Forest Ecology and Management 609 (2026) 123640

Contents lists available at ScienceDirect

Forest Ecology and Management

journal homepage: www.elsevier.com/locate/foreco

Temporal insights into afforestation: A 25-year study of woodland expansion

Julija Fediajevaite [a,*], Aaron Philips [b], John Tucker [b], Peter Buckley [b], Clive Steward [b], Sam Pettman [b], John Crawford [b], Petra Guy [c], Mark Tibbett [d], Nadia Barsoum [c,1], Brian J. Pickles [a,1]

[a] *School of Biological Sciences, University of Reading, Reading, UK*
[b] *Woodland Trust, Kempton Way, Grantham, UK*
[c] *Forest Research, Alice Holt Lodge, Farnham, UK*
[d] *Department of Sustainable Land Management, University of Reading, Reading, UK*

ARTICLE INFO

Keywords:
Restoration
Ectomycorrhiza
Ex-arable land
Indicator species
Soil properties

ABSTRACT

Direct observation of transitions from farmlands to woodlands are rare, and habitat change dynamics are typically inferred indirectly from pseudo-chronosequence observations (space-for-time substitution). The aim of this study was to directly examine the transition of plant communities and soil properties as farmland developed into ex-arable planted woodland, in comparison to nearby ancient woodland. We collected plant identity and abundance records and topsoil cores for chemical analysis between 2001 and 2024. We additionally collected canopy openness data and soil cores for identification of soil fungal communities in 2024, to link plant communities and soil chemical properties with fungal communities and light availability. We modelled differences in species distribution across gradients of distance from ancient woodland. Our results showed that soil properties and plant communities in ex-arable and ancient woodlands remained significantly different 25 years after land-use change. Ex-arable woodland plots had higher pH, more available phosphorus, less total nitrogen, and less total carbon than ancient woodland plots. Changes in plant diversity were mostly associated with carbon to nitrogen ratio. The presence of ancient woodland indicator plant species was associated with higher light availability and proximity to ancient woodland edge. Ectomycorrhizal fungal communities were also significantly different in ex-arable and ancient woodlands, with higher fungal richness positively associated with soil pH and tree species richness. Our study suggests that woodland creation programmes should consider connectivity to ancient woodlands, time lags associated with plant and fungal succession, as well as the history of soil alteration practices, especially within woodland creation and compensatory schemes.

1. Introduction

The UK has committed to creating nearly 200 000 ha of woodland on low grade disused farmland by 2050 as part of the Government's Net Zero Strategy (Climate Change Committee, 2020; Westaway et al., 2023), to mitigate climate change and combat the biodiversity crisis (IPBES, 2019). Current woodland creation practices aim to generate resilient and diverse forest ecosystems (Messier et al., 2022). Compared to ancient woodlands, secondary plantation woodlands often lack structural diversity, such as canopy gaps and variation in tree size, resulting in reduced opportunities for plant species to establish (Waddell et al., 2024). Considerable temporal lags between woodland creation action and the formation of biodiverse ecosystems have been reported (Watts et al., 2020). Chronosequence studies suggest that hundreds of years are needed for a secondary woodland to develop specialist woodland species (Brunet et al., 2021; Waddell et al., 2024), such as ancient woodland vascular plant indicators, which are often adapted to only short-distance dispersal (Wulf, 2004). Minimising spatial isolation has been suggested (Hughes et al., 2023) as a method for mitigating colonisation deficits (also referred to as colonisation/immigration credits by Naaf and Kolk 2015, Watts *et al.* (2016), Hughes et al. 2023 and Waddell et al. 2024) between new and ancient woodland, although

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* Corresponding author.
E-mail address: j.fediajevaite@pgr.reading.ac.uk (J. Fediajevaite).
[1] Senior authors

https://doi.org/10.1016/j.foreco.2026.123640

evidence is limited.

Land use change, such as afforestation of ex-arable land, introduces more recalcitrant litter to the topsoil layer. This affects soil nutrient cycling, resulting in increased soil carbon (C) content (Błonska et al., 2020). Changes in soil nutrient dynamics are in turn associated with shifts in fungal community composition (van der Linde et al., 2018). Agricultural fertilisation and carbon removal legacies (Brudvig et al., 2013), as well as structural diversity and colonisation deficit (Waddell et al., 2024), play a role in a delayed convergence of ex-arable into ancient woodlands. Ex-arable woodlands have been found to contain distinct soil fungal communities, with higher relative read abundance of ectomycorrhizal and arbuscular mycorrhizal fungi compared to saprotrophic fungi (Lutter et al., 2023).

There is limited evidence describing fungal community development during afforestation (Chen et al., 2023; Panico et al., 2025), however there are reports of an increased proportion of ectomycorrhizal fungi (EMF) in relation to other fungal guilds, either linked to an increase in ectomycorrhizal host tree abundance (Wang et al., 2019), or an increase in soil organic C and nitrogen (N) (Panico et al., 2025). EMF play a crucial role in organic matter decomposition (Lindahl et al., 2021) and soil C stabilisation (Hagenbo et al., 2024). They form mutualistic relationships with many temperate tree species (Brundrett and Tedersoo, 2018) and aid plant nutrient acquisition. EMF have been found to outcompete saprotrophs (Gadgil and Gadgil, 1975; Fernandez and Kennedy, 2016), especially in soils with high C/N ratios (Mayer et al., 2023), resulting in lower decomposition rates and higher C storage (Fernandez et al., 2020). Soil nutrient dynamics are closely interlinked with fungal community structure, which can ultimately mediate ground flora composition (Guy et al., 2022).

Understanding of both belowground and aboveground changes during afforestation can help to direct conservation efforts and intervention, however, such knowledge of species and environment relationships in ex-arable settings is lacking (Brudvig et al., 2013). Observations of plant community transitions coupled with insights into soil biotic and abiotic dynamics are much needed, as they would help to inform the efficacy of afforestation efforts for carbon sequestration and nature recovery (Panico et al., 2025).

With increasing afforestation of low-grade agricultural land in temperate regions, studies that monitored plant, microbial, and soil shifts during arable land afforestation are emerging. However, direct observation of succession and changes in biotic and abiotic components over a long period of time, as part of a time-series study, is rare (Harmer et al., 2001; Thomaes et al., 2014; Broughton et al., 2021), and is more often inferred indirectly using a space-for-time approach. This is mainly due to a combination of lack of funding for long-term studies and the empirical challenges of setting up long-term observation experiments at sufficiently large spatial scales (Waddell et al., 2024). Nevertheless, studies conducted at fine temporal resolutions are essential for informing woodland creation practices (Waddell et al., 2024).

Our study goals, therefore, were (i) to examine and compare the evolution of soil and ground flora transitions over a 25-year period in ancient woodland and an adjacent woodland plantation site on former arable land, and (ii) to assess, at a single time-point, any soil fungal community compositional changes along a gradient of ancient woodland into ex-arable woodland and how any such changes relate to environmental gradients. We aimed to undertake a functional traits-based approach to plant and fungi community analyses, and therefore included dispersal related properties, such as seed mass, spore size and sporocarp size, as well as shade light and drought stress related trait of specific leaf area. Based on the previous studies, we predicted a significant distinction between ex-arable and ancient woodland in terms of soil properties, ground flora and soil fungal communities, which, we expected, would decrease over time. We also hypothesised that the distance from ancient woodland, dispersal traits and soil properties would be important factors shaping ground flora and soil fungal communities.

2. Methods

2.1. Site description

The study site is within Hucking Estate, located in Kent County, south-east England (at 51.287631 latitude, 0.64519429 longitude, WGS; Fig. 1). The soils are Eutric Luvic Planosols, seasonally waterlogged and a mixture of fine silt, loam and clay, above a clay sub-soil (National Soil Resources Institute, United Kingdom). The climate is temperate oceanic with mean annual minimum/maximum temperatures of 7.09 °C/ 15.05 °C and 675.27 mm mean annual precipitation (Faversham weather station at 51.3149209674 latitude, 0.892581514554 longitude, data gathered over period of 1991–2020, Met Office, 2006). The site comprises a semi-natural ancient woodland and a former linseed cropland. The former cropland had been continuously farmed since at least 1786, limed and fertilised, hence no persistent woodland plant seed bank remained (Buckley and Howell, 2008). The ancient woodland is listed in the Ancient Woodland Inventory (continuously wooded since at least 1600) (Natural England, 2024). It is dominated by the W10 National Vegetation Classification (NVC) (Rodwell, 1991) community (*Quercus robur - Pteridium aquilinum - Rubus fruticosus*).

In 1999, the cropland (11.95 ha) was planted with oak *Quercus robur* (45 %), ash *Fraxinus excelsior* (30 %), hornbeam *Carpinus betulus* (10 %), small-leaved lime *Tilia cordata* (10 %), and woody shrubs hazel *Corylus avellana* and hawthorn *Crataegus monogyna* (combined 5 %) – these are the typical tree species making up a W10 NVC community, thus matching the adjacent ancient woodland community. Trees were planted 2.1 m apart. The plantation area was sprayed with herbicides and fenced. Permanent observation plots (8 m x 12.5 m) were installed in a grid at 50 m intervals, with a total of 47 plots in the plantation area and 22 within ancient woodland (Fig. 1). Distance from the ancient woodland was determined using QGIS version 3.14 (QGIS Association: http://www.qgis.org) to measure the Euclidian distance between the edge of nearest ancient woodland and ex-arable woodland plots, which was used for the distance gradient analysis

2.2. Surveys

Vegetation surveys were conducted at irregular intervals between 2001 and 2024 during April–May. Surveys were carried out by experienced botanists: PB conducted the assessments from 2001 to 2015, and JF completed the 2024 survey. All vascular plant species were identified to species level, except for *Cotoneaster* sp. due to hybridisation and challenging morphological identification. Percentage cover of groundlayer woody species, forbs, graminoids, and ferns was visually estimated across the entire 8 × 12.5 m plot, without subdivision into smaller subplots. Visual estimation was facilitated by clearly marked plot corners and the relatively low vegetation cover at the study site. Cover was recorded in 5 % increments, with values below 5 % assigned a value of 1 %. The percentage cover of bare ground was also recorded for each plot.

Soil sampling for chemical analysis was completed in 2002, 2012, and 2024. Soil samples were collected using a Dutch auger to 12.5 cm depth, followed by removal of leaf litter, with three samples collected at random points within one plot and then bulked. The same field sampling protocol applied consistently across years.

In 2024, hemispherical canopy photos were taken using a WinSXLScanopy DSLR camera with a fisheye lens and a self-balancing mount, at approximately 0.5 m aboveground (above the shrub layer) at the centre of each plot. Canopy openness was used as a proxy for structural diversity of the woodland. In addition, tree species in the canopy layer were recorded for each survey plot. One additional soil core at the centre of each plot was collected for Internal Transcribed Spacer 2 (ITS2) amplicon sequencing. In order to minimise cross-contamination, the auger was field sterilised (cleaned using chlorine bleach wipes, 0.01 %) between the plots. Details of sampling undertaken

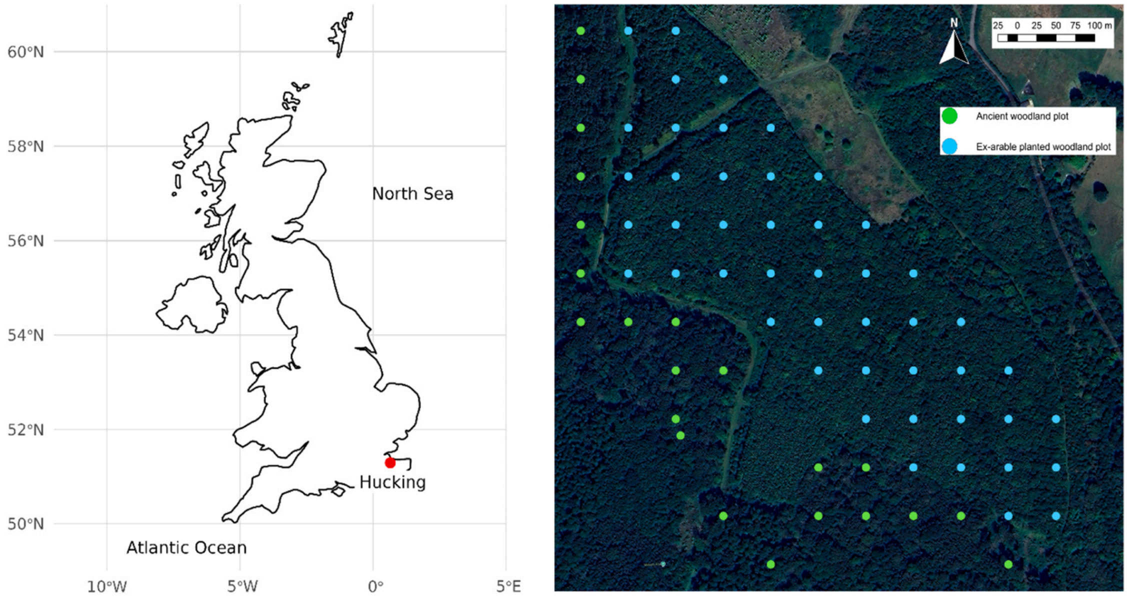

Fig. 1. The study site map: left panel indicates the location of the study site in red (Hucking); right panel shows observation plots within ancient (in green) and planted (in blue) woodland.

and dates are available in Table S1.

2.3. Sample processing

Soil samples for chemical analysis were stored at 4 °C and sent to a commercial laboratory for soil agronomic analysis (Cawood Scientific NRM, Bracknell, UK). Assessments included soil pH (measured in water, reported on a dry basis), available phosphorus (P) (extracted using sodium bicarbonate, reported on a mg/l dry basis), available magnesium (Mg) and potassium (K) (ammonium nitrate soil extract, BS:3882:2015, British Standards Institute, London, 2015), total nitrogen (N) (reported on a % w/w dry basis) and total C (reported on a % w/w dry basis). The same laboratory and methods were used for all soil sampling and analysis occasions.

Soil samples collected for ITS2 amplicon sequencing were stored at −20 °C until DNA extraction. DNA was extracted using the DNeasy PowerSoil Pro Kit (Qiagen: Hilden, Germany), following the manufacturer’s instructions in June 2024. DNA concentration was determined using a NanoDrop 2000 (Thermo-Fisher Scientific: Waltham, US). Extracted DNA samples were shipped on ice to Novogene for ITS2 amplification and sequencing. A total amount of 200 ng of DNA was used for PCR amplification with general fungal primers ITS3-F (GCATCGATGAAGAACGCAGC) and ITS4-R (TCCTCCGCTTATTGATATGC) (White et al., 1990). All PCR reactions were carried out with 15 µL of Phusion High - Fidelity PCR Master Mix; 0.2 µM of forward and reverse primers, and about 10 ng template DNA. Thermal cycling consisted of initial denaturation at 98℃ for 1 min, followed by 30 cycles of denaturation at 98℃ for 10 s, annealing at 50℃ for 30 s, and elongation at 72℃ for 30 s and 72℃ for 5 min. The PCR products were purified using magnetic bead purification. Samples were mixed in equidensity ratios based on the concentration of PCR products. After thorough mixing, the PCR products were detected, and target bands were recovered.

Sequencing libraries were generated and indexes were added. The library was checked with Qubit and real-time PCR for quantification and bioanalyzer for size distribution detection. Amplicon sequencing was performed using a Novaseq 6000 (Illumina: Cambridge, UK) with PE250 (paired-end 250 bp), 50k tags per sample and a Phred score of Q30 > 75 %.

Sequences were processed through a series of computational steps including trimming, quality filtering, dereplication, and clustering into operational taxonomic units (OTUs) at a 97 % similarity threshold by Novogene. Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged using FLASH (V1.2. 1 1, http://ccb.jhu.edu/software/FLASH/) (Magoč and Salzberg, 2011). Quality filtering on the raw tags was performed using the fastp (Version 0.23.1) software. The tags were compared with the Unite reference database (https://unite.ut.ee/) to detect chimera sequences. The effective tags were obtained by removing the chimera sequences with the vsearch package (V2.16.0). Denoising was performed with DADA2. Species annotation was performed using QIIME2 software.

No further removal of OTU occurrences was undertaken. Because of the established ITS metabarcoding biases, relying on sequence reads as a proxy for abundance is problematic (Nilsson et al., 2019), and without having a mock community with known abundance of fungal material, any minimum sequence read thresholds would be arbitrary (Drake et al., 2022). Hence further analyses were performed only on fungal presence/absence data.

Canopy openness was estimated using WinSCANOPY 2022 software, using the default hemisphere detection function. Canopy openness is reported as % of gap in canopy.

2.4. Data analysis

Data was prepared using Python version 3.12.4 package ‘Pandas’ (McKinney et al., 2010). R version 4.4.1 was used for the data analysis. We explored differences in edaphic properties, plant diversity, richness and environmental variables between woodland types, i.e. ancient and planted woodland (henceforth referred to as “ex-arable woodland”) using linear mixed effects models. We used Renyi diversity profiles to obtain diversity values expressed as Hill numbers (Chao et al., 2024) using the ‘vegan’ package (Oksanen *et al.*, 2024). Woodland type, year and their interaction were included as fixed effects, and plot reference was included as a random effect to account for repeated measurements. Linear mixed models and Spearman correlation tests were used to explore interactions between various biotic and abiotic variables. Models were fitted using the lme4 package in R. Model assumptions were checked visually using residual and Q-Q plots. Where homoscedasticity assumption was not met, a generalised linear mixed effects model was fitted. Generalised linear mixed effects model fit was assessed using simulated residual diagnostics. Where violations of uniformity or dispersion were detected, error distribution family was adjusted accordingly. Where non-linear relationship was detected, a generalised additive model was built using the 'gam' package (Hastie, 2015). Temporal autocorrelation in model residuals was assessed using autocorrelation function plots of the residuals. Residuals showed no evidence of temporal autocorrelation. The PERFORMANCE package (Lüdecke et al., 2021) was used to obtain the conditional and marginal R^2 of models with the lowest Akaike Information Criterion (AIC).

We analysed plant and EMF community dissimilarity to explore how these communities differ between woodland types. Bray-Curtis modification of Sorensen's distances were calculated for plant abundance data, and Jaccard distances for EMF presence/absence data. Permutational analysis of variance (PERMANOVA) tests (Anderson, 2001), and pairwise PERMANOVA (Martinez Arbizu, 2020) were performed for Bray-Curtis and Jaccard distances. Significance of effects was assessed using 999 permutations. P-values were adjusted for multiple comparisons using the Benjamini-Hochberg method. To assess variability in community composition within woodland types, the 'betadisper' function in vegan was used (Anderson et al., 2006). The significance of differences in woodland type dispersion was tested using ANOVA. Non-Metric Multidimensional Scaling (NMDS) was used to plot Bray-Curtis and Jaccard distances by years (only applicable to plant and not EMF data) and woodland type, overlaying abiotic and biotic variables on the ordination as a biplot. We also completed indicator species analysis (Dufrêne and Legendre, 1997), using the 'multipatt' function in the indicspecies package, to identify ground flora and EMF species characteristic of ancient vs ex-arable woodland types. Indicator species analysis was undertaken on the presence/absence data of ground flora and EMF communities.

We aimed to explore plant preference to environmental conditions by using Ellenberg plant indicator values of light, soil fertility (nitrogen), soil moisture and soil acidity (reaction) (Hill et al., 1999). These were weighted by plant cover (Zelený and Schaffers, 2012) and averaged across plots (for Ellenberg indicator value range and meaning please see Supplementary Information Table S2). Plant and fungal traits data was incorporated into analysis to explore differences in plant and fungal communities along environmental gradients. Seed mass and specific leaf area of ground flora species were determined using the LEDA database (Kleyer et al., 2008). Values were also weighted by cover and averaged across plots. These functional traits were selected as they are widely used in plant distribution studies (May et al., 2013; Madani et al., 2018) – seed mass as a dispersal related trait, and specific leaf area as a shade tolerance and drought stress related trait. Plants were assigned a woodland specialist, generalist or non-woodland status following Kirby et al. (2012)'s classification. Mycorrhizal status of host tree species was assigned using a review by Guy et al. (2022). The FUNGuild tool was utilized to assign functional groups to fungal OTUs (Nguyen et al., 2016). FungalTraits database (Põlme et al., 2020) was used to assign sporocarp size and spore size to EMF species. Sporocarp size was included in the study as it is a potential adaptation to drought stress (Calhim et al., 2018) and architectural adaptation for higher spore release and longer-range aerial dispersal (Pringle et al., 2015). The spore size trait was added to investigate whether propagule size is related to dispersal distance when shaping fungal communities. Values were averaged across plots based on species presence/absence.

3. Results

3.1. Soil chemical properties

Ex-arable woodland soil properties were significantly distinctive from ancient woodland soil properties (PERMANOVA $p < 0.001$, $R^2 = 0.623$, Figure S1). Ex arable woodland plots had on average circumneutral pH of 6.75 (SD 0.72), while ancient woodland plots were more acidic (Fig. 2), with a mean pH of 4.47 (SD 0.28) (combined mean across years). In the first year of sampling (2002), soil pH in ancient woodland was lower than in ex-arable woodland by 2.93 units. This difference decreased to 1.73 units in 2024 (linear mixed model, conditional R^2=0.945, marginal R^2=0.816, $p < 0.001$). Concentrations of available P and K were also significantly higher in ex-arable woodland, with a trend of decreasing difference over time (for P: linear mixed model, conditional R^2=0.587, marginal R^2=0.167, $p < 0.01$; for K: linear mixed model, conditional R^2=0.754, marginal R^2=0.325, $p < 0.01$). By contrast, total N and C concentrations were higher in ancient woodland (for C: linear mixed model, conditional R^2=0.773, marginal R^2=0.612, $p < 0.001$; for N: linear mixed model, conditional R^2=0.711, marginal R^2=0.528, $p < 0.001$). Available Mg was the only soil property that did not differ between ex-arable woodland and ancient woodland plots in 2002 and 2012 but became significantly higher in ancient than ex-arable woodland in 2024 (linear mixed model, conditional R^2=0.841, marginal R^2=0.645). For more details about the modelling, estimates and goodness of fit measures see Table S3.

3.2. Plants

Ex-arable woodland ground flora communities were significantly distinct from ancient woodland communities throughout the 25-year observation. Dissimilarity of plots between woodland types and different years of sampling is visualised in the NMDS plot (Fig. 3). Within the ex-arable woodland plots, years 2001–2007 are grouped and distinct from years 2015 and 2024, with plots sampled in later years being more similar to plots in the ancient woodland. Ancient woodland and, in later years, ex-arable woodland plant communities were associated with lower soil pH, lower available P, and higher plant seed mass. Ancient woodland plots were associated with higher specific leaf area. For more information about the NMDS, see the Table S4. Pairwise PERMANOVA showed a significant difference between ancient woodland and planted woodland across all survey years ($p < 0.05$). For more information about the PERMANOVA, see the Table S5.

Although ancient woodland plots had higher canopy openness than ex-arable woodland plots (linear model, R^2=0.232, $p < 0.001$), ex-arable woodland had higher species' preference (Ellenberg) value for light (linear mixed model, conditional R^2=0.88, marginal R^2=0.865, $p < 0.001$). Reaction (soil acidity) was also higher in ex-arable (linear mixed model, conditional R^2=0.812, marginal R^2=0.777, $p < 0.001$). Ex-arable woodland plants had higher nitrogen (soil fertility) preference values in years 2001–2003, similar values in 2007–2015, and again higher values in 2025 when compared to ancient woodland plant data from the same survey years (linear mixed model, conditional R^2=0.452, marginal R^2=0.348). Ellenberg moisture values did not differ between the woodland types, except in year 2002, where it was higher in ex-arable woodland (linear mixed model, conditional R^2=0.259, marginal R^2=0.199). For more details about these values, modelling, estimates and goodness of fit measures see Tables S6-S7.

Overall, ground flora Hill's Shannon diversity in ex-arable woodland was higher than in ancient woodland across all survey years (linear mixed model, conditional R^2=0.427, marginal R^2=0.359, $p < 0.05$), with a trend of decreasing difference over time (Table S7). In ancient woodland, non-woodland plants did not occur, and the levels of generalist and specialist plants remained at generally similar levels over time (Fig. 4). In ex-arable woodland, however, non-woodland plants were dominating in early years, but their numbers decreased over time, with no non-woodland plants recorded in 2024. Notably, three species of non-native invasive plants, *Buddleia davidii*, *Prunus laurocerasus* and *Cotoneaster* sp. were found in ex-arable woodland plots, but not in ancient woodland. Generalist plants remained at similar levels over time in ex-arable woodland. Woodland specialist plants did not occur in ex-arable plots over the span of 25-years.

Canopy openness had no effect on specialist richness (linear model, R^2=0.12, $p = 0.048$) or ground flora diversity (linear model, R^2=0.001,

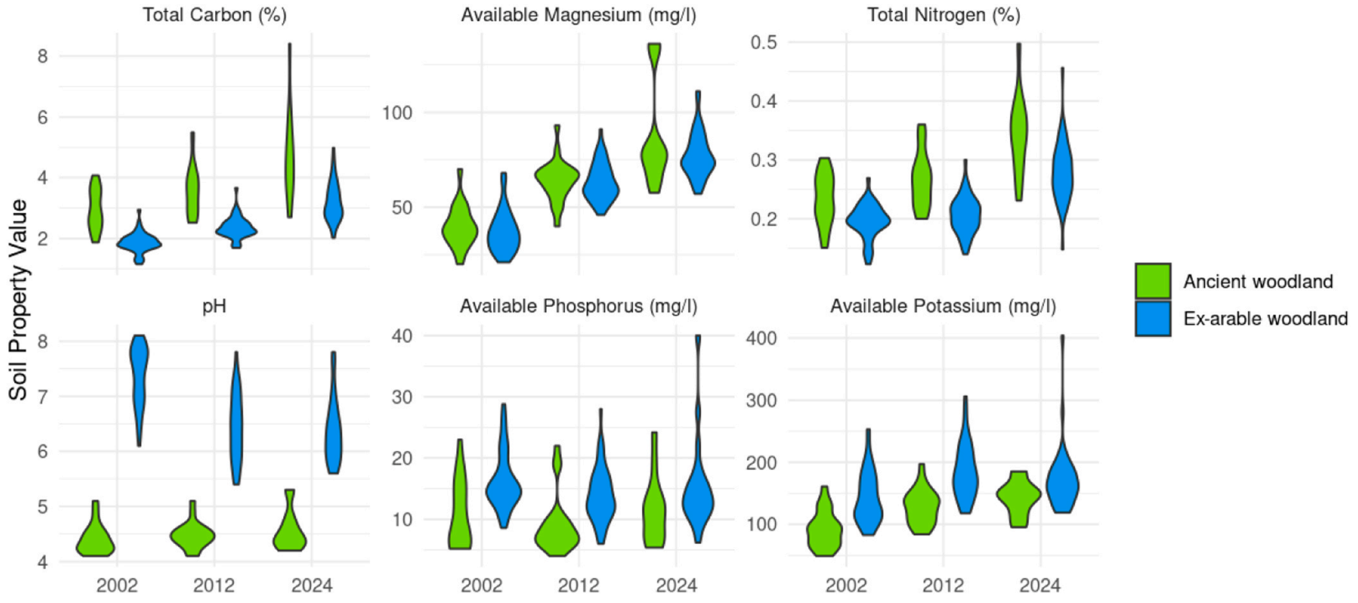

Fig. 2. Total carbon, available magnesium, total nitrogen, pH, available phosphorus and available potassium values of soil samples, collected in 2002, 2012, and 2024 across ex-arable (blue) and ancient (green) woodland observation plots.

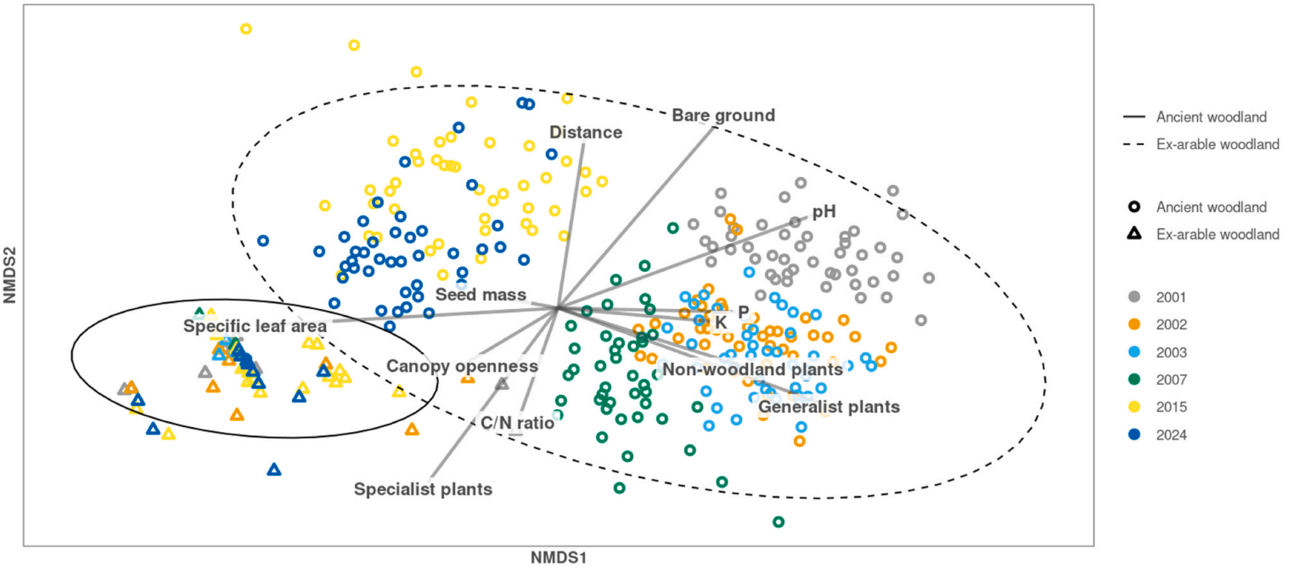

Fig. 3. NMDS plot showing Bray-Curtis dissimilarity of ground flora communities between woodland types and the biplot of environmental vectors. NMDS stress 0.14. All vectors, except seed mass and canopy openness, were significantly associated with NMDS axes.

$p = 0.83$). Of all tested abiotic (soil properties, canopy openness, distance) and biotic (specific leaf area, seed mass) explanatory variables, only C/N ratio (marginal $R^2=0.208$, conditional $R^2=0.072$, $p = 0.003$) and K (marginal $R^2=0.265$, conditional $R^2=0.078$, $p = 0.002$) had an effect on ground flora diversity in linear mixed models. Only the variables that did not significantly correlate with each other were included in the modelling (Figure S2). For more details about modelling, estimates and goodness of fit measures see Table S7. Effect estimates are plotted in the Figure S3.

Indicator species analysis (*sensu* a statistical test as described in the Methods section, rather than an ancient woodland indicator species as listed in Kirby et al., 2012) returned a list (Table S8) of sixteen ancient woodland indicator species: three ferns - bracken *Pteridium aquilinum*, male fern *Dryopteris filix-mas*, buckler fern *Dryopteris dilatata*, three woody species - elder *Sambucus nigra*, bramble *Rubus fruticosus* and sycamore *Acer pseudoplatanus*, and ten forb species, including commonly acknowledged ancient woodland specialists, such as yellow archangel *Lamiastrum galeobdolon*, bluebell *Hyacinthoides non-scripta*, and wood anemone *Anemone nemorosa*. These forbs are perennials, mostly geophytes, forming subterranean wintering buds. Ellenberg values of these ancient woodland indicators suggest that they are adapted to semi-shade, average dampness, acid to weakly acid soil, infertile to intermediately fertile soil conditions.

Forty-two indicators of ex-arable woodland comprised several typical grassland species, such as graminoids - rough meadow grass *Poa trivialis*, Yorkshire fog *Holcus lanatus*, common bent *Agrostis capillaris* and red fescue *Festuca rubra*, and forb species characteristic of nutrient rich open habitats, such as hogweed *Heracleum sphondylium*, creeping thistle *Cirsium arvense*, common sowthistle *Sonchus oleraceus* and white clover *Trifolium repens* These species are mostly perennial hemicryptophytes, forming wintering buds near the soil surface. Ellenberg values indicated that these plants are semi-shade to light loving, adapted to average

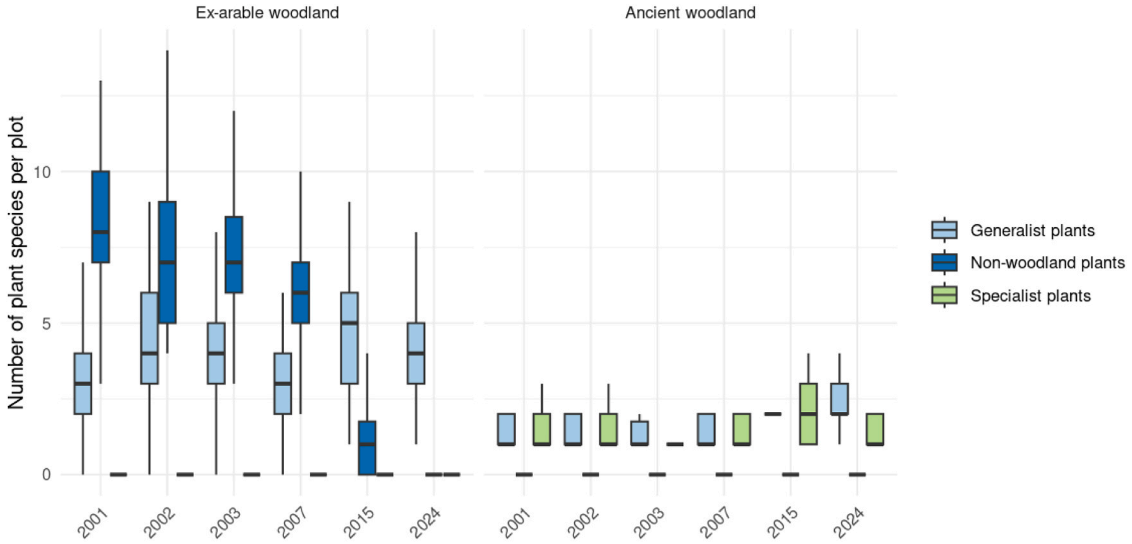

Fig. 4. Boxplot of the richness of generalist, specialist and non-woodland plant species, averaged per plot.

dampness to constantly damp sites, weakly acid to weakly basic and intermediately fertile to richly fertile soils.

3.3. *Fungi*

A list of 921 fungal OTUs was obtained from amplicon sequencing. 156 species were animal pathogens, 29 endophytes, 11 ericoid mycorrhizal fungi, 30 fungal parasites, 5 lichens, 1 lichen parasite, 121 plant pathogens, 1 protist parasite, and 371 saprotrophs. A total of 167 OTUs were ectomycorrhizal fungi. 29 OTUs assigned to various *Cortinarius* species were merged into one genus level entry due to phylogenetic challenges of splitting *Cortinarius* genus into species (Gallone et al., 2024) and shortcomings of molecular identification based on low ITS region variability of this genus (Janowski and Leski, 2023). Following this, a total of 139 OTUs of ectomycorrhizal fungi were obtained.

Ex-arable woodland plots had on average 7.63 more EMF species than ancient woodland plots (linear model, R^2=0.409, $p < 0.001$). Ancient and ex-arable woodland plots contained a higher proportion of ectomycorrhizal (birches *Betula* spp., pedunculate oak *Quercus robur* and hornbeam *Carpinus betulus*) than arbuscular mycorrhizal tree hosts (such as maples *Acer* spp. and ash *Fraxinus excelsior*) (Figure S4). Ex-arable woodland, however, had a higher proportion of ectomycorrhizal trees than ancient woodland (linear model, R^2=0.219, $p < 0.001$).

Ex-arable woodland had on average14.54 more saprotroph species (linear model, R^2=0.143, $p = 0.005$). Ex-arable woodland plots had higher richness of wood saprotrophs (linear model, R^2=0.09, $p = 0.02$, Figure S5). There was no difference in litter saprotroph richness (linear model, R^2=0.002, $p = 0.78$). For more details about these values, modelling, estimates and goodness of fit measures see Tables S11-S12.

PERMANOVA indicated a significant difference between ancient woodland and ex-arable woodland ($p = 0.001$) EMF communities, and the assumption of homogeneity of multivariate dispersions was met ($p = 0.23$). Woodland type explained 15 % of the variation.

Dissimilarity of plots across woodland types is visualised in the NMDS plot (Fig. 5). Ancient woodland EMF communities were associated with low soil pH, high C/N and EM host ratios, high canopy openness, and larger sporocarps.

Among abiotic variables tested, only pH had a significant effect on EMF richness (linear model, R^2=0.298, $p < 0.001$). Variables that did not correlate with each other were included in the modelling

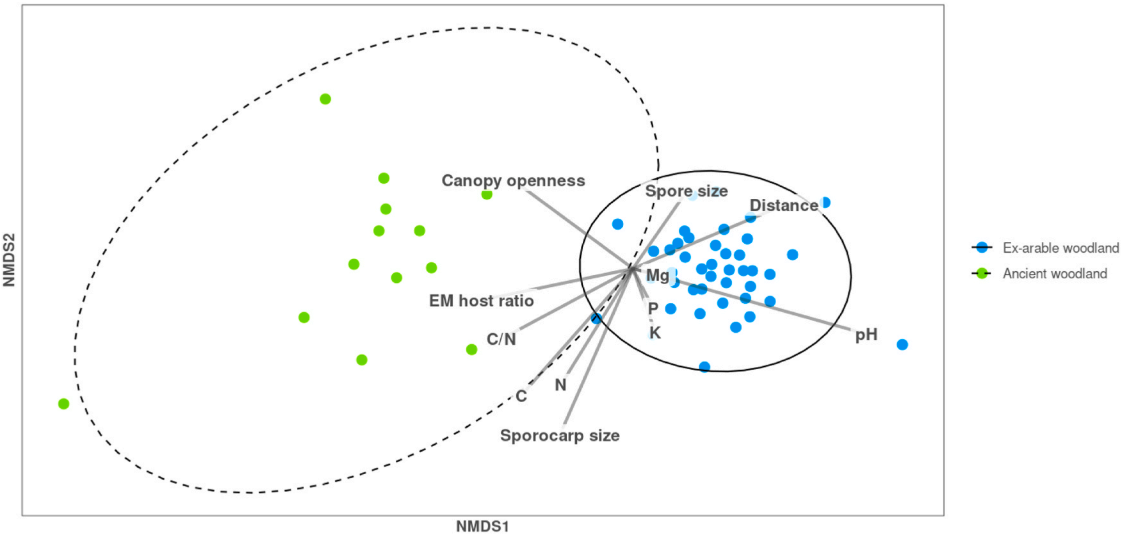

Fig. 5. NMDS plot of EMF community Jaccard dissimilarity, stress 0.13. All variables except potassium and magnesium were significantly associated with an NMDS ordination axis.

(Figure S6). Ground flora diversity had no effect on EMF richness, but tree species richness had a weak positive effect (linear model, $R^2=0.133$, $p = 006$). Effect estimates are plotted in the Figure S7. Distance was not a significant factor in explaining variation in average spore size or in average sporocarp size. Canopy openness had a weak negative effect (Figure S8) on the richness of saprotrophic fungi (negative binomial generalised linear model, deviance explained 11.9 %, $p = 0.006$). A generalised additive model was fitted to explore the relationship between saprotroph richness and EMF richness (deviance explained 40.6 %, $p < 0.001$). A positive asymptotic saturation trend was observed (Figure S9).

Indicator species analysis returned a list of ten ancient woodland indicator EMF species (Table S15). These species are typical in broadleaved and coniferous woodlands, with two species being oak specialists – *Russula ionochlora* and *Lactarius quietus* (Legon *et al*., 2005, Suz *et al*., 2014), and four - *Russula* sp., *Lactarius quietus*, *Russula ochroleuca* and *Hydnotrya tulasnei* - being nitrophilic generalists (Lilleskov et al., 2019).

Fourteen ex-arable woodland indicator EMF species are also typical of broadleaved woodlands (Table S16). Fungi in the *Hebeloma* genus were found to be nitrophobic generalists, while *Laccaria* spp. are nitrophilic generalists (Lilleskov et al., 2019). *Tricholoma scalpturatum* is mainly found with beech, lime and birch (Heap, 2020), *Tricholoma cingulatum* with willows (Hrynkiewicz et al., 2008), *Paxillus involutus* with birch (Blaudez et al., 2001) and *Laccaria tortilis* with alder (Legon et al., 2005).

4. Discussion

4.1. Ex-arable vs ancient woodland

In this woodland succession study, we found that ex-arable woodland ground flora and soil properties progressed towards communities and properties characteristic of ancient woodland over 25 years. However, a significant divergence in soil, ground flora, and ectomycorrhizal fungal communities was detected after more than two decades of woodland development.

Although we only studied one woodland, the temporal resolution of our experiment provides direct evidence, which is often substituted by indirect inferences from chronosequence experiments. Even though our study findings might have implications for providing evidence for woodland creation guidance only in South-East England, the underlying ecological processes studied here and the results of habitat succession observation can be applicable to other regions.

Under Biodiversity Net Gain, which is a habitat loss compensation scheme, applicable to land developments in England (Defra, 2024), developers are required to achieve a certain condition of their created habitats within a constrained period of time (usually within 30 years). Ground flora community is one of the 13 criteria used to determine the condition of woodlands, with the lowest score applicable to a non-recognisable woodland community, medium score to a recognisable woodland community, and a maximum score to a community strongly characterised by ancient woodland indicator species. We argue that based on the results of our experiment, as well as numerous other studies (Naaf and Kolk, 2015; Hughes et al., 2023; Waddell et al., 2024), the characteristic woodland ground flora community and ancient woodland indicator plants take more than a few decades to establish in newly created woodlands.

In our study, colonisation deficit of specialist ancient woodland plants was such, that they did not occur in ex-arable woodland plots 25-years following planting. Even though the ground flora community in ex-arable woodland showed a trend of increasing similarity to ancient woodland, the distinction was still significant. This is comparable to the results of Waddell *et al.*'s (2024) chronosequence study, which reported a difference of 2 specialist species between old (81–160 years) and young woodland creation sites (<30 years), suggesting that even though specialist flora colonisation is increasing over time, it is a slow process, potentially taking centuries.

The results of our study also inform ancient woodland compensatory schemes involving woodland creation adjacent to ancient woodlands. In addition to the temporal lag of specialist plant colonisation, our results indicate importance of previous land-use history, soil alteration practices and distance, which structure plant and fungal community composition. The following sections discuss these results in more detail.

4.2. Soil

In our study, ex-arable woodland pH decreased from an average circumneutral 7.4 (SD 0.52) in 2002 to slightly acidic 6.39 (SD 0.56) in 2024. Year 2024 levels remained significantly higher than in the ancient woodland, where average pH was 4.57 (SD 0.37). A similar trend of ex-arable soil acidification was reported by Harmer et al. (2001) in the Rothamsted long-term observation experiment, where pH of topsoil, sampled at 0–23 cm depth, decreased from 7 in 1886, to 6.1 in 1904 and to 4.2 in 1983. Panico et al. (2025) also observed a progressive pH reduction from 7.09 to 5.91 during ex-arable land afforestation over 70 years in their chronosequence study. This is also consistent with the global trend of acidification during afforestation (Berthrong et al., 2009). Change in soil pH during afforestation is associated with shifts in litter input and altered uptake of exchangeable cations by plants (Hong et al., 2018), which affects the balance of soil hydrogen ions (Binkley and Richter, 1987). A legacy from the addition of lime might also have played an important role in elevated pH of the studied ex-arable woodland.

Similarly to other studies (Deng et al., 2017; Panico et al., 2025), we have observed a reduction in available P within ex-arable land, even though it remained significantly higher than in ancient woodland. Significantly higher P levels are likely persisting due to a fertilisation legacy also observed in study Brudvig et al. (2013). Increasing plant biomass during afforestation might lead to a decrease of available P pools (Vitousek et al., 2010), however shifts in microbial community, i. e. an increase in ectomycorrhizal fungal diversity, can lead to higher P solubilisation (Köhler et al., 2018) and availability. A global meta-analysis of P dynamics during afforestation by Deng et al. (2017) suggested that site specific characteristics, such as climate, tree species planted, soil texture and pH, and stand age, has an effect on total and available P shifts.

Topsoil total C levels reflect both inorganic and organic C, with the former being less significant in the temperate climate zone (Sharififar et al., 2023), hence total C in this study was used as a proxy for soil organic C. Meta-analyses of C stocks during afforestation (Laganiere et al., 2010; Li et al., 2012) showed an initial decrease in soil C following the land use change and a constant increase afterwards. Temporal resolution of our soil sampling was insufficient to capture this, and we observed a consistent increase in total C and N stocks in both woodland types. Total soil C increased by 64.5 % from year 2002 to 2024, which is comparable to the findings of meta-analysis by Guo and Gifford (2002), who found a 53 % increase in soil C from crop to secondary forest. C/N ratio in ex-arable woodland increased over time, as observed by Panico et al. (2025) and in contrast to the findings of Berthrong et al. (2009). Unexpectedly, total C and N concentrations in our studied ancient woodland plots increased significantly over time. This also contrasts with the findings of Peltoniemi et al. (2004), who showed soil C accumulation saturation in woodlands over 50 years of age. Ancient woodland would be expected to have achieved its soil carbon carrying capacity (Guo and Gifford, 2002) at the time of this study, given that woodland land use was continuous in our ancient woodland plots since at least 1600. This might suggest that the observed increase was due to methodological differences of soil analysis, however, the same laboratory and the same protocol was used during all soil sampling and analysis occasions.

4.3. Plants

Multivariate analyses suggested a significant distinction of ex-arable and ancient woodland ground flora communities that persisted over the 25-year study period. This is consistent with the findings of Morel et al. (2020), who also observed a clear distinction in woodlands of > 230 years old compared to ex-arable woodlands of < 120 years old and Hughes et al. (2023), who found distinction between 50–80 years old and 15–21 years old woodlands. However, in our study ex-arable woodland plant communities started to resemble ancient woodland communities over time. Ex-arable woodland plots sampled in the early years of the experiment (2001–2007) were different from later years (2015–2024), the latter being more similar to the ancient woodland plots.

Distance from the ancient woodland, specific leaf area and seed mass significantly contributed to plant community clustering. Specific leaf area represents plant adaptation to light and moisture (Knösche, 2025), and ancient woodland plant community association with higher specific leaf area suggests they are adapted to more shaded and moist conditions. This was also supported by the Ellenberg values: ground flora in the ancient woodland indicated higher shade tolerance. The actual measured light availability, however, was higher in ancient woodland. This might suggest that ex-arable woodland plant community is more light loving in relation to being more ruderal and opportunistic. Similarly, Kirby et al. (2012) found that non-woodland species had on average 46 % higher Ellenberg light value and were more stress-tolerant and ruderal than woodland specialists.

The association between ancient woodland ground flora community and higher seed mass might suggest that, if wind-dispersed, larger seeds are less capable to travel longer distances. Species with low seed mass were shown to travel further and establish in more recent woodlands (Brunet et al., 2021), while woodland specialist ground layer plants were found to be limited to short distance dispersal and hence to the source habitat (Kirby et al., 2012). Ancient woodland plant communities were also associated with acidic soils and lower available P, as in the study of Thomaes et al. (2014), who showed that pH and P are limiting factors of typical ancient woodland plant communities of Western Europe.

Distance, soil C/N ratio and K were the main drivers of plant functional and taxonomic diversity. Higher C/N ratio was associated with higher number of specialists and plants with larger seeds, and with a lower overall ground flora diversity. The relationship between C/N ratio and plant diversity can be reciprocal and diversity has been shown to have a positive effect on C/N ratio through plant biomass input into soil (Spohn et al., 2023).

Overall, ground flora diversity remained significantly higher in ex-arable woodland, as in Barker et al. (2019) and Morel et al. (2020). Our results show that over 25 years of afforestation, the ex-arable woodland plant community shifted from dominant non-woodland plants to a plant community of generalist plants, with a persisting absence of specialist woodland plants. Waddell et al. (2024) also observed a slow colonisation by woodland specialist ground flora and attributed this to life history and dispersal traits. Ex-arable woodlands were dominated by non-woodland and generalist plants, adapted to average dampness, neutral and fertile soils, which was also observed by Waddell et al. (2024).

4.4. Fungi

Ex-arable woodland had higher richness of saprotrophic and ectomycorrhizal fungi than ancient woodland, in contrast to Panico *et al.*'s (2025) findings. It also had on average higher richness of wood saprotrophic fungi than ancient woodland plots, but we found no difference in litter saprotroph richness. This is similar to the findings of Tervonen et al. (2022), who found a positive relationship between time since agricultural land-use abandonment and saprotroph richness in wood-pastures. Our results demonstrated that saprotroph richness was negatively associated with canopy openness. Schreiber et al. (2024) also found a decreasing diversity of wood-inhabiting fungi under open vs closed canopy.

In our study, ancient woodland EMF communities were associated with low soil pH, high C/N ratio, canopy openness, increasing ratio of EM host trees, and larger EMF sporocarp size. The effects of host trees (Guy et al., 2022), soil pH (van der Linde et al., 2018), C/N ratio (Soudzilovskaia et al., 2015), and light availability (Turner et al., 2009) in affecting EMF communities are well documented. However, there are few reports examining how EMF communities are shaped across the gradients of EMF dispersal traits. A study by Peay et al. (2012) showed that EMF species with smaller spores, formed in larger sporocarps, travel further over large landscape-scales. Spore size of fungi has been shown to be negatively associated with distance of aerial dispersal (Norros et al., 2014; Chaudhary et al., 2020). However, in our study larger spores were associated with increasing distance from ancient woodland. Larger EMF spores, having more energy resources for colonisation, have higher chances for successful establishment in resource-depleted or patchy environments (Halbwachs et al., 2017), which in our study might be related to a lower rate of total carbon in ex-arable woodland soil. We also found that larger sporocarps were characteristic to ancient woodland EMF. However, due to a lack of ecological trait information for our studied species in the FungalTraits database, we avoided any further inferences associated with sporocarp and spore size. A systematic literature review to compile fungal traits for more species is necessary for such analyses.

In our study, only pH and host tree richness influenced EMF richness. Ex-arable woodland had on average more ectomycorrhizal tree species than ancient woodland and hence provided a higher range of opportunities for ectomycorrhizal symbionts. With introductions of lime, birch, and beech into the plantation area, specialist EMF, such as *Tricholoma scalpturatum* and *Paxillus involutus* forming associations with these trees, emerged. Ex-arable woodland EMF communities were also comprised by genera described as putatively dispersed and early successional by Collier and Bidartondo (2009): *Hebeloma*, *Inocybe*, *Laccaria*, and *Paxillus* spp. In contrast, ancient woodland EMF indicator species observed in this study, including *Russula* and *Lactarius* spp., are often classified as late successional (Deacon and Fleming, 1992; Nara et al., 2003; Collier and Bidartondo, 2009). Indicator species analysis also suggested that in ancient woodland all indicator EMF species, for which data was available, were nitrophilic, which corresponded with higher total nitrogen levels in ancient woodland soil.

EMF and saprotroph richness was positively associated, suggesting that environmental factors, shaping richness of one guild, had a similar effect on the other, but this trend can vary with geographic regions (Shigyo and Hirao, 2021).

5. Conclusions

In this direct-observation study, we tracked how soil properties and ground flora developed over time and added new evidence for the temporal lag associated with afforesting arable land. As anticipated, soil characteristics and both plant and fungal communities in ex-arable woodland remained distinct from those in ancient woodland 25-years after planting. Nonetheless, several properties - such as pH, available P, available K, and overall plant diversity - showed signs of gradual convergence. Despite this, a substantial colonisation deficit of specialist plant species persisted. Distance from ancient woodland, legacy effects of agricultural soil modification, and species life-history traits all contributed to the continued divergence between ex-arable and ancient woodland communities.

Our findings deepen understanding of how species and environmental factors interact during afforestation. To our knowledge, this is the first direct-observation study of arable land afforestation to integrate plants, fungi, and soil properties. The insights gained can help inform the selection of woodland restoration sites and improve projections of woodland development, particularly where an accelerated transition toward ancient woodland conditions is a management objective.

CRediT authorship contribution statement

Sam Pettman: Writing – review & editing, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization. **John Crawford:** Writing – review & editing, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization. **Petra Guy:** Writing – review & editing, Methodology. **Nadia Barsoum:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization. **Julija Fediajevaite:** Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis. **Brian J. Pickles:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Mark Tibbett:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Aaron Philips:** Investigation. **John Tucker:** Methodology, Data curation, Conceptualization. **Peter Buckley:** Methodology, Data curation, Conceptualization. **Clive Steward:** Writing – review & editing, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Funding

Government's Nature for Climate Fund, Forest Research, Woodland Trust.

Declaration of Competing Interest

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

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.foreco.2026.123640.

Data availability

Data repository link in SI

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