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Root ectomycorrhizal status of oak trees symptomatic and asymptomatic for Acute Oak Decline in southern Britain

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Abstract

Acute Oak Decline (AOD) is a decline-disease that has distinctive symptoms and poses a serious threat to oak. Our understanding of the causal factors of AOD remains poor but it is likely that multiple biotic and abiotic factors contribute to a deterioration in oak condition. There is evidence that indications of above-ground tree health status are frequently reflected below-ground in roots and associated ectomycorrhizal (ECM) fungal communities. However, no study has yet explored these potential relationships specifically in AOD affected trees. In this study, we compare the composition and range of functional exploration types of ECM communities associated with AOD symptomatic oak trees and with AOD asymptomatic trees in three oak-dominated woodlands in southern England. We additionally assess the abundance of fine roots tips in surface soils beneath AOD symptomatic and asymptomatic trees and consider soil physico-chemical effects on ECM communities. The frequency of fine root tips was found to be significantly higher on asymptomatic compared with symptomatic trees in two of the three woodlands studied and long-distance ECM exploration types had a weak positive association with AOD asymptomatic trees. ECM diversity and composition were, however, unaffected by tree symptom status and were not related to the frequency of fine root tips. ECM diversity and compositional (but not exploration type) differences were evident only between the different woodlands and this was related to a small number of soil chemistry variables. This study revealed a relationship between the above-ground symptoms of AOD (i.e. stem lesions and *Agrilus biguttatus* exit holes) and the frequency of live root tips, providing a potential additional diagnostic tool of trees in decline and highlighting the importance of considering belowground rhizosphere microbiome communities.

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Key-words: Acute Oak Decline; ectomycorrhizal fungi; Quercus; exploration types; fine roots

1. Introduction

Oak decline has been reported for more than 250 years in Europe (Thomas et al., 2002, Thomas, 2008). Since the 1980's Acute Oak Decline (AOD) has emerged as a new disorder within the wider oak decline complex, causing rapid decline in tree condition over three to five years and with distinct symptoms that have been described among afflicted trees in Britain (Denman et al., 2014, Brown et al., 2016). The symptoms include weeping stem patches, black exudate emanating from longitudinal splits between bark plates and frequently, at late stages of decline, larval galleries and exit holes on the bark formed by the buprestid beetle, *Agrilus biguttatus* (Denman and Webber, 2009, Denman et al., 2014, Brown et al., 2015, 2016). A deterioration in crown condition can also occur, although this is not considered to be a reliable symptom of AOD (Denman et al., 2014). AOD mainly affects mature trees (DBH of 35-80cm) but can also occur in young oaks and has caused much concern amongst woodland owners due to the threat to oak tree vigour and survival, particularly among native oaks (i.e. *Quercus robur* and *Q. petraea*) (Denman and Webber, 2009). The incidence of AOD-affected woodland is increasing and currently occurs in southern and central England, extending also into Wales (Brown et al., 2018).

No single causal agent of AOD has been identified and current thinking is that as a 'decline-disease', AOD is caused by multiple abiotic and biotic variables that act together in combination, cumulatively and/or sequentially to weaken the tree (Denman et al., 2018). The bacterial species associated with AOD weeping stem lesions (i.e. *Gibbsiella quercinecans*, *Rahnella victoriana* and, particularly, *Brenneria goodwinii*,) are found in much reduced abundances in apparently healthy trees and so it is unclear whether this is a case of a weakened tree becoming more susceptible to bacterial infection or bacteria causing AOD (Broberg et al., 2018, Denman et al., 2018). The role of the *Agrilus* beetle is similarly unclear; i.e. it is not

known whether their association with stem bleeds implicates them as a disease vector, or if they are simply opportunists, taking advantage of weakened trees (Reed et al., 2018). Recent research indicates that the distribution of trees with AOD correlates significantly with sites in Britain that have high dry nitrogen (N) deposition, low rainfall, low elevation and warm temperatures (Brown et al., 2018). However, at the woodland stand scale, oak trees displaying AOD symptoms have been found to co-occur with apparently healthy 'asymptomatic' trees and in some cases, show clear signs of remission (Brown et al., 2016). The distribution of AOD symptomatic trees in woodlands has, nevertheless, been found to cluster at small scales (<40m) in among apparently unaffected trees. This suggests that, as a possible interaction with regional agents of stress (e.g. high temperatures, high N-loading), there may be a localised cause such as: (i) a pest or pathogen that mainly afflicts competitively supressed trees in dense stands (Zhou et al., 1997, Jones et al., 2003, Mosca et al., 2007, Brown et al., 2016), (ii) genetic factors influencing susceptibility to AOD (Harper et al., 2016) and/or (iii) highly localised differences in soil conditions and the wider rhizosphere microbiome that together might predispose individual or small clusters of trees to AOD.

In this study we investigate the belowground rhizosphere microbiome of oak trees and how it relates to local soil conditions under trees showing signs of AOD compared with nearby AOD asymptomatic trees (i.e. without detectable lesions). More specifically, we explore how the presence of lesions on AOD symptomatic trees influences ectomycorrhizal (ECM) fungal communities colonising oak fine roots. ECMs are ancient fungal-plant mutualisms that play important roles in tree growth and health. Comprised of hyphae that, glove-like, cover tree roots, ECMs provide protection against pathogens (Marx, 1972, Sinclair et al., 1982, Duchesne et al., 1988 a, b, Branzanti et al., 1999, Lambers et al., 2018) and drought (Parke et al., 1983, Futai et al., 2008, Smith and Read, 2010), whilst also gathering soil nutrients and water for their tree hosts in exchange for photosynthetic carbon (Read, 1986, Hobbie and Hobbie, 2006).

Many ECM species have evolved functional traits that further enhance soil exploration and resource acquisition capabilities in their host tree. Among these are medium- and long-distance exploration types that send out filamentous hyphae from tree roots. As these hyphae are thinner, longer and able to produce a wider array of enzymes than tree fine roots, they greatly increase the efficiency of resource capture (Hobbie and Agerer, 2010).

The position of ECMs at the interface between the soil and tree roots results in a sensitivity not only to changes in soil chemistry, but also to carbon allocation from the tree host to the ECM. Any impacts on the ECM community are therefore also likely to have a knock-on impact on tree performance. Conversely, a tree in poor condition is likely to have an impact on the ECM community associated with its roots (Kuikka et al., 2003, Swaty et al., 2004, Pena et al., 2010, Treu et al., 2014), potentially serving as an early warning of host stress (Scattolin et al., 2012). Factors that are known to significantly affect ECM communities include high levels of N deposition, low soil pH, available P, K and soil organic deposition rates measured as C:N ratios (Tedersoo et al, 2014, Maghnia et al., 2017, Lilleskov et al., 2019). In high N environments, for example, ECM species richness will tend to decline and the functional traits (e.g. exploration strategies) of species that tolerate high N can differ significantly from those of ECMs present in low N conditions (Cox et al., 2010, Jarvis et al., 2013, Suz et al., 2014, Lilleskov et al., 2019). Such trends have been observed over short distances (e.g. 10m), such as along N gradients from the woodland edge to the woodland interior (Kjøller et al., 2012).

Several studies have explored the composition of ECM communities on oak trees that are displaying clear signs of decline compared with apparently healthy oak trees (Kovacs et al., 2000, Montecchio et al., 2004, Lancelloti and Franceschini, 2013, Corcobado et al., 2014). Most of these studies have been conducted along a gradient of tree decline frequently defined on the basis of levels of ‘crown transparency’, or ‘defoliation’, but tree decline status has also

been assigned based on the detection of cancer and the presence of pathogens in the crown, bark or roots (e.g. Corcobado et al., 2014). The majority of these studies have also relied on the morphological identification of ECMs, with only a small proportion adopting molecular genetic techniques as a recognised method that can facilitate the detection and accurate identification of a wider array of ECM genera and species (Peay et al., 2008). These studies generally report that healthy trees either have similar or significantly greater proportions of fine roots colonised by ECMs than trees showing signs of decline (Przybyl and Pukacka, 1995, Kovacs et al., 2000). ECM species diversity and the abundance of certain ECM species (e.g. *Lactarius chrysorrheus*, *Cenococcum geophilum*) have also tended to be higher on healthy trees (Kovacs et al., 2000, Montecchio et al., 2004, Lancelloti and Franceschini, 2013, Corcobado et al., 2014). Where the abundance of fine roots has additionally been assessed, fine root abundance has been found to either be significantly higher (Corcobado et al., 2014), or significantly lower (Bzdyk et al., 2019) in healthy oak trees compared with trees in decline.

The primary aims of this study were twofold. First, using morphological and molecular genetic identification techniques, we sought to describe the taxonomic and functional composition of ECM communities associated specifically with AOD symptomatic oak trees compared with nearby oak trees showing no symptoms of AOD in three oak dominated woodlands. This involved (i) exploring the potential to identify ECM species, families and/or functional types that are indicators of AOD symptomatic trees, (ii) quantifying the relative abundance of live roots tips available for ECM colonisation on AOD symptomatic and asymptomatic trees and (iii) utilising the currently accepted defining features of trees with AOD symptoms (i.e. stem or bark plate bleeds, *Agrilus* adult exit holes), rather than other criteria used to distinguish oak trees in decline, such as high levels of canopy transparency, or defoliation. Second, we aimed to assess the responses of ECM communities to any variations in soil chemistry (particularly soil pH, and levels of soil N, K, P and C:N ratios) within and between the three woodland

146 locations. We predicted that AOD aymptomatic trees would have a higher abundance of fine
147 roots than AOD symptomatic trees. This would be consistent with numerous studies comparing
148 fine root responses in declining and healthy trees of various tree species (Bauce and Allen,
149 1992, Blaschke, 1994, Power and Ashmore, 1996, Nechwatal and Oβwald, 2008, Corcobado
150 et al., 2014). We expected that a greater availability of colonisable fine roots on AOD
151 asymptomatic tree fine roots would be reflected by greater ECM species richness, diversity and
152 abundance compared with AOD symptomatic trees. In addition, we expected AOD
153 asymptomatic trees to recruit more often ECM exploration types that are thought to have higher
154 plant carbon demands for mycelial growth (e.g. medium fringe and long-distance exploration
155 types) (Lilleskov et al., 2019, Veselá et al 2019). This is based on the assumption that
156 belowground plant C allocation would be greater in AOD asymptomatic trees compared with
157 symptomatic trees of declining health, leading to altered ECM species composition (Saikkonen
158 et al., 1999). Furthermore, we predicted that ECM community composition would be sensitive
159 to levels of soil pH, N, K, P and/or C:N ratios and that any variation in soil chemical properties
160 between symptomatic and asymptomatic trees would be related to ECM community
161 composition responses to tree symptom status. For example, we expected lower ECM species
162 richness and fewer ECMs with long-distance exploration strategies on symptomatic trees
163 present at woodland locations with comparatively high soil N or low soil pH.

164 **2. Material and methods**

165 2.1 Study locations

166 Three oak-dominant woodlands known to have cases of AOD were selected for study in
167 southern England. These were Monks Wood (52˚41'N, 0˚ 23'W), Stratfield Brake (51˚ 80'N,
168 1˚ 28'W) and Writtle Forest (51˚ 70'N, 0˚ 35'E). The three woodlands are at similar
169 developmental stages and experience similar climatic conditions, although at 28m a.s.l.

Monks Wood is at a lower elevation than Stratfield Brake (69 m a.s.l.) and Writtle (90 m a.s.l.) (see Table A.1 in Supplementary material). Soils at Monks Wood are also comparatively fine-textured with higher silt and clay content than the other two woodlands.

At each woodland location, ten trees that showed symptoms of AOD and ten trees that were asymptomatic for AOD were selected for sampling. The selected symptomatic and asymptomatic trees were evenly distributed across each of the three woodlands (Figure 1). AOD symptomatic trees were identified on the basis of the presence of bleeding cracks between bark plates and in many, but not all trees, *Agrilus biguttatus* exit holes on the tree bark (Denman et al., 2014). Crown density of all sample trees was additionally assessed in 5% classes where 0% was equivalent to a fully foliated tree crown. AOD symptomatic and asymptomatic trees selected for sampling were found to have similar average crown density (Table A.1). Sampled trees at Monks Wood and Stratfield Brake had comparable average crown density, while sample trees in Writtle had considerably lower average crown density than the other two woodland locations. We also assessed the average basal area of trees in the vicinity of sample trees and percentage shrub cover around sample trees; these were both found to be similar between symptomatic and asymptomatic trees and between the three woodland locations. With the exception of five trees identified as *Quercus petraea* in Writtle Forest, the trees selected at all three woodland locations were identified as *Quercus robur*.

2.2 Data collection

2.2.1 ECM surveys

In each of the oak woodlands, four soil cores were collected in the four cardinal directions around the perimeter of each of the 10 symptomatic and 10 asymptomatic trees to yield 80 soil cores per woodland location. This sampling intensity was adopted based on previous

sampling of ECMs in oak forests in southern England (Suz et al., 2014, Spake et al., 2016). Soil cores were collected using a 2 cm diameter x 25 cm depth soil auger at distances no greater than 0.5 m from the trunk of each tree. The soil auger was cleaned between each soil core to avoid cross-sample contamination. Soil cores were moistened slightly with distilled water to prevent desiccation of roots and transported in sealed plastic bags in cool boxes to the laboratory where they were stored at 4°C for up to seven days. All soil cores were collected from each woodland location on a single day. Sampling dates for each woodland location were 8/10/2018 for Writtle, 18/10/2018 for Monks Wood and 6/11/2018 for Stratfield Brake.

In the laboratory each soil core sample was gently rinsed with water in a clean 0.5 mm sieve to separate soil particles and organic material from roots. To reduce sample bias and maximise sample independence, three of the longest live ectomycorrhizal oak fine roots (<2mm diameter) were removed during a five-minute timed search per sample using a binocular microscope (10-40X) following Cox et al. (2010). Taking each ECM root in turn, a single live ECM tip was removed, and its morphology (colour, ramification, shape and mantle surface) described; photographs were taken for each morphologically distinct morphotype. The ECM root tip was then placed in ethanol in a labelled Eppendorf tube for molecular identification. Using the remaining soil core root samples, the number of live oak root tips (<2mm diameter) were counted using a binocular microscope and their dry weight recorded. Oak roots were identified based on their morphological characteristics such as surface structure, colour of the periderm and ramification pattern (Meinen, 2008, Rewald et al., 2012). Live roots were distinguished from dead roots largely on the basis of their turgidity and intact appearance. Grass and herb roots were distinguished from tree roots by their smaller diameter, non-lignified structure and lighter colour.

Out of a total of 720 possible samples (three fine roots x four soil cores x a total of 60 trees at three woodland locations), 33% (234) of the samples had either no visible ECM fungi on roots (9%) or no roots available for sampling (23%). This occurred most frequently among the asymptomatic trees at Monks Wood where 37% of samples had no fungi or roots to sample. This compared with between 12% and 22% of samples with no roots or fungi to sample among the sample trees at Writtle and Stratfield Brake, respectively (Table 1). There were only two soil cores that had no roots and fungi to sample; these were a soil core under an AOD symptomatic tree at Writtle and a soil core under an AOD asymptomatic tree at Monks Wood.

2.2.2 Molecular identification of ECMs and categorisation into exploration types

ECMs were air-dried prior to extraction. Fungal DNA was extracted using the Extract-N-AmpTM Plant PCR kit (Sigma-Aldrich, St.Louis, USA). ECMs were incubated at 95˚ C for 10 minutes in 10µl of extraction solution and subsequently diluted in 10µl of dilution solution (Extract-N-Amp™ Plant PCR Kit, Sigma-Aldrich, St. Louis, MO, USA). 1µl of a 1:10 sterile distilled water dilution of this mix was used as the DNA template in a 20µl PCR reaction using primers ITS1-F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990).

DNA was amplified using the following reaction mixture: 1X NH PCR buffer (pH 8.8, 0.1% Tween 20, 20mM $MgCl_2$ (Bioron, Germany), 4µM of each primer, 0.2mM of each dNTP and 0.25U SuperHot Taq DNA polymerase (Bioron, Germany). PCR was carried out using the following program: initial denaturation of 94˚C for 2 minutes, followed by 35 cycles of denaturation at 94˚ C for 30 seconds, primer annealing at 53˚C for 55 seconds and elongation at 72˚C for 50 seconds. The cycle was finished with a final elongation step of 7 minutes at 72˚C. PCR products were checked on a 1.4% agarose gel and samples that produced a band

were cleaned up with EXOSAP-IT (Affymetrix Inc., Santa Clara, USA) following the manufacturer's protocol and then sent for sequencing at Edinburgh Genomics. The sequences were edited and trimmed using Sequencher v5.4 (Gene Codes Corporation, USA). All sequence chromatograms were visually checked prior to inclusion in the analysis to ensure accuracy of calls e.g. bases masked by dye peaks and corrected manually where necessary. The edited fungal sequences were identified using the Basic Local Alignment Search Tool (BLAST) against the National Centre for Biotechnology Information (NCBI) GenBank public sequence database. Fungal sequences ranged from 129 bp – 853 bp in length, with mean, median and mode lengths of 520 bp, 566 bp and 656 bp, respectively. We assigned species or genus names to each morphotype where pairwise identity (i.e. the amount of nucleotide that matches exactly between two different sequences) was equal to or higher than 96%. Most sequences (84%) returned an identical (100%) match. 12% of samples produced a similarity match of 98-99% and a further 3% of samples had a similarity match of 96-97%. To confirm the sequencing matches, morphotype characteristics were additionally compared to reference photos (where these were available) on the DEEMY database (Agerer and Rambold, 2020) and, to a lesser extent, the Ectomycorrhizae Descriptions Database (BCERN, 2020). A total of 69% of the ECM root tip samples could be identified to species or genus levels using molecular and morphological identification. The remaining 31% of the ECM root tip samples could not be identified morphologically, yielded no PCR result or did not generate sufficiently high-quality sequences to be used to match against library sequences. In 37 cases, the fungi sampled on roots were removed from the dataset because they were found to be saprotrophic fungi (e.g. Trichocomaceae) or fungi not proven to be ectomycorrhizal including several members of the Ascomycota such as Eurotiales (1), Heliotiales (6), Leotiomycetes (1), Pezizales (2) and Pezizomycetes (3). Samples of this kind were evenly distributed across all woodland locations and among symptomatic and asymptomatic trees,

making up no more than 19% of ectomycorrhizal root tip samples (Table 1). Once these non-ECM fungi had been removed from the dataset, only two remained that had a sequence similarity match of 97% and four had a sequence similarity match of 98%. The identity of these six morphotypes could be confirmed by morphological identification to species or genus level.

Mycorrhizal exploration types were assigned to each identified taxon following Agerer (2001, 2006), Suz et al. (2014) and the DEEMY database (Agerer and Rambold, 2020). They were further classified as low biomass (contact, short- and medium-distance smooth exploration types) and high biomass (medium-distance fringe, medium distance mat and long-distance exploration) based on Hobbie and Agerer (2010).

2.2.3 Soil assessments

For each of the ten symptomatic and ten asymptomatic trees selected per woodland location, four soil samples (40-50 g field moist) were collected using a soil auger at a depth of 5-15 cm from the four cardinal directions at 1.5-2.0 m away from the tree trunk. Soil samples were subsequently pooled to produce one composite sample per tree. Pooled soil samples were homogenized by sieving (<2 mm) and then air-dried prior to analysis of soil physico-chemical properties. Soil particle size distribution was determined using a Malvern Mastersizer 3000 hydro laser granulometer: i.e. samples were dispersed in 3.3% (m/v) $Na_6O_{18}P_6$ and 0.7% (m/v) Na_2CO_3 and measured in blue and red light and data reported using a Fraunhofer size distribution model. Soil pH was determined in deionised water (soil: water, 1: 2.5 (m/v)). Total C and N contents were determined on ground samples (Pulverisette 5 Planetary Mill) using a FLASH CN elemental analyser (ThermoFisher Scientific). Extractable phosphorus was determined by the Olsen method (Olsen et al., 1954). For

291 determination of exchangeable cations (K^+ and Al^{3+}), soil samples (2.5 g) were extracted with
292 3 x 30 ml 0.1M $BaCl_2$ and pooled extracts (made up to 100 ml) analysed using a Perkin
293 Elmer 3000 ICP-OES fitted with a cross flow nebuliser (Cools and De Vos, 2016). Technical
294 replication was included for the analysis as follows: particle size distribution (5), pH (3), total
295 C and N (2), Olsen P (2) and exchangeable cations (2). In the case of $BaCl_2$ extractions for
296 Al^{3+}, a low percentage of samples recorded concentrations in the extract below the detection
297 limit (2.55 µg L^{-1} $BaCl_2$ extract) for the analysis. Where this was the case, concentrations
298 were entered into calculations as half the detection limit.

299

300 2.3 ECM data preparation

301 Raw data were pooled at two levels: 1) tree AOD symptom status (i.e. ten asymptomatic or
302 ten symptomatic trees combined at each woodland location) and 2) individual tree level. At
303 the tree AOD symptom status level, an ECM species occurring on a sample tree was
304 attributed a score value of 1, with all non-occurring species scoring a zero (0,1 matrix).
305 Therefore, at the tree symptom status level, the maximum count for any one ECM species
306 was 10. At the individual tree level, a single ECM species could score a maximum count of
307 12 occurrences if present in all samples (three roots x four positions around each tree). As
308 well as establishing tree and tree symptom status level matrices for ECM 'Species', this same
309 process was repeated considering ECMs grouped by 'Family' or by 'Exploration Type',
310 resulting in a total of six data matrices.

311

312 2.4 Data analysis

313 All analyses were conducted in R version 3.5.1 (R Core Team, 2018), with graphics produced
314 using ggplot2 in R (Wickham, 2016). To assess how well the sampling intensity captured the

diversity of ECM species present, species accumulation curves at the three woodland locations were estimated using the specaccum() function in vegan() package in R. The effects of tree AOD symptom status and woodland location effects on ECM assemblages were undertaken by fitting multivariate general linear models (GLiMs) to the datasets at the individual tree level using the R package mvabund (Wang et al., 2018), with Poisson errors and log link function (residual plots confirmed a reasonable fit of this model structure). Along with the interaction of woodland location and tree symptom status, the following soil measurements were included in the multivariate GLiMs: pH, C:N ratio, Total N (%), Olsen's P ($mgkg^{-1}$), exchangeable K content ($mg\ kg^{-1}$) and exchangeable Al content ($mg\ kg^{-1}$). Any ECM species, families or exploration types with only a single occurrence were removed from the data sets prior to analysis to aid model fit. Analysis of deviance was conducted on each factor, with pit-trap resampling, 999 iterations and score tests used to determine the significance of woodland location, tree symptom status and soil measurements.

To examine tree-level ECM species/families/exploration type assemblages, a Bray-Curtis dissimilarity matrix was calculated for each data set, using the vegan package in R (Oksanen et al., 2019). Nonmetric Multidimensional Scaling (NMDS), using 1,000 random starts, were performed on the Bray-Curtis dissimilarity matrices, with appropriate numbers of dimensions determined based on stress levels. Stress plots were used to determine goodness-of-fit, and the first two axes were plotted to visualise the data set. Ordination spider plots were colour-coded by significant factors, and ordination spiders plotted (using mean ordination points per woodland location/tree symptom status) to visualise factors.

Soil physico-chemical characteristics were compared among symptomatic and asymptomatic trees at each woodland location and between woodland locations using analysis of variance with robust standard errors using Box-Cox transformed data. Where analysis of variance indicated an overall main effect of woodland location on soil physico-chemical properties,

Games-Howell post-hoc testing was used for pairwise comparisons between means for woodland location across tree symptom status. At the tree level, one-way analysis of variance and Tukey tests were used to test for significant differences in ECM species richness, diversity (Shannon-Weaver, 1949) and the root characteristics (dry weight, number of root tips) of AOD symptomatic and asymptomatic trees at each woodland location. Root datasets were log-transformed prior to analyses.

3. Results

3.1 ECM species abundance, richness and community composition across woodland locations

A total of 90 ECM species belonging to 26 genera and 18 families were identified across all woodland locations. 45 of the ECM root tip samples were identified to species level and the remaining 45 to genus level. At all three woodland locations evenness of the ECM communities was low and species abundance followed a Zipf distribution, indicating that communities had a few species that were very abundant and a long tail of rare species (See Figure A.1 in Supplementary material). Species accumulation curves for each woodland indicate an adequate sampling intensity to capture ECM diversity present (Figure A.2). The average species richness across all three woodland locations was 33 with the lowest number of species recovered at Stratfield Brake (17) and the highest number at Writtle (45) (Table 1). The Russulaceae made up the greatest proportion of ECM species at all three woodland locations (51% of all ECM samples), within which the genera Lactarius (39%) and Russula (11%) dominated. Also present at all woodland locations but in lower abundances were members of the Boletaceae (12%), Gloniaceae (represented exclusively by *Cenococcum geophilum* - 11% of ECM root samples), Thelephoraceae (10%) and Cortinariaceae (3%) (Figure A.3). Several ECM families were found only at Writtle (i.e. Amanitaceae,

Discinaceae, Elaphomycetaceae, Hydnangiaceae, Paxillaceae, Pezizaceae), or only at Monks Wood (i.e. Entolomataceae, Inocybaceae, Sclerodermataceae, Tuberaceae), while no ECM families occurred uniquely at Stratfield Brake. The most abundant species overall were *Lactarius quietus* (33.8%), *C. geophilum* (10.4%), *Boletus rubellus* (6.2%), *Tomentella sublilacina* (5.2%) and *Lactarius subdulcis* (2.7%); the remainder of species were present in proportions of <2% of all species (Figure A.4).

The multivariate GLiM outputs indicated that ECM communities at the three woodland locations differed significantly from one another at the species and exploration type levels, but not at the family level (Table 2a-c).

3.2 ECM communities and AOD symptom status of sample trees

At each of the three woodland locations, levels of ECM species richness and diversity were similar between AOD symptomatic and asymptomatic trees (Table 1). AOD symptomatic and asymptomatic trees also showed no differences in the composition of ECM species, families, or exploration types (Table 2). However, when considering ECMs categorised by exploration type, GLiMs revealed a weak significant interaction effect between woodland location and the AOD symptom status of sample trees ($p > 0.01$) (Table 2c). ECM species with a long-distance exploration strategy tended to be associated more often with asymptomatic trees and ECM species categorised as contact-medium smooth types were associated more often with AOD symptomatic trees (Figure A.5).

3.3 Soil properties and ECM communities

386 We found that soil pH, P and Al were all significantly ($p<0.05$) affected by tree symptom
387 status as a main effect, although effects depended on woodland location for pH and Al. The
388 effects of tree symptom status on N was marginal ($p=0.052$). Woodland location exerted a
389 comparatively strong influence on soil physico-chemical properties. Writtle soils were
390 composed of significantly higher proportions of sand compared with Monks Wood and
391 Stratfield Brake, while Monks Wood had comparatively fine textured soils with significantly
392 higher silt and clay content (silt-clay content ~70%) (Table A.2). Monks Wood additionally
393 had significantly higher soil pH and lower exchangeable P than the other two woodlands,
394 while the C:N ratio at Writtle was significantly higher than Monks Wood and Stratfield
395 Brake. Exchangeable K concentrations were higher at Monks Wood than at Writtle, with
396 intermediate concentrations at Stratfield Brake. Highest concentrations of exchangeable Al
397 were found at Stratfield Brake (almost two- and three-fold higher than Writtle and Monks
398 Wood, respectively), with no significant difference in Total N found between the three
399 woodland locations.

400 The GLiM outputs indicated that ECM communities were significantly influenced at the
401 species level by soil C:N ratios, Total N and levels of exchangeable Al. At the family level,
402 ECM communities were significantly influenced only by levels of Al. When categorised by
403 exploration type, ECMs showed no clear response to any of the measured soil chemistry
404 variables (Table 2). NMDS ordination spiders illustrate a consistent separation of species
405 assemblages by woodland location, C:N ratios and levels of exchangeable Al (Figures 2 and
406 3, respectively), but no clear separation of species assemblages according to tree AOD
407 symptom status.

408 3.4 Root characteristics

No significant root dry weight differences were found between AOD asymptomatic and symptomatic trees, but significantly more root tips were found per soil core in asymptomatic compared with symptomatic trees in Stratfield Brake ($p<0.05$) and Writtle ($p<0.01$). The dry weight and frequency of root tips per soil core was noticeably lower at Monks Wood compared with Stratfield Brake and Writtle (Table 1).

4. Discussion

4.1 Are there significant differences in the number of root tips and composition of ECM communities on fine roots of AOD symptomatic and asymptomatic oak trees?

This study found no difference in the species richness, diversity and composition of ECM communities on AOD symptomatic and asymptomatic trees. However, there was a weak interaction between woodland location and the symptom status of trees that resulted in a positive association between ECMs with a long-distance exploration type and AOD asymptomatic trees; this positive association was only evident at woodland locations with coarser sediment texture, lower soil pH and higher soil P and Al (i.e. Stratfield Brake and Writtle). Soil cores collected at the base of AOD asymptomatic trees were additionally found to have significantly more root tips per soil core compared with soil cores collected at the base of symptomatic trees at two out of the three woodland study locations (Stratfield Brake and Writtle). The third woodland, Monks Wood, had considerably less roots in each soil core, regardless of tree symptom status, which may have been due to the fine-textured soils at this woodland location that have a greater potential to become water-logged.

These results align well with our prediction that asymptomatic trees will have the advantage of a greater capability to explore and exploit resources belowground than symptomatic trees by virtue of a higher number of root tips hosting greater proportions of long-distance ECM exploration types. It is unclear though whether the higher proportions of long-distance ECM

exploration types and higher frequency of root tips found on asymptomatic trees are reflections of the better condition of these trees, or instead, are due to a more favourable soil environment under asymptomatic trees compared with symptomatic trees. As discussed below (section 4.2), we were not able to detect any soil physico-chemical differences, consistent across forest locations in the surface soils around AOD symptomatic and asymptomatic trees.

With the exception of a much higher abundance of Boletaceae at two of the woodland locations (Stratfield Brake and Writtle), the ECM fungal communities recorded at the three study locations were composed of the same dominant genera (i.e. *Russula*, *Lactarius*, *Cenococcum*, *Tometella*) and had similar ECM richness as previous studies of ECM communities based on *Q. robur*/*Q. petraea* in England (Suz et al. 2014, Spake et al., 2016) and further afield in Europe (Van Driessche and Piérart, 1995, Causin et al., 1996, Kovacs et al., 2000, Mosca et al. 2007, Bzdyk et al., 2019). Nevertheless, some caution is required when making comparisons between the results of our study and other similar studies comparing ECM communities on 'healthy' and 'declining' oak trees. One of the main difficulties' rests in the definition of what constitutes a symptomatic tree. In contrast to many studies, we applied a definition of the symptoms we considered to indicate an AOD symptomatic tree (i.e. stem lesions - Denman et al., 2014) and did not use crown condition and levels of defoliation as part of the distinguishing features. Thus, proceeding cautiously with study comparisons we find that, in terms of root tip abundance, our results concur with Corcobado et al. (2014) who also found that fine root abundance was significantly higher in healthy compared with declining oak (*Quercus ilex*) trees (but see opposite results in Bzdyl et al., 2019). The same finding has been observed in a host of other studies comparing root tip frequency in declining and healthy trees, although these involved other, non-oak tree species (Bauce and Allen, 1992, Blaschke, 1994, Power and Ashmore, 1996, Nechwatal and Oβwald,

458 2008). In comparing our findings of ECM richness, diversity and community composition on
459 AOD symptomatic and asymptomatic trees, our study results align with the findings of
460 Causin et al. (1996) and Lancellotti and Franceschini (2013). As in our study, Causin et al.
461 (1996) found no relationship between the health status of sampled *Q. robur* trees sampled and
462 ECM species richness and community composition. Similarly, Lancellotti and Franceschini
463 (2013) found no difference in ECM richness and diversity on the fine roots of healthy and
464 declining *Quercus suber* trees, although they report significant differences in the evenness
465 and taxonomic distinctness (Clarke and Warwick, 1998) of ECM communities across a tree
466 decline gradient. In contrast to these results are numerous other studies that have observed
467 significant differences in the compositions of ECM communities on healthy and declining *Q.*
468 *robur* /*Q. petraea (*Kovacs et al., 2000, Mosca et al., 2007, Bzdyk et al., 2019) and on healthy
469 and declining *Q. ilex* (Montecchio et al., 2004, Corcobado et al., 2014, 2015). Significantly
470 lower species diversity in declining trees is reported by Mosca et al. (2007) and Bzdyk et al.
471 (2019). Bzdyk et al. (2019) also found a significantly lower diversity of ECM exploration
472 types in declining compared with apparently healthy oak trees. Most of the other studies
473 showing significant ECM compositional differences between declining and healthy trees have
474 reported significantly different proportions of dominant ECM species on declining and
475 healthy trees (Kovacs et al., 2000, Montecchio et al., 2004, Corcobado et al., 2015). For
476 example, Kovacs et al. (2000) found that *Amanita rubescens*, *Russula* spp., *Lactarius* spp.
477 and *C. geophilum*, were significantly more abundant on the fine roots of *Q. robur*/ *Q. petraea*
478 trees that displayed good health compared with declining trees (i.e. high levels of
479 defoliation). Similarly, Montecchio et al. (2004) found that *Russula* spp., *C. geophilum* and
480 the oak specialist ECM species *Lactarius chrysorrheus* were more abundant on healthy
481 compared with declining *Q. ilex* trees (i.e. high levels of defoliation). We found no difference

in the relative abundances of any of these dominant ECMs in our AOD symptomatic and asymptomatic trees.

4.2 Are ECM communities influenced by any significant differences in soil physico-chemical conditions under symptomatic and asymptomatic trees and across woodland locations?

ECM community composition was influenced by significant differences in soil physico-chemical conditions between woodland locations rather than any detectable soil variable differences between symptomatic and asymptomatic trees. Woodland locations differed significantly in terms of soil pH, C:N ratios, P, exchangeable Al and sediment textural properties with significant ECM community effects observed in relation to a number of these differences in soil variables. Across the three woodland locations, shifts in ECM community composition could be related, as in previous studies (e.g. Suz et al 2014, Maghnia et al., 2017, Bzdyk et al., 2019, Defrenne et al., 2019), to significant differences in C:N ratios, Total N and exchangeable Al and may explain variations in ECM species richness and diversity among the woodlands. For example, the comparatively low ECM species richness and diversity at Stratfield Brake could be associated with the significantly higher levels of exchangeable Al at Stratfield Brake compared with the other two woodland locations; Al is known to have a negative impact on many ECMs (Entry et al., 1987, Rühling and Söderström, 1990, Jongbloed and Borst-Pauwels, 1992). Also noteworthy are the relatively high C:N ratios at Writtle which are indicative of lower rates of decomposition than at the other two woodland locations. This may be a reflection of the comparatively high ECM species richness and diversity at this site contributing to a suppression of saprotrophic fungal activity and consequently reduced levels of decomposition (i.e. a weakening of the 'Gadgil effect' - Avril and Hawkes, 2016, Fernandez and Kennedy, 2016).

As well as compositional differences between woodland locations, we also observed a number of taxa-specific responses to the significant differences in soil properties between woodland locations. For example, *C. geophilum* occurred in higher abundances at Writtle compared with the other two woodland locations. This ubiquitous species has been reported to be associated with high soil P (Maghnia et al., 2017) and demonstrates a tolerance, and possibly a preference, for drier soil conditions (Kovacs et al, 2000, Di Pietro et al., 2007, Corcobado et al, 2015). Writtle had the highest levels of soil P of the three woodland locations and was likely the driest of the three woodland locations with high proportions of sand in surface mineral layers. *Tomentella* species also demonstrated a taxa-specific response to the differences in soil P with higher species richness and abundance of *Tomentella* at the significantly lower levels of P encountered at Monks Wood. This trend reflects observations by Maghnia et al. (2017) for *Tomentella*.

Contrary to expectations, ECM community compositions were unaffected by the significant differences in soil pH recorded at Monks Wood (pH 4.7) and Stratfield Brake/ Writtle (at both woodlands the mean soil pH was 3.6). This is despite evidence from other studies (e.g. Suz et al., 2014) that ECM species composition and functional exploration types are sensitive to changes in soil pH. It is possible that the pH ranges across our woodland locations were not sufficiently great (mean pH of 3.6 to 4.7) to induce a response in ECM communities as in previous studies where pH gradients studied were much greater (e.g. range of pH3 to pH7 in Suz et al., 2014).

5. Conclusions

A key finding of this investigation was the significantly lower number of root tips present in soil cores collected under AOD symptomatic trees compared with soil cores collected

beneath asymptomatic trees at two of the three woodland study locations. This revealed a relationship between above-ground symptoms used to identify AOD-afflicted trees (i.e. stem lesions and *Agrilus* beetle exit holes) and the abundance of root tips in surface soil layers, providing a potential additional diagnostic feature of trees in decline. Fewer root tips on symptomatic trees suggested that there would be a reduced capacity for ECMs to form mycorrhizal associations compared with the asymptomatic trees, but our results showed no evidence this. We found no differences in ECM composition, richness or diversity between symptomatic and asymptomatic trees, although ECMs with a long-distance exploration type were more commonly found on asymptomatic trees in more free-draining soils. The composition of ECM communities was nevertheless clearly related to the differing soil physico-chemical conditions at the tree woodland locations and specifically to differences in exchangeable Al, Total N and C:N ratios. While we recorded some significant differences in soil chemistry (soil pH, P and exchangeable Al) between symptomatic and asymptomatic trees, these differences may have been distilled by the strength of woodland location effects. A higher replication rate of AOD symptomatic and asymptomatic trees at each woodland location is recommended for a future study to explore any potential important soil chemistry differences between symptomatic and asymptomatic trees and related effects on ECM communities as well as the wider tree-associated soil microbiome. Further work is also required to assess the cross-regional extent of AOD using a standardised definition; this would enable more reliable comparison of results between studies than is currently possible.

CRediT authorship contribution statement

Nadia Barsoum: Conceptualization, Investigation, Writing - original draft, Writing - review & editing. Stuart A'Hara: Investigation, Writing - review & editing. Joan Cottrell: Writing - review & editing. Jack Forster: Investigation, Writing - review & editing. Liz Shaw: Writing - review & editing. Karsten Schonrogge: Writing - review & editing. Mateo Garcia: Writing - review & editing.

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.

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Figures

Fig. 1. Map showing three woodland sample locations (Monks Wood - MW, Stratfield Brake – SB and Writtle-W) and the distribution of symptomatic (+) and asymptomatic (●) trees at each woodland location. Note the differing scales between panels, adjusted to show the distribution of trees in each woodland.

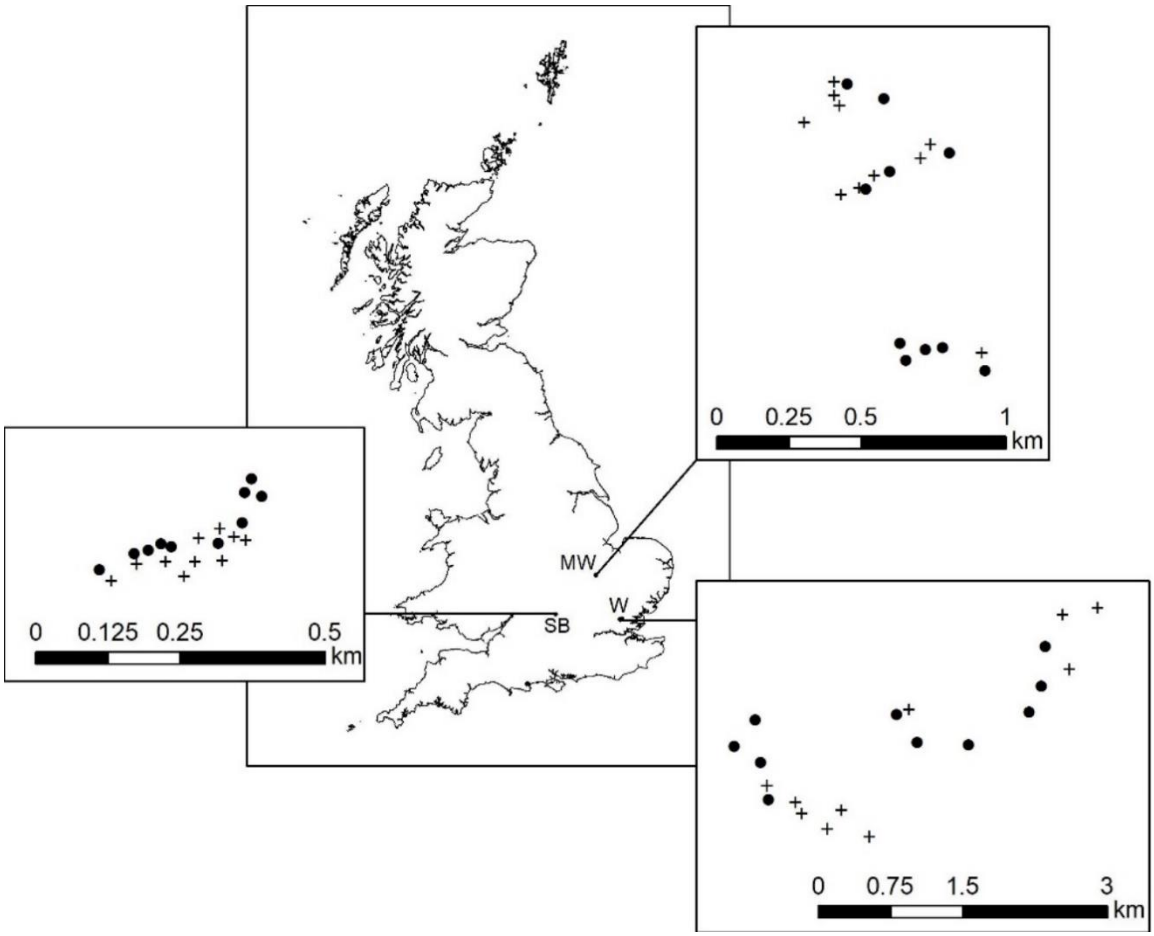


Figure 2 Non-metric multidimensional scaling (NMDS) ordination of ECM species showing samples grouped by woodland location and tree symptom status. Surface plot shows C:N ratios.

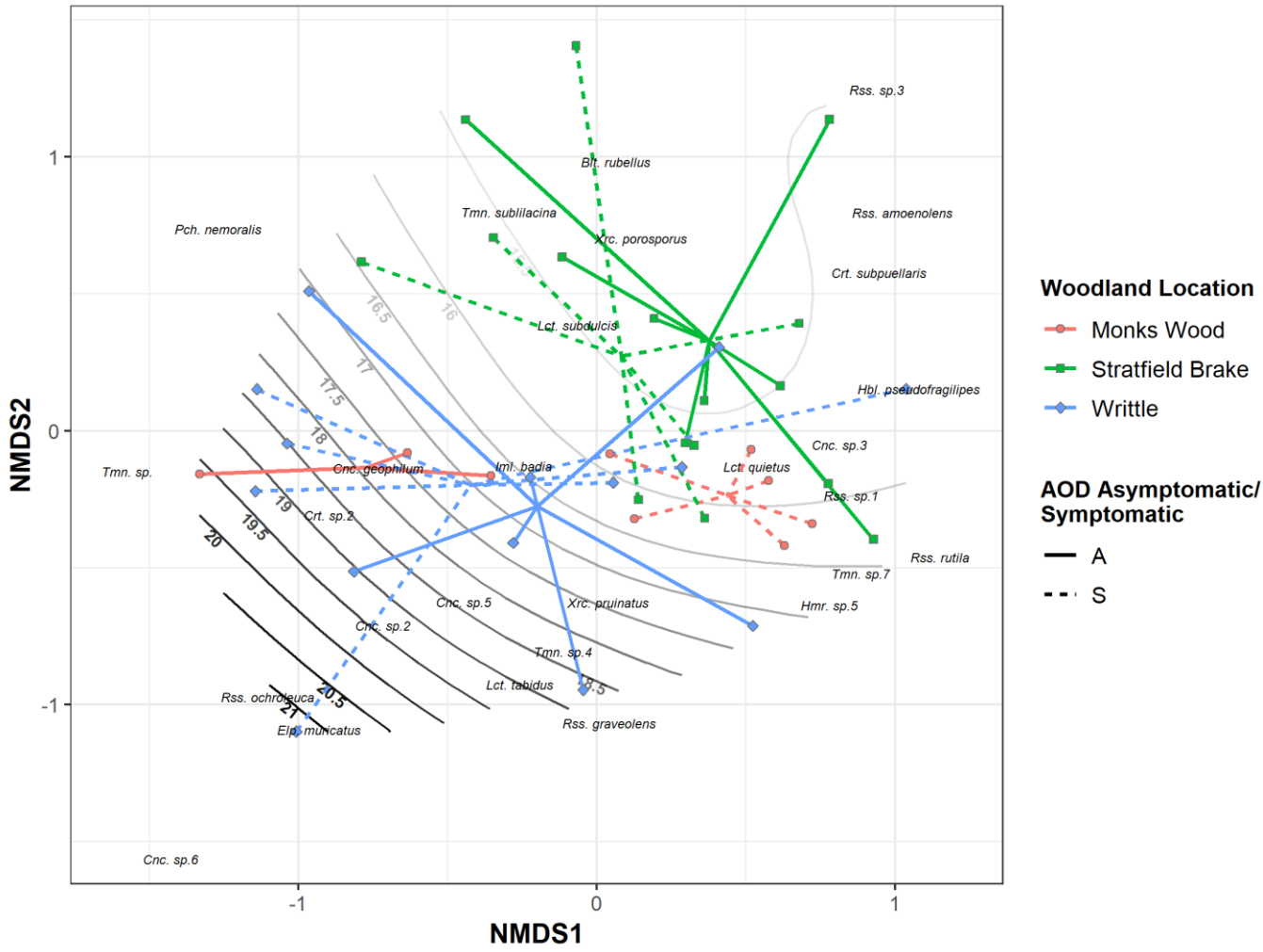
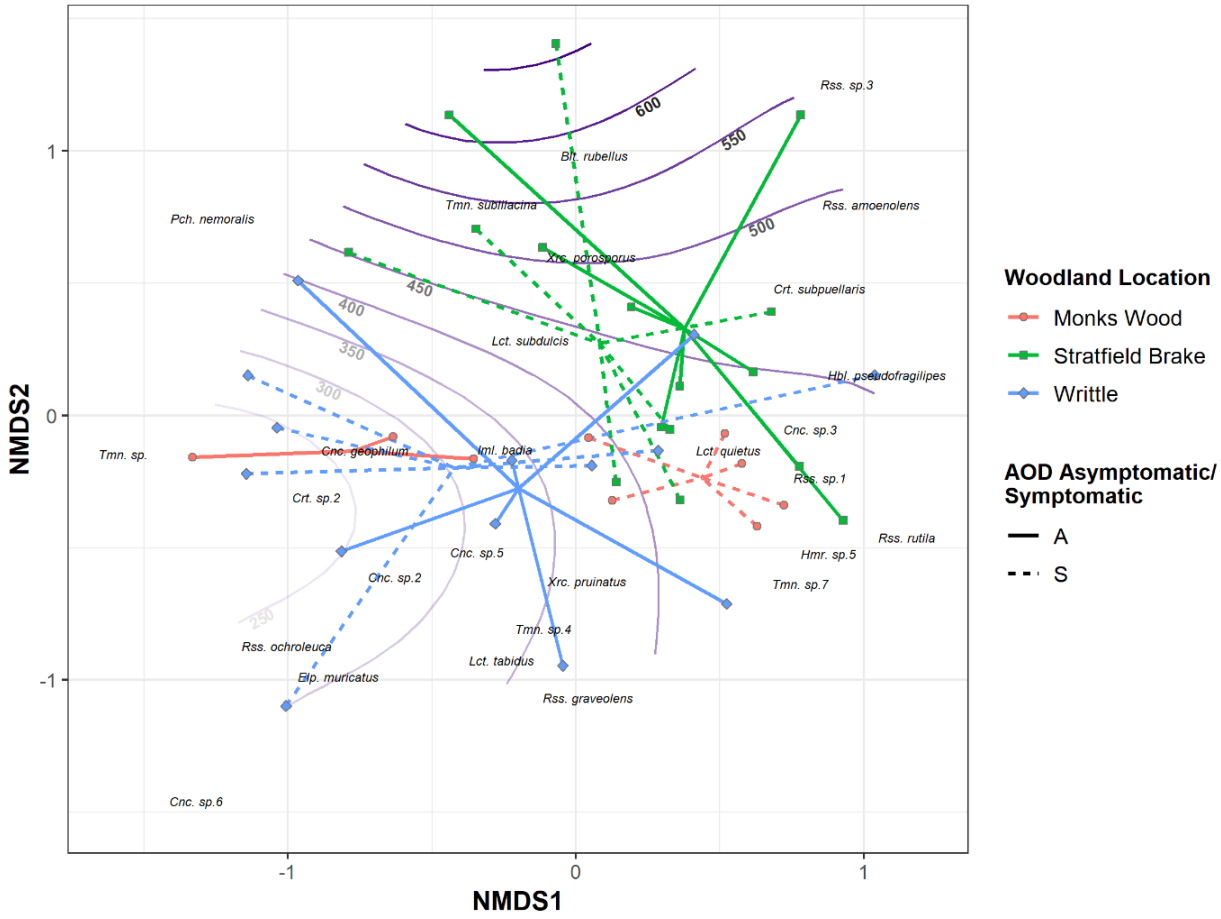


Figure 3 Non-metric multidimensional scaling (NMDS) ordination of ECM species showing samples grouped by woodland location and tree symptom status. Surface plot shows exchangeable Al concentrations (mg kg^{-1}).



872 **TABLES**

873

874 **Table 1** Summary of differences in ECM richness, diversity (*H'*) and root characteristics between woodland locations and for asymptomatic (A)
875 and symptomatic (S) trees at each woodland location. The values expressed for roots (< 2 mm diameter) are the average dry weight or number of
876 root tips per soil core (i.e. per 80cm3 of soil sampled). One-way ANOVAs tested for significant differences between A and S trees in terms of
877 ECM richness, Shannon-Weaver diversity and root characteristics. The root data were log-transformed prior to analysis and back-transformed
878 for presentation. There were no significant differences in ECM richness, diversity or root dry weights between A and S trees. ECM richness and
879 H' values shown combine all sample trees per woodland location. * and ** indicate that S trees have significantly fewer root tips per soil core
880 than A trees at $p<0.05$ and $p<0.01$, respectively. Percentage of soil core samples with no roots/fungi and percentage with positive ECM
881 identifications are also given for each woodland location.

882

	ECM species richness		**ECM family richness**		**ECM species diversity**		**Root dry weight (g)**		**Number of root tips**		**Sample failure**[1]	**Samples with positive ECM ID** [2]
	Total	A or S	Total	A or S	Total	A or S	Total	A or S	Total	A or S	(%)	(%)
Monks Wood	38	A 20	12	A 9	2.81	A 2.46	0.18	A 0.15	469	A 535	36.7	81.7
		S 19		S 9		S 2.39		S 0.21		S 403	10.8	84.5
Stratfield Brake	17	A 12	6	A 6	1.9	A 1.89	0.38	A 0.32	1060	A 1060	15.8	92.1
		S 9		S 5		S 1.61		S 0.44		S 890*	21.7	90.4
Writtle	45	A 28	14	A 12	2.63	A 2.83	0.35	A 0.33	1084	A 1320	11.7	89.6
		S 27		S 10		S 2.68		S 0.36		S 848**	17.5	82.2

[1] Percentage of samples without roots and fungi out of 120 possible samples (three fine root samples x four soil cores x ten A or S trees).

883 [2] Percentage of samples with fungi that have a positive ECM ID. Rejected if not an ECM fungi or could not be identified.

Table 2 Effect of tree symptom status, woodland location and soil variables on the composition of ECM (a) species, (b) families and (c) exploration types assessed by multivariate GLiMs. Analysis of deviance was conducted on each factor, with pit-trap resampling, 999 iterations and score tests, used to determine the significance of tree symptom status and woodland location. Significant *P*-values (≤0.01) are shown in bold.

(a) Effect on ECM species

	Res.Df	*Df.diff*	*Deviance*	*P*-value
Tree symptom status	56	1	47.26	0.402
C:N ratio	55	1	97.20	**0.002**
Total N	54	1	69.73	**0.004**
Exchangeable Al	53	1	119.85	**0.001**
Woodland location	51	2	100.56	**0.002**

(b) Effect on ECM families

	Res.Df	*Df.diff*	*Deviance*	*P*-value
Tree symptom status	53	1	23.78	0.548
Exchangeable Al	52	1	54.68	**0.006**
Woodland location	50	2	56.53	0.097

(c) Effect on ECM exploration type

	Res.Df	*Df.diff*	*Deviance*	*P*-value
Tree symptom status	55	1	2.07	0.561
Woodland location	53	2	9.62	0.145
Tree symptom status x Woodland location	51	2	9.33	**0.010**

Appendix A: Supplementary material

Additional Supplementary tables and figures associated with this article are listed below.

Tables:

Table A.1 Climate, soil characteristics and dimensions of asymptomatic (A) and symptomatic (S) trees sampled at each woodland location. DBH is the diameter at breast height (1.3m above ground level).

Table A.2: Physico-chemical characteristics of soil samples collected from a depth of 5-15 cm around the 20 sampled trees (10 symptomatic, 10 asymptomatic) at each woodland location. Means (± SD) are provided for each soil characteristic. Means sharing a letter in common are not significantly different ($p<0.05$) among woodland sites, according to Games-Howell Pairwise Comparisons. Soil pH, P and Al were all significantly ($p<0.05$) affected by tree symptom status as a main effect, although effects depended on woodland location for pH and Al. The effects of tree symptom status on N was marginal ($p=0.052$).

Figure A.1: Ranked abundance Zipf distributions of ECM species in the three woodland locations. Abundance values represent the number of trees that ECM species were associated with. Rank abundance curves were constructed using the radfit() function of the 'vegan' package using the Zipf-Mandelbrot distribution (Oksanen et al., 2013).

Figures:

Figure A.2: Species accumulation curves at the three woodland locations estimated using the specaccum() function in vegan() package in R. Method = "exact" (finds the expected (mean) species richness), permutations = 9999.

Figure A.3: Matrix plot of ECM families recovered by each sample tree, with fill colour (white to dark purple) indicating presence and abundance of families (maximum count = 12).

Figure A.4: Matrix plot of ECM species recovered by each sample tree. Colour indicates species presence/ abundance (maximum count = 12).

Figure A.5. Matrix plot of ECM exploration types recovered beside asymptomatic (A) and symptomatic (S) trees at each woodland location, with fill colour (white to green) indicating presence and abundance of different exploration types (maximum possible count = 10).

941 **Table A.1** Climate, soil characteristics and dimensions of asymptomatic (A) and symptomatic (S) trees sampled at each woodland location.
942 DBH is the diameter at breast height (1.3m above ground level).

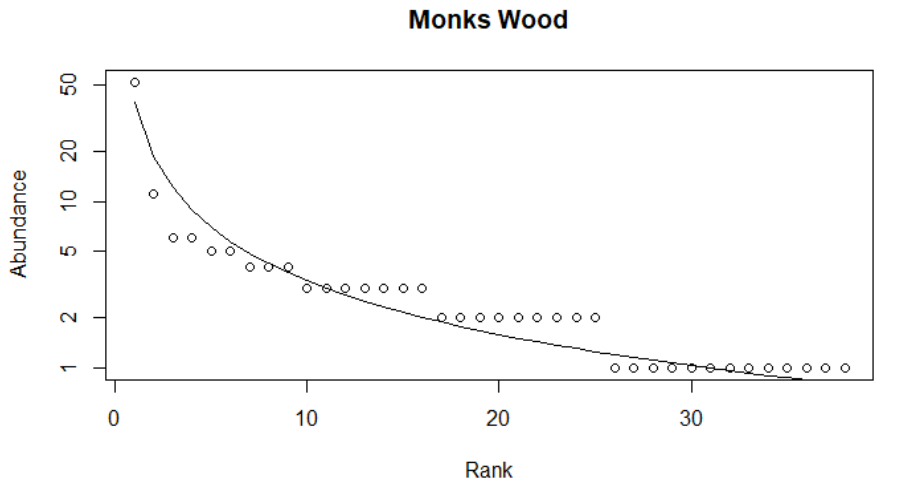
Woodland characteristics	Monks Wood		Stratfield Brake		Writtle Forest	
Mean annual precipitation (mm)*	586		660		592	
Max. annual temperature (^{o}C)*	14.4		14.6		14.6	
Min. annual temperature (^{o}C)*	5.9		6.9		6	
Soil type**	Lime-rich loamy and clayey soils with impeded drainage		Slowly permeable seasonally wet base-rich loamy and clayey soils		Slowly permeable seasonally wet acid loamy and clayey soils	
	A	S	A	S	A	S
Mean crown density^{+}	40.5	44.0	45.5	58.0	8.5	24.5
Shrub cover (%)$^{++}$	33.4	30.7	25.4	29.5	20.1	24.3
Mean DBH of sample trees (cm)	49.3	51.1	55.0	59.1	58.5	64.4
Mean tree basal area (m^2)$^{+++}$	33.4	30.7	25.4	29.5	20.1	24.3
Mean sample tree height^{++++} (m)	18.5	18.2	19.6	19.0	16.1	15.5

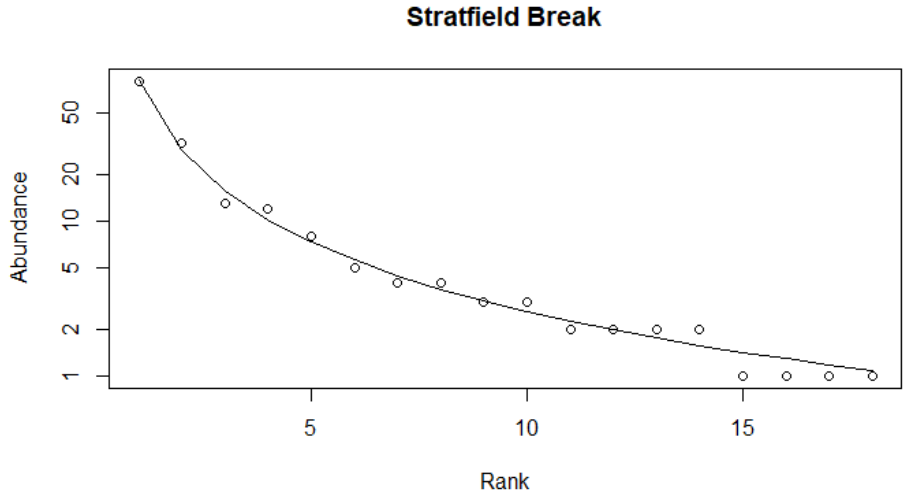
943 * Met Office averages 1981-2010 taken at Monks Wood, Oxford and Stratfield Brake weather stations.
944 ** Soil descriptions from soil maps at http://www.landis.org.uk/soilscapes/
945 $^{+}$ The absolute crown density was recorded in 5% classes where 0% = fully foliated crown and 100% = no leaves present.
946 $^{++}$ Shrub cover was the estimated average percentage cover of woody shrubs and tree sapling in 2m x2m quadrats placed at two random positions
947 within 5m of each sample tree.
948 $^{+++}$ Average tree basal area is based on the average basal area of all trees along four 15m transects running in the four cardinal directions away
949 from each sample tree.
950 $^{++++}$ Tree height was assessed with a clinometer to 0.1m.

Table A.2: Physico-chemical characteristics of soil samples collected from a depth of 5-15 cm around the 20 sampled trees (10 symptomatic, 10 asymptomatic) at each woodland location. Means (± SD) are provided for each soil characteristic. Means sharing a letter in common are not significantly different ($p<0.05$) among woodland sites, according to Games-Howell Pairwise Comparisons. Soil pH, P and Al were all significantly ($p<0.05$) affected by tree symptom status as a main effect, although effects depended on woodland location for pH and Al. The effects of tree symptom status on N was marginal ($p=0.052$).

Soil characteristics	Monks Wood	Stratfield Brake	Writtle Forest
Clay (%)	1.6 ± 0.8^{A}	0.6 ± 0.3^{B}	0.4 ± 0.5^{B}
Silt (%)	68.7 ± 7.8^{A}	56.2 ± 5.3^{B}	47.6 ± 10.1^{C}
Sand (%)	30.0 ± 7.9^{C}	43.1 ± 5.6^{B}	52.0 ± 10.7^{A}
pH (H_2O)	4.7 ± 0.7^{A}	3.6 ± 0.2^{B}	3.6 ± 0.3^{B}
C:N ratio	13.2 ± 1.5^{C}	14.6 ± 0.9^{B}	20.0 ± 2.9^{A}
Total N (%)	0.47 ± 0.1^{A}	0.52 ± 0.1^{A}	0.42 ± 0.2^{A}
Olsen P (mg kg^{-1})	9.4 ± 5.6^{B}	20.9 ± 12.5^{A}	24.8 ± 22.6^{A}
Exchangeable K (mg kg^{-1})	310.7 ± 90.0^{A}	289.4 ± 69.0^{AB}	227.7 ± 143.7^{B}
Exchangeable Al (mg kg^{-1})	190.2 ± 174.2^{B}	531.8 ± 141.3^{A}	306.7 ± 116.6^{B}

Figure A.1: Ranked abundance Zipf distributions of ECM species in the three woodland locations. Abundance values represent the number of trees that ECM species were associated with. Rank abundance curves were constructed using the radfit() function of the 'vegan' package using the Zipf-Mandelbrot distribution (Oksanen et al., 2013).





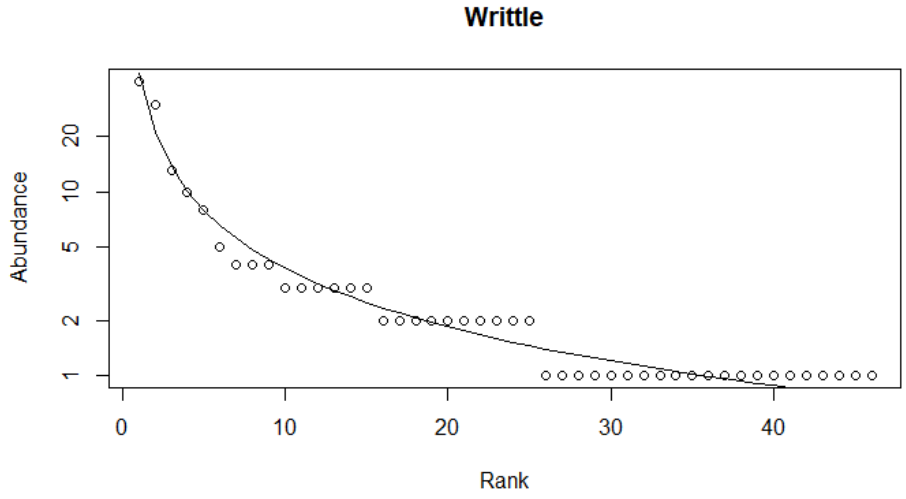
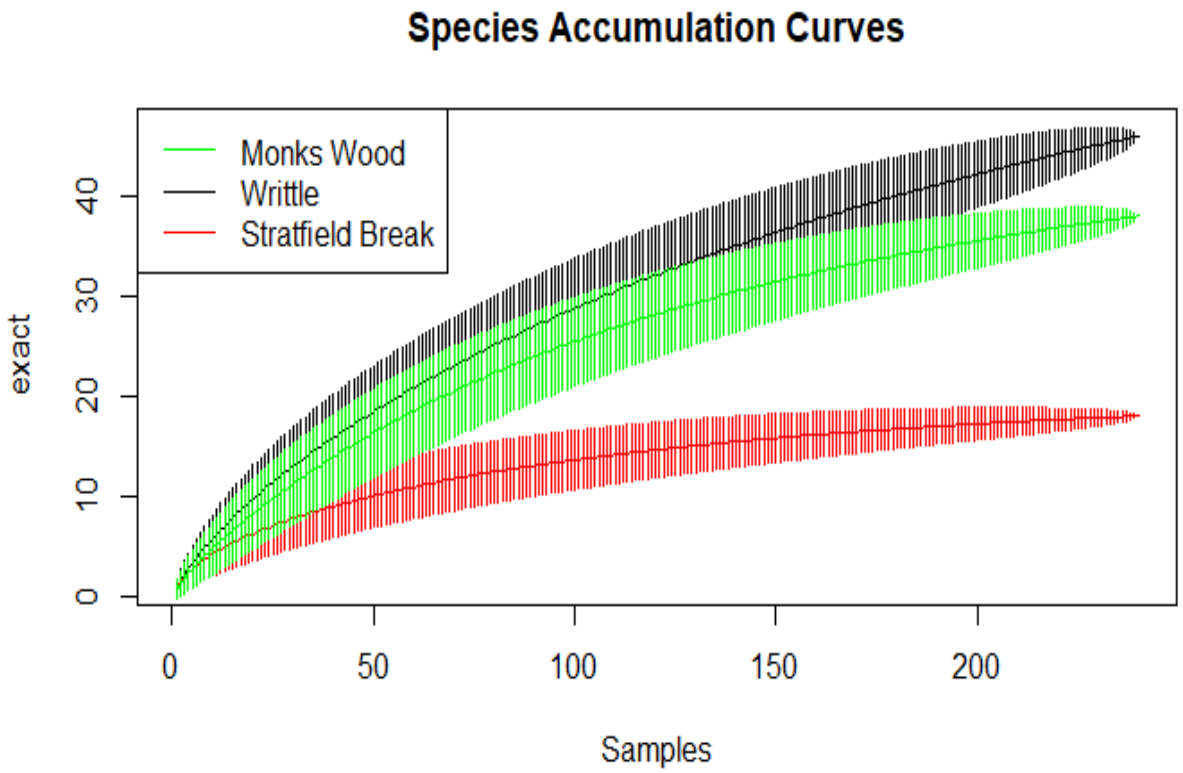


Figure A.2: Species accumulation curves at the three woodland locations estimated using the specaccum() function in vegan() package in R. Method = "exact" (finds the expected (mean) species richness), permutations = 9999.



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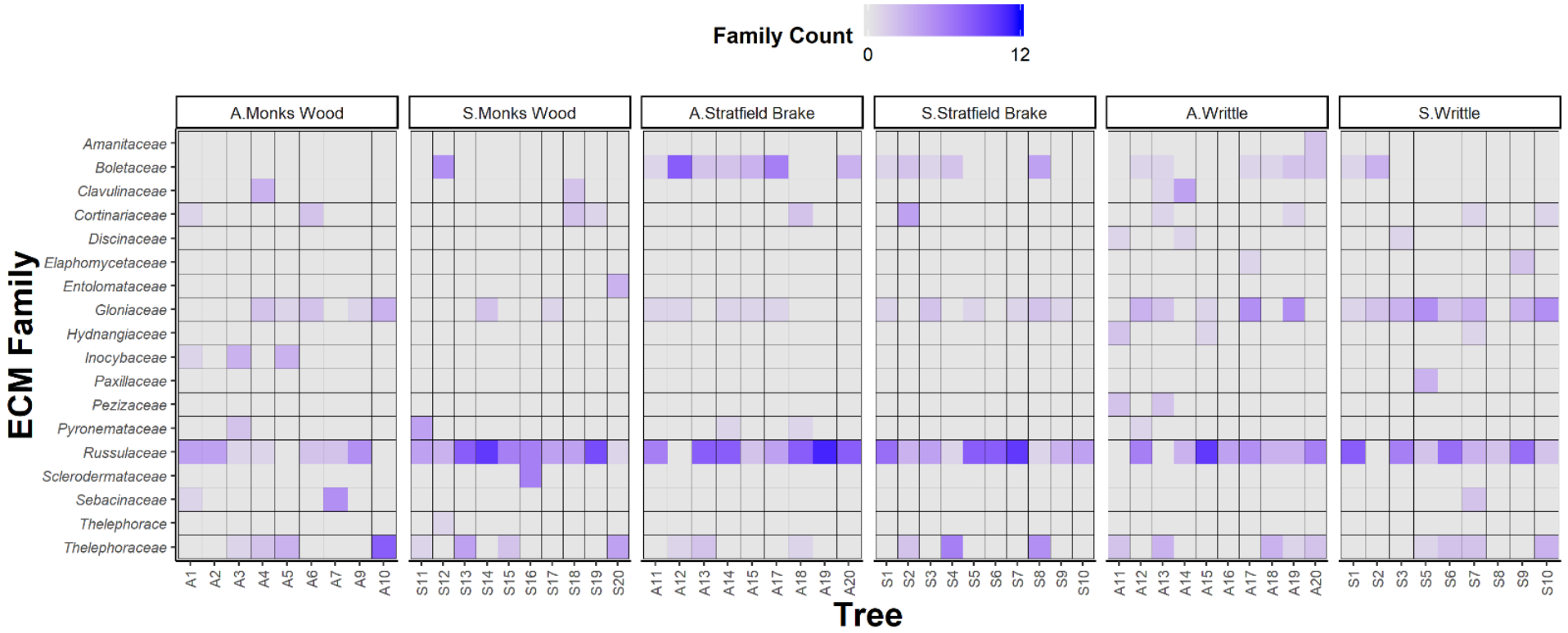


Figure A.4: Matrix plot of ECM species recovered by each sample tree. Colour indicates species presence/ abundance (maximum count = 12).

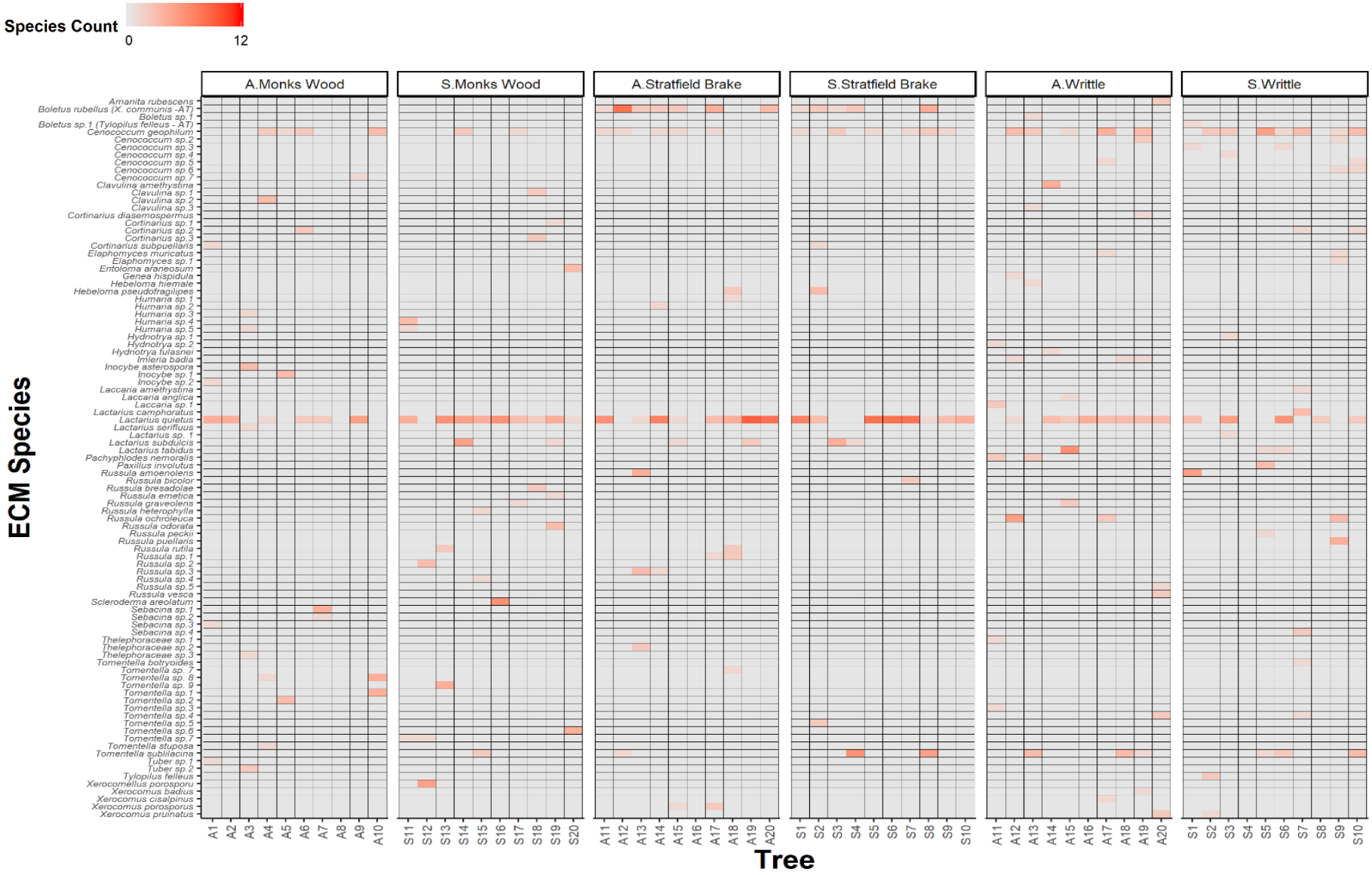
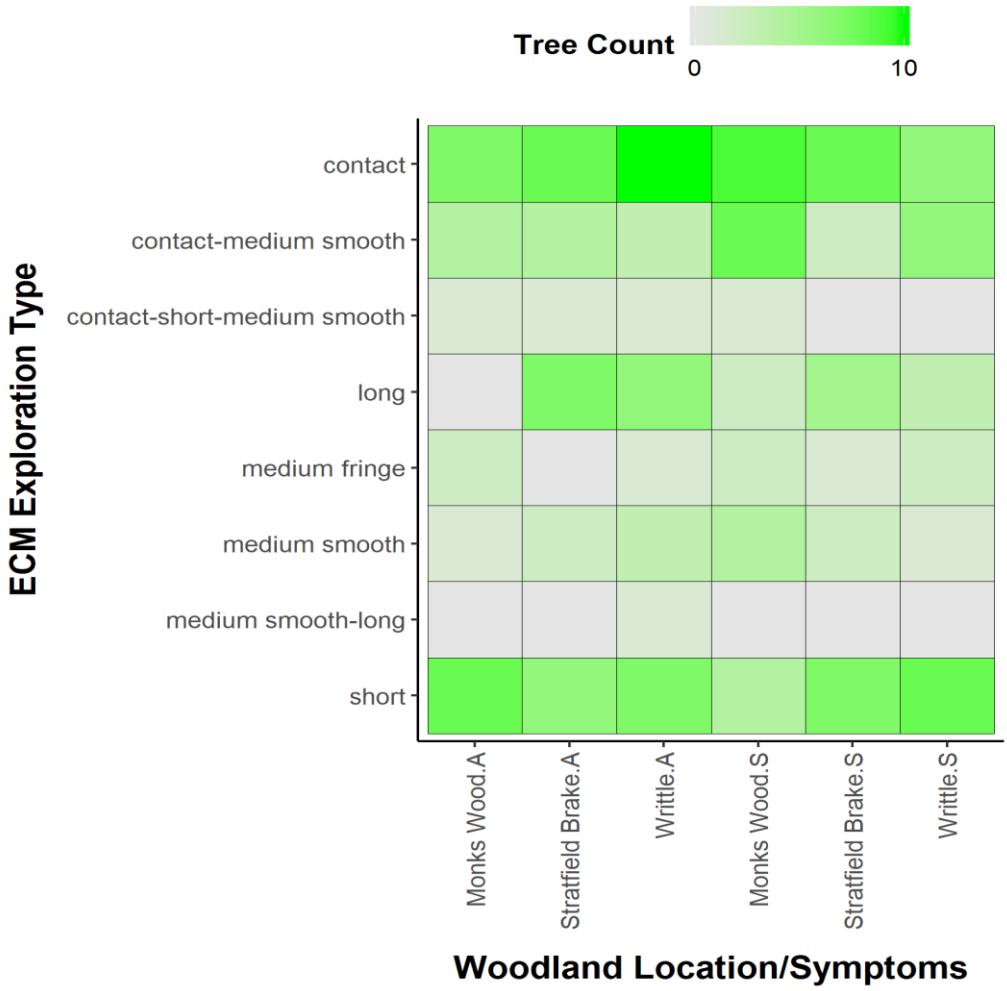


Figure A.5. Matrix plot of ECM exploration types recovered beside asymptomatic (A) and symptomatic (S) trees at each woodland location, with fill colour (white to green) indicating presence and abundance of different exploration types (maximum possible count = 10).



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