Soil Legacies of Tree Species Composition in Mature Forest Affect Tree Seedlings' Performance

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ABSTRACT

Trees affect the biotic and abiotic properties of the soil in which they grow. Tree species-specific effects can persist for a long time, even after the trees have been removed. We investigated to what extent such soil legacies of different tree species may impact tree seedlings in their emergence and growth. We performed a plant–soil feedback experiment, using soil that was conditioned in plots that vary in tree species composition in Białowieża Forest, Poland. Soil was taken from plots varying in proportion of birch, hornbeam, pine, and oak. In each soil, seeds of the same four target species were sown in pots. Seedling emergence and growth were monitored for one growing season. To further explore biotic implications of soil legacies, ectomycorrhizal root tip colonization of oak, a keystone forest species, was determined. We found no effect of soil legacies of tree species on the emergence measures.

We, however, found a clear negative effect of pine legacies on the total biomass of all four seedling species. In addition, we found relationships between the presence of pine and soil fertility and between soil fertility and root tip colonization. Root tip colonization was positively correlated with the biomass of oak seedlings. We conclude that tree species can leave legacies that persist after that species has been removed. These legacies influence the growth of the next generation of trees likely via abiotic and biotic pathways. Thus, the choice of species in today's forest may also matter for the structure and composition of future forests.

Key words: FunDivEUROPE; Pot experiment; Soil legacy; Plant–soil feedback; *Betula pendula*; *Carpinus betulus*; *Pinus sylvestris*; *Quercus robur*.

Author Contributions ED, LB, PDS, and KV conceived the conceptual idea. LB, KV, and BJ contributed to designing the observational platform. ED and LB analyzed the data. ED wrote the manuscript with input from LB, PDS, KV, and BJ.

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Highlights

- Total biomass of tree seedlings is negatively affected by soil legacies of pine.
- Mycorrhizal root tip colonization correlates with total biomass of oak seedlings.
- Legacies of tree species affect seedlings via biotic and abiotic pathways.

Introduction

Trees affect the environment in which they grow, including the soil. The nature in which trees impact the abiotic and biotic soil characteristics greatly depends on the species' identity. This divergence in species effects can be attributed to various plant characteristics, including differences in chemical and physical litter quality (Hobbie and others 2006; Makkonen and others 2013), rooting depth (Bardgett and others 2014), root exudates (Eviner and Chapin III 2003; Bardgett and others 2014), their microbial associations (Dastogeer and others 2020), and interception of atmospheric depositions (De Schrijver and others 2007). Consequently, the identity of the species can in part modulate the soil fertility, by altering the soil chemical composition, soil hydraulic properties, and biotic community (Jost and others 2012; Khlifa and others 2017; Chandler and others 2018; Stefanowicz and others 2021), which can persist long after those individual trees have disappeared (Cuddington 2011). These so-called soil legacies will, subsequently, affect the establishment and performance of plant species in the understory, including the next generations of tree seedlings. These legacies can provoke plant–soil feedbacks, which can be positive, meaning that the plant-induced changes to the soil promote the performance of a next generation of individuals, or negative (Bever 1994). The soil legacies can also be divided into abiotic and biotic legacies (Ehrenfeld and others 2005).

Abiotic imprints of tree species are known to result in part from differences in leaf and root litter quality and quantity. In soil under species with high-quality litter, that is, low C:N ratio and high concentration of calcium, we expect a greater availability of essential nutrients (Augusto and others 2002), which is beneficial for the growth of tree seedlings (Ericsson 1995). The response of tree seedlings to nutrient availability is in part dependent on the seed size, where larger effects to differences in nutrients can be expected in species with smaller seeds that have fewer seed reserves (Milberg and others 1998). Nutrient conditions can also affect the expression of traits and other seedling properties (for example, fine root biomass), with potential implications for ecosystem function (Lavorel and Garnier 2002). As nutrients become more limiting, relatively more biomass is allocated to the roots, resulting in a larger root:shoot ratio (Poorter and others 2012). Studies have also found a greater investment in fine root biomass under nutrient-poor conditions (Yuan and Chen 2010). Next to belowground traits, specific leaf area has also been found to positively relate to nutrient availability (Meziane and Shipley 1999). Trees also produce secondary chemicals that may inhibit the growth of the same species (autotoxicity) or other species (allelopathy) (Muller 1966).

The soil biotic community is also influenced by the species identity of the trees, which can lead to various plant–soil feedbacks. According to the Janzen–Connell hypothesis (Janzen 1970; Connell 1971) seedlings growing in soil originating from stands of the same species are expected to show reduced establishment due to accumulation of host-specific pathogens. On the other hand, host-specific mycorrhiza might facilitate these conspecific seedlings. In addition, ectomycorrhiza provide physical protection against antagonistic effects and lead to an increased growth in conspecific soil (Bennett and others 2017). Furthermore, mycorrhizal seedlings allocate relatively more biomass to the shoot than the roots (Colpaert and others 1996).

The biotic legacies do not exist in isolation from the abiotic context and by extension abiotic legacies (Bennett and Klironomos 2019). Therefore, the abundance and composition of the soil biotic community is not only related to the presence of host species, but also to the abiotic conditions of the soil. Studies have demonstrated a negative effect of soil acidity on mycorrhizal colonization, which may lead to reduced absorption of essential nutrients (Bakker and others 2000; Kluber and others 2012). Resource availability is another strong driver of the composition of the microbial community (Bennett and Klironomos 2019).

While plant–soil feedback research has been predominantly conducted in grassland ecosystems, studies have shown evidence of plant–soil feedbacks in forest ecosystems as well (Crawford and others 2019; Jiang and others 2024). Additionally, in the majority of plant–soil feedback experiments the soil is conditioned using single plants or monocultures. Our study uses soil conditioned by mature forest stands that vary in level of species dominance and species composition. The aim of

our research was to find the relationship between the soil legacies of these forest stands and the performance of tree seedlings growing in that soil. Previous studies did not find evidence for species interaction (that is, diversity) effects at our study site (Dawud and others 2016; Dhiedt and others 2022). Therefore, we focused on additive species effects in this study, that is, where individual species influence the soil proportional to their abundance, without additional interactive effects. A plant–soil feedback pot experiment was established with soil originating from the abovementioned mature forest stands. Because we used soil from one single study site, our research questions and hypotheses are framed within the context of that forest, one of the best examples of mature forest in Europe (Jaroszewicz and others 2019).

We investigated the following research questions: (1) do soil legacy effects on the emergence and growth of tree seedlings exist and do they depend on the proportion of the conditioning tree species in the mature forest stands; (2) is the seedling's response to the soil legacies mediated by biotic interactions through mycorrhizal root tip colonization. We pose the following hypotheses. (1) In soil originating from stands with increasing proportion of species with low-quality litter, we expect seedlings to have a reduced emergence success, a larger emergence duration, a smaller total biomass, and specific leaf area and a larger root:shoot ratio and fine root ratio and we expect these legacies to be explained in part by the chemical soil composition. We expect a larger emergence success, smaller emergence duration, larger total biomass, and smaller root:shoot ratio in conspecific soil. (2) We expect a positive relation between soil acidity and the proportion of mature species with low-quality litter; furthermore, we expect that the root tip colonization of seedlings is negatively related to soil acidity but positively to their total biomass.

MATERIALS AND METHODS

Study Site and Plot Selection

The study site for soil collection from mature forest stands was located in the Białowieża Forest, Poland (52.7°N, 23.9°E; altitude between 134 and 202 m a.s.l.). The site was designed as part of the tree-diversity oriented exploratory platform of the FunDivEUROPE project (Baeten and others 2013). The mean annual temperature is 6.9 °C and the mean annual precipitation is 627 mm (Boczoń and others 2018). The forest is classified as hemiboreal,

nemoral coniferous, and mixed broadleaved-coniferous (EEA 2007).

The original exploratory platform design included 43 plots (30 m × 30 m) in this study site. In the present study, we selected seventeen plots that have a varying proportion of the following species as a dominant species: *Pinus sylvestris* L., *Betula pendula* Roth, *Carpinus betulus* L., and *Quercus robur* L. We will further refer to these "target" species as pine, birch, hornbeam, and oak, respectively. These tree species are economically and/or ecologically important across Europe. To ensure a gradient in species proportion, we selected plots based on a number of criteria relating to the relative basal area of the target species. First, for each target species, two plots were selected for which that species had a relative basal area of at least 60% and all other species had a relative basal area of less than 30%. These plots were thus not pure monocultures of only one tree species, but the proportion in basal area of the plot of one species is distinctly larger than the other species. These are referred to as monodominant plots in this study. There were two replicates for each of the four target species, resulting in eight monodominant plots in total. Second, for each combination of two target species, two plots were selected that consisted of two dominant target species with a relative basal area of at least 25% each and the relative basal area of one dominant species was not more than double of the other dominant species. The proportion of the other species in that plot was less than the two target species. We will refer to these as two-species plots. There were nine two-species plots: two replicates of all possible combinations of the four target species, except for the combination birch–pine (zero replicates) and the combination oak–pine (one replicate), for the reason that the combination birch–pine did not occur and the oak–pine combination only occurred once in the FunDivEUROPE design of the Białowieża Forest. See Baeten and others (2013) for all other formal design criteria. See Supplementary A for the absolute and relative basal areas of each species in each plot. The soil types of the plots are classified as cambisol and luvisol (IUSS Working Group WRB 2015).

The target species differ in the quality of their litter. A low C:N ratio and a high concentration of Ca in the leaf litter promote decomposability and are an indication of a high litter quality. The litter quality of the target species is ranked in increasing litter quality: pine < oak < birch < hornbeam. The chemical composition of the leaf litter of each of the target species at this site are show in Supplementary B.

The shrub layer was dominated by hornbeam and was present in all the observational plots. It is integral to the forest system where the observational plots are situated (Brzeziecki and others 2020) and was not confounded with the overstory species composition. Because hornbeam was also one of the target species, the effect of the other target species in the absence of hornbeam could not be observed in these observational plots. However, the range in the proportion of hornbeam across the different plots was sufficiently large (relative basal area of 4% to 95%) to test whether a hornbeam effect existed in this forest system. A note should be made to the fact that only trees with a diameter at breast height (DBH) of larger than 7.5 cm were measured and included in the calculation of the relative basal area. Readers should be aware that legacies were provoked not only by the plants we measured and were directly included in our analyses, that is, the overstory trees, but also by other plants, including hornbeam trees with a DBH of less than 7.5 cm and should be interpreted accordingly.

Soil Collection

In each plot soil was collected from six subplots in autumn 2019. These subplots were positioned along two transects between two target species. These target trees were of the same species in the monodominant plots and of two distinct species in two-species plots. One subplot was laid out close to each of the two trees (0.5 m to 1 m to the center of the tree stem) and one midway between the two trees (Figure 1). Three liters of soil were collected up to a depth of 10 cm in each subplot with a clean spade after removing the forest floor. The soil of each subplot was sieved with a 5 mm mesh size to remove rocks and large organic material, and then homogenized. The tools to collect, sieve, and homogenize the soil were sterilized with 70% alcohol in between subplots, to avoid cross contamination of soil biota.

Tree Influence Index

The diameter at breast height was measured for each tree in each forest plot with a threshold of 7.5 cm in July 2018. For each subplot we determined a ''tree influence index'' (TII), based on a competition index (Daniels 1976). The TII was based on the distance to and the diameter of a nearby tree. For each subplot $i = 1, \ldots, 102$ and every species j, $TII_{ij} = \sum_{k=1}^{n_{ij}} \frac{d_{jk}}{D_{ijk}}$ was calculated, with d_{jk} being the diameter at breast height of tree k, D_{ijk} being the distance between subplot i and tree $k = 1, \ldots, n_{ij}$. The number of trees n_{ij} per subplot and species was determined by the vertex angle α_{ijk} of the isosceles triangle with height D_{ijk} and base d_{jk}. When α_{ijk} exceeds a threshold of 3°, the diameter over distance ratio of tree k was added to the TII. In essence, this means that only taller trees are included in the composition of a subplot as the
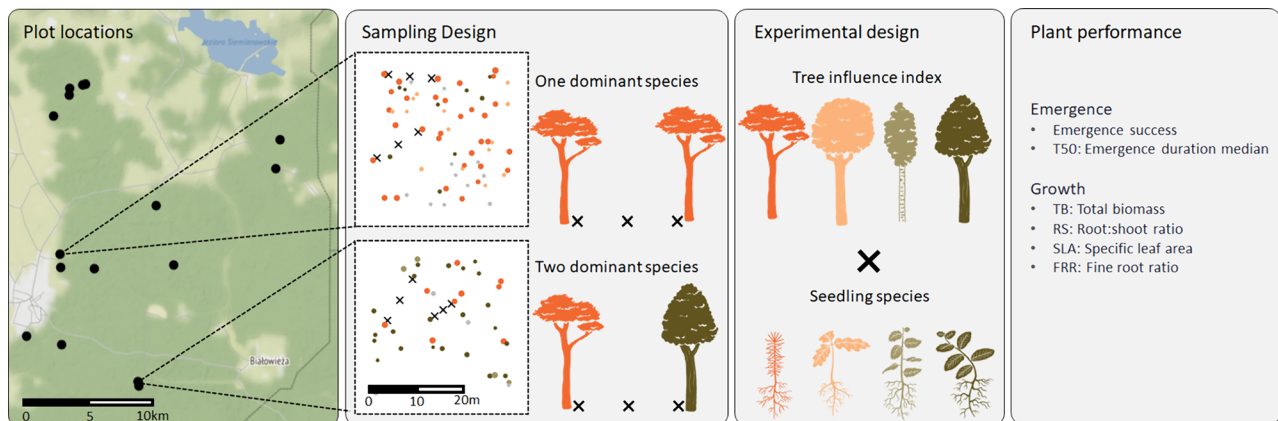
Figure 1. Schematic overview of the design. Panel 1: Locations of the 17 plots in the Białowieża Forest, Poland. Panel 2: Sampling design shown on a plot map. The dots denote the tree positions within a 30 × 30 m² plot, with a different color for each species and a size proportional to the diameter at breast height. The crosses denote the soil collection locations. In each of the 17 plots, two transects were laid out between two target trees (two of the same species in monodominant plots and two different species in two-species plots). In each transect, we sampled at three locations: close to each target tree and once midway between the two target trees. Panel 3: Four species (seedling species: pine, oak, birch, hornbeam) were grown separately in the soil collected from each subplot with varying proportions of the target species, calculated by a tree influence index. Panel 4: six plant performance measures were determined on each seedling.

distance from the subplot increases. Next, the relative TII was calculated of each target tree species to the total TII of each subplot. A large relative TII thus indicates many trees of that species in close proximity to the subplot that have a potential to affect the soil chemistry of this subplot. A visual representation of the calculation of the TII can be found in Supplementary C. The TII represents the proportion of a target species in a subplot. We assume this proportion to be positively related to that species' proportion in the stands' canopy layer and belowground biomass, as well as the amount of leaf and root litter and root exudates.

Experimental Design

The field sampling design consisted of six subplots within seventeen plots, summing up to 102 subplots in total. Seeds of each of the target species present in the study site (that is, pine, oak, birch, and hornbeam) were sown in the soil originating from each subplot. Each combination of subplot and seedling species was replicated twice, resulting in 816 study subjects (= 102 subplots × 4 species × 2 replicates) (Figure 1).

In mid-March 2020, the seeds were sown in the collected soil of each plot in tree trays with cups of 350 ml. In each cup a fixed number of seeds of one species was sown (pine: 9, oak: 2, birch: 15, hornbeam: 2). Numbers varied to account for differences in average emergence success. Seed provenances were Belgium, The Netherlands, Germany, and Germany for pine, oak, birch, and hornbeam, respectively. The pots were positioned randomly in the greenhouse to minimize the effects of potential different climatic conditions in the greenhouse. No significant spatial pattern of the position in the greenhouse were found in the seedling response. After 80 days the seedlings were thinned to the strongest seedling in each cup.

The minimum, mean, and maximum air temperature per month in the greenhouse can be found in Supplementary D. Seedlings were watered twice a week *at libitum* with demineralized water. In the beginning of October until the end of the experiment (start of November 2020) lamps were lighted. This lengthened the growing season, to prevent the seedlings from losing their leaves before they were harvested. The lamps activated when the solar irradiation dropped below 200 Wm^{-2} and deactivated at values higher than 250 Wm^{-2}. They were not operational for 12 h after sundown. Powdery mildew on oak seedlings was frequently treated with potassium hydrogen carbonate (Karma, Certis). All oak pots, regardless of the level of powdery mildew infestation, were treated the same. This way, fungicide that might flow along the stem into the pot, was equalized over the treatments. Directly spraying on the soil was avoided.

Measuring Plant Performance

Emergence

Emergence was measured weekly for 80 days. The emergence success is the number of seeds germinated between the start of the experiment and day 80. Emergence failure is the number of seeds that did not germinate by day 80 and was calculated as the total number of seeds subtracted by the emergence success. Next, the emergence duration median (T50) was determined by estimating the day 50% of the emergence occurred based on the weekly measurements.

To calculate these measures, we corrected for tree seeds that were already present in the soil and were smaller than our 5 mm mesh size, that is, birch, pine and hornbeam seeds. This was done by counting the seedlings in the pots where no birch, pine, or hornbeam was sown. The mean per subplot was calculated and subtracted from the amount that emerged in the birch and hornbeam pots, respectively, assuming that the seeds are homogeneously mixed in the soil. After averaging over all pots that were filled with soil of the same subplot, birch was the only species to have germinated from the soil and was found in the soil of 10 subplots.

Measurements at Harvest

At the beginning of November, after 225 days since the start of the experiment, the seedlings were harvested. A selection of pictures of the seedlings just before harvest can be found in Supplementary E. No evidence was found for plants being pot bound based on two height measurements, meaning that they were likely not restricted in growth due to the size of the pots (Supplementary E). For each individual that survived until the end of the experiment (number of seedlings was 71, 154, 141, 78 for pine, oak, birch, and hornbeam, respectively) several growth measures were measured and calculated. The dry weight of the stem and the leaves was measured (dried for 48 h at 60 °C). Additionally, the dry weight (dried for 48 h at 60 °C) was measured for the largest leaf as well as the total projected surface of the fresh leaves using the Li-Cor Portable Area Meter Li-3100 (Li-Cor Biosciences, Lincoln). For pine seedlings, these leaf

measurements were performed on a subset of ten secondary needles. Roots were cleaned on a sieve with a 0.5 mm mesh size with a water hose. After drying, visible substrate was removed with tweezers. Dry weight (dried for 48 h at 60 °C) was determined for the roots belonging to root order 1 to 3 (starting from the terminal root segments) and the roots belonging to root orders higher than 3 (McCormack and others 2015).

The total biomass (TB) was determined by aggregating the dry weight of the stem, the leaves and the roots. The root:shoot ratio (RS) was calculated by dividing the root biomass by the sum of the dry weight of the stem and the leaves. The specific leaf area (SLA) was defined as the ratio of the leaf area and the dry weight of the largest leaf (Pérez-Harguindeguy and others 2016). Dry weight of the roots of root order 1 to 3 was divided by the dry weight of the roots of root orders larger than 3 to calculate the fine root ratio (FRR).

Ectomycorrhizal Root Tip Colonization

The ectomycorrhizal root tip colonization was determined for a subset of oak seedlings. Oak was chosen over the other three of the sown species, because it is well-studied, commercially important, and a key species in many forest ecosystems. In addition, it was the species that had emerged in most cups. The subset consisted of one seedling per subplot and each seedling was randomly chosen from the two seedlings that were growing in the soil of that subplot. Ultimately, the mycorrhizal measurements were performed on 96 seedlings, due to the fact that not in all cups seedlings emerged. Until measurements were performed, roots were stored in separate plastic bags at 4 °C without washing. After carefully rinsing the roots, ten random root tips were evaluated, on ten random lateral roots under a stereo microscope (hundred root tips in total). Each root tip received a score of one if colonized or zero if uncolonized. The scores were added up to obtain a root tip colonization percentage, similar to Aleksandrowicz-Trzcińska and others (2019). The visual classification is explained in more detail in Supplementary F.

Chemical Analysis

Chemical analyses of the soil were performed at the subplot level. A subsample was taken of the soil collected in each subplot. The soil samples were dried to constant weight at 40 °C for 48 h, ground, sieved over a 2 mm mesh and homogenized. To analyze the total C and N concentration, the sam-

ples were combusted at 1150 °C and the gases were measured by a thermal conductivity detector in a CNS elemental analyzer (vario Macro Cube, Elementar, Germany). Bioavailable P which is available for plants within one growing season (Gilbert and others 2009) was evaluated by extraction in NaHCO$_3$ (Olsen-P; according to ISO 11263:1994(E)) and colorimetric measurement according to the malachite green procedure (Lajtha and others 1999) (Norm: ISO 11263:1994(E)). Samples were analyzed for pH-H$_2$O by shaking a 1:5 ratio soil/H$_2$O mixture for 5 min at 300 rpm and measured with a pH meter Orion 920A with pH electrode model Ross sure-flow 8172 BNWP, Thermo Scientific Orion, USA. Exchangeable K$^+$, Ca^{2+}, and Mg^{2+}, concentrations were measured by inductively coupled plasma optical emission spectroscopy (Thermo Scientific iCAP 7400 ICP-OES) after extraction in 0.1 M BaCl$_2$ (according to NEN 5738:1996). For calculation of base cation concentration (BC) of the soils, all extracted exchangeable cations (K$^+$, Ca^{2+}, and Mg^{2+} in mEq/kg) were summed. One sample in a hornbeam-pine plot showed an exceptionally high concentration of Ca (> 4000 mg kg-1, and more than four times higher than the second highest observation) and pH. Since this is likely an artifact, caused by inclusion of very localized calcareous material, it was decided to exclude this sample from our analysis.

Statistical Analysis

To test whether the plant performance measures are related to the soil legacies of tree species (Hypothesis 1), these measures were fitted as a function of the relative TII of each of our four target species as fixed effects. Separate univariate general linear mixed models were fitted for emergence and emergence duration median (T50), because in numerous cups no seedlings emerged and it was therefore not possible to calculate T50 for those cups. Emergence success (number of emerged seedlings) and failures (number of seeds that did not emerge) were fitted assuming a binomial model with a logit link function. Emergence duration median was fitted assuming a Gaussian distribution with an identity link function. Multivariate linear mixed models were used for the (scaled) growth measures (total biomass (TB), root:shoot ratio (RS), specific leaf area (SLA), and fine root ratio (FRR)), assuming Gaussian distributions with an identity link function. Separate models were fitted for each of the tree seedling species and included plot ID and subplot ID as group-level (random) effects. The
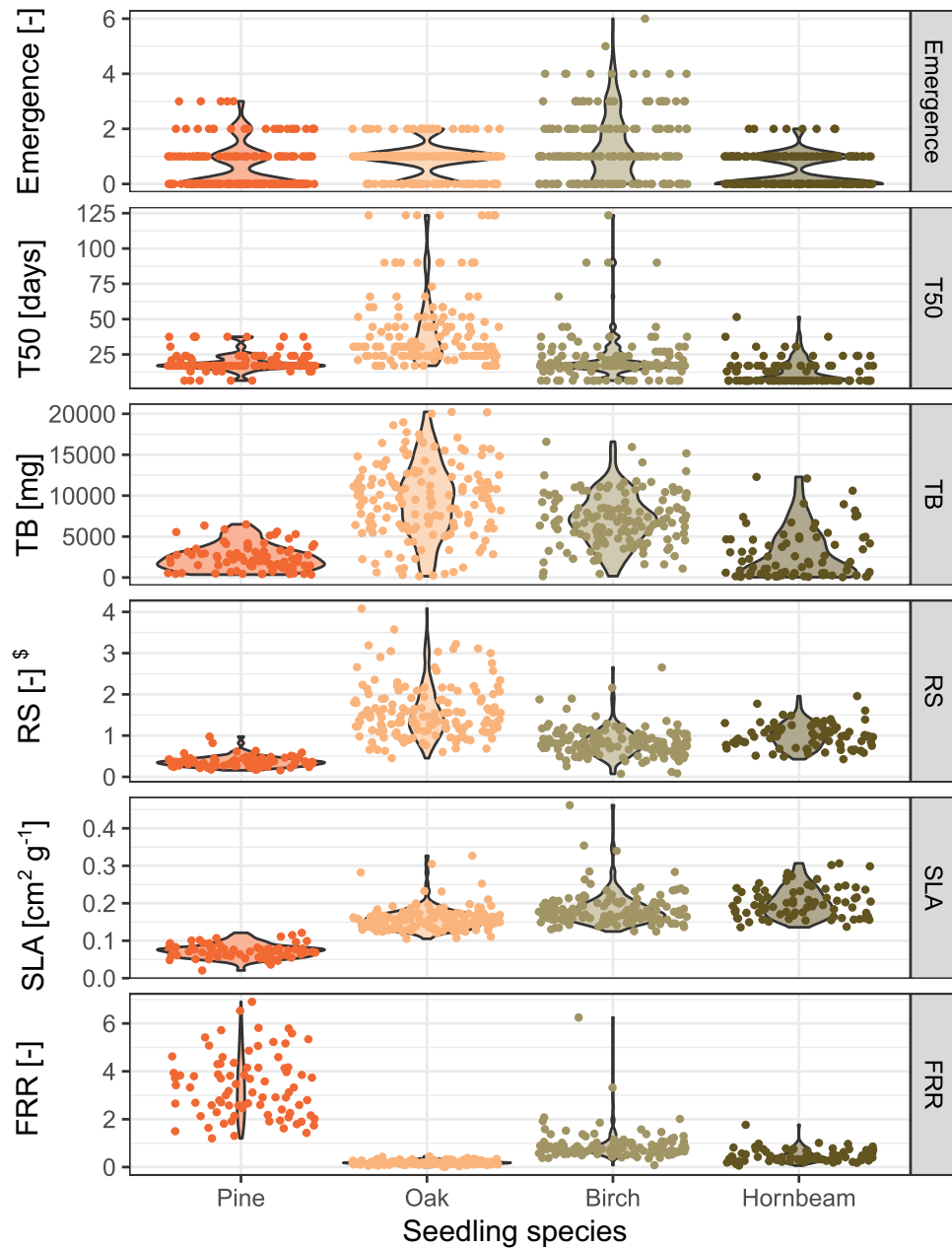
Figure 2. Distribution of the observations of the plant performance measures (emergence success; *T50* emergence duration median, *TB* total biomass, *RS* root:shoot ratio, *SLA* specific leaf area, *FRR* fine root ratio) per seedling species. The points denote the observations for which the y value represents the value of the performance measure. Random noise is added along the *x* axis for each seedling species, to make it possible to distinguish between points (jitter plot). The distribution of the observations is shown by a violin plot. $^\$$Note that for RS the y axis is constrained between 0 and just over 4 and three outliers are not visualized to enhance discernability of the distributions of RS for the four seedling species. Outliers not shown are: hornbeam: RS = 7.7, oak: RS = 10.9, birch: RS = 17.9.

growth model, being multivariate, also contains information on the (co)variation between the responses, which can be found in the (co)variance structure of the random variables and the residuals. The responses were allowed to be correlated between subplots and we assumed the variances between plots and within subplots to be independent, but different. A mathematical representation of the variance structure of the random effects can be found in Supplementary G.

To test whether these soil legacies of tree species can be explained by the soil chemical composition
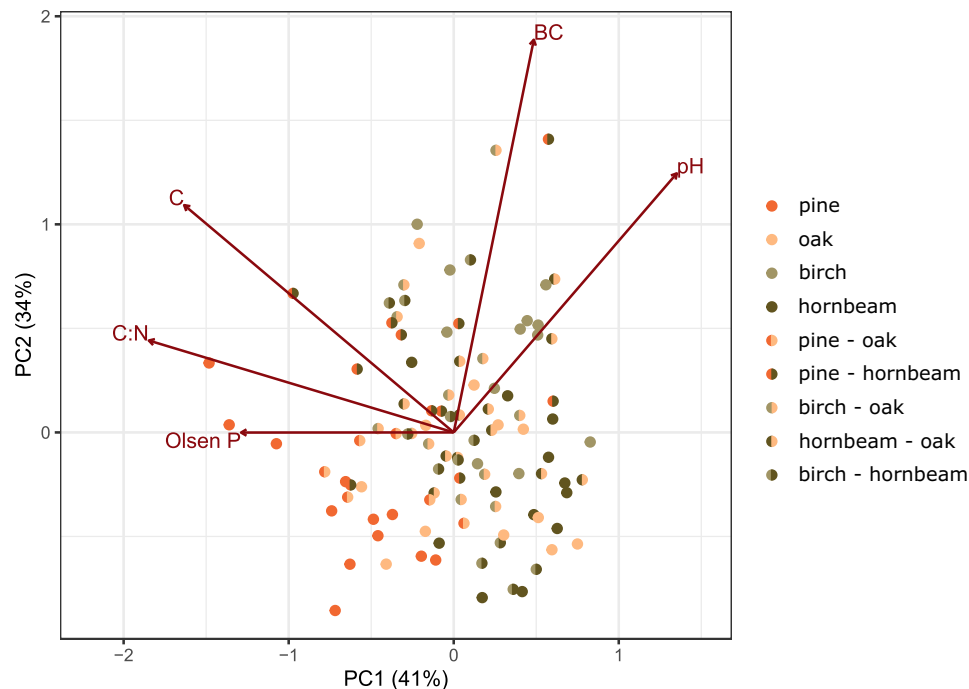
Figure 3. Biplot of the first two principal component axes (PC1 and PC2) of the soil variables (C, C:N ratio, Olsen-P, base cation concentration (BC), and pH-H2O). The first and second axis explain 41% and 34% of the variation, respectively. Each species is presented by a different color. Points that represent monodominant plots are colored in one solid color denoting the target species. Points that represent two-species plots are split in two halves, each color denoting one of the two target species.

(Hypothesis 1) a soil chemistry variable was included as an additional fixed variable to the previous model. A principal component analysis was performed using the measured soil variables (total C, C:N ratio, Olsen-P, BC, and pH-H$_2$O), to avoid adding all five highly correlated variables as single independent variables. We used the first component of this principal component analysis (PC1). Models with versus models without the soil chemistry variables were compared based on the deviance information criterion (DIC).

To test whether the soil legacies can in part be explained by the root tip colonization (Hypothesis 2), three consecutive models were fitted for the seedlings for which the root tip colonization percentage was calculated. First, PC1 was fitted as a function of the relative TII of each of our four target species assuming a Gaussian distribution with an identity link function. Second, the root tip colonization percentage was fitted as a function of PC1 assuming a Gaussian distribution with an identity link function. Third, the growth variables (TB, RS, SLA, and FRR) were fitted as a function of the root tip colonization percentage making use of a multivariate linear mixed model assuming Gaussian distributions with an identity link function. In each

of these three models, plot ID was included as a group-level (random) effect. Subplot ID was not included, since there is only one observation per subplot. The fit was tested by comparing these models with an intercept-only model based on the DIC.

The statistical analyses were performed in R version 4.4.0 (R Core Team 2024). The *MCMCglmm* package (Hadfield 2010) was used to model the univariate and multivariate linear mixed models. In each model an improper flat prior was used for the fixed effects and a weakly informative prior for the variance structure of the random effects.

RESULTS

Distribution of the Performance Measures and Soil Variables

The mean emergence (and standard deviation, sd), based on the binomial distribution was 0.56 out of 9 seeds (sd = 0.72) for pine, 0.85 out of 2 seeds (sd = 0.70) for oak, 1.25 out of 15 seeds (sd = 1.07) for birch, 0.43 out of 2 seeds (sd = 0.58) for hornbeam respectively; in a large number of pots no seedling emerged. A mean of 26 days (sd = 21 days) was estimated for median emergence dura-
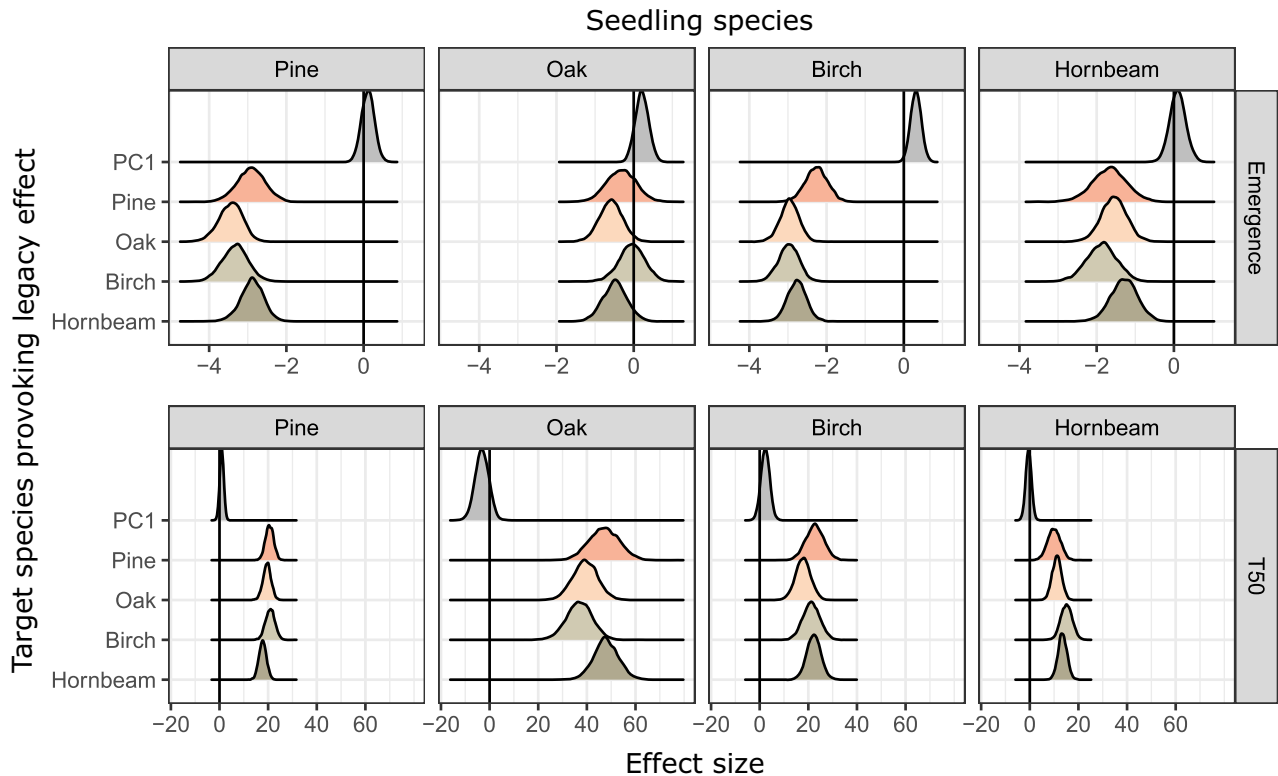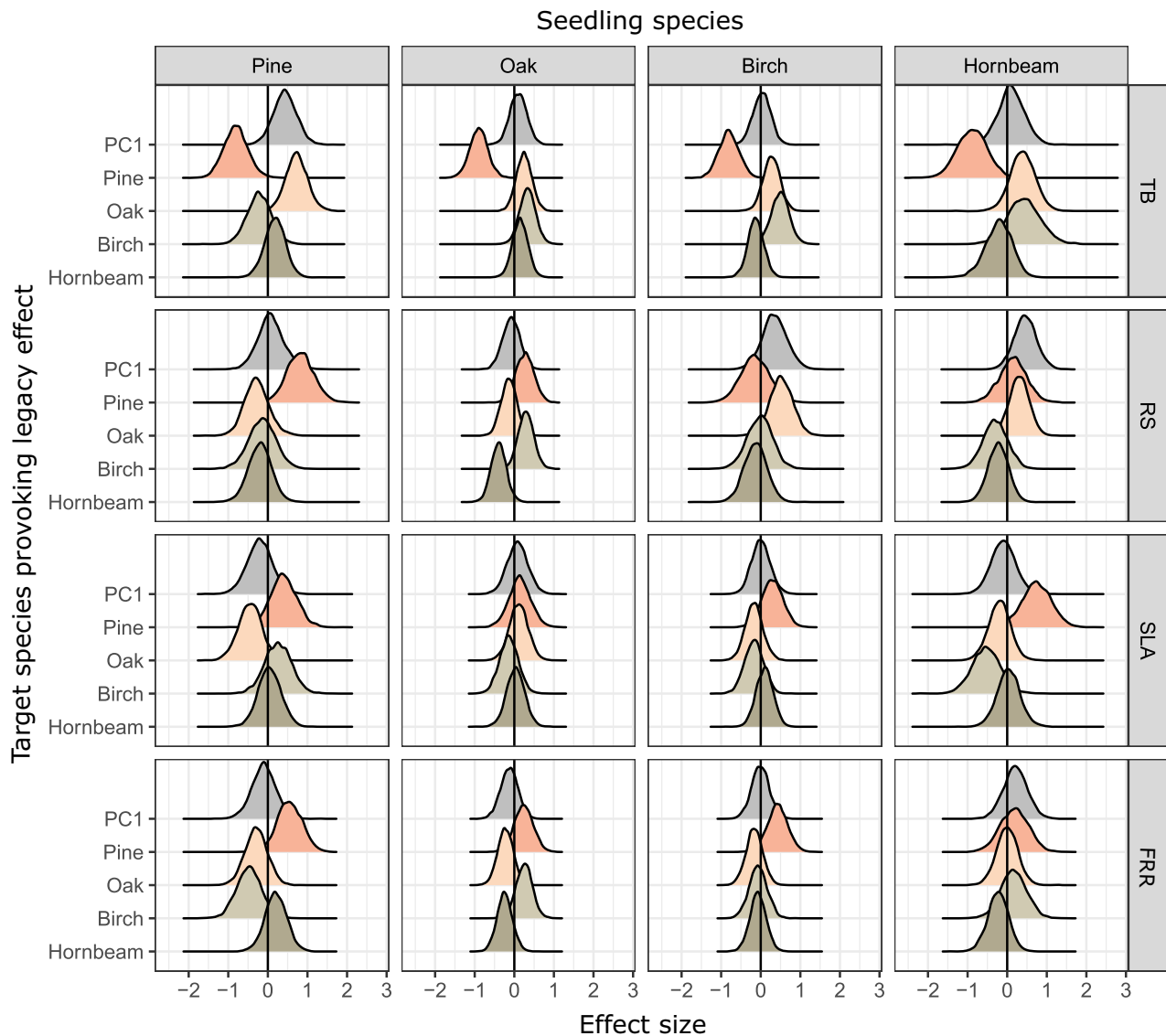
Figure 4. Posterior distribution of the effect sizes when the proportion of a target species in a subplot is 100% (rows) on the emergence measures (emergence success; T50: emergence duration median) for seedlings growing in the soil of that subplot. The first principal component of the principal component analysis of the soil variables (PC1; Figure 3) was included as an additional covariate. Seedling species are shown from left to right: pine, oak, birch, and hornbeam. Soil legacy effects of tree species are given on the y axis, ranked from lower (top: pine) to higher (bottom: hornbeam) litter quality. For emergence success, the effect size is presented on a logit scale. Therefore, a value of x is equal to the inverse logit of x [$= e^x/(1 + e^x)$] on an arithmetic scale. For T50, the effect sizes are presented on an arithmetic scale.

tion (T50) across all seedling species where emergence occurred. The mean total biomass (TB) across all species equated to 6.4 g (sd = 4.5 g), with the largest mean value for oak (9.5 g, sd = 4.6 g) and the smallest for pine (2.4 g, sd = 1.6 g). The mean root:shoot ratio (RS) is 1.1 (sd = 1.1). The mean specific leaf area (SLA) equated to 0.16 cm^2 g^{-1} (sd = 0.06 cm^2 g^{-1}) and 0.07 cm^2 g^{-1} (sd = 0.02 cm^2 g^{-1}) for the broadleaved species (hornbeam, birch, and oak) and pine, respectively. The mean fine root ratio (FRR) was 1.0 (sd = 1.3) across all seedling species. The largest mean FRR was found for pine seedlings (3.4, sd = 1.3) (Figure 2).

The first axis of the principal component analysis of the soil variables was negatively correlated with C ($p < 0.0001$), C:N ($p < 0.0001$), and Olsen-P ($p < 0.0001$), positively correlated with pH ($p < 0.0001$) and BC ($p = 0.021$) (Figure 3). The distribution of the soil variables with the absolute values can be found in Supplementary H.

Soil Legacy Effects on Tree Seedling Emergence

For each of the seedling species, emergence as a function of mature tree species-specific legacies did not fit the data better than a model with just a random intercept. In other words, there was no evidence for an effect of tree species legacy on emergence for any seedling species (Figure 4 and see also Supplementary I for the deviance information criterion (DIC) of the various models). PC1 of the soil variables had a positive effect on emergence for birch, which means lower Olsen-P and C:N in the soil was related to higher emergence success. The addition of PC1 as an explaining variable did not affect the effect sizes of the proportion of the target species (Supplementary I). We used binomial models with a logit link function to fit the emergence. For this reason, an approximate mean effect size of -3 on birch seedling emergence where the proportion of hornbeam is 100% is to be interpreted as a probability p of 4.7% (= inverse
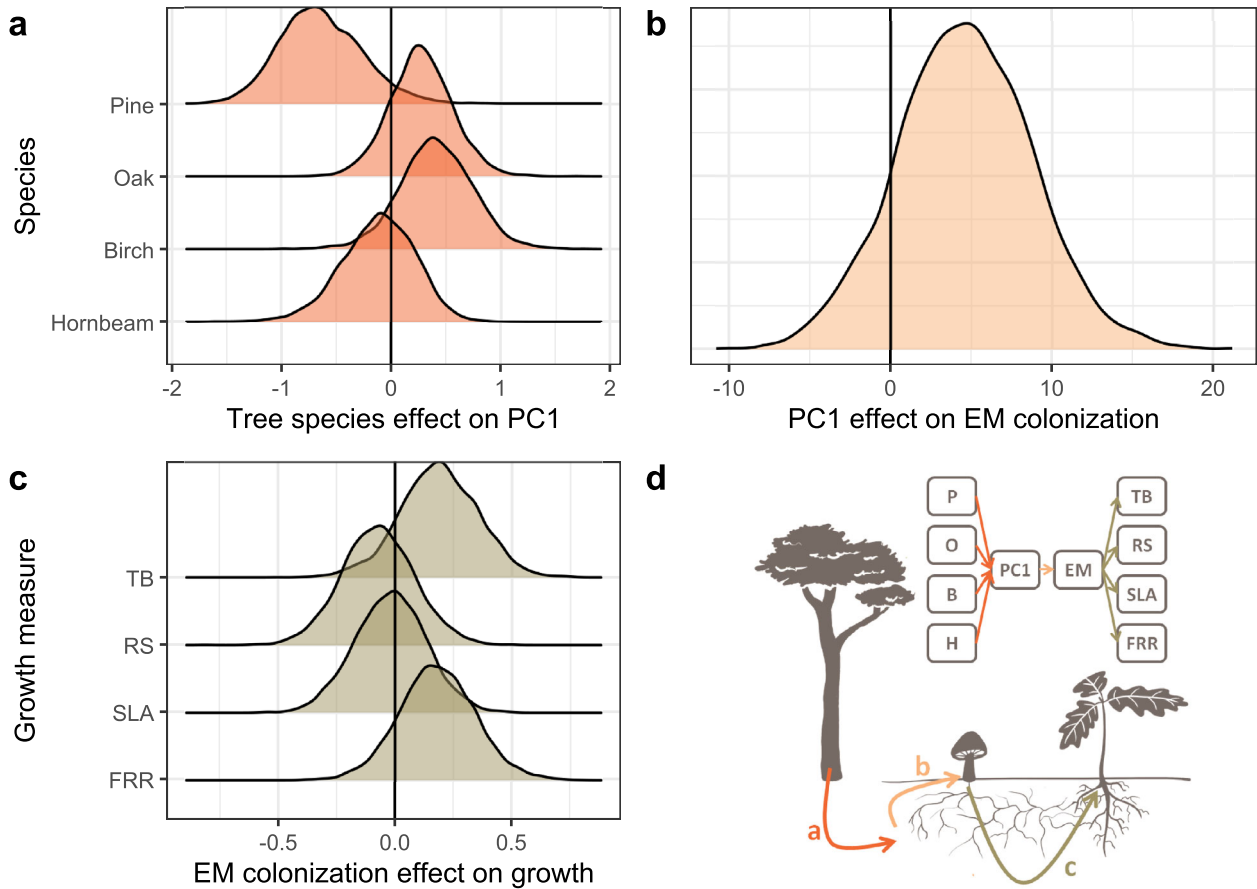
Seedling species

Figure 5. Posterior distribution of the effect sizes when the proportion of a target species in a subplot is 100% (rows) on the growth measures (TB: total biomass; RS: root:shoot ratio; SLA: specific leaf area; FRR: fine root ratio) for seedlings growing in the soil of that subplot. The first principal component of the principal component analysis of the soil variables (PC1; Figure 3) was included as an additional covariate. Seedling species are shown from left to right: pine, oak, birch, and hornbeam. Soil legacy effects of tree species are given on the y axis, ranked from lower (top: pine) to higher (bottom: hornbeam) litter quality. A positive effect means that the growth measure was higher with a greater proportion of a target species influencing the soil as compared to the average across all soils for that seedling species. For the PC1 axis, a positive effect means the growth measure of the seedling species correlates positively with the PC1 axis.

logit of -3 or $e^{-3}/(1 + e^{-3})$) for a birch seedling to emerge in soil where hornbeam is 100%. For mixtures of target species, the proportion of the target species is to be multiplied with the effect size and subsequently summed over all target species and inverse logit determined to calculate the predicted probability. When using birch seedlings growing in soil that was dominated by both hornbeam and pine at 50% as an example, the proba-

bility p would be the inverse logit of -3 (effect size of hornbeam) * 0.5 (proportion of hornbeam) + -2.5 (effect size of pine) * 0.5 (proportion of pine), which equals to 9.4% ($e^{-2.75}/(1 + e^{-2.75})$).

The model fitting the emergence duration median (T50) as a function of the four target species did not perform better than the model fitting only the random intercept (Figure 4 and Supplementary I).

Figure 6. **a** Posterior distribution of the effect sizes when the proportion of target species in a subplot is 100% (rows) on the first component of the principal component analysis of the soil variables (PC1). **b** Posterior distribution of the effect size on root tip colonization when the value of PC1 is increased by one standard deviation. **c** Posterior distribution of the effect size on growth measures (*TB* total biomass, *RS* root:shoot ratio, *SLA* specific leaf area, *FRR* fine root ratio) when the value of root tip colonization is increased by one standard deviation. **d** Visual representation of the structure of the three models. From left to right: proportion of target tree species (P: pine, O: oak, B: birch, H: hornbeam), PC1, root tip colonization (EM), growth measures. The lowercase letters are referring to the previous panels.

The soil variables (PC axis) did not explain variation in the T50. Similar to the emergence success models, the addition of PC1 did not change the effect sizes of the proportions of target species (Supplementary I).

Soil Legacy Effect on Tree Seedling Growth

For every seedling species, the model fitting the growth measures as a function of the four target species performed better than the model fitting only the random intercept based on the deviance information criterion. This means there was evidence in favor of an effect of tree species legacy on growth measures of all four seedling species (Supplementary I). An approximate mean of the effect size of -1 on the total biomass of oak in soils with a pine proportion of 100% can be interpreted as a decrease in one standard deviation from the average total biomass of oak seedlings across all soils in soils dominated by pine. For mixtures of target species, the proportion of the target species is to be multiplied with the effect size and subsequently summed over all target species to calculate the effect size.

The total biomass (TB) of seedlings of all four species was smaller in soil originating from plots with a larger proportion of pine (Figure 5). TB of pine seedlings was larger in plots with a larger proportion of oak. Root:shoot ratio (RS) of pine seedlings was larger in plots with a larger proportion of pine, the RS of birch seedlings is larger in plots with a larger proportion of oak, and the RS of oak seedlings was smaller under a larger proportion of hornbeam. The specific leaf area (SLA) of pine

seedlings was smaller in soils originating from plots with a larger proportion of oak. The SLA of hornbeam seedlings was larger in plots with a larger proportion of pine. Equivalently to the emergence success and T50 models, PC1 did not affect the effect sizes of the proportion of the four target species very much (Supplementary I). PC1 had a positive effect on the RS of hornbeam seedlings (Supplementary I).

When the soil legacy effects of the different target trees were compared, pine had a consistent negative effect on the total biomass of seedlings. We also identified a positive pine effect on the other growth measures of the four seedling species. The effect of hornbeam, however, was rather average for most measures and seedling species. Birch and oak had more diverging legacy effects on the growth measures of the various seedling species (Figure 5).

Mediation by Biotic Interactions Through Mycorrhizal Root Tip Colonization

The negative association between pine and PC1 showed that soil collected under a larger proportion of pine is expected to have higher C:N, C and Olsen-P concentrations (Figure 6a). The effect was, however, not significantly different from zero based on the deviance information criterion (Supplementary I). The other three species did not differ so much in these soil characteristics. While oak seedlings tended to show somewhat higher root tip colonization by mycorrhiza when growing in soil with lower C:N and Olsen-P, this effect was not significantly different from zero (Figure 6b and Supplementary I). Contrary to the previous two models, we found support for our second hypothesis that root tip colonization affects the growth measures of oak seedlings. Root tip colonization was positively correlated with TB and FRR, but not the other growth measures (Figure 6c).

DISCUSSION

Legacies of Tree Species Affect Seedling Growth, but not Emergence

No significant soil legacy effects of the proportion of species in forest stands on the emergence of seedlings were found. In agreement with these results, studies have shown that PSFs are weak to nonexistent at the seed stage, especially in comparison with the seedling stage (Comita and others 2014; Song and Corlett 2021). It is important to note that emergence was measured, which is strongly related to seed survival and germination, but occurs at a later stage of the life history. At that time, predators and pathogens that act at the seedling stage, may also be driving the soil legacy effects on the emergence measures. Similarly, mycorrhizal associations may have already been initiated after the germination of the radicle but before the seedling emerges. Regardless of this fact, no significant legacy effects were found. Root pathogens and mutualists likely did not have sufficient time between the emergence of the radicle and the emergence of the plumule to cause noticeable effects on our emergence measures. Because only a small number of seeds was sown per cup, especially for hornbeam and oak, the variation in observations of emergence might be large. This could also be a reason for not seeing any significant legacy effects on emergence. In addition, studies have shown the importance of the seed microbiome in the germination and the initial performance of plants. Seeds were not sterilized prior to sowing. Therefore, the seed microbiome might have overwhelmed any existing legacy effects. However, since all seeds of the same species originated from the same location, they are expected to have a similar microbiome.

In contrast to the emergence measures, significant soil legacy effects of species proportion on the growth measures were found. Total seedling biomass had the strongest response to the proportion of tree species which for the most part consisted of a negative heterospecific PSF of pine. This negative effect could be explained by biotic and abiotic drivers. An abiotic driver could be the low-quality litter produced by pine trees, both above and belowground (Silver and Miya 2001; Hobbie and others 2006), which causes soil acidification and a reduction in availability of essential nutrients (Augusto and others 2002). This may have a negative impact on the growth of the seedlings. Pine is more distantly related in the phylogenetic tree than the three other species. In pine dominated stands, this could result in a smaller abundance of microbiota that form mutualistic relations with the broadleaved species and may thus result in a negative biotic heterospecific PSF. However, this does not explain the negative effect of pine legacies on pine seedlings, since we could expect a greater abundance of mutualistic microbiota specific to pine. This could be evidence supporting the Janzen–Connell hypothesis that describes negative conspecific PSFs (Janzen 1970; Connell 1971).

A plant generally reacts to a reduction in growth rate due to resource limitation by allocating more resources to the part that experiences the limitations or by changing morphology (Kramer-Walter

and Laughlin 2017). Our results indeed show that pine and oak seedlings invested more biomass to belowground parts in soil originating from pine stands. Considering the negative effect of pine on soil fertility, this was to be expected. Similarly, a somewhat larger fine root ratio for pine and birch seedlings in soils originating from a pine dominated neighborhood was found.

Next to the negative legacy effects observed in soils originating from pine dominated plots, a positive soil legacy effect of oak on the total biomass of pine seedlings was also detected. This positive heterospecific PSF may be ascribed to a better soil fertility in oak dominated stands or a lack of pine specific pathogens. However, we would expect a similar result for pine seedlings in soils with a birch or hornbeam legacy, which was not the case. This may suggest that other mechanisms are at play, which were not measured in this study. A negative legacy effect of oak dominated soil on SLA for pine seedlings was also revealed. The negative correlation with total biomass was surprising based on the leaf economics spectrum that generally associates higher SLA with fast-growing plants (Wright and others 2004). However, the evidence in literature supporting this hypothesis within a species is not consistent (Fajardo and Siefert 2018).

A positive conspecific PSF for birch seedlings was found. Considering that birch is a pioneer species, this was contrary to what has been reported regarding succession. Kardol and others (2013) hypothesized that early stages of succession show negative PSFs, unlike slow-growing species in later stages of succession, which are dominated by positive PSFs. Moreover, Semchenko and others (2022) note how negative PSFs are stronger in fast-growing species with efficient dispersal, two characteristics of birch. However, Semchenko and others (2022) also described how species that collaborate with mycorrhiza, show more positive PSFs, especially ectomycorrhizal species (Bennett and others 2017), which could explain the positive effect found here.

The seeds sown in the experiment did not originate from a provenance local to the site where the soil was collected. Seedlings grown from locally harvested seeds might show a different response to the species legacy effects to those found in our experiment, due to intraspecific PSFs (Schweitzer and others 2018). A positive intraspecific PSF leads to local adaptation, where we might expect stronger positive conspecific PSFs where local genotypes are grown. In contrast, a negative intraspecific PSF would likely reduce positive conspecific PSFs.

We also would like to point out the high maximum temperatures in the greenhouse during the experiment (Supplementary D). Leaf temperatures of more than 42 °C are damaging to the photosynthetic apparatus (Ludlow and Björkman 1984). In a hot environment, the leaf temperature is lower than the environment, due to evapotranspiration, which will prevent stress and damage to some extent (Mahan and Upchurch 1988). However, seedlings are generally more sensitive to environmental stress, like extreme temperatures, as they have not built up sufficient reserves to cope (Niinemets 2010). Therefore, the seedlings in our experiment were possibly subjected to heat stress during the summer months, which might have overwhelmed any legacy effects. On the other hand, plant–soil feedbacks have also been shown to be increased under environmental stress (van der Putten and others 2016).

Seedling Response Cannot be Explained by Soil Chemistry

The soil variables, represented by the first axis of the principal component analysis (PC1), did not explain additional variation of the emergence or growth measures, except for the emergence success of the birch seedlings. Further, the estimates of effect size of the legacies of tree species did not change by including PC1. This suggests that the soil legacy effects we described in the previous section are likely driven by mechanisms other than the abiotic soil variables we measured.

The stands from which the soil originated are reasonably fertile, with a median Olsen-P of approximately 39 mg kg^{-1} and median Ca, Mg, and K concentration (BaCl$_2$ extraction) of 1.57, 0.27, and 0.15 cmol$_c$ kg^{-1}, respectively, and over all plots (Supplementary H). It may be that nutrient availability was not the limiting factor, even in stands dominated by pine trees that are known to acidify the soil and lead to limitations of nutrients in soil with low buffering capacity (Augusto and others 2002). This would explain why the growth measures of the seedlings did not react to the measured soil variables.

However, a high C:N ratio was observed in litter of the mature pine trees in the forest plots (for example, compared to Hobbie and others (2006)) (Supplementary B), which might indicate nitrogen limitation (Berg and others 1987). On the other hand, the C:N ratio of the leaf litter of the other three studied species is not out of the norm (Supplementary B). In addition, Cholewińska and others (2020) observed an increase in forest canopy

cover and N-demanding understory species during the last decades in the Białowieża forest, which could be a result of increased N deposition, and hence indicate N limitation of the forest soil. The fact that PC1, which is negatively correlated with soil C:N, does not significantly affect the emergence and growth, could mean that the C:N ratio might not be the best indicator of N limitation if the seedling were limited by nitrogen.

Oak Seedling Response can in Part be Explained by Root Tip Colonization

Although not significantly better than the intercept-only model, a clear negative trend of soil legacies of pine on PC1 was found. This indicated that the soil underneath pine trees contained slightly more C and Olsen-P, has a greater C:N ratio, and is more acidic. This is to be expected based on its low-quality litter (Hobbie and others 2006). As stated in the previous section, the site was situated on relatively fertile soil, and negative effects of pine on soil fertility may be buffered. Historical documentation and palynological records strongly indicate that these pine trees were the first generation of this species at this site (Jaroszewicz and others 2019). Their influence may not have had sufficient time to degrade the soil yet. This may explain why the proportion of pine had only a small and non-significant effect on PC1.

Previous studies have shown evidence that mycorrhiza are negatively affected by a low pH (Bakker and others 2000; Kluber and others 2012), which was indicated by a small value of PC1 in our study. Although a positive trend was found, this relation was not significant in this study. The soils at our study site (cambisol and luvisol) were moderately acidic, but had a high buffering capacity and the negative effect of a low pH may not be as severe. Also, PC1 was only weakly correlated with pH.

PC1 was also negatively correlated with Olsen-P. Studies have shown a negative relation between the availability of soil P and ectomycorrhizal mycelium production and colonization (Ekblad and others 1995; Torres Aquino and Plassard 2004; Wallander and Nylund 1992), since it is energetically unfavorable for the plant to invest in symbiosis in soils that are not limiting in P. Despite this known relation, the positive correlation between PC1 and root tip colonization was not significant.

The mycorrhizal community was also determined by soil variables that may not have been affected by the proportion of the species growing at the site. Plots were carefully selected to minimize differences between plots in management and other abiotic factors, but unwanted covariation with environmental factors may exist.

Another factor may be the distance to the tree, since a higher ectomycorrhizal abundance and diversity may be expected near the base of a tree where the root density is high (O'Hanlon 2012). The distance to the nearest tree had no significant effect for most of our seedling species (Supplementary J), so we decided not to include this in our study. Other spatial variation at a local scale could be attributed to soil legacies of previous generations. Although these trees were no longer present, their effect on the soil composition may persist for a long time (Trumbore 2000) and may cause variation in mycorrhizal composition and abundance that we did not account for.

We may also take a look at the seedlings themselves to explain the root tip colonization. Not only is there literature showing evidence of the positive effect of root tip colonization on total biomass (for example, Lewis and others 2008), there is also an argument in favor of the positive effect of productivity on mycorrhizal abundance, since mycorrhiza rely on carbohydrates, assimilated by the plant, as an energy source (Druebert and others 2009). This may suggest that the root tip colonization can be driven by the growth measures and not the other way around. In our study we were not able to distinguish between cause or consequence.

The results revealed a positive relation between root tip colonization on the one hand and TB and FRR on the other hand. A greater percentage of root tips that were colonized may lead to a greater reach to explore the soil for essential nutrients. This greater supply of nutrients may result in a greater TB (Lewis and others 2008). Mycorrhizal seedlings may allocate more biomass to the fine roots to accommodate the symbiotic relationship, which could explain the positive correlation between root tip colonization and FRR. Additionally, mycorrhizal sheaths have a mass of their own. An increase in root tips that are colonized, will likely lead to an increase in biomass of these tips, and the fine roots as a whole (Rygiewicz and Andersen 1994).

CONCLUSION

Our results showed no significant soil legacy effect of tree species on the emergence of tree seedlings. However, significant legacy effects of tree species on the performance of all four seedling species were found. There was a consistent negative effect of soil originating from pine stands on the total biomass. The results also revealed a positive con-

specific legacy effect of birch soil on total biomass of birch seedlings and a positive heterospecific legacy effect of oak soil on total biomass of pine seedlings. No clear mediating effects of soil chemistry on the emergence and growth of tree seedlings were found, which we attributed to the fact that the soils are relatively nutrient-rich and nutrient availability may not be the limiting factor. Nor did our study reveal significant soil legacy effects on the mycorrhizal root tip colonization, but a clear trend between presence of pine and soil fertility as well as between soil fertility and root tip colonization could be distinguished. A positive correlation between root tip colonization and total biomass of the oak seedlings was also identified. These results suggest that soil legacies of tree species were not purely abiotic and that biotic legacies are also driving the seedlings' response. Our study highlights that species choice is not only important for the current forest functioning but may also have consequences for the growth of the future generations of tree seedlings.

DATA AVAILABILITY

DOI of data and code are available at: https://doi.org/10.6084/m9.figshare.26240264.

Declarations

Conflict of interest   The authors declare that they have no conflict of interest.

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