

RESEARCH ARTICLE

Soil legacies of tree species richness in a young plantation do not modulate tree seedling response to watering regime

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Keywords

afforestation; FORBIO; irrigation frequency; pot experiment; soil legacy; tree seedlings; tree species diversity; TreeDivNet.

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Editor

M. Zhou

Received: 27 March 2023;

Accepted: 1 November 2023

doi:10.1111/plb.13597

ABSTRACT

- Trees have a strong and species-specific influence on biotic and abiotic properties of the soil. Even after the vegetation is removed, the effect can persist to form so-called soil legacies. We investigated the effects of soil legacies of tree species richness on the emergence and growth of tree seedlings, and how these legacy effects modulate the seedling responses to irrigation frequency.
- We used a 9-year-old tree plantation on former agricultural land in Belgium, which is part of a biodiversity-ecosystem functioning experiment (FORBIO). Soil originating from monocultures and four-species plots, with different species combinations, was translocated to a greenhouse. Five tree species (*Betula pendula*, *Fagus sylvatica*, *Pinus sylvestris*, *Quercus robur*, and *Tilia cordata*) were sown and grown for one growing season in these soils. We performed a watering treatment (low and high irrigation frequency) to measure any potential interaction effects between the soil legacies and irrigation frequency.
- There was no evidence for soil legacy effects of species richness on plant performance or their response to the irrigation frequency. However, the effect of irrigation frequency was dependent on species identity of the tree seedlings. Despite the lack of clear legacy effects, performance measures did show correlated responses that are likely due to species composition effects.
- We ascribe these patterns to the young age of the forest and the agricultural past land use. At this early stage in forest development, the land-use history likely has a more important role in shaping soil characteristics that affect plant growth and their response to drought, than species diversity.

INTRODUCTION

Trees affect the environment in which they grow. The nature and strength of this effect depends on the identity of the tree species involved, since tree species differ in productivity, litter quality and quantity, root exudates, and canopy and root structure (Augusto *et al.* 2002). When different species are grown together, their effect can differ from the sum of their monoculture effects, through non-additive effects, referred to as diversity effects. An important compartment of the ecosystem that is affected by species composition is the soil (Binkley & Giardina 1998). The effects on soil properties can express themselves abiotically, by altering soil nutrient concentrations, pH, carbon content, and structure (Jacob *et al.* 2009; Dawud *et al.* 2016), and biotically, by modifying the microbial and faunal communities (Cesarz *et al.* 2007, 2013; Khelifa *et al.* 2017). Such tree influences gradually build up when trees age and could affect the environment for a long time (Leuschner *et al.* 2009). At the same time, even when the trees are no longer present, their effects on soil composition are expected to persist and influence the next generation of trees. These so-called soil legacies have received little attention to date.

There is increasing interest in the ecosystem functioning of young tree plantations (Verheyen *et al.* 2016). Since large-scale

land conversions to forests have occurred in recent years, and substantial afforestation efforts are expected in the years to come (*e.g.*, Messier *et al.* 2021), it is important to understand the impact of afforestation, so that foresters can make informed management decisions at the start of an afforestation project, also in the context of soil legacies. In a previous study (Dhiedt *et al.* 2021), we sampled soil *in situ* and found clear and context-dependent effects of species composition on the chemical soil properties in a recently planted forest. An alternative approach to evaluate how trees alter the abiotic and biotic conditions of the soil in which they grow is by examining how these changes influence the ability of the next generation of trees to grow in this soil. This is a plant–soil feedback approach applied in the context of young forest plantations. The germination, establishment, and growth of tree seedlings is indeed greatly affected by the environment in which they grow (De Lombaerde *et al.* 2020).

The way plants affect other individuals by altering soil properties (plant–soil feedback) takes various forms. The Janzen–Connell hypothesis (Janzen 1970; Connell 1971) states that establishment of seedlings growing in soil originating from stands with the same species is reduced because of the accumulation of host-specific pathogens, herbivores, and seed predators. However, host-specific mycorrhiza may facilitate these

conspicuous seedlings. Moreover, physical protection by ectomycorrhiza reduces potential antagonistic effects and leads to increased growth in conspecific soil (Bennett *et al.* 2017). Plants at seedling stage, the focus of this study, are particularly vulnerable to pathogens or can be assisted by mycorrhiza (Kardol *et al.* 2010). Differences in soil abiotic fertility caused by tree species composition (Augusto *et al.* 2002) may also affect growth and biomass allocation (Ericsson 1995; Leuschner *et al.* 2004) of tree seedlings. The presence of secondary chemicals produced by the previous generation may inhibit growth of the same species (autotoxicity) or other species (allelopathy) (da Silva *et al.* 2017; Vincenot *et al.* 2017).

Not only species identity, but also tree species diversity is expected to affect seedling growth. A higher diversity of tree species is thought to promote a higher diversity and functioning of soil organisms (Korboulewsky *et al.* 2016; Cesarz *et al.* 2020), which in turn improves nutrient uptake, nutrient cycling, and pest and disease control (Barrios 2007). This is especially important in relation to environmental stressors, since the effects of differences in composition and functioning of food webs strengthen under stress (de Vries *et al.* 2012b).

The environment in which new generations of trees are now growing is changing (IPCC 2021). Previous studies have hypothesized that diversity effects might mitigate the negative effects caused by global change, through more complete resource partitioning in species mixtures and through a larger probability of the presence of resilient species (Chapin III *et al.* 1997). Here, we focus on drought, which we can expect to be more severe and frequent in large parts of the world because of climate change (IPCC 2021). Climate change has been shown to reduce forest growth and lower tree survival (Allen *et al.* 2015). Similarly, seedling germination and growth are negatively affected (Brunner *et al.* 2015; Yigit *et al.* 2016). Furthermore, drought affects biomass allocation, resulting in a higher root:shoot ratio, fine:coarse root ratio, and specific leaf area under drought (Marron *et al.* 2003; Poorter *et al.* 2009, 2012; Brunner *et al.* 2015; Zhou *et al.* 2018).

The literature on the effect of tree species diversity on vulnerability to drought reports conflicting results, ranging from positive to negative effects (Ammer 2019; Grossiord 2020). Only considering the legacy effects of tree species on the soil, the interaction effects with drought remain complex. First, abiotic soil legacies can affect the response to drought stress in multiple ways (Gessler *et al.* 2017). Several studies report reduced performance under drought stress when more nutrients are available (Wendler & Millard 1996; Wu *et al.* 2008). A larger leaf surface area is linked to more nutrient availability but also causes increased transpirational loss of water (Wendler & Millard 1996). Moreover, seedlings growing in more nutrient-limited conditions have a larger root:shoot ratio (Ericsson 1995) and fine root biomass (Leuschner *et al.* 2004) and are likely to more effectively explore the soil for water. These arguments favour the hypothesis that nutrient stress strengthens the tolerance of plants to drought (Chapin III 1991). On the other hand, it can be argued that under high nutrient supply, reserves can be built up, which will enable the tree to more easily recover from drought stress (Waring 1987; Demchik & Sharpe 2000). When nutrient availability is only reduced during drought, it is likely to enhance negative effects of drought stress, since both nutrient and water limitation will impair similar processes within the plant (Gessler *et al.* 2017).

In addition, soils with higher nutrient content often hold a larger organic matter content, which improves the waterholding capacity of the soil, enabling plants to take up water, even though the water input into the system is low (Bouyoucos, 1939).

Second, the interaction between biotic soil legacies and drought is even more complex. The resilience and resistance of the soil food web to drought depends on its land use (de Vries *et al.* 2012a). Therefore, tree species composition can alter the resilience and resistance of the food web (Rivest *et al.* 2015). A reduction in soil moisture reduces microbial biomass and increases the fungal:bacterial growth ratio (Evans & Wallenstein 2012). Drought also affects the interaction of plants and pathogens (Desprez-Loustau *et al.* 2006) or symbionts (Mohan *et al.* 2014). Drought stress can cause a reduction in the ability of the plant to produce energy required to defend itself against pathogens (Schoeneweiss 1986), or cause changes in tissue chemistry that stimulate pathogen growth (Wargo 1996), creating a positive interaction. In this manner, microorganisms that were previously harmless may become pathogenic in stressed plants. Previous studies report opposing results on the response of ectomycorrhizal performance (Mohan *et al.* 2014). Some found a negative effect on root colonization (Lehto 1992; Kennedy & Peay 2007; Alvarez *et al.* 2009), likely because of a reduction in carbon assimilation and hence release of photosynthetic products (Dosskey *et al.* 1991) and direct negative effects of reduced soil moisture (Coleman *et al.* 1989). Others found no effect of drought on ectomycorrhizal colonization (Lehto 1992; Bogeat-Triboulot *et al.* 2004; Domínguez Núñez *et al.* 2009). It has also been demonstrated that ectomycorrhizal relations can alleviate drought stress in plants, although no or negative effects have also been found (Mohan *et al.* 2014).

To study these legacy effects on tree diversity, we performed a pot experiment with soil originating from young stands which varied in tree species identity and species richness, and for which we know from a previous study that at least the chemical conditions bear the imprint of differences in tree species composition after 9 years of forest development (Dhiedt *et al.* 2021). To measure the interacting effect of these soil legacies with irrigation frequency, we subjected half of the pots to a high irrigation frequency and half to a low irrigation frequency treatment. Since the above-described interactions between soil conditions and drought are highly species dependent (Osonubi & Davies 1981; Wendler & Millard 1996), we evaluated the response of five different ectomycorrhizal tree seedling species. Moreover, seed size affects the response to soil fertility, because smaller seeds contain less resources, resulting in a greater reliance on resources from the environment at a young growth stage (Milberg *et al.* 1998), and not all species are equally tolerant to drought stress (Niinemets & Valladares 2006). We address the following research questions: (i) do species richness and irrigation frequency affect emergence and growth of tree seedlings, and do these treatment effects interact; (ii) do seedlings of different species react differently to the treatments; and (iii) how do various emergence and growth variables correlate (co-vary) and is this driven by species composition, instead of species richness *per se*?

We expected a smaller emergence percentage and duration, specific leaf area (SLA), and total biomass, and a larger root:shoot ratio and fine:coarse root ratio under low irrigation frequency. In soil originating from mixtures, we expected a larger

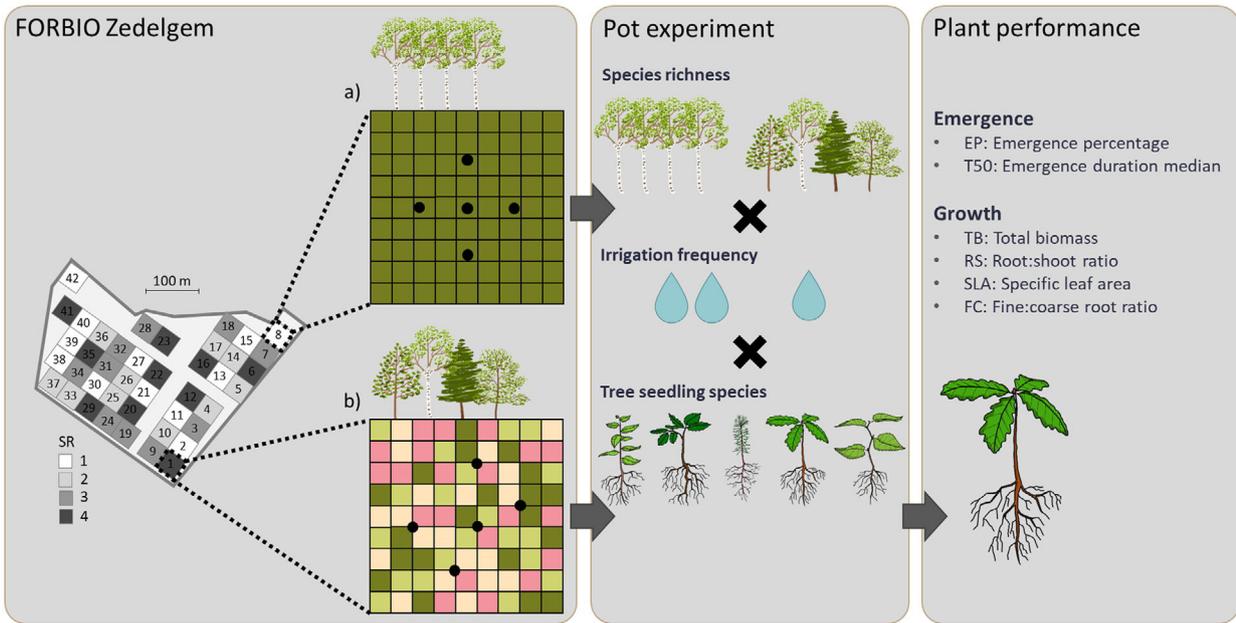


Fig. 1. Schematic overview of the experiment. Left: Sampling design for soil collection for the pot experiment. Soil was collected in plots with a species richness (SR) of (a) one and (b) four. Each of the five distinct species combinations of both SR levels and their replicates was sampled. Each coloured square represents a small monocultural patch of 3×3 trees. Note that sampling points in the four-species plots are situated at the intersections of at least three different species. Middle: Five species (birch, beech, pine, oak, lime) were grown separately in soil collected from each of the 10 compositions for one growing season with two water treatments (high and low irrigation frequency). Right: six plant performance measures determined on each seedling during or after the experiment.

emergence percentage and duration, SLA, and total biomass, and a smaller root:shoot ratio and fine:coarse root ratio. Furthermore, we expected an interaction effect between both treatments, where there would be a stronger effect of irrigation frequency in soils originating from monocultures. We expected that smaller-seeded species (birch and pine) will have a stronger reaction to species richness and that the reaction to irrigation frequency will strengthen with drought sensitivity (pine < oak < lime < beech < birch; Niinemets & Valladares 2006). Finally, we hypothesized that the relationship between the plant performance measures will be partly explained by species composition, irrespective of species richness.

MATERIAL AND METHODS

Site description

The study site is part of a large tree diversity experiment in Belgium [FORBIO, FORest BIOdiversity and Ecosystem Functioning (Verheyen *et al.* 2013)], which is part of a larger network of tree diversity experiments, TreeDivNet (Verheyen *et al.* 2016; treedivnet.ugent.be). The FORBIO experiment consists of multiple sites with a replicated design. The present study made use of a site situated in Zedelgem, Belgium ($51^{\circ}9' N$ $3^{\circ}7' E$), which is the oldest of the three sites. It is situated on relatively dry sandy soil (Podzol) to moderately wet loamy soil (Cambisol) (IUSS Working Group WRB 2015). The experiment was planted in the winter of 2009–2010 on an agricultural field, where arable crops (potatoes and maize) had been grown. The site was ploughed prior to planting. The site-level design of the experimental plantation follows a classical synthetic

community approach. It was planted with five species in different tree species richness levels (1–4 species). The species pool consisted of silver birch (*Betula pendula* Roth), European beech (*Fagus sylvatica* L.), Scots pine (*Pinus sylvestris* L.), pedunculate oak (*Quercus robur* L.), and small-leaved lime (*Tilia cordata* Mill.), further referred to as birch, beech, pine, oak, and lime, respectively. These ectomycorrhizal species are well adapted to the local environment, functionally dissimilar, and of importance for forest management in Belgium. In total 20 species combinations were established. Only the five monocultures and all five possible four-species mixtures are included in this study. Each plot was 42×42 m in size and trees were planted on a 1.5×1.5 m grid. In mixed plots, trees were planted in small monospecific patches of 3×3 trees and randomly mixed in the four-species mixtures (Fig. 1 Left). More information can be found in Verheyen *et al.* (2013).

Soil collection

We collected soil in the monoculture and four-species mixtures, *i.e.* the extremes of the species richness gradient. At least 40 L of soil per plot were collected up to a depth of 10 cm, after removing the forest floor or vegetation, according to the scheme in Fig. 1. Points were chosen in the middle of the plot and in the four wind directions at a distance of 10 m from the middle of the plot. Hence, all points were well away from the edges of the plot to avoid spill-over effects of adjacent plots. In the four-species plots, the selected points were situated in between at least three different species. The soil was sieved to a 0.5-cm mesh size to exclude large organic material and

Table 1. Provenance and stratification strategy of the five species. Sowing, thinning (two strongest seedlings left per pot), and harvest date are also given.

species	provenance	warm stratification (room temperature) (days)	cold stratification (1 °C) (days)	sowing date	number sown per pot	seedling thinning date	harvest date
<i>Pinus sylvestris</i>	Belgium	–	–	23/04/2019	15	01/08/2019	25/11/2019
<i>Quercus robur</i>	Belgium	–	–	01/03/2019	6	01/07/2019	02/12/2019
<i>Tilia cordata</i>	Poland	161	148	23/04/2019	15	02/07/2019	10/12/2019
<i>Fagus sylvatica</i>	Slovakia	–	62	23/04/2019	10	05/07/2019	18/11/2019
<i>Betula pendula</i>	Germany	–	21	19/04/2019	30	16/07/2019	12/11/2019

stones, then homogenized. The material to collect, sieve, and homogenize the soil was sterilized with 70% alcohol between the different plots in order to avoid cross-contamination of the soil biota.

Experimental design

In the collected soil of each plot, stratified seeds (for stratification procedure see Table 1) of each of the species present in the experimental site (*i.e.* birch, beech, pine, oak, lime) were sown in separate pots of 2 l. We also conducted an irrigation frequency treatment (low frequency and high frequency). We created a full factorial design that consists of all possible combinations of the three factors: two species richness levels (five monocultures + five four-species mixtures) × five sown species × two irrigation frequencies (N = 100 treatment combinations). Every tree species composition was represented by two replicate plots in the field experiment, and we replicated each combination of irrigation frequency and experimental plot once more in the greenhouse, resulting in 400 pots. The pots were positioned randomly in the greenhouse to minimize the effects of potential different climate conditions in the greenhouse. The design is visualized in Fig. 1. In each pot, several seeds were sown, depending on their average emergence percentage. Oak was sown 7 weeks prior to the other species, because of earlier availability of the seeds (Table 1). After 3–4 months, the seedlings were thinned until the two strongest seedlings remained (dates given in Table 1).

Irrigation frequency treatment

Before the seeds were sown, all pots were watered to field capacity by applying water until the soil was saturated, covering the pot with a wet cloth to prevent evaporation, and waiting for 2 days to allow excess water to drip from the bottom of the pot. Next, all pots were weighed at field capacity. Hence, the amount of water that has been evaporated and transpired since the last irrigation could be derived simply by weighing the pots. For the first two times, all pots were weighed when irrigated and thereafter; based on variations in these measurements, only four random pots per species per treatment were weighed to determine the irrigation need of the other pots in the same species and treatment combination. The average amount of water lost in this subset was given to all pots of that species and treatment. Next, four sensors were installed in four unplanted pots (two in each treatment) to measure the volumetric moisture content every 15 min, to assess the moisture content at a more detailed level (Supplementary A). The pots were watered with demineralized water, to prevent changes in soil nutrient concentration from traces of nutrients in rain- or tapwater.

Two different watering regimes were used, one at a higher frequency than the other: “high irrigation frequency” and “low irrigation frequency”, respectively. Because of the artificial conditions in the greenhouse (little to no competition for resources, little to no wind, no connection to groundwater), it was more important to water the seedling sufficiently so that they would survive in the greenhouse environment, rather than imitate a supply of water that is expected in nature. The two treatments commenced at the start of the experiment, after all pots were watered to field capacity. The pots in high irrigation frequency were watered to field capacity every 3–4 days; the low irrigation frequency pots received water to field capacity every 2 weeks.

Greenhouse climate and pest and disease control

The seedlings were grown in the controlled environment of a greenhouse. The minimum, mean, and maximum temperatures per month are given in Supplementary B. Plants were watered by hand according to the protocol described above. Shade cloth was used to simulate a cooler and shadier forest environment. At the end of September, the area was artificially illuminated. This lengthened the growing season, preventing the seedlings from losing their leaves before they were harvested. The lamps activated when solar irradiation dropped below 200 Wm⁻² and deactivated at values >250 Wm⁻². No lighting was operational for 12 h after sundown.

Powdery mildew on oak seedlings was frequently treated with KHCO₃ (Karma, Certis). All oak pots, regardless of the level of powdery mildew infestation, were similarly treated. Hence, product that might flow down the stem into the pot was equal over treatments. Direct spraying on the soil was avoided. To control for damage caused by thrips (Thysanoptera) and spider mites (Tetranychidae), Swirski-Mite (*Amblyseius swirskii*, Koppert) was applied three times during the experiment.

Measuring plant performance

Emergence

The number of emerged seedlings in a pot was determined every week until July 2019, when they were thinned to two seedlings per pot. Because the soil was not treated, it contained small seeds, including birch seeds, that could not be sieved out with 0.5-cm mesh sieve. Hence, we corrected for these extra birch seedlings (ranging from zero in approximately half of the pots to >10 in three pots). Assuming the birch seeds were homogeneously distributed in the soil of each plot, we calculated the mean number of birch seedling per treatment and soil in non-birch pots and subtracted this amount from the

number of emerged birch seedlings in the birch pots of that same treatment and soil.

We calculated two variables with these data. First, emergence percentage: the number of seedlings that emerged after 64, 64, 78, 82, 117 days for lime, beech, pine, birch, and oak, respectively, the time at which the seedlings were thinned. By that time, virtually no more seedlings emerged for any of the species. The second variable was the emergence duration median. For a given species, this is the day at which 50% of total recorded emergence occurred. We estimated emergence duration median based on weekly emergence measurements.

Measurements at harvest

The two remaining seedlings per pot of birch, beech, lime, pine, and oak were harvested after 213, 216, 223, 231, 256 days, respectively (dates given in Table 1), at the end of the growing season. The difference in time was caused by differences in sowing date between oak and the other four species and the processing duration during harvesting, which required 1 week per species. On every seedling that emerged and survived until the end of the experiment (amount can be found in Supplementary C), we performed multiple measurements.

We measured the dry weight of the stem (dried for 48 h at 65 °C). For the leaves, we measured the total dry weight (48 h at 65 °C) and the total projected surface of fresh leaves using a Li-Cor Portable Area Meter Li-3100 (Li-Cor Biosciences, Lincoln, NE, USA). In the case of pine, we calculated leaf variables on secondary needles and only on a subset of 10 needles. We also measured total dry weight (48 h at 65 °C) of primary (short single needles preceding secondary needles on seedlings) and secondary needles (typical pine needles). Roots were cleaned in a sieve with 0.5-mm mesh size using a water hose. Visible particles and stones, if present, were removed with tweezers after drying. Dry weight (48 h at 65 °C) of fine roots (defined as roots belonging to root orders 1–3, starting from terminal root segments) and dry weight of coarse roots (defined as roots belonging to root orders >3) were determined (McCormack *et al.* 2015).

The total biomass was calculated as the aggregated dry weight of leaves, stem, and root. The root:shoot ratio is defined as the ratio between total dry root weight and the sum of dry weight of the stem and leaves. We calculated the SLA as total leaf area divided by total dry weight (Pérez-Harguindeguy *et al.* 2016). Finally, the fine:coarse root ratio was calculated.

Statistical analysis

There were two groups of response variables: emergence variables, which are observed on a pot level, and growth variables, which are observed at the level of the individual plant. To answer our research questions, we used multi-response multivariate linear mixed models in a Bayesian framework. For each seedling species, we performed two separate models: one with the two emergence measures as response variables and one with the four growth measures as response variables. To assess whether there are any species richness and/or irrigation frequency effects on the plant performance measures, we fitted both explanatory variables and their interaction as fixed effects. Using a stepwise procedure, and through consideration of the deviance information criterion (DIC), we tested whether the consecutive inclusion of the irrigation frequency factor, species

richness factor, and their interaction in the model helped explain the data better than a simpler model without the respective factor. We retained a simpler model with the smallest DIC. To visualize and evaluate the effects of each treatment combination of species richness and irrigation frequency on each plant measure for each of the species, we made predictions from the models for the different levels of the two treatments and accounting for their interaction effects.

Being multivariate models, the models not only quantify the effects of the treatments on the different plant responses, but also include information on how these responses co-vary, after accounting for the treatment effects. This information can be found in the (co)variance structure of the random variables and residuals.

The emergence model has two levels of variation, *i.e.* between Zedelgem (FORBIO) experimental plots (*i.e.* accounting for soil taken from the same stands), and between pots in the greenhouse (*i.e.* residual variation). We assumed that the two plant performance measures have different but independent between-plot variances. In contrast, we allowed the responses to be correlated across the treatments (species richness and irrigation frequency), *i.e.* the co-variances for the between-pot level effects were estimated.

The growth model had three levels of variation: between experimental plots, between pots, and between individual plants within a pot (residual variation). Similar to emergence, we assumed the plant performance measures have different but independent (and therefore uncorrelated) between-plot variances, but that responses could be correlated at the between-pot level. The within-pot level variances were assumed to be different and independent. This structure allowed us to study the correlation between plant performance measures across pots. The strength of the correlation between plant measures is related to whether the plant measures respond to the same treatments or are forced to change together due to intrinsic trade-offs. To identify to what extent this correlation is driven by the experimental treatments, we compared the full model with the intercept-only model.

A mathematical representation of the model and its priors can be found in Supplementary D. All models were fitted in R version 4.1.0 (R Core Team 2021) using the *MCMCglmm* package (Hadfield 2010). The data and R code can be found on figshare (Dhiedt *et al.* 2023).

RESULTS

The data showed no significant effect of species richness, nor a significant interaction effect between species richness and irrigation frequency, based on the fact that the models that include the species richness effect and/or the interaction effect did not explain the data better than a model fitting only the irrigation frequency effect (deviance information criteria (DIC) can be found in Supplementary E). The main effect of irrigation frequency was clear on all the seedlings for both the emergence and growth measures, except for the emergence measures for lime (see Fig. 2 for measured observations and Figs. 3 and 4 for model predictions).

Looking at emergence of the seedlings in monocultures, both pine and beech showed higher emergence percentage and lower emergence duration median at lower irrigation frequency, *i.e.* more seedlings but slower emergence. In contrast, oak, and to

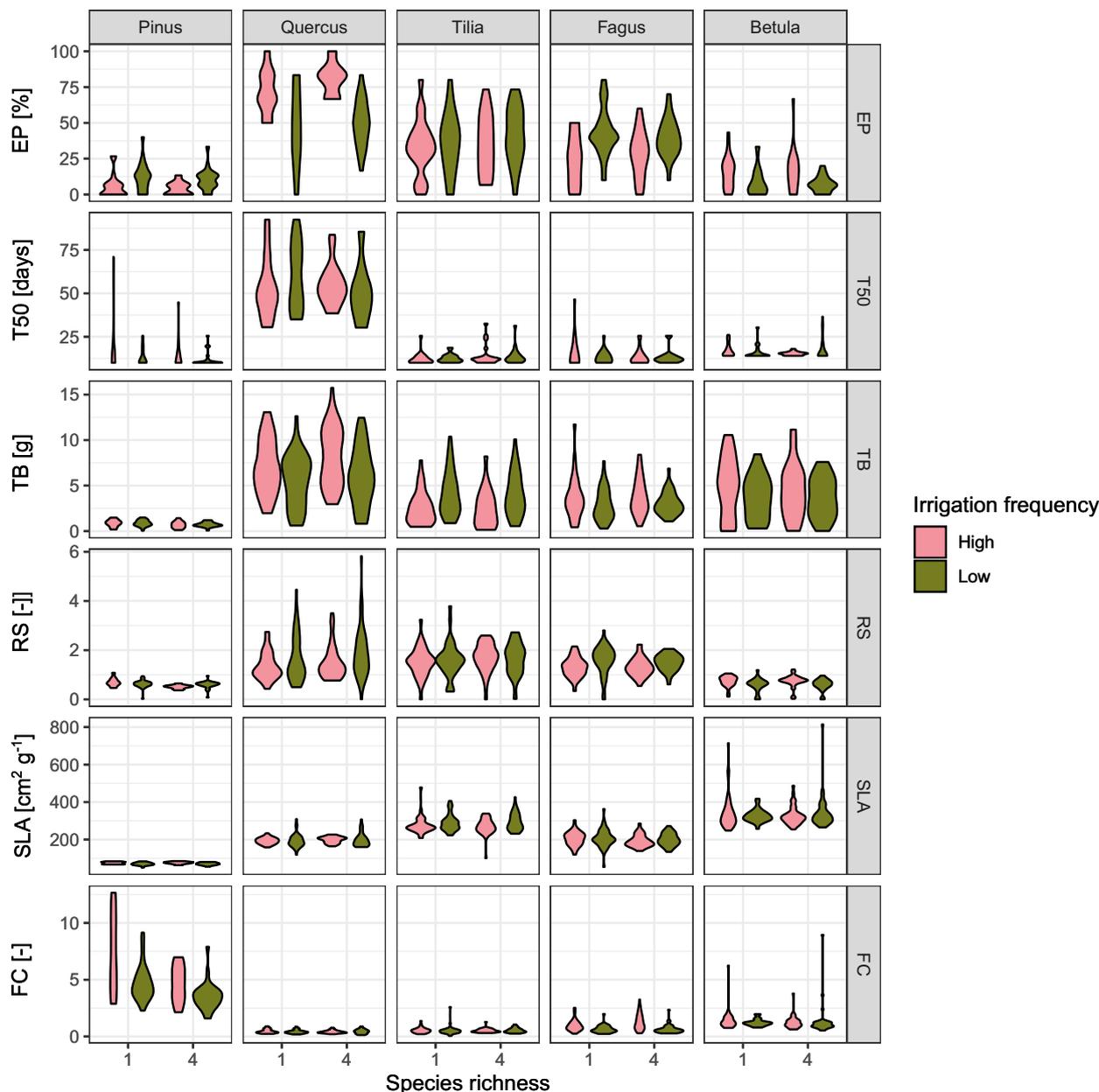


Fig. 2. Distribution of observations of plant performance measures (EP: emergence percentage; T50: emergence duration median; TB: total biomass; RS: root:shoot ratio; SLA: specific leaf area; FC: fine:coarse root ratio) as a function of species richness and irrigation frequency.

a lesser extent birch, showed higher emergence percentage and lower emergence duration median at higher irrigation frequency (Fig. 3).

When focusing on growth performance, oak, and to some extent beech and birch, reduced their total biomass when the irrigation frequency was lowered. Lime, however, had a larger total biomass under low irrigation frequency. Beech had a larger root:shoot ratio under low irrigation frequency, indicating that this species invests more biomass into belowground tissue when the irrigation frequency is lowered. There was no strong effect of a reduced irrigation frequency on SLA for any of the species, except for a negative effect in pine seedlings. A reduced irrigation frequency negatively affected fine:coarse root ratio for all five species, meaning that there were relatively fewer fine roots under a low irrigation frequency, but the

magnitude of this effect was substantial only for pine and beech (± 1 SD) (Fig. 4).

Figures 5 and 6 show covariances between the different measures at the pot level for the emergence model and the growth model, respectively. First, the covariances between plant performance measures of intercept-only model provide insight in the way the measured responses are related to each other. For the emergence measures, there was a negative covariance for all species. In other words, in pots in which more seedlings emerged, it took less time for 50% of the seedlings to emerge. The direction of covariances between the growth measures depended on the species, except for the correlation between total biomass and root:shoot ratio. For all species, seedlings that had a larger biomass invested relatively more into belowground biomass.

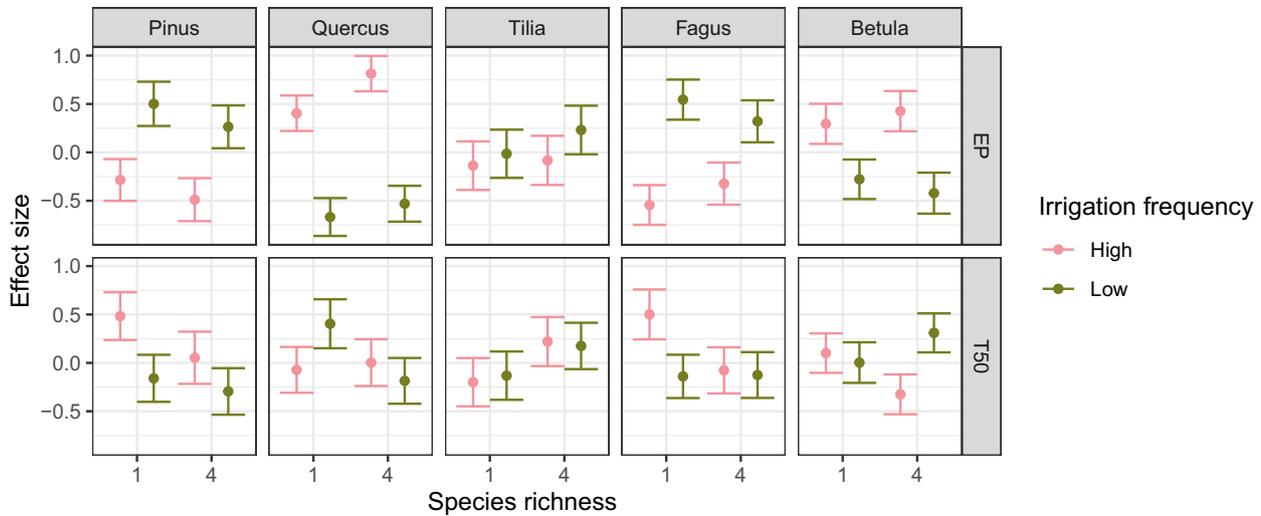


Fig. 3. Model predictions showing treatment effects (effect size) across tree seedling species (top row – species names) of emergence measures (EP: emergence frequency; T50: emergence median duration), *i.e.*, effects of species richness in the FORBIO experiment, and irrigation frequency. The SD is indicated by error bars. Response variables were standardized to allow comparison between predictions of different measures of one species (column) and between predictions of one response measure across different seedling species (row). A prediction of zero means no difference as compared to the mean of all observations of one species and one emergence measure. A prediction of one means that this measure is 1 SD larger/smaller than the overall mean. Species of tree seedlings are ordered from high to low tolerance to drought stress (Niinemets & Valladares 2006).

Second, we were also interested in the extent to which this covariation is driven by the treatments (species richness and irrigation frequency). For this, we compared the covariances of the intercept-only model and the full model, below and above the diagonal, respectively. The treatments explained the part of the covariance that differs between the two models. Any covariance that remains was explained by other variables, one of which was the species composition. For both the emergence and growth measures, there were no large differences, suggesting that the correlation between plant performance measures is driven by something other than the two treatments used.

DISCUSSION

We measured soil legacy effects of species richness and its interaction with irrigation frequency by measuring plant responses in a pot experiment to evaluate the ecological effects, in contrast to direct measurements of soil characteristics. We found that irrigation frequency had a strong effect on the emergence and growth measures of the five species. However, species richness and its interaction with irrigation frequency did not have a strong effect. We also found that the direction and strength of the irrigation frequency effect was dependent on the species. Finally, the covariance between plant performance measures across pots was also dependent on the species and was mostly driven by variables other than our two treatments (species richness and irrigation frequency). The latter indicated that species composition, irrespective of species richness, largely determined the relationship between the plant performance measures. Below, we discuss these findings in more detail.

No species richness effect

The full model did not support our data better than the model that fitted only the effect of irrigation frequency. Hence, the

soil legacy effect of species richness of the stand where the soil originated did not have a strong effect on the plant measures for any of the seedling species, regardless of seed size, in contrast to our hypothesis (Figs. 3 and 4). Legacies that are specific to a tree species composition, irrespective of species richness, may, however, affect the emergence and growth and/or mitigate or strengthen effects caused by irrigation frequency. Because of the focus of our design on species richness, we were not able to distinguish these effects. The covariances, discussed below, provide an indication of what happens at composition level. First, we discuss possible reasons for the lack of any species richness effects.

A first reason could be the age and land-use history of the forest from which the soil originated. The soil was collected only 9 years after the forest had been planted. In a previous study at this site, we found little evidence for tree species richness effects on the chemical composition of the soil at plot level on this site (Dhiedt *et al.* 2021). This suggested that the seedlings experience only subtle differences in abiotic condition of the soil in which they were growing. In that study, we did not look at biotic differences between compositions, which could also affect seedling growth. Furthermore, it is unlikely that nutrients would be limiting, since the concentrations of essential nutrients were high because of the site's agricultural legacy (Dhiedt *et al.* 2021). Cesarz *et al.* (2020) found that the strength of species richness effects on microbial functioning increases with age of the forest, hence it might be too early in forest development to find strong differences in biotic legacies. It is possible that the agricultural land-use legacy overwhelms any potential tree species richness legacies at this young stage. Indeed, in a previous study (Dhiedt *et al.* 2021), we found stronger effects of species composition on the chemical composition at another site of the same experiment, which was afforested after a clear-cut. More generally, effects of species richness are likely very context-dependent (Cesarz *et al.* 2020).

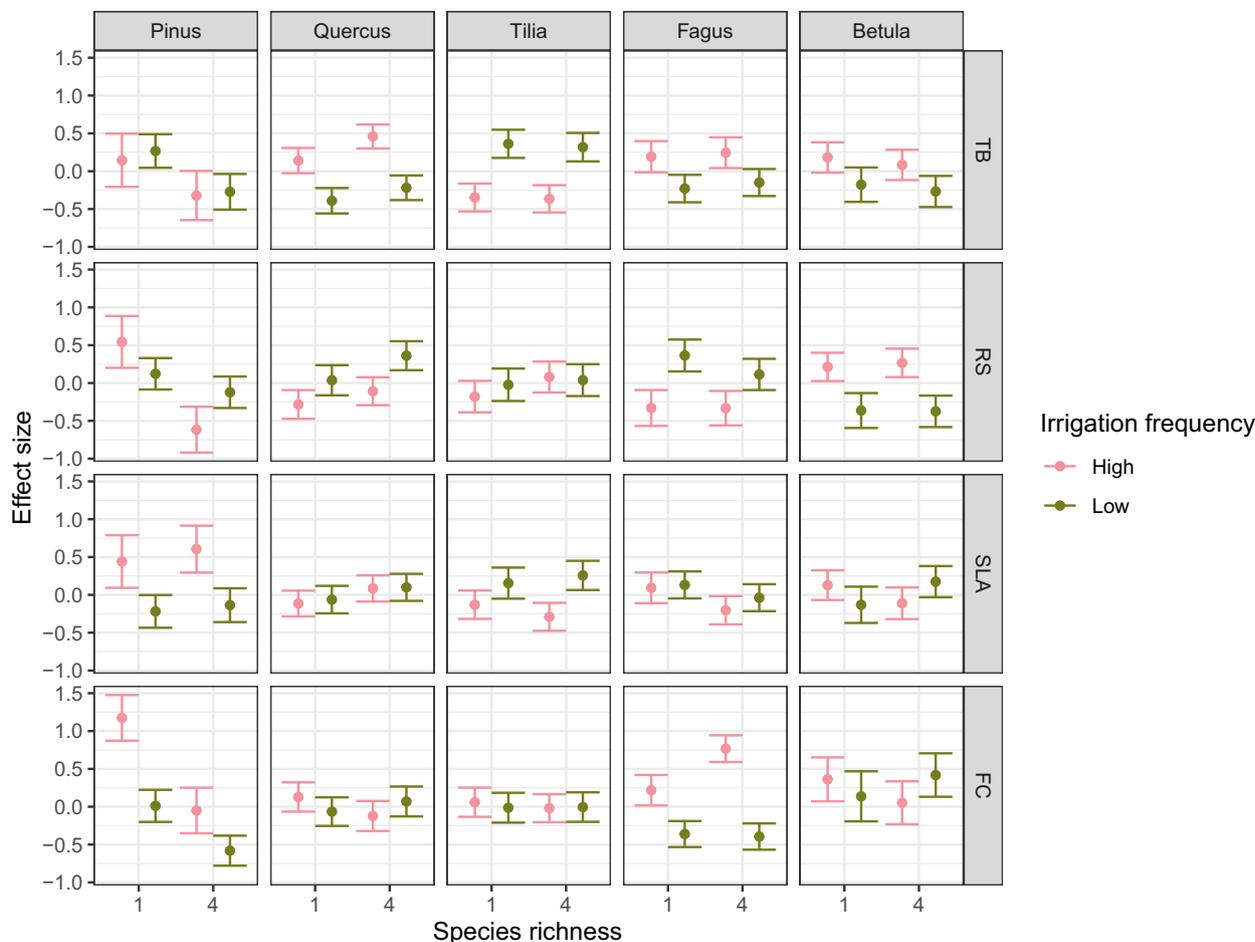


Fig. 4. Model predictions showing treatment effects (effect size) across tree seedling species (top row – species names) of growth measures (TB: total biomass; RS: root:shoot ratio; SLA: specific leaf area; FC: fine:coarse root ratio), i.e., effects of species richness in the FORBIO experiment, and irrigation frequency. The SD is indicated by error bars. Response variables were standardized to allow comparison between predictions of different measures of one species (column) and between predictions of one response measure across different seedling species (row). A prediction of zero means no difference as compared to the mean for all observations of one species and one growth measure. A prediction of one means that this measure is 1 SD larger/smaller than the overall mean. Species of tree seedlings are ordered from high to low tolerance to drought stress (Niinemets & Valladares 2006).

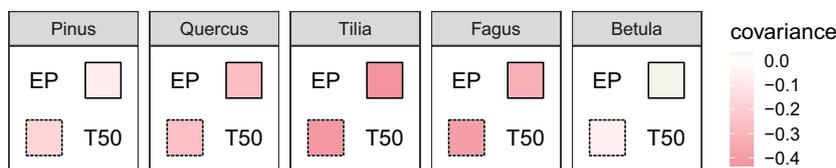


Fig. 5. Co-variances between different emergence measures (EP: emergence percentage; T50: emergence median duration) at pot level. Co-variances are given for the full model with fixed effects of both species richness and irrigation frequency and their interaction (top half of graph and solid lines) and intercept only model (bottom half of graph and dashed lines). Colour denotes direction: green for positive co-variance and pink for negative co-variance and intensity denotes strength of the effect. Since co-variances are mostly negative, green is only slightly visible. Species are ordered from high to low tolerance to drought stress (Niinemets & Valladares 2006).

Previous studies have found long-term effects of past agricultural land use, reporting higher pH and P concentration and lower organic matter content (Falkengren-Grerup *et al.* 2006; Valtinat *et al.* 2008). Such nutrient-rich conditions are favourable for plant growth, improving plant health and thus plant defence against soil pathogens. Under nutrient-rich conditions, plants also benefit less from mutualistic relationships. Both

mechanisms will alter plant–soil feedbacks and limit the biotic soil legacy effect (Bennett & Klironomos 2019).

The duration of the experiment is a potential second explanation. The seedlings were grown for only one growing season before measurements were made. This allowed us to detect soil legacies by using young seedlings as a phytometer, corresponding to a plant–soil feedback approach. The duration is thus in line

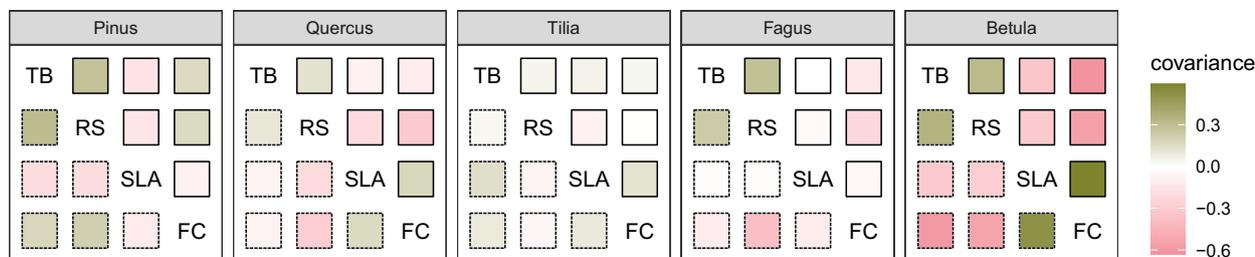


Fig. 6. Co-variances between different growth measures (TB: total biomass; RS: root:shoot ratio; SLA: specific leaf area; FC: fine:coarse root ratio) at pot level. Co-variances are given for the full model with fixed effects of both species richness and irrigation frequency and their interaction (top half of graph and solid lines) and the intercept only model (bottom half of graph and dashed lines). Colour denotes direction: green for positive co-variance and pink for negative co-variance and intensity denotes strength of the effect. Species are ordered from high to low tolerance to drought stress (Niinemets & Valladares 2006).

with our objective, since trees are especially vulnerable to environmental stresses at this early stage (Kardol *et al.* 2010). However, a more long-term experiment would give seedlings more time to react and would reduce season-specific effects (Kawaletz *et al.* 2014).

A third reason could be related to manipulation of the soil before the experiment started. A limitation of this study results from the fact that, prior to installing the experiment, soil was sieved to exclude large organic material and stones and was homogenized. Both manipulations partially destroy the structure of the soil. Soil structure is an important soil legacy (Oades 1993) affecting plant growth, especially under dry conditions (Dexter 1988). Indeed, higher species richness can lead to improved soil structure, as indicated by larger soil aggregates and lower bulk density. This is related to larger above- and belowground biomass and a more complex food web (Fischer *et al.* 2015). It is possible that interaction effects with irrigation frequency treatment may have been found in undisturbed soil, especially considering that soil at the site has a low clay content and hence formation of soil structure is more dependent on living organisms and organic material (Oades 1993). Soil sieving can also impact the microbial soil communities, especially that of filamentous fungi (Petersen & Klug 1994). Also, transfer of the soil from the field to the greenhouse means that most macrofauna were absent from the pots. Many biotic processes leading to soil aeration and aggregate formation would thus be lost (Jones *et al.* 1994).

A fourth reason could be related to results of Smith & Reynolds (2015) that plant–soil feedbacks are stronger in high light conditions, as in a greenhouse experiment. They argued that a higher photosynthetic rate would deplete soil nutrients faster and become limiting. However, as mentioned above, it is unlikely that nutrients were limiting during our experiment because of past agricultural use. Smith & Reynolds (2015) also hypothesized that in high light, plant–soil feedbacks could intensify through increased release of photosynthetic products to mycorrhiza. Although both mechanisms suggest potential legacy effects would be strengthened by the high availability of light in the greenhouse, we did not find any effects on plant growth and emergence. Heinze *et al.* (2016) compared the importance of plant–soil feedbacks in a greenhouse with those in the field. They found weaker plant–soil feedbacks in the greenhouse, and concluded that these are likely overwhelmed by increased diversity of above- and belowground interactions in the field. A further consequence of working in a greenhouse is the high temperatures. A leaf temperature $>42^{\circ}\text{C}$ damages the photosynthetic apparatus

(Ludlow & Björkman 1984), and such a temperature was frequently reached in the greenhouse during summer (Supplementary B). Even though plants can lower their leaf temperature in a hot environment (Mahan & Upchurch 1988), this mechanism may be impaired in dry conditions, when evapotranspiration is reduced (Gardner *et al.* 1981). Therefore, the seedlings may have suffered from heat stress, which could have overwhelmed the expected legacy effects of species richness.

Recent studies have shown the importance of the seed microbiome on germination and initial plant performance (Wassermann *et al.* 2021). Since seeds were not sterilized prior to sowing, their microbiome could have affected the expected legacy effects. All seeds of a given species, however, originated from the same location and thus likely to have a similar microbiome, *i.e.*, no differences between treatments and having little influence on germination patterns.

Irrigation frequency effect depends on species

Since we did not find any interaction effects, we conclude that the legacy of species richness does not affect tolerance of seedlings to the irrigation treatment, to the degree that it can be revealed by our analysis. Similar reasons as discussed above can also be ascribed to this outcome. Moreover, it is possible that our irrigation treatment did not cause sufficient stress for any species richness effect to be apparent. A limitation of our study is that we did not make any physiological measurements, so we cannot ensure that the seedlings growing under low irrigation suffered from drought stress, although various individuals did show visible signs of drought stress, including temporary or permanent leaf wilting, scorched leaves, and defoliation (Kozłowski 1976). Drought may strengthen plant–soil feedbacks (Bennett & Klironomos 2019), but if plants are not stressed, this interaction effect will likely be hidden. However, in contrast to the soil legacy effect of species richness, we did find a strong effect of irrigation frequency. Hence, the lack of interaction between species richness and irrigation frequency effects is likely related to very subtle differences in soil legacy. Below, we discuss irrigation frequency effects.

For both emergence variables, only two species follow our hypothesis of both reduced seedling emergence percentage and duration under low irrigation frequency. Interestingly, there seems to be a trade-off between emergence percentage and duration. On the one hand, for oak the percentage was smaller and the duration larger under low irrigation frequency. This can be explained by the fact that ideal conditions in terms of water

availability for germination were not present and the soil was too dry for the seeds to absorb sufficient water (Hillel 1972). Alternatively, we might be measuring the emergence and not the germination. Low water availability might have slowed seedling growth, hence it could take longer to form a shoot, even if a root had already formed (Gazanchian *et al.* 2006). On the other hand, pine and beech had high emergence percentages and smaller durations under low irrigation frequency. An explanation for reduced emergence percentage under high irrigation frequency could be seed rot (Schafer & Kotanen 2003), but rot does not explain longer median emergence duration. This could be related to higher moss cover in high irrigation frequency (data in Supplementary F), which can slow emergence (Serpe *et al.* 2006), and also explain the smaller emergence percentage.

Consistent with our hypothesis, oak, beech, and birch had smaller total biomass, while oak, beech, and lime had larger root:shoot ratios under low irrigation frequency. Similar results have been reported previously (*e.g.* Brunner *et al.* 2015). The model results also show a small reduction in SLA for pine and birch under low irrigation frequency, but not for the other species. Scheepens *et al.* (2010) described how intraspecific variation of SLA is not only shaped by environmental variation, but also partly by genetic variation. Although all seeds of the different species originate from the same provenance, there will be some genetic variation that may overwhelm variation related to irrigation frequency. Poorter *et al.* (2009) also reported a moderate response to water availability relative to other environmental variables, and hence potentially too small to be statistically distinguishable. In contrast to our expectations, all species showed a slight to strong decrease in fine:coarse root ratio under low irrigation frequency. Since total root biomass was smaller under low irrigation, coarse root biomass decreased relatively less than that of fine roots. More permanent root structures remained similar under both irrigation frequencies, and the reduction in root biomass was mainly related to reduced fine root biomass. This can be related to reduced production of fine roots under water limitation, or increased mortality, not accompanied by increased production. Increased fine root mortality as a response to drought has indeed been described (Zhou *et al.* 2018). This is often followed by increased fine root production, leading to a larger root turnover rate (Meier & Leuschner 2008), which ensures sufficient water acquisition. Zang *et al.* (2014) reported an increase in fine root production under moderate drought stress, compared to a control, but a decrease in production under severe drought stress. Various studies also report smaller fine root production under lower water availability (Yuan & Chen 2010; Herzog *et al.* 2014), which would confirm our results.

The response to irrigation frequency is strongly dependent on the species identity of the seedlings. We ordered the species in Figs. 3 and 4 from most to least tolerant to drought stress, following Niinemets & Valladares (2006). Consistent with our hypothesis, we did not see a negative effect of low irrigation frequency on total biomass for the drought-tolerant pine; we even saw a slight, but not significant, positive effect. Pine is well adapted to dry conditions, explaining the larger total biomass under low irrigation frequency. Lime also had a larger total biomass under low irrigation frequency. This species is also drought tolerant. Furthermore, the provenance of the seed material was Poland, which has a moderately dry climate,

hence these seedlings will be better adapted to drier conditions. Birch and beech, the two most drought-sensitive species (according to Niinemets & Valladares 2006), did not show a strong negative effect on total biomass, in contrast to oak. Oak was sown almost 7 weeks before the other species. The longer growing period and related longer exposure to the irrigation treatment can explain the stronger effect. van Hees (1997) reported that oak seedlings can better adapt their morphology to drought than beech. We, however, saw a stronger effect of irrigation frequency on the root:shoot ratio of beech but no difference in effect on SLA for both oak and beech. The root:shoot ratio of birch was negatively affected by low irrigation frequency. Wendler & Millard (1996) described how birch avoids, rather than tolerates, stress from limited water availability through stomatal closure rather than morphological adaptation. This also explains the negative effect on total biomass, since this will restrict leaf gas exchange.

Coordinated responses of plant measures

In regard to how plant measures were related across pots (Figs. 5 and 6), there were relatively strong patterns of covariation, which were consistent across species. Importantly, these correlated responses across pots were not explained by irrigation frequency or species richness treatment effects. Such effects could be expected if two plant measures respond to the same treatment or if one measure responds to the treatment and, due to intrinsic trade-offs, another measure is forced to change with it. The observed parallel changes are likely related to other sources of between-pot variation. Pots were filled with soil from different experimental plots in the FORBIO experiment, representing different species compositions. The correlations in plant measures might thus be partly explained by species composition effects. Studies have shown that effects of tree diversity on the soil are mostly driven by species identity rather than diversity *per se* (Dawud *et al.* 2016; Tedersoo *et al.* 2016). This pattern has also been seen in early years after afforestation (Gunina *et al.* 2017). Our design did not lend itself to study these identity and composition effects in detail. Further studies could provide a deeper understanding, particularly how soil legacy effects relate to species composition effects.

Similar to the negative correlation between emergence percentage and duration median when comparing differences in species richness and irrigation frequency, there was a negative covariance at pot level (Fig. 5). Seedlings grown in soil originating from the same plot with the same species composition took longer to emerge if a smaller percentage emerged, and *vice versa*. The change in direction of the co-variance between emergence percentage and duration for birch, when comparing co-variances for the intercept-only model and the model including both treatments, may reveal that the treatments explain some of the variance. Lime showed the strongest covariation between emergence percentage and duration at pot level. A large portion of the co-variance was not explained by the treatment effects, as expected by the small effect sizes (Fig. 3).

For growth variables, there was a consistent positive covariance between total biomass and root:shoot ratio, unlike the correlation for irrigation frequency (Fig. 6). Seedlings growing in soil from a given plot were larger, when they allocated

relatively more biomass to the roots. A seedling with large roots can take up and transport more nutrients and water and hence support a larger total biomass. Most species also showed a negative co-variance between total biomass and SLA. This is in contrast to expectations based on the leaf economics spectrum, which states that SLA is positively correlated with photosynthetic capacity and hence is found in fast-growing plants (Wright *et al.* 2004). However, such relationships are often unclear within a species (Fajardo & Siefert 2018), which is the level that we investigated. The other co-variances differ in direction between different species, suggesting that the response of one species cannot be translated to all species. The strongest co-variance was between response variables of the growth model for birch. As with lime for the emergence model, there was no strong effect of species richness or irrigation frequency on any of the response variables and hence more co-variance is explained by the plot location, which is associated with species composition. Surprisingly, birch is the species that is most vulnerable to drought and with the smallest seed, yet most of the co-variance was explained by something other than the treatment variables.

CONCLUSION

Using a pot experiment, we investigated the legacy effects of species richness of a young tree plantation on tree seedling emergence and growth. We also examined how possible richness effects interact with an irrigation frequency treatment. There was no strong soil legacy effect of species richness on any of the plant performance measures for any of our tree species. This lack of evidence is likely because the trees in the plantation did not have sufficient time to substantially affect the soil, and because the agricultural land-use legacy may have overwhelmed any species richness legacy effects. However, there were strong effects of irrigation frequency on most plant performance measures. The direction and strength of these effects were dependent on the tree species. A large portion of the co-variance was explained by the species composition, irrespective of the species richness. Previous studies have shown that soil legacies are highly context dependent. Hence, further studies examining the soil legacy effect of species richness in different contexts are necessary in order to generalize our results.

AUTHOR CONTRIBUTIONS

E.D., L.B., P.D.S., and K.V. conceived the conceptual idea and outline. K.V. and L.B. designed the FORBIO experiment. E.D. set up the pot experiment and collected data. E.D. and L.B. performed the statistical analysis. E.D. wrote the manuscript with support from L.B., P.D.S., and K.V. All authors discussed the results and contributed to the final manuscript.

ACKNOWLEDGEMENTS

E.D. held a doctoral fellowship and P.D.S. holds a post-doctoral fellowship of the Research Foundation Flanders (FWO). We thank the many people that made this study

possible. Kris Ceunen, Robbe De Beelde, and Shengmin Zhang helped in collecting soil. Kris Ceunen, Robbe De Beelde, Shengmin Zhang, Brecht Vandendriessche, Maarten Volckaert, and Luc Willems helped with experimental setup, practically and/or advisory. Stefan Vidts was responsible for the greenhouse and always ready with advice on pest control and greenhouse climate regulation. Kris Ceunen, Robbe De Beelde, Iris Moeneclay, Margot Vanhellemont, and Luc Willems assisted with irrigation. Tobias Malfroy, Haben Blondeel, Kris Ceunen, Eva DeCock, Frederik Gerits, Dries Landuyt, Margot Vanhellemont, Bin Wang, and Luc Willems helped during the harvest and measurements at the end of the experiment. We are grateful to Mike Perring who commented on the English grammar and spelling and gave helpful comments on the content. We also thank anonymous reviewers for their insightful comments. In addition, we thank Ellen Desie, Quentin Ponette, Eduardo de la Peña, and Wannes Hubau, who provided their time and gave helpful comments during the review of E.D.'s PhD thesis, of which this manuscript forms part.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. Supporting information.

Figure A.1. Volumetric soil moisture as a function of time. Soil moisture was measured every 15 min by the TMS-4 sensor of TOMST. The line type denotes the irrigation frequency (solid = high, dashed = low). For each irrigation frequency two sensors were installed, hence the two lines for each frequency (sensor a and b for high irrigation frequency; sensor c and d for low irrigation frequency). The sensors were placed in pots without seedlings, to not hinder the seedlings in their growth, but were watered with the same volume of water at the same frequency as the pots with seedlings. Due to a lack of transpiration by plants, the fluctuations in soil moisture are less extreme than in pots with seedlings, but show a similar pattern. From mid-August until mid-September the sensor pots in the high irrigation frequency treatment were not irrigated due to an oversight, hence the large drop in soil moisture during that time. This oversight did not affect the pots containing tree seedlings.

Table B.1. Minimum, mean, and maximum greenhouse temperature per month.

Table C.1. Number of seedlings per species richness (SR) and irrigation frequency (IF) treatment and per species that germinated and grew until the end of the experiment. We measured growth properties on these seedlings.

Table E.1. Deviance information criteria (DIC) of the models (Intercept = intercept-only model, IF = model with irrigation frequency (IF) as main fixed effect, IF + SR = model with irrigation frequency and species richness (SR) as main fixed effects, IF × SR = model with irrigation frequency, species richness, and their interaction as fixed effects). The smallest DIC is highlighted in bold.

Figure F.1. Distribution of the moss cover data for each species as a function of the irrigation frequency.

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