongst the three topsoils, seedlings grown on LO material produced significantly higher shoot and total biomass compared to seedlings grown on the other two topsoil materials. For top- and subsoil material, root biomass of the seedlings grown in PS material was lower than that of seedlings grown in material from LO or RS sites. Root/shoot ratios of seedlings were significantly lower when grown in soil material from the LO site than when grown in material from the RS site, while seedlings grown on material from the PS site had intermediate root/shoot ratios (Table 3).

The elemental analysis of leaves and roots revealed that seedlings grown in subsoil material had significantly lower concentrations of most plant nutrients including N, K, Mg, Ca, Fe and P. Significant nutrient reductions of seedlings grown on subsoil material were more pronounced in leaf compared to root tissue. However, C concentrations of both leaves and roots were significantly higher across all three sites when seedlings were grown on subsoil as compared to topsoil material (Soil depth main effects: p=0.002 for leaf C, p=0.037 for root C). This led to a strong significant increase in the C/N ratios, especially in the roots but also in the leaves (Table S1 and S2). The leaf sodium (Na) concentrations were significantly higher for plants grown in subsoil than those grown in topsoil (Table S2).

## pH, OC and N contents of bulk soils

The rooting treatment increased pH values significantly in the acidic substrates, except for subsoil LO (Table 4).

The OC concentrations in the LO subsoil significantly increased by 15 % from 2.1 to 2.4 mg g-1 in the rooted samples, causing an overall significantly positive effect of rooting on SOC when averaged across sites for the subsoils. Nitrogen concentrations decreased with rooting in the RS topsoil. C/N ratios increased due to rooting in all soils, except for the PS subsoil (Table 4).

## Distribution of Water-Stable Aggregate Size Classes

The distribution of aggregate size classes in the topsoils was quite similar for the three sites, as topsoil material was mainly made up of water-stable macroaggregates (>250 µm). On average, wet sieving yielded approx. 80 % particles >250 µm in the three topsoils. The mass contribution of the other size classes decreased with their size (Figure 2). For the topsoils, significant treatment effects were observed across sites: Rooting decreased the amount of particles > 250 µm, while it led to a concomitant increase in the size fraction 250-53 µm. Particles 53-20 µm in size also tended to increase in the rooted treatment (p < 0.1), whereas the size fraction < 20 µm did not respond to rooting (Figure 2a).

In the subsoils of the two finer textured sites (LO and RS), the inherent aggregate size distribution was quite different from the topsoils: In the unrooted samples particles 250-53 µm made up most of the soil mass (57 % for LO and 64 % for RS), followed by particles 53-20 µm in size for the LO site (21 %) and macroaggregates > 250 µm for the RS site (30 %). In the subsoils of these two sites, rooting strongly increased macroaggregation by 53 % for LO and 66 % for RS soil material, respectively. This was accompanied by a decrease in the size fraction 250-53 µm at both sites (Figure 2f, 2g). The PS subsoil, having a very coarse soil texture with > 40% mineral particles > 250 µm in size (Table 2), had a particle size distribution similar to the associated topsoil with > 80 % of the particles being > 250 µm. At this site, the subsoil response to rooting was a small, but significant decrease in the size classes < 53 µm from 2.6 to 1.9 % (Figure 2h).

## OC and N Contents of Aggregate Size Classes

The amount of some aggregate size fractions was too small to be in the range for OC and N analyses. These were the fractions 53-20 µm and < 20 µm for the RS topsoil as well as the fraction < 20 µm for the PS site (topsoil and subsoil).

In the topsoils, OC and N concentrations of the aggregate size class 250-53 µm decreased across sites due to rooting (Figures 3a, S1a). This was, however, mainly due to the LO topsoil material, where this effect was very pronounced (Figure 3b, S1b). In the PS topsoil, N concentrations of the size class 53-20 µm were significantly lower in the rooted compared to the unrooted samples (Figure S1d). For OC, the same trend occurred in this size fraction (p < 0.1) (Figure 3d). For the subsoils, the OC concentration of aggregates 250-53 µm in size decreased significantly across sites in the rooted pots (Figure 3e). In the RS subsoil the macroaggregates showed a higher OC concentration in the rooted compared to the unrooted samples (Figure 3g). The N concentration of the respective subsoil aggregate size classes showed the same treatment effects for the RS and PS sites (Figures S1f, S1h) but not for the LO site, where the N concentration of the size class 250-53 µm was not significantly different between rooted and unrooted pots (Figure S1f). However, the macroaggregate N concentration increased in the rooted pots (Figure S1f).

We estimated the overall amount of OC and N stored in aggregate size classes by multiplying elemental concentrations by aggregate mass fractions. In topsoils, by far most of OC and N was stored in macroaggregates, but the contribution significantly decreased in the rooted samples across sites (Figures 4a, S2a). Furthermore, in the RS topsoil material, the amount of OC and N stored in the large macroaggregates (250-53 µm) increased in the rooted pots (Figure 4c, S2c). While rooting did not affect DOC in the topsoils (Figure 4a), it had a significantly negative effect on dNt storage in the topsoils of LO and RS sites (Figures S2b, S2c). In the subsoils of the two finer-textured sites (LO and RS), the amount of OC and N stored in macroaggregates strongly increased with rooting. This was accompanied by a decrease of both elements stored in the large microaggregates (250-53 µm) (Figures 4f, 4g, S2f, S2g). The contribution of DOC to overall SOC storage significantly increased in the PS subsoil (Figure 4h) and tended to do so in the RS subsoil (p < 0.1) (Figure 4g). The resulting C/N ratios decreased upon rooting in the particles > 250 µm from LO and PS topsoils as well as RS subsoil and in the partciles 250-53 µm in size of the LO subsoil material. A higher C/N ratio was measured in the particles > 250 µm of the RS topsoil and in de DOM obtained from LO and RS topsoils and PS subsoil (Figure S3).

## Potential Enzyme Activities normalised to OC content

Rooting generally had a positive effect on potential enzyme activities and produced similar effects in topsoils and subsoils when observed across sites: In both soil depths, the potential activities of β-glucosidase, chitinase and thyrosine-aminopeptidase were significantly increased in the rooted samples. Additionally, rooting strongly enhanced α-glucosidase activity in the topsoils of all sites and arginine-aminopeptidase in the topsoil of the PS site. In the PS topsoil, no activity of β-cellobiosidase could be detected. In the subsoils of the RS and PS sites, potential arginine-aminopeptidase activity was also increased by rooting. The only significant decrease in a potential enzyme activity due to rooting occurred in the RS subsoil for acid phosphatase (Figure S4).

## Extracellular Polymeric Substances

Rooting significantly increased the concentrations of EPS saccharides in the soils from the LO site by 62 % and 114 % for topsoil and subsoil, respectively. EPS protein content of topsoil material increased across sites due to rooting by 71% on average (Table 5).

## Microbial Abundance and Community Composition

Microbial abundance measured as total microbial PLFA concentration was not significantly affected by rooting in the LO and PS topsoils, whereas in the RS topsoil it was reduced by 18% in rooting treatments by the end of the experiment. The lowered microbial abundance in RS topsoil was driven by reductions of bacteria, including gram positive and negative ones, whereas rooting did not affect fungal PLFAs. In the PS topsoil, a root-induced decrease in bacterial PLFAs, specifically of gram positive markers was observed, while fungal PLFAs increased with rooting (Table 6). This shifted the microbial community composition towards fungi in RS and PS topsoils, as indicated by corresponding increases in the F:B ratio (Figure 5).

In the subsoils, rooting led to a strong increase in microbial abundance. There, total microbial PLFA concentrations were 87, 70, and 30 % higher than in the unrooted controls of LO, RS, and PS, respectively. This general increase in microbial biomass was mainly driven by a very strong increase in fungal abundance due to rooting: Rooting increased concentrations of fungal biomarkers by 348, 195, and 82 % in LO, RS, and PS subsoil, respectively. In the LO subsoil material, rooting additionally resulted in significantly higher concentrations of gram negative and total bacterial PLFAs (increases by 58 % and 31 %, respectively). In the RS subsoil, additionally to the fungi, all three classes of bacterial PLFAs contributed to the increased total microbial PLFA content (gram positive, gram negative, and total bacteria increased by 54 %, 50 %, and 52 %, respectively). In the PS subsoil in contrast, fungi were the only taxonomic group that was significantly affected by rooting (Table 6). The strong effect of rooting on fungal PLFAs became also visible in the F:B ratios of the subsoils that were significantly increased for all three sites. Overall, the shift towards fungi in the microbial community composition was most pronounced in the LO subsoil, where the relative contribution of fungi to total microbial PLFAs was 2.4-fold enhanced from 15 to 36 mol% in the rooted as compared to the unrooted samples (Figure 5).

The contents of individual PLFA biomarkers are given in Table S4.

## Principal Component Analysis of Microbial Parameters

For both soil depths, the PCA score plots revealed a considerable level of differentiation between sites and rooting treatments. Principal component  1 explained 71.8 % and 48.2 % of total variance for topsoil and subsoil, respectively, and separated the soil material derived from the PS site (negative contribution) from that derived from the two other sites (positive contribution) (Figures 6a, 6b). Principle Component 2 explained 13.4 and 25.2 % of total variance in topsoil and subsoil, respectively, and separated rooted from unrooted samples fairly well in the topsoil (Figure 6a) and even better in the subsoil (Figure 6c). In the topsoils, differentiation of rooting treatments along PC2 was mostly influenced by the F:B ratio (Figure 6b), whereas separation of treatments in subsoils along PC2 was strongly correlated with fungal PLFA abundance (Figure 6d).

# Discussion

## The Interplay of Plant Roots, Soil Microbes, and Aggregate Formation in Subsoils vs. Topsoils

Rhizosphere development had a strong positive effect on microbial abundance in the subsoils of all three studied forest sites (Table 6). We assume that rhizodeposition provided easily available OM and thereby relieved microbial C limitation within these soils that are generally characterized by low OC concentrations and microbial activity. Rhizosphere development thus created a much more favourable environment for microbial growth in the subsoils. On the contrary, microbial abundance was unaffected by rooting at the end of our experiment in the LO and PS topsoils and even decreased in the rooted samples of the RS topsoil material (Table 6). Although microbial abundance in the topsoils was generally much higher than in the subsoils (Table 6), this finding was surprising as we hypothesised to find a positive rooting effect for microbial abundance in topsoils as well. However, the results are only a snapshot after five months of rooting within a defined volume of soil. The significantly higher root biomass in the topsoil compared to the subsoil samples (Table 3) likely comes along with higher nutrient removal in the topsoils. Thus, the observation of unaltered or reduced microbial abundance compared with the unrooted soil might be explained by beginning nutrient deficiency and the resulting competition between plants and microbes in the densely rooted pots. In fact, shortly before the harvest, beginning signs of nutrient deficiency were observed in these samples (starting necroses at the edges of older leaves, reddish coloration of young leaves). Root exudation and thereby substrate supply to microbes might also decrease with root age and beginning senescence ([Bertin et al., 2003](#_ENREF_7)), thus providing an explanation for lower microbial biomass in rooted topsoils.

Also, the effects on microbial community composition varied between the two studied soil depths: While rooting had only minor effects on most microbial groups in the topsoils, it led to an enormous shift toward fungi in all three subsoils (Figure 5). This particularly strong response of fungi to rooting can be related to several processes: On the one hand, mycorrhizal associations with plant roots which were evident on several of the root samples at harvest surely played an important role in increasing fungal biomass measured by PLFA markers. On the other hand, fungi generally tend to utilise rhizodeposits more intensely than bacteria ([Butler et al., 2003](#_ENREF_11)) and can outcompete bacteria especially at high rates of root exudates ([Griffiths et al., 1998](#_ENREF_28)), which very likely occurred in our densely rooted experimental pots (Figure 1). Interestingly, the soil materials with the lowest levels of microbial biomass (PS topsoil, LO, RS, and PS subsoil) showed the strongest increase in fungal abundance upon rhizosphere development (Figure 5, Table 6). The three subsoils additionally responded with significant increases in overall microbial abundance (Table 6). Thus, soils poor in microbial biomass appear to be especially responsive towards the addition of easily available OM induced by rooting, resulting in an alteration of microbial community composition in favour of fungi.

We assume that this increase in fungal biomass was the main reason for another observation we made in the rooted subsoil material: Rooting lead to a considerable increase in macroaggregation in the subsoils of the two finer textured sites (LO and RS). This effect was likely caused by a root-induced boost of fungal abundance (Figure 5), since mycorrhiza ([Rillig and Mummey, 2006](#_ENREF_71)) and fungal hyphae in general ([Tisdall, 1991](#_ENREF_87); [Chantigny et al., 1997](#_ENREF_13); [Degens, 1997](#_ENREF_17); [Lehmann et al., 2017](#_ENREF_43)) are well-known binding agents for this type of aggregates. It is possible that the roots themselves had also an aggregating effect by applying physical force which pushed soil particles together to form macroaggregates ([Nichols and Halvorson, 2013](#_ENREF_55)). To analyse, whether roots and fungal hyphae have a synergistic effect on macroaggregation in the subsoil rhizosphere, we suppose experiments with fungal as well as antifungal treatments in a next step.

In the PS subsoil, no effect of rooting on macroaggregation was found. Compared to the other sites, this one had the most coarse-textured soil with subsoil material consisting of 40 % primary particles > 250 µm (coarse and middle sand) (Table 2). The PS subsoil material used for filling the pots was comprised of 64 % particles and aggregates > 250 µm prior to the start of the experiment (Table S3) and during the experiment, the proportion of material > 250 µm increased to more than 80 % in both treatments (Figure 2). Thus, a formation of additional macroaggregates must have taken place during the experiment in both treatments. Yet, [Degens et al. (1994)](#_ENREF_18) found that fungal hyphae were not effective in enmeshing coarse sand grains to form macroaggregates. Moreover, the energy applied to the aggregates by wet-sieving might be more destructive for newly built macroaggregates in coarse-sandy soils as compared to finer-textured ones. Furthermore, although the fungal abundance and F:B ratio were statistically significantly increased by rooting in the PS subsoil, the increase in fungal PLFAs was not as strong as in the two other subsoils (Table 6 and Figure 5). A possible explanation could be that rhizodeposits cannot be retained in sandy soils to the same extent as in finer textured soils, although we could not find evidence for this hypothesis in the literature. However, the significantly enhanced DOC amount of the rooted compared to the unrooted PS subsoil material could support this hypothesis. Furthermore, some studies report an overall lower rhizodeposition in coarse-textured soils ([Nguyen, 2003](#_ENREF_54); [Scandellari et al., 2007](#_ENREF_76)). This might have limited the growth of fungi and thereby their influence as binding agents for the formation of macroaggregates in the coarse-textured PS subsoil. However, PCA separated the rooted and unrooted PS subsoil samples clearly along PC 2 which correlated with fungal biomass (Figure 6). Yet, differences in e.g. nutrient availability among the individual soil materials could have caused different fungal species to thrive and since not all fungi are good soil aggregators, high fungal abundance may not always improve aggregation ([Rillig et al., 2015](#_ENREF_70); [Lehmann et al., 2020](#_ENREF_44)).

The distribution of water-stable aggregate size classes in the topsoils contradicted the effect in the subsoils and was also contrary to our hypothesis: Rooting generelly decreased rather than increased water-stable macroaggregates, being significant for the LO and RS topsoils (Figure 2). These were the soils highest in OC and microbial abundance (Table 6). Due to this high content of OM and the concomitant high biological activity, they already had undergone strong inherent structure formation which was evident due to high relative proportions of macroaggregates prior to the start of the experiment (75 and 64 % macroaggregates in the topsoil material from LO and RS sites, respectively (Table S1)). The negative effect of rooting on macroaggregation might thus be related to the high physical pressure exerted by the very dense root system which can lead to a fragmentation of inherent or newly built macroaggregates ([Amézketa, 1999](#_ENREF_1)). A similar decline in macroaggregation caused by disintegration due to living roots was observed in samples that were densely planted with maize as compared to bare fallow samples by [Kumar et al. (2017)](#_ENREF_41).

Compared to fungal abundance, EPS seem to have played a less important role in formation or stability of aggregates. The observed increase in EPS polysaccharides might have influenced the tendency towards higher contribution of intermediate microaggregates (53-20 µm) in the LO topsoil and subsoil (Figure 2a, 2f), as it is frequently reported that exopolysaccharides affect microaggregate formation ([Puget et al., 1999](#_ENREF_65); [Six et al., 2004](#_ENREF_82); [Verchot et al., 2011](#_ENREF_90); [Nichols and Halvorson, 2013](#_ENREF_55)). Although most studies focus on the role of EPS saccharides on soil aggregation, structural EPS proteins are known to play a role in stabilizing and strengthening the EPS architecture and are therefore assumed to also influence soil aggregate stability ([Fong and Yildiz, 2015](#_ENREF_23)). This could explain the increased contribution of large microaggregates (250-53 µm) in the rooted topsoil materials (Figure 2).

Potential enzyme activities (Figure S4) did not show very pronounced differences between topsoils and subsoils. The PCA shows that C-, N- and P-acquiring enzymes seem to correlate well with each other as well as with other microbial parameters (Figure 6). Rhizosphere development generally increased enzyme activities.

Our study adds further evidence to the growing comprehension that processes involved in SOC dynamics can not be translated 1:1 from top- to subsoils but may differ substantially between the two soil depth compartments. Apparently, rhizosphere development, which is a major input source of OM to soils, has diverging effects in top- and subsoils: In subsoils, plant roots lead to a boost in fungal abundance which in turn can strongly enhance macroaggregate formation and thereby OM occlusion. In contrast, dense rooting of topsoil material appears to lower the contribution of macroaggregates compared to unrooted topsoil material.

## The Potential of root-derived OM Input to SOC-Poor Soils Regarding Aggregate Formation and C Storage

A previous experiment using artificial roots (i.e. without mycorrhizal interaction), carried out with soil from the LO site, demonstrated the same phenomena in the subsoil: exudate-induced macroaggregation in conjunction with increased F:B ratio. However, this was only the case after the addition of experimentally high doses of artificial root exudates ([Baumert et al., 2018](#_ENREF_6)). In the present experiment, we can show that root-induced macroaggretaion in subsoils driven by fungi also happened in a much more natural system and for differently textured soils. The influence of plant roots (i.e. natural rates of exudation, full diversity of rhizodeposits, mycorrhization) triggers shifts in microbial community composition towards fungi and thereby fosters macroaggregation in subsoils. Our results show that rhizosphere development has an overall stronger effect in subsoils than in topsoils. This can be attributed to the lower amount of OM as compared to OM-rich topsoils. In soils with low OM contents, such as subsoils, root influence can favour the physico-chemical protection of OC by either direct sorption to mineral surfaces or occlusion of coarse OM within aggregates ([Rasse et al., 2005](#_ENREF_68); [Poirier et al., 2014](#_ENREF_63)). Accordingly , [Poirier et al. (2014)](#_ENREF_63) observed a greater effect of plant residue addition on the formation of water-stable macroaggregates in subsoil than in topsoil in an incubation study. [Keidel et al. (2018)](#_ENREF_37) found an increase of macroaggregates only in the subsoil due to supposedly higher rhizodeposition after long-term elevated atmospheric CO2 concentrations. Also in artificial soils that were incubated with either particulate or dissolved OM, rapid macroaggregate formation within one month of incubation was observed ([Bucka et al., 2019](#_ENREF_10)). Thus, water-unsaturated soils low in OC tend to react with rapid macroaggregate formation following input of fresh OM ([Poirier et al., 2014](#_ENREF_63)).

Clearly, the present experimental setup did not account for the entire set of subsoil characteristics that differ from surface-near topsoil horizons with respect to e.g. temperature, pressure, and aeration regimes. Also the higher root density in our approach might lead to more pronounced effects compared to in-situ subsoils.

Occlusion of SOM within soil aggregates has long been considered less important for OC stabilization in deep soil horizons than sorption to the mineral phase ([Lorenz and Lal, 2005](#_ENREF_46); [von Lützow et al., 2006](#_ENREF_92)). However, evidence is growing that aggregation plays a crucial role for SOM stabilization also in subsoils ([Rasmussen et al., 2005](#_ENREF_67); [Moni et al., 2010](#_ENREF_53); [Salomé et al., 2010](#_ENREF_74); [Sanaullah et al., 2010](#_ENREF_75); [Schrumpf et al., 2013](#_ENREF_77); [Baumert et al., 2018](#_ENREF_6)). Although macroaggregates are typically characterised by faster turnover rates than microaggregates and therefore contribute less to the long-term persistence of OM ([Six and Jastrow, 2002](#_ENREF_83); [De Gryze et al., 2006](#_ENREF_16); [Segoli et al., 2013](#_ENREF_78)), their formation can be the first and crucial step towards longer-term stabilisation of OM ([Six et al., 2004](#_ENREF_82); [Poirier et al., 2014](#_ENREF_63)), since new microaggregates might be formed within macroaggregates ([Oades, 1984](#_ENREF_57); [Oades and Waters, 1991](#_ENREF_58)).

Our results show that the contribution of aggregates to SOC storage in subsoils is clearly determined by the input of fresh OM into the rhizosphere. In the LO and RS subsoils, macroaggregates contributed significantly more to total OC storage in rooted treatments compared to unplanted controls (Figures 4f, 4g). While this was the result of an increased number of macroaggregates in the LO subsoil (Figure 2f), in the RS subsoil the OC concentration of the macroaggregates themselves increased significantly due to the root influence (Figure 3g).

In summary, we show that five months of root growth and associated rhizodeposition significantly increased the bulk SOC content of subsoils across sites with different parent materials in a pot experiment. This was specifically true for the fine-textured LO subsoil (Table 4), which might indicate a higher capacity of fine-textured and C-poor soils to store additional OC ([Hassink, 1997](#_ENREF_29)). Fungal abundance seems to play a crucial role for macroaggregate formation in subsoils and thereby might help to foster SOC storage. This is underlined by the fact that high F:B ratios can be associated with an increased C storage potential by altering OM decomposition and C turnover ([Malik et al., 2016](#_ENREF_49)).

The rapid response of subsoil macroaggregation following OM-input via rhizodeposition in our experiment highlights a high potential for targeted OC storage following suitable changes in soil management ([Degens, 1997](#_ENREF_17); [Andruschkewitsch et al., 2014](#_ENREF_2)). This potential could be put into practice by a variety of different measures such as planting of deep-rooting species or varieties ([Kell, 2011](#_ENREF_38)), direct injection of OM into deep soil layers, as suggested by [Sosa-Hernandez et al. (2019)](#_ENREF_86), and other methods of sustainable subsoil management ([Frelih-Larsen et al., 2018](#_ENREF_25)). However, these methods still need to be tested more thoroughly for their effects on subsoil OC dynamics and potential unwanted side effects. In forest ecosystems might be less possibilities to implement management strategies that increase the OM content of subsoils than in an agricultural setting. Yet, especially the effects of different tree species and their interplay in mixed forests as well as different types of mycorrhiza they are associated with should be investigated further.

In the face of global warming and elevated CO2 concentrations, rooting and rhizodeposition are expected to increase ([Phillips et al., 2006](#_ENREF_61)). Considering our results, root expansion and concomitant rhizodeposition might thus become a highly important mechanism determining the C sink strength of forest subsoils.

# Conclusions

Our results clearly demonstrate that soil aggregation, a central process in the cycling of soil carbon, reacts differently to rhizosphere development in subsoils than in topsoils. We show that rhizosphere development in subsoils can strongly enhance macroaggregate formation and thereby occlusion and physical protection of OM, leading to subsoil OC sequestration. This process is likely triggered by an increased abundance of fungi in the rhizosphere which act as active binding agents for aggregates. Our results add to the growing perception that SOC-poor soils, such as subsoils, react with rapid macroaggregate formation to the input of fresh OM, such as rhizodeposits.

The discrepancy between low concentrations and large stocks of SOC in deep soil layers together with the demonstrated strong reaction to OM addition indicates that subsoils might provide promising C sinks given appropriate management. Still, subsoils remain understudied and are often excluded from experimental studies and ecosystem monitoring programs. We thus appeal for a stronger scientific consideration of subsoils with special regard to SOC dynamics and thorough investigation of sustainable subsoil management practices as a promising measure for mitigating climate change.

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# Tables

|  |  |  |  |
| --- | --- | --- | --- |
|  | Loess (LO) | Red Sandstone (RS) | Pleistocene Sands (PS) |
| Location | Rüdershausen51° 34‘ 51.52“ N10° 14‘ 43.03“ E | Ebergötzen51° 34’ 45.89” N10° 03’ 59.52” E | Linsburg (Grinderwald)52° 34‘ 22“ N9° 18‘ 51“ E |
| Soil type (WRB) | Haplic Luvisol | Haplic Cambisol | Dystric Cambisol |
| Horizons | Oi, Ah1, Ah2, E, Btg1, Btg2, C | Oi, Oe, Ah, Bw, C | Oi, Oe, Oa, AhE, Bsw, Bw, BwC, C |
| MAP, MAT (1981-2000) | 650 mm, 9.2 °CDeutscher Wetterdienst, station “Göttingen” | 650 mm, 9.2 °CDeutscher Wetterdienst, station “Göttingen” | 762 mm, 9.7 °CDeutscher Wetterdienst, station “Nienburg” |
| Altitude | 200 m a.s.l. | 280 m a.s.l. | 66 m a.s.l. |
| Topsoil | Ah1-horizon:P1: 0-5 cmP2: 0-5 cmP3: 0-5 cm | Ah-horizon:P1: 0-15 cmP2: 0-10 cmP3: 0-7 cm | AhE-horizon:P1: 0-3 cmP2: 0-1.5 cmP3: 0-3.5 cm |
| Subsoil | Btg1-horizon:P1: 30-60 cmP2: 40-65 cmP3: 45-70 cm | Bw-horizon:P1: 15-55 cmP2:10-58 cmP3: 7-43 cm | Bw-horizon:P1:6-36 cmP2: 5-55 cmP3: 11-45 cm |

**Table 1: General characterization of the study sites and the sampled profiles**

WRB: World Reference base of Soil Resources (IUSS Working Group WRB, 2015),

MAP: mean annual precipitation, MAT: mean annual temperature

**Table 2: Basic characteristics of the composite substrates used for the planting experiment.**

The original soil material was sampled in two soil depths (topsoil and subsoil) at the three sites Loess (LO), Red Sandstone (RS), and Pleistocene Sands (PS). Values are means (n=5) with standard errors of the mean given in brackets.

|  |  |  |  |
| --- | --- | --- | --- |
|  | Topsoil |  | Subsoil |
|  | LO | RS | PS |  | LO | RS | PS |
| soil texture (%) |  |  |  |  |  |  |  |
| CSa + MSa (> 250 µm) | 0.3 | 9.8 | 37.6 |  | 0.2 | 10.2 | 40.2 |
| FSa (250-53 µm) | 7.3 | 60.2 | 31.0 |  | 6.5 | 58.8 | 29.2 |
| Si (53-2 µm) | 68.0 | 15.0 | 22.5 |  | 61.1 | 14.7 | 20.2 |
| Cl (< 2 µm) | 24.4 | 15.0 | 8.9 |  | 32.2 | 16.3 | 10.4 |
| pH | 5.0 (0.02) | 4.8 (0.01) | 3.8 (0.03) |  | 4.9 (0.01) | 4.9 (0.02) | 4.6 (0.01) |
| OC (mg g-1) | 39.0 (0.31) | 34.4 (0.58) | 27.9 (0.15) |  | 2.1 (0.05) | 4.3 (0.05) | 6.8 (0.03) |
| N (mg g-1) | 2.8 (0.03) | 2.1 (0.03) | 1.2 (0.01) |  | 0.3 (0.00) | 0.4 (0.00) | 0.3 (0.01) |
| C/N | 14.0 (0.03) | 16.1 (0.05) | 23.3 (0.21) |  | 7.1 (0.18) | 12.3 (0.25) | 25.1 (0.7) |

CSa: coarse sand, MSa: middle sand, FSa: fine sand, Si: silt, Cl: clay

**Table 3: Biomass and root/shoot ratio of the *F. sylvatica L.* seedlings as influenced by site and soil depth.**

Values are means averaged across study sites (n=15) and within sites loess (LO), red sandstone (RS), and pleistocene sands (PS) (n=5 for each site). Standard errors of the mean are given in brackets. Across sites, bold pairs of values indicate significant main effects of soil depth in the absence of a significant soil depth by site interaction. Within sites, bold pairs of values indicate significant post-hoc differences between soil depths. Different lower case letters indicate statistically significant post-hoc differences between the study sites within one soil depth. Significance level p < 0.05.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Across Sites | LO | RS | PS |
| shoot biomass (g) |  |  |  |  |
| Topsoil | 0.98 (0.08) | **a 1.35 (0.12)** | b 0.84 (0.03) | b 0.76 (0.04) |
| Subsoil | 0.70 (0.03) | **0.76 (0.04)** | 0.73 (0.05) | 0.59 (0.04) |
| root biomass (g) |  |  |  |  |
| Topsoil | **1.19 (0.08)** | **a 1.45 (0.16)** | **a 1.19 (0.07)** | **b 0.92 (0.05)** |
| Subsoil | **0.86 (0.04)** | **a 0.93 (0.03)** | **a 0.93 (0.06)** | **b 0.72 (0.08)** |
| total biomass (g) |  |  |  |  |
| Topsoil | 2.17 (0.15) | **a 2.79 (0.27)** | b 2.03 (0.09) | b 1.68 (0.07) |
| Subsoil | 1.56 (0.07) | **1.69 (0.07)** | 1.67 (0.11) | 1.31 (0.11) |
| root/shoot ratio |  |  |  |  |
| Topsoil | 1.24 (0.06) | a 1.07 (0.04) | b 1.43 (0.09) | ab 1.23 (0.08) |
| Subsoil | 1.24 (0.04) | a 1.22 (0.04) | b 1.28 (0.05) | ab 1.23 (0.08) |

**Table 4: Mean pH, OC, and N concentrations, and C/N ratios as influenced by rooting treatment in topsoil and subsoil.**

Values are means across study sites (n=15) and within sites loess (LO), red sandstone (RS), and pleistocene sands (PS) (n=5 for each site). Standard errors of the mean are given in brackets. Across sites, bold pairs of values indicate significant main effects of rooting treatment in the absence of a significant treatment by site interaction. Within sites, bold pairs of values indicate significant post-hoc differences between rooting treatments. Significance level p < 0.05.

|  |  |  |  |
| --- | --- | --- | --- |
|  | Topsoil |  | Subsoil |
|  | Across Sites | LO | RS | PS |  | Across Sites | LO | RS | PS |
| OC (mg g-1) |  |  |  |  |  |  |  |  |  |
| unrooted | 31.6 (1.0) | 35.8 (1.2) | 31.4 (0.6) | 27.6 (0.2) |  | **4.3 (0.5)** | **2.1 (0.0)** | 4.1 (0.1) | 6.5 (0.0) |
| rooted | 31.2 (0.8) | 34.2 (0.4) | 32.0 (0.5) | 27.4 (0.3) |  | **4.4 (0.5)** | **2.4 (0.1)** | 4.3 (0.0) | 6.5 (0.1) |
| N (mg g-1) |  |  |  |  |  |  |  |  |  |
| unrooted | 1.92 (0.2) | **2.56 (0.1)** | 2.03 (0.0) | 1.16 (0.0) |  | 0.31 (0.0) | 0.31 (0.0) | 0.36 (0.0) | 0.25 (0.0) |
| rooted | 1.82 (0.1) | **2.37 (0.0)** | 1.95 (0.0) | 1.13 (0.0) |  | 0.31 (0.0) | 0.32 (0.0) | 0.36 (0.0) | 0.25 (0.0) |
| C/N |  |  |  |  |  |  |  |  |  |
| unrooted | 17.8 (1.2) | **14.0 (0.2)** | **15.5 (0.1)** | **23.8 (0.1)** |  | 15.0 (2.3) | **6.9 (0.0)** | **11.4 (0.1)** | 26.8 (0.1) |
| rooted | 18.4 (1.1) | **14.4 (0.1)** | **16.4 (0.1)** | **24.2 (0.1)** |  | 15.5 (2.2) | **7.8 (0.3)** | **12.1 (0.1)** | 26.8 (0.1) |
| pH |  |  |  |  |  |  |  |  |  |
| unrooted | 5.0 (0.1) | **5.1 (0.05)** | **5.1 (0.03)** | **4.6 (0.06)** |  | 5.6 (0.1) | 5.1 (0.03) | **5.6 (0.07)** | **6.2 (0.05)** |
| rooted | 5.4 (0.1) | **5.7 (0.04)** | **5.6 (0.03)** | **4.8 (0.04)** |  | 5.8 (0.1) | 5.1 (0.03) | **6.0 (0.04)** | **6.4 (0.05)** |

**Table 5: Mean concentrations of EPS saccharides and proteins as influenced by rooting treatment in topsoil and subsoil.**

Values are means across study sites (n=15) and within sites loess (LO), red sandstone (RS), and pleistocene sands (PS) (n=5 for each site). Standard errors of the mean are given in brackets. Across sites, bold pairs of values indicate significant main effects of rooting treatment in the absence of a significant treatment by site interaction. Within sites, bold pairs of values indicate significant post-hoc differences between rooting treatments. Significance level p < 0.05.

|  |  |  |  |
| --- | --- | --- | --- |
|  | Topsoil |  | Subsoil |
|  | Across Sites | LO | RS | PS |  | Across Sites | LO | RS | PS |
| EPS saccharides (µg glucose eq. g-1) |  |  |  |  |  |  |  |  |  |
| unrooted | 139.1 (11.6) | **158.6 (27.5)** | 160.3 (5.4) | 98.3 (3.9) |  | 90.3 (11.5) | **60.5** **(9.9)** | 146.8 (8.4) | 63.4 (3.7) |
| rooted | 174.7 (18.4) | **256.8 (18.1)** | 170.5 (3.9) | 96.9 (4.2) |  | 119.7 (10.1) | **130.1 (21.8)** | 147.0 (3.4) | 81.9 (3.3) |
| EPS proteins (µg g-1) |  |  |  |  |  |  |  |  |  |
| unrooted | **34.3** **(5.0)** | **44.9 (10.8)** | 42.3 (1.8) | **15.7** **(3.0)** |  | 16.5 (3.0) | 8.7 (1.5) | 17.5 (2.4) | 23.4 (7.5) |
| rooted | **58.5** **(7.0)** | **87.1** **(7.2)** | 57.0 (6.1) | **31.3** **(5.7)** |  | 23.6 (2.4) | 18.8 (5.0) | 22.0 (1.8) | 30.1 (4.0) |

**Table 6: PLFA concentrations indicative for microbial groups and total microbial PLFAs as influenced by rooting tr****eatment in topsoil and subsoil.**

Values are means across study sites (n=15) and within sites loess (LO), red sandstone (RS), and pleistocene sands (PS) (n=5 for each site except for Subsoil/LO/Unrooted where n=4). Standard errors of the mean are given in brackets. Across sites, bold pairs of values indicate significant main effects of rooting treatment in the absence of a significant treatment by site interaction. Within sites, bold pairs of values indicate significant post-hoc differences between rooting treatments. Significance level p < 0.05.

|  |  |  |  |
| --- | --- | --- | --- |
|  | Topsoil |  | Subsoil |
|  | Across Sites | LO | RS | PS |  | Across Sites | LO | RS | PS |
| gram positive (nmol g-1) |  |  |  |  |  |  |  |  |  |
| unrooted | 21.2 (2.1) | 22.8 (1.1) | **29.5 (1.1)** | **11.3 (0.4)** |  | 1.25 (0.17) | 0.63 (0.03) | **1.02 (0.12)** | 1.97 (0.12) |
| rooted | 18.1 (1.9) | 23.0 (0.8) | **22.2 (2.3)** | **9.1 (0.7)** |  | 1.53 (0.18) | 0.75 (0.06) | **1.57 (0.17)** | 2.26 (0.11) |
| gram negative (nmol g-1) |  |  |  |  |  |  |  |  |  |
| unrooted | 3.0 (0.4) | 4.0 (0.0) | **4.0 (0.1)** | 0.9 (0.0) |  | 0.28 (0.04) | **0.12 (0.01)** | **0.24 (0.02)** | 0.46 (0.02) |
| rooted | 2.6 (0.4) | 3.9 (0.1) | **3.1 (0.3)** | 0.9 (0.1) |  | 0.36 (0.04) | **0.19 (0.01)** | **0.36 (0.02)** | 0.53 (0.03) |
| bacteria (nmol g-1) |  |  |  |  |  |  |  |  |  |
| unrooted | 30.5 (3.1) | 34.6 (1.2) | **42.2 (1.1)** | **14.8 (0.4)** |  | **2.10 (0.27)** | **0.98 (0.05)** | **1.82 (0.17)** | 3.27 (0.16) |
| rooted | 26.5 (2.9) | 34.5 (1.2) | **32.7 (3.0)** | **12.4 (0.9)** |  | **2.67 (0.31)** | **1.28 (0.10)** | **2.77 (0.23)** | 3.96 (0.17) |
| fungi (nmol g-1) |  |  |  |  |  |  |  |  |  |
| unrooted | 9.8 (1.2) | 12.3 (0.2) | 13.8 (0.4) | **3.4 (0.1)** |  | 0.65 (0.06) | **0.35 (0.05)** | **0.73 (0.03)** | **0.81 (0.02)** |
| rooted | 10.8 (1.2) | 14.2 (0.5) | 13.1 (1.4) | **5.2 (0.5)** |  | 1.74 (0.12) | **1.57 (0.17)** | **2.16 (0.20)** | **1.48 (0.06)** |
| total microbial PLFAs (nmol g-1) |  |  |  |  |  |  |  |  |  |
| unrooted | 73.6 (8.5) | 88.0 (1.4) | **103.3 (2.0)** | 29.5 (0.9) |  | 5.22 (0.59) | **2.30 (0.21)** | **5.20 (0.19)** | **7.58 (0.25)** |
| rooted | 67.3 (7.6) | 87.8 (2.9) | **85.3 (6.5)** | 28.9 (1.9) |  | 7.63 (0.68) | **4.33 (0.39)** | **8.79 (0.62)** | **9.76 (0.30)** |

# Figure Captions

**Figure 1: Setup of the potting experiment.**

(A) *F. sylvatica* seedlings after transfer to the potting trays, (B) plant development two months after planting, (C) densely rooted soil material which can be assumed to be transformed to rhizosphere soil five months after planting, (D) careful removal of rhizosphere soil material upon harvest, (E) European beech plant after rhizosphere sampling, (F) pooling scheme: three rooted samples were pooled to one composite sample and compared with individual unrooted control samples.

**Figure 2: Distribution of water-stable aggregate size classes (mass%) as influenced by rooting treatment in topsoil (a-d, upper panel) and subsoil (e-h, lower panel).**

Values are means across study sites and within sites loess (LO), red sandstone (RS), and pleistocene sands (PS). Bars are standard errors of the mean. Sample size n is 5 except for Topsoil/RS/Unrooted (n=4), Topsoil/RS/Rooted (n=4), Topsoil/PS/Rooted (n=3), Topsoil/PS/Unrooted (n=4), Subsoil/LO/Unrooted (n=4), Subsoil/RS/Unrooted (n=4), Subsoil/RS/Rooted (n=4), Subsoil/PS/Rooted (n=4). Across sites, symbols indicate significant main effects of rooting treatment in the absence of a significant treatment by site interaction. Within sites, symbols indicate significant post-hoc differences between rooting treatments. Significance levels are as follows: ▪ P < 0.1, \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001.

**Figure 3: Mean OC concentration of water-stable aggregate size classes as influenced by rooting treatment in topsoil (a-d, upper panel) and subsoil (e-h, lower panel).**

Values are means across study sites and within sites loess (LO), red sandstone (RS), and pleistocene sands (PS). Error bars are standard errors of the mean. Sample size n is 5 except for Topsoil/RS/Unrooted (n=4), Topsoil/RS/Rooted (n=4), Topsoil/PS/Rooted (n=3), Topsoil/PS/Unrooted (n=4), Subsoil/LO/Unrooted (n=4), Subsoil/RS/Unrooted (n=4), Subsoil/RS/Rooted (n=4), Subsoil/PS/Rooted (n=4). Across sites, symbols indicate significant main effects of rooting treatment in the absence of a significant treatment by site interaction. Within sites, symbols indicate significant post-hoc differences between rooting treatments. Significance levels are as follows: ▪ P < 0.1, \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001. n.a. = not available.

**Figure 4: Amount of OC stored in bulk soil, water-stable aggregate size classes, and DOM as influenced by rooting treatment in topsoil (a-d, upper panel) and subsoil (e-h, lower panel).**

Values are means across study sites and within sites loess (LO), red sandstone (RS), and pleistocene sands (PS). Error bars are standard errors of the mean. Sample size n is 5 except for Topsoil/RS/Unrooted (n=4), Topsoil/RS/Rooted (n=4), Topsoil/PS/Rooted (n=3), Topsoil/PS/Unrooted (n=4), Subsoil/LO/Unrooted (n=4), Subsoil/RS/Unrooted (n=4), Subsoil/RS/Rooted (n=4), Subsoil/PS/Rooted (n=4). Across sites, symbols indicate significant main effects of rooting treatment in the absence of a significant treatment by site interaction. Within sites, symbols indicate significant post-hoc differences between rooting treatments. Significance levels are as follows: ▪ P < 0.1, \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001. n.a. = not available.

**Figure 5: Microbial community composition as influenced by rooting treatment in topsoil (upper panel) and subsoil (lower panel).**

Bars depict means of the relative abundance of different taxonomic groups (mol%, left-hand axis). Dots depict the fungal-to-bacterial ratio (F:B, right-hand axis). Values are means across study sites (n=15) and within sites loess (LO), red sandstone (RS), and pleistocene sands (PS, n=5 for each site except for Subsoil/LO/Unrooted where n=4). Error bars are standard errors of the mean. Only F:B ratios were statistically analysed. Within sites, black scatter symbols indicate significant post-hoc differences between rooting treatments. Significance level p < 0.05.

**Figure 6: Unconstrained ordination of rooted and unrooted samples by principle component (PC) analysis for topsoil (a-b, upper panel) and subsoil (c-d, lower panel) samples.**

For each PCA samples from all three sites loess (LO, circles), red sandstone (RS, squares) and pleistocene sands (PS, diamonds) were used. The first two PCs (dimensions) together capture 86 and 66 % of total variance for topsoil and subsoil, respectively. **a,c** Score plots of samples along the first two dimensions (principle components). **b,d** Correlations circles of microbial loading variables. Loading variables are visualized by their correlations with the first two dimensions.