# Effects of tree species richness on topsoil carbon and fungal diversity in European planted mixed forests are modulated by environmental conditions

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# ABSTRACT

Mixed-species forests have emerged as a promising approach to mitigate climate change impacts through enhanced carbon (C) sequestration while maintaining productivity, biodiversity, and other ecosystem services. However, we still have a poor understanding of the context-dependency of soil C sequestration in tree mixtures, particularly how it is influenced by plant-soil-microbe interactions and environmental conditions.

Using soil samples collected from nine European sites within the global network of tree diversity experiments, TreeDivNet, we examined how tree species richness is associated with topsoil C stocks, fungal community composition and diversity, and their interactions. We further investigated the influence of biotic, edaphic, and climatic factors on the relationship between tree richness and topsoil C stocks. We hypothesised that increased tree species richness leads to increased topsoil C stocks and fungal diversity, and that this effect is modulated by site-specific interactions between biotic and abiotic factors.

Overall, we found topsoil C stocks in stands with high tree diversity to be greater than in monocultures across the study sites. Lower soil fertility, cooler mean annual temperatures, and lower interannual variability of temperature and precipitation were found to correlate with positive effects of tree diversity on soil C stocks. While tree diversity did not directly influence fungal diversity, topsoil C stocks were positively correlated to fungal species richness. In addition, fungal richness showed a positive correlation with the net diversity effect of tree mixtures on topsoil C, suggesting that fungal diversity may be one of several factors contributing to the context-dependency of tree diversity effects on soil C stocks.

Our study shows that tree species diversity can increase topsoil C storage across Europe, influenced both directly and indirectly by fungal diversity and environmental conditions. The mediation of direct and indirect linkages between tree diversity, fungal diversity and topsoil C stocks by local abiotic context highlights the need to improve our mechanistic understanding for site-specific management of soil C sequestration in tree mixtures to promote climate change mitigation in European forests.

## 1. Introduction

Soils harbour the largest terrestrial pool of organic carbon (C; Scharlemann et al. 2014) and support around 59 % of global biodiversity, including 90 % of fungal biodiversity (Anthony et al. 2023). Globally, forest soils constitute one of the largest terrestrial C pools, and while regional contributions vary, they are increasingly threatened by land-use change and forest degradation (Dixon et al. 1994; Lal 2005; Hu et al. 2021). To combat this, afforestation, reforestation and restoration programmes are being implemented globally. In the last decades, there has been a growing focus on mixed-species forests (Messier et al. 2022) to enhance productivity (Pretzsch & Schütze 2016; Urgoiti et al., 2023), biodiversity (Carnus et al. 2006), and resilience (Jactel et al. 2017) through increased complementarity, substrate and habitat diversity and the spreading of risks from both biotic agents (e.g., specialist pests) and abiotic disturbances (e.g., windthrow; Forrester, 2019; Jactel et al., 2017). Restoration efforts significantly affect soil, as land-use changes such as changing tree species or planting trees on former agricultural lands alter soil chemistry (Guo & Gifford 2002; Foster et al. 2003), impact soil C stocks (Guo & Gifford 2002; Rytter & Rytter 2020; Zang et al. 2024), and influence the diversity and composition of microbial communities (Szoboszlay et al. 2017; Balami et al. 2020). Tree species identity plays a key role in influencing soil organic carbon (SOC) dynamics, as different species influence litter input, root turnover, and microbial activity in distinct ways (Vesterdal et al. 2008; Angst et al. 2019). For example, Dawud et al. (2017) found that conifer presence increased topsoil SOC more than species richness, and Osei et al. (2021) showed that species identity had a greater effect on SOC storage than species mixing *per se* in common European two-species mixtures. Similarly, Jandl et al. (2007) emphasize species-specific effects on carbon inputs and soil organic matter (SOM) decomposition, while also noting that mixed-species forests may enhance forest stability and help mitigate SOM losses. Accordingly, while some studies report positive effects of tree diversity on SOC levels (Gamfeldt et al. 2013; Chen et al. 2018; Mayer et al. 2020; Chen et al. 2024), others suggest that SOC stocks in mixed stands often fall between those of the corresponding monocultures (Wiesmeier et al. 2013; Cremer et al. 2016). Tree mixtures producing more biomass than equivalent monocultures (i.e., overyielding), has been found to increase SOM (Augusto & Boča 2022) and is attributed to complementary resource use and facilitative species interactions (Leuschner et al. 2001; Pretzsch 2014; Williams et al. 2017; Mayer et al. 2020). However, despite this potential mechanism, empirical findings on diversity effects on SOC remain highly variable across studies, suggesting that additional processes are involved. These may include biomass quantity (Viana et al. 2014), quality (Angst et al. 2019; Desie et al. 2023), frequency of litter inputs (Zhou et al., 2022), soil chemical properties (Cesarz et al. 2022), climatic factors like temperature and moisture (Berg & McClaugherty 2020), and historical land-use (Guo & Gifford 2002; Deng et al. 2016). For example, Ratcliffe et al. (2017) found that tree diversity increased soil C under conditions of lower water availability and longer growing seasons, while Pretzsch et al. (2014) and Toïgo et al. (2015) noted that positive tree diversity effects on soil C increased with decreasing soil fertility. The stress-gradient hypothesis (Bertness & Callaway 1994) suggests that species interactions become more complementary under harsher conditions, as competition decreases for limited resources like nitrogen, water, or light, with facilitative interactions becoming more important (Forrester 2014; Ratcliffe et al. 2017). On the other hand, Chen et al. (2024) found that the positive relationship between tree functional diversity and soil C accumulation was more pronounced at sites with greater water and nutrient availability. Climatic variability, expected to rise with climate change, can also affect species interactions (Kitzberger et al. 2000; Kikvidze et al. 2006; Carnwath & Nelson 2016). Greater biomass in diverse stands can also reduce surface evaporation, increasing soil moisture during droughts (Wright et al. 2015). Consequently, tree diversity effects depend heavily on species interactions and environmental conditions in which they coexist (i.e., context-dependency), including resource availability and heterogeneity (Ratcliffe et al. 2017). Although crucial, this environmental context-dependency remains understudied (Depauw et al. 2024), yet a deeper understanding could directly inform climate change mitigation and adaptation strategies.

Fungal biodiversity in forest ecosystems is vital, as soil fungi play key roles in litter decomposition, C turnover, and the formation of mineral-stabilized SOM (Zak et al. 2019; Zang et al. 2024). In addition to their role in decomposition, fungi also contribute to long-term C sequestration through producing recalcitrant fungal necromass. The recalcitrance of hyphal structures, for example, contributes to the accumulation of SOM

(Godbold et al. 2006; Ekblad et al. 2013). Thus, the diversity and composition of soil fungal communities significantly impact soil C stocks, as different fungal groups vary in their contribution to decomposition and necromass accumulation. Higher tree diversity has been linked to higher soil microbial diversity (Weißbecker et al. 2018; Liu et al. 2020), through both direct and indirect effects. Recent studies further suggest that higher fungal functional diversity in mixed tree stands amplifies the tree diversity-productivity relationship (Luo et al. 2024) and enhances forest resilience to stress, boosting productivity (Sachsenmaier et al. 2024). Additionally, positive correlations between fungal diversity and soil organic C (Yang et al. 2017; Zang et al. 2024) suggest a triangular relationship between tree and fungal diversity and soil C. However, tree species identity can also strongly influence fungal and bacterial communities (Gunina et al., 2017), potentially modifying the effects of tree diversity on fungal diversity and soil C stocks. Direct effects have been observed for ectomycorrhizal (EM) fungi, where a wider range of host species leads to greater EM fungal diversity (Singavarapu et al. 2023). Indirect effects on fungal diversity occur via changes in litter, exudates, and soil chemistry (Nguyen et al. 2016; Gillespie et al. 2021) or through increases in SOM (Bending et al. 2002), which can alter resource availability and habitat conditions. Yet, the response of fungal communities to changes in tree diversity varies strongly by fungal functional guilds (Tedersoo et al. 2016). For example, Griffin et al. (2019) reported decreased fungal richness for endophytes, pathogens, and saprotrophs with higher tree species richness, while Tedersoo et al. (2016) found positive effects on EM richness in Finland and fungal richness, especially for saprotrophic fungi, in Estonia. It has been found that tree species identity and composition, rather than species richness alone, are the key drivers of fungal diversity and composition (Waldrop et al. 2006; Tedersoo et al. 2016; Gillespie et al. 2021), but in addition to plant variables there are also other important drivers commonly identified such as land-use history, climatic, spatial and edaphic variables (Tedersoo et al. 2014; Djemiel et al. 2024).

Research examining the combined effects of tree diversity on both soil fungal diversity and topsoil C stocks remains limited (e.g., see Chen et al. 2018 for tree diversity-soil C relationships and Gunina et al. 2017 for tree diversity-soil microbial interactions). While the mechanistic context-dependency of diversity effects on ecosystem functions has been highlighted in previous research (Wardle & Zackrisson 2005; Ratcliffe et al. 2017), it remains poorly understood. This study investigates the complex relationships among tree species diversity, fungal diversity, and topsoil C stocks in young European forest plantations, and examines how edaphic, vegetation and climatic conditions influence these interactions. We hypothesize that:

I. increased tree species diversity increases topsoil C stocks and fungal diversity,
II. the increase in soil carbon stocks with tree species diversity is stronger under harsher edaphic and climatic conditions, i.e., context-dependent
III. soil fungal diversity correlates positively with the positive effect of tree diversity on topsoil C stocks.

## 2. Materials and Methods

### 2.1. Study sites and experimental set-up

This study was conducted within the MixForChange project as part of the global Tree Diversity Network (TreeDivNet, http://www.treedivnet.ugent.be/; Verheyen et al. 2016), designed to investigate the effects of tree diversity on ecosystem functions. Sampling was conducted on 198 plots at nine experiments (sites) across Europe, covering various soil types, climates, and land-use histories, with 25 deciduous and coniferous tree species or varieties (Table 1). The experimental design includes species richness gradients from one to six species per site. Sites feature monocultures, 2-species mixtures, and higher richness mixtures

of 4–6 species. For standardization, richness levels were categorized as 'mono' (monocultures), 'low' (2–3 species), and 'high' (4 + species). Species composition refers to the specific admixture of tree species present within a stand, i.e., encompassing the identity of the tree species. At the ECOLINK-Salix Uppsala site, *Salix* varieties were used, which have been shown to significantly differ in their effects on soil biology and properties such as soil C accumulation (Baum et al. 2020). One additional site in Brazil (MataDIV; Table 1) with monocultures, 3 and 4-species mixtures was sampled (22 plots), but omitted from the main analyses due to its unique environmental conditions, though it was included in a supplementary analysis to test hypothesis (I) regarding C stocks.

In 2022, the year of sampling, stand ages ranged from 8 to 23 years, with stand densities between 2,500 and 49,400 trees per hectare. Mean annual temperature (MAT) since establishment varied between + 6.0 °C and + 13.4 °C and mean annual precipitation (MAP) ranged from 511 mm and 1168 mm across sites (Table 1). Climate data were collected from nearby climate stations for five sites while national data triangulation was used for the three FORBIO sites and ECOLNIK-Salix Rostock. To capture climatic variability, we calculated the interannual coefficient of variation (CV) for both temperature and precipitation based annual averages.

### 2.2. Basal area inventories

Values for tree standing stock, expressed as basal area per hectare (BA ha$^{-1}$), were obtained from stem inventories for all sites, except Satakunta, where a single angle-count sample using a relascope was taken from the of each plot. Inventories were conducted between 2020 and 2023.

### 2.3. Soil sampling and physicochemical analysis

Soil sampling was conducted in spring 2022 across 198 plots, with each tree species composition (i.e., plot) replicated twice per site in a block design. Additionally, 22 plots were sampled at the tropical site. Ten subsamples were taken per plot (0–10 cm depth, after removing surface litter) to cover all tree species combinations while avoiding plot borders. The sampled depth represents the uppermost soil layer and may include both organic and mineral horizons, depending on site-specific conditions. Subsamples were pooled and homogenized, dried at 40 °C for 24 to 48 h, sieved (2 mm), and stored dry until further analysis. A subset was taken for fungal community analysis (see below). Sampling was limited to the top 10 cm due to its typically high fine-root biomass, fungal activity, and sensitivity to tree richness effects (Spohn et al. 2016; Wambsganss et al. 2021; Prescott & Grayston 2013).

Chemical soil analysis was performed on all samples, further dried at 105 °C for 24 h. Soil pH (logarithmic scale) was measured in 0.1 mol CaCl$_2$ at a 1:2.5 soil-to-solution ratio. Carbon and nitrogen concentrations were analyzed using a CN analyzer (Vario EL III, Elementar Analysensysteme, Hanau, Germany). Throughout the manuscript, we present organic carbon content, subtracting carbonate-C from total C in carbonate-containing soils to determine organic C. C stocks were calculated by multiplying soil organic C content (g 100 g$^{-1}$), bulk density (g cm$^{-3}$), and sampling depth (cm).

Bulk density (BD) of the top 0–10 cm mineral soil was measured at each site using three randomly distributed, non-compressed soil cores, expressed in g dry soil cm$^{-3}$ (Grossman & Reinsch, 2002). Soil texture was determined using the sieve and pipette method (Hartge & Horn, 1999).

To quantify tree richness effects on soil C stocks, we calculated the net diversity effect on soil C stocks (NDE C) by comparing observed soil C stocks in mixtures with expected values derived from monocultures at each site. This metric represents the relative difference between observed and expected values (Wardle & Nicholson 1996; Hector et al. 2002; Scherer-Lorenzen et al. 2007; Dawud et al. 2016). Relative basal

**Table 1**
Site characteristics, planting design, tree species and varieties, richness gradient and climate data since site establishment.

| Site | Country | Planting year | Plot size [m²] | Planting density [trees ha⁻¹] | MAT [°C] | MAP [mm] | Soil type | Former land-use | Richness gradient | Planted tree species and varieties |
|---|---|---|---|---|---|---|---|---|---|---|
| B-Tree | Austria | 2013 | 170–300 | 12,500 | +11.5 | 657.0 | Chernozem | pasture | 1, 2, 4 | *Acer platanoides, Carpinus betulus, Quercus robur, Tilia cordata* |
| ECOLINK-Salix Rostock | Germany | 2014 | 92 | 15,625 | +10.5 | 584.0 | Cambisol | crop | 1, 2 | *Salix* varieties:'Tora' (SW 910007, *S. schwerinii × S. viminalis*),'Loden' (SW 890,129 *S. dasyclados*) |
| ECOLINK-Salix Uppsala | Sweden | 2014 | 92 | 15,625 | +7.6 | 511.0 | Cambisol | crop | 1, 2, 3, 4 | *Salix* varieties:'Tora' (SW 910007, *S. schwerinii × S. viminalis*),'Björn' (SW 910006, *S. schwerinii × S. viminalis*),'Jorr' (SW 880013, *S. viminalis*),'Loden' (SW 890129, *S. dasyclados*) |
| FORBIO Gedinne | Belgium | 2010 | 1,575–1,764 | 4,444 | +8.6 | 1168.0 | Cambisol | plantation | 1, 2, 4 | *Acer pseudoplatanus, Fagus sylvatica, Larix × marschlinsii, Pseudotsuga menziesii, Quercus petraea* |
| FORBIO Hechtel-Eksel | Belgium | 2012 | 1,296 | 4,444 | +11.2 | 1058.5 | Podzol | plantation | 1, 2, 4 | *Betula pendula, Larix kaempferi, Pinus sylvestris, Pseudotsuga menziesii, Quercus petraea* |
| FORBIO Zedelgem | Belgium | 2009 | 1,764 | 4,444 | +11.1 | 1022.0 | Cambisol | crop | 1, 2, 4 | *Betula pendula, Fagus sylvatica, Pinus sylvestris, Quercus robur, Tilia cordata* |
| IDENT Freiburg | Germany | 2013 | 10 | 49,438 | +11.6 | 839.5 | Cambisol | pasture | 1, 2, 6 | *Acer platanoides, A. saccharum, Betula papyrifera, B. pendula, Quercus robur, Q. rubra* |
| ORPHEE | France | 2008 | 400 | 2,500 | +13.4 | 985.5 | Podzol | plantation | 1, 2, 4 | *Betula pendula, Quercus pyrenaica, Q. robur, Q. ilex, Pinus pinaster* |
| Satakunta | Finland | 1999 | 400 | 4,444 | +6.0 | 693.5 | Podzol | plantation | 1, 2, 5 | *Alnus glutinosa, Betula pendula, Larix sibirica, Picea abies, Pinus sylvestris* |
| MataDIV* | Brazil | 2019 | 460 | 1,887 | +19.8 | 1070 | Ferralsol | plantation | 1, 3, 6 | *Cariniana estrellensis, Cecropia pachystachya, Guazuma ulmifolia, Hymenaea courbaril, Handroanthus impetiginosus, Syagrus romanzoffiana* |

\* = not included in main analyses.

areas of species were used as weights to prevent dominance by high-performing species. For statistical analyses, NDE C was modelled as a ratio, but values are presented as percentage change for interpretability. The NDE C was logarithmically transformed for linear statistical analyses due to its non-linear nature.

### 2.4. DNA extraction and high-throughput sequencing

DNA extraction and community analyses were performed on a subset of 152 samples (Table S1), prioritizing sites with at least three richness levels and excluding species compositions/stands unique to a single site. This approach was done to balance downstream processing effort and sequencing cost and allowing us to retain the full dataset for broader analyses of soil C stocks and environmental drivers, thereby maintaining statistical power for context-dependency testing.

DNA was extracted from 250-350 mg soil using Qiagen DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) according to manufacturer's recommendations and confirmed via 1 % agarose gel electrophoresis. The fungal internal transcribed spacer (ITS) region (hereafter ITS2) was amplified for high-throughput sequencing using primers with Illumina adapter overhangs (Tedersoo et al., 2015; supplementary Table S2). We focused on ectomycorrhizal (EM) fungi, as most trees in the study form EM associations, while arbuscular fungi, associated with both trees and understory plants, are likely driven by understory composition and light availability.

PCR for fungal amplicons was performed in triplicates in 25 µl reaction with 2 µl DNA template (10 ng µl⁻¹), 5 µl 5X Buffer with 1.5 mM MgCl₂ (Promega, Charbonnières-les-Bains, France), 2 µl BSA at 10 mg ml⁻¹ (Promega), 0.5 µl of 10 mM dNTPs, 0.3 µl of Promega Go Taq G2 (5 U ml⁻¹), 1.25 µl of 5 pmol ITS3 tagmix forward primer (equally

mixed ITS3-Mix1 to 5), 1.25 µl of 5 pmol ITS4 tagmix reverse primer (equally mixed ITS4-Mix1 to 4), 12.7 µl of ultrapure distilled water (Invitrogen). PCR conditions were 2 min at 95 °C, 30 cycles [30 s at 95 °C, 30 s at 50 °C, 60 s at 72 °C], 45 s at 50 °C and 10 min at 72 °C. PCR triplicates were quality checked, pooled, and purified (for details see Supplementary Information). The indexing PCR was performed in a total volume of 18 µl (5 µL of ITS-PCR products, 9 µl Phusion High-Fidelity PCR Master Mix (NEB, France), 2 µl I5 index-adapter, 2 µl I7 index-adapter). Cycling conditions were 3 min at 95 °C, 8 cycles [30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C] and 5 min at 72 °C. After final purification, products were sequenced on a MiSeq Illumina sequencer using a v3 Kit (Illumina, San Diego, CA, USA). Full details are in Supplementary Information.

### 2.5. Bioinformatical processing of fungal ITS2 data

FASTQ files from the Illumina MiSeq system were processed using the default setting of PIPITS 3.0 (Gweon et al. 2015), involving read pair joining, quality filtering, and ITS2 extraction with ITSx (Bengtsson-Palme et al. 2013). Operational taxonomical units (OTUs) were assigned at 97 % identity (Gweon et al. 2015) using VSEARCH (Rognes et al. 2016), with chimeras removed via the UNITE v9.0 UCHIME database (Abarenkov et al. 2022). Taxonomic classification was done using the RDP classifier (Wang et al. 2007) against the UNITE fungal ITS database (v 27.10.2022; Kõljalg et al. 2013), resulting in 8,123,021 sequences categorized into 5,100 fungal OTUs, mostly from Ascomycota (75.6 %) and Basidiomycota (17.7 %; Supplementary Fig. S1), with minor fractions from Rozellomycota, Mucoromycota, and others (see Supplementary Information). After excluding low-read samples (<20,000 reads), data were rarefied with 1,000 repetitions to 22,730 sequences per

sample using R (v 4.3.2; R Core Team 2022) with RStudio (2023.09.1 Build 494; Rstudio Team 2023) and the R package 'GUniFrac' (Chen et al., 2023).

FungalTraits database (Põlme et al. 2020) was used to categorize OTUs at the species level or, where species data was unavailable, at the genus level (primary lifestyle only), classifying 48 % of the OTUs, with 7 % identified as EM fungi and 30 % as saprotrophic fungi. Full methodology and soil characteristics are provided in Supplementary Information and Table S2.

### 2.6. Statistical analyses

Data was analysed using R with RStudio and graphs were generated using the 'ggplot2' package (Wickham et al. 2023). Estimated marginal means were used to account for unbalanced sample sizes between sites. Where applicable, model assumptions of normality, homoscedasticity and multicollinearity were assessed visually. A mixed effects linear regression model (function *lme()* in package 'nlme'; Pinheiro & Bates 2023) was used to assess the response of C stocks and fungal diversity to tree species richness, with log-transformed topsoil C stocks or fungal diversity as response variables and tree species richness as the main predictor. C:N ratios and pH (with a quadratic term to capture its non-linear effect) were included as fixed covariates. Random effects for site and block, i.e., replicates within sites, were added to account for non-independence, and group-specific variance weights were applied to account for heteroscedasticity across sites (Zuur, 2009), ensuring that differences in residual variance between sites did not disproportionately affect model estimates. ANOVA (type III) using the function *Anova()* in the package 'car' (Fox et al. 2012) was used to test for effects in an unbalanced design and Tukey-adjusted pairwise comparisons of estimated marginal means (function *emmeans()* in package 'emmeans'; Lenth, 2016) were used to assess differences between richness levels. Marginal and conditional $R^2$ values were calculated with *r.squaredGLMM()* in package 'MuMIn' (Barton 2015), representing the variance explained by fixed factors and by both fixed and random factors, respectively (Nakagawa & Schielzeth 2013).

To investigate the context-dependency of the NDE C, we used a mixed-effects multiple linear regression model with standardized predictors (z-transformation). The z-transformation standardizes variables by centering them around a mean of zero and scaling them by their standard deviation. This process enables direct comparability of effect sizes across variables measured on different scales, preventing variables with larger absolute ranges from disproportionately influencing the results. Random effects for site and block (nested within sites) were included to account for the hierarchical structure, and group-specific variance weights were applied to address heteroscedasticity, which was observed as varying residual variances across sites. The model included fixed environmental variables such as climatic factors (MAT, MAP) and edaphic factors (clay content) at site level (n = 9), along with plot-level variables (fertility index, standing stock as BA ha$^{-1}$, n = 110). MAT and MAP indicated overall growing conditions (King et al. 2006; Wiesmeier et al. 2019; Kohyama et al. 2023), while variability in temperature and precipitation represented climatic stress, with greater variability suggesting higher stress on plants (Mearns et al. 1996; Goulden et al. 1996; Fay et al. 2011). Soil clay content was included to reflect soil air permeability (Niu et al. 2012) and soil water and nutrient retention capacity (Brady et al. 2008), while soil pH, and C:N ratios were included to reflect nutrient availability (Brady et al. 2008). Due to collinearity, pH and C:N ratios were combined into a fertility index using principal component analysis (Fertility index; Supplementary Fig. S2). Standing stock (BA ha$^{-1}$) was included as a proxy for accumulated aboveground biomass and potential organic matter inputs (Viana et al. 2014), while acknowledging it does not directly reflect productivity.

For fungal diversity analysis, we calculated Bray-Curtis dissimilarities for the fungal community and the Jaccard Index for the tree community, alongside indices for α-diversity, specifically Shannon index,

OTU (i.e., species) richness, and evenness, using the package 'vegan' (Oksanen 2022). We prioritized fungal OTU richness for its direct biological relevance and simplicity (Chiarucci et al. 2011) and as it demonstrated the strongest correlations with soil C stocks across fungal groups (Supplementary Fig. S3). Multivariate analyses, including non-metric multidimensional scaling (NMDS) for visualization, permutational multivariate analysis of variance (PERMANOVA), and Mantel test (using Spearman's rank correlation to account for non-linearity), were based on Bray-Curtis and Jaccard dissimilarity matrices, and were also performed with package 'vegan'. Since 'site' showed a strong effect on fungal community composition, this factor was subsequently decomposed into a series of descriptive variables, namely former land-use, edaphic, and climatic conditions (i.e., 'global model'). To account for the nested experimental design, PERMANOVA was performed with 1,000 permutations, stratified by site using the *strata* argument within the *adonis2()* function of the 'vegan' package to ensure that permutations occurred within sites rather than across them. All statistical analyses involving fungal data were conducted on the subsetted dataset of 152 samples, as described in the 'DNA extraction and high-throughput sequencing' section.

We used a Random Forest regression model to assess the relative importance of variables in explaining topsoil C stocks across nine study sites. This analysis helped to identify the primary drivers of topsoil C stocks and guided the selection of variables for evaluating the context-dependency of tree diversity effects. Our dataset included a range of variables relevant to soil C storage, and related to climatic factors (MAT, MAP, interannual temperature variability (CV), and interannual precipitation variability (CV)), edaphic factors (pH, C:N ratio, and clay content), site characteristics (plantation age, former land-use, species composition, planting densities and tree species richness levels), and vegetation (BA ha$^{-1}$). For a more detailed methodology see Supplementary Information.

## 3. Results

### 3.1. Effects of tree species richness on topsoil C stocks and fungal diversity

There was a significant overall positive effect of tree species richness on topsoil C stocks (Fig. 1A). Specifically, C stocks in stands with high tree richness were significantly higher (23.7 Mg C ha$^{-1}$; p = 0.028) compared with monocultures (21.7 Mg C ha$^{-1}$), corresponding to an increase of 9.2 % (Supplementary Table S3). C stocks in low tree richness stands (21.9 Mg C ha$^{-1}$) were not found to significantly differ from either high tree richness stands or monocultures (p > 0.05). Incorporating our additional tropical site into the analysis confirmed the trend of higher topsoil C stocks in high tree richness stands compared to monocultures (p = 0.022) and revealed a significant difference between high and low richness stands across sites (p = 0.049; Supplementary Table S4). Tree species richness did not significantly affect overall fungal OTU richness (Fig. 1B; Supplementary Table S5) or other α-diversity measures for the total or saprotrophic communities (Supplementary Fig. S3), with the notable exception of Shannon index and evenness in the EM fungal communities, which were higher in low tree richness stands compared to high tree richness stands (Shannon: p = 0.045; evenness: p = 0.029; Fig. 1C, Supplementary Fig. S3D).

### 3.2. Effects of sites and tree communities on fungal community composition

The NMDS analysis showed that site-specific factors, including former land-use type, predominantly shaped fungal community composition. Specifically, samples were clustering strongly by site and were grouped by land-use history along the x axis (Fig. 2A). Site, when used as a single predictor, accounted for 53 % of the variation in fungal community composition (PERMANOVA $R^2$ = 0.53, F = 22.7, p < 0.001). While the effect of site was predominant, in a global model excluding
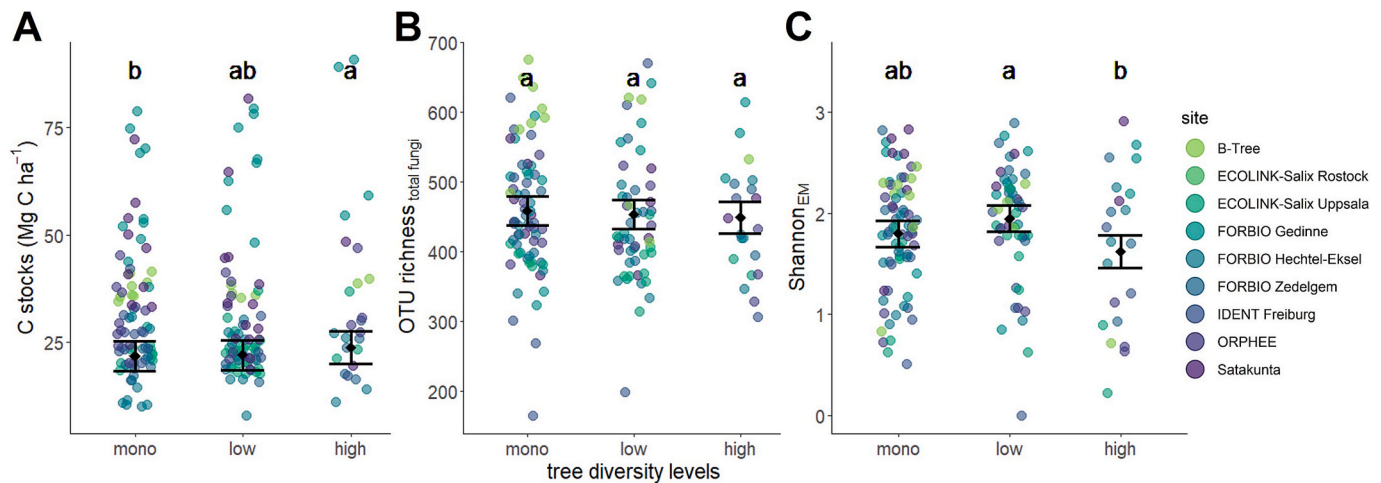
**Fig. 1.** Estimated marginal means of A) topsoil C stocks, B) total fungal OTU richness, and C) Shannon index for the ectomycorrhizal fungi (EM) subgroup across tree richness levels categorized as monoculture ('mono'), low (2–3 species), and high (4–6 species) diversity. Each point represents an individual sample and is color-coded according to experiment (site). Letters denote significant differences (LMM with Tukey's post-hoc test; $p < 0.05$; $n_A = 189$ [mono = 88, low = 85, high = 25], $n_{B,C} = 147$ [mono = 73, low = 53, high = 21]). Black circles with error bars indicate mean ± SE for each diversity level.

site, former land-use also emerged as a significant predictor, explaining c. 21 % of the variance in fungal community composition. While other factors (namely pH, interannual temperature and precipitation variability, MAP, and C:N ratio) significantly influenced community composition as well, their influences were less pronounced (Fig. 2A, Table 2).

Although PERMANOVA detected no significant variation in fungal community composition across different levels of tree diversity, a positive correlation was observed between tree and fungal community compositions (Fig. 2B), i.e. plots that were more similar in their tree species composition were also more similar in their fungal community composition.

### 3.3. Drivers and environmental context-dependency of richness effects on C stocks

To identify the key drivers of topsoil C stocks and to guide variable selection for the NDE C analyses, we used a Random Forest regression model (Supplementary Fig. S4). Variables with the highest relative importance (>10 %) were pH, MAP, interannual temperature variability (CV) and C:N ratio. Variables of lower relative importance (<10 %) included standing stock (BA ha$^{-1}$), interannual precipitation variability (CV), species composition, MAT, clay content, former land-use, plantation age, tree species richness level, and planting densities.

Using these variables, we examined how edaphic, climatic, and vegetation factors modulated the net diversity effect on topsoil C stocks (NDE C). The NDE C was significantly influenced by both edaphic and climatic factors (Fig. 3; Table 3). Specifically, a negative relationship was observed between soil fertility (Fertility index; Supplementary
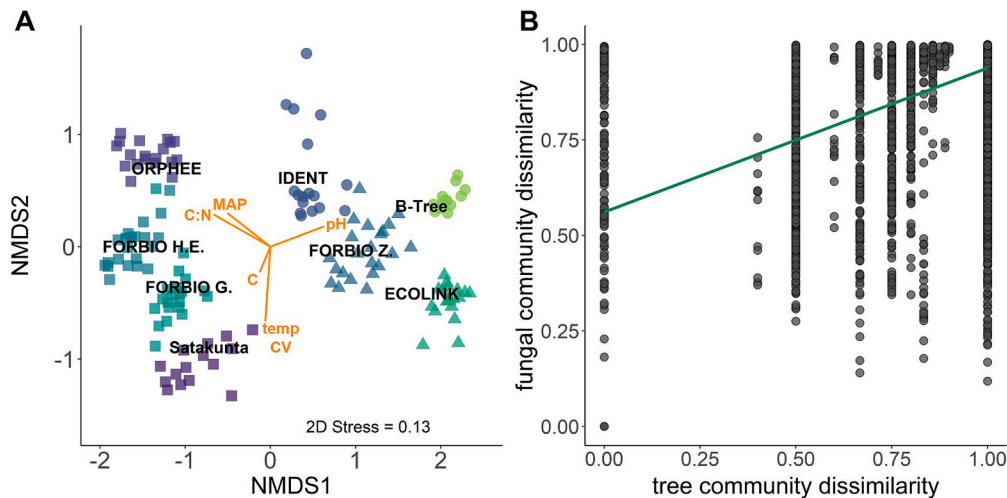


**Fig. 2.** A) Non-metric Multidimensional Scaling (NMDS) ordination plot illustrating fungal community compositions across different sites. Each point represents one sample, with shape corresponding to former land-use type (circle = pasture, square = plantation, triangle = crop). Samples closer together indicate higher similarity than those further apart. Arrows indicate the direction and strength of environmental variables correlated with community composition (temp CV = interannual temperature variability, C = soil C stocks). Abbreviated site names: ECOLINK = ECOLINK-Salix Uppsala, FORBIO G. = FORBIO Gedinne, FORBIO H.E. = FORBIO Hechtel-Eksel, FORBIO Z. = FORBIO Zedelgem, IDENT = IDENT Freiburg; n = 147. B) Relationship between tree and fungal community composition. Each point represents a pair of plots, comparing the dissimilarity of their tree and fungal communities. Dissimilarity values range from 0, indicating identical species compositions between samples, to 1, denoting completely distinct species assemblages. The line represents a linear regression fit to the data. Mantel test r = 0.268, p = 0.001.

**Table 2**

Summary of PERMANOVA results for fungal community composition by predictor variables (e.g. tree species richness level, soil pH, climatic variables), including degrees of freedom (df), $R^2$, F statistics and associated p values. Asterisks denote the level of significance (*** = p < 0.001, ** = p < 0.01, * = p < 0.05, n.s. = not significant).

| Variable | df | $R^2$ | F statistic | p value | |
|---|---|---|---|---|---|
| *Site only* | | | | | |
| Site | 7 | 0.533 | 22.652 | 0.001 | *** |
| *Global Model* | | | | | |
| Former land-use | 2 | 0.206 | 30.61 | 0.001 | *** |
| pH | 1 | 0.077 | 22.90 | 0.049 | * |
| Interannual temperature variability (CV) | 1 | 0.071 | 21.19 | 0.001 | *** |
| Interannual precipitation variability (CV) | 1 | 0.060 | 17.88 | 0.041 | * |
| MAT | 1 | 0.054 | 16.02 | 0.056 | n.s. |
| MAP | 1 | 0.055 | 16.32 | 0.030 | * |
| Topsoil C stocks | 1 | 0.009 | 2.59 | 0.104 | n.s. |
| C:N ratio | 1 | 0.006 | 1.85 | 0.001 | *** |
| Tree species richness level | 2 | 0.008 | 1.23 | 0.063 | n.s. |
| Residual | 135 | 0.454 | | | |
| Total | 146 | 1 | | | |

Fig. S2) and NDE C (p = 0.005). Interannual variability in both temperature and precipitation (CV), showed a negative correlation with NDE C (p = 0.003 and p = 0.043, respectively). Mean annual temperature (MAT) correlated negatively with NDE C (p = 0.031), while mean annual precipitation (MAP) and NDE C showed no significant relationship. Neither standing stock (BA ha$^{-1}$), mean annual precipitation (MAP) nor clay content were found to significantly correlate with NDE C (p > 0.05). The fixed effects explained 66 % of the variance in the model ($R^2_{marginal} = 0.66$), while the fixed and random effects together explained 88 % of the variance ($R^2_{conditional} = 0.88$).

### 3.4. Linking soil C stocks with fungal biodiversity

We observed a significant positive relationship between topsoil C stocks and fungal OTU richness (Fig. 4, Supplementary Fig. S5). Mixed-effects models confirmed a positive correlation with the total and the saprotrophic community (Fig. 4A, B), with higher soil C stocks corresponding to higher OTU richness (total fungi: $R^2_{marginal} = 0.07$, $R^2_{conditional} = 0.29$; saprotrophic fungi: $R^2_{marginal} = 0.05$, $R^2_{conditional} = 0.42$).

Furthermore, we observed a significant relationship between total fungal OTU richness and the NDE C (Fig. 4C). Based on mixed-effects model predictions, a higher fungal OTU richness modulated the NDE C weakly but positively ($R^2_{marginal} = 0.01$, $R^2_{conditional} = 0.15$).

## 4. Discussion

Our study investigated interactions between tree species richness, soil fungal diversity, and topsoil C stocks in mixed forest plantations across Europe. We found that higher tree species richness was associated with higher topsoil C stocks compared to monocultures, but not to greater fungal diversity. Tree species richness influenced fungal guilds differently: EM fungal diversity decreased with higher tree species richness, while saprotrophic fungi taxa richness was indirectly linked to tree species richness through its positive correlation with topsoil C stocks. We also showed that low soil fertility, stable interannual climatic conditions as well as low temperatures positively correlated with a net tree diversity effect on topsoil C stocks.
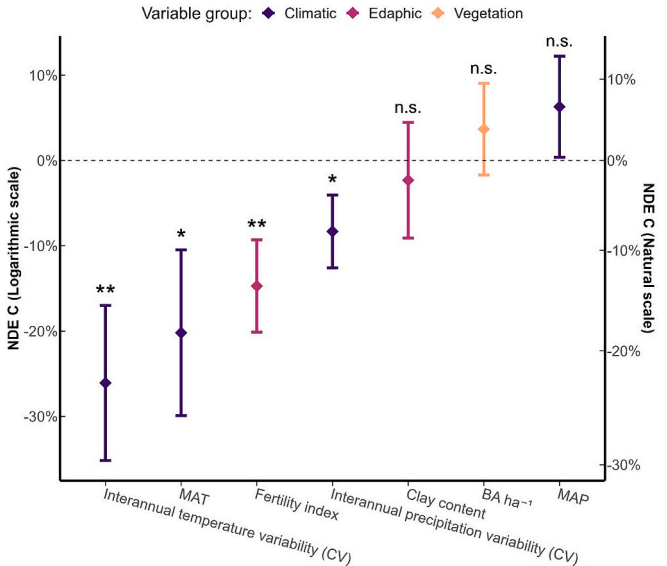


**Fig. 3.** Standardized effects of climatic, edaphic, and vegetation variables on the net diversity effect on topsoil C stocks (NDE C) across nine sites (n = 110) on a logarithmic scale (first y-axis; left) and natural scale (second y-axis; right). Error bars denote standard errors. Climatic variables include mean annual temperature (MAT), mean annual precipitation (MAP), as well as variability in temperature and precipitation (interannual CV). Edaphic variables include the inverted first principal component axis for soil pH and C:N ratio (Fertility index) as well as soil texture (based on clay content). Effects of standing stock is represented by basal area per hectare (BA ha-1). Significant effects on NDE C are indicated as *** = p 0.05, n.s. = not significant. Values below 0 indicate negative correlation between NDE C and predictors.

**Table 3**

Summary of multiple linear regression analysis results for context-dependency of the net diversity effect on topsoil C stocks (NDE C) of climatic and edaphic variables, including variable coefficients, standard error (SE), degrees of freedom (df), t-value and associated p-values. Asterisks denote significant correlations with NDE C for each variable (*** = p < 0.001, ** = p < 0.01, * = p < 0.05, n.s. = not significant).

| Predictor | Coefficient | SE | df | t-value | p-value | |
|---|---|---|---|---|---|---|
| Intercept | −0.2 | 3.3 | 88 | −0.07 | 0.943 | n.s. |
| MAT | −20.2 | 9.7 | 3 | −2.08 | 0.031 | * |
| MAP | 6.3 | 6.0 | 3 | 1.06 | 0.271 | n.s. |
| Interannual temperature variability (CV) | −26.1 | 9.1 | 3 | −2.90 | 0.003 | ** |
| Interannual precipitation variability (CV) | −8.3 | 4.3 | 3 | −1.95 | 0.043 | * |
| Fertility index (pH + C:N) | −14.7 | 5.4 | 88 | −2.72 | 0.005 | ** |
| Clay content | −2.3 | 6.8 | 3 | −0.34 | 0.722 | n.s. |
| Standing stock (BA ha$^{-1}$) | 3.7 | 5.4 | 88 | −0.68 | 0.478 | n.s. |

### 4.1. Higher tree species richness increases topsoil C stocks

Our analysis revealed a significant increase in topsoil C stocks understands with high tree species richness compared to monocultures stands, supporting our hypothesis (I) and aligning with previous research linking diverse forests to increased soil C storage (Chen et al. 2018; Augusto & Boča 2022). Such increases in soil C storage have been linked to increases in biomass production and litter inputs in mixtures, often attributed to more efficient use of available canopy and rooting space (Leuschner et al. 2001; Williams et al. 2017; Forrester et al. 2013; Barry et al. 2019). Beyond biomass quantity, several mechanisms may
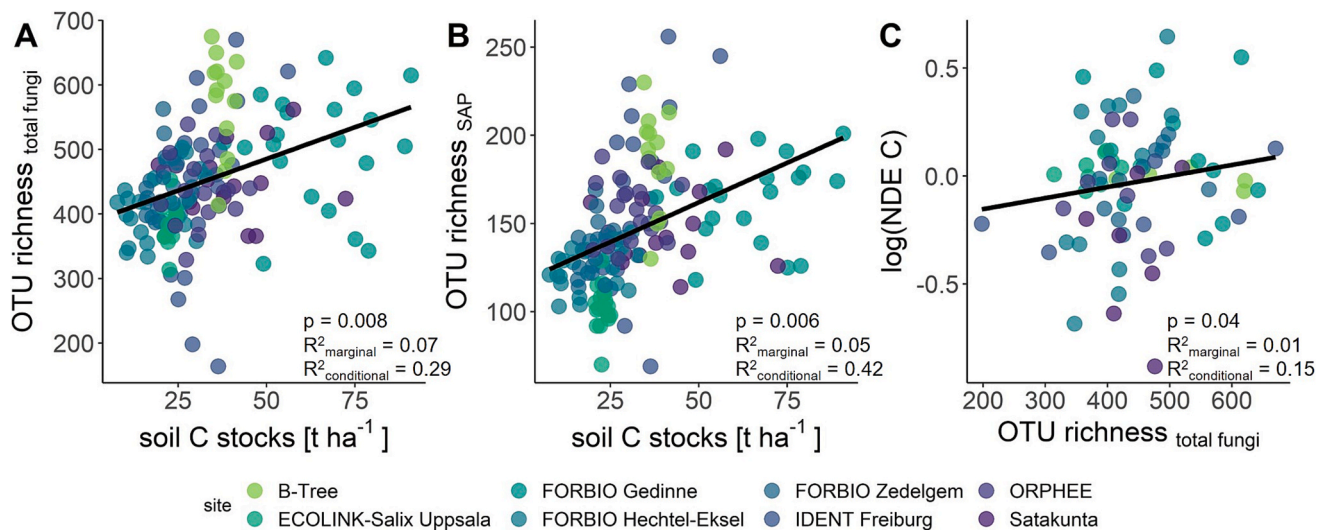
**Fig. 4.** Relationships between topsoil carbon (C) stocks and OTU richness for A) total fungi, and B) saprotrophic (SAP) fungi. C) Correlation between the log-scaled net diversity effect on soil C stocks (NDE C) and the total fungal OTU richness. Each point represents a unique soil sample, color-coded by site. The lines represent a linear fit to the model. nA & B = 147, nC = 71.

contribute to diversity effects on soil C accumulation. Evidence from grassland biodiversity experiments shows that higher plant diversity can stimulate microbial activity and contribute to greater soil C stocks through increased microbial necromass contributions to the soil (Lange et al. 2015; Steinbeiss et al. 2008). However, tree species mixing may also reduce the rate of soil organic matter decomposition by lowering microbial efficiency (Manzoni et al. 2008; Zhang et al. 2008), for example through increased soil C:N ratios (Spohn et al. 2023), thereby promoting soil C accumulation (Tipping et al. 2016; Lehmann et al. 2020). Similarly, El Moujahid et al. (2017) found a positive relationship between the diversity of soil organic compounds and plant species richness, potentially lowering decomposability of SOM in tree species mixtures due to a higher cost of consumption for decomposers (Lehmann et al. 2020; Nunan et al. 2015; Spohn et al. 2023). Although we did not have the means to investigate decomposition mechanisms in our study, reduced decomposition rates in mixed stands, as reported in other studies, could explain the observed diversity effects on soil C stocks. Notably, we observed significant differences only between high tree species richness levels and monocultures, while low tree species richness showed no clear difference to either group. Potentially, significant influences of tree species richness on topsoil C stocks may manifest earlier in stands with a higher species richness. Moreover, our Random Forest model identified climatic and edaphic conditions, such as soil pH and MAP, not tree species richness, as the primary predictors of soil C stocks. While these more fundamental drivers exert stronger influence soil C accumulation, the fact that tree species richness still explained variation after accounting for them provides evidence of its importance. Nevertheless, it is important to recognize that although climatic and edaphic conditions are largely beyond human control, species mixing remains a critical management strategy. Given that effects of tree diversity on soil C often increase over time (Chen et al. 2020) and that the stands of the experimental network are relatively young, it is plausible that this effect will increase as the stands mature. Furthermore, consistent with other studies, species composition was found to be more influential on topsoil C stocks than species richness *per se* (Dawud et al. 2016; Mayer et al. 2020).

### 4.2. Effects of tree species richness on soil C stocks are modulated by environmental factors

The reported influence of tree diversity on soil C stocks varies between studies (Gamfeldt et al. 2013; Dawud et al. 2016; Chen et al.

2018), reflecting context-dependency that may be contingent on abiotic factors (Ratcliffe et al. 2017). Our analyses also indicate that the effect of tree species richness on soil C stocks can be influenced by both climatic and edaphic factors, specifically soil fertility, mean annual temperature, and the interannual variability (CV) of temperature and precipitation, thus partially aligning with our hypothesis (II) that positive effects of tree diversity on soil C stocks are stronger under harsher environmental conditions.

If greater biomass inputs were solely responsible for the increase in soil C stocks, we would expect the effects of tree species richness on soil C stocks to positively correlate with standing stock, which we used as a proxy for stand biomass and organic inputs (Viana et al. 2014), but this was not observed. Similarly, Bryant et al. (2024) reported no link between aboveground biomass accumulation and soil C stocks in a 12-species diversity experiment, despite large differences in biomass. Such discrepancies may derive from temporal lags between aboveground growth and soil C accumulation or altered above- to belowground C allocation patterns in mixtures (Forrester et al. 2006; Martin-Guay et al. 2020). Additionally, species-rich stands may have higher fine root turnover due to increased belowground competition for resources or higher fine root density (Jacob et al., 2014; Lei et al., 2012; Wambsganss et al., 2021). Such belowground inputs, which were not captured in our aboveground biomass measurements, could contribute to a greater accumulation of organic matter in the soil and help explain why we did not detect a direct relationship between standing stock and the effects of tree species richness on soil C stocks. Moreover, changes in soil C stocks result not only from C inputs, but also from C losses due to decomposition of SOM. A higher biomass input in mixed stands compared to monocultures, for example due to niche complementarity or increased fine root turnover in mixtures, would lead to a significant difference in soil C stocks more quickly if the decomposition rate is slower. There is evidence to suggest that increased soil C stocks due to tree species mixing is more common in colder and less fertile conditions (Augusto & Boča, 2022; Bertness & Callaway, 1994; He et al., 2013). Microbial activity and therefore decomposition rates tend to decrease under drought, low temperatures, low pH, and high C:N ratios (Gabarrón-Galeote et al., 2015; González-Domínguez et al., 2019; Kirschbaum, 1995; Le Roux et al., 2013). Thus, even if the relative increase in C inputs from tree diversity does not rise under harsher conditions, the decreased decomposition rates in these conditions may result in larger carbon stocks in mixed stands compared to monocultures. In contrast, under more favorable conditions with faster decomposition rates, this difference

may be less pronounced. The negative relationship between NDE C and climatic variability might result from species-specific responses favoring more adaptable tree species (Reyer et al. 2013; Hipler et al. 2020; Giberti et al. 2022), potentially leading to the domination of certain species, diminishing associated benefits of tree diversity on soil C stocks. Further research is needed to investigate the specific species dynamics within each stand.

### 4.3. Tree species richness affects EM diversity and saprotrophic fungal richness, but not total fungal richness

We found a positive correlation in β-diversity between tree and fungal communities, suggesting that tree species identity in mixtures influences fungal community composition. Species-specific traits, such as litter quality, root exudates, and root architecture may create ecological niches, supporting the resource diversity and niche differentiation hypothesis (see Yang et al. 2017). Unexpectedly, tree α-diversity (tree species richness) did not significantly affect fungal richness contradicting our hypothesis (I) and the common assumption that increased tree species richness translates to increased microbial diversity (Liu et al. 2020). Our findings support evidence that tree-fungi diversity relationships can be neutral or even negative (Waldrop et al. 2006; Griffin et al. 2019). For example, Griffin et al. (2019) proposed that higher tree species richness may promote competitive generalist fungi, reducing overall fungal diversity. Instead, tree species identity (broadleaf vs conifers) and functional traits, rather than richness, strongly influence fungal community composition and diversity (Nguyen et al. 2016; Sanaei et al. 2022). Additionally, site-specific differences in fungal richness, consistent with Weißbecker et al. (2018), highlight the importance of special variables and soil properties in driving soil fungal α- and β-diversity. In addition, tree-fungi diversity relationships vary by fungal guild (Tedersoo et al. 2016). In our study, EM fungal richness was unaffected by tree species richness, possibly due to a dilution effect when mixing non-EM and EM host species (Singavarapu et al. 2024). However, EM fungal evenness decreased in high-richness stands, suggesting more low-abundance, narrow-host taxa and fewer dominant, broad-host taxa, likely due to an increase in shared EM communities (Ferlian et al. 2021). Other factors, such as forest age (Lim & Berbee 2013) and land-use history, which we found to be the second most influencing factor, may mask effects of tree diversity on fungal communities. The limited richness gradient and young stand age in our study may have also constrained detectability of effects on fungal diversity (Nguyen et al. 2016).

### 4.4. Tree diversity, fungal richness, and topsoil C stocks are positively correlated

While we did not find direct links between tree species richness and fungal diversity, we observed a positive correlation between total and saprotrophic fungal richness with topsoil C stocks, aligning with the role of saprotrophs as primary decomposers driving SOM turnover (Bödeker et al. 2016). In contrast, EM fungi may influence soil C dynamics more indirectly through necromass accumulation due to high hyphal biomass (Godbold et al. 2006; See et al. 2022), alterations in root exudation patterns (e.g., Meier et al. 2013; Liese et al. 2018), or potential interactions with saprotrophs that may either suppress or enhance their activity, depending on site conditions (Fernandez & Kennedy 2016; Mayer et al. 2021; Fanin et al. 2022). Although we found fungal richness to correlate with topsoil C stocks, causality remains difficult to infer: fungal communities influence SOM dynamics, but they also respond to SOM quantity and quality (see, e.g., Feng & Wang, 2023; See et al., 2022; Zak et al., 2019). The generally positive relationship between fungal diversity and soil C (Bastida et al. 2021) may reflect increased heterogeneity in organic matter inputs that can provide a broader range of ecological niches. This is supported by findings that soils with higher humus content tend to have higher total C stocks (Bonifacio et al. 2011)

and also support greater fungal diversity (Trap et al. 2011), as well as evidence that variation in the molecular composition of soil carbon is shaped by the functional potential of soil microbial communities (Davenport et al. 2025). However, it is important to note that our sampling was restricted to the upper 10 cm of soil, which represents a more labile carbon pool compared to deeper mineral horizons where long-term C stabilization occurs (Vormstein et al. 2020).

We found a positive correlation between NDE C and total fungal richness, which, despite the high variability typical of such complex ecological data, supports our hypothesis (III). Additionally, the NDE C was negatively correlated with soil fertility and MAT, indicating higher NDE C under conditions that promote slower decomposition (Berg & McClaugherty 2020). Slower decomposition leads to humus accumulation, potentially increasing organic matter in the top 10 cm of the soil (Getino-Álvarez et al. 2023). Sites with slower decomposition and diverse litter input from mixed stands (Bonifacio et al., 2011; Trap et al., 2011; Queiroz et al., 2021) may develop a more heterogeneous soil layer, creating niches for greater fungal richness. A more diverse fungal community, in turn, can promote soil aggregate stability (Lehmann et al., 2020; Sae-Tun et al., 2022), ensuring a sustained C accumulation.

## 5. Conclusion

Our study sheds light on the complex relationships between tree species richness, fungal diversity, and topsoil C stocks in European mixed forests. We show that higher tree species richness can potentially enhance topsoil C stocks, and that this effect is modified by climatic and edaphic conditions. Specifically, the positive effects of species mixing on soil C stocks may diminish under high temperatures, high soil fertility and climatic variability. This suggests that the potential for climate change mitigation through diverse forest plantations may be particularly pronounced in less fertile and more climatically stable environments, while under changing or extreme conditions, expected benefits could be reduced. Future research should investigate whether the contribution of tree species richness to topsoil C stocks in young plantations increases over time, as well as the efficacy of specific species mixtures in relation to a changing climate. Although tree species richness did not directly affect fungal diversity, we found a positive correlation between fungal richness and NDE C. This suggests that fungal diversity may be one of several factors influencing the variability in NDE C, and that tree species richness, fungal diversity, and topsoil C stocks are interconnected through both direct and indirect pathways.

**CRediT authorship contribution statement**

**Ramona Werner:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation, Conceptualization. **Joel Jensen:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation, Conceptualization. **Petra Fransson:** Writing – review & editing, Supervision, Data curation, Conceptualization. **Christel Baum:** Writing – review & editing, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Hans Sandén:** Writing – review & editing, Supervision, Methodology, Data curation, Conceptualization. **Boris Rewald:** Writing – review & editing, Visualization, Methodology, Data curation, Conceptualization. **Douglas L. Godbold:** Writing – review & editing, Supervision, Methodology, Data curation, Conceptualization. **Mathias Mayer:** Writing – review & editing, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Joannès Guillemot:** Writing – review & editing, Data curation. **Agnès Robin:** Writing – review & editing, Methodology, Data curation. **Pedro H.S. Brancalion:** . **Julia Koricheva:** Writing – review & editing, Data curation. **Quentin Ponette:** Writing – review & editing, Data curation. **Bart Muys:** Writing – review & editing, Data curation. **Kris Verheyen:** Writing – review & editing, Data curation. **Michael Scherer-Lorenzen:** Writing – review & editing, Data curation. **Jürgen**

**Bauhus:** Writing – review & editing, Data curation. **Friderike Beyer:** Writing – review & editing, Data curation. **Peter Hajek:** Writing – review & editing, Data curation. **Hervé Jactel:** Writing – review & editing, Data curation. **Martin Weih:** Writing – review & editing, Supervision, Data curation, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.geoderma.2025.117591.

## Data availability

Soil and tree data, fungal diversity metrics, and site metadata at Zenodo (DOI: 10.5281/zenodo.17514655). ITS2 amplicon sequence data at Zenodo (DOI: 10.5281/zenodo.17496974).

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