Journal of Ecology

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Article type : Research Article

Handling Editor: Liesje Mommer

Tree species mixing reduces biomass but increases length of absorptive fine roots in European forests

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Abstract

1. Mixed-species forests often enhance the provision of ecosystem functions, both above and below-ground. Several of these effects are mediated by the amount and spatial distribution of tree tissues. However, previous studies on tree diversity effects on fine-root biomass (FRB) have returned inconsistent results and did not distinguish between absorptive and transport fine roots. Furthermore, owing to the lack of species-specific data, it is not well understood whether complementarity or selection effects contribute more to these mixing effects.
2. Here, we analysed tree species mixing effects on fine-root traits while considering the respective tree species contributions and root functional types. Specifically, we tested whether tree species mixing increases FRB and root length density (RLD) and results in vertical root stratification. We quantified FRB and RLD in 30 cm deep soil profiles for 13 tree species in mixed and pure stands across four wide-spread European forest types. The differentiation of different fine-root species in mixtures allowed us to disentangle complementarity and selection effects.
3. Across all sites, mixtures supported on average less FRB than pure stands, which was reflected in negative complementarity and selection effects. RLD of absorptive fine roots did not change across the soil profile and even increased in the topsoil, which was associated with positive complementarity effects. There was no evidence for vertical root stratification. Conifer proportion, which was mainly associated with selection effects, dampened net diversity effects. Root functional type further influenced tree species mixing effects.
4. **Synthesis:** Despite the underyielding of FRB in mixtures, overall soil occupation by absorptive fine roots (RLD) did not decrease in mixtures, pointing to morphological root trait adaptations associated with higher resource-use efficiency. Increased RLD in the most nutrient-rich layer in mixtures further indicates complementary interactions among species and a greater resource uptake capacity. This work illustrates that considering only one aspect of trait-functioning relationships, e.g. root biomass, may not capture the full effect of plant diversity on ecosystem functioning. The integration of a larger range of relevant traits is required. Moreover, traditional classification of fine roots based on the 2-mm diameter cut-off may obscure responses of roots to environmental changes.

Key-words: additive partitioning, biodiversity and ecosystem functioning, root length density, SoilForEUROPE, species identity effects, tree species richness, vertical root distribution

Introduction

Mixed-species forests have often been shown to enhance above-ground ecosystem properties and processes (e.g. Scherer-Lorenzen 2014). They can be more productive (Zhang, Chen, & Reich, 2012), provide a greater diversity and a higher degree of ecosystem services (van der Plas et al., 2016), harbour more biodiversity at multiple trophic levels (Ampoorter et al., 2020), and are more resistant to biotic and abiotic disturbances than their mono-specific counterparts (Jactel et al., 2017). In addition, they are an important management approach to adapt forest ecosystems to the consequences of climate change (Ammer, 2019). The underlying mechanisms driving these positive mixing effects are often attributed to complementarity effects (CE), i.e. positive species interactions resulting in competitive reduction, owing to niche differentiation and/or facilitation, and selection effects (SE), i.e. when mixing effects are mainly determined by dominance of more productive species (Forrester & Bauhus, 2016; Loreau & Hector, 2001). However, most previous studies examining tree diversity effects focused on above-ground processes. Consequently, studies complementing our understanding of above-ground mechanisms in mixtures by investigating below-ground processes are urgently needed.

Despite their crucial role for ecosystem functioning (Bardgett, Mommer, & De Vries, 2014), fine-root responses to changing tree species diversity and their interactions with environmental conditions are still not well understood (Ammer, 2019; Laliberté, 2017). Yet, knowledge on below-ground diversity effects are of major importance for ecosystem functioning as the below-ground and above-ground compartments are strongly interlinked (Bardgett & Van Der Putten, 2014). In particular, below-ground plant biomass and the root length density (RLD) encapsulate key aspects of positive biodiversity-ecosystem functioning relationships (Bardgett & Van Der Putten, 2014; Cardinale et al., 2011). Indeed, the acquisition of below-ground resources by plants relies largely on maintaining a sufficiently high surface of absorptive fine roots, which can be achieved by increasing fine-root lifespan and/or increasing fine-root productivity (Eissenstat & Yanai, 1997, Hodge 2004).

Below-ground overyielding in terms of standing fine-root biomass (FRB) can be regarded as an example of a positive net diversity effect (NE). Overyielding occurs when FRB in species mixtures is greater than expected from the species' corresponding pure stands. It may be the result of increased soil space occupation (i.e. increased root length deployed and more even root distribution) and can facilitate higher resource exploitation by fine roots. While above-ground vertical crown stratification may increase canopy packing (Jucker, Bouriaud, & Coomes, 2015), vertical root stratification (i.e. more even vertical root distribution, for instance, due to increased root biomass at depth) owing to complementary rooting pattern of different species can result in an enhanced soil space occupation by fine roots (Bakker, Mommer, & van Ruijven, 2019; Brassard et al., 2013; Sun et al., 2017). A greater soil space occupation and thereby a greater resource capture in any soil layer could also be achieved by complementary resource acquisition strategies (Sun et al., 2017) related to mycorrhizal symbionts (Chen, Koide, & Eissenstat, 2018) and/or (abiotic) facilitative effects enhancing soil nutrient (Prieto et al., 2015; von Felten et al., 2009) or water availability (Prieto, Armas, & Pugnaire, 2012).

Reported biodiversity effects on standing FRB appear to be inconsistent across plant types and ecosystems. Although across all terrestrial ecosystems, FRB was 28.4% higher in mixtures than in monocultures, the results in forests are quite variable. Studies reported a higher (e.g. Brassard *et al.,* 2013; Valverde-Barrantes *et al.*, 2015, Sun *et al.,* 2017; Zeng *et al.,* 2020) or lower biomass (Archambault et al., 2019; Bolte & Villanueva, 2006), or no changes in mixtures compared to pure stands (e.g. Bauhus, Khanna, & Menden, 2000; Finér et al., 2017; Ma & Chen, 2016; Peng & Chen, 2020). These inconsistencies may have occurred for a number of reasons. First, they may be attributable to differences in methodologies of root sampling and analyses (Freschet & Roumet, 2017). Very few studies have considered different functional fine-root types, i.e. absorptive fine roots (first three most distal orders) vs. transport roots (those of the fourth or higher orders) (Liao et al., 2019; McCormack et al., 2015; Yan et al., 2019). Consequently, diversity effects on fine roots may have been obscured in some cases, as, in particular, responses of absorptive roots can be expected to be more pronounced, owing to their greater plasticity (Kou et al., 2018; Salahuddin et al., 2018) and their direct involvement in resource uptake and tree-to-tree interactions (McCormack et al., 2015). Secondly, inconsistencies may have resulted from the questionable use of FRB as a proxy for soil space occupation, which may be better represented by other root characteristics such as root length (Freschet, Swart, & Cornelissen, 2015). Owing to these issues, in many studies the underlying mechanisms of diversity effects on fine roots remained unclear. Third, species identity can play a more important role than diversity effects for FRB (Domisch, Finér, Dawud, Vesterdal, & Raulund-Rasmussen, 2015; Finér et al., 2017; Jacob, Hertel, & Leuschner, 2013, 2014). In particular, the presence of conifer species has been shown to increase standing FRB (Archambault et al., 2019; Finér et al., 2017). Owing to the lack of appropriate experimental designs and species-specific root data, it is often impossible to disentangle SE and CE, which could, however, provide valuable insights into the underlying mechanisms behind diversity effects.

There are comparatively few biodiversity-ecosystem functioning studies in natural, mature forests, and most recent insights stem from young tree experiments with controlled conditions (e.g. Grossman *et al.*, 2018). However, results from these experiments can hardly be extrapolated to forested landscapes, as functioning of complex, naturally assembled communities differs substantially from that of more artificially formed communities (Duffy, Godwin, & Cardinale, 2017). Therefore, comparative observational studies in managed forests are also required to test for diversity effects in near-natural situations and to provide recommendations for decision-makers in forestry (Baeten et al., 2013).

In view of the abovementioned potential methodological shortcomings and existing knowledge gaps, the overall objective of this study was to analyse tree species mixing effects on standing FRB, RLD, and vertical root stratification in mature European forests. The following research hypotheses were tested:

1. Standing fine-root biomass and root length density are higher in mixtures than in mono-specific stands (overyielding) owing to the complementary use of space and resources among species.
2. Net diversity effects are mainly driven by complementarity rather than selection effects.
3. Species identity influences mixing effects so that conifers increase standing FRB and RLD and amplify mixing effects.
4. Absorptive fine roots are more strongly influenced by tree species mixing than transport fine roots.

Materials and Methods

Study Design

To conduct this study, we took advantage of an existing network of mostly mature, uneven-aged forest plots along tree species richness gradients from the pan-European FunDivEUROPE project (Baeten et al., 2013). Plots (30 x 30 m) were selected according to predefined criteria on evenness, tree age, density, species composition, richness, and environmental factors. Except for tree species composition and richness, all other variables were kept as constant as possible to minimise confounding factors (see Baeten et al., 2013). Here, we used a subset of these plots, consisting of three-species mixtures (33 plots) and the corresponding mono-specific stands (30 plots), distributed across four sites representative of major European forest types, i.e. boreal (Finland), hemiboreal (Poland), mountainous beech (Romania) and thermophilous deciduous (Italy) forests (Table 1). In total, 13 tree species were studied. Each species occurred in single-species stands (replicated twice, except for *Quercus robur* and *Picea abies* in Poland where only one pure stand was available for sampling, respectively), while mixtures consisted of different species compositions. The number of replicate plots for identical species compositions varied from one to four per site.

Table 1. Study sites' soil, forest floor, climate, and forest stand characteristics. Abbreviations: MAT (mean annual temperature), MAP (mean annual precipitation).

Site	**North Karelia**	**Białowieża**	**Râsca**	**Colline Metallifere**
Country	Finland	Poland	Romania	Italy
Latitude/ Longitude (°)	62.6, 29.9	52.7, 23.9	47.3, 26.0	43.2, 11.2
Elevation range (m)	80-200	135-185	600-1000	260-525
MAT (°C)	2.1	6.9	6.8	13
MAP (mm)	700	627	800	850
Forest type	**Boreal**	**Hemiboreal**	**Mountainous beech**	**Thermophilous deciduous**
Average stand age (y)	51	104	88	67
Target tree species	*Betula pendula/*	*Betula pendula*, *Carpinus*	*Abies alba*, *Acer*	*Castanea sativa*, *Ostrya*

	pubescens, Picea abies, Pinus sylvestris	*betulus, Picea abies, Pinus sylvestris, Quercus robur*	*pseudoplatanus, Fagus sylvatica, Picea abies*	*carpinifolia, Quercus cerris, Quercus ilex, Quercus petraea*
Average conifer proportion (% basal area)	70	48	48	0
Soil type	Podzol	Cambisol/ Luvisol	Eutric Cambisol	Cambisol
Mean max. soil depth (cm)	80	80	75	67
Topsoil texture class	Sandy loam	Sandy loam	Silty clay loam	Silt loam
Topsoil clay %	5	6	27	18
Topsoil pH	3.9	3.8	4.6	4.6
Topsoil carbon (mg g^{-1})	37.8	28.4	49.2	50.4
Topsoil nitrogen (mg g^{-1})	1.7	1.7	3.5	2.6
Forest floor total phenols (mg g^{-1} dry litter)	6.86 ± 1.54	4.1 ± 1.48	5.25 ± 0.97	4.12 ± 1.47
Forest floor condensed tannin (%)	1.56 ± 0.51	0.73 ± 0.42	1.07 ± 0.28	0.82 ± 0.36
Forest floor lignin (g kg^{-1} dry litter)	564 ± 184	917 ± 192	658 ± 136	810 ± 201
Forest floor C/N	40.5 ± 8.3	29.3 ± 6.9	30.9 ± 4.5	35.8 ± 5.2
Forest floor P (%)	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.03 ± 0.01
Number of mono-specific / mixed stands	6/3	6/13	8/8	10/9

Within each plot, we selected five subplots, each consisting of three neighbouring similar-sized target trees, so-called *triplets*, following the approach of Vivanco & Austin (2008). We collected soil samples at a point of approximately equal influence of the three trees shaping the *triplets*. This point was determined based on a visual estimate of the individual trees' diameter at breast height (DBH) and crown area. We selected similar-sized (DBH, height, and crown size), dominant, or codominant (Kraft, 1884), and healthy individuals for the *triplet* trees. When *triplet* trees had different dimensions, we moved the position of the sampling point towards the tree(s) with the smaller dimensions. The average distance between sampling spots and tree individuals was 2.7±0.05 m (the minimum was 0.9 m; the maximum was 5.2 m). We chose this neighbourhood approach instead of a random sampling design to facilitate quantification of the influence of the target trees on our sampling spots. Hence, we additionally measured tree DBH and distances between target trees and sampling points. Usually, the five tree neighbourhoods were evenly distributed across the plot and representative of the plot's microtopography. We further ensured that no shrubs occurred in the vicinity of sampling spots.

Root Sampling and Processing

Fine-root sampling took place in spring 2017 at, or close to, spring leaf emergence at each site, as we expected a peak in FRB then. For each tree *triplet*, one soil core was taken with a split-tube sampler (Eijkelkamp, inner diameter 5.3 cm) after removal of the litter layer. The 315 soil cores were divided into 0-10 cm, 10-20 cm and 20-30 cm soil depth layers.

All samples were frozen at -20°C upon arrival in the laboratory until further processing. Samples were thawed and soaked in water at 4°C for at least two hours before being washed with tap water over a cascade of sieves (2 and 1 mm) to separate roots from mineral soil particles and other organic material. We used the flotation method to separate stones from roots (Bauhus & Bartsch, 1996). Stones were weighed, and their volume was determined for the calculation of the fine-earth (i.e. soil particles <2 mm) volume of each sample. All root fragments >1 cm in length were separated by diameter into coarse (>2 mm) and fine roots (≤2 mm). Fine roots were sorted into tree roots and understory (herbaceous) roots under a binocular. Herbaceous roots can easily be identified by their light, transparent colour and simple structure. Tree roots were subsequently divided into dead (brittle, non-elastic, dark, hollow) and live (elastic, lighter) roots. Live tree fine roots were further manually sorted by species using a binocular and intact reference samples (stored in 40% ethanol) from all sites. Criteria for distinguishing species included periderm structure, cross-section, architecture, colour and mycorrhizal associations (for further details, see Table S1). Only the three *Quercus* species in Italy could not be reliably separated from each other and were therefore pooled as *Quercus* spec. for subsequent analyses. Furthermore, in Finland, *Betula pendula* and *Betula pubescens* were combined into a single group (called *Betula pendula*, as this species was the dominant birch species) owing to their close relatedness and indistinguishable fine-root characteristics.

We applied the functional classification approach (McCormack et al., 2015) and divided fine roots ≤2 mm into absorptive (first three root orders) and transport (higher-order) roots. Short root fragments (<1 cm in length) can constitute a large amount of the total FRB in root samples extracted with small-diameter soil cores (Bauhus & Bartsch, 1996). To account for these root fragments, we followed a subsampling approach of 10% of the remaining sample for root fragments <1 cm in length (Bauhus & Bartsch, 1996). Fragments were not distinguished by species, functional type or status (dead or live). As they belonged mainly to the first three root orders, their mass was later only allocated to the absorptive root pool. Roots were scanned at a resolution of 800 dpi using a flat-bed scanner. Root length and volume were calculated using the software WinRhizo (Regent Instruments, Quebec, Canada, 2009), and root volume was re-calculated using individual diameter classes (Rose, 2017). The coarse root volume was used to obtain the fine-earth content by subtracting the coarse root and the stone volume from the total soil volume of each sample. The fine-root length was used for the calculation of the RLD (cm cm^{-3} fine-earth volume). All root fractions were dried for 72 h at 40°C and weighed with a high-precision scale. Mass of live and dead tree fine roots and of understory fine roots was calculated as g dry mass per m^2 soil surface area.

Calculations

Tree neighbourhood index

In addition to adjusting sampling points based on individual trees' visually estimated influence in the field, we calculated a tree neighbourhood index (NHI), similar to a competition index. This index mathematically quantifies the influence of individual trees of each *triplet* on the root sampling point to test for imbalanced effects of individual trees on FRB and RLD. Therefore it allows for possible corrections of these values.

In line with Yanai *et al.* (2006), the following equation was used:

$$NHI = \sum_{i=1}^{n=3} \frac{DBH_i^2}{d_i} \qquad (1)$$

where n is the number of trees considered, i.e. $n=3$ (the number of trees composing one *triplet*), DBH_i is the diameter at breast height of tree i, and d_i is the distance of tree i to the sampling points.

We calculated the NHI both per species and per *triplet*, i.e. subplot. The index values did not significantly differ between the two richness levels for any site, neither did they significantly differ for any of the tree species, except for *Carpinus betulus* in Poland (which had significantly lower NHI values in mixed compared to mono-specific plots, $P = 0.02$, i.e. *C. betulus triplet* tree individuals in mixtures had either lower DBHs and/or were further away from the sampling points than in mono-specific stands, Table S2). These results show that our sampling approach ensured a balanced influence of tree individuals on our sampling point. Consequently, we did not mathematically adjust FRB or RLD values of the corresponding tree species.

Quantifying vertical fine-root distribution

To quantify vertical evenness of fine-root distribution among the three soil layers, we calculated the inverse coefficient of variation (CV) based on each species' FRB and RLD per soil depth, respectively (Barry et al., 2020; Zeng et al., 2021).

The following equation was used:

$$inverse\ CV = \frac{\mu}{SD} \qquad (2)$$

where μ is the mean value of the respective trait (either FRB or RLD) across soil depths, and SD represents the standard deviation of the trait (either FRB or RLD) across depths. High and low inverse CV values indicate a more even and heterogeneous distribution of roots across soil depths, respectively.

Diversity effects

The additive partitioning method of Loreau & Hector (2001) was applied to calculate net diversity effects (NE) and individual contributions of selection and complementarity effects (SE, CE) for biomass and length density of absorptive roots, respectively, both for overall values, i.e. aggregated across the entire soil profile and separately for the topsoil layer (0-10 cm).

For NE, the following equation was used:

$$\Delta Y = Y_O - Y_E \tag{3}$$

where ΔY is the net effect, Y_O is the observed yield in mixture, and Y_E is the expected yield, i.e. based on the observed average yield of the component species in mono-specific stands. Yield refers to either FRB or RLD.

If ΔY equals 0, the effects of the component species are additive. If ΔY deviates from 0, effects are non-additive (i.e. negative if <0 or positive if >0).

CE is calculated as:

$$CE = N \times mean(\Delta RY_i) \times mean(M_i) \tag{4}$$

where N is the number of species (in this study N=3),

ΔRY_i is the difference between the observed relative yield of species i in mixture and expected relative yield of species i in mixture;

M_i is the yield in the mono-specific stand of species i.

SE is calculated as:

$$SE = N \times cov(\Delta RY_i, M_i) \tag{5}$$

where *cov* stands for covariance.

SE is thereby determined by the covariance between species' yield in mono-specific stands and in mixtures.

Statistical Analyses

All statistical analyses were performed using RStudio version 1.2.1335 (R Core Team, 2018). Significance levels were set at P = 0.05. To account for the nested design structure and the non-independence of data points at the sub-plot level, linear mixed-effects models (LMMs) were used (package *LME4*, Bates *et al.*, 2015).

To assess broad effects across all four sites, models were fitted with random slopes and intercepts with plot nested within site as random effect structure. R^2 values were retrieved using the R package *r2glmm* (Jaeger, 2017). The model structures are given in the corresponding result tables of the supplement. To quantify overall mixing effects on FRB and RLD, we modelled total values of FRB and RLD, respectively, as functions of tree species richness for each root functional type and depth layer separately. In addition, we tested whether tree species richness affected the distribution of FRB and RLD by fitting the inverse CV of FRB and RLD across depths, respectively, in response to tree species richness. To test species-specific responses to mixing, we computed models for each of the tree species with their (a) FRB, (b) RLD and inverse CV of (c) FRB, and (d) RLD, all of absorptive fine roots, as functions of tree species richness. To test the effects of conifer proportion, we modelled (a) overall values, (b) inverse CV across depths, (c) NE, (d), CE, and (e) SE, for both FRB and RLD, separately, as functions of *triplet* conifer proportion (% basal area) across sites. We only performed these analyses for the sites where conifers were present (i.e. Italy was excluded from the analyses). In addition, we computed models including site as fixed effect to test whether the conifer proportion effect was still significant, even when using site as a covariate, thus indicating that it was not solely a site effect. We included the NHI as covariate in the models to test whether it affected response variables (except for the models with diversity effects as response variables). Wilcoxon signed-rank tests were used to determine the significance of NE, SE, and CE. We also modelled fine-root necromass and understory mass as functions of tree species richness, across all sites and for each site separately.

Results

Total fine-root biomass values across forest types

Average tree FRB to a soil depth of 30 cm across all plots and species compositions and including both absorptive and transport roots was 410±22 g m^{-2}. Mean total standing FRB, necromass and understory mass values differed among sites (Fig. S1; Table S3). The boreal forest plots had the highest total FRB (493±69 g m^{-2}), followed by the mountainous beech forests (460±44 g m^{-2}). Hemiboreal and thermophilous deciduous forests had the lowest total FRB (341±27 and 398±41 g m^{-2}).

Overall tree species mixing effects (H1)

Across all four forest types and species compositions, tree species mixing had a negative effect on biomass of absorptive roots (hereafter referred to as FRB) across the entire soil profile and within all individual depth layers (Fig. 1a). In contrast to biomass, length density of absorptive roots (RLD) across the soil profile was not significantly affected by tree species mixing (Fig. 1b). Yet, RLD in the 0-10 cm soil depth layer was significantly higher in mixtures compared to mono-specific stands (Fig. 1b). Whereas the vertical distribution of FRB across soil depths did not significantly differ between mixtures and mono-specific stands (Fig. 1c), the RLD was more heterogeneously distributed across soil depths in mixtures, indicated by a lower inverse CV of RLD across depths in mixtures compared to mono-specific stands (Fig. 1d).

Overall, fine-root necromass (≤2 mm diameter) was significantly higher in mixtures, while fine-root understory mass (≤2 mm diameter) did not significantly differ between mixed and mono-specific stands (Fig. S2).

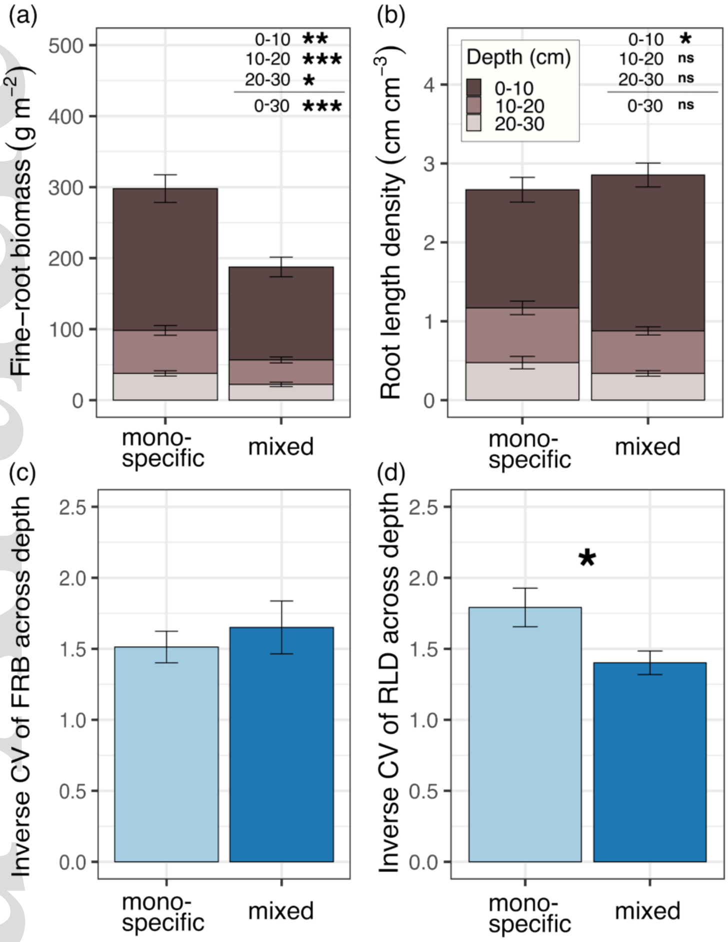

Figure 1. (a) Biomass (FRB) and (b) length density (RLD) of absorptive tree roots by soil depth as well as the inverse coefficient of variation (CV) of (c) FRB and (d) RLD across soil depths, all in mixed and mono-specific stands across the four study sites. Error bars represent one standard error of the mean. Asterisks indicate significant differences between mono-specific and mixed stands (***P<0.001, **P<0.01, *P<0.05, ns P>0.1). For FRB and RLD significance levels are provided for the entire soil profile (0-30 cm) and for each depth layer separately (i.e. 0-10, 10-20, and 20-30 cm).

Complementarity vs. selection effects (H2)

Overall negative net effects (NE) of mixing on FRB across the entire soil profile were associated with both negative complementarity (CE) and selection effects (SE) (Fig. 2a). Yet, the contribution of CE to NE was 77%, while SE only contributed 23%. Neither the NE nor CE or SE were significant for RLD aggregated across soil depths (Fig. 2b). However, when considering only the 0-10 cm depth layer, overall NE in terms of RLD were significant and reflected in positive CE (70%) and negative SE (30%) (Fig. 2c).

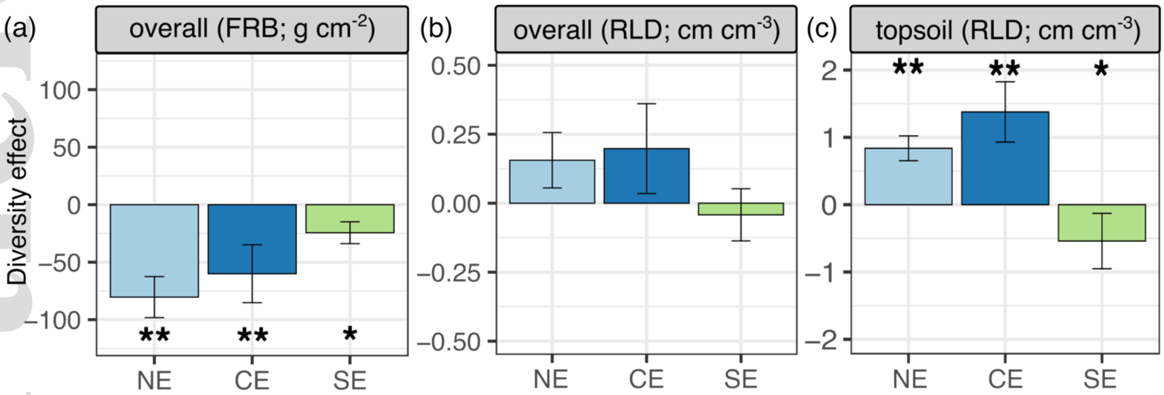

Figure 2. Overall net diversity (NE), complementarity (CE), and selection effects (SE) for (a) biomass (FRB) and (b) length density (RLD) of absorptive fine roots of the entire soil profile combined (overall), and (c) RLD of the topsoil only (0-10 cm depth) across all four sites. Asterisks indicate significant differences from zero (**P<0.01, *P<0.05). Error bars represent one standard error of the mean.

Species-specific responses and the role of conifer proportion (H3)

Species-level FRB analyses across different species compositions showed that *P. abies* (boreal and hemiboreal forests; Finland and Poland), *C. betulus*, *C. sativa*, *O. carpinifolia* and *Q. spec* were all underyielding in terms of overall FRB in mixtures compared to their respective mono-specific stands; in *A. alba* the differences were significant only at P <0.1 (Fig. 3a). At the boreal site (Finland), *P. sylvestris* was the only species significantly overyielding. Identical species-specific patterns emerged when only considering the 0-10 cm soil depth layer.

A significantly negative effect of mixing on RLD was found only for *O. carpinifolia*. Negative trends (at P<0.1) were observed for *C. sativa* and *P. abies* (only in hemiboreal forests; Poland) and an opposite trend, positive response to mixing for *P. sylvestris* in boreal forests (Finland) (Fig. 3b). When only considering RLD at 0-10 cm soil depth, similar patterns emerged, though not significant for some of the species.

FRB of *A. alba*, *P. abies* (mountainous beech forests; Romania), and *O. carpinifolia* was distributed more heterogeneously along the soil profile in mixtures than in mono-specific stands (Fig. S3a). Moreover, *B. pendula*, *C. betulus* (only at P <0.1), *A. alba*, *A. pseudoplatanus*, *P. abies* (in mountainous beech forests; Romania), *C. sativa* and *Q. spec* all had higher inverse CV of RLD across soil depths in mixed compared to mono-specific stands (Fig. S3b), indicating a more heterogeneous vertical distribution of RLD in mixtures than in pure stands.

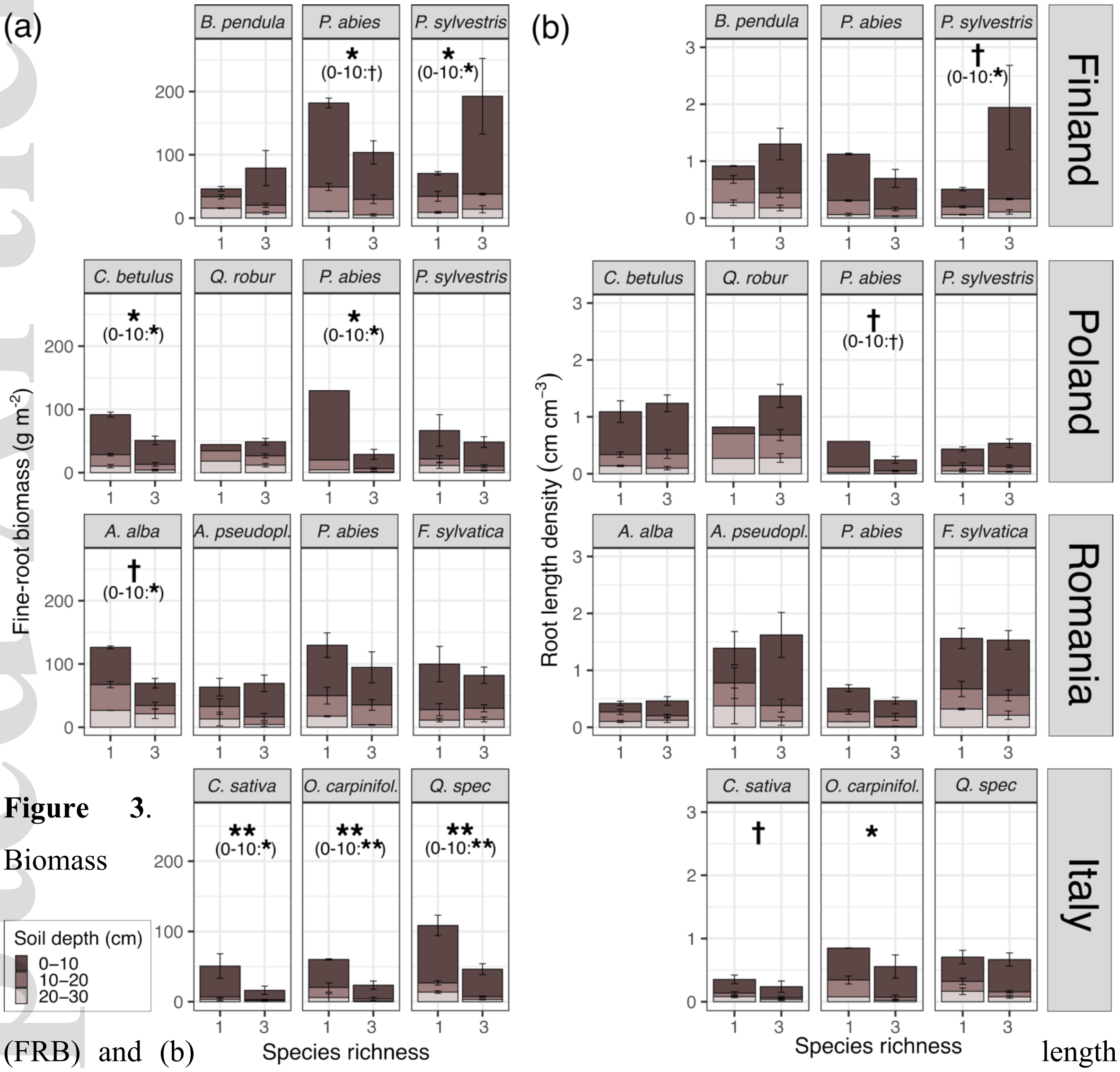

Figure 3. (a) Biomass (FRB) and (b) length density of absorptive fine roots (RLD) by species, soil depth, richness level (mono-specific vs. mixed), and site. Values in mono-specific stands were adjusted to a single tree individual to facilitate comparability between mixed and mono-specific yields of each species. Asterisks indicate significant differences within species between the two richness levels (bold asterisks indicate data for the entire 0-30 cm soil depth combined, and for 0-10 cm samples in brackets): **P<0.01, *P<0.05, †P <0.1. Error bars represent one standard error of the mean.

Across the three sites where conifer tree species were present in our plots, i.e. Finland, Poland, and Romania, *triplet* conifer proportion positively affected overall FRB and overall NE and SE in terms of FRB (Table S4). In addition, RLD was negatively influenced by *triplet* conifer proportion across sites (Table S4). Overall average NE and CE in terms of RLD were not affected by *triplet* conifer proportion, whereas NE for the 0-10 cm depth layer tended to be negatively related to *triplet* conifer proportion. Both overall and topsoil average SE in terms of RLD were also negatively related to *triplet* conifer proportion (Table S4). In addition, when including site as fixed effect in the LMMs, *triplet* conifer proportion still significantly affected overall FRB, RLD, NE, and SE, except for NE in terms of overall FRB (Table S5).

Overall, the biomass share of absorptive and transport roots across broadleaved species did not significantly differ and was on average 51 and 49%, respectively, while conifer species had a significantly higher proportion of absorptive (70%) than transport roots (30%) (Fig. S5a). Yet, the share of the lengths of absorptive and transport roots neither differed for broadleaved (on average 78% vs. 22%) nor conifer trees (on average 85% vs. 15%) (Fig. S5b).

The role of root functional type (H4)

In contrast to absorptive roots (Fig. 1a), tree diversity effects on the biomass of transport roots were not significant (Fig. 4a). The pattern for all live tree fine roots (≤2 mm diameter) followed that of absorptive roots (Figs 1a and 4a). Total length density of all fine roots combined (≤2 mm diameter) was not affected by tree species mixing, whereas total length density of transport fine roots was significantly higher in mixtures compared to pure stands (Fig. 4b).

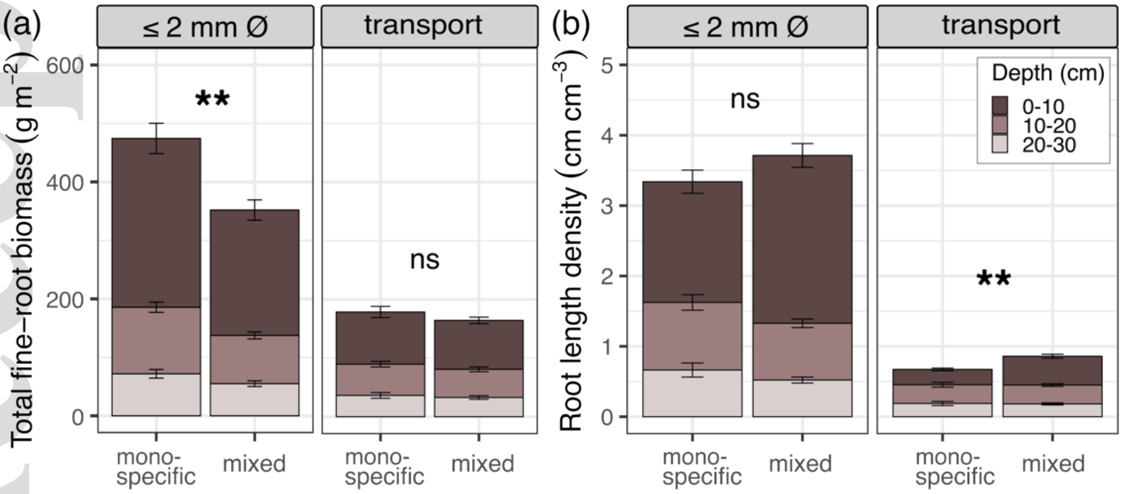

Figure 4. Total mean standing tree (a) biomass and (b) length density of transport roots (i.e. higher-root orders ≤2 mm in diameter) as well as transport and absorptive roots combined (all roots ≤2 mm in diameter) in mixed and mono-specific stands by soil depth, respectively. Error bars represent one standard error of the mean. Asterisks indicate significant differences between mono-specific and mixed stands for the entire soil profile (***P*<0.01, ns *P*>0.1).

Discussion

The overall objective of this study was to investigate tree species mixing effects on biomass, length density, and vertical stratification of fine roots of different functional types in European forests, while also considering the influence of individual tree species. Across four major European forest types and irrespective of differences in broad environmental conditions (e.g. climate, soil types) and species composition, tree species mixing had a consistently negative effect on standing biomass of absorptive fine roots (FRB). These negative mixing effects across all sites were mainly reflected in negative complementarity effects (CE) (77% of the total) and less so in selection effects (SE) (23%). Yet, length density of absorptive roots (RLD) did not significantly change across the soil profile and even increased in the uppermost sampling depth. The positive CE in terms of topsoil RLD indicate positive species' interactions in mixtures. The conifer proportion in mixtures and root functional type influenced these tree species mixing effects. The discussion focuses on the results related to absorptive fine roots, which were more responsive to mixing effects than the bulk fine-root pool.

Mixing effects

In our first hypothesis, we expected that standing FRB and RLD are higher in mixtures than in mono-specific stands owing to positive species interactions. Yet, our results show underyielding of FRB (Figs 1a and 2a) and no overall changes in RLD (Figs 1b and 2b) across all four forest types. These results contrast with several previous studies in mature (semi-)natural forests where either a higher FRB in mixtures or no differences across richness levels have been observed (i.e. Brassard et al., 2013; Finér et al., 2017; Meinen et al., 2009a; Peng & Chen, 2020).

The overyielding in above-ground wood production measured across six European forest types, including the four study sites investigated here (Jucker, Bouriaud, Avacaritei, & Coomes, 2014), suggests that trees in mixtures may require more water and nutrients to sustain the higher above-ground productivity. Consequently, the underyielding of FRB in mixtures across sites points to higher below-ground resource-use efficiency in mixed compared to mono-specific stands and possibly a higher C allocation to above-ground than below-ground compartments (Poorter et al., 2012). This has also been indicated by Archambault *et al.* (2019), who observed below-ground underyielding despite above-ground overyielding of trees in mixtures. Our results point to several different ways in which an increased below-ground resource uptake by trees in mixtures could have been achieved despite underyielding of FRB.

First, the increased RLD in the most nutrient-rich soil layer (0-10 cm) in mixtures compared to mono-specific stands indicates an increased resource-uptake capacity by fine roots (Fig. 1b). This higher fine-root soil occupation in the topsoil supports results from other studies (e.g. Mommer *et al.*, 2010; de Kroon *et al.*, 2012) and partially corroborates our first hypothesis. The overyielding in RLD was mainly reflected in positive CE (Fig. 2c), which supports our second hypothesis and indicates complementary interactions among species. However, as the calculated CE only represents a consequence of mixing but cannot be directly interpreted as an underlying cause (Barry et al., 2019), several ecological mechanisms, including resource partitioning or facilitation, may explain the results. Yet, species greatly differed in RLD (Fig. 3b) and likely also other functional traits, pointing to complementary resource-acquisition strategies (e.g. Bauhus & Messier, 1999; Sun *et al.*, 2017) and/or resource uptake efficiencies (Nijs & Impens, 2000) that could have resulted in competitive reduction (Mahaut, Fort, Violle, & Freschet, 2020). In addition, positive biotic feedbacks may have played a role (Barry et al., 2019). A reduction in pest- or pathogen-related pressure on fine-root growth in mixtures (de Kroon et al., 2012) or a lower reliance on mycorrhizal symbionts (Bergmann et al., 2020) could explain the higher rooting density. Alternatively, root exudates and specific neighbour recognition were also shown to result in increased RLD (Semchenko, Saar, & Lepik, 2014). Without further analyses of other functional fine-root traits related to soil exploitation and symbionts, the exact underlying mechanisms are difficult to ascertain.

Second, increased or constant RLD paired with reduced FRB of absorptive fine roots in mixtures compared to mono-specific stands (Figs 1a and 1b) point to plastic changes in fine-root morphology (Hodge, 2004), possibly enhancing root resource-uptake efficiency in the topsoil and across the entire soil profile. These adaptations could be driven by a reduction in tissue density and/or diameter. Reduced root tissue density could result from a lower pathogen or root herbivore pressure (Jactel & Brockerhoff, 2007), decreasing the requirement of structural defence tissue (as described aboveground, Bumb *et al.*, 2018). Thinner fine roots could indicate more acquisitive roots capable of fast root proliferation and efficient nutrient capture (increased absorptive area per unit C invested) (Eissenstat, 1991). Yet, thinner and/or lower-density roots can be expected to have a reduced lifespan (McCormack, Adams, Smithwick, & Eissenstat, 2012; Reich, 2014;

Weemstra et al., 2016) and thereby a faster turnover, which could result in a higher necromass (given a constant decomposition rate) in mixtures, as indicated by our results (Fig. S2a) and those of others (Jacob et al., 2014; Zilong Ma & Chen, 2018; Meinen et al., 2009a). In this case, overall C cost would not necessarily be lower but could actually increase, as the production of new roots can be relatively more expensive in terms of C cost (Weemstra et al., 2020). Without measuring all root-related C fluxes such as turnover, respiration, exudation, and mycorrhizae relative to nutrient acquisition, fine-root resource acquisition efficiency is difficult to ascertain. In addition, it further remains to be tested whether root morphology changes in response to mixing and, if so, whether these morphologically modified roots (e.g. thinner roots) in mixtures can actually exploit the same quantity and quality of soil resources as roots in pure stands.

Third, C allocation to fine roots may have been reduced owing to increasing nutrient availability in mixtures (Forrester, 2017; Richards, Forrester, Bauhus, & Scherer-Lorenzen, 2010), possibly enhancing resource-use efficiency (Binkley, Stape, & Ryan, 2004). One such facilitative mechanism operating in mixtures may be the nutrient pump effect (Berger, Swoboda, Prohaska, & Glatzel, 2006), where deep-rooted species make nutrients such as Ca or Mg accessible to shallow-rooted species. Moreover, an increased or more diverse litter input and/ or more favourable micro-climatic conditions resulting from a denser canopy in mixtures (Joly et al., 2017) can accelerate nutrient cycling (Hättenschwiler, Tiunov, & Scheu, 2005; Richards et al., 2010), and consequently increase pools of plant-available nutrients. Here, forest floor P concentrations were significantly higher, and C/N ratios were significantly lower in mixtures compared to mono-specific stands across all four forest types (Gillespie, L., unpublished data). Furthermore, changes in micro-environmental conditions accelerating decomposition rates were observed in response to mixing for the same sites (Joly et al., 2017). Altogether, this indicates that increased nutrient availability in mixtures may partially explain our observations.

Regarding the entire soil profile, our results do not provide evidence for spatial resource partitioning as a result of vertical root stratification that could have supported increased above-ground productivity in mixtures compared to mono-specific stands. Although we did not sample below 30 cm depth, no changes in FRB distribution both across (Fig. 1c) and within most species (Fig. S3a), and the lack of an increased RLD below the topsoil in mixed stands (Fig. 1b) suggests that species did not occupy different soil depths. These findings agree with several other studies in forests (e.g. Meinen *et al.*, 2009b; Valverde-Barrantes *et al.*, 2015; Archambault *et al.*, 2019) and suggest that vertical root stratification may not be a common phenomenon and that the overlap between fine roots and zones of high nutrient availability likely plays a more important role than fine-root distribution itself (Richards et al., 2010). However, our study that focussed on the top 30 cm of mineral soil was limited to address the question of stratification of fine-root systems in a robust manner. For that purpose, soil profiles in mixed-species forests need to be explored to a greater depth, ideally where vertical root growth is not restricted by high subsoil rock content, as could have been the case in our study.

Species identity effects

The net diversity effects (NE) in relation to FRB and RLD (in the topsoil) were associated with negative SE across all sites (Figs 2a and 2c). According to Loreau & Hector (2001), negative SE indicate that mixtures are either dominated by species with comparatively low yields (i.e. FRB or RLD) in pure stands or that species with relatively high yields in pure stands show the strongest underyielding in mixtures. The species-specific responses (Fig. 3) could theoretically indicate which species drive these SE (Tobner et al., 2016). Yet, as values are averaged across different species compositions that differ among sites, we cannot draw any overall conclusions related to individual species. Owing to the lack of sufficient replication, species-specific analyses for each species composition were not possible in our study. Nevertheless, significant overall SE indicate that species' relative performance in mixtures differed.

Our results suggest that SE but also FRB, RLD, and NE were related to conifer proportions in *triplets* across the three sites, where conifers were present in our plots (Table S4). In addition, the site in Italy (thermophilous deciduous forests), where conifers did not occur in our plots, was characterised by the most negative NE and CE in terms of FRB (Fig. S4a). In other words, the presence and a high proportion of conifers in mixtures dampened both a negative diversity effect on FRB across the entire soil profile and a positive diversity effect on RLD in the topsoil. These results point to species identity effects, in support of our third hypothesis.

In addition, we need to consider that the magnitude of CE and SE may also be influenced by environmental conditions (e.g. Morin, Fahse, Scherer-Lorenzen, & Bugmann, 2011), and our study design and analyses do not allow for a clear separation of the effects of conifer proportion from site conditions. Moreover, this species identity effect is likely related to distinct effects of conifer and broadleaf species on soil resource availability (Richards et al., 2010). Across all

FunDivEUROPE sites, including the sites addressed in this study, conifer proportion significantly altered topsoil properties (increasing C stocks, C/N ratio, and reducing soil pH; Dawud et al., 2016, 2017), affected decomposition rates (Joly et al., 2017), and evergreen litter reduced earthworm abundance and richness (De Wandeler et al., 2018), altogether suggesting slower nutrient cycling. Consequently, decreasing soil nutrient availability with increasing conifer proportion may cause greater biomass allocation to roots. A recent study along a soil fertility gradient further showed that conifers allocated an increasingly larger share of biomass to their absorptive fine roots in response to increasing soil C:N ratios compared to the broadleaved birch (Ostonen et al., 2017), supporting our explanation.

The fact that conifer proportion still significantly affected our response variables, even when using site as a fixed effect in the models, shows that conifer proportion is likely not solely a site effect (Table S5). Our results are also in agreement with other studies that have described similar effects of conifers on fine roots in mixtures (e.g. Finér et al., 2017; Archambault et al., 2019), including a study across the FunDivEUROPE plot network, which showed that conifer proportion was an even more important predictor of FRB than tree diversity and site factors (Finér et al., 2017). Consequently, the species' phylogenetic background appears to be relevant for below-ground diversity effects.

The observed species identity effect may also point to the different inherent below-ground resource acquisition strategies of gymnosperms and angiosperms and the frequently observed lower root plasticity of conifers compared to broadleaved species (Ma et al., 2018). Conifer tree species were found to form thicker and longer-lived fine roots that are slow in root proliferation and rely more on mycorrhizal fungi to acquire soil nutrients (Bauhus & Messier, 1999; Kong et al., 2014; Liu et al., 2015; McCormack et al., 2012; Weemstra et al., 2016). All three conifer species studied here had significantly larger shares of absorptive than transport fine roots, while the broadleaved species had approximately equal proportions of the two functional types (Fig. S5a). This may be attributable to the fact that thicker rooted species usually have fewer total root orders than thinner rooted species (Eissenstat, Kucharski, Zadworny, Adams, & Koide, 2015), and according to the functional classification approach, the share of absorptive fine roots should obviously be higher if the same fine-root (>2 mm) cut-off is considered. However, it also suggests that conifers had much larger biomass of absorptive fine roots (or generally larger, heavier absorptive roots) than broadleaved species. Nevertheless, lengths of absorptive relative to transport fine roots of broadleaved and conifer species were similar (Fig. S5b). Therefore, the lower share of absorptive roots in broadleaved species appears to be compensated for by a relatively greater length of absorptive roots.

Role of root functional type

In agreement with the fourth hypothesis, our results show that tree species mixing effects on fine roots depended on root functional types (Fig. 4). Lower-order roots, i.e. absorptive roots, have often been found to respond stronger to changes in soil conditions than higher-order roots (e.g. Kou et al., 2018; Wang et al., 2013), likely because they are the most physiologically active roots with the highest plasticity. Here, we demonstrate that this also applies to the response of biomass of absorptive and transport fine roots to tree species mixing, though responses of length density showed inverse patterns. Consequently, inconsistent results of tree species mixing effects on fine-root biomass among previous studies may be, at least partially, attributable to the inadequate classification of fine roots.

Conclusions

In this study, we assessed below-ground diversity effects across a broad environmental gradient in four major European forest types. Our results suggest that despite overall relatively consistent tree diversity effects on fine roots across forest types, species identity, and functional root types played an important role. Despite the overall lower FRB in mixtures, the higher RLD in the most nutrient-rich soil depth actually indicates a higher resource uptake capacity in mixed compared to mono-specific stands, which could be linked to the commonly observed positive (above-ground) diversity-productivity relationship. This highlights that FRB is not an adequate proxy for soil occupation by fine roots, and other root traits, such as RLD, may provide a better estimate. Yet, as the link between root traits and functions, and thereby overall ecosystem functioning, is still not well understood, future studies should consider additional fine-root traits to obtain a better picture of the underlying mechanisms linked to below-ground species interactions. Owing to the distinct responses of absorptive and transport roots observed in this study, we advocate an order-based approach for the classification of fine roots instead of the arbitrary 2-mm-diameter cut-off when investigating below-ground diversity effects. In addition, our study highlights the context-dependency of tree diversity effects on below-ground properties related to fine roots, possibly explaining ambiguous results of previous studies. Owing to the design of our study with observational plots in existing forests, we cannot entirely rule out any *a priori* differences in soil conditions between plots of different tree species richness, although conditions were kept as constant as possible between plots. Therefore, we encourage future studies in controlled tree-diversity experiments to employ similar fine-root traits to quantify below-ground responses to tree diversity.

Acknowledgements

This study was funded by the German Research Foundation (Grant numbers: BA 2821/16-1 and SCHE 695/10-1). This research was part of the SoilForEUROPE project funded through the 2015-2016 BiodivERsA COFUND call for research proposals, with the national funders Agence Nationale de la Recherche (ANR, France), Belgian Science Policy Office (BELSPO, Belgium), Deutsche Forschungsgemeinschaft (DFG, Germany), Research Foundation Flanders (FWO, Belgium), and The Swedish Research Council (FORMAS, Sweden). FB was supported by the Margarete-von-Wrangell Fellowship of the Ministry of Science, Research and the Arts Baden-Württemberg and European Social Fund. We thank Lauren Gillespie and her team for the measurement of forest floor characteristics at the experimental platform PACE at CEFE Montpellier, France. We further thank Bart Muys and Karen Vancampenhout at KU Leuven University for the soil texture data, the site managers Leena Finér, Natural Resource Institute Finland (LUKE), Bogdan Jaroszewicz, University of Warsaw, Olivier Bouriaud, Forest Research and Management Institute (ICAS), Filippo Bussotti, University of Florence, and Jakub Zaremba, Ewa Chećko, Iulian Dănilă, Timo Domisch, as well as the SoilForEUROPE consortium for their assistance with soil sampling, Renate Nitschke and the many student assistants for their help with root processing in the laboratory. We also thank three anonymous reviewers for their constructive and helpful comments. The authors declare no conflict of interest.

Author Contributions

JB and MSL conceived the study and acquired funding for the project. JB, MSL, JW, GF and FB designed the study. JW carried out data collection, performed the analysis and drafted the manuscript. All authors revised the manuscript.

Data Availability Statement

The datasets used for this study are archived on a data portal associated with the FunDivEUROPE and SoilForEUROPE projects and available after a 1-year embargo (fine-root data: https://data.botanik.uni-halle.de/fundiveurope/datasets/518; *triplet* tree data: https://data.botanik.uni-halle.de/fundiveurope/datasets/517).

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