Evaluating the impact of tree provenance, tree phenotype and emergent disease on microbial and insect populations in tree ecosystems

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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Abstract

Trees host complex microhabitats, supporting a wide diversity of organisms. This study focuses on two of these communities: the insect herbivores and the microbial endophytes. Studies in the field of community genetics have demonstrated that intraspecific genetic variation in a foundation species can have cascading effects on the composition and diversity of associated communities. Using a provenance trial, which excludes confounding environmental factors, the effects of tree phenotypic traits on the insect herbivore and microbial endophytic community were tested, in the first instance, using the *Quercus* study system. A leaf morphometric analysis allowed for accurate differentiation of the two native oak species, *Quercus robur* and *Q. petraea.* Interspecific variation in the composition of insect herbivore species and in the composition of bacterial and fungal endophyte species were recorded in the two oak species for the first time. The plant vigour hypothesis, which states that insect herbivores would preferentially feed on the most vigorous plant, was supported by the galling and mining insect feeding guilds in this study system but had varying effects on the endophytic species. Tree budburst phenology, another phenotypic trait, also had varying effects on the abundance of insects and endophytes.

Quercus trees in the UK are expected to experience profound changes in climate. In an effort to maintain oak species, assisted migration schemes may be implemented, which involves the planting of seed stock from exotic provenances that match the predicted climate of the planting site in 50-100 years. This study considers the effect of this approach on the associated biodiversity. Insect and endophyte composition, richness and diversity were not strongly correlated with tree provenance, so it seems unlikely that climate matching strategies would strongly negatively impact these associated communities.

As the UK climate warms, walnut trees (*Juglans* spp.) may become a more commercially viable source of hardwood timber. This study characterised the endophytic community of two introduced walnut trees in the UK: *Juglans nigra* and *J. regia*. A number of latent pathogenic fungi and bacteria were associated with asymptomatic tissues of both *Quercus* and *Juglans*, these species have the potential to cause disease if the tree is subjected to stress such as climate change and should therefore be monitored.

Finally, the effect of a decline syndrome, Acute Oak Decline (AOD) which causes bleeding cankers among other symptoms on native oak species in the UK, on the endophytic community of oak was recorded. Bacterial endophyte communities did not differ between symptomatic and asymptomatic trees. However, differences in the fungal endophytic community associated with the inner bark of trees showing symptoms of AOD were significantly different from asymptomatic trees. This result suggests that (1) there could be a fungal component(s) to AOD that has not been reported or (2) asymptomatic trees harbour beneficial fungal endophytes that protect against AOD.

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Abbreviations & acronyms

AOD Acute oak decline

BAN Brown apical necrosis

Bg *Brenneria goodwinii*

BLAST Basic local alignment search tool

BSO Breeding seedling orchard

cfu Colony forming units

CTAB Hexadecyltrimethylammonium bromide

DBC Deep bark canker

DBH Diameter at breast height

DED Dutch elm disease

DNA Deoxyribonucleic acid

EDTA Ethylenediaminetetraacetic acid disodium salt dihydrate

GA Gibberellic acid

GLM Generalised linear model

GLMM Generalised linear mixed effect model

Gq *Gibbsiella quercinecans*

IAA Indole-3-acetic acid

ISR Induced systemic resistance

ITS Internal transcribed spacer

LA Luria Bertani agar

LB Luria Bertani broth

MAC MacConkey agar

NA Nutrient agar

NGS Next generation sequencing

NMDS Nonmetric multidimensional scaling

OTU Operational taxonomic unit

PBS Phosphate buffered saline

PCA Principal component analysis

PCR Polymerase chain reaction

PDA Potato dextrose agar

PERMANOVA Permutational multivariate analysis of variance

PVP Polyvinylpyrrolidone

RNA Ribonucleic acid

ROS Reactive oxygen species

Rp *Raoultella planticola*

rpm Revolutions per minute

Rv *Rahnella victoriana*

SBC Shallow bark canker

TBE Tris-borate-EDTA

TCD Thousand canker disease

TRF Terminal restriction fragment

TRFLP Terminal restriction fragment length polymorphism

Xaj Xanthomonas arboricola pv. juglandis

CHAPTER 1 - *Introduction*

Trees in the UK are facing a challenging combination of pressures, principally from climate change, increasing pollution and expanding urban development, all of which interact with emerging pests and diseases to cause devastating losses. A decline in a particular tree species, particularly in a foundation species such as oak (*Quercus* spp.), will have cascading effects on the associated biodiversity, ecosystem functions and ecosystem services of the forest or woodland.

This chapter begins by highlighting the importance of forests in the UK and the vast biodiversity that they support, with particular focus on insect herbivores and the microbes that live within the tree tissues (endophytes), two major groups that are likely to be affected by changes in tree health. Challenges facing UK forests are then addressed. Understanding the underlying tree characteristics that influence associated organisms is particularly important, if we are to predict how climate change and related pressures will affect forest ecosystems. The effect that tree phenotypes, for example phenology and vigour, have on associated species assemblage is described first, and then the possible impact that tree disease may have on this local biodiversity. The study genera, *Quercus* and *Juglans*, will then be introduced to address the following questions: Does tree origin (provenance) influence the insect herbivore or microbial endophytic communities that are associated with trees, and how one climate change mitigation strategy, climate matching, might affect these communities? Which tree characteristics: species, phenology, vigour or stress influence the organisms associated with these trees? Does the endophytic microbial community associated with the host tree influence the insect community? And does tree disease/decline alter the endophytic community of a tree?

1.1. *Importance of forests in the UK*

Woodland cover represents 13% of the total land area in the UK, this equates to around 3.2 million hectares (Forest Research, 2019b). Around 55% of this area is coniferous woodland such as Sitka spruce (*Picea sitchensis*, 51%), Scots pine (*Pinus sylvestris*, 17%) and Larches (*Larix* spp., 10%); the remaining 45% consists of broadleaved species such as oaks (*Quercus robur* and *Q. petraea*, 16%), birch (*Betula* spp., 18%) and ash (*Fraxinus excelsior*, 12%) (Forestry Commission, 2018). These woodlands provide a wide range of ecosystem services (Boyd et al., 2013). Carbon sequestration is one of the most significant ecosystem service provided by trees, in the UK alone the total carbon stock in forests (including soil) is approximately 800 megatons (Quine et al., 2011). Trees also provide shade, reduce soil erosion and provide valuable food, fuel and timber. Their cultural services are also of consequence, providing both physical and mental health benefits. The capitalised value of the social and environmental benefits provided by woodlands and forests in the UK was estimated to be over £29 billion (Willis et al., 2003). Trees are also of great ecological importance, they provide food and habitat to a wide variety of animals, plants and microbes.

The management of existing forest and the creation of further forests in the UK has become an important objective of reducing UK emissions. The Committee on Climate Change argues that at least 30,000 hectares of forest area should be planted each year and 5% of agricultural areas should be turned into forest to achieve net-zero emissions by 2050 (Committee on Climate Change, 2019).

1.2. *Challenges facing forests in the UK*

The number of exotic pests and pathogens entering and establishing in the UK has increased dramatically in the last half century (Smith et al., 2007, Santini et al., 2013). Spread of these

invasive pests and pathogens can be rapid due to low levels of host resistance and lack of 'natural enemies' in the introduced environment. Therefore, they have the potential to cause long term negative impacts to the UK natural environment, economy, culture and heritage (Brasier, 2008). Global movement of plants and plant products by human activity is now widely accepted as the primary mode of introduction of these novel pests and pathogens (Brasier, 2008, Smith et al., 2007). For example, the second Dutch elm disease epidemic started with the introduction of the fungal pathogen *Ophiostoma novo-ulmi* on imported Canadian elm (*Ulmus* spp.) logs in 1970, resulting in the death of 30-50 million elm trees in the UK (Brasier and Gibbs, 1973, Brasier, 2008). *Thaumetopoea processionea* or oak processionary moth was accidentally introduced into the UK in 2005, most probably due to the trade of live oak trees with central and southern Europe (Tomlinson et al., 2015, Forest Research, 2019a).

Environmental changes brought about by climate change are also thought to contribute to pest and pathogen epidemics in the UK. Over the next 50 years, the global average temperature is likely to increase by 2-5°C (IPCC, 2014). Climate change models have predicted that the UK will experience drier summers and wetter winters (Broadmeadow et al., 2005). The long lifespan and immobility of trees hinders rapid adaptation to these environmental variations, rendering forest ecosystems particularly susceptible to climate change (Lindner et al., 2010). Some aspects of climate change are likely to be beneficial, for example elevated temperatures and carbon dioxide concentrations could improve productivity (Broadmeadow et al., 2003, Broadmeadow et al., 2005). However, changes in temperature and precipitation are likely to alter the geographic distributions of both hosts and pests and pathogens and may increase the suitability of the UK climate for non-native pests and pathogens (Roy et al., 2017). Increasing abiotic stresses, such as drought and flooding, are also likely to predispose trees to pest and disease attack. Climate change will also likely affect the phenology of hosts and pest/pathogens; for example the timing of budburst and the timing of pest emergence and

spore release of pathogens, this may increase incidence and severity of attacks (Sturrock et al., 2011, Tubby and Webber, 2010, La Porta et al., 2008).

1.2.1. *Insects associated with trees*

Insects are essential components of forest ecosystems, they play a role in plant reproduction, they are important decomposers, they provide food for various groups of animals, but they are also major tree pests. The group of interest here are the herbivorous insects i.e. the insects that, for at least at one part of their life cycle, feed on plant tissue. Herbivorous insects can be divided into polyphagous insects which feed on plants of different families, oligophagous insects which feed on plants of different species from the same family and lastly the monophagous insects which feed mainly on plants of one particular species (Schoonhoven et al., 2005).

Forest herbivorous insects can be crudely grouped into the foliage feeders, the bark and ambrosia beetles, the wood boring insects, phloem and xylem sucking insects and galling insects. Foliage feeding can take many forms; some feed on the entire leaf, others just the tender leaf tissue between the veins (skeletonisers) and others mine internally. Free feeders can be solitary or feed in colonies (Ciesla, 2011). Some roll themselves in the leaf, like the oligophagous green oak leaf roller, *Tortrix viridana* (Hunter, 1998). Others, like the oak processionary moth (*Thaumetopoea processionea*), build nests of webbing in which they live gregariously, moving in large processions to feed in the crowns of trees at night (Groenen and Meurisse, 2011). Leaf miners feed, for at least part of their lifecycle, between the layers of epidermis in the leaves. The pattern of feeding and frass deposition together with the plant species and layer of the leaf being mined are often diagnostic of mining species (or genus). Of most recent concern to urban trees in the UK is the recent introduction of *Cameraria ohridella*, the horse chestnut leaf miner (Thalmann et al., 2003, Pocock and Evans, 2014).

Members of the Coleoptera order that utilise the bark and wood of trees usually attack dead or dying trees (Ciesla, 2011). Some of these species have a symbiotic relationship with fungi, which they transport from one tree to another as spores on their bodies, some of which are tree pathogens. For example, elm bark beetles in the genus *Scolytus* vector the fungi responsible for Dutch elm disease, *Ophiostoma novo-ulmi* (Anderbrant and Schlyter, 1987). *Agrilus biguttatus,* another bark beetle, has been found associated with declining oak trees in the UK (see *Chapter 6*). Larvae of this beetle create galleries in the inner bark as they feed on the vascular tissues, restricting the flow of nutrients within the tree (Brown et al., 2014).

Insects in the Hemipteran order have mouthparts for piercing and sucking, these include aphids, psyllids and plant hoppers, many of which are important tree pests, such as the green spruce aphid (*Elatobium abietinum*) which can cause needle abscission and growth loss in Sitka spruce (*Picea sitchensis*) (Straw et al., 2005) and some aphid species are vectors for plant pathogenic viruses (Gildow et al., 2004).

The last group, the gallers, stimulate plants to form abnormal tissue growth (Redfern and Askew, 1992). The insect disrupts normal plant cell growth and through enlargement and proliferation of plant host cells a gall is formed which acts as both the habitat and the food source for the gall maker (Redfern and Askew, 1992, Stone et al., 2002). Galling insects include gall midges (Diptera: Cecidomyiidae), gall wasps (Hymenoptera: Cynipidae) and aphids (Hemiptera: Aphididae). Oaks in particularly are prone to insect galling, with around 60 species causing galls in the UK on leaves, acorns, roots, trunk, twigs and catkins (Redfern, 2011).

Most of these forest insects are of little or no consequence to tree survival as they remain at low densities, others however can go through periods of extremely high numbers that can cause devastating destruction. For example, the spruce budworm (*Choristoneura fumiferana*) can remain at low levels for many years but when outbreaks develop, they can cause millions

of hectares of damage (Blais, 1983). Insects may also play a significant role in the occurrence of declines and diebacks of trees, for example, the wood boring beetle *Agrilus biguttatus* has been implicated as a contributing factor of oak decline in the UK (see *Chapter 6*).

1.2.2. *Endophytic communities associated with trees*

Any organism that colonises the internal tissue of a plant may be described as an endophyte. Most, if not all, plant species in natural ecosystems possess endophytic organisms (Strobel, 2018, Strobel et al., 2004), the most commonly encountered belonging to the fungal and bacterial kingdoms. These biologically diverse organisms have been isolated from every organ of a plant; roots, stems, leaves, flowers, fruits and seeds (Schulz and Boyle, 2005). This study focuses on the endophytes associated with the phyllosphere, the above-ground portions of the plant.

Of the plants studied to date, plant phyllosphere bacterial communities are dominated by members of a few phyla: Actinobacteria, Bacteroidetes, Proteobacteria and to some extent Firmicutes (Terhonen et al., 2019, Hardoim et al., 2015). For fungi, the majority belong to the phyla: Ascomycota, Basidiomycota and Glomeromycota (Terhonen et al., 2019, Hardoim et al., 2015). Endophytes of tree species mainly colonise their host through horizontal transmission i.e. via transfer of inoculum in the environment (Frank et al., 2017) but some have also been shown to transmit vertically from parent to progeny through host seeds (Bright and Bulgheresi, 2010). The mode of transmission is likely to be determined by ecological and evolutionary relationships between the symbiotic partners (Frank et al., 2017). Once inside the plant the endophyte may grow inter- or intracellularly and is either restricted locally or able to grow systemically (Schulz and Boyle, 2005).

The term 'endophyte' in the literature has conventionally been used to describe only those organisms residing in the endosphere that cause no apparent harm i.e. they do not cause disease symptoms (Wilson, 1995). However, the endosphere of a seemingly healthy plant is likely to also include commensalistic, latent pathogenic or dormant saprophytic organisms (Saikkonen et al., 2004, Schulz and Boyle, 2005, Delaye et al., 2013). It appears also, that endophytes can shift between these life strategies depending on changes in environment or host characteristics (Schulz and Boyle, 2005, Schulz and Boyle, 2006). For example, *Discula quercina* lives as an endophyte in healthy *Quercus cerris* trees, but if the oak is subjected to physiological or environmental stress this fungus becomes pathogenic, causing damage to host structures and functions (Moricca and Ragazzi, 2008, Ragazzi et al., 2001). It has been suggested, therefore, that a new definition be presented for endophytes as the set of microbial genomes present inside symptomless plant organs (Bulgarelli et al., 2013).

In a true mutualistic endophyte-host relationship, the symbiosis may confer host fitness benefits through: heat tolerance (Redman et al., 2002), tolerance to salt stress (Waller et al., 2005, Rodriguez et al., 2008), drought tolerance (Khan et al., 2016, Waller et al., 2005), protection against insect herbivores (Azevedo et al., 2000) (see also *Chapter 5*) and resistance against pathogens (Ren et al., 2013, Arnold et al., 2003, Waller et al., 2005, Ganley et al., 2008) (see also *Chapter 6*).

Environmental conditions have important effects on the species distribution and frequency of microbial endophytes. Latitude (Terhonen et al., 2011) and altitude (Hashizume et al., 2008) have been shown to alter endophytic communities, attributed to the indirect effects of temperature, precipitation and other environmental factors (Zimmerman and Vitousek, 2012). The distribution and abundance of endophytes has been shown to vary with host species (Petrini and Fisher, 1990, Peršoh, 2013, Shen and Fulthorpe, 2015) and also among genotypes of the same species (Balint et al., 2013). At the individual plant level, different plant parts are

also shown to host different compositions of endophytes, as species are likely to adapt better to the physiology and biochemistry of particular plant organs. For example, common forest trees (*Betula pendula*, *Pinus sylvestris* and *Sorbus aucuparia*) host different bacterial endophytic populations in their phyllosphere compared to their roots (Izumi et al., 2008).

Only 1-2% of the 391,000 known plants (RBG Kew, 2016) have been studied for their endophytic community, there is great potential to find new and interesting species (Strobel, 2018). Research concerning endophyte biology has seen considerable increase in scientific attention due in part to their potential to influence the ability of the plant to defend against abiotic and biotic agents. *Box 1* outlines the past and current methods used for studying tree endophytic communities.

BOX 1 – Methods used to record the endophytic communities associated with trees

The first step of any endophytic analysis must be to surface sterilise the plant material of interest, to remove the fungi or bacteria that live epiphytically. Typically, plant material will be surface sterilised by: (1) washing in tap water, to remove debris (2) washing with a surfactant, such as ethanol or Tween, to remove hydrophobic substances that may hinder the sterilising agent (3) washing with a sterilising agent, usually sodium hypochlorite, used to eradicate bacterial and fungal epiphytes and to denature DNA of epiphytes (4) and several washes with sterile water to wash away the epiphytic debris (Schulz and Boyle 2005, Hallmann et al. 2006). It may be necessary to use physical treatment in addition to chemical treatment, this is usually achieved through sonication or vortexing (Burgdorf et al. 2014, Gweon et al. 2015). The sterilisation method must be optimised for the plant species, age and tissue of interest so as to effectively remove all epiphytes without damaging plant cells and potentially losing endophytes (Hallmann et al., 1997). To ensure that the surface is thoroughly sterile it is common to either plate an aliquot of the final water wash onto general purpose media (Trivedi et al. 2010) or to imprint the sterilised plant material onto agar. Imprinting treated plant tissue is preferable as it tests the ability of the surface sterilant to remove epiphytes hidden in plant structures such as trichomes (Schulz and Boyle 2005). To test the effectiveness of the sterilisation technique to denature the DNA of epiphytes, total DNA must be extracted from the final wash solution and amplified using universal bacterial and fungal primers (Burgdorf et al. 2014).

Traditionally, endophytes have been isolated from plants using culture dependent techniques. For bacteria, plant material is macerated in a buffer solution and the extract is serially diluted onto general purpose media usually amended with fungicides (Hallmann et al., 1997). For fungi, small pieces of plant material are plated directly onto general purpose media typically containing antibiotics. Pure endophyte cultures are obtained using sub-culturing and macro and microscopic examinations are performed to group the endophytes into morphotaxa based on shared morphological traits, such as colour, colony shape and texture (Morrica et al., 2012, Ragazzi et al., 2001). As morphotaxa is not a real taxonomic entity, it is common to sequence a representative isolate of each morphotype to more accurately determine endophyte diversity (Arnold et al., 2003, Martín et al., 2013).

BOX 1 – continued…

Culture-dependent methods have a long history of use in the isolation of endophytes from their plant hosts, but it has become apparent that results using these methods may not truly represent the diversity of the entire endophytic community (Zabalgogeazcoa, 2008). For example, slower growing or rarer endophytic species are likely to be outcompeted or inhibited in the medium by more dominant rapidly growing species (Martín et al., 2013). Other species may be unculturable i.e. they may not be able to utilize the nutrients available in the media, this is especially true for obligate biotrophs that rely on their host plant for survival (Schloss and Handelsman, 2005). Culture-dependent methods can be improved by restricting rapidly growing fungi and by optimising isolation conditions, but more recent focus has been on the development of culture-independent molecular methods.

All culture-independent methods begin with total DNA isolation from surface sterilised plant tissue. Extracted DNA must be of a high quality with little contamination from proteins, RNA, polysaccharides and polyphenols (Healey et al. 2014). For many plant hosts, commercially available DNA extraction kits have proven successful (Gweon et al. 2015, Fort et al. 2016, Borruso et al. 2018). However, these kits are expensive and are not suitable for all plant species or plant tissues, especially if they have high concentrations of secondary metabolites. Since the publication of the CTAB (cetyl trimethylammonium bromide) plant DNA extraction protocol by Doyle and Doyle (1987) many have modified this method for working with difficult plant tissues and species (Porebski et al. 1997, Khanuja et al. 1999, Sahu et al. 2012).

Once high-quality DNA has been extracted, the regions of interest can then be amplified, for bacteria this is usually the 16S rRNA gene and for fungi the ITS region (or 18S rRNA gene), followed by various downstream methods that analyse the endophytic community. One such method uses terminal restriction fragment length polymorphisms or TRFLPs. The TRFLP method requires one (or both) of the amplification primers to be labelled on their 5’ end with a fluorescent marker, after amplification the PCR product is digested using restriction enzymes and the sizes of the different terminal fragments are separated by electrophoresis on automated sequencers. The result is a graph, with each peak representing a genetic variant and the height of the peak representing relative abundance.

BOX 1 – continued…

To obtain specific information on the composition of the community a clone library can be constructed. TRFLP analysis has been used extensively for community profiling, presenting accurate and reproducible results (Ulrich et al., 2008, Shen and Fulthorpe, 2015, Osborn et al., 2000, Yu et al., 2009). However, like any PCR based method, there may be a degree of amplification bias and artefact formation (Becker et al., 2000, Wang and Wang, 1997), the construction of clone libraries is also a time-consuming process.

The recent development of high-throughput sequencing (or next generation sequencing, NGS) has greatly advanced the study of endophytes by allowing enormous amounts of sequence data to be processed at a fraction of the cost of traditional methods (Knief, 2014, Rastogi et al., 2013). NGS methods use array-based sequencing in order to process a large number of reactions in parallel. A number of sequencing technologies are included under the term NGS, for example Roche 454 sequencing, Ion Torrent sequencing and the method used in this study: Illumina sequencing. Illumina technologies work by adding adaptors to the DNA fragments of interest, the DNA fragments are washed across a flowcell and the adaptors bind to complimentary primers on the flowcell surface. The DNA is separated into single strands and the flowcell is flooded with fluorescently labelled nucleotides and polymerase. These nucleotides have a terminator, so only one base is added at a time, when the base has been added a fluorescent signal is transmitted and an image is recorded. The nucleotide terminators are removed, and the next base is added, this process is continued for a set number of cycles, resulting in reads of the same length across all samples (Illumina, 2017). The read lengths are often short and therefore less accurate compared to Sanger sequencing, which is the main disadvantage of using Illumina technologies. However, Illumina technologies have been used successfully to characterise endophytic communities of a range of plant samples (Akinsanya et al., 2015, Caporaso et al., 2012, Gweon et al., 2015).

1.3. *Effects of tree phenotype on the associated tree community*

Trees, as foundation species, have the potential to shape associated community structures (Whitham et al., 2006). It is now a well understood concept in community genetics that genes which are expressed in a dominant species will have extended phenotypes at the community level (Wimp et al., 2005, Whitham et al., 2006). Thus, different genotypes and phenotypes of the same tree species should support a different assemblage of microbial and insect species, as shown with insect species associated with *Quercus robur* in Finland (Pohjanmies et al., 2015) and with *Q. petraea* in a common garden trial in France (Sinclair et al., 2015) and the fungal microbiomes of different genotypes of *Populus balsamifera* (Balint et al., 2013).

The phenotype of an individual organism is determined by an interaction between their genotype and their environment. Tree phenotypic traits, such as vigour, are expected to have an impact on the abundance and diversity of species associated with them. The plant vigour hypothesis predicts that organisms will be more abundant on plants that grow more rapidly and ultimately reach a larger size compared to the mean growth of the population (Price, 1991). These more vigorous plants are likely to have increased resources, less defence chemicals and higher food quality. Conversely, the plant stress hypothesis proposes that higher numbers of organisms will be associated with stressed plants due to reductions in defence chemicals and increased availability of nutrients (White, 1969). Although, there have been supporting evidence for both theories (Price, 1991, Price et al., 2004, Cornelissen et al., 2008, White, 1984, Miles et al., 1982, Wagner and Frantz, 1990, Mopper and Whitham, 1992), it is thought that these hypothesis should be regarded as a continuum, with some organisms preferring vigorous plants, others stressed plants and others choosing the comparative average plant (Price, 1991).

Timing of budburst, a partially genetically controlled plant trait (Scotti-Saintagne et al., 2004), seems to be particularly influential for insect herbivores. For organisms such as leaf miners, leaf defoliators and gallers, their survival depends on synchronicity with leaf flushing in the spring and leaf abscission in the autumn (Crawley and Akhteruzzaman, 1988, Mopper and Simberloff, 1995, Pearse and Karban, 2013, Tikkanen and Julkunen-Tiitto, 2003).

1.4. *Tripartite interactions in the tree system*

Tree communities contain many different species, interactions between these species varies depending on the evolutionary context and environmental conditions in which they occur but can be generally grouped into the following interactions: (1) commensalistic, where one party benefits and the other(s) is neither impaired or aided (2) mutualistic, where all parties benefit, (3) amensalistic, where the presence of one party negatively effects the other, but the first is unaffected, (4) competition, where multiple organisms vie for the same limiting resource and (5) predation/parasitism, where one party benefits to the detriment of the other. These interactions between members of the ecological community can be further subcategorised into (1) direct effects and (2) indirect effects, where the impact of one species on another is mediated or transmitted by a third party. The following sections highlight examples of these types of interactions in plant ecosystems.

1.4.1. *Interactions between plants, microbial endophytes and herbivorous insects*

During feeding and development, insect herbivores are likely to encounter microbes within their plant host. For example, larvae of the herbivorous moth *Lobesia botrana* are attracted to grape vines (*Vitis* spp.) that are infected by the fungal pathogen *Botrytis cinerea* (Mondy et al., 1998a, Mondy et al., 1998b). The fungal mycelium is a good source of water and nutrients and

the moths exhibit higher survival and faster development on infected plants. This microbe-insect relationship is truly mutualistic as the moth vectors the fungus to new hosts and creates entry wounds for infection (Mondy and Corio-Costet, 2004).

Indirect responses between microbes and insects arise when the occurrence of the first organism alters the host plant in a way that affects the second, this may take place when the two organisms are separated spatially or temporally (Hatcher, 1995, Tack and Dicke, 2013). For instance, spider mites (*Tetranychus urticae*) grow less well on cotton plants (*Gossypium* spp.) infected with the fungus *Verticillium dahliae* (Karban et al., 1987). It is possible that fungal infection causes a reduction in quantity and quality of leaf tissue available to the spider mites. Reductions in photosynthesis, changes in plant cellular structure and nutrition partitioning in infected plants often results in increased indigestible fibre and lignin and reduced levels of available water, carbohydrate and nitrogen (Hatcher, 1995). Another explanation is that fungal infection evokes chemical changes in the plant host that deters herbivory (Karban et al., 1987). In principle, wounding by herbivores or infection by pathogens activates the biosynthesis of phytohormones, these phytohormones govern the networks of defence signalling pathways which lead to the expression of the appropriate defence genes and subsequently the induction of suitable metabolite deterrents (Biere et al., 2013). It is thought that these signalling pathways may 'cross-talk', so for instance a pathogen induced response following the salicylic acid pathway may limit the herbivore induced response through the jasmonic acid pathway and vice versa (Schoonhoven et al., 2005, Thaler et al., 2012).

Indirect interactions can be further subcategorised as locally plant tissue mediated or systemic (Rostás et al., 2003). For example, infection of cabbage leaves (*Brassica oleracea*) by the necrotrophic fungus *Alternaria brassicae* deters feeding by leaf beetles (*Phaedon cochleariae*) but the host response was localised to the leaf where both antagonists were active (Rostás et al., 2003, Rostás and Hilker, 2002). Conversely, infection of the herbaceous plant *Adenostyles*

alliariae by the rust fungus *Uromyces cacaliae* induces systemic acquired resistance against a secondary attack by alpine leaf beetles (*Oreina* spp.) (Röder et al., 2007).

Plant pathogen infection may impact insect herbivores through changes in natural enemy behaviour. The presence of oak powdery mildew (*Erysiphe alphitoides*) in oak increased the risk of parasitism of the leaf miner *Tischeria ekebladella (Tack et al., 2012)*. Changes in larval phenology, changes in nutritional quality of the larvae and altered volatile emissions produced by the plant in response to pathogen infection could all influence herbivore natural enemy response (Tack et al., 2012).

Tripartite interactions among plant, microbial endophytes and herbivorous insects are being recognised for their importance and have received increasing attention by researchers. However, particularly in trees there is a paucity of evidence of the relative importance of such interactions in relation to tree performance and health even though some mechanistic understanding exists (Stout et al., 2006, Fernandez-Conradi et al., 2018). These interactions between endophytic microorganisms and insect herbivores will be addressed later in this study (*Chapter 5*).

1.4.2. *Interactions between plants, microbial endophytes and pathogens*

Endophytes and plant pathogens are likely to interact in the plant endosphere as they exploit a similar or the same limiting resource. Microbes may compete directly for space and nutrients (Arnold et al., 2003) or they can produce antimicrobial metabolites such as terpenoids, alkaloids or extracellular enzymes such as cellulases and proteases that directly antagonise competitors (Liu et al., 2001, Ren et al., 2013, Gao et al., 2010).

Endophytes have been known to protect the host from pathogen attack through induced systemic resistance (ISR), i.e. the presence of the endophyte primes the host for pathogen attack which results in increased physical or chemical barriers of resistance (Waller et al., 2005, Ganley et al., 2008). Plant pathogens may also induce changes in the host plant that alter the endophytic community. For example, grapevines (*Vitis* spp.) infected by phytoplasma disease (Bulgari et al., 2011) and citrus trees infected by citrus greening disease (or Huanglongbing) (Trivedi et al., 2010) have higher levels of reactive oxygen species (ROS), essential components of signal transduction cascades that lead to plant defences, such as the hypersensitive response and the salicylic acid pathway. Only endophytes that can overcome ROS stress can succeed in the endosphere (Kniskern et al., 2007).

Another category of interaction, commonly called facilitation, can occur in a community where one party indirectly improves the local environment for another. For example, pathogens such as *Erwinia carotovora* subsp. *atroseptica* and *Phytophthora cinnamomi* produce cell wall degrading enzymes that cause rot in potato (*Solanum tuberosum*) and avocado (*Persea americana*) respectively. This degradation of the cell wall may facilitate entry or may increase the accessibility of nutrients for endophytes (Trivedi et al., 2010).

Predicting the outcomes of pathogen-endophyte-host interactions can be challenging as plant hosts may be under attack from multiple enemies at any given time and environmental conditions are constantly changing. Traditionally, studies in plant pathology, plant-insect interactions and endophyte-host interactions have operated independently, future research needs to be cross-disciplinary as evidence shows that multipartite exchanges cannot be predicted by extrapolating from pair-wise interactions (Hatcher, 1995, Biere et al., 2013).

1.5. *Thesis outline*

This study aims to further our understanding of the bipartite interactions between trees and their insect herbivore community and between trees and their microbial endophytic community. Also considered are the much less studied tripartite interactions between trees, insect herbivores and microbial endophytes. Lastly, special consideration is given to the impacts of endophytes becoming pathogenic, comparing the tree-microbiome interactions in symptomatic and asymptomatic trees. The study systems include native oak, iconic for various reasons in the UK and elsewhere, and two species of walnut regarded by some as a tree species for the future in the UK considering future climates. The study is divided into the following sections:

1. Characterising the insect herbivore communities associated with healthy *Quercus* trees in the UK and the effect of tree species, tree provenance and tree phenotype on these communities (*Chapter 2*).

2. Documenting the microbial endophytic communities associated with healthy *Quercus* trees in the UK and the effect of tree species, tree provenance and tree phenotype on these communities (*Chapter 3*).

3. Documenting the microbial endophytic communities associated with healthy *Juglans* trees in the UK and the effect of tree species, tree provenance and tree phenotype on these communities (*Chapter 4*).

4. Revealing the interactions between the microbial endophytic community and insect herbivores associated with *Quercus* trees in the UK (*Chapter 5*).

5. Comparing the microbial endophytic communities associated with asymptomatic *Quercus* trees with those displaying signs of decline (*Chapter 6*).

1.6. *Study systems*

The following section introduces the tree species of interest for this study, why they were chosen, the organisms that are known to be associated with these trees and the challenges facing these trees in the UK today and in the future.

1.6.1. *Quercus robur and Q. petraea*

The genus *Quercus* in the family *Fagaceae*, comprises at least 500 species distributed in the northern hemisphere (Jones, 1959, Manos et al., 1999). Two species of *Quercus* are native to Britain, *Quercus robur* L. and *Q. petraea* (Matt.) Liebl.. These two large broadleaved species occur in most of lowland Europe, with the range of *Quercus robur* extending further north east than *Q. petraea*. In the UK, *Quercus robur* is more dominant in southern England, west Wales, central and northern England and southern Scotland (Cottrell et al., 2002) (*Figure 1.1*). Both species tolerate a wide range of soil conditions but thrive on moist, fertile soil. *Quercus robur*, is slightly better suited to heavier soils and will tolerate a degree of waterlogging, while *Q. petraea* is more drought tolerant preferring well-drained, more acidic soil (Jones, 1959, Morris and Perring, 1974). *Quercus robur* is more commonly found in valleys and lowlands while *Q. petraea* prefers slopes and hilltops. Both are light-demanding species but *Quercus petraea* is significantly more shade tolerant than *Q. robur* (Jones, 1959). Nevertheless, *Quercus robur* and *Q. petraea* are sympatric across most of their distribution.

Both species are extremely long-lived trees (up to 800 years or more) and have the potential to grow to above 30 metres in height (Phillips, 1978). Leaves appear in April-May with a second or third flush, called lammas growth, appearing in July or August. Leaves are abscised in late October-November. Budburst phenology is temperature sensitive and has advanced as

temperatures have increased as a result of climate change in recent decades (Parmesan, 2007, Roberts et al., 2015).

Leaf and acorn morphology of the two species are very similar, with a few subtle differences. Leaves of *Quercus robur* are glabrous and almost sessile, leaves of *Q. petraea* are borne on long petioles and veins on the underside are pubescent with some stellate hairs on the lamina. *Quercus robur* acorns are borne on long stalks (peduncles) and *Q. petraea* are sessile (or sub-sessile) (Aas and Riedmiller, 2008). However, these species show a wide range of natural variation and therefore there is no single morphological feature that can be used to unambiguously distinguish between them with sufficiency certainty (Rushton, 1993). This is further complicated as natural hybridisation between *Quercus robur* and *Q. petraea* occurs, due in part to their overlapping distributions. Nevertheless, it is possible to determine two distinct groups using multivariate analyses of morphological traits (Kremer et al., 2002; Curtu et al., 2007; Boratynski et al., 2008).

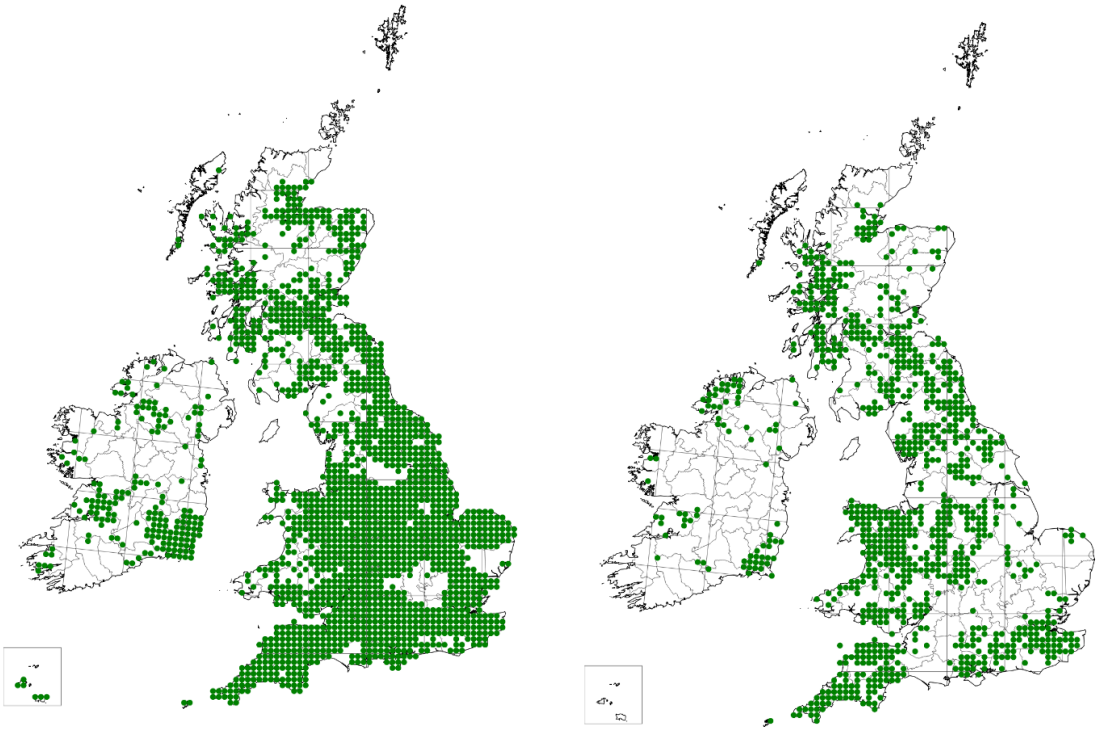

Figure 1.1 – distribution of *Quercus robur* (left) and *Q. petraea* (right) in the UK and Ireland (post 2010). Each dot represents a record in a 10km square of the national grid. Map from www.bsbidb.org.uk/maps

Oaks were chosen as the focal system for this study due to their ecological, historical and cultural value. One of Britain's most loved tree species, the oak is the national tree of England with a long history of importance in British culture. The hard and durable timber has been used in construction for centuries, traditionally used for ship building it is now used mainly for furniture and fencing. The wood is reasonably resistant to insect and fungal attacks due to the high tannin content and is particularly valuable for making wine and spirit casks as it is also resistant to liquids. The bark has been used in the past in leather tanning. Acorns are a valuable food source for many birds and mammals, especially in mast years, and are fed to livestock (Jones, 1959, Eaton et al., 2016, Ducousso and Bordacs, 2004). Due to their large size and longevity, oak trees are also of major ecological importance, supporting a wealth of biodiversity. In the UK, native oaks support the highest levels of biodiversity of all UK tree species, 326 of which are obligate associates (Mitchell et al., 2019, Kennedy and Southwood, 1984).

Oak species are model candidates for studying the influence of tree phenotype on insect herbivores as there are thought to be more insect species associated with native oak trees in Britain than any other British tree (Kennedy and Southwood, 1984). The most recent account found 1178 invertebrates supported by *Quercus robur*, *Q. petraea* and their hybrids in the UK. Of these, 257 invertebrate species were obligate, and 104 species were highly associated with oak (Mitchell et al., 2019). These insects can be divided into several different feeding guilds, the ones of interest to this study are free feeders, leaf webbers, leaf rollers, leaf miners and gallers. These feeding guilds are described in more detail in the above *Section 1.2.1* and in *Chapter 2*. These feeding types all represent different levels of intimacy of interaction with the host tree; free feeders encounter the external environment of the host, leaf miners and gallers experience both the external and the internal environment and galling insects manipulate the morphology and physiology of the host tissues surrounding them. Tree phenotypic traits, for

example vigour and phenology, are likely to influence these feeding guilds in different ways due to the differences in their interaction with the host. Oak trees are an ideal study system to understand these bipartite interactions due to the richness and diversity of herbivore feeding guilds associated with them.

Oak trees in Europe, and elsewhere, are increasingly affected by decline. Tree decline can be defined as a progressive deterioration in health of a mature tree, the etiology is often complex involving multiple biotic and abiotic forces (Manion, 1981, Haavik et al., 2015). The first recorded decline episode of oaks in the UK was in the 1920s (Gibbs and Greig, 1997). This decline episode was believed to have started with intensive defoliation by *Tortrix viridana*, coupled with subsequent infection of secondary growth by oak powdery mildew and a possible attack by *Armillaria mellea*, a fungal pathogen that causes root and butt rot (Robinson, 1927, Osmanston, 1927, Day, 1927). Oak powdery mildew is one of the most common diseases of European oak forests (Marçais and Desprez-Loustau, 2014). Caused by the biotrophic fungal pathogen *Erysiphe alphitoides* and related species (Mougou et al., 2008), the white mycelium of this pathogen covers the surface of the leaves, absorbing nutrients from the plant host (Hewitt and Ayres, 1976), often resulting in leaf distortion (*Figure 1.2*).

This decline episode subsided in 1925 but since the 1980s several oak tree species in the UK, Europe and the US are once more in decline (Biosca et al., 2003, Gibbs and Greig, 1997, Thomas et al., 2002, Leininger, 1998, Brady et al., 2014). The symptoms are not the same in all regions, however most display yellowing of leaves and dieback of branches (Denman and Webber, 2009, Gibbs and Greig, 1997). The causal agents are also not likely to be the same in all regions, however oak decline syndrome is typically triggered by an abiotic stress for example: drought, soil compaction, poor soil drainage, poor air quality and excessive soil nitrogen. These abiotic stressors reduce the carbohydrate reserves of the tree (Bréda et al., 2006) leaving the tree unable to defend against subsequent attack by biotic forces such as

opportunistic insects, bacteria and root and butt rot fungi (*Armillaria*, *Collybia* and *Phytophthora* species for example) (Denman and Webber, 2009, Gibbs and Greig, 1997, Thomas et al., 2002).

Another oak decline symptom often observed is the presence of dark exudates on the bark, trees with these bleeding cankers typically dieback rapidly (within 3-5 years) and are associated with high rates of tree mortality (Denman and Webber, 2009, Denman et al., 2014). This oak decline syndrome, termed acute oak decline (AOD) in the UK (Denman and Webber, 2009) will be the focus of the latter part of this study (*Chapter 6*).

Figure 1.2 – (above) symptoms of oak powdery mildew (*Erysiphe* sp.) on oak leaves in the UK, (right) shows the bleeding bark lesion characteristic of Acute Oak Decline (AOD). Wimbledon Common, London, photos taken by S. Roy

1.6.2. *Juglans regia and J. nigra*

Two deciduous species belonging to the large plant family *Juglandaceae* are commonly grown in the UK: *Juglans regia* L. (or common walnut) and *J. nigra* L. (or black walnut). *J. regia* originates from south-eastern Europe through to China (Phillips, 1978). However, as this

species has been widely cultivated for nut and timber production throughout Europe it is thought to be somewhat naturalised in these areas (Aas and Riedmiller, 2008) (*Figure 1.3*). *Juglans nigra* is native to eastern and central US, but is also commonly grown in Europe (Phillips, 1978). The hard shell of *Juglans nigra* makes it a less attractive tree for nut production and is grown primarily as a timber or ornamental tree (Ginzel, 2010).

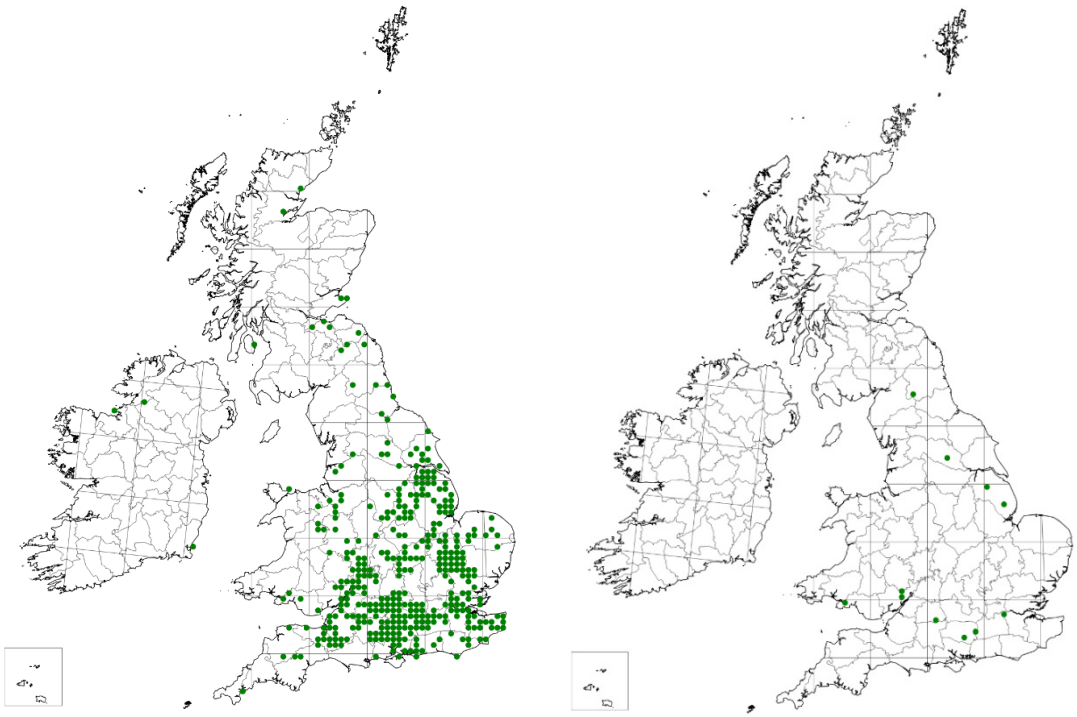

Figure 1.3 – distribution of *Juglans regia* (left) and *J. nigra* (right) in the UK and Ireland, post 2010. Each dot represents a record in a 10km square of the National Grid. Map from: https://bsbi.org/maps

The two species can be easily differentiated based on bark and leaf morphology (*Figure 1.4*). Bark of *Juglans regia* is pale grey and smooth between deep fissures, while bark of *J. nigra* is dark brown with a network of diamond shaped furrows (Mitchell, 1974). Leaves of both species are pinnate, but *Juglans regia* usually has seven large elliptic leaflets and *J. nigra* 11 to 23 smaller narrowly elliptic leaflets (Aas and Riedmiller, 2008) (*Figure 1.4*).

Figure 1.4 - *Juglans regia* bark (top left), *J. nigra* bark (top right), *J. regia* leaves (bottom left) and *J. nigra* leaves (bottom right). Paradise Wood, Oxfordshire, photos taken by S. Roy.

Juglans species will be the focus of one part of this study as interest in growing walnut trees for timber in the UK is increasing (Hemery, 2004). Under the future environmental conditions predicted by climate change models (Broadmeadow et al., 2005), there is great potential for *Juglans* species to thrive in the UK and to produce high quality, valuable timber. However, walnut trees, are susceptible to bleeding cankers. These bleeding cankers are caused by similar pathogens to those causing AOD (*Brenneria* sp.), potentially sharing a similar aetiology and are therefore of interest to this study (*Chapter 4*).

CHAPTER 2 - *Evaluating the influence of tree phenotype on insect herbivore communities of Quercus species in the UK*

2.1. *Introduction*

Trees are vital components of forest ecosystems, shaping community structures and ecosystem processes. Oak trees are of great ecological importance, they provide food and habitat to a wide variety of animals, plants and microbes. In the UK, native oaks support the highest levels of biodiversity of all UK tree species (Mitchell et al., 2019, Kennedy and Southwood, 1984). Not only are they of ecological significance they are one of the most economically important deciduous forest trees in Europe. The hard, durable timber has been used in construction for centuries, the bark used in tanning and the acorns as food for livestock (Jones, 1959, Eaton et al., 2016, Ducousso and Bordacs, 2004).

2.1.1. *Quercus robur and Q. petraea*

Quercus robur L. and *Q. petraea* (Matt.) Liebl. are large long-lived broadleaved trees. Native to most of lowland Europe, *Quercus robur* and *Q. petraea* are sympatric across most of their distribution, but the range of *Q. robur* extends further north east (EUFORGEN, 2009a, EUFORGEN, 2009b). In Great Britain, they represent around 23% of the wooded area (Forestry Commission, 2003). Natural hybridisation between *Quercus robur* and *Q. petraea* is particularly common, due in part to their overlapping distributions (Curtu et al., 2007). However, despite interspecific gene flow, multivariate analyses of leaf morphological traits have been used successfully to differentiate between the two species (Kremer et al., 2002, Curtu et al., 2007, Boratynski et al., 2008). More recently, molecular techniques have been used to differentiate the two oak species but finding a single specific marker to differentiate between the species

has proved difficult (Muir et al., 2000, Cottrell et al., 2002, Guichoux et al., 2011). Given the morphological (and genetic) similarities of these species it might be assumed that organisms associated with the two UK native oak species would show few differences, and very few have studied this. Southwood et al. (2004) compared the richness of all insect herbivore orders on the two native oak species and found them to be very similar. The following study compares the insect herbivore communities of *Quercus robur* and *Q. petraea* in a provenance trial in the UK. Hypothesis 1: *Quercus robur* and *Q. petraea* would show no interspecific differences in their insect herbivore communities.

2.1.2. *Insects associated with oak in the UK*

More insect species are thought to be associated with native oak trees in Britain than any other UK tree (Kennedy and Southwood, 1984, Southwood, 1961). The most recent account found 1178 invertebrates supported by *Quercus robur, Q. petraea* and their hybrids in the UK. Of these, 257 invertebrate species were obligate, and 104 species were highly associated with oak (Mitchell et al., 2019). These insects can be divided into three different feeding guilds that also reflect differing degrees of intimacy of interaction between host and insect: (1) defoliating insects (2) mining insects and (3) galling insects.

Oak trees are particularly vulnerable to attack from free-feeding larvae of the Lepidoptera order, many of which are recognised pests, e.g. *Operophtera brumata*, *Tortrix viridana, Lymantria dispar* and *Thaumetopoea processionea*, a recently arrived alien species in the UK. If present in sufficient numbers these pests can defoliate trees. Single spring defoliation events have limited effects on tree growth however, as oak trees are able to refoliate within the same season, referred to as lammas growth (Jones, 1959). Defoliating insects have different feeding behaviours that have been differentiated into the following feeding guilds for this study: (1) leaf chewing insects, these include members of the coleopteran and lepidopteran order that

feed externally, removing sections of leaf tissue (2) leaf webbing insects, members of the lepidopteran order e.g. *Acrobasis consociella* that spin a silk web around multiple leaves within which they feed and (3) leaf rolling insects e.g. *Tortrix viridana* that use webbing to manipulate a single leaf.

Many insects reside for part of their life cycle within the plant tissues of oak trees and can therefore be described as endophytic; these include members of the mining and galling feeding guilds. This endophytic relationship provides a number of advantages to the insect. Firstly, galls in particular contain tissues that are higher in nutrients with fewer defence chemicals than non-galled tissue (the nutrition hypothesis). Secondly, gall and mine structures protect the insect from unfavourable abiotic conditions such as water stress (the microenvironment hypothesis). Lastly, these structures provide protection from attack from natural enemies (the enemy hypothesis) (Stone and Schönrogge, 2003).

There are a number of leaf miners associated with oak trees in the UK and they span multiple orders, including Coleoptera, Diptera, Hymenoptera and most commonly Lepidoptera (Claridge and Wilson, 1982). Leaf miners feed, for at least part of their lifecycle, between the layers of epidermis in the leaves. Adult miners lay eggs on the leaf surface, after hatching the larvae enter the leaf through the epidermis and feed on the spongy mesophyll layer or the palisade tissue between the veins of the leaf before pupating and exiting from the leaf surface (Hausman, 1941, Askew and Shaw, 1974). The most commonly encountered mines on UK native oaks belong to the *Phyllonorycter* genus. These species are identified by tentiform mines usually with a crease in the lower epidermis, the fully-grown larvae pupate inside a white silk cocoon (Askew and Shaw, 1974). Members of the *Phyllonorycter* genus often have two generations in a year, the eggs of the first-generation hatch in spring and adults emerge in mid-summer. The second-generation over-winters as pupae and adults emerge the following spring (Miller, 1973). Also common on oaks in the UK, members of the *Coleophora* genus

produce mines of a very different morphology. The larvae construct a cigar-like case that is attached to the leaf surface and penetrates the epidermis, they mine as far as they can reach without leaving their case (Askew and Shaw, 1974, Csóka, 2003).

Approximately 60 species of insect are known to form galls on oak species native to the UK and they can be formed on all plant parts: leaves, buds, acorns, roots, trunk, twigs and catkins (Redfern, 2011). A plant gall is an abnormal growth of the host plant induced by another organism (Redfern and Askew, 1992). The insect disrupts normal cell growth and through enlargement and proliferation of plant host cells creates shelter and food (Chinery, 2013). The most well recognised gall forming insects on oak trees belong to the hymenopteran superfamily Cynipoidea. Gall formation can be divided into three stages (1) the initiation phase starts when the female cynipid wasp oviposits into the host plant, the plant cells surrounding the egg lyse and produce a chamber in which the larvae develops (2) the growth phase begins when the insect manipulates the plant host cells into forming a gall (3) maturation, the larvae feed on the nutritive cells that now line the inside of the gall, the gall will eventually be shed from the host and the adult wasp will emerge (Stone and Schönrogge, 2003, Stone et al., 2002). Gall morphologies are often not just species specific, but where species have more than one alternating agamic (asexual) and sexual generations per year, they are also generation specific (Stone et al., 2002). The female of the agamic generation will oviposit eggs into the host plant early in the year, producing the gall of the sexual generation in spring. Adults emerge late spring, mate and lay eggs producing the gall of the agamic generation in autumn (Stone et al., 2002). For example, *Andricus quercuscalicis*, the knopper gall, which arrived in southern England in the 1950s and has since spread across the UK, has two very different generations (Schönrogge et al., 2012). The spring sexual generation develops in small galls on the catkins of the introduced tree *Quercus cerris*, while the second agamic generation develops waxy ridge protrusions on the acorns of native oak. Given these morphological differences

between the sexual and agamic generations of galling insects, they will be considered separately in this study.

2.1.3. *Impact of phenotypic variation on herbivory*

In oak woodlands, there is noticeable variation in the susceptibility of individual trees to herbivore attack. Even amongst oak trees growing in close proximity, some trees consistently support fewer or no folivores, whilst others are heavily attacked (Crawley and Akhteruzzaman, 1988). Phenotypic variation in the host plant can have cascading effects on higher trophic levels, such as insect herbivores (Wimp et al., 2005, Whitham et al., 2006). A number of theories have been devised to explain how insect herbivore abundance and diversity is correlated to host phenotype.

2.1.3.1. *Impact of tree vigour and stress on herbivory*

Tree health is expected to have an impact on the presence and abundance of organisms associated with them. There are two well-known conflicting theories linking aspects of plant phenotype to the observed variations in insect herbivore densities. The first is the plant stress hypothesis, which proposes that herbivore abundance will be higher on stressed plants due to reductions in defence compounds (Rhoades, 1985) and/or increased availability of nutrients (White, 1984, White, 1969). Experiments testing this theory have produced conflicting results (White, 1984, White, 1969, Miles et al., 1982, Watt, 1986, Wagner and Frantz, 1990, Mopper and Whitham, 1992). A more recent meta-analysis has suggested that the consequences of host plant stress on phytophagous insects will vary depending on their mode of feeding (Larsson, 1989, Koricheva et al., 1998). Boring and sucking insect guilds will, in general, perform better on stressed trees but gallers and chewing insects will be negatively affected by plant stress (Koricheva et al., 1998). White (2009) revised their plant-stress hypothesis, so that

it is confined to insects that feed on mature tissue that are in the process of senescing and to exclude insects that solely feed on young developing modules.

When testing this plant stress hypothesis many researchers also found that some insects more frequently fed and/or oviposited on younger, more vigorous plants compared to older, mature plants. These observations led to the plant vigour hypothesis, that predicts insect herbivores will be more abundant on the most vigorous plants or the most vigorous organs of a plant (Price, 1991). These more vigorous plants are likely to have more available feeding or oviposition sites, higher resource quality and lower concentrations of defensive compounds (Price, 1991). The plant vigour hypothesis is thought to be of most significance to the galling insects due to their more intimate relationship with plant growth (Price, 1991, Cornelissen et al., 2008). While, there is support for the plant vigour hypothesis across a range of herbivore taxa and feeding guilds, some studies found no supporting evidence that herbivore abundance correlates with vigour (Price et al., 2004, Cornelissen et al., 2008). It has therefore been suggested that the above two hypotheses should be regarded as a continuum, with some insect herbivores attacking vigorous plants, others attacking stressed plants, and some choosing a comparative 'average' plant (Price, 1991, Koricheva et al., 1998).

In this study, the effect of tree vigour and tree stress on herbivore densities in the *Quercus* host system will be tested. It is predicted that host vigour and host stress will impact the galling insects to a greater degree than the mining and defoliating insects, due to the stronger intimacy of interaction between this guild and their host plant. Hypothesis 2: tree vigour and tree stress would be significant drivers of gall insect abundance and would be less important drivers of leaf mining and defoliating insect abundance.

2.1.3.2. *Impact of leaf phenology on herbivory*

UK native oaks display high intraspecific variability in the date of budburst, a range of almost three weeks between individuals (Jones, 1959). This timing of budburst in the spring is a partially genetically controlled trait but may also be influenced by climate and by age (Crawley and Akhteruzzaman, 1988, Parmesan, 2007).

It is well established that host phenological variation can influence the abundance and performance of insect herbivores on oak trees (Hough, 1953, Askew, 1962, Rosenthal and Koehler, 1970, Crawley and Akhteruzzaman, 1988). In the case of generalist defoliating insects (e.g. *Tortrix viridana* and *Operophtera brumata*), early budburst appears to be preferable (Jones, 1959, Satchell, 1962, Hunter, 1992, Tikkanen and Julkunen-Tiitto, 2003). Leaves in the early spring have a high nitrogen content and low tannin concentration. As the season progresses leaves accumulate defence compounds and become less digestible by chewing insects (Feeny, 1970, Forkner et al., 2004). The survival of these defoliating insects therefore depends highly on the synchronicity of their emergence with leaf flushing in the spring (van Asch et al., 2007). The associations between date of budburst and abundance of galling and mining insects, in the literature are invariably discordant. Leaf miners associated with valley oak (*Quercus lobata*) were at higher densities on trees that budburst earlier in the season (Pearse and Karban, 2013, Pearse et al., 2015). This is in accordance with *Dyseriocrania subpurpurella* on UK native oak trees (Crawley and Akhteruzzaman, 1988). However, Mopper and Simberloff (1995) found that oak trees suffered higher rates of herbivory, by the leaf miner *Stilbosis quadricustatella*, if they budburst later in the spring. Regarding the galling insects, the conclusions are also varied (Askew, 1962), with some studies showing no effect of host phenology on cynipid abundance (Ito and Hijii, 2001). There is even evidence of spring and autumn generations of the same *Neuroterus* galling species differing in their response to budburst (Sinclair et al., 2015).

Quercus robur and *Q. petraea* are deciduous, a habit that is believed to be an adaptation to better survive winter conditions, to conserve water over winter, and to maximise photosynthesis when conditions are optimal (Chabot and Hicks, 1982). Many members of the family Fagaceae, including *Quercus robur* and *Q. petraea*, are known to retain senescent leaves through winter, a habit termed leaf marcescence. The evolutionary explanation for why this phenomenon occurs remains unclear. It is conceivable, as with budburst in the spring, that the timing of leaf drop is influenced by insect herbivory. It has been suggested that early leaf abscission of *Populus* trees is an adaptive response triggered by herbivory by gall aphids (Williams and Whitham, 1986). Stiling and Simberloff (1989) reported similar findings with leaf miners on *Quercus nigra*.

The effect of budburst date and the tendency for leaf marcescence on the different feeding guilds and species associated with *Quercus* trees will be tested. Hypothesis 3: budburst phenology will influence damage caused by defoliating insects, the effect of phenology on galling and mining insects would be variable. Hypothesis 4: the retention of senescent leaves would affect insect herbivory by galling and mining insects

2.1.4. *Climate change effects and assisted migration*

Climate change models forecast increasing summer temperature and drought scenarios for the UK, while winter precipitation and the frequency of extreme events may also increase (IPCC, 2014). This will be a challenge to locally adapted trees, potentially inducing increasing amounts of stress with potential implications for tree vigour. In an effort to conserve forest species, human-aided relocation of species or 'assisted migration' schemes may be implemented. Assisted migration (or climate matching) involves planting seed stock from exotic provenances that match the predicted climate of the planting site in 50-100 years (Broadmeadow et al., 2005, Hubert and Cottrell, 2007). Perceived disadvantages include issues around biosecurity

when moving tree material (Ricciardi and Simberloff, 2009, Simler et al., 2018), but also that introduced trees would differ in their phenotypes from local provenances and potentially in ways that might affect interactions with local associated species or taxa (Sinclair et al., 2015, Edmunds and Alstad, 1978). Hypothesis 5: local and nonlocal provenances would differ in their phenotypic traits including tree vigour and budburst phenology. Hypothesis 6: insect herbivore abundance would be highest on local provenances, in accordance with the local adaptation hypothesis.

This study addresses questions about tree origin (provenance), tree phenotype and associated herbivore abundance using an experimental trial plantation of oak in the UK. Firstly, leaf morphometric characteristics were tested to see if oak species can be identified and then the variation in phenotypic characters (vigour and phenology) between tree species and between provenances for indications of interspecific and intraspecific adaptation. Finally, the distribution of the abundance patterns in different feeding guilds of insect herbivores were assessed against predictions from the plant vigour hypothesis and plant stress hypothesis.

2.2. *Materials and Methods*

2.2.1. *Site description*

Paradise Wood is a collection of trial plantations located in Oxfordshire, UK (lat: 51.637, long: -1.198) (*Figure 2.1a*). Established by Earth Trust in 1993, the main aim of the trials is to improve the quality and productivity of hardwood timber species of commercial importance to the UK (Clark and Hemery, 2009). Seeds were collected, by Earth Trust, from global 'plus trees', characterised as displaying desirable traits for timber production: straight stems, resistance to disease and fast growth.

Paradise Wood is located at an altitude of 50m, with a mean temperature of about 11°C, an average monthly rainfall of 95mm and only three months of the year (June-August) free from air frost (Met Office, 2019). Soil in Paradise Wood is described as luvisol, with a clay enriched subsoil. The soil is very slightly acidic with a pH of between 6.5-7.2 and a sandy to sandy loam texture (IUSS Working Group WRB, 2015).

The trial of interest here is the oak breeding seedling orchard (BSO) trial. Planted in 2003, this trial includes both *Quercus robur* and *Q. petraea* progeny from 56 parent 'plus trees' from across the UK, Netherlands, Ireland and France (*Figure 2.1b*). Offspring of each individual tree represents a half-sibling family i.e. with a shared parent female that potentially was pollinated by multiple males. Planted in a randomised complete block design, this trial includes 39 blocks each containing one progeny from each of the 56 'plus trees', planted with 2 by 2 metre spacing. Fourteen of these blocks were randomly selected for this study (*Appendix D.1*). Due to tree mortality, the blocks are unbalanced. Trees were also removed from the analysis if they were growing outside of their protective tree guard, due to uncertainty of provenance, and if they exhibited severe dieback.

Figure 2.1 – (a) map of the UK showing location of Paradise Wood (white cross). Map created using R version 3.5.2 (R Core Team 2018), maps (version 3.3.0, Becker et al. 2018), and ggmap (version 3.0.0, Kahle and Wickham 2013) **(b) aerial photograph of Earth Trust Paradise Wood, shaded box indicates location of the BSO trial.** Photo taken from Google Maps.

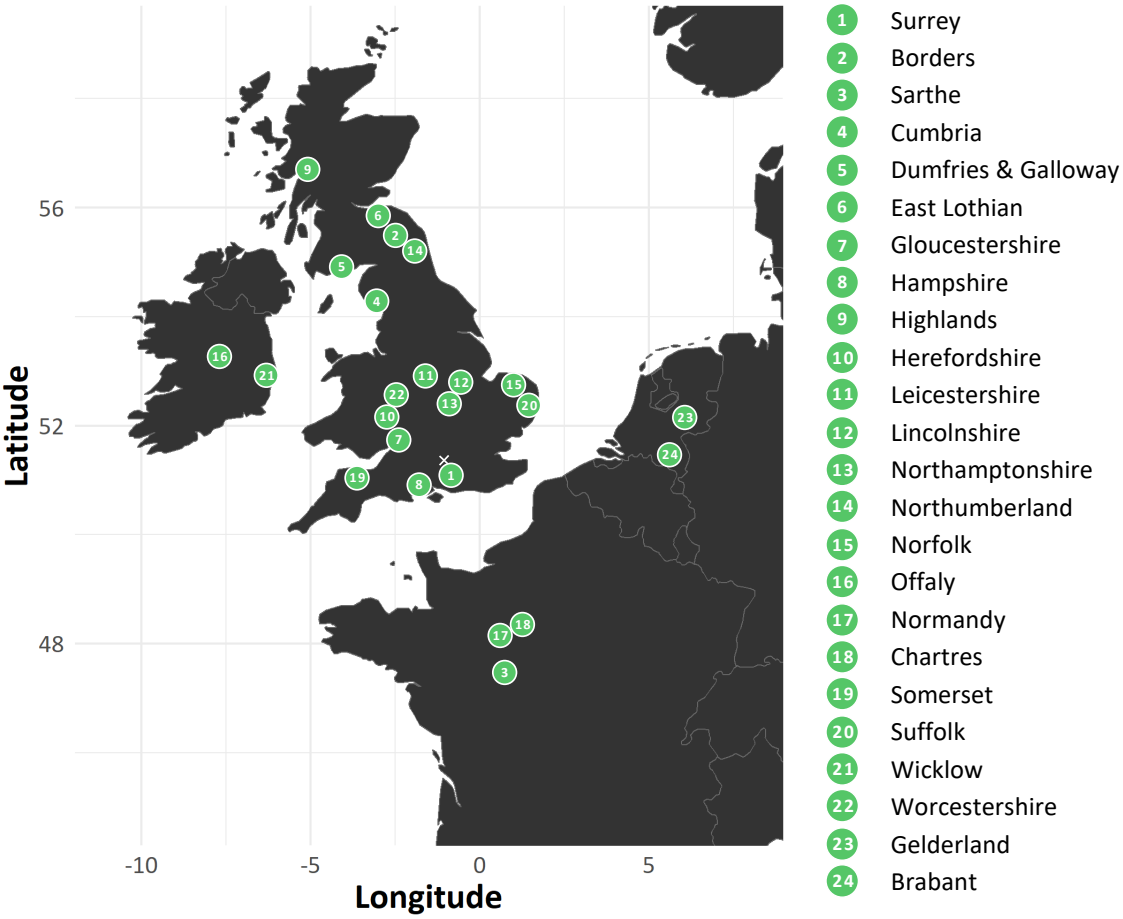

Figure 2.2 - geographic locations of parent trees planted in the BSO oak trial. Map created using R version 3.5.2 (R Core Team 2018), package maps (version 3.3.0, Becker et al. 2018) and ggmap (version 3.0.0, Kahle & Wickham, 2013). White cross indicates location of Paradise Wood.

2.2.2. *Determining oak species using leaf morphometrics*

The BSO trial in Paradise Wood contains both UK native oak species, *Quercus robur* and *Q. petraea*, but there was no record of which trees belong to which species. Using a method developed by Kremer et al. (2002) it is possible to determine oak species using leaf measurements, which is a cheaper alternative to DNA barcoding. Five fully expanded, insect and disease-free leaves, from the first flush, were selected at random from the mid canopy of each oak tree of interest. Using a ruler, five characters were measured (*Figure 2.3*): (1) lamina length, (2) petiole length, (3) lobe width, (4) sinus width and (5) length of lamina at largest width. The number of lobes and number of intercalary veins were counted. Five variables were then calculated from these measurements:

$$\boldsymbol{Laminar\ shape}\ or\ \boldsymbol{obversity} = 100 \times \ (length\ of\ lamina\ at\ largest\ width)/(lamina\ length)$$

$$\boldsymbol{Petiole\ ratio} = 100 \times \ (petiole\ length)/(lamina\ length + petiole\ length)$$

$$\boldsymbol{Lobe\ depth\ ratio} = 100 \times \ (lobe\ width\ - sinus\ width)/(lobe\ width)$$

$$\boldsymbol{Percentage\ venation} = 100 \times \ (number\ of\ intercalary\ veins)/(number\ of\ lobes)$$

$$\boldsymbol{Lobe\ width\ ratio} = 100 \times \ (lobe\ width)/(lamina\ length)$$

The basal shape of the lamina was scored from 1-9 according to the index of Kremer et al. (2002). Using a card with a 2mm x 2mm square window, a stereomicroscope (x30) was used to score abaxial laminar pubescence within the square, according to the grading system of Kissling (1980): (1) no pubescence (2) 10% pubescence coverage, (3) 30% pubescence coverage, (4) 50% pubescence coverage, (5) 70% pubescence coverage and (6) above 90% pubescence coverage.

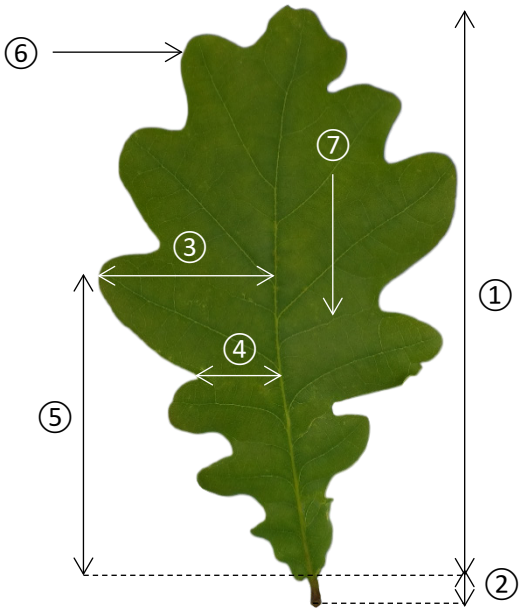

Figure 2.3 – five measured leaf traits: ① lamina length, ② petiole length, ③ lobe width, ④ sinus width and ⑤ length of lamina at largest width. The two counted variables: ⑥ number of lobes and ⑦ number of intercalary veins. These values were used to calculate five derived variables, which were used together with the basal shape and abaxial laminar pubescence in a PCA to differentiate between the two native oak species in the UK. Diagram adapted from Kremer et al. 2002. Photo taken by S. Roy.

The *MASS* package (version 7.3-51.3, Venables and Ripley (2002)) in R (version 3.5.2, R Core Team (2018)) was used to perform a principal component analysis (PCA). The PCA was used to determine which of the seven leaf variables: lamina shape (or obversity), petiole ratio, lobe depth ratio, percentage venation, lobe width ratio, basal shape and abaxial laminar pubescence best explained the differences between individual oak trees. Using the first two components of the PCA, K-means clustering was used to cluster the individual trees into groups based on their similarity. A discriminant function analysis was used to determine the accuracy of the PCA.

2.2.3. *Estimating tree vigour*

Tree traits, such as height and diameter at breast height (DBH), have long been used by forestry experts to measure tree vigour (Innes, 1990). The circumference of the trunk at breast height (1.3m) was measured using a tape measure. DBH was calculated using the following equation:

$$\boldsymbol{DBH} = (Circumference\ of\ trunk\ at\ 1.3m)/\pi$$

To estimate tree height a smartphone tilt meter application (Bubble Level, Version 3.12) was used to measure the angle of elevation of the top of the tree crown from the horizontal. To account for measurement error, this reading was taken three times and an average calculated. Considering the height of the measurer's eye to the ground, and the distance from the measurer to the base of the tree, the following calculation was used to estimate tree height:

$$\boldsymbol{Height} = \tan(average\ angle\ of\ elevation) \times distance\ of\ the\ measurer\ to\ tree + height\ of\ measurer's\ eye\ above\ ground$$

The length of ten primary shoots of each tree were also measured from the current ring bark scar using a measuring tape and an average length per tree was calculated. Correlation between height and DBH and between shoot length and DBH was tested using a Pearson product-moment correlation test. Trees showed a strong correlation between their height and DBH (*Section 2.3.2*) so it was decided that only DBH would be used in further analysis. Shoot length showed a weak correlation with DBH so will be included in further analysis (*Section 2.3.2*). Generalised linear mixed effect models (GLMM) were run to test for differences in tree vigour between provenances for DBH and shoot length separately, as described in *Section 2.2.6*.

2.2.4. *Measuring leaf phenology*

2.2.4.1. *Date of budburst and full flush*

The date of budburst of each of the 590 trees was recorded to determine whether provenance and vigour of the host correlates with phenology, which in turn could affect insect herbivore density and composition. Budburst was assessed by visual examination approximately every three days starting in late March and continuing into early June 2016. At each survey, leaf phenology was classified on an ordinal scale ranging from 0 to 5, according to the furthest developmental stage of >50% of buds on each individual tree (*Figure 2.4*). The scale was

modified from Crawley and Akhteruzzaman (1988) and Ducousso et al. (1996). Two timings were used in the analyses: the number of days from the first day of meteorological spring (1st March 2016) to budburst (stage 1) and the number of days taken from budburst (stage 1) to full flush (stage 5).

The trees were ranked based on their date of budburst; the first trees to burst were given a rank of 1, the second trees to burst a rank of 2 and so on, as described by Crawley and Akhteruzzaman (1988). Similarly, flush date was ranked in the same way. Correlation between budburst date and flush date was tested using a Pearson product-moment correlation test. There was a strong correlation between date of budburst and date of full flush (*Section 2.3.3.1*) so it was decided that only budburst date (ranked) would be used in further analysis. GLMMs were run to test for differences in tree phenology between provenances, as described in *Section 2.2.6*. The dates of budburst were previously recorded on the same set of trees in 2008 (Peters, 2008, unpublished). These results were compared to those of 2016 to see if the variability in date of budburst within this oak population is consistent each year using a Pearson's product-moment correlation test.

2.2.4.2. *Leaf marcescence*

In winter 2015/2016 it was observed that some of the oak trees in the BSO trial retained their senescent leaves over winter, a phenomenon known as leaf marcescence, while others abscised their leaves in autumn. This was recorded as either 0 (leaves were abscised) or 1 (leaves were retained). The distance of each tree from the edge of the trial was determined to examine whether the variability in leaf retention was due to increased weather exposure at the edges. Trees on the edge of the trial were given the value 0, the next row in a 1 and so on until the middle was reached. GLMMs were used to test for the effect of tree phenotypic variables on the marcescence habit, as described in *Section 2.2.6*.

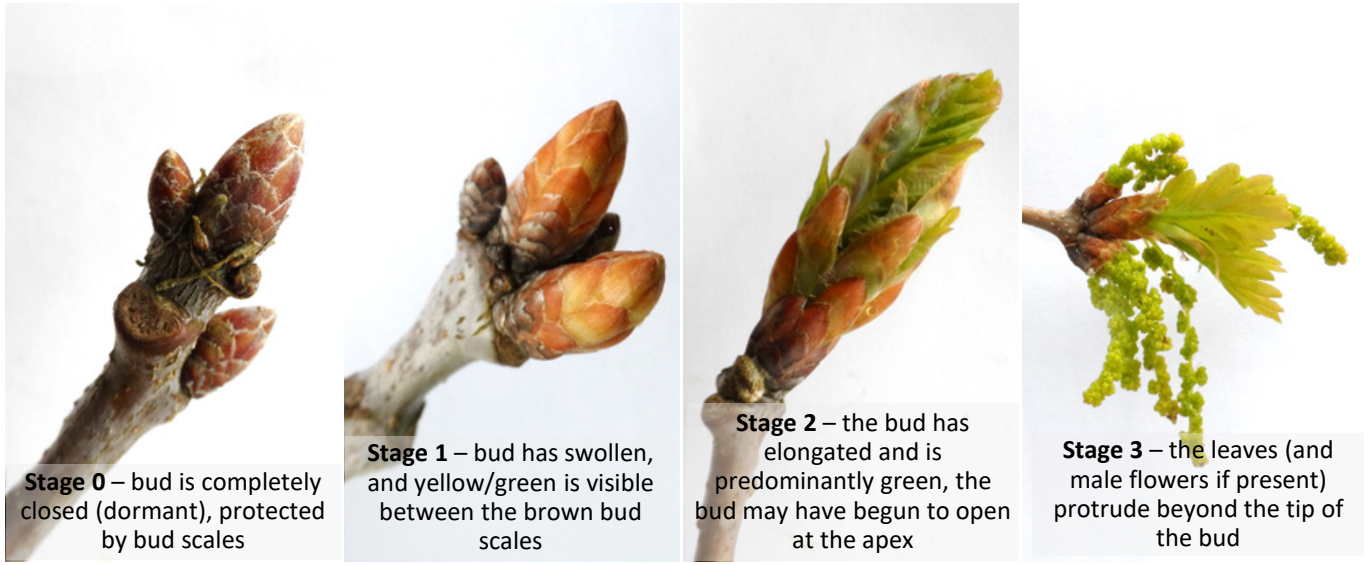

Figure 2.4 - 6 developmental stages of budburst recorded in the BSO oak trial in Paradise Wood in spring 2016. Photos taken by S. Roy.

2.2.5. *Measuring insect abundance*

All 590 viable trees in the selected 14 blocks of the BSO trial were assessed for their insect herbivore assemblage. Five main branches were randomly selected from each tree. One shoot from this year's growth (identified using ring scars) were selected from two areas of each branch, one from the inner canopy and one from the outer, totalling 10 shoots per tree. Insect herbivores from all buds, leaves and acorns were identified using keys (Csóka, 2003, Chinery, 2013). Insects were characterised into five guilds: gallers, miners, webbers, rollers and chewers. All galling insects were recorded at species level due to ease of identification. The following leaf miners were recorded to genus level as immature stages and feeding traces are more difficult to identify to species level without DNA barcoding: *Phyllonorycter, Coleophora*, *Stigmella* and *Caloptilia*. *Dyseriocrania subpurpurella* and *Orchestes pilosus* were difficult to distinguish in the field so were recorded as one. Leaf chewing insects were recorded as the percentage area of each leaf consumed, split into the following categories: (1) less than 5% of the total leaf consumed, (2) 5-10% consumed, (3) 10-25% consumed, (4) 25-50% consumed and (5) 50-100% consumed, estimated by eye. Average damage per leaf was calculated and used in analysis. Leaf webbers, characterised as those insect larvae that manipulate multiple leaves with webbing, were also recorded as percentage damage per leaf in the same way as above. Leaf rollers, larvae that use webbing to manipulate a single leaf, were counted to guild level, but are most likely to be *Tortrix viridana*.

Insects were assessed in both spring (May/June 2016) and autumn (September 2016) and blocks were assessed at random. See *Figure 2.5* for photos of all insect species encountered in the BSO trial.

2.2.5.1. *Analysis of insect abundance*

Only insects that were encountered more than 50 times were included in the following analysis to test the influence of host phenotype on insect herbivore abundance. Insect abundance below 50 presented convergence problems in the statistical models and therefore was considered a suitable cut-off point. Galling, mining and rolling insects were expressed as abundance per 10 shoots per tree. Leaf webbers and chewers were presented as average percentage damage per leaf. GLMMs with negative binomial errors were used to test for the effect of: tree vigour (DBH and shoot length), tree species (*Quercus robur* or *Q. petraea*), marcescence habit (abscised or retained), tree provenance (latitude and longitude) and tree phenology (budburst date ranked) on the abundance of insect herbivores, as described fully in *Section 2.2.6*. Models were tested first on the combined abundance within the following guilds: spring gallers, autumn gallers, leaf miners, leaf rollers, leaf webbers and leaf chewers. Each insect species (or genus) within the galling and mining groups were then analysed separately.

2.2.5.2. *Analysis of insect community richness and alpha diversity*

All insect species, families and guilds encountered in the BSO trial were used in the following analysis to determine the effects of phenotype on the richness and diversity of the insect community. Results were calculated using the *vegan* package (version 2.5-3, Oksanen et al. (2018)). Species richness gives an estimate of the number of insect herbivore species (e.g. orders or guilds) encountered in the BSO trial. The common measure of species richness is simply the number of species that have more than one individual recorded.

Species diversity is a measure that considers species richness and also the evenness of the species in the community. First to be considered is alpha diversity, this is the within sample diversity. A Shannon-Wiener diversity index was calculated for the insect community in the

BSO trial also using the vegan package. A GLMM was then used to determine which factors contributed to the variation in species richness and species diversity of the insects recorded in the BSO trial, as discussed in *Section 2.2.6*.

2.2.5.3. *Analysis of insect community, beta diversity*

To test whether tree species, tree provenance or tree phenotype act as sufficient ecological filters resulting in different communities of associated insect herbivores, beta diversity was considered. Beta diversity metrics compare how different each sample is from every other sample; they are pairwise data in the form of a similarity matrix. The Bray-Curtis index was calculated using the insect data from the BSO trial. Non-metric multidimensional scaling (NMDS) was used with 4 axis (k=4) and ensuring that the stress value was below 0.2. NMDS scores can then be used to visualise the dissimilarity between samples, where each point represents the total insect community of that sample. To test for statistical differences PERMANOVA tests were computed using the NMDS score and the *Adonis* function in the *vegan* package. Explanatory terms included DBH, shoot length, tree species, marcescence habit, longitude and latitude of the parent tree and budburst date (ranked).

2.2.6. *Statistical modelling – generalised linear mixed effect models (GLMMs)*

Table 2.1 summarises each of the GLMM models used in this study. The *lme4* (version 1.1-18-1 Bates et al. (2015)) package was used for Gaussian models and *glmmTMB* (version 0.2.2.0, Brooks et al. (2017)) packages for non-normal models. To account for similarities between families within each provenance, family was included as a random factor nested within provenance. Trial block number was also included as a random factor. Continuous variables were tested for linearity and normality before model fitting, transformations were applied as necessary. Quadratic terms were also included to account for nonlinear relationships.

Non-significant terms were removed from the model using likelihood ratio testing to achieve the minimal adequate model, *F* tests for Gaussian models and Wald χ^2 for non-normal models. Normality of the residuals was confirmed, where necessary, using a Shapiro-Wilk test, for homoscedasticity by plotting the residuals versus the fitted values for each model (Crawley, 2007) and for overdispersion using the *sjstats* package (version 0.17.6, (Ludecke, 2019). All graphs in the following section were plotted using packages: *ggplot2* (version 3.1.0, Wickham (2016)), *extrafont* (version 0.17 Chang (2014)) and *viridis* (version 0.5.1, Garnier (2018)).

Table 2.1 – a summary of the generalised linear mixed effect (GLMM) models used in this study

Response variable	Transformation	Explanatory variables	Random variables	Model type
DBH *Section 2.2.3*	Square rooted	Tree species, budburst date, latitude, latitude (quadratic), longitude, longitude (quadratic)	Block number Provenance/Family	Gaussian GLMM
Shoot length *Section 2.2.3*	Square rooted	Tree species, budburst date, latitude, latitude (quadratic), longitude, longitude (quadratic)	Block number Provenance/Family	Gaussian GLMM
Budburst date (ranked) *Section 2.2.4.1*	Square rooted	DBH, tree species, marcescence habit, latitude, latitude (quadratic), longitude, longitude (quadratic)	Block number Provenance/Family	Gaussian GLMM
Marcescence habit (Retained or abscised) *Section 2.2.4.2*	None	DBH, tree species, latitude, longitude, budburst date, distance from edge of trial	Block number	Logistic GLMM
Insect species abundance *Section 2.2.5.1*	None	DBH, shoot length, tree species, marcescence habit, latitude, longitude, budburst date	Block number Provenance/Family	Negative binomial GLMM
Insect species richness *Section 2.2.5.2*	None	DBH, shoot length, tree species, marcescence habit, latitude, longitude, budburst date	Block number Provenance/Family	Gaussian GLMM
Insect species diversity *Section 2.2.5.2*	None	DBH, shoot length, tree species, marcescence habit, latitude, longitude, budburst date	Block number Provenance/Family	Gaussian GLMM

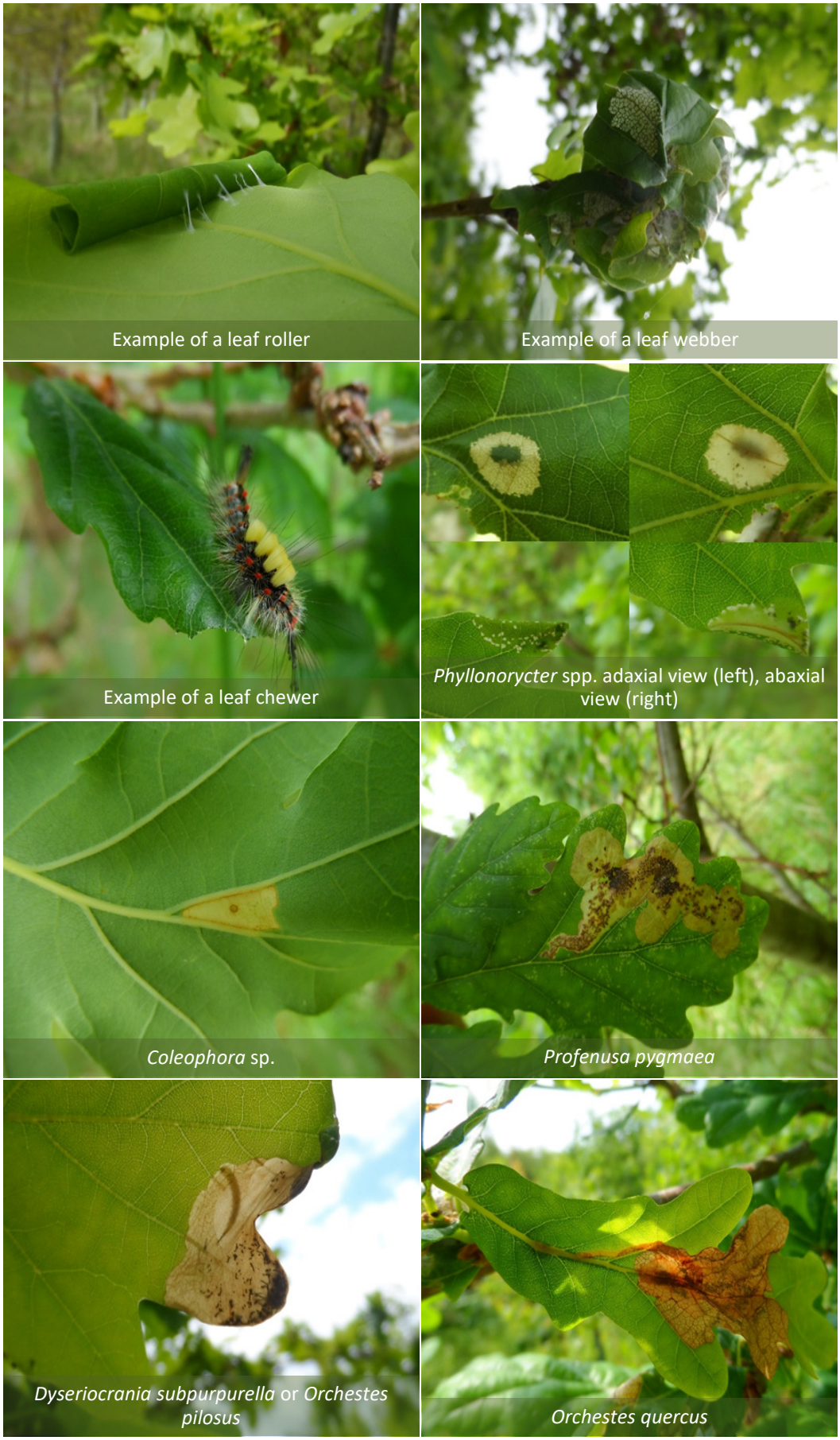

Figure 2.5 – spring and autumn insect herbivores identified in the BSO oak trial. All photos taken by S. Roy

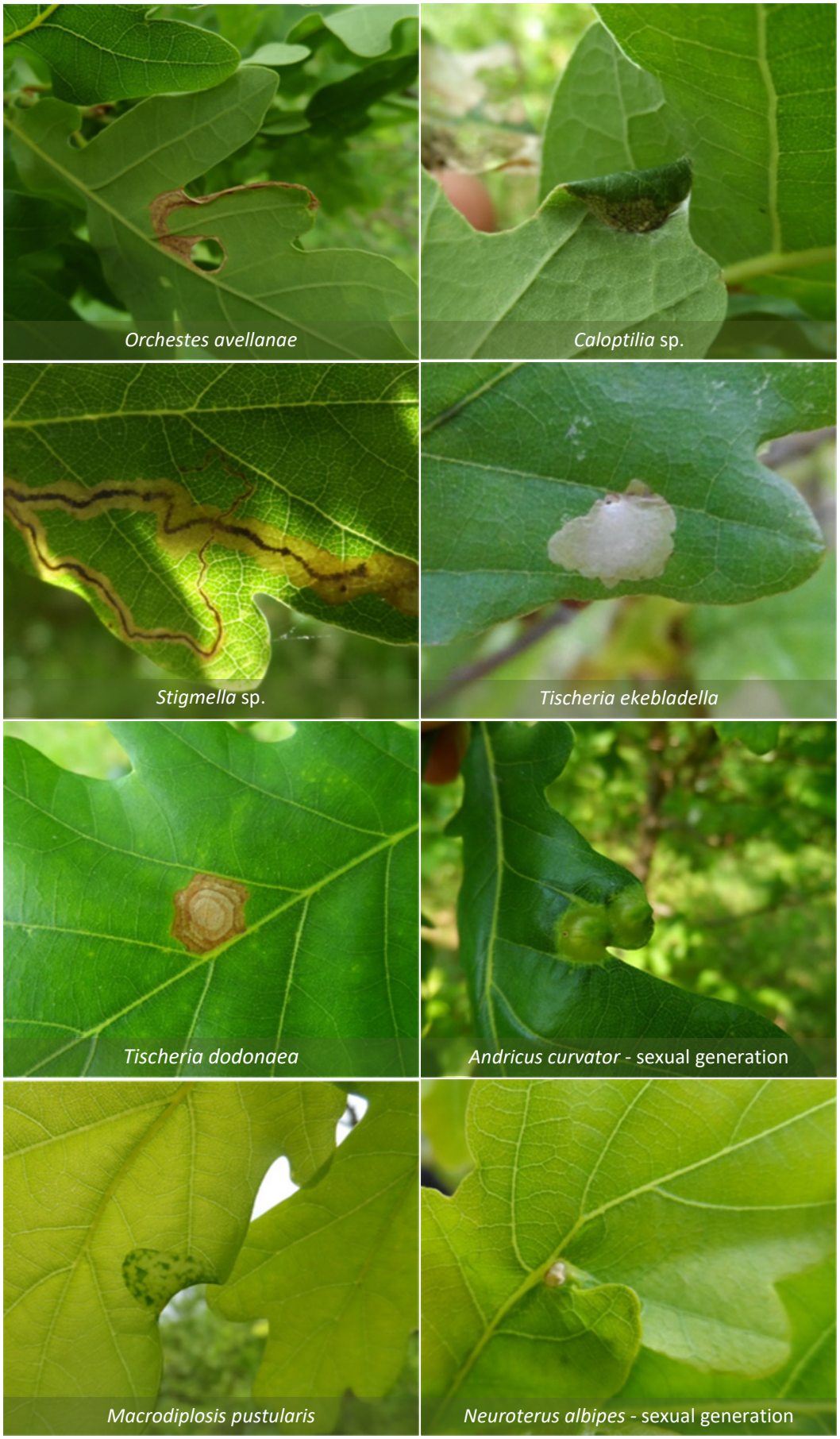

Figure 2.5 continued – spring and autumn insect herbivores identified in the BSO oak trial. All photos taken by S. Roy

Figure 2.5 continued – spring and autumn insect herbivores identified in the BSO oak trial. All photos taken by S. Roy

Figure 2.5 continued – spring and autumn insect herbivores identified in the BSO oak trial. All photos taken by S. Roy

2.3. *Results*

2.3.1. *Oak species*

Using seven measurements of leaf morphology it was possible to group the 604 individual oak trees into two species clusters using a principal component analysis (PCA) (*Figure 2.6a*). The result from K-mean clustering confirmed a bimodal distribution. The variables contributing most to the first component of the PCA, laminar pubescence and basal shape, are variables traditionally used for species identification. Therefore, it was interpreted that those with a negative first component score represents *Quercus robur* and those with a positive score *Q. petraea* (Kremer et al., 2002).

Fourteen individuals in the BSO trial were assigned to a different species to the rest of their family and were thus assumed not to be true offspring. Errors could have been made at the time of seed collection, in the nursery where these trees were germinated or when the trees were planted into the BSO trial. These 'wrong' individuals were removed from further analysis. Thus, 590 trees remained for analysis, 343 individuals assigned to *Quercus robur*, and 247 to assigned to *Q. petraea* (*Figure 2.6b*). A discriminant function analysis was used to determine the accuracy of determining species identity with the morphometric measurements at 99.8%.

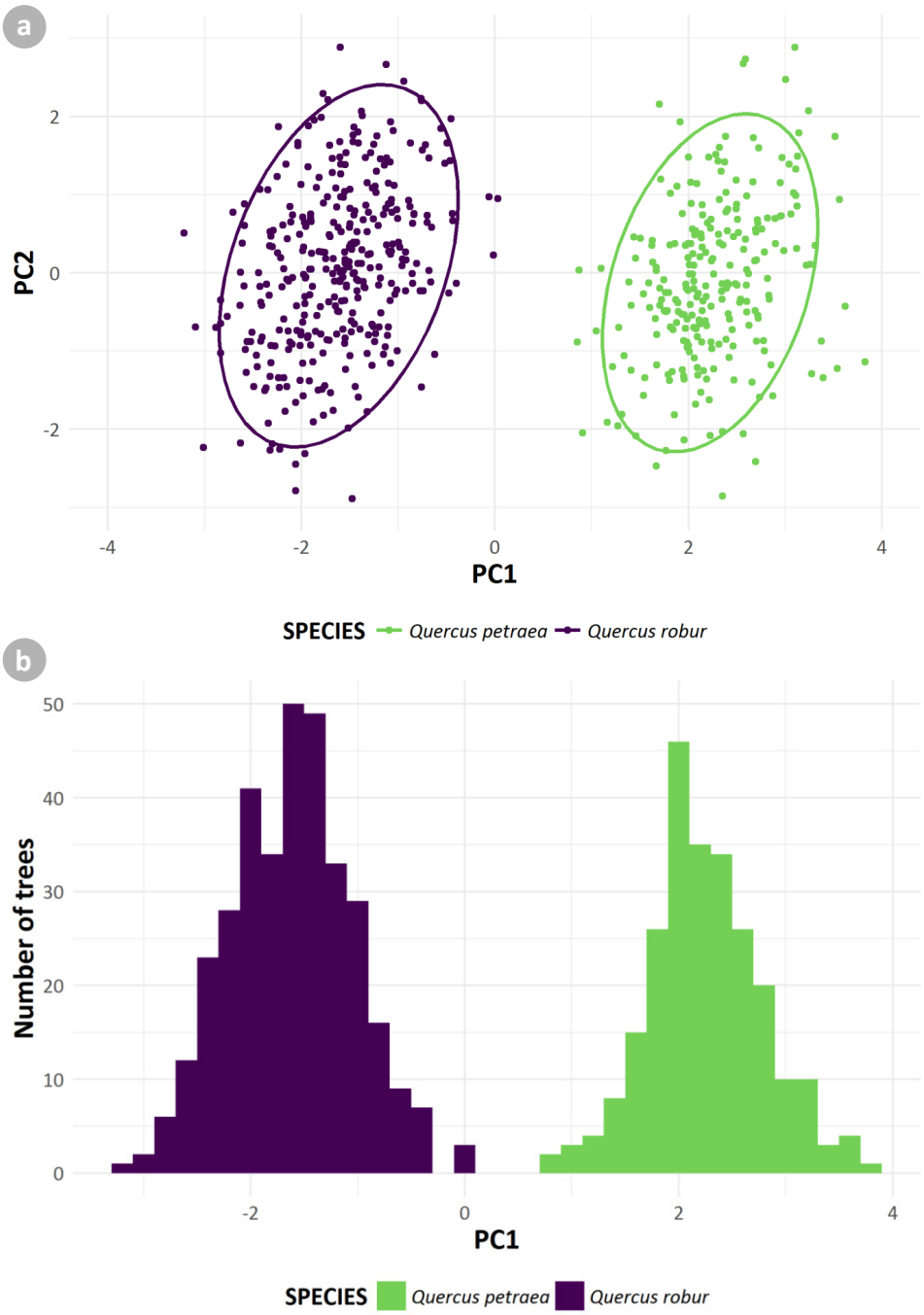

Figure 2.6 – (a) a principal component analysis (PCA) was used to assign the trees in the BSO oak trial to a species based on their leaf morphology. The first two components of the PCA grouped the 590 individual oak trees into two groups. The group on the right represents *Quercus petraea* and on the left *Q. robur*. Each point represents one tree. Ovals represent 95% confidence intervals. **(b) a histogram showing the number of trees from the BSO trial allocated to each PC1 score from the PCA.**

2.3.2. *Tree vigour*

Of the 659 trees measured in the BSO trial, 42 trees were removed as they were considered significantly stunted at less than 1.5 metres in height. Thirteen trees were removed as their DBH was below 1.5cm, considerably different from the average DBH of 4.6cm. Of the

remaining trees the DBH varied considerably from 1.6cm to 11.15cm and from 1.5m to 9.1m in height. Trees showed a strong correlation between their height and DBH (df=588, Pearson's cor=0.81, p<0.001) so it was decided that only DBH would be used in further analysis (*Figure 2.7a*). A very weak correlation existed between DBH and shoot length (df=588, Pearson's cor=-0.16, p<0.001) (*Figure 2.7b*). Shoot length was therefore used in further analysis.

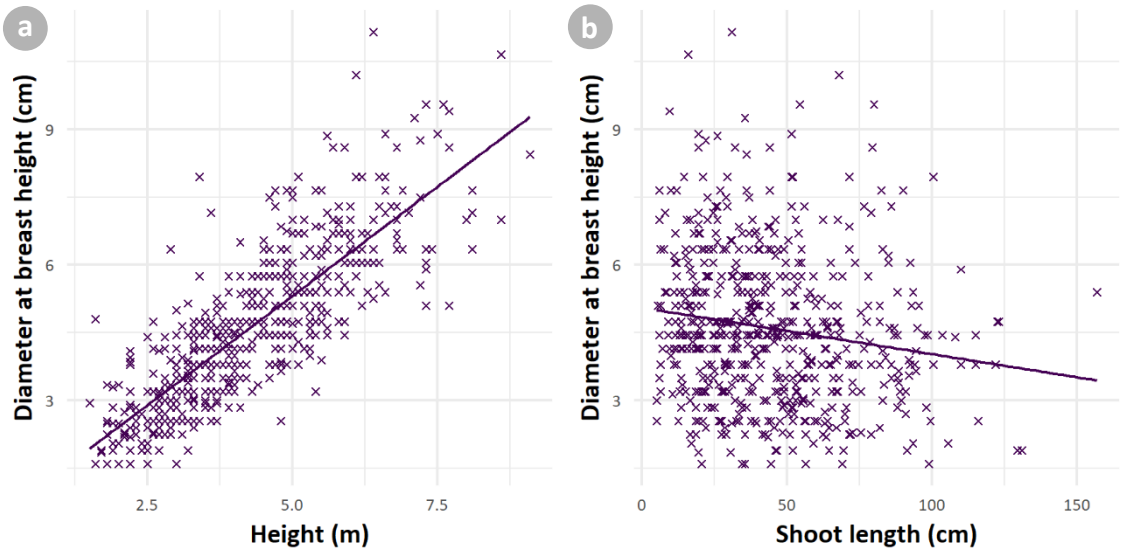

Figure 2.7 – correlations between (a) height and DBH of the oak trees in the BSO trial and (b) between shoot length and DBH (right). Each point represents one tree.

Latitude of the parent tree did influence the shoot length and DBH of *Quercus* trees. A non-linear relationship is shown with trees from lower latitudes and higher latitudes than Paradise Wood having a smaller DBH (df=7, F=4.13, p<0.05) (*Figure 2.8b*) and shorter shoots (df=7, F=4.24, p<0.05) (*Figure 2.8a*).

Although the heavier, waterlogged soil found in Paradise Wood should favour the growth of *Quercus robur*, there was no difference in the DBH of the two species. Conversely, *Q. petraea* did have longer shoots (df=7, F=8.99, p<0.01) (*Figure 2.8e*). Phenology contributed to variations in the diameter of the trees (df=5, F=6.40, p<0.05) (*Figure 2.8d*). Trees that budburst earlier also had shorter shoots (df=7, F=15.56, p<0.001) (*Figure 2.8c*).

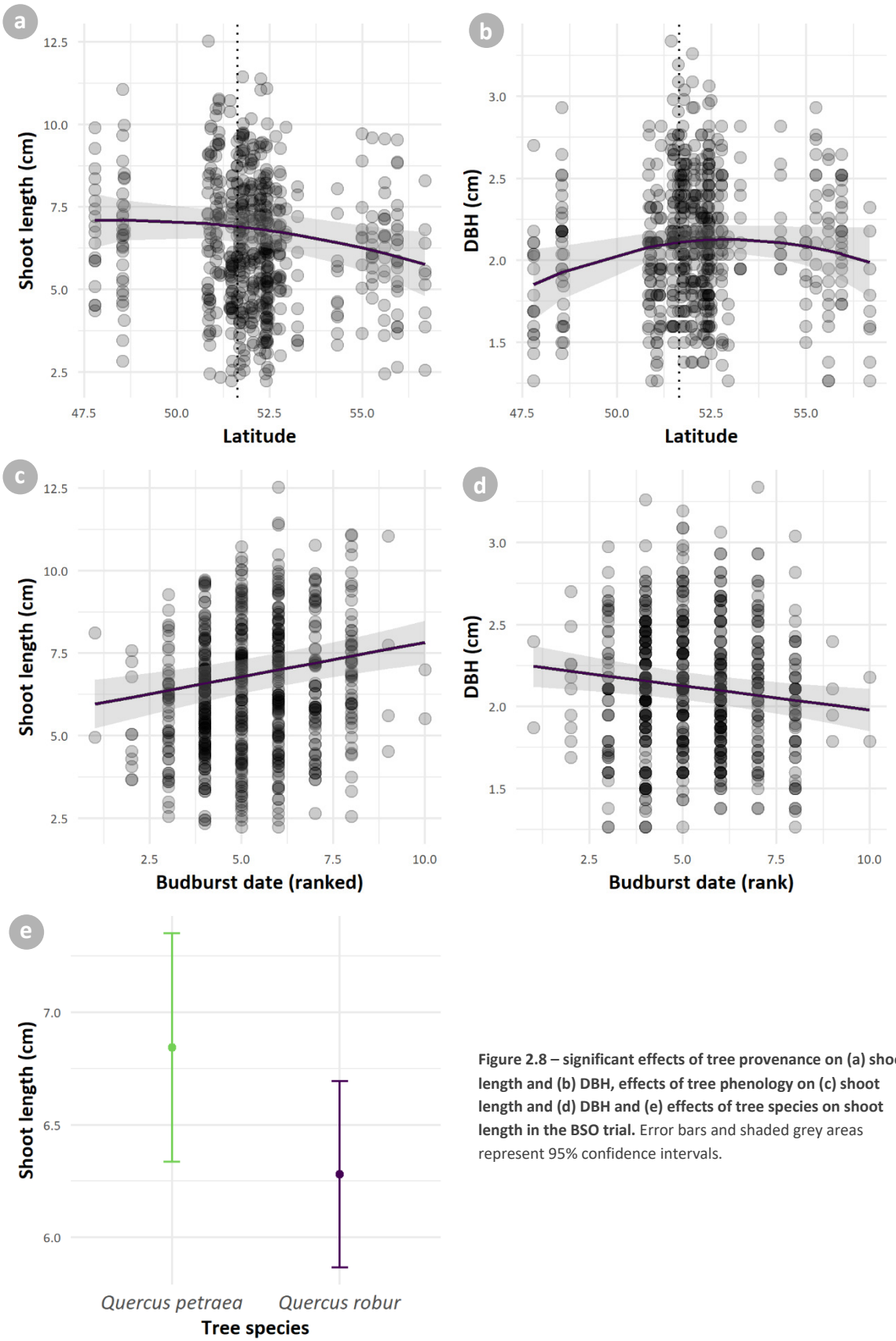

Figure 2.8 – significant effects of tree provenance on (a) shoot length and (b) DBH, effects of tree phenology on (c) shoot length and (d) DBH and (e) effects of tree species on shoot length in the BSO trial. Error bars and shaded grey areas represent 95% confidence intervals.

2.3.3. *Leaf phenology*

2.3.3.1. *Date of budburst and full flush*

Two measurements of phenology were considered; the date of budburst and the time taken from budburst to full leaf expansion. These two measurements exhibited a strong negative correlation (df=588, Pearson's cor=-0.79, p<0.001) (*Figure 2.9a*). Due to the strong correlation between budburst and full flush date it was decided that only budburst date (ranked) would be used in further analysis.

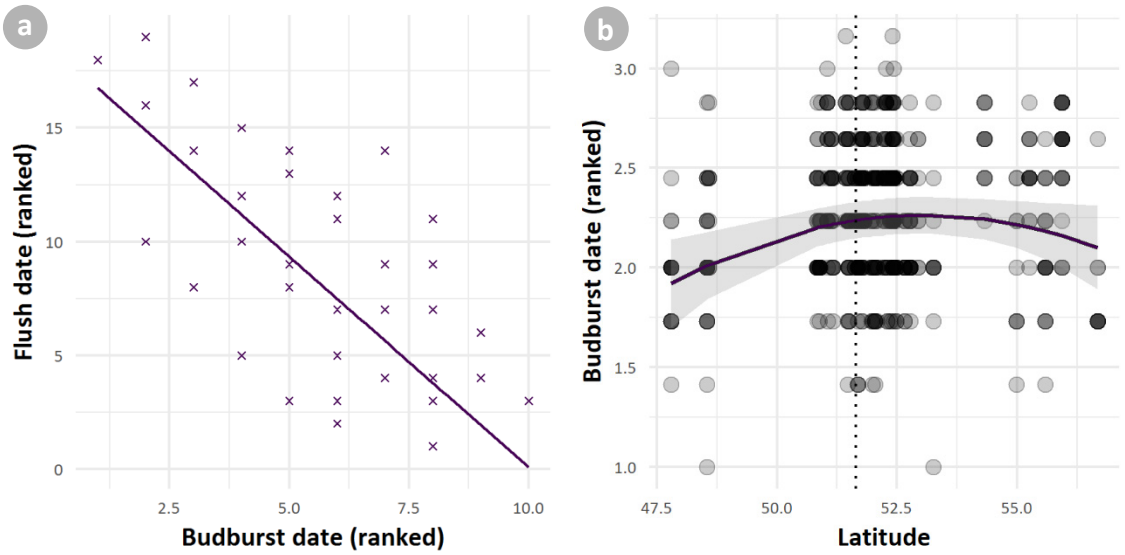

Figure 2.9 – (a) strong negative correlation between ranked budburst date and ranked flush date. Each point represents one tree in the BSO trial. **(b) significant interaction between latitude and ranked budburst date of the oak trees in the BSO trial.** Shaded grey areas represent 95% confidence intervals.

Latitude of the parent tree (on the quadratic scale) influenced the date of budburst of the *Quercus* trees (df=8, F=5.28, p<0.05). There was a non-linear correlation between latitude of the parent tree and budburst date. Trees from lower latitudes than Paradise Wood burst earlier and trees from further north of Paradise Wood also burst earlier (*Figure 2.9b*).

Taller trees budburst earlier then shorter trees (df=8, F=5.98, p<0.05) (*Figure 2.10a*). Also, trees that retained their senescent leaves overwinter tended to budburst later the following spring (df=8, F=17.08, p<0.001) (*Figure 2.10b*).

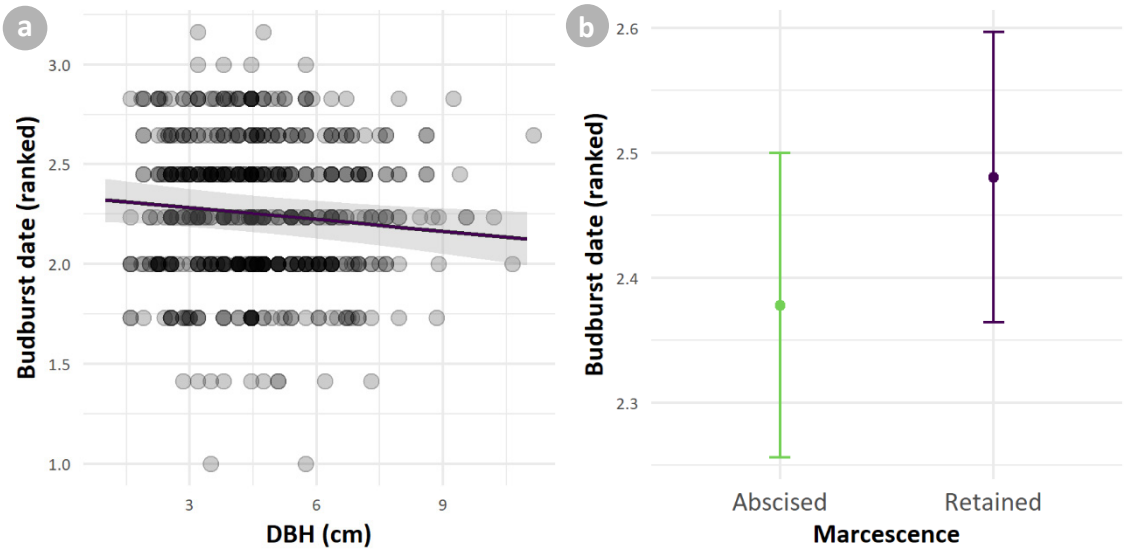

Figure 2.10 – significant effects of tree vigour (a) and marcescence (b) on date of budburst of the oak trees in the BSO trial. Error bars and grey shaded areas represent 95% confidence intervals.

The same individual trees were examined for budburst in 2008 (Peters, 2008, unpublished), these results were compared to 2016 using Pearson's product-moment correlation. The correlation in rank order between the two years was positive and significant (df=586, Pearson's cor=0.56, p<0.001), suggesting that the order of budburst among individuals in a population is consistent between years (*Figure 2.11*).

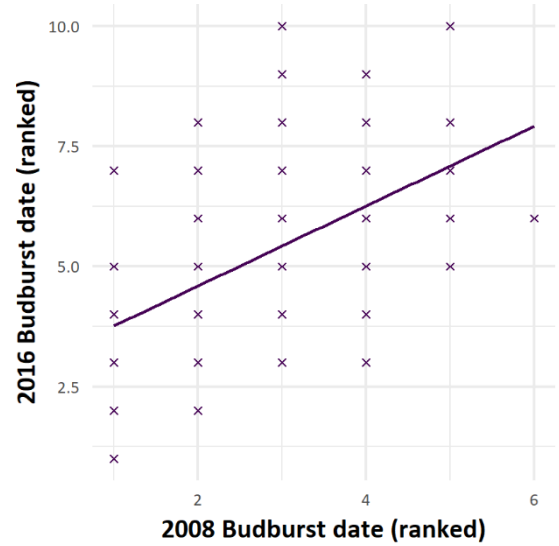

Figure 2.11 – positive correlation between date of budburst recorded in 2008 (Peters, 2008, unpublished) compared to 2016 of the same trees in the BSO trial. Each point represents one tree.

2.3.3.2. *Leaf marcescence*

Results on leaf marcescence from the logistic mixed effect model found *Quercus petraea* trees were more likely to retain their leaves overwinter than *Q. robur* (df=6, Wald χ^2=44.27, p<0.001) (*Figure 2.12c*). Leaf marcescence was also correlated with the timing of budburst in spring, trees that budburst later were more likely to retain their leaves (df=6, Wald χ^2=9.44, p<0.001) (*Figure 2.12a*). Additionally, trees originating from higher longitudes retained their leaves more readily (df=6, Wald χ^2=5.18, p<0.05) (*Figure 2.12b*).

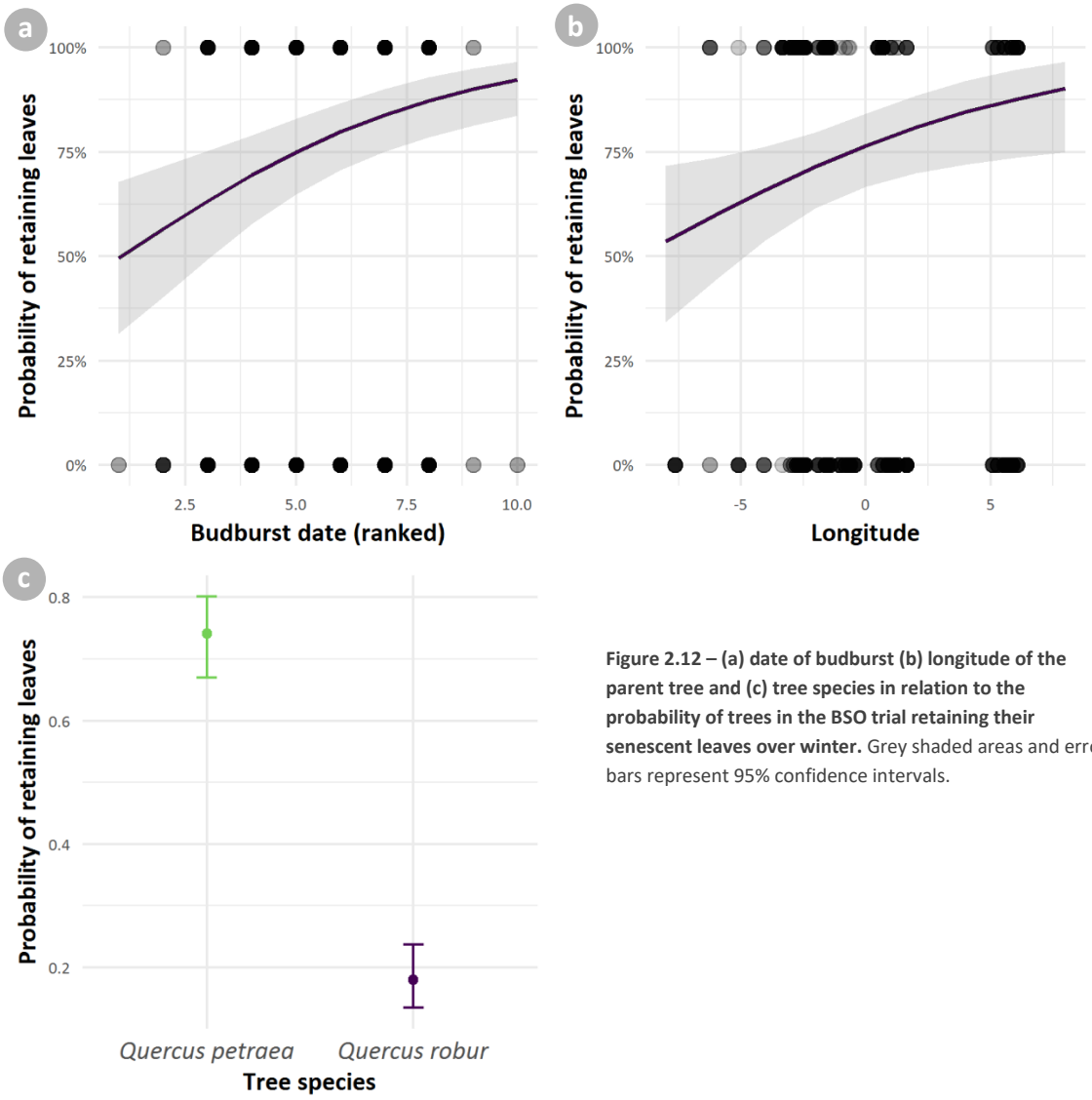

Figure 2.12 – (a) date of budburst (b) longitude of the parent tree and (c) tree species in relation to the probability of trees in the BSO trial retaining their senescent leaves over winter. Grey shaded areas and error bars represent 95% confidence intervals.

2.3.4. *Insect abundance*

In spring and autumn 2016 the total number of insect herbivores recorded in the BSO trial was 29,150, comprising 17 species of galling insect, 4 genera and 6 species of leaf miner and the guild of leaf rollers. On average, 12% of the area of each leaf in the BSO was defoliated by leaf chewers, and 5% of the area removed by leaf webbers. The insects were considered at both guild level and also at species/genus level (*Tables 2.2, 2.3 & 2.4*).

Table 2.2 – total abundance of the galling insects recorded in the BSO trial. * insects recorded fewer than 50 times were not analysed for their abundance, but were included in community analysis

Galler species	Common name	Generation	Season recorded	Total abundance
Neuroterus albipes	Smooth spangle gall	Agamic	Autumn	6875
Neuroterus anthracinus	Oyster gall	Agamic	Autumn	5841
Neuroterus quercusbaccarum	Common spangle gall	Agamic	Autumn	3708
Macrodiplosis pustularis		Sexual	Spring	3101
Neuroterus numismalis	Silk button gall	Agamic	Autumn	2384
Trioza remota		Sexual	Spring	1905
Neuroterus albipes	Schenck's gall	Sexual	Spring	103
Andricus curvator	Curved leaf gall	Sexual	Spring	95
Andricus foecundatrix	Artichoke gall	Agamic	Autumn	90
Andricus kollari	Marble gall	Agamic	Autumn	77
Macrodiplosis roboris		Sexual	Spring	51
Andricus lignicolus*	Cola nut gall	Agamic	Autumn	42
Andricus quercuscalicis*	Knopper gall	Agamic	Autumn	33
Neuroterus numismalis*	Blister gall	Sexual	Spring	24
Cynips quercusfolii*	Cherry gall	Agamic	Autumn	21
Cynips longiventris*	Striped pea gall	Agamic	Autumn	4
Andricus testaceipes*	Leaf vein gall	Sexual	Spring	4
Andricus aries*	Ram's horn gall	Agamic	Autumn	3
Andricus inflator*	Twig gall	Sexual	Spring	1

Table 2.3 - total abundance of leaf manipulating insects recorded in the BSO trial. Leaf webbers and chewers were recorded as percentage damage per leaf (not included here).

Leaf manipulator guild	Season recorded	Total abundance
Leaf rollers	Spring	291
Leaf webbers	Spring	N/A
Leaf chewers	Spring & Autumn	N/A

Table 2.4 – total abundance of all mining insects recorded in the BSO trial. * insects recorded fewer than 50 times were not analysed for their abundance but were included in community analysis.

Miner species/genus	Season recorded	Total abundance
***Phyllonorycter* spp.**	Spring & Autumn	3068
***Coleophora* spp.**	Spring	1034
***Stigmella* spp.**	Autumn	133
Tischeria dodonaea	Autumn	86
Orchestes quercus	Spring	79
***Orchestes avellanae* ***	Spring	34
***Profenusa pygmaea* ***	Spring	23
***Caloptilia* spp. ***	Autumn	19
***Tischeria ekebladella* ***	Autumn	14
***Dyseriocrania subpurpurella* or *Orchestes pilosus* ***	Spring	7

The minimal adequate models of the linear mixed effect modelling on the insect herbivore guilds are found in *Table 2.5*, i.e. every explanatory parameter shown was found to be significant at $p<0.05$. Results for the analysis of insect abundance at species or genus level are shown in *Table 2.6*.

Table 2.5 - significant explanatory terms from the generalised linear mixed effect models of herbivore guild abundance in the BSO trial using negative binomial errors.

Insect group	Significant term	df	Wald χ^2	p	Direction of effect
Spring gallers	Budburst date	7	28.67	<0.001	Positive
	Marcescence	7	6.22	<0.05	Abscised > retained
	Shoot length	7	4.26	<0.05	Positive
Autumn gallers	Tree species	9	27.76	<0.001	*Q. robur* > *Q. petraea*
	Shoot length	9	24.81	<0.001	Positive
	Budburst date	9	11.73	<0.001	Positive
	DBH	9	6.89	<0.01	Positive
	Longitude	9	6.04	<0.05	Negative
Miners	Shoot length	7	28.72	<0.001	Positive
	DBH	7	13.51	<0.001	Positive
	Budburst date	7	7.82	<0.01	Negative
Webbers	Shoot length	7	58.61	<0.001	Negative
	Tree species	7	26.50	<0.001	*Q. robur* > *Q. petraea*
	Budburst date	7	7.64	<0.01	Positive
Chewers	Budburst date	7	52.91	<0.001	Negative
	Shoot length	7	4.63	<0.05	Negative
	Tree species	7	6.10	<0.05	*Q. petraea* > *Q. robur*
Leaf rollers	Tree species	4	21.56	<0.001	*Q. petraea* > *Q. robur*

Table 2.6 – significant explanatory terms from the generalised linear mixed effect model of individual herbivore species abundance using negative binomial errors. Top part of the table are the galling insects, bottom part mining insects

Insect species	Significant term	df	Wald χ^2	p	Direction of effect
Neuroterus albipes (agamic)	Longitude	8	4.17	<0.05	Negative
	DBH	8	4.72	<0.05	Positive
	Tree species	8	57.83	<0.001	*Q. robur* > *Q. petraea*
	Shoot length	8	23.19	<0.001	Positive
Neuroterus anthracinus (agamic)	Longitude	7	10.97	<0.001	Negative
	Tree species	7	8.64	<0.01	*Q. petraea* > *Q. robur*
	Budburst date	7	3.73	<0.05	Negative
Neuroterus quercusbaccarum (agamic)	DBH	6	3.92	<0.05	Positive
	Shoot length	6	6.47	<0.01	Positive
Macrodiplosis pustularis	Marcescence	7	5.10	<0.05	Retained > abscised
	Budburst date	7	12.21	<0.001	Negative
	DBH	7	4.90	<0.05	Positive
Neuroterus numismalis (agamic)	Tree species	6	6.44	<0.05	*Q. robur* > *Q. petraea*
	Budburst date	6	25.77	<0.001	Positive
Trioza remota	Tree species	8	4.14	<0.05	*Q. robur* < *Q. petraea*
	Marcescence	8	3.95	<0.05	Abscised > retained
	Budburst date	8	48.77	<0.001	Positive
	Shoot length	8	9.41	<0.01	Positive
Neuroterus albipes (sexual)	Tree species	5	15.44	<0.001	*Q. robur* < *Q. petraea*
Andricus curvator (sexual)	Tree species	6	27.42	<0.001	*Q. robur* < *Q. petraea*
	Budburst date	6	20.32	<0.001	Negative
Andricus foecundatrix (agamic)	Tree species	7	10.27	<0.01	*Q. petraea* > *Q. robur*
	Budburst date	7	10.89	<0.001	Positive
	Shoot length	7	5.30	<0.05	Positive
Andricus kollari (agamic)	Longitude	5	6.26	<0.05	Positive
Macrodiplosis roboris	No significant variables				
***Phyllonorycter* spp.**	DBH	6	5.90	<0.05	Positive
	Shoot length	6	13.90	<0.001	Positive
***Coleophora* spp.**	DBH	7	10.06	<0.01	Positive
	Budburst date	7	9.84	<0.01	Negative
	Shoot length	7	15.03	<0.001	Positive
***Stigmella* spp.**	No significant variables				
Tischeria dodonaea	Shoot length	5	5.89	<0.05	Positive
Orchestes quercus	Tree species	6	14.33	<0.001	*Q. robur* < *Q. petraea*
	Shoot length	6	5.23	<0.05	Positive

Results of the mixed effect modelling indicate that tree vigour explains the variation in insect abundance both at the guild and individual level for many insect groups. Trees with a larger DBH supported a higher density (i.e. abundance per tree) of autumn gallers, in particular *Neuroterus albipes* and *N. quercusbaccarum* (*Figure 2.13a*). These larger trees also supported higher densities of the leaf mining guild, specifically *Phyllonorycter* spp. and *Coleophora* spp. (*Figure 2.13b*).

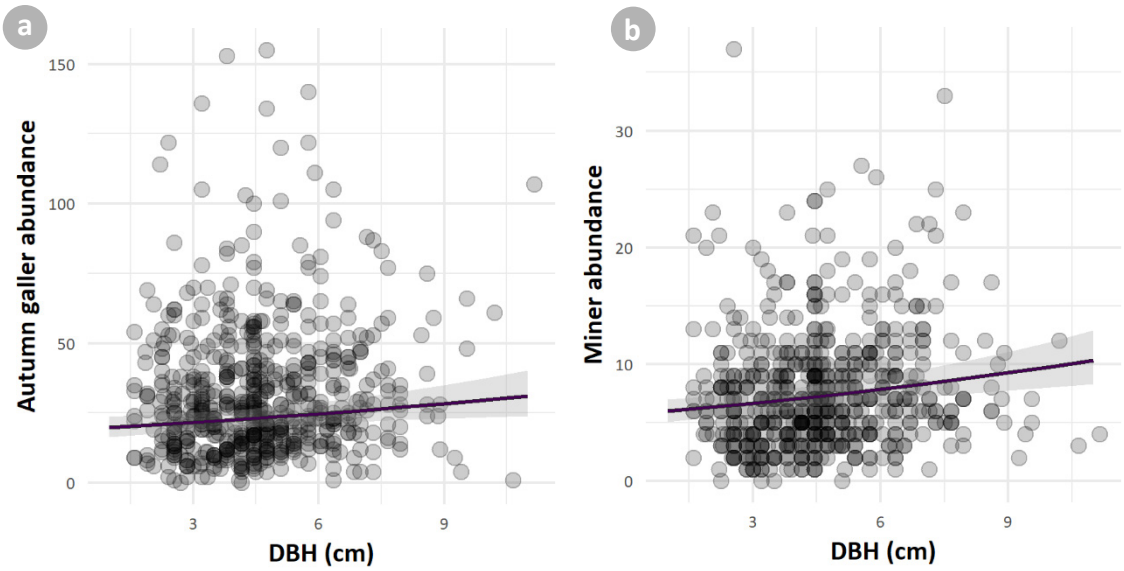

Figure 2.13 - significant effects of tree diameter (DBH) on abundance of (a) autumn galling insects (b) mining insects in the BSO trial. Grey shaded areas represent 95% confidence intervals.

Shoot length appears to be of major importance in determining insect abundance, influencing four out of the five insect guilds. Leaf webbers and chewers preferentially fed on trees with shorter shoots (*Figures 2.14a & b*). Longer shoots sustained higher numbers of gallers, in particular: *N. albipes* (agamic generation), *N. quercusbaccarum*, *Trioza remota* and *Andricus foecundatrix* (*Figure 2.14c & d*). Leaf miners also preferred longer shoots, specifically *Phyllonorycter* spp., *Coleophora* spp., *Tischeria dodonaea* and *Orchestes quercus* (*Figure 2.14e*).

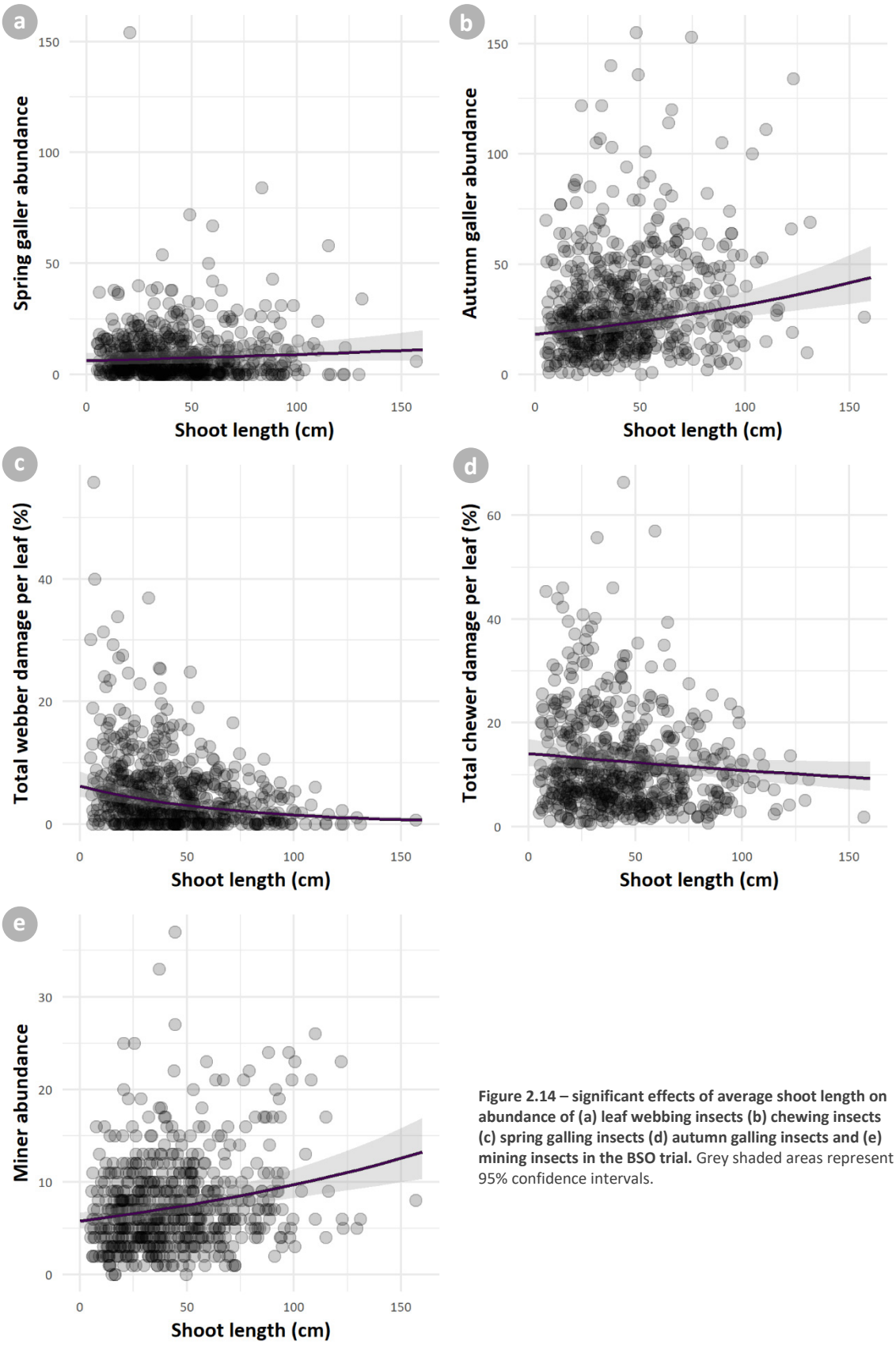

Figure 2.14 – significant effects of average shoot length on abundance of (a) leaf webbing insects (b) chewing insects (c) spring galling insects (d) autumn galling insects and (e) mining insects in the BSO trial. Grey shaded areas represent 95% confidence intervals.

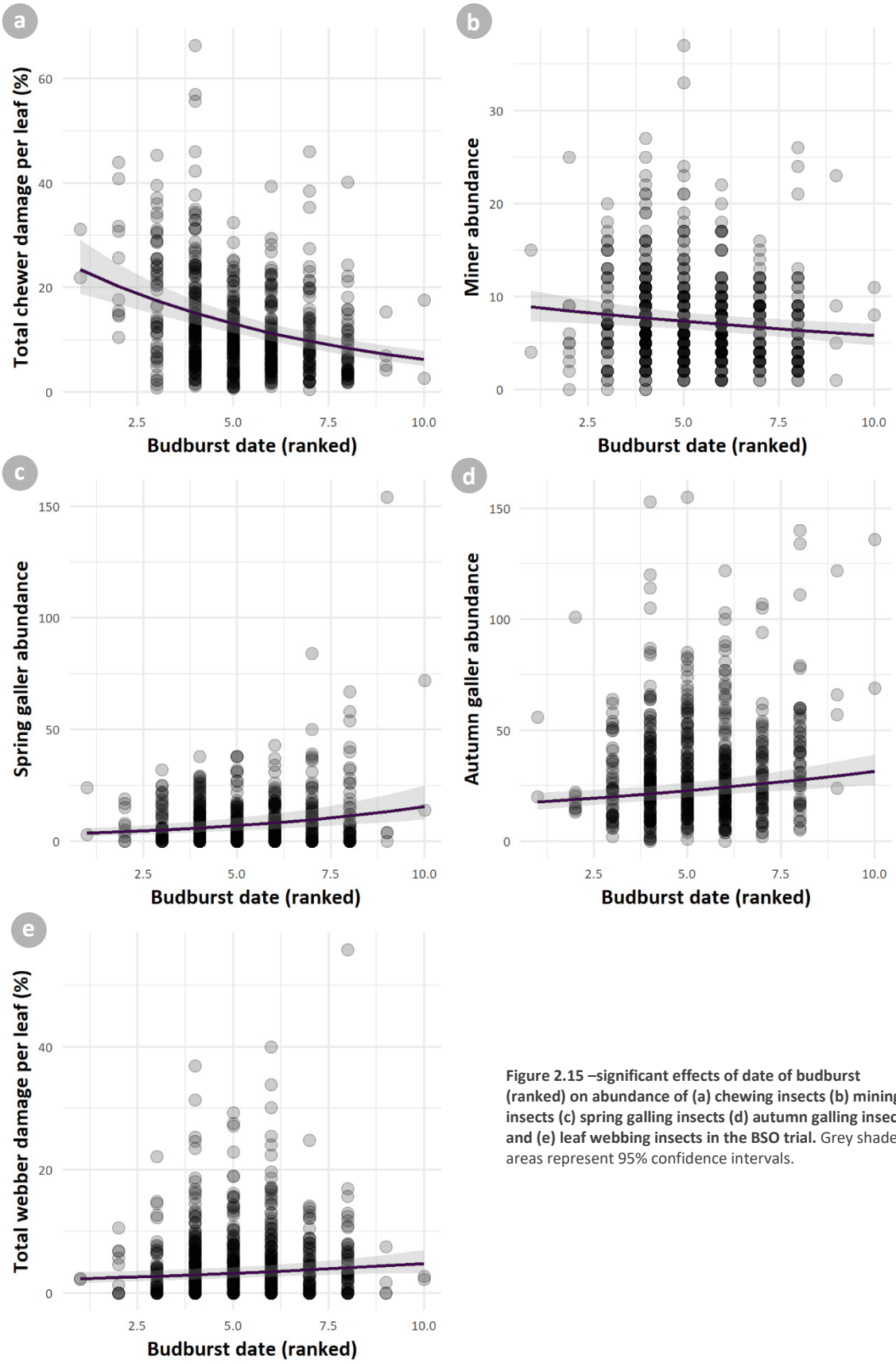

Figure 2.15 –significant effects of date of budburst (ranked) on abundance of (a) chewing insects (b) mining insects (c) spring galling insects (d) autumn galling insects and (e) leaf webbing insects in the BSO trial. Grey shaded areas represent 95% confidence intervals.

Earlier budburst in the spring corresponded with higher rates of defoliation by leaf chewers and also higher abundances of leaf miners, in particular *Coleophora* spp. (*Figure 2.15a & b*). Conversely, at guild level gallers preferred later bursting trees (*Figure 2.15c & d*). When considering the gallers individually, *Neuroterus numismalis* (agamic), *Trioza remota* and *Andricus foecundatrix* all preferred later bursting trees, while *N. anthracinus*, *Macrodiplosis pustularis* and *A. curvator* preferred earlier bursting trees. Leaf webbers also preferred trees that burst later (*Figure 2.15e*).

Provenance effects on insect abundance were few. However, trees from lower longitudes supported a higher abundance of autumn gallers, specifically: *N. albipes* and *N. anthracinus* (*Figure 2.16*). Conversely, the autumn galler, *Andricus kollari* preferred trees from higher longitudes. The leaf miner, *Tischeria dodonaea* was recorded at a higher abundance on trees from lower latitudes, but no effect was found at the guild level.

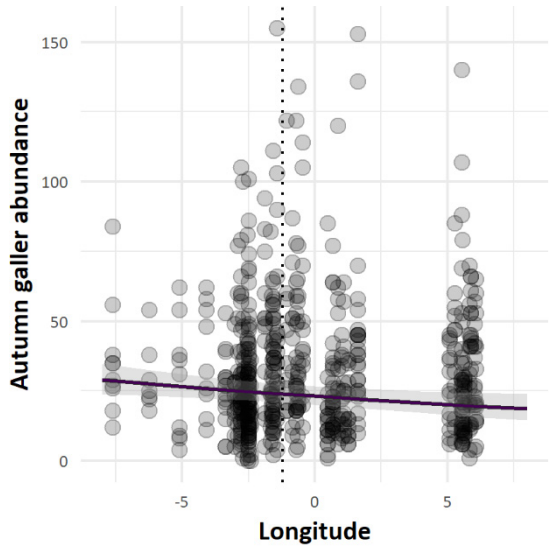

Figure 2.16 – significant effects of tree provenance on the abundance of autumn galling insects. Grey shaded areas represent 95% confidence intervals.

Oak tree species explained much of the variation in insect abundance at the guild and species level. Autumn gallers, in particular *N. albipes*, *N. numismalis* and *T. remota* preferred to feed on *Q. robur* (*Figure 2.17a*). Contrary to the rest of the guild, the autumn gallers, *N. anthracinus* and *A. foecundatrix*, were found in higher numbers on *Q. petraea*. The spring gallers, *N. albipes*

and *A. curvator* were at higher numbers of *Q. robur* but no relationship was found at guild level. The leaf miner *Orchestes quercus* was recorded at higher abundances on *Q. robur* but no preference was recorded at the guild level. Leaf webbers preferentially fed on *Q. robur* (*Figure 2.17c*) and leaf chewers and leaf rollers on *Q. petraea* (*Figure 2.17b & d*).

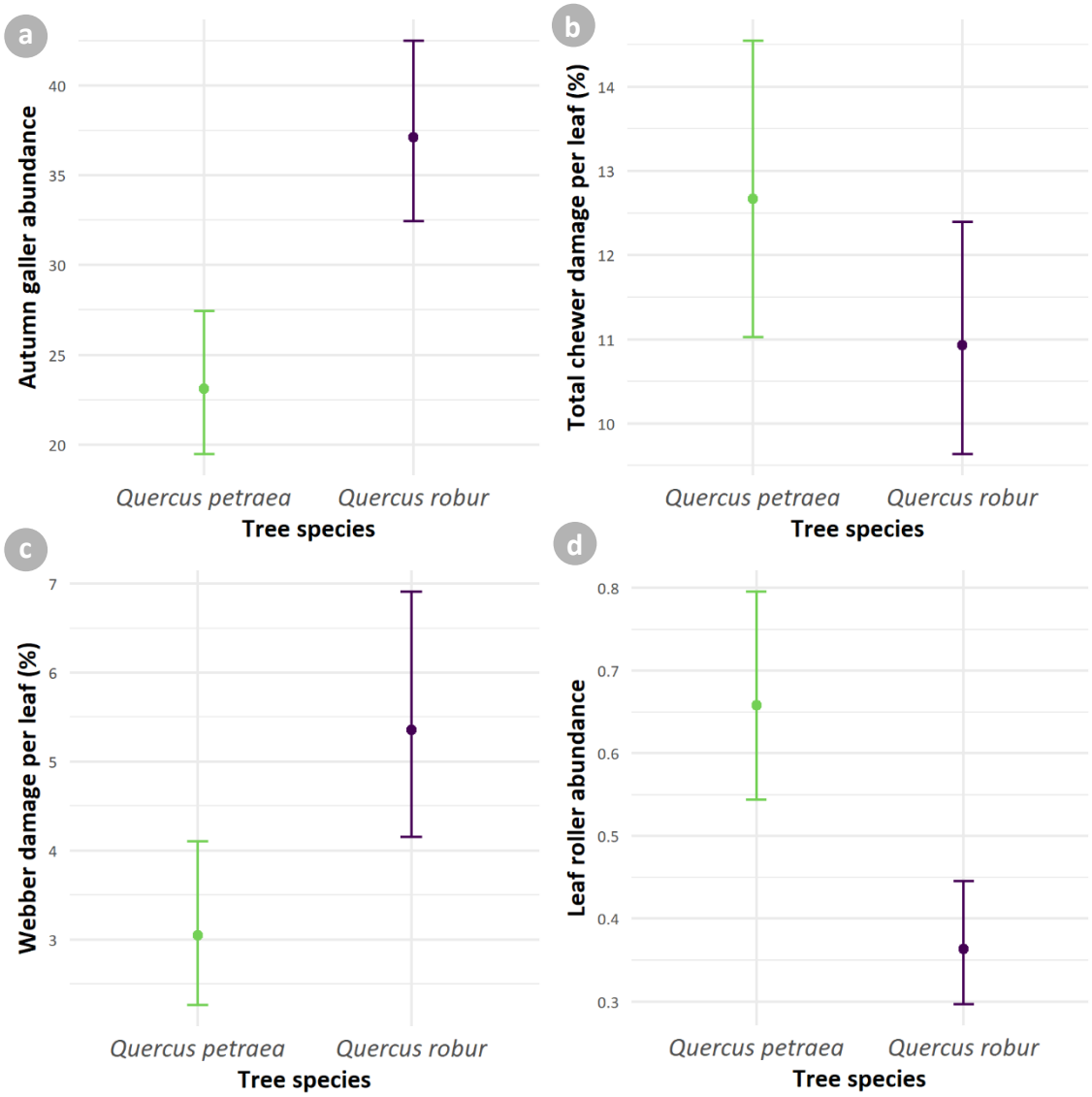

Figure 2.17 - significant effects of tree species on abundance of (a) autumn galling insects (b) chewing insects (c) webbing insects and (d) leaf rolling insects in the BSO trial. Error bars represent 95% confidence intervals.

Fewer spring gallers, in particular *Trioza remota* were associated with trees that retained their leaves over winter, the opposite was recorded for *Macrodiplosis pustularis* (*Figure 2.18*).

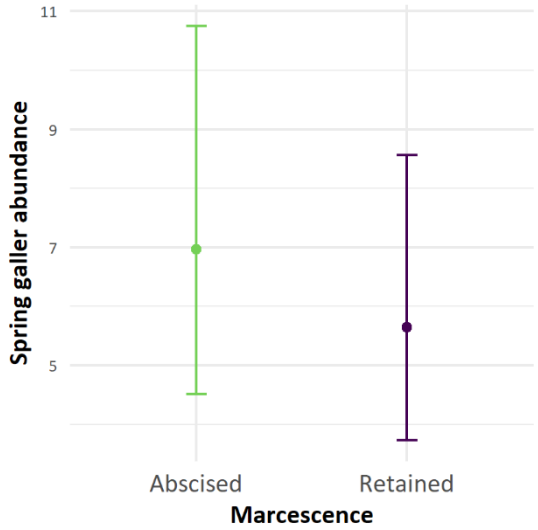

Figure 2.18 – significant differences in spring galler abundance in relation to marcescence habit in the BSO trial. Error bars represent 95% confidence intervals.

2.3.5. *Insect community richness and diversity*

2.3.5.1. *Alpha diversity*

Quercus robur supported a higher herbivore species richness (df=8, F=25.23, p<0.001) and higher Shannon-Wiener diversity of herbivorous insects (df=8, F=11.47, p<0.001) than *Q. petraea* (*Figures 2.19a & b*). Independent from tree species, taller trees supported a higher richness (df=8, F=30.82, p<0.001) and diversity (df=8, F=17.60, p<0.001) of herbivores (*Figure 2.19c & d*). Longer shoots also supported a higher richness (df=8, F=10.87, p<0.001) and diversity of insect herbivores (df=8, F=5.84, p<0.05) (*Figure 2.19g & h*). Tree phenology influenced both richness and diversity (df=8, F=3.75, p<0.05) of insect herbivores (*Figure 2.19e & f*).

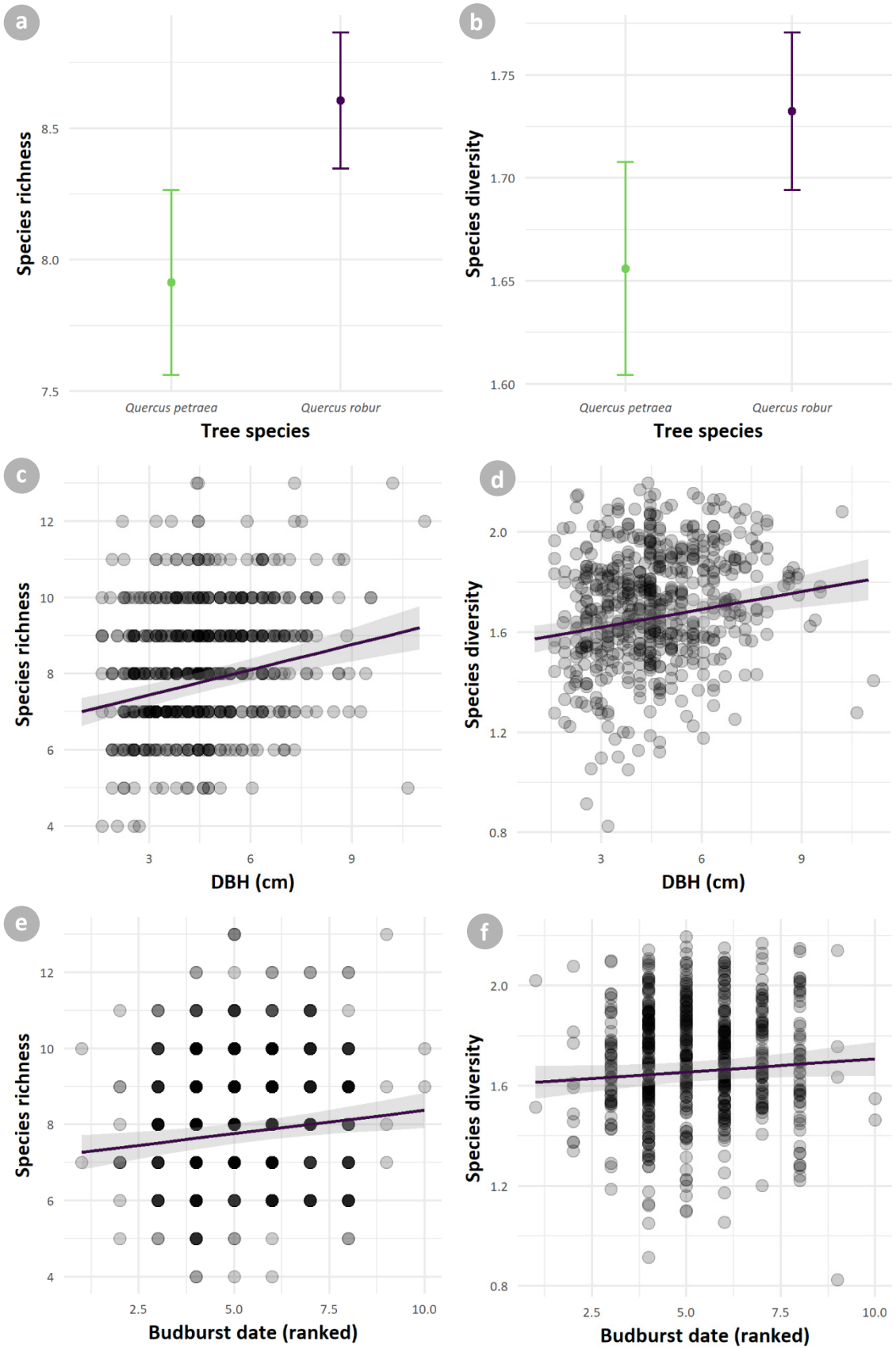

Figure 2.19 – significant effects of (a) Tree species (c) tree diameter (e) date of budburst and (g) shoot length on insect species richness and (b) tree species (d) tree diameter (f) date of budburst and (g) shoot length on insect species diversity in the BSO trial. Error bars and shaded areas represent 95% confidence intervals.

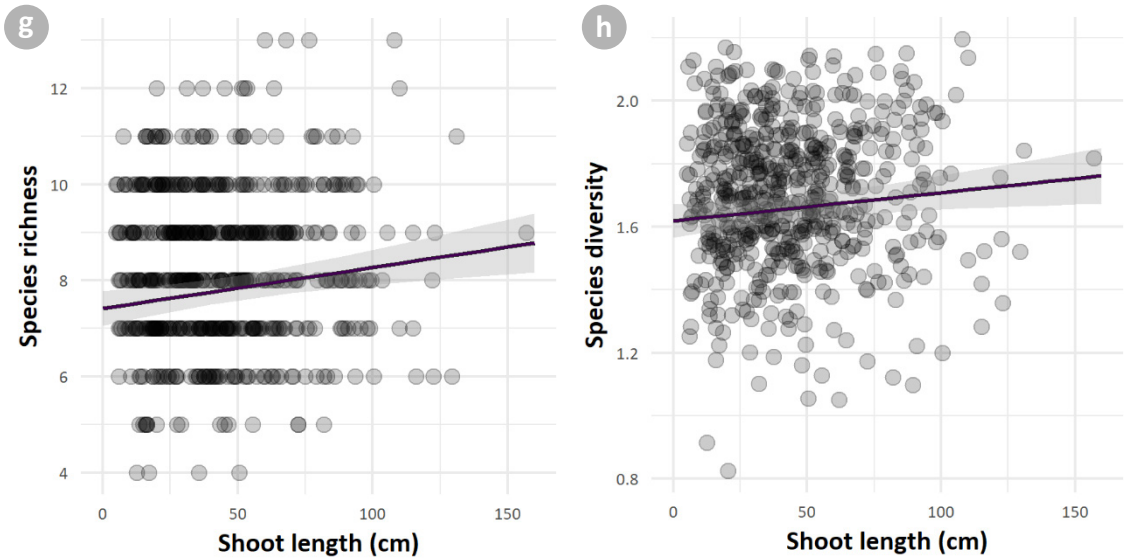

Figure 2.19 cont. - significant effects of (a) Tree species (c) tree diameter (e) date of budburst and (g) shoot length on insect species richness and (b) tree species (d) tree diameter (f) date of budburst and (g) shoot length on insect species diversity in the BSO trial. Error bars and shaded areas represent 95% confidence intervals.

2.3.5.2. *Beta diversity*

Results of the Bray-Curtis analysis (*Figure 2.20*) found that insect herbivore communities associated with *Quercus robur* and *Q. petraea* differ in species composition and relative abundance ($F=28.44$, $p<0.001$). Taller oak trees and those with longer shoots also support different herbivore communities than smaller oak trees ($F=7.43$, $p<0.001$) with shorter shoots ($F=12.13$, $p<0.001$). Trees originating from higher longitudes shared a different herbivore community than those from lower longitudes ($F=2.36$, $p<0.05$). Trees that budburst earlier also shared a different assemblage of herbivores than those trees that took longer to budburst ($F=20.85$, $p<0.001$).

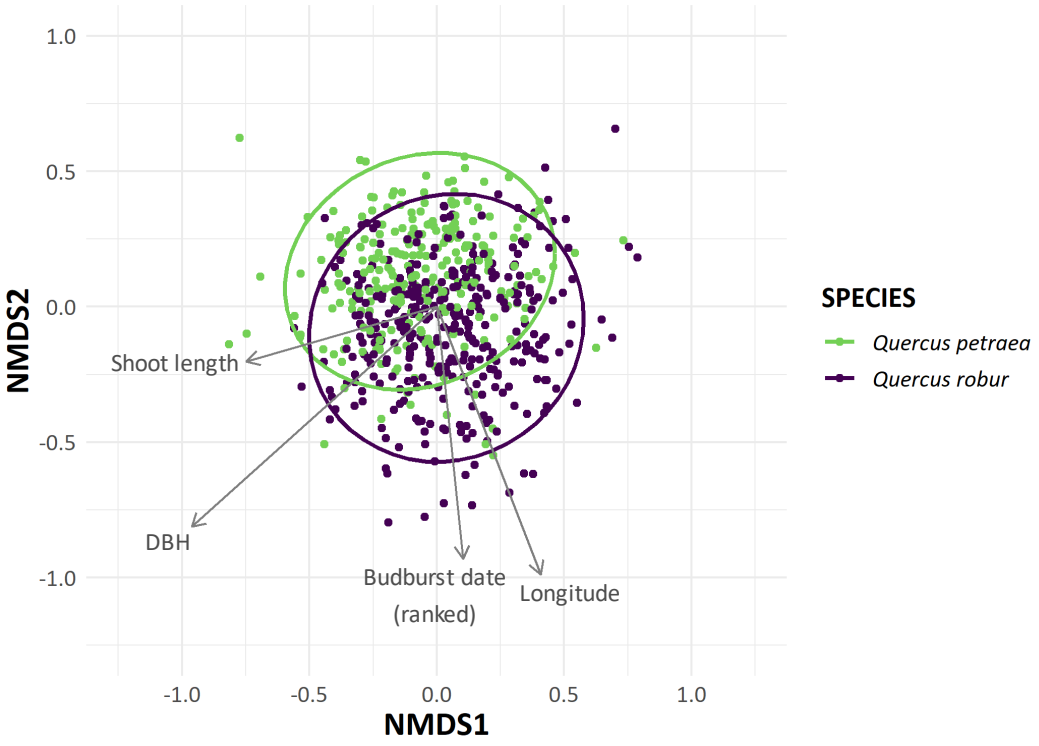

Figure 2.20 – a two axes NMDS plot of Bray-Curtis among tree dissimilarities of herbivore communities. Fitted environmental effects are those identified by the *Adonis* analysis as significant. Ovals represent 95% confidence intervals.

2.4. *Discussion*

Trees of both *Quercus robur* and *Q. petraea* were identified in the BSO trial and there were differences in insect herbivore abundance and diversity associated with host species. Phenotypic differences in the host plant, such as vigour and leaf phenology independently explained variation in both abundance and diversity of insect herbivores. In the following section the effects of each phenotypic trait on the different insect herbivore species, genera and guilds are discussed.

2.4.1. *Does oak species status influence insect herbivore abundance, richness and diversity?*

The leaf morphometric analysis revealed two distinct groups of individuals, classed as *Quercus robur* and *Q. petraea*. The two native oak species are closely related and ecologically similar, yet subtly different. Very few insect herbivores have been described to differentiate the two,

such as *Andricus quercuscalicis*, an acorn galling cynipid, that attacks *Quercus robur* and the *Q. robur* x *petraea* hybrid but has been reported to be absent or exceedingly rare on *Q. petraea* (Schönrogge et al., 1994).

The insect herbivore community recorded in the BSO trial associated with *Quercus robur* was both more species rich and diverse than *Q. petraea*. This conflicts with Southwood et al. (2004) who found that both species supported a very similar species richness of insect herbivores. Differences across these studies may be due to the sampling method used, this study used visual observation of herbivory whereas Southwood et al. (2004) used mist blowing to 'knock down' the insect species a method which could perhaps underestimate internal insects such as gallers and miners. Considering community structure, results here show that *Quercus robur* and *Q. petraea* share a different assemblage of insect species and at different relative abundances. This result suggests that at the species level there is evidence for oak species to act as ecological filters.

In general, *Quercus robur* supported a higher abundance of agamic gallers; this was particularly evident for *Neuroterus albipes* and *N. numismalis*, and also supported a higher number of leaf webbers. *Quercus petraea*, on the other hand, were attacked more frequently by leaf chewing insects and leaf rollers. There are morphological traits in which *Quercus robur* and *Q. petraea* differ that have been described as defensive against herbivores. For example, *Quercus petraea* leaves are noticeably more pubescent than *Q. robur* leaves. These trichomes may provide a barrier to insect feeding or to oviposition (Chiang and Norris, 1983). In contrast, some insects use the pubescence to hold onto, and to make themselves inaccessible by predators and parasitoids (Schoonhoven et al., 2005).

It is also likely that the two species of oak exhibit chemical differences. There are higher levels of tannins and lower levels of oak lactones in the heartwood of *Quercus robur* compared to

that of *Q. petraea* (Mosedale and Savill, 1996). Tannins and lactones are known deterrents of insect herbivores and it is assumed that as these differences in concentration occur in the wood, that they are likely to also be present in the leaves and other plant parts. In other oak species, condensed tannin levels are known to vary in leaves depending on the host species and this in turn effects the selection and performance of insect herbivores (Makkar et al., 1991, Hata et al., 2011). Ovipositing females have been shown to use plant volatiles, such as flavonoids and carboxylic acids, as host recognition cues (Roininen et al., 1999, Kagata and Ohgushi, 2002), these compounds may vary in composition or concentration between the two oak species.

It is clear that some insect herbivores respond to oak species status and it is thought that these differences would be even more distinct if hybrids were discernible. According to other studies, there are three possible scenarios (1) hybrids exhibit a form of outbreeding depression as they are more susceptible to insect herbivores than either parent species, (2) hybrids exhibit outbreeding enhancement and support fewer herbivores or (3) they could be intermediary (Fritz et al., 1994, Boecklen and Spellenberg, 1990, Pearse and Baty, 2012). However, hybrid individuals were undetectable in this study. The results of the leaf morphometric analysis gave no indication of an intermediate form i.e. no suggestion of a third group and no overlaps between the two groups. This corresponds with Kremer et al. (2002), who suggest that hybrids of *Q. robur* and *Q. petraea,* in particular juveniles, should display the morphologies of their maternal parent tree. The PCA used in this study shows no evidence to suggest hybrids are present in the trial and therefore their relationship with associated insect herbivore communities could not be inferred. However, the analyses of insect abundance, species richness and diversity do not suggest that a third population of trees were present, for example the NMDS plot showing the composition of insect herbivores associated with the *Quercus* trees does not show a third group. More research is needed to support these findings,

potentially with the use of molecular studies that have had some reported success in differentiating between these species and their hybrids (Petit et al., 1993, Dumolin et al., 1995, Muir et al., 2000, Guichoux et al., 2011).

2.4.2. *Was there evidence supporting the local adaptation hypothesis?*

According to the local adaptation hypothesis, native insect herbivores will perform better on trees from local provenances as they share a co-evolutionary history and are therefore better adapted to overcome host defences (Pearse and Hipp, 2012) and share a tighter phenological synchrony (Egan and Ott, 2007, van Asch et al., 2007, Pearse and Karban, 2013). In this study, few effects of provenance on insect abundance were found and it was not on local provenance trees that the highest diversity of insects was recorded. The widespread historical movement of oak around Europe and the UK could explain why these results do not follow the expected trend (Worrell, 1992, Petit et al., 2002). The provenances used in the BSO trial are not from as far south as might be necessary for climate matching in the UK, Broadmeadow et al. (2005) proposes that trees should be sourced from southern Italy to match the UK climate in 2050. If trees from more southerly latitudes were sourced, then it is possible the local adaptation hypothesis for insect herbivores would be better supported. However, tree provenance did significantly affect tree vigour and phenology, factors which in turn influence herbivore abundance and diversity.

2.4.3. *Does tree phenology influence insect herbivore richness and diversity?*

Using a provenance trial (BSO) of equal age trees reduces the impact of microclimate and host developmental stage on leaf phenology, so it is possible to study the implications of genotype on the budburst of oak trees. In this study, date of budburst varied with tree provenance

(latitude of the parent tree), suggesting heritable differences in leaf phenology. Trees from more southerly, generally warmer, latitudes should burst earlier in the season than those from northerly latitudes (Ducousso et al., 1996). As differences in phenology are partially genetically controlled, these trends should remain when the offspring are planted in the BSO trial. In this study, latitude of the parent tree influenced budburst in the spring, but the relationship was non-linear. As expected, trees from further south than Paradise Wood burst earlier in the season, consistent with other studies of oak (Ducousso et al., 1996, Deans and Harvey, 1995), however trees from further north than Paradise Wood also had earlier budburst times. As stated earlier, the historical movement of oak trees around Europe may help to explain this deviation from other studies. Trees in this oak trial were phenologically consistent year on year, the first to burst in 2008 were the first to burst in 2016. This corresponds well with other studies of oak in the UK (Crawley and Akhteruzzaman, 1988).

Consistent with the community genetics hypothesis, leaf phenology has been shown to be an important tree trait that influences the abundance and performance of insect herbivores on oak trees (Hough, 1953, Askew, 1962, Rosenthal and Koehler, 1970, Pearse et al., 2015). In this study, date of budburst influenced the percentage damage of leaves by leaf chewing insects and by leaf webbing insects, this correlates well with other studies of free-feeding insects (Jones, 1959, Satchell, 1962, Hunter, 1992, Tikkanen and Julkunen-Tiitto, 2003). Leaves in the early spring have a high-nitrogen content and reduced tannin concentrations, as the season progresses the leaves accumulate defences and become less digestible by free-feeding insects (Feeny, 1970, Forkner et al., 2004). The survival of these defoliating insects therefore depends highly on synchronicity of their emergence with leaf flushing in the spring and are therefore expected to be highly influenced by budburst date, as was shown in this study. Contrary to these results, Crawley and Akhteruzzaman (1988) found no such relationship between

defoliator performance and phenology, it is possible that these relationships may be affected additionally by spatial and temporal variability (Hunter, 1992).

The leaf webbers in the BSO trial preferred trees that budburst later. These webbing insects tended to feed on the apical parts of the shoot (personal observation), where they would be susceptible to frost damage in early flushing trees. Leaf webbers (e.g. *Acrobasis* spp.) are generally specialist feeders and may therefore be less sensitive to the defensive compounds in older leaves and will also encounter less competition from other free feeding insects later in the season.

The associations between date of budburst and abundance of galling and mining insects, in the literature, are invariably discordant. Regarding leaf miners, Mopper and Simberloff (1995) found that earlier burst trees escaped defoliation from leaf miners while other studies found conflicting results (Crawley and Akhteruzzaman, 1988, Pearse and Karban, 2013, Pearse et al., 2015). In this study, trees that burst earlier supported a greater abundance of leaf miners. As is the case with leaf chewers, the higher nutritional quality and reduced defences of the earlier leaves could aid colonisation and development of the leaf miners. It could, therefore, be assumed that assisted migration will positively influence the abundance of leaf mining insects as those trees transplanted from further south are likely to have earlier budburst times.

Agamic gallers were recorded at a higher abundance on those trees that burst later in the season, this is in accordance with agamic *Neuroterus* species in other studies (Askew, 1962, Crawley and Akhteruzzaman, 1988, Sinclair et al., 2015). The agamic generation of gall wasps develop in the autumn, so it is likely that they will preferentially select trees that have budburst later in the spring as the leaves will still have a high nutritional content and lower defences by the autumn. However, this does not explain why the spring generation of galling insects also preferred late bud-bursting trees, not in accordance with other studies (Sinclair et

al., 2015). It could be possible that selection pressures other than phenology, such as competition with other herbivores or natural enemies, are local selection regimes and lead to different adaptive outcomes in different localities. Regardless, the galling insects as studied in the BSO trial are likely to be negatively affected by climate matching, as trees from further south are likely to burst earlier in the season.

The intraspecific variation in leaf phenology is thought to be far greater than the interspecific variation (Jones, 1959, Satchell, 1962), however, in this study *Q. robur* trees burst consistently earlier in the season than *Q. petraea*.

Oak trees in the BSO trial also varied considerably in their tendency for leaf marcescence. The evolutionary explanation for the marcescent habit of oak trees is debated. The nutrient cycling hypothesis, whereby leaf marcescence guarantees a gradual release of nutrients, proposed by Otto and Nilsson (1981) seems improbable, because it suggests an advantage of marcescence in species with leaves that decompose quickly, however oak leaves are particularly slow to decompose (Steffen et al., 2007). Leaf retention may also be an adaptation to late or deep frosts, the senescent leaves protecting the new buds in the spring from frost damage (Nilsson, 1983). In this study, trees that retained their senescent leaves budburst later in the spring, another adaptation to avoid spring frosts. In might be expected that trees from more northerly latitudes would be more likely to retain senescent leaves overwinter but this was not shown here. It was found, however, that *Q. petraea* was more likely to exhibit marcescence than *Q. robur.* This provides support for the frost protection hypothesis as *Quercus petraea*, in general, is found growing in higher altitudes than *Q. robur* (Eaton et al., 2016) where later and deeper frosts are more common.

Spring galling insects, in particular *Trioza remota*, were negatively affected by leaf marcescence. It has been proposed that early leaf abscission is an induced response triggered

by herbivore attack (Williams and Whitham, 1986, Stiling and Simberloff, 1989, Fernandes et al., 2008). Early leaf fall could make the leaves nutritionally unsuitable for the developing insect (Kahn and Cornell, 1983), or could leave the insect more susceptible to predation or pathogen attack on the ground (Faeth et al., 1981). However, as galling insects tend to be at low densities on oak trees it may seem improbable that they could impose such selective pressures on their host. It seems more likely that leaf marcescence is merely an injury response of the host to herbivory (Faeth et al., 1981, Stiling and Simberloff, 1989).

On the other hand, leaf marcescence was shown to have a positive effect on the density of the gall midge, *Macrodiplosis pustularis*. In a similar study, *Q. lobata* trees that retained their leaves over winter experienced a three-fold increase in herbivore colonisation, especially by cynipid gall wasps, the following year (Karban, 2007). It is possible that retained leaves act as oviposition cues for the spring generation (Karban, 2007).

2.4.4. *Was there evidence supporting the plant vigour hypothesis or plant stress hypothesis?*

A curvilinear relationship between tree vigour (both DBH and shoot length) and latitude of the parent tree was recorded, with the largest trees originating from more local provenances. As might be expected, local adaptation of natal oak trees to their home environments means that trees from exotic provenances did not grow as well in Paradise Wood as the more local provenances.

According to the plant vigour hypothesis, trees that grow more rapidly, relative to the rest of the population, should support a higher density of insect herbivores due to their higher food quality and/or lower concentrations of induced defences (Price, 1991). In this study the DBH of the tree and average primary shoot length were used as estimates of tree vigour. In accordance with the plant vigour hypothesis, trees with a large diameter at breast height

supported a higher richness and greater diversity of insect herbivores. Larger trees and those with longer shoots also supported a different community structure of insect herbivores than smaller trees and those with shorter shoots. The agamic (autumn) galling and leaf mining guilds in particular, were recorded at higher numbers on trees with larger diameters and longer shoots. This is also in accordance with the expectation that late season (senescent) feeders will be influenced more by the plant vigour hypothesis (White, 2009). The sexual (spring) galling insects were unaffected by DBH but showed a strong preference for trees with longer shoots. The larvae of developing gall wasps and leaf miners are sessile, the larvae are solely dependent on plant quality chosen by the ovipositing female. Larger trees with longer shoots are more attractive and present an easier target for oviposition (Price, 1991). As galling and mining insects have this intimate relationship with their host plant, they are more likely to support the plant vigour hypothesis than free-feeding insects that are able to move between suitable feeding sites (Price, 1991, Cornelissen et al., 2008).

The free-feeding insects (leaf webbers, chewers and rollers) in the BSO trial, were unaffected by DBH but were recorded in higher numbers on shorter shoots, contrary to the plant vigour hypothesis. These free-feeding insects, in particular the external leaf chewers, are particularly exposed to predation by birds. By choosing shorter, less apparent shoots these insects are decreasing their possibility of detection, although there is no support of this theory reported in the literature.

This study found no evidence in support of the plant stress hypothesis, which suggests that herbivores would show a higher abundance on stressed trees that may have reduced chemical defences and elevated nutritional quality (White, 2009, White, 1969). Non-native provenances may experience more stressful growing conditions in the local climate in Paradise Wood, this was evident by the reductions in vigour from latitudes further south and north of the

provenance trial. This reduction in vigour did not increase herbivory in any of the feeding guilds.

2.4.5. *Conclusions*

In the UK, climate change predictions have forecast hotter drier summers and milder wetter winters (Hulme et al., 2002). These climatic changes are predicted to have detrimental effects on trees, for example reductions in growth rate, reductions in timber quality, increased chances of soil waterlogging in winter and drought in summer (Broadmeadow et al., 2003, Broadmeadow et al., 2005) and advanced phenology e.g. budburst (Kuster et al., 2014). Local provenance trees may not be able to adapt to this fast-changing climate. One solution is to select trees from exotic provenances which are currently experiencing warmer temperatures, higher winter rainfall and lower summer rainfall than the UK. However, as results here have shown, these trees are likely to have earlier budburst times and currently lower growth rates. As the results of this study have shown, these phenotypic variations will greatly influence the insect herbivore abundance, richness and community structure, either negatively or positively. Additionally, as many predators and parasitoids are dependent on these folivores, one may expect the impacts of climate matching to cascade up to these higher trophic levels.

In summary: (1) morphometric analysis is an efficient method for differentiating between the two oak species (*Quercus robur* and *Q. petraea*) and interspecific variation in insect herbivory was recorded for the first time in this study. *Quercus robur* is host to a higher richness and higher diversity of insect herbivores, and differences between hosts occurs at species and also at guild herbivore level. (2) this study provides support for the tree vigour hypothesis of galling and mining insects, with higher DBH and longer shoots supporting higher densities of both guilds. There was no support for the plant stress hypothesis. (3) tree budburst phenology influenced the damage caused by defoliating insects and had variable effects on galling and

mining insects. In general, trees that burst early in the season supported a higher richness and diversity of insect herbivores. (4) the theory that leaf marcescence is an adaptation to frost damage is supported and the effect of marcescence on two non-cynipid gallers is reported. (5) trees from non-local provenances had a smaller DBH and shorter shoots and differed in their budburst phenology to local provenances. (6) little evidence to support the local adaptation hypothesis of insect herbivores to their host, trees from local provenances did not support a higher abundance, richness or diversity of insect herbivore

CHAPTER 3 - *Evaluating the influence of tree phenotype on microbial endophytic communities of Quercus species in the UK*

3.1. *Introduction*

Microorganisms, including fungi, bacteria, archaea and protists, are ubiquitously associated with trees (Baldrian, 2017). These microbial communities can be divided into groups depending on the plant tissue they are associated with. The rhizosphere includes the microbial communities associated with the root surface and the adjoining soil, the phyllosphere includes those found on the outer surfaces of aerial plant parts and the endosphere includes those living within the plant tissues (Turner et al., 2013). The full set of microbial genomes associated with any plant is referred to as the plant microbiome. Just as the human microbiome has received increased attention (Turnbaugh et al., 2007, Hadrich, 2018), interest in the plant microbiome has escalated in recent years due to reports of the benefits these microbes may confer to the host (Turner et al., 2013). Given the important role that phyllosphere endophytes might play in plant health, relatively little is known of the mechanisms that shape these plant-associated microbial communities. The focus of this chapter is on the microbial organisms that reside in the endosphere of native *Quercus* species in the UK and whether changes in host identity influence these microbial communities.

3.1.1. *What are endophytes?*

The term 'endophyte' was originally used to describe any organism that resides within plant tissues i.e. the endosphere (Bary, 1879) (translated by Stergiopoulos and Gordon (2014)). Today, however, the term is primarily used for organisms that invade internal plant tissues without causing any apparent effect to the host (Wilson, 1995). Endophytes have been isolated

from virtually all plant species and the most commonly detected ones belong to the fungal and bacterial kingdoms (Strobel, 2018).

3.1.2. *How do endophytes affect tree success?*

The majority of endophytes are commensalistic i.e. they have no apparent effect on plant performance but use the plant for nutrients and protection from the external environment (Schulz and Boyle, 2005, Hardoim et al., 2015). Some endophytes are known to confer benefits to the host plant. They have the potential to offer tolerance to various abiotic stresses (Singh et al., 2011), such as thermotolerance (Redman et al., 2002), salt stress (Waller et al., 2005, Rodriguez et al., 2008) or drought tolerance (Waller et al., 2005, Khan et al., 2016). Endophytes may even provide protection against insect herbivores (Preszler et al., 1996, Azevedo et al., 2000, Miller et al., 2008) (see also *Chapter 5*) and resistance against pathogens (Arnold et al., 2003, Waller et al., 2005, Ganley et al., 2008, Ren et al., 2013) (see also *Chapter 6*). This protection against pests and pathogens could be due to the endophyte producing inhibiting bioactive compounds (Liu et al., 2001, Gao et al., 2010, Ren et al., 2013), through direct competition for space and nutrients (Arnold et al., 2003) or through the induction of plant defence reactions i.e. systemic resistance (Waller et al., 2005, Ganley et al., 2008).

It is also possible for endophytes themselves to be latent pathogens (Scortichini and Loreti, 2007, Kuo et al., 2014). Endophytes may switch from a neutral or beneficial relationship to a pathogenic relationship if the balanced interaction with the host is disrupted. For example, *Discula quercina* lives as a beneficial/neutral endophyte in symptomless oak trees (*Quercus cerris*), but if the tree is subjected to climatic stress this fungus becomes pathogenic, causing oak anthracnose (Moricca and Ragazzi, 2008). Many endophytes may also be latent saprobes, with some contributing to nutrient cycling as decomposers of leaf litter in the early stages of decomposition (Osono, 2006, Korkama-Rajala et al., 2008).

The nature of the plant-endophyte relationship ranges from mutualistic to pathogenic on what has been described as a symbiotic continuum (Schulz and Boyle, 2005). The relationship depends on abiotic and biotic factors including host genotype, microbe genotype, environmental conditions and the interactions between organisms in the endosphere (Hardoim et al., 2015).

3.1.3. *What environmental or tree characteristics influence endophytic communities?*

Community assembly is the study of the processes that shape the composition and abundance of species in a community (Weiher et al., 2011, Kraft and Ackerly, 2014). In the context of the endophytic community, the 'species pool' would refer to all the potential colonists in the environment outside of the plant (Kraft and Ackerly, 2014). Abiotic and biotic barriers then act as 'filters' to the successful establishment of a subset of this species pool (Diamond, 1975, Weiher et al., 2011) (*Figure 3.1*). These filters can be divided into two groups: habitat filters and species interactions (Saunders et al., 2010). Environmental factors act as habitat filters preventing the establishment of endophytes that are unable to survive. Variation in endophyte communities have been recorded across latitudes and altitudes, with differences attributed to variations in temperature and precipitation (Terhonen et al., 2011, Millberg et al., 2015, U'Ren et al., 2012, Zimmerman and Vitousek, 2012, Cordier et al., 2012b). In general, endophytic communities increase in diversity from arctic to tropical sites (Arnold and Lutzoni, 2007, Terhonen et al., 2011), although this may not be true for coniferous species (Millberg et al., 2015).

If the endophyte is able to tolerate the abiotic conditions in the environment, their success is then dependent on the outcome of interactions with other species in the community. Plant-imposed filters, such as variations in defence mechanisms are likely to influence the microbial

endophyte community, as are interactions between microbes that range from competitive (Arnold et al., 2003) to facilitative (Lawrey, 2000). Different host species, even those living in close proximity, have been shown to have different endophytic communities associated with them (Morrica et al., 2012, Redford et al., 2010, Whipps et al., 2008, Lambais et al., 2006). Host genotype is another factor driving the structure and composition of endophytic communities in the endosphere (Balint et al., 2013, Todd, 1988, Cordier et al., 2012a, Bodenhausen et al., 2014).

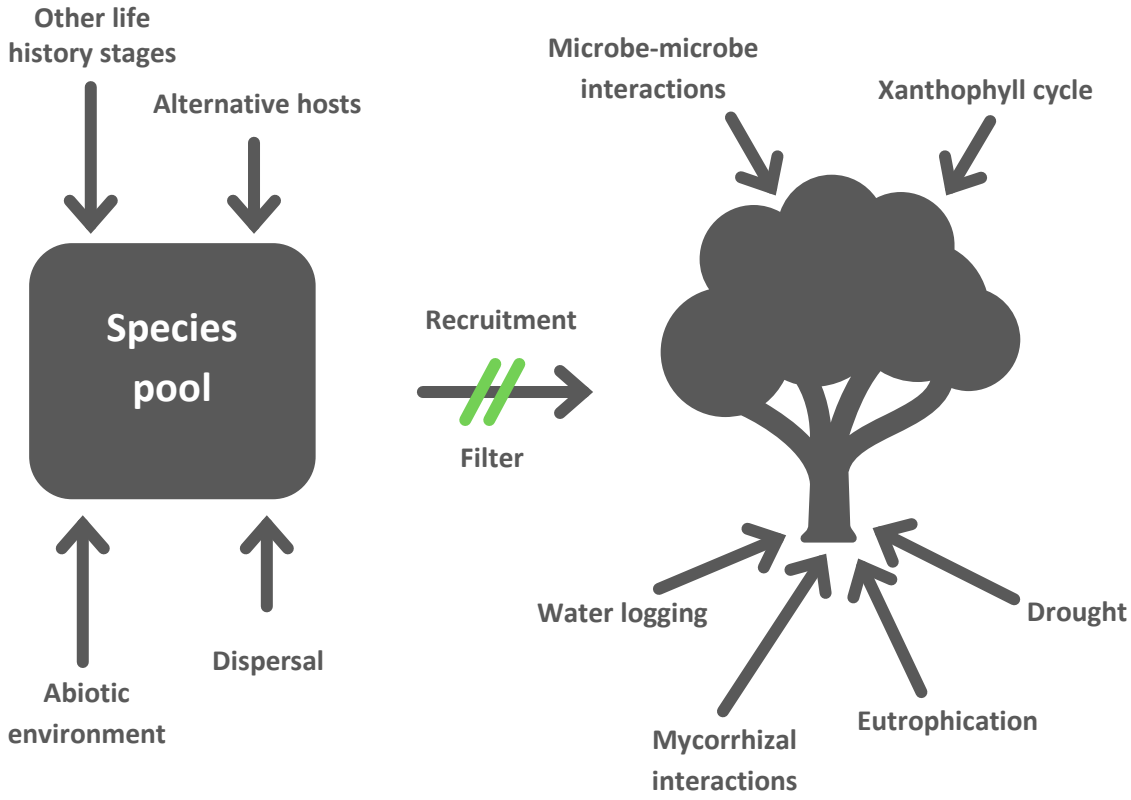

Figure 3.1 – factors influencing the community assembly of endophytic species in the tree ecosystem

Within an individual tree there may be variation in endophytic communities between plant organs e.g. between leaves and twigs (Sieber, 1989, Ragazzi et al., 2001) as endophytic species are likely to adapt to the physiology and biochemistry of particular plant organs. Dissimilarity between endophyte communities may even exist between leaves on the same plant (Cordier et al., 2012a). Leaf age may influence the microbial endophytic community, with differences

likely due to the time of exposure to infection and colonisation by the endophytes i.e. window of opportunity and changes in leaf physiology with age (Hata et al., 2011). The health status of the tree has also been shown to affect endophyte communities. Declining Scots pine (*Pinus sylvestris*) trees harboured a higher diversity of microbes within the sapwood compared to seemingly healthy trees (Giordano et al., 2009), the same result was found for declining oak trees (*Quercus cerris* and *Q. pubescens*) (Morrica et al., 2012). *Chapter 6* addresses the impact of tree health on the microbial endophytic community of *Quercus* species in the UK. Forest structure and surrounding vegetation have also been shown to influence endophytic communities (Saikkonen, 2007, Nguyen et al., 2016, Nguyen et al., 2017).

This study considers the two UK native oaks: *Quercus robur* and *Q. petraea*, species of great cultural, historical and ecological significance (Ducousso and Bordacs, 2004). The endophytes of these two tree species have been studied previously in a different context. Agostinelli et al. (2018) recorded the fungal endophytes associated with *Q. robur* in Sweden, they found a marked difference in endophyte diversity of xylem tissues in low vitality trees using culture dependent methods. For a deeper analysis of endophytes, culture independent methods have been developed (Knief, 2014). This study uses next generation sequencing to characterise both the fungal and bacterial endophytic communities of *Quercus* species in the UK. This is believed to be the first time that the fungal endophyte community of *Quercus robur* and *Q. petraea* has been determined using culture independent techniques. The bacterial endophyte community of these oak species have been recorded previously using next generation sequencing (Meaden et al., 2016, Sapp et al., 2016, Denman et al., 2017), however these studies have focused on bark and woody tissues of mature trees. This study focuses on the effect of host phenotypic traits on the fungal and bacterial endophyte community of leaf and twig samples of young oak trees. Interspecific differences in the endophytic community between *Quercus robur* and *Q. petraea* are compared for the first time.

3.2. *Materials and methods*

3.2.1. *Site description and tree selection*

The oak provenance trial (BSO trial) in Paradise Wood, Oxfordshire was established in 2003 by Earth Trust with the aim of improving the quality and productivity of UK native oak trees (Clark and Hemery, 2009) (see *Chapter 2* for further details of the trial design).

Nineteen oak families belonging to seven provenances spanning a wide geographic range, were selected from the BSO to examine the diversity and abundance of leaf and twig endophytes associated with oak trees (*Figure 3.3*). Of the 19 families, 10 were *Quercus robur* according to the morphometric analysis and nine were *Q. petraea* (*Chapter 2, Section 2.3.1*). Included in the 19 families were four families from Hampshire, used here as a local 'control' as the parent trees were closest geographically to the trial site and included both oak species. These 19 families were sampled from 3 replicate blocks (giving a total of 57 trees (*Appendix D.1*).

3.2.2. *Measured tree traits*

3.2.2.1. *Oak species*

Species assignment of each tree to either *Quercus robur* or *Q. petraea* was established using leaf morphometric measurements, a method developed by Kremer et al. (2002). The full details of how these measurements were determined are provided in *Chapter 2, Section 2.2.2*.

Two trees (22_18_HAM012 and 35_56_HAM013) were removed from analysis as their species assignment did not match the rest of their family, leaving 55 trees for analysis.

Figure 3.2 - (a) map of the UK showing location of Paradise Wood (white cross). Map created using R packages: maps (version 3.3.0, Becker et al. 2018), and ggmap (version 3.0.0, Kahle and Wickham 2013) **(b) aerial photograph of Earth Trust Paradise Wood, shaded box indicates location of the BSO trial.** Photo taken from Google Maps.

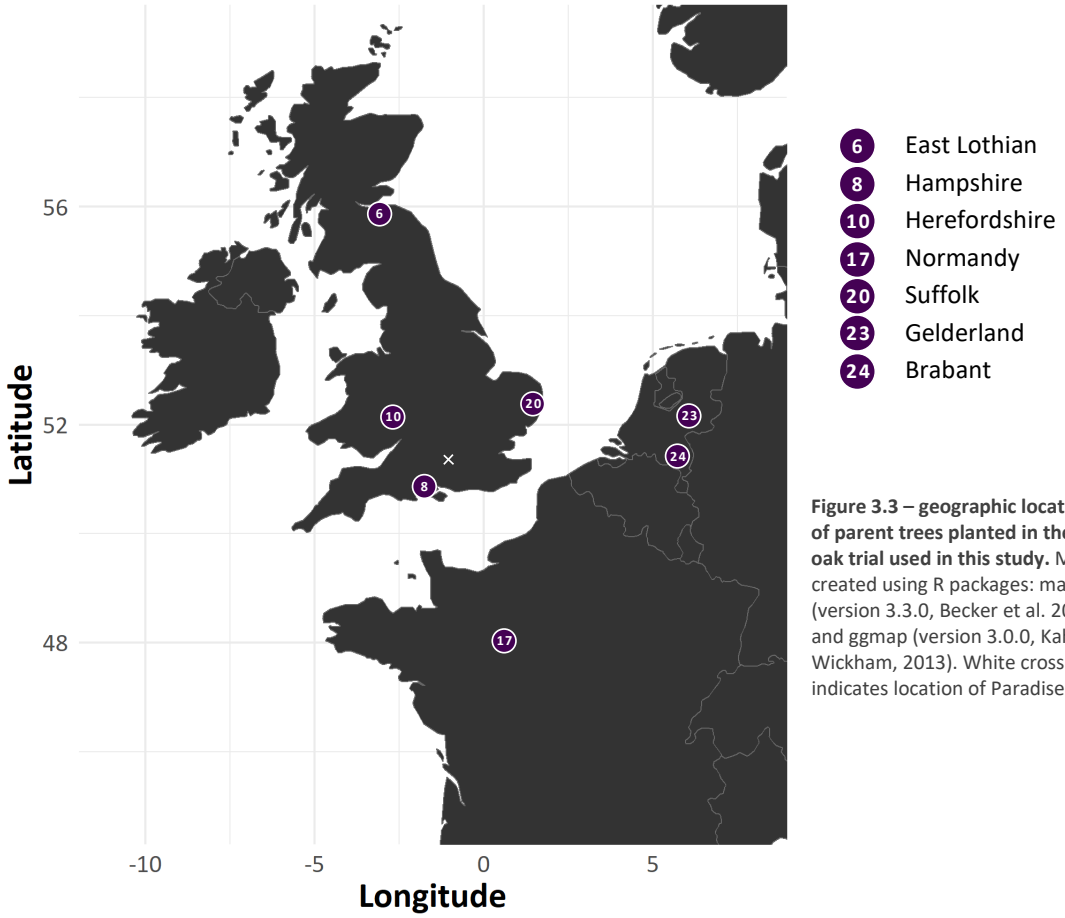

Figure 3.3 – geographic locations of parent trees planted in the BSO oak trial used in this study. Map created using R packages: maps (version 3.3.0, Becker et al. 2018) and ggmap (version 3.0.0, Kahle & Wickham, 2013). White cross indicates location of Paradise Wood

3.2.2.2. *Phenology*

The timing of budburst of the oak trees in Paradise Wood was recorded in spring 2016 (see *Chapter 2, Section 2.2.4.1* for a full description of data collection). Leaf retention in the autumn (known as marcescence) was also recorded in autumn 2016 (see *Chapter 2, Section 2.2.4.2*). The effect of these phenological traits on the endophyte population will be studied.

3.2.2.3. *Determining tree vigour using DBH and shoot length*

Diameter at breast height and average shoot length of 10 shoots per tree were used to gauge tree vigour (see *Chapter 2, Section 2.2.3* for full details of how these measurements were performed). The influence of tree vigour on the microbial endophyte population of oak trees will be assessed.

3.2.2.4. *Determining tree health using chlorophyll fluorescence*

Another measure that is commonly used to determine tree health is to measure chlorophyll fluorescence (Percival, 2005). A continuous excitation chlorophyll fluorometer (Hansatech Handy PEA+) was used to take measurements of 15 leaves evenly distributed over each tree. Care was taken to only select fully expanded, similar sized leaves from the first flush with no visible insect herbivore or disease damage. To dark adapt the leaves, leaf clips were attached for 20 minutes prior to measurement. The measurements were all taken on the same clear, sunny day (24th August 2016), to reduce the possible impact of weather on the readings. The Fv/Fm value was used for further analysis as this is the most common technique for measuring tree stress (Percival, 2005). This measurement indicates the maximum efficiency of photosystem II chemistry if all capable centres are functional (Murchie and Lawson, 2013).

3.2.3. *Sample collection*

On 27th September 2016 four fully expanded leaves from the first flush were collected from each tree, one from each cardinal point. These were pooled together in a sterile zip-lock bag and were transported back to the laboratory on ice. Care was taken to select leaves that were insect and disease free and were not noticeably damaged. Twig samples were also taken; one 2cm section of twig was cut from each tree and transported in the same way as above. All plant tissues were removed with scissors sterilised with 100% ethanol and gloves were used to avoid contact with the hands. All samples were stored at 4°C until processed.

3.2.4. *Surface sterilisation*

To remove those fungi or bacteria that live epiphytically on the plant surface, all plant material were surface sterilised within 48 hours of collection using method A (*Appendix B*). For twig samples they were shaken in the bleach for 8 minutes, all other steps remain the same. A 5x5mm square of each leaf was cut using a sterile scalpel and pooled (four leaves per tree) into a 2mL microcentrifuge tube and stored at -80°C for DNA extraction. A 10mm section was cut from the centre of each twig and stored in 2mL microcentrifuge tubes at -80°C.

3.2.5. *Sequencing preparation*

3.2.5.1. *DNA extraction*

Leaf and twig samples were removed from -80°C storage and the tubes were immediately immersed in liquid nitrogen. Total DNA was extracted from leaf and twig samples using method A (*Appendix C*). An extraction blank was included. To test for integrity, DNA was run on a 1% w/v agarose gel at 90V for 40 minutes. DNA concentration and quality were measured using a UV-Vis spectrophotometer (NanoDrop™ ND-1000, Thermo Fisher Scientific).

For most samples a clear extract was produced of high-quality DNA, however, for 11 of the twig samples with suboptimal nanodrop quality readings (and a noticeable brown colour to the pellet) it was necessary to remove possible PCR inhibitors using gel extraction. A 1% w/v agarose gel was cast with a wide gel comb. 20µL of each DNA sample, mixed with loading buffer, was loaded into the gel, making sure to leave a blank well either side of the sample to avoid contamination between samples. The gel was run at 50V for 80 minutes. Using a dark reader transilluminator (Clare Chemical Research, DR-48B) the defined DNA band was excised from the top of the gel using a sterile scalpel, making sure to avoid the lower smear which could contain inhibitors. The gel piece was transferred into a 1.5mL tube and weighed. DNA was extracted from the gel using the Monarch® DNA gel extraction kit (New England BioLabs) following the manufacturer's protocol, and DNA was eluted in 20µL molecular grade water.

DNA was extracted from colonies of four microbial species of interest: *Brenneria goodwinii*, *Gibbsiella quercinecans*, *Rahnella victoriana* and *Raoultella planticola* (provided by Oliver Booth and Mateo San José Garcia, University of Reading) to run alongside the oak samples on the Illumina Miseq platform. These four bacterial species have been implicated in the decline syndrome, Acute Oak Decline (AOD) (Brady et al., 2017) but their presence in asymptomatic trees has not been established. All strains were isolated from the bleeding lesion of *Quercus* tree WD01195 in Writtle Forest, Essex using MacConkey agar. Species identity was confirmed by Oliver Booth and Mateo San José Garcia using 16S amplicon sequencing (Booth, 2019, unpublished) and by gyrB sequencing (*Appendix I*). Strains were grown from -80°C stocks (overnight culture in Luria Bertani broth (LB) and 20% glycerol) on Luria Bertani agar for 24 hours at 27°C, single colonies were picked and grown in LB overnight, shaking at 27°C. DNA was extracted from the overnight culture of each bacterium using Gentra® Puregene® kit (Qiagen) following manufacturer's instructions. These samples were included with the oak samples in the following sections.

3.2.5.2. *PCR and Sequencing*

The following two step polymerase chain reaction procedure was developed and optimised by Dr Anna Oliver at the Centre for Ecology and Hydrology (CEH), Wallingford. For bacterial species, the V5 and V6 regions of the 16S rRNA gene were targeted for amplification and for fungal species the ITS region 2 (ITS2). The first PCR step produces a single amplicon (either 16S or ITS) and uses gene specific primer pairs with overhanging adapter sequences appended to them (Illumina adapter primers, shown in *Table 3.1*). The second step PCR adds on the indexes and the sequence the Illumina sequencing primers bind to (Illumina tag primers, shown in *Appendix E.1*); these indexes are unique forward and reverse barcodes for each sample.

All 110 DNA samples (55 leaf and 55 twig) were diluted to 10ng/µL with nuclease-free water and were arranged into 96-well plates (*Appendix E.2-E.5*). The four bacterial species of interest were included on the plate, as was a negative water control. The first step PCR mix contained 10µL of 5X PCR buffer (Q5® reaction buffer, New England Biolabs), 0.5µL each of forward and reverse primers (100µM, Eurofins Genomics, *Table 3.1*), 1µL dNTP mix (each 10mM), 0.5µL Q5® high-fidelity DNA polymerase (5U, New England Biolabs), 2µL of DNA (10ng/µL) and made up to 50µL with molecular grade water. PCR conditions consisted of an initial denaturation at 98°C for 30 seconds, followed by 25 cycles of 98°C for 10 seconds, 50°C for 10 seconds and 72°C for 20 seconds and a final elongation step at 72°C for 10 minutes. PCR products were purified using ZR-96 DNA clean up kit (Zymo Research) according to kit instructions, and samples were eluted with 40µL molecular grade water.

For the second step PCR reaction a unique forward and reverse primer combination was created for each sample. Four primer arrays were created using the primers in *Appendix E.1-E.5*. The 50µL PCR reaction mix contained 10µL 5X PCR buffer (Q5® reaction buffer, New England Biolabs Inc.), 5µL of the primer mix from the array (10µM, Eurofins Genomics), 1µL

dNTP mix (each 10mM), 0.5µL Q5® high-fidelity DNA polymerase (5U, New England Biolabs), 10µL of cleaned step 1 template and made up to 50µL with nuclease-free water. PCR conditions consisted of an initial denaturation at 98°C for 30 seconds, followed by 10 cycles of 98°C for 10 seconds, 62°C for 20 seconds and 72°C for 30 seconds and a final elongation step at 72°C for 2 minutes. PCR products were run on a 2% w/v agarose gel, at 90V for 60 minutes. A band at 400bp was expected for 16S samples and 500bp for ITS samples.

25µL of each sample was normalised using a SequelPrep™ kit (Applied Biosystems) according to manufacturer's instructions. 10µL of each sample in each 96-well plate were pooled to make libraries, resulting in four libraries: 16SA, 16SB, ITSC and ITSD. Each library was concentrated down to approximately 50µL using a vacuum concentrator (Eppendorf, 5301), set at room temperature for around 3 hours. The remaining 50µL of each library was mixed with loading dye and run on a 2% w/v agarose gel for 45 minutes at 85V. Bands were excised at 400bp for 16S and 500bp for ITS and DNA was extracted using QIAquick gel extraction kit (QIAGEN), following kit instructions. Samples were eluted in 30µL of kit elution buffer. The concentration of these libraries was quantified using a Qubit fluorometer and Qubit dsDNA HS assay kit, the DNA concentration was converted to nM based on the size of the DNA amplicon. 16SA and 16SB were combined into one library and ITSC and ITSD into another library. The following steps were performed by Anna Oliver, CEH. The two libraries were run on one flow cell with the addition of 10% PhiX on the Illumina MiSeq platform using V3 chemistry.

Table 3.1 – amplification primers used in the first step PCR for Illumina Miseq sequencing for *Quercus* samples

Primer name	Primer sequence	Target region
16SV5IAF	5'- ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGATTAGATACCCTGGTA -3'	V5-V6 region of 16S gene
16SV6IAR	5'- AGACGTGTGCTCTTCCGATCTCGACRRCCATGCANCACCT -3'	
ITS2IAF	5'- ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCCTCCGCTTATTGATATGC -3'	ITS2
ITS2IAR	5'- AGACGTGTGCTCTTCCGATCTGTGARTCATCGAATCTTTG -3'	

3.2.6. *Bioinformatic analysis*

The following bioinformatic analysis was performed by Dr Soon Gweon, University of Reading for the 16S samples. Sequenced paired-end reads were joined using VSEARCH (Rognes et al., 2016), quality filtered using FASTX tools (hannonlab.cshl.edu), length filtered with the minimum length of 300bp, presence of PhiX and adapters were checked and removed with BBTools (jgi.doe.gov/data-and-tools/bbtools/), and chimeras were identified and removed with VSEARCH-UCHIME_REF (Rognes et al., 2016) using Greengenes Release 13_8 (at 97%) (DeSantis et al., 2006). Singletons were removed and the resulting sequences were clustered into operational taxonomic units (OTUs) with VSEARCH_CLUSTER (Rognes et al., 2016) at 97% sequence identity (Tindall et al., 2010). Representative sequences for each OTU were taxonomically assigned by RDP Classifier with the bootstrap threshold of 0.8 or greater (Wang et al., 2007) using the Greengenes Release 13-5 (full) (DeSantis et al., 2006) as the reference. Unless stated otherwise, default parameters were used for the steps listed. For the ITS samples, Dr Soon Gweon, University of Reading used the PIPITS pipeline (Gweon et al., 2015) which uses the UNITE fungal ITS reference data set (Nilsson et al., 2018) for taxonomic assignment.

All operational taxonomic units (OTUs) assigned to chloroplast, mitochondrial or organisms other than fungi and bacteria were removed. Also, all OTUs that were unassigned were removed.

3.2.7. *Targeted search for AOD pathogens*

As the 16S rRNA gene of members of the *Enterobacteriaceae* family is highly conserved, culturing methods and more targeted PCR approaches were used to search for the pathogens thought to be involved in Acute Oak Decline in the Paradise Wood oak samples: *Brenneria goodwinii* (*Bg*), *Gibbsiella quercinecans* (*Gq*), *Rahnella victoriana* (*Rv*) and *Raoultella planticola*

(*Rp*). Tissue samples were chosen for re-sampling based on the results from the Illumina Miseq sequencing. Samples were collected in the same way as above (*Section 3.2.3*) on the 19th July 2019. Samples were stored at 4°C and were processed within 24 hours. Samples were surface sterilised using method A (*Appendix B*). Four 5x5mm squares of each leaf and one 10mm section of twig were cut with a sterile scalpel and placed into 1mL lysing matrix tubes with a ¼ inch ceramic sphere (Lysing Matrix M, MP Biomedicals), 50mg of autoclaved acid washed sand and 1mL of phosphate buffered saline (PBS). Samples were homogenised using a bead beating system (FastPrep-24™ 5G Instrument, MP Biomedicals) for 20 seconds at top speed. Samples were centrifuged at 5000rpm for 5 seconds to gently pellet the leaf and twig tissue. 100µL of each sample (taken from the top of the tube) was transferred to MacConkey agar and spread using a sterilised glass spreader. Each sample was also diluted 1:10 with PBS and 100µL was spread onto another MacConkey plate. The plates were incubated at 27°C for 48 hours. After 48 hours, all pink colonies (potential members of the *Enterobacteriaceae* family) were re-streaked onto fresh MacConkey agar and incubated for 24 hours at 27°C. *Figure 3.4* shows the morphology of the four bacterial controls used as comparison.

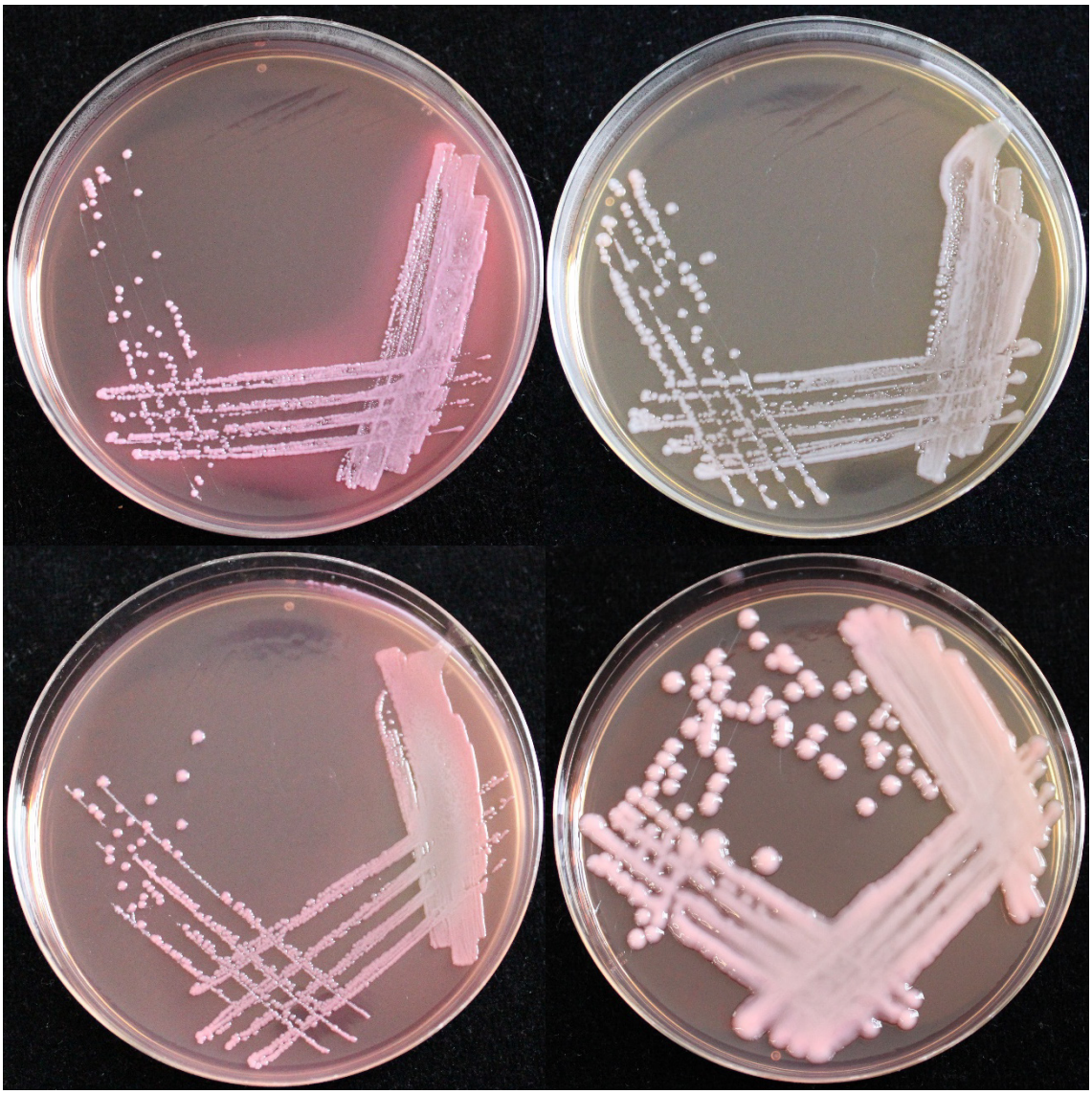

Figure 3.4 – colony morphology of the 4 bacterial species of interest grown on MacConkey agar, clockwise from top-left: *Brenneria goodwinii*, *Gibbsiella quercinecans*, *Rahnella victoriana* and *Raoultella planticola*. These strains were isolated from a *Quercus* tree in Writtle Forest displaying bleeding symptoms.

To further narrow the search for AOD pathogens, a rapid, inexpensive and accurate method developed by (Doonan et al., 2015) was used to ribotype the bacteria using the ITS1 region. PCRs were performed in 50µL reactions containing: 25µL 2X PCR Buffer (MyTaq™ Mix, Bioline), 2.5µL each of forward (ITSF) and reverse (ITSReub) primer (10µM, Eurofins Genomics, *Table 3.2*) and made up to 50µL with molecular grade water. One colony of bacterial cells was picked from the MacConkey agar and added directly to the PCR mix. PCR conditions consisted of initial denaturation at 95°C for 60 seconds, followed by 25 cycles of denaturation at 95°C for

15 seconds, annealing at 55°C for 15 seconds and elongation at 72°C for 10 seconds, a final elongation step at 72°C for 10 minutes was included. PCR products were visualized on a 3% agarose gel at 120V for 120 minutes (Doonan et al., 2015). PCR products of known cultures of BG, GQ, RV and RP were run alongside the samples on the gel. Amplicon banding patterns of the samples were compared to the positive controls.

Amplicon banding patterns matching the positive controls were selected and sequenced using the type II topoisomerase *gyrB* gene to verify the species. Single colonies of interest were picked and grown in King's B broth overnight in a shaking incubator set at 27°C. To extract the DNA, 1mL of the overnight culture was centrifuged at 13000rpm for 3 minutes, supernatant was discarded, and pellet was re-suspended in 200µL of TE buffer (1M tris, 0.5M EDTA, pH8). Samples were heated in a heat block at 100°C for 10 minutes, and then cooled on ice for 10 minutes. Samples were centrifuged again at 13000rpm for 3 minutes and the supernatant was used in the PCR. Primers gyrB01F and gyrB02R were used for amplification, as shown in *Table 3.2*. The PCR mix consisted of: 25µL 2X PCR mix (PCRBIO Taq Mix Red, PCR Biosystems), 2µL forward and reverse primer (10µM), 5µL of template DNA; made up to 50µL with molecular grade water. The PCR conditions were: 95°C for 5 minutes, 3 cycles of 95°C for 1 minute, 55°C for 2 minutes 15 seconds and 72°C for 1 minute 15 seconds followed by 30 cycles of 95°C for 35 seconds, 55°C for 1 minute 15 seconds and 72°C for 1 minute 15 seconds and with a final elongation step of 72°C for 7 minutes (Brady et al., 2008). PCR products were run on a 1% w/v agarose gel at 90V for 40 minutes, a band was confirmed at 742bp using a 1kb ladder (Hyperladder 1kb, Bioline). PCR products were purified using Monarch® PCR & DNA Cleanup kit (New England Biolabs) following kit instructions and DNA was sent to Eurofins Genomics for sequencing using primers gyrB 07-F and gyrB 08-R listed in *Table 3.2*. Sequences were trimmed and forward and reverse reads were aligned using BioEdit (version 7.2.6, Hall, 1999). Concatenated sequences were BLAST against the NCBI nucleotide database.

Table 3.2 – PCR and sequencing primers used to identify isolates from asymptomatic *Quercus* trees from Paradise Wood using culture-dependent techniques

Primer name	Primer sequence	Target region	Source
ITSF	5'-GTVGTAAACAAGGTAGCCGTA-3'	ITS	Cardinale et al. (2004)
ITSReub	5'-GCCAAGGCATCCACC-3'		
gyrB 01-F	5'-TAARTTYGAYGAYAACTCYTAYAAAGT-3'	DNA gyrase	Brady et al. (2008)
gyrB 02-R	5'-CMCCYTCCACCARGTAMAGTT-3'		
gyrB 07-F	5'-GTVCGTTTCTGGCCVAG-3'	DNA gyrase	Brady et al. (2008)
gyrB 08-R	5'-CTTTACGRCGKGTCATWTCAC-3'		

3.2.8. *Statistical analysis*

Rarefaction curves were produced for each OTU table to see whether sufficient sequencing depth was reached to allow a reasonable estimate of the number of different OTUs. For fungi, the majority of samples converged i.e. reached asymptote, suggesting that the sequencing depth achieved here provides a good estimate of the number of different OTUs in the full community (*Appendix F.2*). For 16S however, the sequencing depth of the samples differed considerably (*Appendix F.2*). To adjust for these differences in sequencing depth in the analysis, OTU tables were rarefied to an even sequencing depth prior to analysis using the *vegan* package (version 2.5-3, Oksanen et al. (2018)) in R to avoid biases resulting from differences in sample size. Data were rarefied to the lowest sequencing depth. There has been recent debate in the scientific community about the efficacy of rarefying (McMurdie and Holmes, 2014, Hughes and Hellmann, 2005), that it can lead to loss of statistical power so the raw OTU table was also analysed and results compared.

The extraction blank contained 29 OTUs and the negative control 17 OTUs, this contamination could be from the laboratory environment, the equipment or from the user. It is difficult to distinguish between this 'real' contamination and the possible cross contamination between samples during the sequencing process (Fort et al., 2016). There appears to be no agreement in the literature on how to deal with OTUs found in negative controls. One option is to delete any OTUs that appear in the negative controls across all samples, however as in other studies

(Fort et al., 2016, Nguyen et al., 2015) two of these OTUs were abundant in the experimental samples. In the present study, the taxa represented by the two OTUs present in the negative controls are known to be associated with trees so were considered to be ecologically valid. Additionally, these OTUs were markedly lower in abundance in the negative controls compared to the experimental samples so it was decided that they would be retained in the dataset for analysis (after Fort et al., 2016, Nguyen et al., 2015). Also, when the negative control samples were included in the NMDS plot based on a Bray-Curtis similarity index, their NMDS values were considerably different, suggesting different composition of OTUs than the experimental samples.

Before sequencing, the DNA of 11 samples were gel extracted to remove PCR inhibitors: 22_02_HRF013, 22_04_REN001, 22_06_HRF004, 22_34_ELT004, 22_40_ELT005, 35_01_HAM007, 35_24_ELT004, 35_32_ELT005, 35_54_HRF017, 37_07_ELT005 and 37_39_REN003. It was noticed during analysis that the diversity between these gel extracted *Quercus petraea* OTU samples and the other *Q. petraea* twig samples appeared different when considering the NMDS plot based on Bray-Curtis similarity indices (*Appendix F.1*). Although, these samples were not statistically significantly different from the other *Quercus petraea* samples, they were removed from further analysis. This decision was made, as interpreting whether potential differences are in fact genuine differences within the *Quercus petraea* population or a result of the gel extraction process would be difficult.

OTU richness was calculated as the number of observed species in each sample, the Shannon-Wiener diversity index was used to estimate species diversity and Pielou's evenness index was used to measure evenness using the *vegan* package. Linear mixed effect models were used to analyse OTU richness, diversity and evenness correlations with host plant species (*Quercus robur* versus *Q. petraea*), host plant provenance (latitude and longitude), host vigour (DBH and shoot length), host phenology (budburst date ranked), host leaf marcescence habit (abscised

or retained), host health (chlorophyll fluorescence based on Fv/Fm values) and tissue type (leaf or twig) nested within species as fixed effects and block number and the families within each provenance (provenance/family) as random variables in the model. OTU tables for fungal and bacterial species were analysed separately. The residuals were tested for normality using a Shapiro-Wilk normality test and for heteroscedasticity by plotting the residuals versus the fitted values for each model (Crawley, 2007).

Dissimilarities in OTU composition between samples (beta diversity) was calculated based on the Bray-Curtis dissimilarity index. Dissimilarities between samples were visualised in a non-metric multidimensional scaling (NMDS) plot using 2 axes (k=2) and ensuring that the stress value was close to or below 0.2. Bray-Curtis dissimilarities were analysed using permutational multivariate analysis of variances (PERMANOVA) using the *adonis* function in the vegan package (Oksanen et al., 2018), with 999 permutations and block number as a stratifying factor. The fixed effects used were the same as for alpha diversity.

The 20 most abundant fungal and bacterial OTUs were identified. The taxonomy of these OTUs was assigned according to the UNITE reference database for fungi (Nilsson et al., 2018) and Greengenes reference database for bacteria (DeSantis et al., 2006). Their identity was confirmed by comparing the representative OTU sequence with the NCBI GenBank database using the BLAST function (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The GenBank database was used to see if these OTUs of interest matched closely to any taxa isolated from similar studies of endophytes. Linear mixed effect models were used to test the effects of a number of variables (same as alpha diversity above) on the abundance of these OTUs. The square root of the OTU abundance was included in the Gaussian model, and the residuals of the model were tested for normality using the Shapiro-Wilk test and for heteroscedasticity by plotting the fitted versus residual values of the model (Crawley, 2007).

The OTU tables were also searched for potential pathogenic or saprophytic species and the relative abundance of these species across all sample types were recorded.

3.3. *Results*

The total number of joined reads for 16S was 6,117,252 which after quality filtering resulted in 6,113,724 reads. For ITS the number of joined reads was 8,044,812 and after quality filtering 8,034,269 remained. The sequencing reads clustered into 2955 OTUs for fungi (ITS) and 1535 OTUs for bacteria (16S). The fungal OTU matrix was rarefied to the lowest sequencing depth of 10989, leaving 2542 OTUs for analysis. The bacterial OTU matrix was rarefied to 147, leaving 790 OTUs for subsequent analysis. Rarefying both OTU tables made no differences to the results of the statistical tests, the results reported here are the results after rarefying.

Of the remaining fungal OTUs, 62.2% were assigned to the Ascomycota phylum, 13.2% to the Basidiomycota divisions and 23.6% remained unassigned. *Figure 3.5* shows the relative abundance of the 10 highest abundant classes within each tissue type and each species of *Quercus*. The bacterial sequences assigned to the Proteobacteria phylum accounted for 57.6% of all the OTUs, 28.2% were classed as Actinobacteria, 8.5% Bacteroidetes, 1.7% Acidobacteria, 1.4% Firmicutes and 9.3% remained unassigned. *Figure 3.6* shows the relative abundance of the top 10 class divisions between tissue type and *Quercus* species.

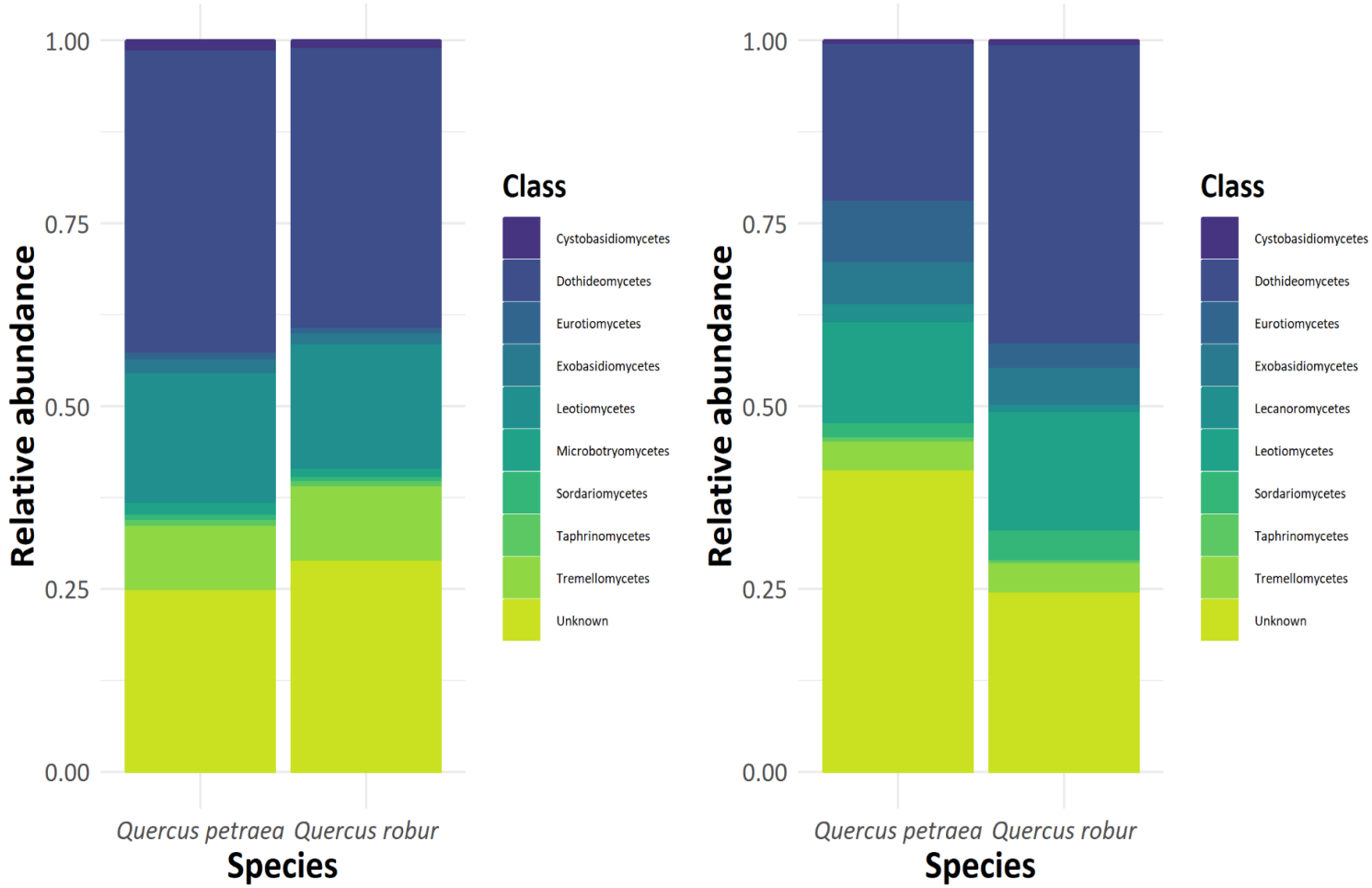

Figure 3.5 – relative abundance of the top 10 class divisions for fungal OTUs, leaf (left) and twig (right)

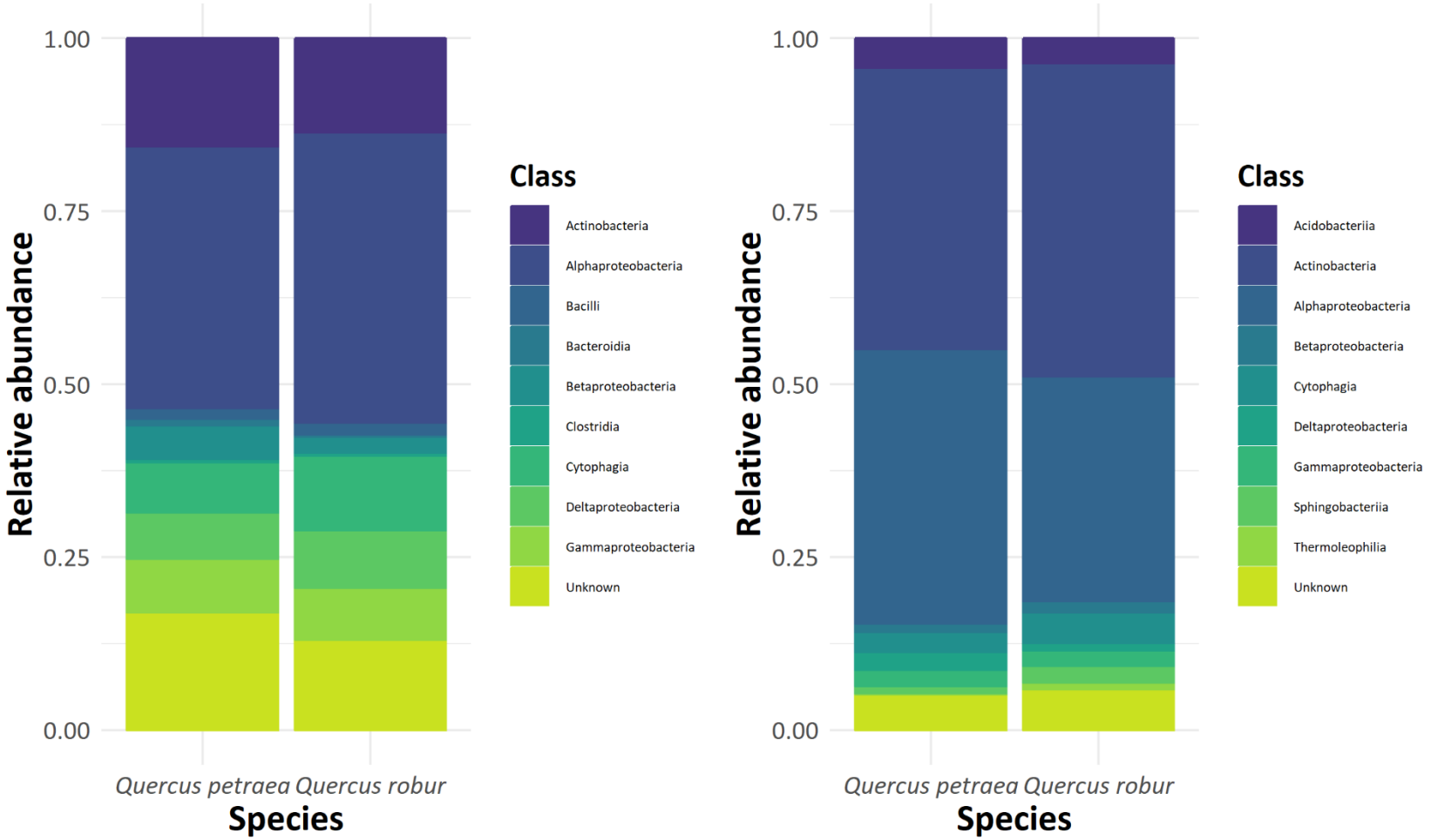

Figure 3.6 – relative abundance of the top 10 class divisions for bacterial OTUs, leaf (left) and twig (right)

3.3.1. *Variations in species richness and diversity of fungal and bacterial endophytes*

There were no statistically significant differences in fungal species (OTU) richness for any of the variables tested. However, fungal OTU diversity correlated significantly with DBH (df=6, F=3.90, p<0.05) (*Figure 3.7a*) and marcescence habit (df=6, F=4.80, p<0.05) (*Figure 3.7c*), and fungal OTU evenness correlated significantly also with DBH (df=6, F=4.00, p<0.05) (*Figure 3.*7b) and marcescence habit (df=6, F=6.36, p<0.05) (*Figure 3.7d*). Bacterial OTU richness was influenced by tissue type (df=6, F=28.11, p<0.001) and bacterial OTU diversity was influenced by both tissue type (df=7, F=16.67, p<0.001) and DBH (df=8, F=3.9, p<0.05).

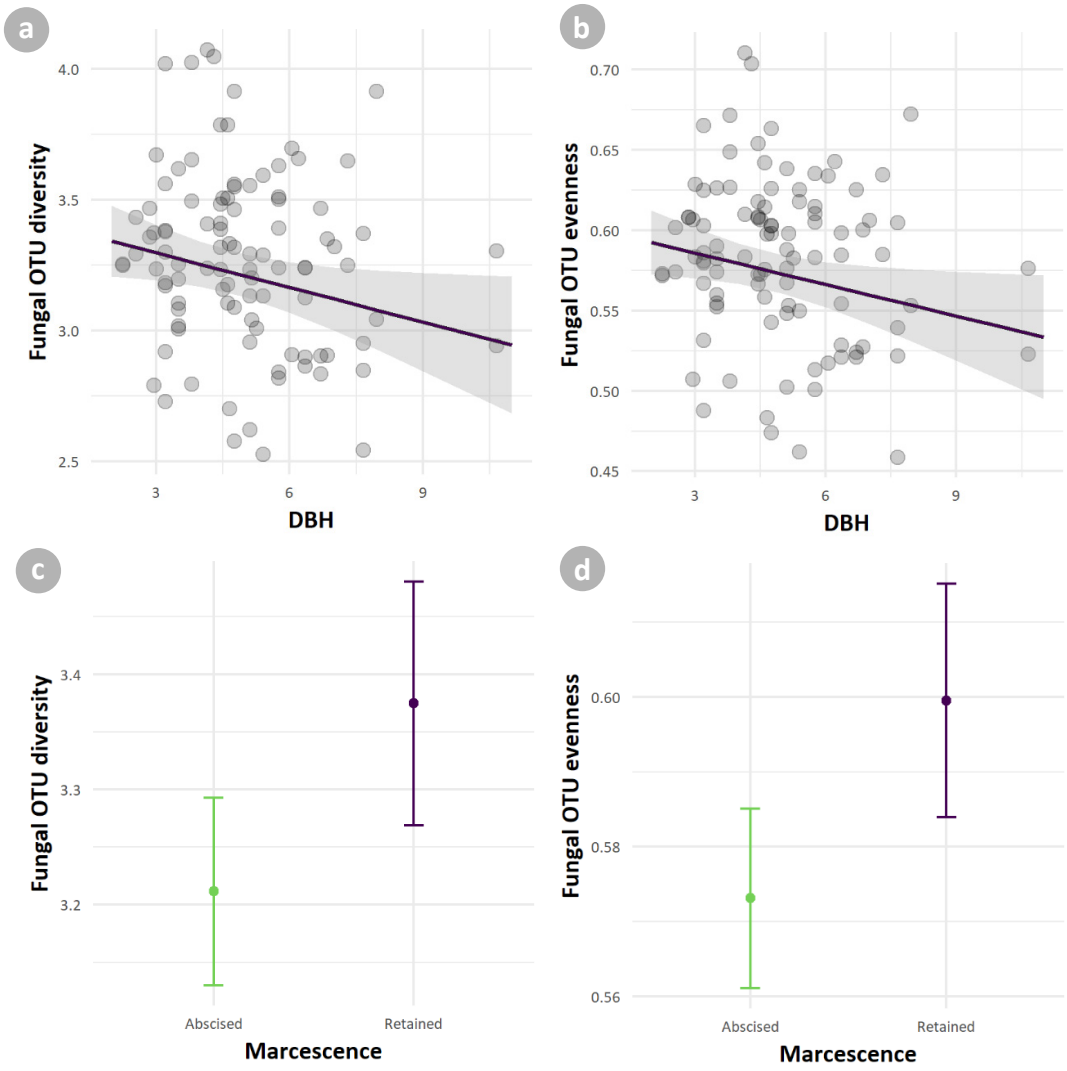

Figure 3.7 – significant effects of DBH and marcescence habit on fungal endophyte OTU diversity and evenness. Shaded grey areas and error bars represent 95% confidence intervals

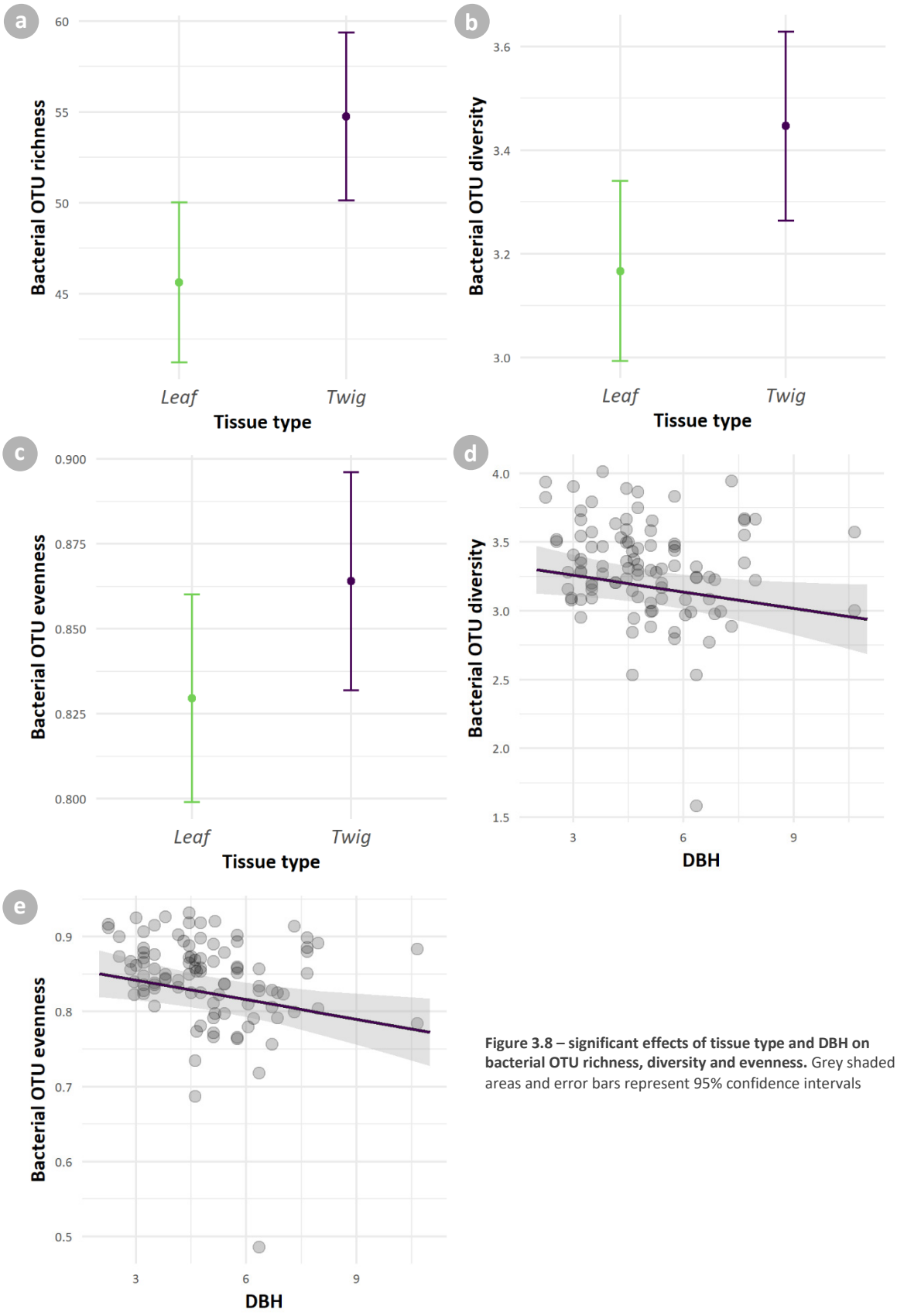

Figure 3.8 – significant effects of tissue type and DBH on bacterial OTU richness, diversity and evenness. Grey shaded areas and error bars represent 95% confidence intervals

3.3.2. *Variations in the composition of fungal and bacterial endophytes (beta diversity)*

PERMANOVA revealed a significant difference in the composition of the fungal communities between *Quercus* species ($F=5.74$, $p<0.01$) and between tissue types ($F=42.82$, $p<0.001$) (*Figure 3.9*). The composition of bacterial endophytes also differed considerably between the two *Quercus* species ($F=5.84$, $p<0.001$) and tissue type ($F=12.43$, $p<0.001$) (*Figure 3.10*).

575 fungal OTUs and 166 bacterial OTUs were shared between all tissue types and all *Quercus* species. Each tissue type and each *Quercus* species also included a number of unique OTUs not shared by any other tissue type or host species (*Figure 3.11*).

3.3.3. *Factors affecting the most abundant fungal and bacterial endophytes*

The influence of all host factors on the abundance of the top 20 bacterial and fungal endophytes are shown in *Table 3.3 & 3.5*. A number of pathogens were identified such as *Erysiphe* sp. that causes powdery mildew of *Quercus* leaves and *Colpoma* sp. that causes dieback of *Quercus* branches (*Table 3.4 & 3.6*). A number of the highest abundant OTUs were unidentified, uncultured fungi and bacteria that have previously been found as endophytes in other tree species.

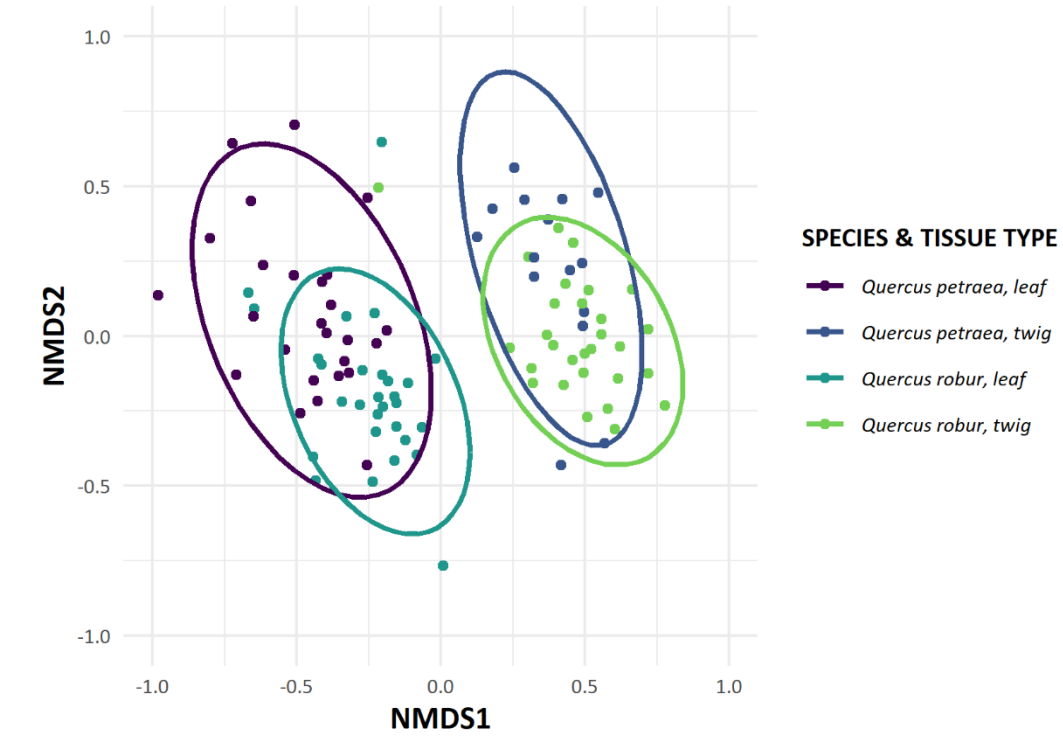

Figure 3.9 – NMDS score computed using the Bray-Curtis index, representing dissimilarities in the rarefied fungal species (OTU) community between host species and host tissue type. The stress value associated with this representation was 0.148. The ellipsis represents the 95% confidence interval.

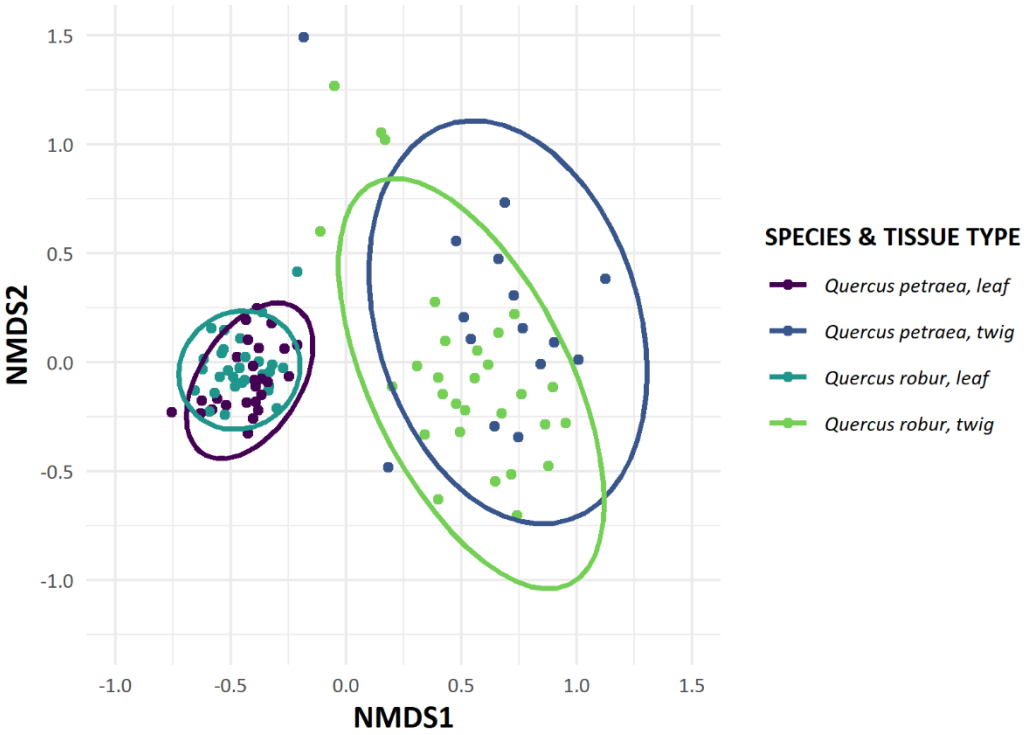

Figure 3.10 – NMDS score computed using the Bray-Curtis index, representing dissimilarities in the rarefied bacterial species (OTU) community between host species and host tissue type. The stress value associated with this representation was 0.212. The ellipsis represents a 95% confidence interval.

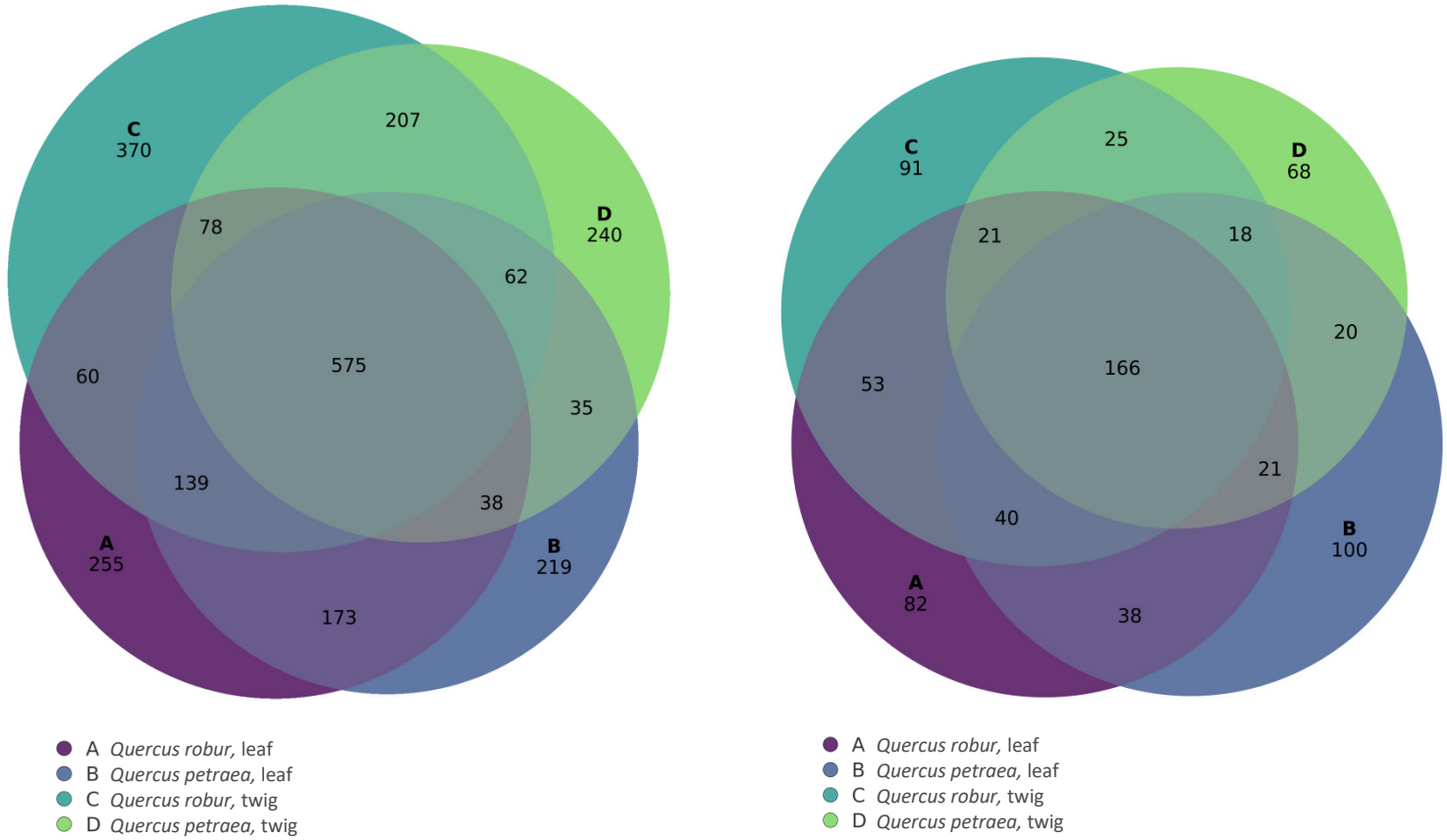

Figure 3.11 - number of OTUs shared by each tissue type and each *Quercus* species, fungi (left), bacteria (right)

Table 3.3 – top 20 abundant fungal OTUs. Taxon identiy was determined using the BLAST analysis against the GenBank database. Identity is the percentage identity between OTU representative sequence and the closest matches in GenBank. Taxa unassigned at species or order level indicate OTUs assigned to multiple species or orders with identical e-values. Relative abudance is the percentage abundance of each OTU in the full dataset (including both *Quercus* species and both tissue types). Statistical results from linear mixed effect models.

OTU number	% Identity	Taxon	Tissue	Significant variable	df	F	p	Direction of effect
OTU_F1433	96.95-100.00	**Uncultured fungi**	Leaf	Shoot length	5	6.28	<0.05	Positive
OTU_F1760	96.79-98.71		Leaf	No significant variables				
OTU_F2725	99.30-100.00		Twig	Marcescence	6	5.35	<0.05	Abscised > Retained
				Tree species	6	20.39	<0.001	Robur > Petraea
OTU_F1053	85.48-98.83		Twig	DBH	6	10.37	<0.01	Negative
				Tree species	6	16.26	<0.001	*Q. petraea* > *Q. robur*
OTU_F939	94.81-96.30		Twig	No significant variables				
OTU_F591	100.00		Twig	Fv/Fm	6	7.22	<0.01	Negative
				Tree species	6	7.56	<0.01	*Q. robur* > *Q. petraea*
OTU_F1552	99.38		Twig	Tree species	5	8.89	<0.01	*Q. robur* > *Q. petraea*
OTU_F890	99.4-100.00	***Erysiphe* sp.**	Leaf	No significant variables				
OTU_F2984, OTU_F3047	99.28, 100.00	***Ramularia endophylla***	Leaf	No significant variables				
OTU_F2729, OTU_F2834	97.14, 100.00	***Colpoma* sp.**	Twig	No significant variables				
OTU_F528	100.00	***Vishniacozyma* sp.**	Leaf	Budburst	5	4.47	<0.05	Positive
OTU_F577	98.99	***Exobasidium bisporum***	Both	No significant variables				
OTU_F2749	100.00	***Devriesia fraserae***	Twig	Budburst	5	5.17	<0.05	Positive
OTU_F2349	100.00	**Pleosporales**	Twig	Tree species	6	11.01	<0.001	*Q. robur* > *Q. petraea*
OTU_F2687	93.92			Marcescence	6	5.02	<0.05	Abscised > Retained
				DBH	8	6.96	<0.01	Positive
				Tree species	8	5.92	<0.05	*Q. robur* > *Q. petraea*
				Shoot length	8	9.14	<0.05	Positive
OTU_F2406	100.00	***Cladosporium* sp.**	Both	Longitude	7	4.24	<0.05	Positive
				DBH	7	5.85	<0.05	Negative
				Marcescence	7	4.40	<0.05	Retained > Abscised
OTU_F1185	100.00	***Ascomycota***	Twig	Longitude	6	7.24	<0.01	-1.62
				Tree species	6	9.50	<0.01	12.95
OTU_F1807	99.36	***Phaeomoniella* sp.**	Twig	Tree species	5	17.34	<0.001	-16.52

Table 3.4 - potential pathogenic or saprophytic fungal OTUs. Taxon identiy was determined using the BLAST analysis against the GenBank database. Taxa unassigned at species or order level indicate OTUs assigned to multiple species or orders with identical e-values. Relative abudance is the percentage abundance of each OTU in the full dataset (including both *Quercus* species and both tissue types).

OTU number	Closest identity match using NCBI database	Relative abundance (%)	Potential disease symptoms in *Quercus* and other tree species
OTU_F2449, 2654, 2820, 2902, 2984, 3034, 3047, 3082, 3098, 3111, 3129, 3152, 3175, 3197, 3198, 3202, 3223, 3224, 3225, 3250, 3251, 3257, 3266, 3268, 3272, 3279, 3282, 3285, 3289, 3304, 3343, 3347, 3449	***Ramularia* sp.**	15.0	*Ramularia* sp. (formally *Mycosphaerella*) (Videira et al., 2015) are important phytopathogens causing leaf spots on a number of important crop species (Videira et al., 2016). There are some reports of this species causing leaf spots on *Quercus* species in the UK, but it is of negligible importance (Phillips and Burdekin, 1982).
OTU_F890	***Erysiphe* sp.**	7.5	*Erysiphe alphitoides*, *E. hypothylla* and *E. quercicola* cause powdery mildew on *Quercus* species in Europe (Mougou et al., 2008).
OTU_F2729, 2769, 2890, 2934, 2974, 3107, 3143, 3153, 3182, 3185, 3187, 3190, 3291, 3319, 3369, 3493	***Colpoma* sp.**	5.4	*Colpoma quercinum* causes minor dieback in branches of *Quercus* (Phillips and Burdekin, 1982).
OTU_F223, 308, 320, 328, 337, 347, 358, 469, 510, 520, 536, 547, 551, 554, 556, 558, 563, 566, 587, 588, 592, 594, 3664	***Taphrina* sp.**	0.6	*Taphrina caerulescens* causes leaf blisters on *Quercus* species, only of minor significance in the UK (Phillips and Burdekin, 1982)
OTU_F1594, 1706, 1763, 1801, 1848, 1919, 1935	***Diaporthe* sp.**	0.3	Common saprophytes on small *Quercus* branches, they may become pathogenic, causing cankers in weakened trees (Phillips and Burdekin, 1982)
OTU_F1757	***Apiognomonia errabunda***	0.006	Causes anthracnose of *Quercus* and other species e.g. *Fagus* and *Tilia* (Boron et al., 2019).
OTU_F1622, 1759, 1799, 1540	***Hypoxylon* sp.**	0.003	Opportunistic fungi which cause canker in weakened or dying trees (Lee and Whalley, 2018).
OTU_F531	***Stereum hirsutum***	0.001	Fungi causing white-rot decay in *Quercus* species and other trees, most likely saprophytic (Swift, 1978).
OTU_F597	***Ganoderma adspersum***	0.001	Fungi causing white-rot decay in *Quercus* species and other trees e.g. *Tilia, Fagus, Platanus* and *Aesculus* (Schwarze and Ferner, 2012).

Table 3.5 – top 20 abundant bacterial OTUs. Taxon identiy was determined using the BLAST analysis against the GenBank database. Identity is the percentage identity between OTU representative sequence and the closest matches in GenBank. Taxa unassigned at species or order level indicate OTUs assigned to multiple species or orders with identical e-values. Relative abudance is the percentage abundance of each OTU in the full dataset (including both *Quercus* species and both tissue types). Statistical results from linear mixed effect models.

OTU number	% Identity	Taxon	Tissue	Significant variable	df	F	p	Estimate
OTU_B1444	99.28	***Friedmanniella* sp.**	Twig	No significant variables				
OTU_B402	99.29	**Actinomycetales**	Leaf	Tree species	6	5.02	<0.01	*Q. petraea* > *Q. robur*
				Fv/Fm	6	7.25	<0.01	Negative
OTU_B1681	98.54		Twig	No significant variables				
OTU_B1377	98.91		Twig	Shoot length	5	6.2748	<0.05	Negative
OTU_B682	99.64	**Rhiziobiales**	Leaf	Fv/Fm	5	4.30	<0.05	Positive
OTU_B467	99.30		Both	Longitude	6	4.22	<0.05	Negative
				Shoot length	6	3.89	<0.05	Negative
OTU_B1684	99.29		Leaf	Longitude	5	5.94	<0.05	Positive
OTU_B2259	97.64		Leaf	DBH	5	4.19	<0.05	Negative
OTU_B1770	99.64		Leaf	DBH	7	5.15	<0.05	Negative
				Tree species	7	5.64	<0.05	*Q. robur* > *Q. petraea*
				Shoot length	7	5.74	<0.05	Positive
OTU_B2439	99.63	***Hymenobacter* sp.**	Leaf	DBH	5	4.5814	<0.05	Negative
OTU_B2550	96.63		Leaf	No significant variables				
OTU_B675	92.55	**Myxococcales**	Leaf	No significant variables				
OTU_B1790	99.63	***Microbacteriaceae***	Twig	No significant variables				
OTU_B1771	99.63		Twig	Marcescence	5	5.5735	<0.05	Abscised > Retained
OTU_B1668	98.57	***Pseudomonas* sp.**	Leaf	No significant variables				
OTU_B2738		**Unknown bacterium**	Leaf	Longitude	5	5.3524	<0.05	Negative
OTU_B2763			Leaf	No significant variables				
OTU_B522	100.00	***Ralstonia* sp.**	Leaf	Longitude	6	5.125	<0.05	Negative
				Marcescence	6	12.756	<0.001	Retained > Abscised
OTU_B679	97.17	***Acetobacteraceae***	Twig	No significant variables				
OTU_B1370	98.57	***Actinomycetospora* sp.**	Twig	DBH	6	11.416	<0.001	Negative
				Budburst	6	8.3514	0.01	Negative

Table 3.6 - potential pathogenic bacterial OTUs. Taxon identiy was determined using the BLAST analysis against the GenBank database. Taxa unassigned at species or order level indicate OTUs assigned to multiple species or orders with identical e-values. Relative abudance is the percentage abundance of each OTU in the full dataset (including both *Quercus* species and both tissue types).

OTU number	Closest identity match using NCBI database	Relative abundance	Potential disease symptoms in *Quercus* and other tree species
OTU_B1435	*Brenneria goodwinii* (99.64%)	0.7	*B. goodwinii*, *G. quercinecans, Rah. victoriana* and *R. planticola* are possible agents contributing to a decline syndrome called acute oak decline (AOD) in both UK native oak species (Denman et al., 2014). Symptoms include bleeding cankers and canopy dieback.
OTU_B32, 2154, 2318, 1693	*Gibbsiella* sp., with 97.86-99.64% match to G. *quercinecans*	0.4	
OTU_B1549, 1680	*Rahnella* sp., with 96.02-98.93% match to *R. aquatilis*	0.2	
OTU_B2159, 1773	*Raoultella* sp., with 91.14-99.29% match to *R. terrigena* and 97.14-99.29% to *R. ornithinolytica*	0.4	
OTU_B1427, 2001	*Brenneria* sp., with 98.94% match to *B. rubrifaciens,* 96.45% match to *B. salicis*, 92.99% to *B. nigrifluens* and 92.99% to *B. goodwinii*	0.1	*B. rubrifaciens* causes deep bark canker (McClean et al., 2008 and *B. nigrifluens* causes shallow bark canker (Wilson et al., 1957) of walnut trees (*Juglans* sp.)) and *B. salicis* causes watermark disease in willow (Hauben et al., 1998).
OTU_B1176, 1511, 1430, 1460, 1785, 1766	*Pantoea* sp.	1.2	*Serratia* spp. have been isolated from declining *Quercus* species in Spain (Poza-Carrión et al., 2008). Other members of the *Enterobacteriaceae* family are known to cause disease symptoms in other plants such as *Erwinia amylovora* that causes the disease fire blight of members of the *Rosaceae* plant family (Beer and Norelli, 1977).
OTU_B1451	*Erwinia* sp., with 99.64% with *E. iniecta* and *E. billingiae*	0.2	
OTU_B1550	*Yersinia* sp.	0.1	
OTU_B1432	*Serratia* sp. (99.29%)	0.04	
OTU_B1166, 1534, 1433, 1456, 1471	Enterobacteriaceae bacterium	0.4	
OTU_B1468, 2135, 363, 1668, 1475	*Pseudomonas* sp. (95.71-100%)	1.8	*Pseudomonas* species, in particular *P. syringae* are common pathogens of woody plants, for example *Prunus* species (Hulin et al., 2018) and *Aesculus hippocastanum* (Green et al., 2010).
OTU_B1454	*P. syringae* (99.29%)	0.05	
OTU_B1651	*P. aeruginosa* (99.64%)	0.01	
OTU_B1288, 1056,999	Xanthamonadaceae bacterium	0.03	Members of the *Xanthomonadaceae* family are also plant pathogens, for example *Xanthomonas arboricola* pv. *Juglandis* causes vertical oozing canker (VOC) in walnut (*Juglans* sp.) (Hajri et al., 2010).

3.3.4. *Targeted search for AOD pathogens*

Results of the Illumina sequencing showed that there were OTU matches to the *Bg*, *Gq*, *Rv* and *Rp* sequences included in the sequencing run as positive controls. Tissue samples that contained OTU matches to any of the four pathogens at an abundance of over 20, were chosen for resampling using culturing and PCR techniques (*Table 3.7*).

Table 3.7 – abundance of the four bacterial species of interest: *Bg, Gq, Rv* and *Rp* found in the Paradise Wood *Quercus* trees using next generation sequencing, these samples were chosen for further analysis using culture dependent techniques

TREE NAME	TISSUE	OTU	OTU MATCH	ABUNDANCE
22_10_HAM007	Twig	OTU_B1435	*Brenneria goodwinii*	96
		OTU_B32	*Gibbsiella quercinecans*	35
		OTU_B1166	*Rahnella victoriana*	75
22_11_ZE11-1	Twig	OTU_B32	*Gibbsiella quercinecans*	32
22_33_HAM013	Twig	OTU_B1435	*Brenneria goodwinii*	67
		OTU_B32	*Gibbsiella quercinecans*	63
22_47_HAM014	Twig	OTU_B1435	*Brenneria goodwinii*	68
		OTU_B32	*Gibbsiella quercinecans*	32
		OTU_B1166	*Rahnella victoriana*	70
22_50_REN003	Twig	OTU_B1435	*Brenneria goodwinii*	38
		OTU_B2159	*Raoultella planticola*	99
35_12_REN003	Twig	OTU_B1435	*Brenneria goodwinii*	29
		OTU_B32	*Gibbsiella quercinecans*	35
35_12_REN003	Leaf	OTU_B1435	*Brenneria goodwinii*	25
35_23_ZE11-1	Leaf	OTU_B1435	*Brenneria goodwinii*	24
35_24_ELT004	Leaf	OTU_B1435	*Brenneria goodwinii*	20
35_52_HRF013	Leaf	OTU_B1435	*Brenneria goodwinii*	27
35_53_REN001	Leaf	OTU_B1435	*Brenneria goodwinii*	42
		OTU_B2159	*Raoultella planticola*	22
37_23_ZE142-1	Leaf	OTU_B1435	*Brenneria goodwinii*	108
37_53_ZE46-1	Leaf	OTU_B1435	*Brenneria goodwinii*	34

After streaking onto MacConkey agar, 56 pink colonies were sub-cultured and analysed using ITS ribotyping. The gel banding patterns of the four control species of interest is shown in *Figure 3.12* gyrB sequencing was used on the samples that closely matched the banding pattern of the controls, 12 samples in total. The closest matches to sequences in the NCBI

GenBank database are listed in *Table 3.8*. These samples are also included in a phylogenetic tree in *Chapter 6* together with a description of how the tree was constructed.

Table 3.8 – sequencing results of bacteria isolated from asymptomatic *Quercus* tissue, closest match in the NCBI GenBank database is shown

TREE ID	CLOSEST MATCH	IDENTITY MATCH (%)
37_ZE142-1	*Rahnella variigena*	100.00
	Rahnella variigena	99.84
	Rahnella variigena	100.00
35_ELT004	*Serratia fonticola*	99.44
	Serratia fonticola	99.31
	Rahnella variigena	99.86
35_HRF013	*Serratia fonticola*	99.04
35_REN003	*Rahnella variigena*	100.00
	Serratia fonticola	99.58
35_ZE11-1	*Rahnella variigena*	99.67
22_HAM013	*Rahnella variigena*	100.00
35_REN001	*Rahnella variigena*	99.73

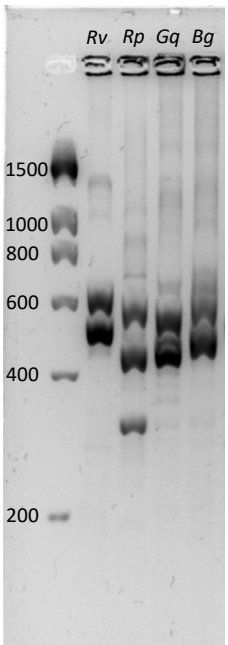

Figure 3.12– gel banding pattern of four bacterial controls: *Rahnella victoriana* (*Rv*), *Raoultella planticola* (*Rp*), *Gibbsiella quercinecans* (*Gq*) and *Brenneria goodwinii* (*Bg*) using ITS primers. First lane is 1kb ladder.

3.4. *Discussion*

The composition of fungal and bacterial endophytes in the phyllosphere varied between the two oak species (*Quercus robur* and *Q. petraea*) and between the tissue types (leaf and twig). The abundances of the most dominant bacterial and fungal taxa were also influenced by host parent provenance, tree vigour, marcescence habit, phenology and tree health to varying degrees.

3.4.1. *Does oak species status influence endophyte abundance, richness and diversity?*

Quercus robur and *Q. petraea* possess distinct assemblages of bacterial and fungal endophytes in their phyllosphere. Considering that trees of both oak species were recruiting their microbiomes from the same species pool, assuming all are horizontally transferred, only 54% of fungal OTUs and 48% of bacterial OTUs were shared by all samples irrespective of host species. 19% of fungal OTUs and 24% bacterial OTUs were unique to *Q. petraea* and 27% of fungal and 29% of bacterial OTUs were specific to *Q. robur*. These results suggest that there are qualitative differences in how the two oak species acts as ecological filters.

In another study that compared fungal endophyte composition in the rhizosphere of *Quercus robur* and *Q. petraea* using culture dependent techniques they reported no significant differences in relation to species (Halmschlager and Kowalski, 2004). Halmschlager and Kowalski (2004) attributed the few differences they found in endophytic composition between the two *Quercus* species to site characteristics and the different ecological requirements of the host. As described in *Chapter 2*, the site characteristics of Paradise Wood were not favourable to either *Quercus* species and are therefore unlikely to be influencing the endophytic composition of these trees.

When considering the top 20 most dominant fungal and bacterial OTUs in isolation, many of these OTUs differed in abundance between the two host *Quercus* species. The reference OTU sequence of the top 20 most dominant endophytes were matched to sequences in the GenBank database to determine whether similar sequences have been found in other studies. OTU_F2725, OTU_F1053, OTU_F591 and OTU_F1552 are likely to be generalist endophytes of forest trees as they were identical (or very close matches) to uncultured fungi found in the phyllosphere of horse chestnut (*Aesculus hippocastanum*) (Menkis et al., 2018) and Norway spruce trees (*Picea abies*) (Menkis et al., 2015) in Lithuania and inside the European elm bark beetle (*Scolytus multistriatus*) associated with elm trees (*Ulmus minor*) in Sweden (Menkis et al., 2016). These species may be considered highly generalist as they are found spanning a large division of plants: from angiosperms to gymnosperms. Although found in both oak species here, their abundance varied. OTU_F2725, OTU_F591 and OTU_F1552 were found at a higher abundance in *Q. robur* and OTU_F1053 at a higher abundance in *Q. petraea*. These fungi are likely to be as yet undescribed species, and as they are at such high abundance in *Quercus* trees and other species it is likely that these have not been named to date as they are not culturable, either because they are obligate biotrophs or they are outcompeted by other endophytes when grown in culture.

Two possible members of the Pleosporales order, OTU_F2349 which closely matched to *Lophiostoma coricola* a generalist endophyte (Agostinelli et al., 2018) and OTU_F2687, are present at a higher abundance in *Quercus robur*. OTU_B1770, a *Methylobacterium* species which as a genus are abundantly found as endophytes of plants (Knief et al., 2012), was found at a higher abundance in *Quercus robur*.

OTU_F1185 which is identical to an unassigned Ascomycete fungus cultured from the twigs of *Quercus robur* in a previous study in Sweden (Agostinelli et al., 2018), was found here to be at a higher abundance in *Q. robur* than *Q. petraea*. OTU_F1807, most likely a *Phaeomoniella*

species, found in the above study (Agostinelli et al., 2018) and also in the cork (*Quercus suber*) stopper manufacturing process (Barreto et al., 2012), was more abundant in *Quercus petraea*. It is possible that these are more specialist endophytes, having a narrow host range potentially confined to the *Quercus* genera. However, as many of the OTUs were not resolved to species level, it is difficult to establish which endophytes show host preference for *Quercus*.

Bacterial endophyte OTU_B402, most likely to be *Cutibacterium acnes* was found at a higher abundance in *Q. petraea*. The causal agent of acne in humans, it is possible that OTU_B402 is a laboratory contaminant; however this bacterium has been found in a number of other organisms, for example in coral (Ziegler et al., 2019) and in the digestive tract of bees (Alberoni et al., 2019). It is possible that all the above studies were contaminated by bacterial species commonly associated with the human skin microbiome. However, it is not uncommon for human pathogens to be found in the endosphere of plants (Tyler and Triplett, 2008) and this bacterium in particular was thought to be a recent horizontal inter-kingdom transfer from human to grapevine plant, during grapevine domestication (Campisano et al., 2014).

The results shown here support the idea that tree species act as ecological filters and that differences in microbial endophytic community composition are the result of differences in tree traits rather than local species pools of dispersal ability. Colonisation of the phyllosphere by endophytes is dependent on tissue characteristics and plant defences which are likely to differ between the two oak species. As discussed in *Chapter 2*, the two *Quercus* species have different leaf morphology and chemical defences, with *Q. robur* typically possessing higher levels of tannins and *Q. petraea* higher levels of lactones (Mosedale and Savill, 1996). Leaf traits such as photosynthetic capacity, nutrient content, leaf wettability and leaf mass may differ between the species which may also influence the endophyte assemblage (Cordier et al., 2012b).

It has been suggested that as *Quercus robur* and *Q. petraea* are interfertile, they lack a single morphological trait that distinguishes them and they have different ecological requirements, that they should only be considered subspecies or ecotypes (Kleinschmit et al., 1995). This view, however, has been challenged and most taxonomists agree that they should be considered separate species. Some studies have found genetic markers that are able to discriminate between the two species (Muir et al., 2000, Cottrell et al., 2002, Guichoux et al., 2011) but in general, interspecific differentiation based on genetic markers is weak (Gomory et al., 2001, Petit et al., 1993). However, the use of multivariate analysis is able to differentiate the two species based on morphology (Kremer et al., 2002, Curtu et al., 2007, Boratynski et al., 2008) (see also *Chapter 2*). In addition, the ecology of these two species is distinct with different communities of insect herbivores and bacterial and fungal endophytes associated with the two *Quercus* species, as is shown in this chapter and *Chapter 2*.

As explained in *Chapter 2*, hybrid species were not considered in this study, as species assignment was determined by leaf morphometric measurements and hybrids do not show intermediate forms. It is likely that if hybrid trees, if there are any, be removed from the study that differences in endophytic composition between the two host species may be even stronger. It is unknown how the composition of endophytes in a hybrid individual would compare to that of *Quercus robur* or *Q. petraea*. One study found that hybrid individuals had an intermediate endophyte frequency compared to the two parent *Quercus* species (Gaylord et al., 1996). It might also be possible that the hybrid individual could possess the same endophytic assemblage as one of the parents.

3.4.2. *Are endophytic species organ specific?*

Major differences in fungal and bacterial endophyte composition were recorded in the two tissue types (leaf and twig) as has been shown in multiple other studies (Sieber, 1989, Ragazzi

et al., 2001, Leff et al., 2015). Two of the dominant endophytic taxa, OTU_F2406 and OTU_B467, associated with the *Quercus* trees in this study were found in almost equal proportions between the plant tissues. These taxa can be classed as systemic endophytes, as they are able to colonise multiple parts of the same plant (Zabalgogeazcoa, 2008). Although not completely limited to one organ, the majority of the top 20 bacterial and fungal endophytes, however, were found at a higher abundance in one tissue type over the other. These taxa are considered localised endophytes. The biotic and abiotic characteristics of leaves are very different from those of twigs. Twigs and branches are longer living elements, with the tissues changing over time from epidermal primary tissues to bark (Leff et al., 2015). This could explain why twig samples in this study harboured a higher species richness, diversity and evenness of bacterial endophytes compared to transient leaves.

3.4.3. *Were latent pathogens or saprophytes found as endophytes in Quercus?*

Many potential fungal and bacterial pathogens and saprophytes were found associated with the *Quercus* hosts. As the plant material used here was apparently healthy i.e. asymptomatic, it can be assumed that these were latent pathogens and saprophytes. These taxa are present inside the host without needing an entry wound and their close and continuous association with the host plant means that any slight changes in metabolism will be detected and they would be ready to take advantage of any weakness (Gonthier et al., 2006). *Colpoma quercinum,* for example, has been found in other studies as one of the most dominant fungi in thin branches or twigs of *Quercus robur* (Kehr and Wulf, 1993). This species is thought to act as a weak parasite, attacking twigs of reduced vitality and performing natural pruning (Butin and Kowalski, 1983). Another example is *Ganoderma adspersum* found here in healthy tissue, it is thought to later develop saprophytically (Schwarze and Ferner, 2012).

The four positive controls used in this study matched to the following OTUs: *Bg* to OTU_B1435, *Gq* to OTU_B32, *Rv* to OTU_B1166 and *Rp* to OTU_B2159. Thought to be involved in the decline syndrome of *Quercus*, referred to as acute oak decline (AOD), these species have rarely been isolated from healthy trees in woodlands where AOD is not present (Meaden et al., 2016, Denman et al., 2017). However, these OTUs were represented in the *Quercus* samples from asymptomatic trees in Paradise Wood, but at a low abundance. Given the low abundance any statistical analysis for differences in abundance in relation to tree species and tree traits were not possible. Trees that were shown to have a relatively high abundance of these four bacteria were re-sampled and culture dependent methods were used to confirm the presence of *Bg, Gq, Rv* and *Rp*. Using culturing methods, none of the species of interest were found in the *Quercus* samples. Further research is required to establish whether these potential pathogens could exist as commensalistic or mutualistic endophytes in healthy plants for a part of their lifecycle, becoming pathogenic when the tree is under stress, this theory will be discussed in further detail in *Chapter 6*.

Members of the *Erysiphe* genera are fungal pathogens that cause powdery mildew on *Quercus* species in the UK (Marçais and Desprez-Loustau, 2014, Mougou et al., 2008, Desprez-Loustau et al., 2018). Hyphae grow epiphytically on the leaf surface, while specialised cells extend into the epidermal surface to obtain nutrients from the host plant. The leaves collected here for analysis were free of any visible signs of powdery mildew and were surface sterilised to remove any possible epiphytic mycelium and DNA. It may be assumed therefore that *Erysiphe* might live as an asymptomatic endophyte within *Quercus* leaves before symptoms appear. In this study the abundance of *Erysiphe* sp. was not correlated with any of the tree phenotypic traits measured, this contrasts with a recent study of *Erysiphe* sp. on *Quercus* which found that more vigorous trees i.e. taller trees with longer shoots had a higher incidence of powdery mildew (Field et al., 2019). The abundance of *Erysiphe* detected here was thought to be in the

early stages of infection as no symptoms were present which may explain the differences between studies.

Another example, *Ramularia endophylla* which causes leaf spots on *Quercus* species (Phillips and Burdekin, 1982), has a known endophytic phase in asymptomatic *Quercus* leaves (Verkley et al., 2004) and beech (*Fagus sylvatica*) leaves (Cordier et al., 2012a). *Apiognomia errabunda* is also found as an endophyte in healthy trees but is also the causative agent of oak anthracnose disease (Boron et al., 2019).

Trees are under increasing stress from the changes in temperature and precipitation brought about by climate change, weakening trees and making them more vulnerable to pathogen attack. It is clear that *Quercus* trees are inhabited by many endophytic species that are potentially weak parasites, that may become more virulent as the tree is weakened by other biotic or abiotic stresses. Tree health is discussed further in the following *Section 3.5.7* and in *Chapter 6.*

3.4.4. *Does host provenance influence endophytic species, and how might this relate to climate change?*

Few studies have considered the effect of planting trees from non-local provenances on the abundance, diversity and composition of microbial endophytes. The local adaptation hypothesis posits that local provenances will perform better in local environmental conditions (Edmunds and Alstad, 1978). It is likely that co-evolution of trees with their associated endophytes has resulted in local adaptation where the growth and fitness of both parties is greatest when they share their local environment (Thrall et al., 2007). However, it is unknown whether coadaptation arises as a result of increased mutualism between host and endophytes or due to decreased antagonism (Revillini et al., 2016). It might be expected therefore that trees from different provenances would filter microbial communities differently and therefore

would present differences in their endophytic community. However, latitude and longitude of the parent tree had no influence on the composition, richness or diversity of the bacterial and fungal endophytes associated with *Quercus robur* and *Q. petraea* in Paradise Wood. This corresponds with a study of the foliar endophytes of a grass species which found that communities of endophytes were not locally adapted, and instead local environmental conditions were the primary force structuring endophytic communities (Whitaker et al., 2018)

3.4.5. *Does tree phenology influence endophyte composition?*

Very few studies have addressed the influence of phenology on the endophytic community of tree species. At the time of budburst, it is believed that leaves are nearly free from endophytes (Scholtysik et al., 2012), endophytes then colonise horizontally from the environment and as leaves get older more endophytes accumulate (Hata et al., 2011, Scholtysik et al., 2012). With this in mind, it could be assumed that trees that burst earlier in the spring should accumulate a higher richness and diversity of endophytes in their leaves. However, no such overall trend was observed here, budburst time did not influence the richness or diversity in bacterial or fungal endophytes in the samples tested. The abundance of two dominant OTUs (OTU_F528 and OTU_B1370) was higher on trees that burst earlier in the season, and it is possible that if endophyte assembly is dispersal-driven then early budburst would increase the time of exposure to infection and colonisation by these taxa. On the other hand, OTU_F2729 was at a higher abundance on trees that burst later in the season. This may be explained by changes in microclimate e.g. avoidance of frost, competition from other endophytes, insects or pathogens or from variations in the chemical and physiological traits of the tree. Stomata are thought to be the main entry point for bacterial pathogens, and it is believed that the same is true for endophytic species (Underwood et al., 2007, Borruso et al., 2018). Changes in stomata morphology with leaf age may influence the colonisation of bacterial endophytes (Borruso et

al., 2018). The carbon and nitrogen nutrients within the leaves also diminishes with leaf age and is likely to also influence the endophytic community (Borruso et al., 2018).

Phenology is partly genetically controlled but is also driven by climate cues. Climate change is likely to influence these budburst dates, potentially affecting the length of the growing season of the tree and in turn this may affect the availability of nutrients for the endophytic communities that rely upon them.

3.4.6. *Does the leaf marcescence habit influence the endophytic community?*

The reason for why some *Quercus* trees retained their senescent leaves overwinter has not been resolved, although many explanations have been presented (as discussed in *Chapter 2*). There are no known studies addressing the influence of the marcescence habit on the endophytic community. Results from this study show that trees that retained their leaves overwinter had, at the time of sampling, a higher diversity and evenness of fungal endophytes associated with them i.e. trees that retained leaves had fungal species in equal proportions and trees that abscised their leaves had dominant species and rare species. One of the most abundant fungal OTUs (OTU_F2406) and a bacterial OTU (OTU_B522) were at a higher abundance on trees that retained senescent leaves. It is possible that these endophytes are able to overwinter in the canopy of the tree, providing primary inoculum when the buds open in the spring. However, some of the dominant endophytes (OTU_F2725, OTU_F2687, OTU_B1771) were more abundant on trees that abscised their leaves in autumn.

3.4.7. *Does plant vigour and plant health influence the endophytic composition*

More vigorous trees i.e. those with a larger DBH and longer shoot length, are likely to have higher carbohydrate resources (Dobbertin, 2005) and may therefore be a more favourable

host for endophytic species. Two of the most dominant fungal endophytes, OTU_F2687 and OTU_F1433, were indeed at a higher abundance on larger and therefore more vigorous trees. However, the majority of the most abundant bacterial and fungal endophytes (OTU_F1053, OTU_F2406, OTU_B1377, OTU_B467, OTU_B2259, OTU_B1770, OTU_B2439 and OTU_B1370) preferred trees that were less vigorous. These less vigorous trees may lack the resources required to produce defensive mechanisms, providing the endophytes with energy for higher growth or reduced barriers to colonisation (Wargo, 1996). Another study of *Quercus robur* trees found that phyllosphere fungal endophytic communities were higher in diversity in trees showing reduced vitality (Agostinelli et al., 2018) and a similar result was found in the roots of oaks stressed by floods (Kwaśna et al., 2016).

The diversity and evenness of fungal and bacterial endophytes was higher on trees with a smaller diameter (DBH), however, there were no differences in species richness. In other words, less vigorous trees have a more even distribution of fungal endophytes. It can be assumed therefore, that more vigorous trees are host to a few dominant fungal endophytes and many rare taxa, whereas less vigorous trees have a more equal abundance of each species, with no dominant or rare taxa.

Measures of Fv/Fm used here, and in other studies (Percival, 2005), as an indicator of tree stress was correlated with OTU abundance. An unknown fungi OTU_F591 and an Actinomycetales bacterium, OTU_B402, were recorded at a higher abundance on stressed trees. This result agrees with the plant stress hypothesis which suggests that stressed trees should support a higher abundance of organisms due to increased nutrients and decreased defence compounds (White, 1984). Conversely, OTU_B682 was at a higher abundance on less stressed trees i.e. trees with a higher Fv/Fm. OTU_B682 matched closely to the order Rhizobiales, members of this order have been known for their plant growth promoting abilities (Santoyo et

al., 2016) and it is possible they can mitigate stressful conditions for the trees, but further research is needed to confirm this.

3.4.8. *How do these culture-independent results compare to culture-dependent methods in the literature?*

The fungal endophytic species associated with twigs of *Quercus* have previously been studied using culture-dependent methods, the number of fungi recorded ranging from just 15 taxa to 126 (Petrini and Fisher, 1990, Kehr and Wulf, 1993). As in other studies the Ascomycota represent the most dominant phyla with fewer Basidiomycota (Kwaśna et al., 2016). These numbers are significantly lower than the 2542 potential fungal taxa found in this study using culture-independent techniques and shows how culture-dependent techniques have underestimated the full endophytic diversity in the phyllosphere. The bacterial endophytes associated with the two native UK oaks have only been characterised very recently using culture-independent techniques (Meaden et al., 2016, Uroz et al., 2010, Borruso et al., 2018). The top three most dominant phyla found in other studies of UK native *Quercus* were Actinobacteria, Proteobacteria and Acidobacteria in both bark (Meaden et al., 2016) and roots (Uroz et al., 2010). This study found the same three dominant phyla but also found the Bacteroidetes to be equally dominant in the leaf and twig samples.

As was observed here, species accumulation curves (or rarefaction curves) of endophytic species rarely reach an asymptote, meaning that if more plant samples were analysed, more endophyte species would be discovered (Zabalgogeazcoa, 2008). In these non-asymptotic analyses, it is likely that the most dominant species have been discovered but that the full scope of rare or singleton species is yet to be revealed (Neubert et al., 2006, Zabalgogeazcoa, 2008).

The results shown here are a snapshot view of the endophytic community at one timepoint late in the season. As endophyte communities change temporally (Shen and Fulthorpe, 2015, Borruso et al., 2018), for a more thorough analysis, multiple sample dates should be analysed and compared.

3.5. *Conclusions*

The trees in this study shared the same endophyte species pool, however different ecological filters determined the establishment of these endophytes in *Quercus* trees, in support of the community assembly principles (*Figure 3.1*). Results show that although *Quercus robur* and *Q. petraea* share some generalist endophytes they host distinct assemblages of bacterial and fungal endophytes in leaves and twigs. Differences also lie within the tissue types, with leaf and twig tissues also possessing distinct communities of endophytes. Individual endophytes are influenced by tree phenotype, in particular tree vigour, tree health and tree phenology. Tree provenance has little impact on the endophyte community directly but may influence species composition and abundance through changes in host phenology and vigour.

It is clear that many potential latent pathogens and saprophytes are found in the endosphere of *Quercus* species in the UK. In a changing climate, trees are likely to become weakened by various biotic and abiotic stressors that may make them vulnerable to attack from these symbionts that have the ability to become pathogenic. On the other hand, these trees may possess beneficial endophytes that could alter their interactions with plant pathogens. *Cladosporium* species for example, found here as an endophyte can produce antimicrobial compounds that inhibits the growth of pathogenic fungi in the phyllosphere (Wang et al., 2006). These endophytes may also confer other benefits such as thermotolerance (Redman et al., 2002) or drought tolerance (Waller et al., 2005, Khan et al., 2016), both of which are important in a changing climate. The ability of a plant to recruit a diverse range of endophytes

from their environment may be beneficial, especially for a sessile organism, in order to tolerate a changing climate. In accordance with the 'insurance hypothesis' maintaining a high diversity of, in this case endophytes, may support the success of an ecosystem (Terhonen et al., 2019, McCann, 2000, Bengtsson et al., 2000).

Understanding the underlying environmental and host traits, such as host species, tree vigour, tree phenology and tree provenance that influence these important endophytic communities is necessary, if we are to understand how to conserve the endophytic biodiversity of trees and to appreciate how endophytes can affect the interactions of plants with their environment. These interactions between endophytic microorganisms and the host plant are thought to be important drivers of plant community structure and dynamics (Clay and Holah, 1999, Aguilar-Trigueros and Rillig, 2016). Endophytic microbes are also likely to influence the diversity and dynamics of other organisms associated with plants such as, herbivorous insects (Omacini et al., 2001, Field et al., 2019, Tack et al., 2012) and this will be addressed in *Chapter 5*.

CHAPTER 4 - *Evaluating the influence of tree phenotype on microbial endophytic communities of introduced Juglans species in the UK*

4.1. *Introduction*

Common walnut (*Juglans regia*), originating from south-eastern Europe through to China (Phillips, 1978), is considered an ancient introduction to the UK. Black walnut (*Juglans nigra*), native to eastern and central North America (Phillips, 1978), was introduced to the UK in the early 17th century. Both species have been widely cultivated for timber, and common walnut for nut production, in Europe and the United States (Bernard et al., 2017). Walnut produces high-quality, valuable timber in a comparatively short rotation period (around 60 years) and is worth about three times the value of *Quercus* in the UK (Clark and Brocklehurst, 2011). However, interest in walnut planting declined in the UK in the early nineteenth century due to the availability of tropical hardwood timber (Hemery et al., 2005). It has also been disregarded by British foresters as it is a site-demanding species i.e. requires significant space, usually displaying poor form and is particularly vulnerable to late spring frosts (Kerr, 1993). However, under the future environmental conditions predicted by climate change models (Broadmeadow et al., 2005), there is great potential for *Juglans* trees to thrive in the UK.

Genetic and silvicultural improvement research programs are ongoing to determine whether *Juglans* species could support home grown timber in a changing climate and reduce importation into the UK (Hemery, 2004, Hemery et al., 2005). Earth Trust's Paradise Wood is one such research woodland; *Juglans* seeds were collected from various global provenances to test their suitability for timber production in the UK (Clark and Hemery, 2009).

Although *Juglans* species seem an ideal candidate for timber, and potentially nut, production in the UK a number of diseases affect walnut trees, which can result in a slow decline in productivity and vigour. One of the most economically important diseases of walnut is a blight caused by the bacterium *Xanthamonas arboricola* pv. *juglandis* (*Xaj*). Although not fatal to the tree, this bacterium causes blackish greasy spots on leaves, stems and nuts which may lead to premature abscission (Frutos, 2010, Burokiene and Pulawska, 2012). *Fusarium* species and occasionally *Alternaria* species have been known to interact with the *Xaj* bacterium in certain environmental conditions to cause brown apical necrosis (BAN) which leads to severe fruit drop (Moragrega and Özaktan, 2010).

Walnut species are also susceptible to canker diseases. Shallow bark canker (SBC), caused by *Brenneria nigrifluens*, causes irregular cankers in the bark, from which dark reddish-brown coloured bleeds emanate (Moretti et al., 2007, Wilson et al., 1957). The disease was first reported in California, USA (Wilson et al., 1957) and has since been reported in Spain (López et al., 1994), Iran (Harighi and Rahiman, 1997), Italy (Morone et al., 1998), France (Ménard et al., 2004) and most recently in Hungary (Végh et al., 2014). Another *Brenneria* species, *B. rubrifaciens* causes deep bark canker (DBC) of walnut. DBC can be distinguished from SBC, as necrosis extends deep into the region of the phloem (Wilson et al., 1967). Although first recorded in North America (Wilson et al., 1967), it seems to have spread to Europe and has been detected most recently in Spain (González et al., 2002). Symptoms of SBC and DBC have not yet been reported in the UK and are not currently listed in the UK Plant Health Risk Register (Department for Environment Food and Rural Affairs, 2019). However, with the predicted changes in climate (IPCC, 2014) and the increased global movement of plant and plant products (Brasier, 2008, Sardain et al., 2019), there is potential for these pathogens to affect walnut trees in the UK in the future.

Limited data shows that *Brenneria rubrifaciens* may persist for long periods of time in walnut trees without showing signs of disease i.e. as an endophyte (McClean et al., 2008, Teviotdale et al., 1991, Thapa et al., 2010). Endophytes are defined as microorganisms that are capable of colonising the internal tissues of plants without showing symptoms of disease (Wilson, 1995). This general definition includes pathogens during their latency period (Verhoeff, 1974) and a number of phytopathogens have been reported as endophytes in healthy tissues of woody plants (Sessa et al., 2018, Slippers and Wingfield, 2007). In the case of *Brenneria rubrifaciens*, water stress has been shown to be a key predisposing factor in disease progression from an endophyte (Teviotdale and Sibbett, 1982). However, to date neither *Brenneria* species has been isolated from asymptomatic trees using culture-based plating. This study uses targeted PCR based methods (Thapa et al., 2010, Loreti et al., 2008) that were developed for symptomatic tissue but will be used to see of these pathogens are present in asymptomatic tissues. This will aid understanding of the aetiology of these diseases and could establish whether these pathogens are present in the UK.

This study also characterises the fungal and bacterial endophytes found in asymptomatic walnut tissues using next generation sequencing technologies. Most studies of endophytes associated with *Juglans* species have relied on culture-dependent techniques e.g. (Pardatscher and Schweigkofler, 2009, Ghorbani et al., 2018). The new developments in high-throughput technologies have allowed for a much deeper analysis of microbial communities (Knief, 2014). Only two culture-independent studies have analysed the endophytes of *Juglans* species, one study developed a bioinformatics pipeline using a very small sample size of *Juglans regia* to study fungal endophytes (LaBonte et al., 2018) and the other considered *in vitro* tissue culture shoots of *J. regia* and *J. nigra* to study bacterial endophytes (Pham et al., 2017). A more in-depth study is therefore needed to understand the endophyte-host relationships in *Juglans* species.

It might be expected that *Juglans* species would have a unique composition of endophytes due to the presence of high polyphenolic compounds in their tissues. All plant parts of *Juglans nigra* and *J. regia* can produce the aromatic phytochemical Juglone (Cosmulescu et al., 2011). Juglone is an allelopathic compound i.e. a compound that is synthesised by the plant and affects the performance of another organism (Babula et al., 2009). Juglone is known to have an inhibitory effect on other plants (Ercisli et al., 2005, Kocaçaliskan and Terzi, 2015, Topal et al., 2007) and may even have insecticidal activities (Sun et al., 2007). Juglone has been used for centuries in herbal remedies (Thakur, 2011, Erdemoglu et al., 2003) and the antimicrobial activities of this chemical have been studied more recently. *In vitro*, walnut leaf extracts have been shown to inhibit the growth of bacterial and fungal species associated with humans (Kocaçaliskan et al., 2018, Wang et al., 2016, Clark et al., 1990) and it is thought that juglone may contribute to resistance against tree disease (Hedin et al., 1979, Cline and Neely, 1983). Juglone has even been studied as a potential biological control agent against the fire blight pathogen *Erwinia amylovora* (Fischer et al., 2012).

Although the exact interactions between endophytes and their host are not well known, it is thought that some have the ability to induce plant growth promotion effects. For example, an *Enterobacter* sp. was shown to increase biomass production in poplar cuttings (Taghavi et al., 2009). Endophytic bacteria have also been shown to fix nitrogen for the plant host (Doty et al., 2016, Doty, 2011, Anand et al., 2013) and have been known to produce phytohormones such as indole acetic acid (IAA) (Madmony et al., 2005). Other endophytes protect against herbivores (see *Chapter 5*) and some show mechanisms of activity against phytopathogens (see *Chapter 6*). This study characterises the fungal and bacterial endophytes associated with host plants as a first step of identifying potentially beneficial microbes, further study can then be targeted towards understanding how these microbes help the plant tolerate abiotic and

biotic stresses. With even further study, these microbes could be harnessed as biological control agents or plant growth promotors to benefit forestry practices.

4.2. *Materials and methods*

4.2.1. *Site description and tree selection*

Three walnut trials were selected in Paradise Wood, Oxfordshire to study the endophytic community of *Juglans* species (see *Chapter 2* for a full site description of Paradise Wood) (*Figure 4.1a* & *b*). The 'common walnut provenance trial' was used to study the endophytes associated with *Juglans regia* and allowed for the study of host provenance and host phenotypic influences. The trial was planted in 1999, with the aim of determining the suitability of various genotypes of *Juglans regia* for timber production in the UK. Seed material was collected from 18 provenances across the species natural range: Spain, Tajikistan, Kyrgyzstan, Iran, Romania, Slovakia and Turkey. Multiple progeny were collected from each maternal tree, considered here as families. Trees were planted in a randomised block design with 5 by 5 metre spacing; three blocks were selected at random to be used in this study (*Appendix D.2*). The two provenances of interest used in this study were: K11 from Kyrgyzstan, the native range of *Juglans regia* and E1 from Spain, representing the introduced range of this tree species. Two families were selected from each of these provenances (*Figure 4.2*). Trees in this trial were cut back to the base, known as stumping, in 2014 in the hope of improving tree form (Clark and Brocklehurst, 2011, Hemery and Savill, 2001).

The 'black walnut provenance trial' was used to examine the endophytes associated with *J. nigra*. The trial was established in 2004 using trees of the same age. The seed material was collected from 45 *J. nigra* trees across their introduced range in Europe: Austria, Czech Republic, UK and Slovakia. Multiple seeds were collected from each tree, considered here as

families. Ten blocks containing 49 individual progenies were planted in a randomised complete block design with 2 by 2 metre spacing (*Appendix D.3*). Three blocks were selected at random to be used in this study (*Appendix D.3*). The two provenances used were AU from Austria and the CR from Czech Republic; two families were selected from each provenance.

The 'walnut nitrogen trial' was selected to compare the endophyte assemblage of *J. regia* with that of *J. nigra*. This trial was used as the trees were collected from the same area of France, they are of the same age and are growing in the same location in Paradise Wood. Established in 2001, the trial was originally set up to test the effects of applying artificial nitrogen to the establishment and growth of walnut trees. The two walnut species were subjected to 6 nitrogen treatments, replicated twice, laid out as a full factorial experiment in a randomised complete block design. Nitrogen treatments had no significant effect on height after 1 and 3 years and the experiment was concluded in 2004 (Hemery, 2004). Eight *Juglans regia* blocks and eight *J. nigra* plots were selected that spanned the greatest range in nitrogen concentration applied: 0, 100, 200 and 400kg/ha replicated twice (*Appendix D.4)*.

Figure 4.1 - (a) map of the UK showing location of Paradise Wood (white cross). Created using R packages: maps (version 3.3.0, Becker et al. 2018), and ggmap (version 3.0.0, Kahle and Wickham 2013) **(b) aerial photograph of Paradise Wood, shaded box indicates locations of the 3 trials: (1) common walnut provenance trial, (2) black walnut provenance trial and (3) walnut nitrogen trial.** Photo taken from google.com/maps.

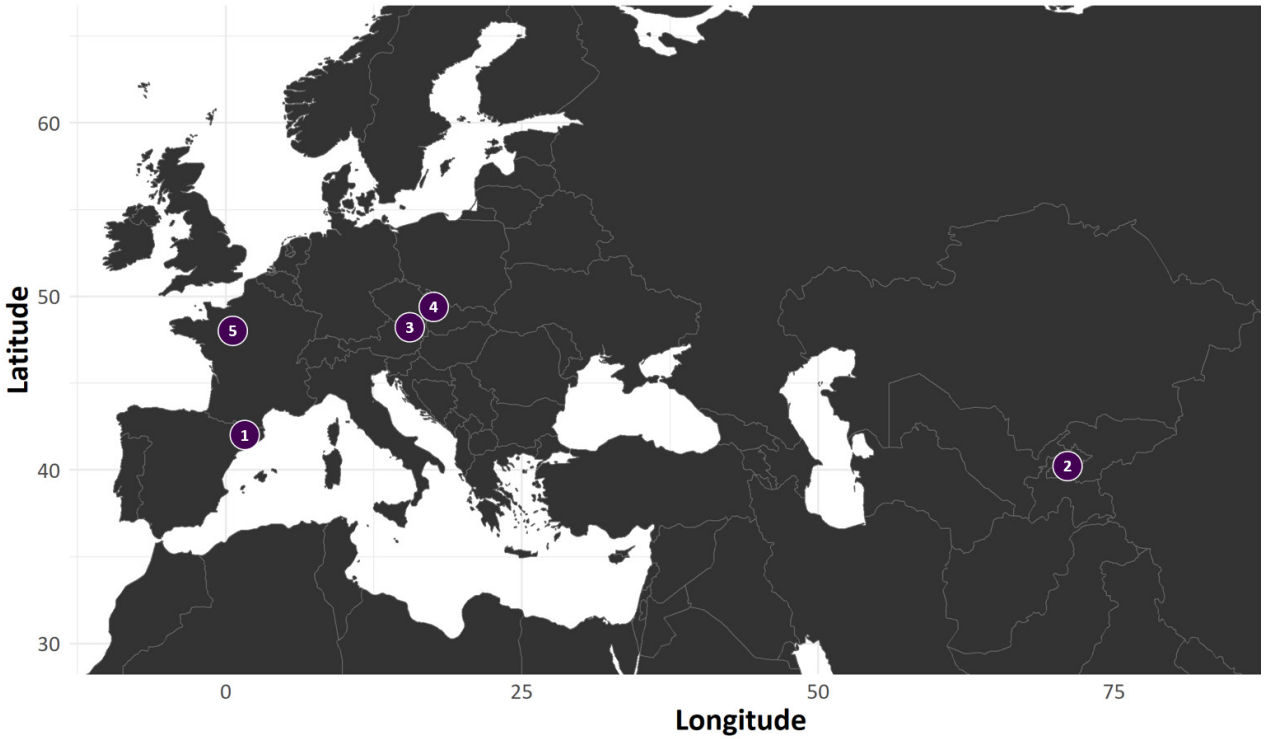

Figure 4.2 - the geographic locations of the parent trees planted in the three walnut trials. Map created using R packages: maps (version 3.3.0, Becker et al. 2018) and ggmap (version 3.0.0, Kahle & Wickham, 2013)

	Trial name	Country	Provenance	Families
1	Common walnut provenance trial	Spain	E1	E1.2 & E1.4
2	Common walnut provenance trial	Kyrgyzstan	K11	K11.1 & K11.16
3	Black walnut provenance trial	Austria	AU	AU06 & AU18
4	Black walnut provenance trial	Croatia	CR	CR02 & CR17
5	Walnut nitrogen trial	France		

4.2.2. *Measured tree traits*

4.2.2.1. *Phenology*

The timing of budburst of the walnut trees was recorded in Spring 2019. Bud burst was assessed by visual examination on one day (27th April 2019) and classified on an ordinal scale ranging from 0 to 5, according to the furthest developmental stage of >50% of buds on each individual tree (*Figure 4.3*) (Hemery et al., 2005, Hemery and Savill, 2001).

4.2.2.2. *Determining tree vigour using DBH and height*

The circumference of the trunk at breast height (1.3m) was measured using a tape measure. DBH was calculated using the equation in *Chapter 2, Section 2.2.3*. Height was measured using a smartphone tilt meter application (Bubble Level, Version 3.12) to measure the angle of elevation of the top of the tree crown and a tape measure to measure the distance from measurer to the base of the tree. Height was calculated using the equation in *Chapter 2, Section 2.2.3*.

4.2.3. *Sample collection*

On the 27th of September 2016, four fully expanded leaves and one 2cm section of twig were cut from each tree. Care was taken to select tissues that were insect and disease free and were not noticeably damaged by insect pests or pathogens. The tissues were removed with scissors sterilised with 100% ethanol and contact with the hands was avoided by using gloves. Samples were transported to the laboratory on ice in sterile zip-lock bags and stored at 4°C until processed.

4.2.4. *Surface sterilisation and DNA extraction*

All tissues were surface sterilised within 48 hours of collection using method A (*Appendix B*). Twig samples were shaken in the bleach step for 8 minutes; all other steps remained the same. A 5x5mm square was cut from the leaves using a sterile scalpel and pooled (4 leaves per tree) into a 2mL microcentrifuge tube and stored at -80°C for DNA extraction. A 10mm section was cut from the centre of each twig and stored in 2mL microcentrifuge tubes at 80°C. Total DNA was extracted from leaf and twig samples using method B (*Appendix C*). An extraction blank was included. For most samples high quality DNA was extracted, however, for some of the twig samples it was necessary to remove possible PCR inhibitors using gel extraction (full details in *Chapter 3, Section 3.3.1*).

Chelex extracted DNA of *Brenneria nigrifluens* (NCPPB no. 564) and *B. rubrifaciens* (NCPPB no. 2020) supplied by the National Collection of Plant Pathogenic Bacteria (NDPPB) were used as positive controls (see *Appendix I* for phylogenetic tree).

4.2.5. *PCR and sequencing*

A two-step PCR procedure developed by Dr Anna Oliver (CEH) was used as described in full in *Chapter 3, Section 3.3.2*. The V5/V6 region of the 16S rRNA gene was targeted for bacterial species and ITS region 2 (ITS2) for fungal species and were run on the same Illumina Miseq run as the oak samples in *Chapter 3, Section 3.3.2*.

4.2.6. *Bioinformatic analysis*

Bioinformatic analysis was performed by Dr Soon Gweon, University of Reading as described in *Chapter 3, Section 3.3.3*. All OTUs assigned to chloroplast, mitochondrial or kingdoms other than fungi and bacteria and all unassigned OTUs were removed.

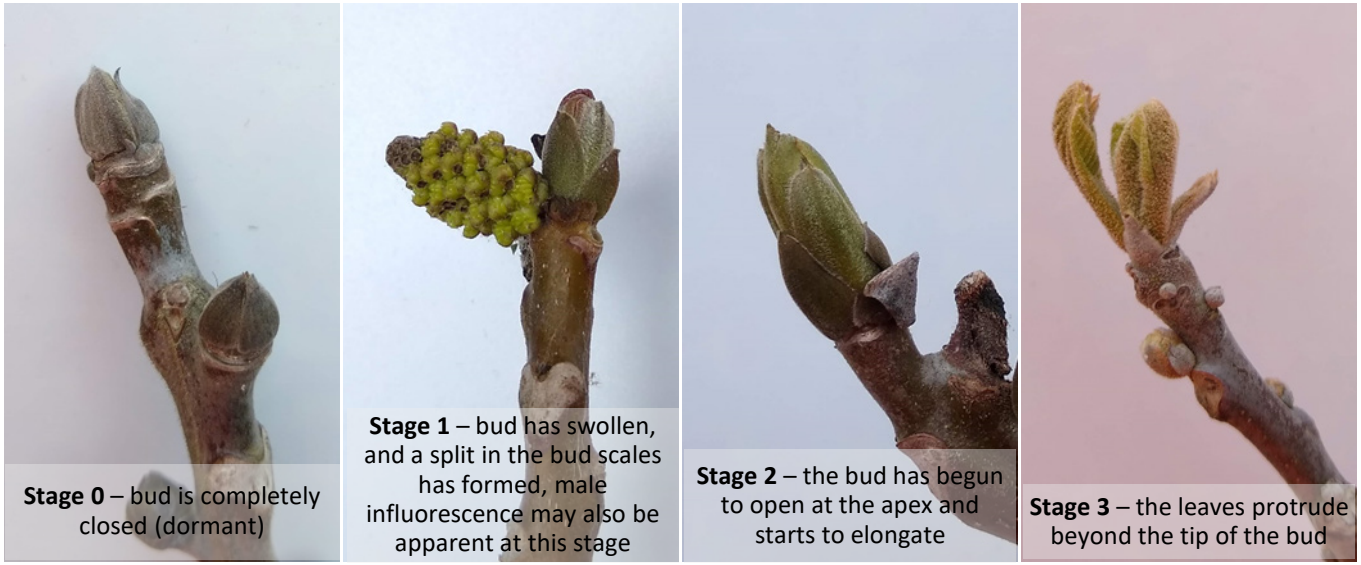

Figure 4.3 - 6 developmental stages of budburst recorded in the walnut trials in Paradise Wood in spring 2016. Photos taken by S. Roy.

4.2.7. *Targeted search for Brenneria species in Walnut*

All leaf and twig walnut samples from all three trials in Paradise Wood were tested for the presence of the causal agents of shallow bark canker (*Brenneria nigrifluens, Bn*) and deep bark canker (*B. rubrifaciens, Br*). Primers used for the detection of *Bn* and *Br* are listed in *Table 4.1*. *Brenneria nigrifluens* (NCPPB no. 564) and *B. rubrifaciens* (NCPPB no. 2020) were used as positive controls. Four walnut samples from Paradise Wood were spiked with an equal volume of *Bn* and *Br* DNA to test for the presence of inhibitors in the walnut extracts that may prevent the detection of the bacterial species of interest, these included: a leaf and a twig from *Juglans nigra* (from block 1 of the nitrogen trial) and a leaf and twig from *J. regia* (from block 2 of the nitrogen trial). The reaction mix contained 25µL 2X PCR buffer (Taq Mix red, PCRBIO), 1.25µL each primer (10µM, Eurofins Genomics), 5µL of DNA template; made up to 50µL with molecular grade water. PCR conditions for *Bn* consisted of an initial denaturation of 96°C for 3 mins, followed by 30 cycles of 94°C for 1 minute, 58°C for 1 minute and 72°C for 45 seconds and a final elongation step at 72°C for 5 minutes (Loreti et al., 2008). Conditions for *Br* were: initial denaturation at 94°C for 2 mins, followed by 30 cycles of 94°C for 15 seconds, 64°C for 15 seconds and 72°C for 1 minute and a final elongation step at 72°C for 7 minutes (Thapa et al., 2010). Amplification products were visualised on a 1% w/v agarose gel at 90V for 40 minutes. A band at 255bp indicated presence of *Bn* and 536bp confirmed presence of *Br.*

Table 4.1 - primers used in the detection of *Brenneria nigrifluens* and *B. rubrifaciens* from *Juglans* trees growing in the three trials in Paradise Wood.

Primer name	Primer sequence	Target species	Source
F1	5'-CCTGCGCCATGTTGCCAGATCGCTAT-3'	*Brenneria nigrifluens*	Loreti et al. (2008)
C3	5'-ACCTGAGTAGCAGTTTCGACTATTT-3'		
BrAF	5'-ATGTACGCAGTCTCTATTTGG-3'	*Brenneria rubrifaciens*	Thapa et al. (2010)
BrAR	5'-CCATCAGCCTGAAATAACTCA-3'		

4.2.8. *Statistical analysis*

During analyses it was noted that the following twig samples that were gel extracted to remove PCR inhibitors showed considerably different NMDS scores after Bray-Curtis analysis from the other samples in the group, these were therefore removed from further analyses: 5_AU11, 5_COMMON, 18_COMMON, 19_COMMON, 13_COMMON, 16_BLACK and 7_COMMON. The resulting two OTU tables (bacterial endophytes: $_{B}$OTU and fungal endophytes: $_{F}$OTU) were split into the three respective trials: (1) common walnut provenance trial and (2) black walnut provenance trial and (3) walnut nitrogen trial and were analysed separately. Samples from the common trial (1) were analysed both together and separately by tissue type. The black trial (2) was analysed in the same way. Samples from the nitrogen trial (3) were analysed firstly according to walnut species and secondly by tissue type. Each OTU table was rarefied to the lowest sequencing depth using the vegan package (version 2.5-3, Oksanen et al. (2018)) in R to avoid biases resulting from differences in sample size (*Table 4.2*). There has been recent debate in the scientific community about the efficacy of rarefying (McMurdie and Holmes, 2014, Hughes and Hellmann, 2005), that it can lead to loss of statistical power, so the following analyses were performed on the raw OTU table also and results were compared. Rarefying the data made few differences to the results; the results from the non-rarefied analyses are shown in (*Appendix G.8*). Two bacterial OTUs were removed from further analysis based on the rarefaction curves (*Appendix G.7*) as they differed considerably from the other samples in the group: 3_BLACK_LEAF and 4_CR04_TWIG.

The extraction blank contained 21 $_{B}$OTUs and 17 $_{F}$OTUs and the negative control 43 $_{F}$OTUs and 14 $_{B}$OTUs with more than 5 sequencing reads. There appears to be no agreement in the literature on how to deal with these OTUs found in negative controls. However, as the number of reads associated to these OTUs was markedly lower in the negative control samples compared to the experimental samples, they were retained in the table (Fort et al., 2016).

Also, when the negative control samples included in the NMDS plot based on Bray-Curtis similarity index, their NMDS values were considerably different, suggesting a different composition of OTUs than the experimental samples.

OTU richness was recorded as the number of observed OTUs in each sample and the Shannon-Wiener diversity index was used to estimate OTU diversity. Linear mixed effect models were used to test the effect of the variables listed in *Table 4.2* on the OTU richness and diversity for each of the trial groupings. There was a very strong correlation between height and DBH for all the trees (df=35, Pearson's cor=0.86, $p<0.001$) so it was decided that only DBH be used in further analysis, as there was a higher degree of accuracy of this measurement in the field. Family nested within provenance did not influence any of the statistical models, so was removed from analyses, and was not presented in the final models. Block number was included in the models as a random factor. The response variable was log transformed before running the Gaussian model. The residuals were tested for normality using a Shapiro-Wilk normality test and for heteroscedasticity by plotting the residuals versus the fitted values for each model (Crawley, 2007).

Dissimilarities in OTU composition between the samples (beta diversity) were calculated based on the Bray-Curtis dissimilarity index, ensuring a stress value below 0.2. Sample dissimilarities were visualised on a NMDS plot. PERMANOVA models were used to test for differences between samples using the *adonis* function in the vegan package, with 999 permutations and block number as a stratifying factor. The variables included in the models are listed in *Table 4.2*.

The five most abundant OTUs from each of the trial groupings were determined. Their identity was confirmed by comparing the representative OTU sequence with the NCBI GenBank database using the BLAST function. Linear mixed effect models were used to test the effects of

a number of variables (*Table 4.2*) on the abundance of these OTUs. The square root of the OTU abundance was included in the Gaussian model, and the residuals of the model were tested for normality using the Shapiro-Wilk test and for heteroscedasticity by plotting the fitted versus residual values of the model (Crawley, 2007).

The bacterial and fungal OTUs obtained from *Juglans* trees in this study were compared to the results of bacterial and fungal OTUs from *Quercus* trees growing in the same trial, Paradise Wood (*Chapter 3*). The samples were collected from *Quercus* and *Juglans* on the same day, and although the DNA was extracted using different methods (*Appendix C*), all other PCR and library preparations were carried out simultaneously using the same methods. All samples were run on the same Miseq sequencing run. All tissue types from all trials were combined. The combined bacterial OTU table was rarefied to 147 and combined fungal OTU table rarefied to 1038, the lowest sequencing depths. Dissimilarities in OTU composition between the samples were calculated based on the Bray-Curtis dissimilarity index, ensuring a stress value below 0.2. Sample dissimilarities were visualised on a NMDS plot. PERMANOVA models were used to test for differences between samples using the *adonis* function in the vegan package, with 999 permutations. Tree genus and tree species nested within genus were included as a fixed variables.

Table 4.2 – subsetting of the OTU table into the three walnut trials of interest, showing the response and explanatory variables for each statistical model. Each OTU was rarefied to the lowest sequencing depth and table shows the number of OTUs remaining after rarefaction.

Trial name	Samples included	16S rarefied to:	No. of 16S OTUs after rarefying	ITS rarefied to:	No. of ITS OTUs after rarefying	Variables included in statistical models
Common walnut provenance trial	**Leaf** + **twig** *J. regia*	891	454	7415	863	Tissue type Provenance DBH Budburst
	Leaf only *J. regia*	891	328	7415	716	Provenance DBH Budburst
	Twig only *J. regia*	4521	466	22172	529	Provenance DBH Budburst
Black walnut provenance trial	**Leaf** + **twig** *J. nigra*	1372	447	3471	625	Tissue type Provenance DBH Budburst
	Leaf only *J. nigra*	1444	327	3471	463	Provenance DBH Budburst
	Twig only *J. nigra*	1372	322	11289	550	Provenance DBH Budburst
Walnut nitrogen trial	***J. regia*** only Leaf + twig	1555	526	4061	507	Tissue type Nitrogen level DBH Budburst
	J. nigra only Leaf + twig	2355	621	1038	462	Tissue type Nitrogen level DBH Budburst
	Leaf only *J. regia* + *J. nigra*	1555	419	1038	403	Walnut species Nitrogen level DBH Budburst
	Twig only *J. regia* + *J. nigra*	6641	705	23457	820	Walnut species Nitrogen level DBH Budburst

4.3. *Results*

Figures 4.4 & 4.5 show the relative abundance of the top 10 class divisions within each tissue type and each species of *Juglans* from the walnut nitrogen trial. For the leaf samples the most abundant bacterial phyla were Proteobacteria (41.7% *Juglans regia*, 30.2% *J. nigra*), Actinobacteria (23.6% *Juglans regia*, 17.4% *J. nigra*) and Firmicutes (26.1% *Juglans regia*, 42.9% *J. nigra*). For twig samples: Proteobacteria (40.3% *Juglans regia*, 46.9% *J. nigra*), Actinobacteria (46.8% *Juglans regia*, 35.4% *J. nigra*) and Bacteroidetes (9.3% *Juglans regia*, 14.2% *J. nigra*).

The most abundant fungal phyla in leaf samples were Ascomycota (41.4% *Juglans regia*, 63.2% *J. nigra*) and Basidiomycota (50.5% *Juglans regia*, 33.9% *J. nigra* and for twig samples Ascomycota (58.9% *Juglans regia*, 70.3% *J. nigra*) and Basidiomycota (2.5% *Juglans regia*, 6.8% *J. nigra*).

4.3.1. *Endophyte richness and diversity*

Bacterial and fungal endophyte richness and diversity were affected by a number of variables, as shown in *Tables 4.3 & 4.4*. Few general inferences can be made from the results as the influence of each variable on OTU richness and diversity was different depending on which walnut species and which tissue type was tested (*Tables 4.3 & 4.4*).

4.3.2. *Endophyte composition*

PERMANOVA tests revealed a significant difference between the two walnut species represented in the 'walnut nitrogen trial' for both bacterial and fungal OTUs. The community composition of both kingdoms of endophytes were different for leaf and twig samples in all of the walnut trials. DBH was also a contributing factor to the composition of bacterial and fungal

endophytes in the 'black walnut provenance trial'. Results are summarised in *Tables 4.5 & 4.6* and NMDS plots are shown in *Figures 4.6 & 4.7*.

4.3.3. *Comparing Juglans to Quercus trees*

PERMANOVA tests revealed a significant difference between the two tree genera: *Juglans* and *Quercus* for both fungal ($F=43.74$, $p<0.001$) and bacterial OTUs ($F=29.16$, $p<0.001$). Species within each genus were also different for fungi ($F=3.67$, $p<0.01$) and bacteria ($F=3.57$, $p<0.001$) supporting the results above for walnut and the results for *Quercus* in *Chapter 3*. *Figure 4.11* show a Venn diagram of the number of shared OTUs between the genera and species.

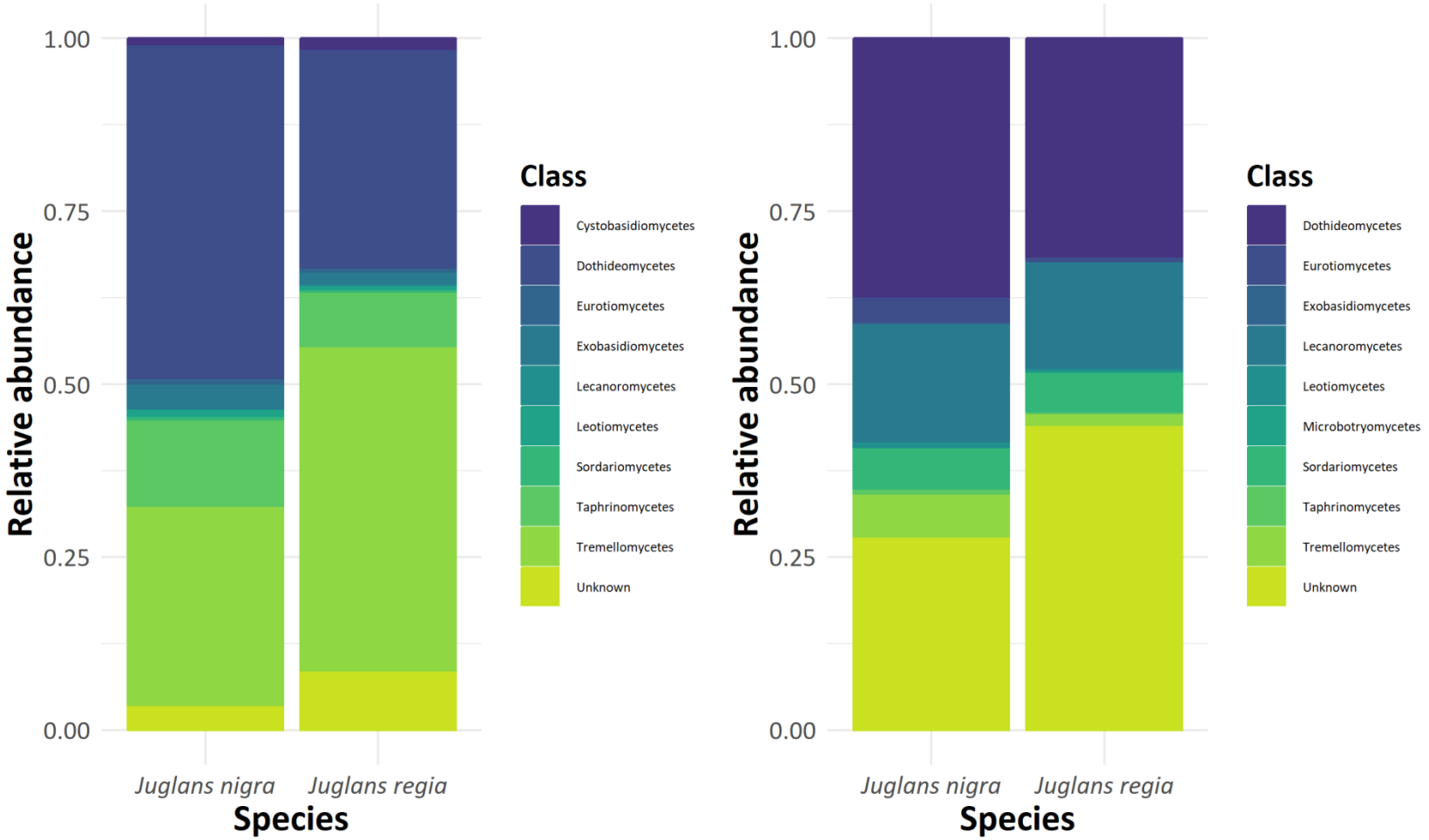

Figure 4.4 - the relative abundance of the top 10 class divisions for fungal OTUs in the two walnut species represented in the 'walnut nitrogen trial', leaf (left) and twig (right)

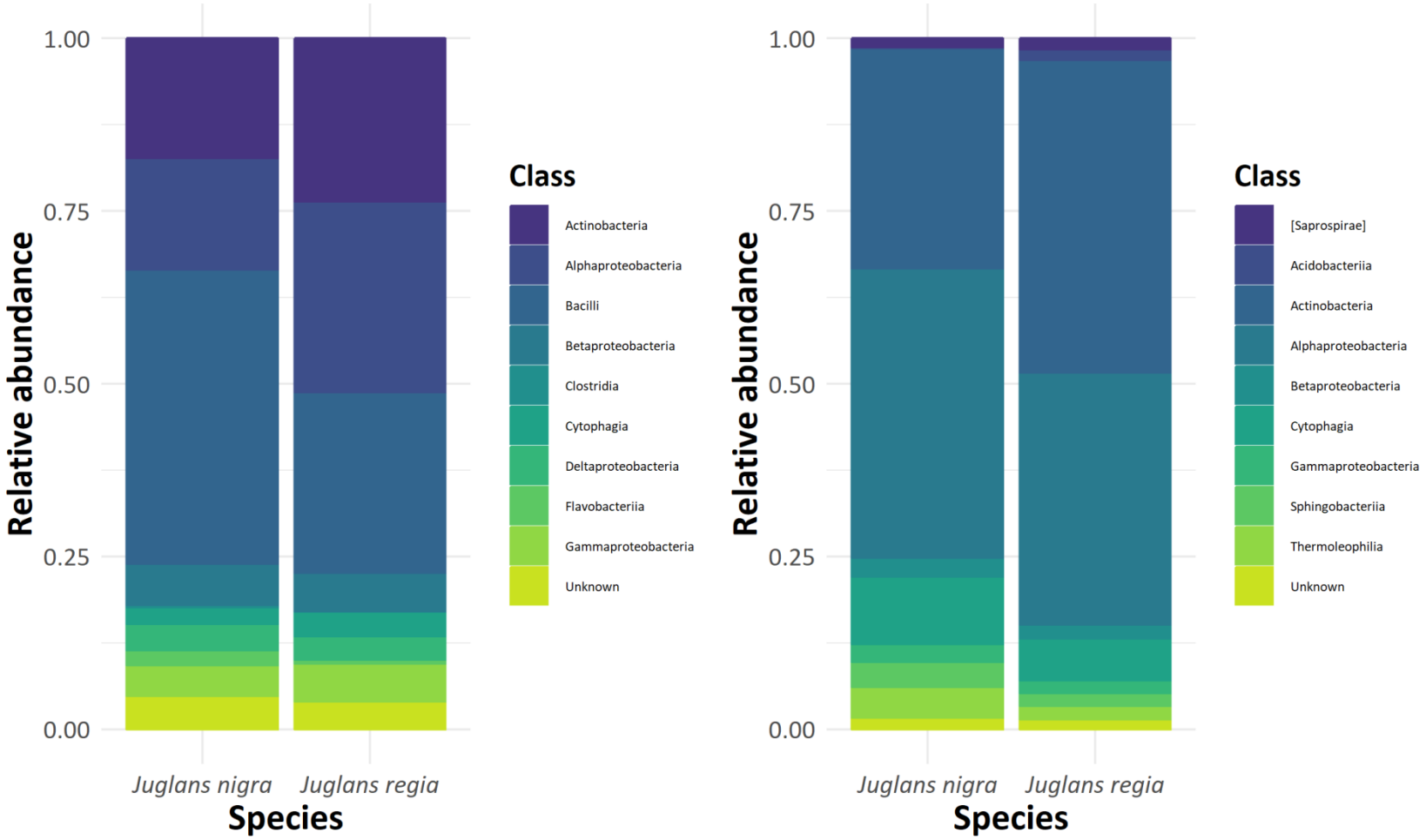

Figure 4.5 - the relative abundance of the top 10 class divisions for bacterial OTUs in the two walnut species represented in the 'walnut nitrogen trial', leaf (left) and twig (right)

Table 4.3 – results from the GLMM for species richness and diversity of fungal endophytes associated with the walnut trees in the different trials. Each OTU table was rarefied to the lowest sequencing depth before analysis. Effects shown here that are close to significant i.e. those with p values close to 0.05 were shown to be significant (p<0.05) on the non-rarefied OTU tables

		RICHNESS					DIVERSITY				
Trial	**Samples included in analysis**	**Signif. variables**	**df**	**F**	**p**	**Direction of effect**	**Signif. variables**	**df**	**F**	**p**	**Direction of effect**
Common walnut provenance trial	**Leaf** + **twig** *J. regia*	**Tissue** **Provenance**	5 5	19.27 2.83	<0.001 0.09	Leaf > twig Kyrgyzstan > Spain	**Tissue** **Budburst**	4 4	14.90 8.28	<0.001 <0.01	Leaf > Twig Positive
	Leaf only *J. regia*	No significant variables					No significant variables				
	Twig only *J. regia*	**Budburst** **Provenance**	4 4	3.60 5.28	0.06 <0.05	Positive Kyrgyzstan > Spain	**Budburst**	3	8.05	<0.01	Positive
Black walnut provenance trial	**Leaf** + **twig** *J. nigra*	**DBH**	3	5.39	0.07	Negative	**DBH**	3	8.10	<0.01	Negative
	Leaf only *J. nigra*	**DBH**	3	4.59	<0.05	Negative	No significant variables				
	Twig only *J. nigra*	**DBH** **Provenance**	4 4	9.27 14.05	<0.01 <0.001	Negative Czech Rep. > Austria	**DBH** **Budburst** **Provenance**	5 5 5	31.37 10.14 18.51	<0.001 <0.01 <0.001	Negative Negative Czech Rep. > Austria
Walnut nitrogen trial	***J. regia*** only Leaf + twig	**Tissue**	9	4.74	<0.05	Twig > Leaf	No significant variables				
	J. nigra only Leaf + twig	No significant variables					**Tissue**	13	5.59	<0.05	Twig > Leaf
	Leaf only *J. regia* + *J. nigra*	No significant variables					No significant variables				
	Twig only *J. regia* + *J. nigra*	**Species** **DBH**	7 7	6.36 9.76	<0.05 <0.05	*J. nigra* > *J. regia* Positive	**Species** **DBH**	8 8	6.26 7.61	<0.05 <0.05	*J. nigra* > *J. regia* Positive

Table 4.4 – results from the GLMM for species richness and diversity of bacterial endophytes associated with the walnut trees in the different trials. Each OTU table was rarefied to the lowest sequencing depth before analysis. Effects shown here that are close to significant i.e. those with p values close to 0.05 were shown to be significant (p<0.05) on the non-rarefied OTU tables

		RICHNESS					DIVERSITY				
Trial	**Samples included in analysis**	**Signif. variables**	**df**	**F**	**p**	**Direction of effect**	**Signif. variables**	**df**	**F**	**p**	**Direction of effect**
Common walnut provenance trial	**Leaf** + **twig** *J. regia*	**Tissue**	4	3.38	0.07	Twig > Leaf	No significant variables				
	Leaf only *J. regia*	No significant variables					No significant variables				
	Twig only *J. regia*	No significant variables					No significant variables				
Black walnut provenance trial	**Leaf** + **twig** *J. nigra*	**Provenance**	3	12.05	<0.001	Czech Rep. > Austria	**Provenance**	3	12.17	<0.001	Czech Rep. > Austria
	Leaf only *J. nigra*	**Budburst**	3	4.14	<0.05	Positive	No significant variables				
	Twig only *J. nigra*	**Provenance**	3	11.49	<0.001	Czech Rep. > Austria	**Provenance**	3	7.66	<0.01	Czech Rep. > Austria
Walnut nitrogen trial	***J. regia*** only Leaf + twig	**Tissue** **Budburst**	8 8	14.66 12.73	<0.01 <0.01	Twig > Leaf Negative	No significant variables				
	J. nigra only Leaf + twig	**Tissue**	12	29.43	<0.001	Twig > Leaf	**Tissue**	12	20.20	<0.001	Twig > Leaf
	Leaf only *J. regia* + *J. nigra*	No significant variables					**Species** **Nitrogen**	12 12	20.58 5.60	<0.001 <0.05	*J. regia* > *J. nigra* Positive
	Twig only *J. regia* + *J. nigra*	No significant variables					No significant variables				

Table 4.5 – results from the PERMANOVA for fungal endophyte beta diversity of walnut trees in the tree trials. OTU tables were rarefied to the lowest sequencing depth before analysis.

Trial	Samples included in analysis	Rarefied to:	Stress value	Significant variables	F	p
Common walnut provenance trial	***Juglans regia*** only Leaf + twig	7415	0.165	**Tissue**	6.34	<0.01
	Leaf only *Juglans regia*	7415	0.302	No significant variables		
	Twig only *Juglans regia*	22172	0.155	No significant variables		
Black walnut provenance trial	***Juglans nigra*** only Leaf + twig	3471	0.192	**Tissue** **DBH**	7.63 2.50	<0.001 <0.05
	Leaf only *Juglans nigra*	3471	0.167	**DBH**	1.90	<0.05
	Twig only *Juglans nigra*	11289	0.182	**DBH**	1.63	<0.05
Walnut nitrogen trial	***Juglans regia*** only Leaf + twig	4061	0.100	**Tissue**	13.28	<0.01
	Juglans nigra only Leaf + twig	1038	0.061	**Tissue**	8.51	<0.001
	Leaf only *Juglans regia* + *Juglans nigra*	1038	0.198	**Species**	2.85	<0.05
	Twig only *Juglans regia* + *Juglans nigra*	23457	0.064	**Species**	2.62	<0.01

Table 4.6 – results from the PERMANOVA for bacterial endophyte beta diversity of walnut trees in the tree trials. OTU tables were rarefied to the lowest sequencing depth before analysis.

Trial	Samples included in analysis	Rarefied to:	Stress value	Significant variables	F	p
Common walnut provenance trial	**Leaf** + **twig** *Juglans regia*	891	0.131	**Tissue**	5.63	<0.01
	Leaf only *Juglans regia*	891	0.144	No significant variables		
	Twig only *Juglans regia*	4521	0.045	No significant variables		
Black walnut provenance trial	**Leaf** + **twig** *Juglans nigra*	1372	0.102	**Tissue** **DBH**	3.86 1.36	<0.001 <0.05
	Leaf only *Juglans nigra*	1444	0.129	**DBH**	2.78	<0.05
	Twig only *Juglans nigra*	1372	0.105	No significant variables		
Walnut nitrogen trial	***Juglans regia*** only Leaf + twig	1555	0.033	**Tissue**	6.63	<0.01
	Juglans nigra only Leaf + twig	2355	0.061	**Tissue**	11.16	<0.001
	Leaf only *Juglans regia* + *Juglans nigra*	1555	0.227	**Species**	2.36	<0.05
	Twig only *Juglans regia* + *Juglans nigra*	6641	0.035	No significant variables		

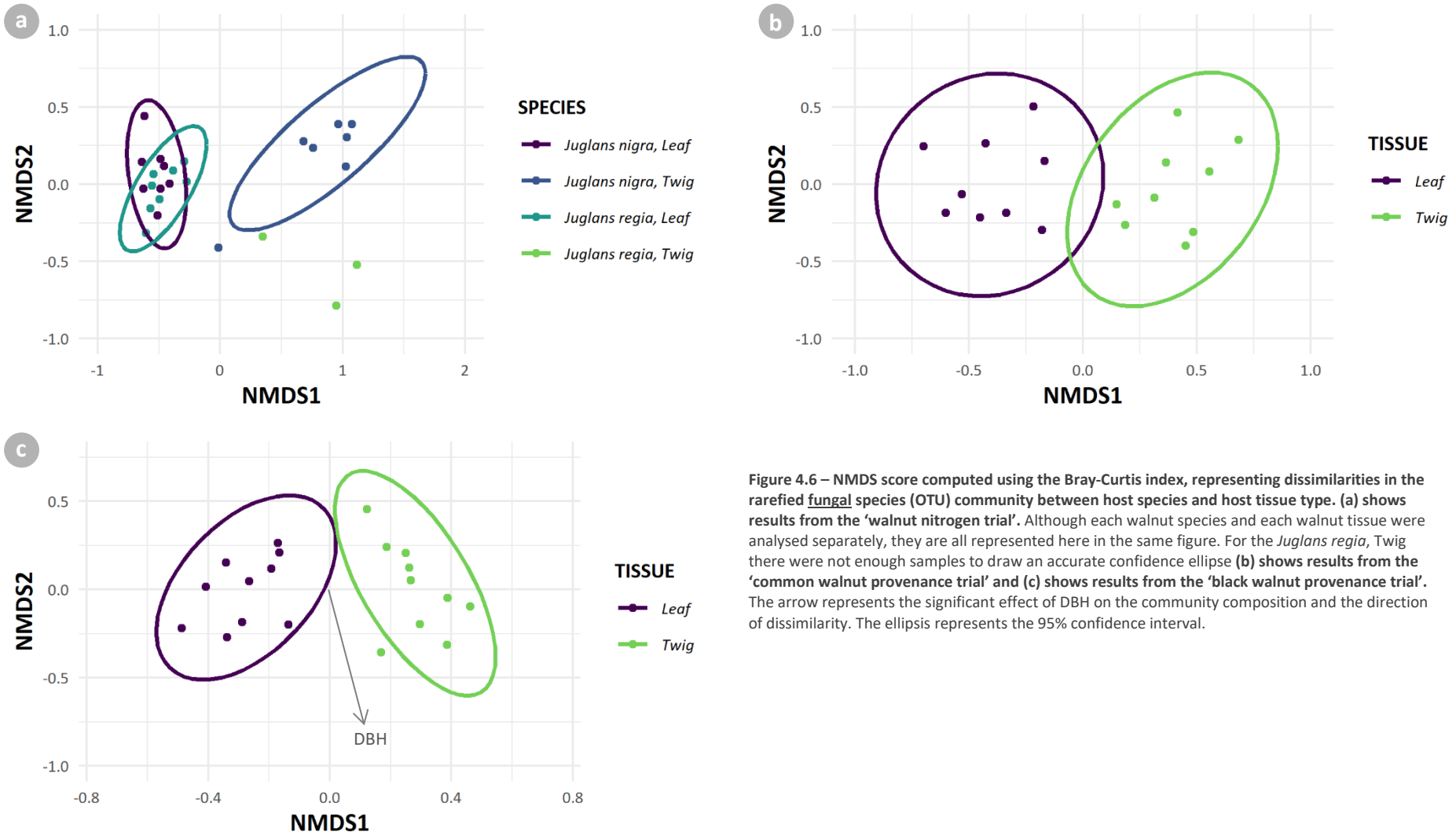

Figure 4.6 – NMDS score computed using the Bray-Curtis index, representing dissimilarities in the rarefied fungal species (OTU) community between host species and host tissue type. (a) shows results from the 'walnut nitrogen trial'. Although each walnut species and each walnut tissue were analysed separately, they are all represented here in the same figure. For the *Juglans regia*, Twig there were not enough samples to draw an accurate confidence ellipse **(b) shows results from the 'common walnut provenance trial' and (c) shows results from the 'black walnut provenance trial'.** The arrow represents the significant effect of DBH on the community composition and the direction of dissimilarity. The ellipsis represents the 95% confidence interval.

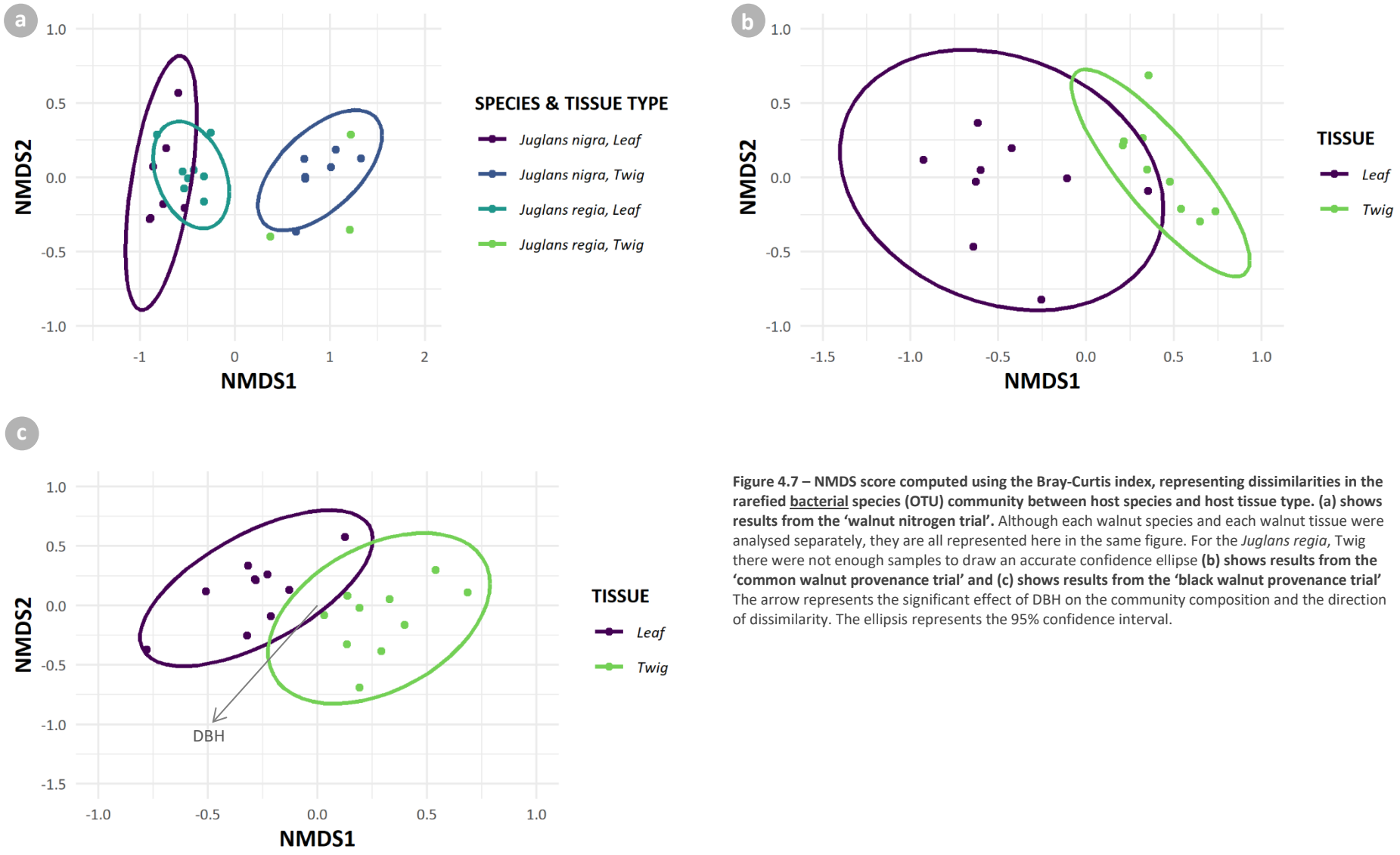

Figure 4.7 – NMDS score computed using the Bray-Curtis index, representing dissimilarities in the rarefied bacterial species (OTU) community between host species and host tissue type. (a) shows results from the 'walnut nitrogen trial'. Although each walnut species and each walnut tissue were analysed separately, they are all represented here in the same figure. For the *Juglans regia*, Twig there were not enough samples to draw an accurate confidence ellipse **(b) shows results from the 'common walnut provenance trial' and (c) shows results from the 'black walnut provenance trial'** The arrow represents the significant effect of DBH on the community composition and the direction of dissimilarity. The ellipsis represents the 95% confidence interval.

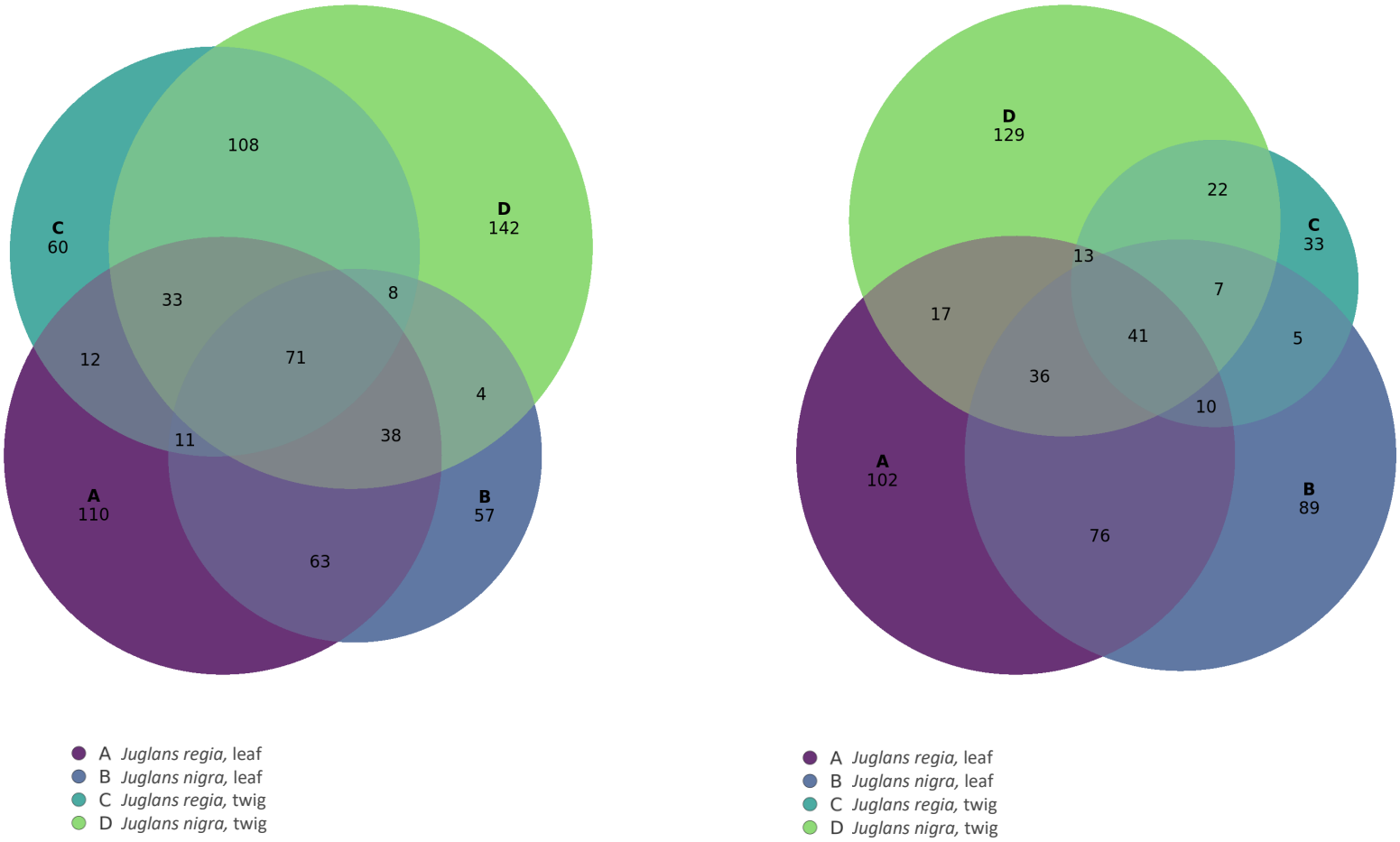

Figure 4.8 – number of OTUs shared by each tissue type and each *Juglans* species represented in the 'walnut nitrogen trial', fungi (left), bacteria (right)

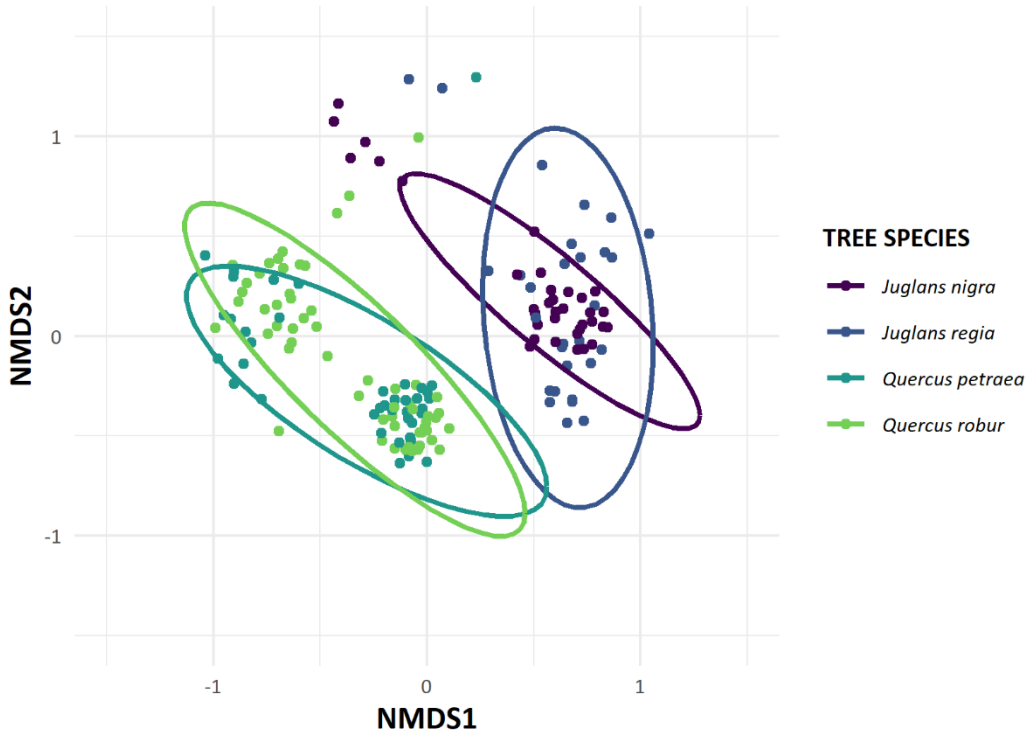

Figure 4.9 – NMDS score computed using the Bray-Curtis index, representing dissimilarities in the rarefied fungal OTU community between tree host species. The ellipsis represents the 95% confidence interval.

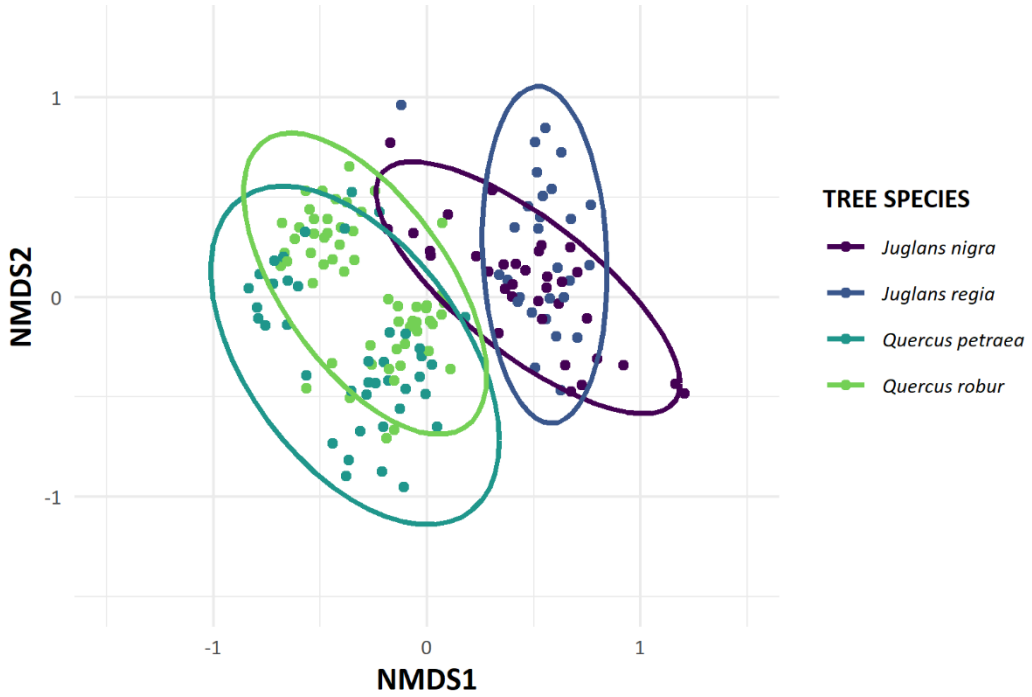

Figure 4.10 – the NMDS score computed using the Bray-Curtis index, representing dissimilarities in the rarefied bacterial OTU community between tree host species. The ellipsis represents the 95% confidence interval.

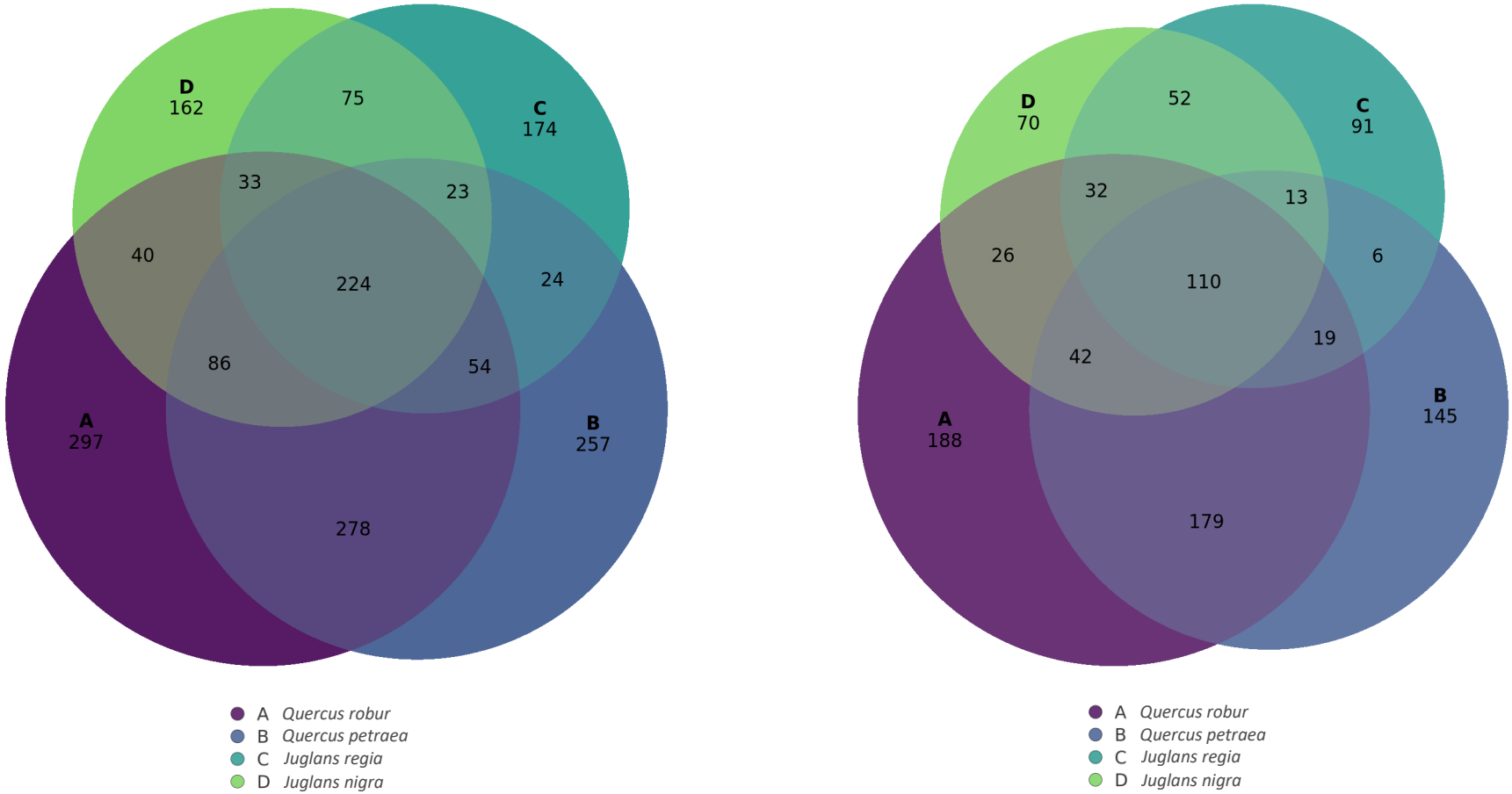

Figure 4.11 – the number of OTUs shared by four tree species: *Quercus robur, Q. petraea*, *Juglans regia* and *J. nigra*. Fungal OTUs (left) and bacterial OTUs (right)

4.3.4. *Factors affecting the most abundant fungal and bacterial endophytes*

Only 71 fungal OTUs and 41 bacterial OTUs were shared between all tissue types and all *Juglans* species represented in the 'walnut nitrogen trial'. Each tissue type and each species have a number of unique OTUs (*Figure 4.8*).

The five most abundant OTUs from each of the trial groupings are listed in *Tables 4.7-4.12*, the influence of all environmental and host factors on the abundance of these OTUs are also shown in the table. The taxonomy of the most abundant OTUs were confirmed by using the BLAST function with the representative OTU sequence against the NCBI GenBank database and recording the top identity match based on percentage identity.

A number of OTUs recorded in asymptomatic tissue were assigned to taxa thought to be pathogenic to *Juglans*, these are listed in *Table 4.13*, with their relative abundance in each of the tissue types and walnut species collected from the 'walnut nitrogen trial'.

4.3.5. *Presence of Brenneria species*

Species specific primers designed for *Brenneria nigrifluens* (Loreti et al., 2008) and *Brenneria rubrifaciens* (Thapa et al., 2010) were unable to detect either species in the paradise wood walnut samples, from either leaf or twig samples. A distinct band was present at around 200bp for the *Brenneria nigrifluens* positive control and at around 500bp for the *B. rubrifaciens* control. Corresponding bands were also present in the spiked walnut samples (*Figure 4.12*).

Table 4.7 – top 5 abundant fungal OTUs from the 'common walnut provenance trial'. Taxa assigment was based on NCBI GenBank BLAST results with the OTU representative sequence, the highest identity match is shown here. Table also shows significant variables effecting the abundance of these OTUs using a linear GLMM

OTU number	% Identity	Taxon	Species	Tissue	Signif. variable	df	χ^2	p	Direction of effect
OTU_F2447	99.31	*Cladosporium* sp.	*J. regia*	Leaf	No significant variables				
OTU_F528	100.00	*Vishniacozyma victoriae*	*J. regia*	Leaf	No significant variables				
OTU_F2406	100.00	*Cladosporium* sp.	*J. regia*	Leaf	**Provenance**	**4**	**4.71**	**<0.05**	**Spain > Kyrgyzstan**
					DBH	**4**	**6.30**	**<0.05**	**Positive**
OTU_F3320	99.29	*Vishniacozyma foliicola*	*J. regia*	Leaf	No significant variables				
OTU_F3025	97.22	*Vishniacozyma haemaeyensis*	*J. regia*	Leaf	**Budburst**	**3**	**4.02**	**<0.05**	**Negative**
OTU_F1989	99.34	*Didymella* sp.	*J. regia*	Twig	No significant variables				
OTU_F2406	100.00	*Cladosporium* sp.	*J. regia*	Twig	**Provenance**	**3**	**10.70**	**<0.01**	**Spain > Kyrgyzstan**
OTU_F1826	99.36	*Alternaria* sp.	*J. regia*	Twig	No significant variables				
OTU_F1414	100.00	*Fusarium* sp.	*J. regia*	Twig	**DBH**	**4**	**12.63**	**<0.001**	**Negative**
					Budburst	**4**	**7.22**	**<0.01**	**Positive**
OTU_F3320	99.29	*Vishniacozyma foliicola*	*J. regia*	Twig	No significant variables				

Table 4.8 – top 5 abundant bacterial OTUs from the 'common walnut provenance trial'. Taxa assigment was based on NCBI GenBank BLAST results with the OTU representative sequence, the highest identity match is shown here. Table also shows significant variables effecting the abundance of these OTUs using a linear GLMM

OTU number	% Identity	Taxon	Species	Tissue	Signif. variable	df	χ^2	p	Direction of effect
OTU_B505	99.29	*Sphingomonas* sp.	*J. regia*	Leaf	No significant variables				
OTU_B1684	99.29	*Methylobacterium adhaesivum*	*J. regia*	Leaf	No significant variables				
OTU_B1772	99.64	*Methylobacteriaceae* family	*J. regia*	Leaf	No significant variables				
OTU_B2166	99.28	*Aurantimonadaceae* family	*J. regia*	Leaf	No significant variables				
OTU_B1510	98.58	*Methylobacterium* sp.	*J. regia*	Leaf	**Provenance**	**3**	**3.81**	**<0.05**	**Spain > Kyrgyzstan**
OTU_B1685	98.93	*Frigoribacterium* sp.	*J. regia*	Twig	**Provenance**	**5**	**24.14**	**<0.001**	**Spain > Kyrgyzstan**
					DBH	**5**	**16.81**	**<0.001**	**Positive**
					Budburst	**5**	**18.40**	**<0.001**	**Positive**
OTU_B2158	98.20	*Nocardioidaceae* family	*J. regia*	Twig	**Provenance**	**3**	**4.88**	**<0.05**	**Spain > Kyrgyzstan**
OTU_B1778	99.28				**Budburst**	**3**	**3.91**	**<0.05**	**Positive**
OTU_B1288	99.64	*Xanthomonadaceae* family	*J. regia*	Twig	No significant variables				
OTU_B505	99.29	*Sphingomonas* sp.	*J. regia*	Twig	No significant variables				

Table 4.9 – top 5 abundant fungal OTUs from the 'black walnut provenance trial'. Taxa assigment was based on NCBI GenBank BLAST results with the OTU representative sequence, the highest identity match is shown here. Table also shows significant variables effecting the abundance of these OTUs using a linear GLMM

OTU number	% Identity	Taxon	Species	Tissue	Signif. variable	df	χ^2	p	Direction of effect
OTU_F2447	99.31	*Cladosporium* sp.	*J. nigra*	Leaf	No significant variables				
OTU_F510	98.51	*Taphrina* sp.	*J. nigra*	Leaf	**DBH**	**3**	**11.06**	**<0.001**	**Negative**
OTU_F528	100.00	*Vishniacozyma victoriae*	*J. nigra*	Leaf	**DBH**	**3**	**4.73**	**<0.05**	**Positive**
OTU_F2406	100.00	*Cladosporium* sp.	*J. nigra*	Leaf	No significant variables				
OTU_F1857	98.09	*Didymellaceae* family	*J. nigra*	Leaf	No significant variables				
OTU_F510	98.51	*Taphrina* sp.	*J. nigra*	Twig	**Provenance** **Budburst**	**4** **4**	**4.03** **9.92**	**<0.05** **<0.01**	**Austria > Czech Rep.** **Positive**
OTU_F528	100.00	*Vishniacozyma victoriae*	*J. nigra*	Twig	**DBH**	**3**	**7.40**	**<0.01**	**Positive**
OTU_F2406	100.00	*Cladosporium* sp.	*J. nigra*	Twig	No significant variables				
OTU_F3025	97.22	*Vishniacozyma haemaeyensis*	*J. nigra*	Twig	**Provenance** **Budburst**	**4** **4**	**7.69** **5.26**	**<0.01** **<0.05**	**Czech Rep. > Austria** **Negative**
OTU_F3264	100.00				No significant variables				

Table 4.10 – top 5 abundant bacterial OTUs from the 'black walnut provenance trial'. Taxa assigment was based on NCBI GenBank BLAST results with the OTU representative sequence, the highest identity match is shown here. Table also shows significant variables effecting the abundance of these OTUs using a linear GLMM

OTU number	% Identity	Taxon	Species	Tissue	Signif. variable	df	χ^2	p	Direction of effect
OTU_B1684	99.29	*Methylobacterium adhaesivum*	*J. nigra*	Leaf	**DBH**	**3**	**4.57**	**<0.05**	**Negative**
OTU_B505	99.29	*Sphingomonas* sp.	*J. nigra*	Leaf	No significant variables				
OTU_B692	100.00				No significant variables				
OTU_B1768	98.92	*Aurantimonadaceae* family	*J. nigra*	Leaf	No significant variables				
OTU_B402	99.29	Actinomycetales order	*J. nigra*	Leaf	**DBH**	**3**	**7.37**	**<0.01**	**Positive**
OTU_B505	99.29	*Sphingomonas* sp.	*J. nigra*	Twig	**DBH** **Budburst**	**4** **4**	**5.81** **8.71**	**<0.05** **<0.01**	**Positive** **Positive**
OTU_B692	100.00				No significant variables				
OTU_B1684	99.29	*Methylobacterium adhaesivum*	*J. nigra*	Twig	**Provenance** **Budburst**	**4** **4**	**11.16** **8.17**	**<0.001** **<0.01**	**Austria > Czech Rep.** **Positive**
OTU_B2439	99.63	*Hymenobacter* sp.	*J. nigra*	Twig	No significant variables				
OTU_B1685	98.93	*Frigoribacterium* sp.	*J. nigra*	Twig	**Provenance**	**3**	**7.68**	**<0.01**	**Austria > Czech Rep.**

Table 4.11 – top 5 abundant fungal OTUs from the 'walnut nitrogen trial'. Taxa assigment was based on NCBI GenBank BLAST results with the OTU representative sequence. Table also shows significant variables effecting the abundance of these OTUs using a linear GLMM

OTU number	% Identity	Taxon	Species	Tissue	Significant variable	df	χ^2	p	Direction of effect
OTU_F528	100.00	*Vishniacozyma victoriae*	Both	Leaf	**Species** **Nitrogen**	**13** **13**	**9.05** **7.72**	**<0.05** **<0.05**	***J. regia* > *J. nigra*** **Negative**
OTU_F2447	99.31	*Cladosporium* sp.	Both	Leaf	No significant variables				
OTU_F510	98.51	*Taphrina* sp.	Both	Leaf	**Species** **Budburst**	**13** **13**	**9.27** **10.77**	**<0.01** **<0.01**	***J. nigra* > *J. regia*** **Negative**
OTU_F2406	100.00	*Cladosporium* sp.	Both	Leaf	No significant variables				
OTU_F3025	97.22	*Vishniacozyma haemaeyensis*	Both	Leaf	No significant variables				
OTU_F2457	98.68	*Lecania cyrtella*	Both	Twig	No significant variables				
OTU_F2358		Unknown	Both	Twig	**Species**	**8**	**22.34**	**<0.01**	***J. regia* > *J. nigra***
OTU_F2349	100.00	*Angustimassarina* sp.	Both	Twig	**Nitrogen** **Budburst**	**7** **7**	**6.88** **7.63**	**<0.05** **<0.05**	**Positive** **Negative**
OTU_F2322		Unknown	Both	Twig	**Species** **Nitrogen**	**7** **7**	**43.98** **14.58**	**<0.001** **<0.01**	***J. regia* > *J. nigra*** **Positive**
OTU_F2725	100.00	Capnodiales order	Both	Twig	No significant variables				

Table 4.12 – top 5 abundant bacterial OTUs from the 'walnut nitrogen trial'. Taxa assigment was based on NCBI GenBank BLAST results with the OTU representative sequence. Table also shows significant variables effecting the abundance of these OTUs using a linear GLMM

OTU number	% Identity	Taxon	Species	Tissue	Signif. variable	df	χ^2	p	Direction of effect
OTU_B1282	98.22	*Bacillus* sp.	Both	Leaf	**DBH**	**12**	**5.29**	**<0.05**	**Negative**
					Budburst	**12**	**8.36**	**<0.05**	**Positive**
OTU_B1117	96.80		Both		**DBH**	**12**	**6.16**	**<0.05**	**Negative**
					Budburst	**12**	**7.44**	**<0.05**	**Positive**
OTU_B1271	97.51		Both		**DBH**	**12**	**7.07**	**<0.05**	**Negative**
					Budburst	**12**	**9.36**	**<0.01**	**Positive**
OTU_B402	99.29	Actinomycetales	Both	Leaf	No significant variables				
OTU_B1818	99.63	Actinobacteria	Both	Leaf	**Budburst**	**13**	**7.61**	**<0.05**	**Negative**
OTU_B1444	99.28	*Friedmanniella* sp.	Both	Twig	No significant variables				
OTU_B2439	99.63	*Hymenobacter* sp.	Both	Twig	**Species**	**7**	**49.18**	**<0.001**	***J. nigra* > *J. regia***
					DBH	**7**	**15.65**	**<0.01**	**Negative**
OTU_B1185	99.28	*Actinomycetospora* sp.	Both	Twig	No significant variables				
OTU_B505	99.29	*Sphingomonas* sp.	Both	Twig	No significant variables				
OTU_B682	99.64	Rhizobiales	Both	Twig	No significant variables				

Table 4.13 – potentially pathogenic or saprophytic fungal and bacterial OTUs. Taxon identiy was determined using the BLAST analysis on the respresentative OTU sequence against the GenBank database. Taxa unassigned at species or order level indicate OTUs assigned to multiple species or orders with identical e-values. Relative abudance is the percentage abundance of each OTU in the 'walnut nitrogen trial' OTU tables.

OTU number	Closest identity match	Relative abundance	Potential disease symptoms in *Quercus* and other tree species
OTU_B1288	***Xanthomonadaceae* family**	***J. regia* leaf – 0.06, *J. regia* twig – 0.92** ***J. nigra* leaf – 0, *J. nigra* twig – 0.002**	*Xanthomonas arboricola* pv. *juglandis* is the causative agent of bacterial blight of walnuts. Black spots develop on leaves, stems and fruit (Burokiene and Pulawska, 2012, Hajri et al., 2010).
OTU_B1427	***Brenneria* sp. (most likely *B. rubrifaciens*)**	***J. regia* leaf – 0, *J. regia* twig – 0.02** ***J. nigra* leaf – 0, *J. nigra* twig – 0.02**	*Brenneria rubrifaciens* causes deep bark canker (Wilson et al., 1967) in *Juglans* species
OTU_B1435	***Brenneria* sp. (most likely *B. goodwinii*)**	***J. regia* leaf – 0.30, *J. regia* twig – 0.01** ***J. nigra* leaf – 0.63, *J. nigra* twig – 0.02**	*Brenneria goodwinii* causes cankers in *Quercus* species (Denman and Webber, 2009)
OTU_F1414 **OTU_F1265** **OTU_F1345**	***Nectriaceae* family**	***J. regia* leaf – 0, *J. regia* twig – 0.01** ***J. nigra* leaf – 0, *J. nigra* twig – 0.02**	*Nectria galligena* produces a perennial target canker on *Juglans* species, in particular *J. nigra*. Cankers are usually formed where a branch has fallen and causes defects in the wood, stunting of growth and if the canker girdles the tree it can lead to tree death (Weber et al., 1980)
OTU_F1333	***Ophiognomonia leptostyla***	***J. regia* leaf – 0, *J. regia* twig - 0** ***J. nigra* leaf – 0.16, *J. nigra* twig – 0.09**	This fungal pathogen causes walnut anthracnose, dark brown to black spots that occur on the leaves, twigs and fruit of walnut leading to general loss of vigour (Strouts and Winter, 2000, Weber et al., 1980)
OTU_F1700 **OTU_F1791**	***Cytospora* sp.**	***J. regia* leaf – 0.01, *J. regia* twig - 0** ***J. nigra* leaf – 0.02, *J. nigra* twig – 0.002**	*Cytospora* species cause cankers and dieback in a number of fruit and nut crops, including *Juglans* species, however they are also known to have an endophytic phase in asymptomatic trees (Lawrence et al., 2018).
OTU_F54	***Microstroma juglandis***	***J. regia* leaf – 0.45, *J. regia* twig – 0.02** ***J. nigra* leaf – 0.04, *J. nigra* twig – 0.11**	*Microstroma juglandis* causes downy leaf spot on *Juglans* species (Wolf, 1927, Kurt et al., 2003)
OTU_F2981 **OTU_F1860**	***Septoria* sp.**	***J. regia* leaf – 4.44, *J. regia* twig – 6.03** ***J. nigra* leaf – 9.24, *J. nigra* twig – 2.73**	Although not yet recorded from *Juglans* species a number of *Septoria* species are known to cause leaf spot infections in other tree species (Zalasky, 1978)
OTU_F1560	***Alternaria* sp.**	***J. regia* leaf – 0.40, *J. regia* twig – 0.15** ***J. nigra* leaf – 1.36, *J. nigra* twig – 0.18**	More likely to be an opportunistic pathogen, interacting with other pathogens to cause cankers (Mazzaglia et al., 2005)

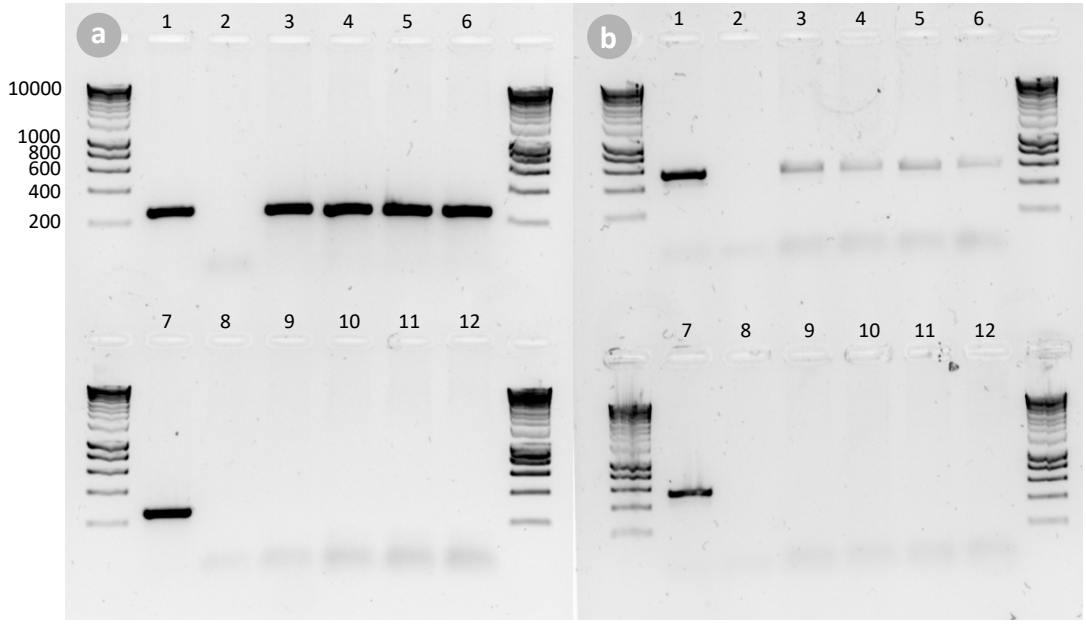

Figure 4.12 – the results of the targeted search for *Brenneria nigrifluens* and *B. rubrifaciens* using species specific primers (a) *Brenneria nigrifluens* and (b) *Brenneria rubrifaciens*. Lanes (1) positive control, (2) negative control, (3) spiked *Juglans regia* leaf sample, (4) spiked *J. regia* twig samples, (5) spiked *J. nigra* leaf sample, (5) spiked *J. nigra* twig sample, (7) positive control, (8) negative control, (9) example *J. regia* leaf sample, (10) example *J. regia* twig sample, (11) example *J. nigra* leaf sample, (12) example *J. nigra* twig sample.

4.4. *Discussion*

The black and common walnut trees in Paradise Wood are host to a wide variety of fungal and bacterial endophytic species. The richness, diversity and species composition of which are affected by tree species, tree tissue, tree vigour, tree phenology and tree provenance.

This study has characterised the set of endophytic bacteria and fungi that reside within *Juglans regia* and *J. nigra* trees in their introduced range in the UK. As the majority of the endophytes associated with woody plants are horizontally transmitted, trees growing outside their native range are likely to be colonised by indigenous bacteria and fungi. The endophytic assemblage of these walnut species may therefore be very different in their native range. For example, other studies have found that trees growing outside of their native range are host to a higher abundance of opportunistic host-generalist endophytes (Fisher et al., 1994, Hoffman and Arnold, 2008). As these introduced walnut species have not co-evolved with the indigenous endophytic species, there is potential for increased antagonistic interactions between host and

endophyte and reduced mutualistic relationships (Revillini et al., 2016). For example, it has been well studied in tree-mycorrhizal associations that trees perform better when grown in their local soil biota (Manzanedo et al., 2018, Pickles et al., 2015).

In accordance with the local adaptation hypothesis, trees growing outside their native ranges are likely to be maladapted to their environment which may result in reduced growth and fitness (Savolainen et al., 2007). Abiotic stress, in particular water stress, often leads to changes in plant physiology such as reduced carbon pools, shoot biomass, stomatal densities etc. (McDowell et al., 2008, Elad and Pertot, 2014) making these trees a less suitable or less easily accessed habitat for biotrophic endophytes. Results from this study show that the richness and diversity of fungal endophytes associated with *Juglans nigra* were higher on trees with a smaller DBH. This result supports the plant stress hypothesis, which states that plants experiencing stress result in increased abundance of pests and pathogens due to reduced chemical defences and increased nutritional quality (White, 1984, Koricheva et al., 1998). The plant stress hypothesis was proposed for insect herbivores but could be applied to endophytes as they share a similar resource. DBH also effected the composition of both bacterial and fungal endophytes in common and black walnut species.

Budburst date in walnut species is partly under genotypic control, trees from different provenances i.e. from different latitudes and altitudes, are likely therefore to exhibit variations in their budburst phenology (Charrier et al., 2011). Differences in phenology may create a mismatch between endophyte dispersal and the optimal stage of plant growth for endophyte colonisation for example. Phenological synchrony between host and endophyte has received little attention, but inferences can be made from host-pathogen and host-pest interactions in the literature (Dantec et al., 2015, Desprez-Loustau et al., 2010, van Asch and Visser, 2007). Phenological synchrony would be particularly important for endophytes with an obligate biotrophic lifestyle with a limited host range and less important for endophytes with a broad

host range, and those that that can switch to a saprophytic lifestyle (Desprez-Loustau et al., 2010). Budburst date was shown here to influence the richness and diversity of fungal endophytes associated with common walnut trees. Common walnut trees that budburst earlier in the season had a higher richness and diversity of fungal endophytes. Until the date of sample collection, the trees that budburst earlier had more time to accumulate fungal endophytes from the environment.

The richness, diversity and community composition of bacterial and fungal endophytes were consistently different between tissue types. Differences in tissue organ colonisation have also been shown in other studies (Sieber, 1989, Ragazzi et al., 2001, Leff et al., 2015, see also *Chapter 3*). In general, twigs hosted a higher richness and higher diversity of endophytes than leaves. This is consistent with the longevity of these organs; twigs are longer living elements and may therefore accumulate a higher richness and diversity of endophytes, leaves of *Juglans* are seasonal and therefore have a narrower window of opportunity for endophytic colonisation (Leff et al., 2015).

Results from this study show that *Juglans regia* and *Juglans nigra* host a very different assemblage of endophytic fungal and bacterial organisms. Different endophytic communities have been found in other studies between closely related tree species growing in the same location (Morrica et al., 2012, Arnold et al., 2000), and was also shown in *Chapter 3* for two *Quercus* species in Paradise Wood. These results suggest that tree species act as ecological filters of endophytes in the surrounding environment. These differences in community composition are a result of differences between tree species, for example in phenology, vigour or differences in plant physical or chemical defences. Differences in phenolic compounds have been recorded in *Juglans regia* and *J. nigra*. *Juglans nigra* contains higher levels of hydrojuglone glucoside, the precursor to juglone, than *J. regia* (Burtin et al., 1998). Juglone has been shown to have antimicrobial properties so is likely to influence the endophyte

composition, as discussed later. *Juglans regia*, on the other hand, contain higher concentrations of methyl salicylate (Farag, 2008), a volatile compound involved in plant defence (Dicke and Hilker, 2003) which is also likely to impact on the colonisation potential of endophytes. The concentrations of phenolic compounds were not measured here but should be considered in future endophyte studies.

A number of latent pathogens were identified in the asymptomatic walnut tissues sampled in the 'walnut nitrogen trial'. *Ophiognomonia leptostyla*, is a fungal pathogen which causes numerous brown spots on the leaves, shoots, twigs and fruits of walnut, more commonly known as walnut anthracnose (Strouts and Winter, 2000). This fungus has been associated with significant economic damage in *Juglans regia* and *J. nigra* worldwide (Belisario et al., 2008). OTU_F1333 matched with 99.39% identity to *Ophiognomonia leptostyla*, using the GenBank (NCBI) database. In the literature, *Ophiognomonia leptostyla* is known to cause disease in both *Juglans regia* and *J. nigra*, however, in this study, it was found associated with only *Juglans nigra*, at a higher relative abundance in the leaves than in the twigs. Using culture dependent methods, this species has not previously been isolated from asymptomatic tissue (Pardatscher and Schweigkofler, 2009). It is believed that this is the first report of *Ophiognomonia leptostyla* in asymptomatic walnut tissues, but many other closely related species fungal species, such as *O. bugabensis* or *O. cryptica*, have been considered endophytes in other tree species (Walker et al., 2012). It is also closely related to *Apiognomonia errabunda* (Sogonov et al., 2008) which causes anthracnose of *Quercus* species and which has also been found as an endophyte in asymptomatic *Quercus* tissues (*Chapter 3*).

OTU_B1288 matched to the *Xanthomonadaceae* family, most likely to be *Xanthomonas arboricola* pv. *juglandis* (*Xaj*) the causal agent of walnut blight. In this study, this bacterium was found at the highest abundance in *Juglans regia* twigs. It was also found in high abundance in asymptomatic *Juglans regia* in other studies (Pardatscher and Schweigkofler,

2009, Giovanardi et al., 2015). *Fusarium* sp. (OTU_F141) and *Alternaria* sp. (OTU_F1560) were also found in the walnut trees in Paradise Wood and are believed to be opportunistic pathogens that interact with *Xaj* to cause brown apical necrosis (BAN) (Moragrega and Özaktan, 2010, Belisario et al., 2002).

Results of the metabarcoding showed two OTUs (OTU_B1427 and OTU_B1435) that were assigned to *Brenneria* species. OTU_B1427 is thought to be *Brenneria rubrifaciens* as it closely matched the *B. rubrifaciens* sample that was included as a control in the sequencing run. The *Brenneria nigrifluens* sample that was included did not successfully sequence so it was not possible to match this to any of the resulting OTUs. *Brenneria rubrifaciens* causes deep bark canker of *Juglans* species, although it has been suggested to also have an endophytic life stage (Teviotdale et al., 1991, Thapa et al., 2010, McClean et al., 2008) this species has not been isolated from asymptomatic tissue to date. However, most studies of walnut endophytes in the literature have used culture-dependent techniques. Endophytes at low concentrations are difficult to detect by plating and *Brenneria* species have been known to be particularly difficult to isolate through culturing (Maes et al., 2009). The use of culture-independent analyses reduces the risk of false negative detections from unculturable stages or outcompeted growth of *Brenneria* species.

As 16S sequencing of members of the *Enterobacteriaceae* family is known to be problematic due to the highly conserved 16S rRNA gene (Janda and Abbott, 2007, Naum et al., 2008), the presence of *Brenneria rubrifaciens* and *B. nigrifluens* was tested using species specific primers. The primers used in this study were unable to detect either bacterial species in the walnut samples from Paradise Wood. However, the primers used to detect *Brenneria rubrifaciens* have only been used to detect the bacteria in artificially inoculated ($1x10^6$ CFU/ml) walnut branches (Thapa et al., 2010). If *Brenneria rubrifaciens* is found at as endophyte, the concentration of bacterial cells will be considerably lower, and potentially undetectable using

these primers. More research is therefore necessary to establish whether *Brenneria rubrifaciens* is found as an endophyte in asymptomatic walnut tissue in the UK. Developing a quantitative PCR (qPCR) method could help to detect low abundant endophytic species such as *Brenneria rubrifaciens* in asymptomatic tissue (Tellenbach et al., 2010).

OTU_B1435 matched to *Brenneria goodwinii* that was included on the sequencing run as part of *Chapter 3* in this project. *Brenneria goodwinii* is associated with a bleeding canker of *Quercus* trees and has not yet been found to be associated with any other tree species. It is possible, just as *Brenneria rubrifaciens* above, that this is an incorrect assignment of species due to the conserved nature of the 16S rRNA region of *Enterobacteriaceae*. On the other hand, *Brenneria* species have been known to reside as true endophytes i.e. as a commensalist or mutualist, in tree species where they are unable to induce disease. For example, *Brenneria salicis* is found as an endophyte in willow, poplar and alder but only willow sap can support high enough densities of *B. salicis* to induce disease symptoms (Maes et al., 2009, Huvenne et al., 2009).

Thousand canker disease (TCD) is an emerging disease of *Juglans* in the United States, it is caused by the fungal pathogen *Geosmithia morbida* and is transmitted by the walnut twig beetle, *Pityophthorus juglandis* (Kolarik et al., 2011, Daniels et al., 2016). A recent discovery of TCD in Italy suggests this disease has the potential to spread to the UK and should be monitored (Department for Environment Food and Rural Affairs, 2019). This disease is often difficult to diagnose due to the absence of noticeable symptoms on the bark surface (Daniels et al., 2016). Reassuringly, this fungal pathogen was not found in the walnut trees in Paradise wood based on the Illumina sequencing, however, it may be more appropriate to perform a targeted detection using species specific primers (Oren et al., 2018).

Endophytic bacteria have been shown to have plant-growth promoting activity; this could be through nitrogen fixation (Oses et al., 2018, Anand et al., 2013), through the production of

phytohormones and/or by providing enzymes necessary for plant growth regulator metabolism (Taghavi et al., 2009, Madmony et al., 2005). In this study, three OTUs: OTU_B1282, OTU_B1117 and OTU_B1271 were assigned to the genus *Bacillus*. *Bacillus* species are well-known for their plant-growth promoting ability (Paz et al., 2012, Falcao et al., 2014, Lopes et al., 2018) and biological control potential (Ren et al., 2013). *Bacillus* strains have been isolated from *Juglans* species in high abundance in other studies also (Pardatscher and Schweigkofler, 2009, Ghorbani et al., 2018). In *vivo*, *Bacillus* strains isolated from *Juglans regia* have been shown to produce the phytohormones, indole-3-acetic acid (IAA) and gibberellic acid (GA) (Ghorbani et al., 2018). However, in this study it was found that trees that possessed a higher abundance of the three OTUs assigned to *Bacillus* sp. were less vigorous i.e. had a smaller DBH. It is possible that other factors are involved in this interaction, and more study is needed to establish whether *Bacillus* species found in *Juglans* trees have plant growth promoting potential. Ghorbani et al. (2018) also found that one *Bacillus* strain had the ability, *in vivo*, to inhibit growth of the bacterial pathogen *Brenneria nigrifluens*. Again, more study is needed to establish whether *Bacillus* species could be used as biocontrol agents against the causal agent of shallow bark canker of walnut (*Brenneria nigrifluens*).

Endophytic yeasts, *Vishniacozyma* species, were found at a high abundance on almost all walnut species and in both tissue types, this yeast has also been found at high abundance in *Juglans* species in other studies (LaBonte et al., 2018). One species in particular, *Vishniacozyma victoriae* was associated with more vigorous i.e. higher DBH, black walnut trees in Paradise Wood. Endophytic yeast species may play an important role in plant growth promotion by producing auxins e.g. IAA (Nutaratat et al., 2014) but more research is needed to establish the relationship between these yeast species and walnut growth. With further research, these potentially plant-growth promoting species could be harnessed to encourage growth of *Juglans* species in the UK.

The compound juglone found in walnut species is thought to have both antibacterial (Dawson and Seymour, 1983, Pereira et al., 2007) and antifungal (Hedin et al., 1979, Cline and Neely, 1983) properties. However, as this study and others have shown, walnut species possess a number of fungal and bacterial species in their plant tissues. It is possible that the endophytes found in walnut tissues are adapted to overcome the antagonistic activity of juglone. If this is the case, it might be expected that walnut endophytes would be host specific. Results from this study show that trees of the genus *Juglans* have a significantly different composition of fungal and bacterial endophytes than the genus *Quercus* growing in the same locality. 27.7% of bacterial OTUs and 30.2% of fungal OTUs were shared between the two tree genera, the majority are therefore thought to be genus or species specific. However, many of the endophyte species found here e.g. *Taphrina* sp. and *Cladosporium* sp. have also been isolated from other woody plant species (LaBonte et al., 2018) (see also *Chapter 3*). Other studies have also shown that the majority of microbes associated with walnuts are not host specific (Pardatscher and Schweigkofler, 2009). There has been some debate in the literature as to whether free juglone is actually present in walnut tissues (Daglish, 1950, Hedin et al., 1980, Hedin et al., 1979, Cline and Neely, 1983). It is thought instead, that the potentially non-toxic, precursor hydrojuglone glucoside is present in walnut tissues and through wounding or invasion by an organism this compound is oxidised to form the active compound juglone (Daglish, 1950). It is possible; therefore, that endophyte colonisation may not be sufficient to activate the biochemical pathway necessary for juglone production. Theoretically, if endophyte growth reaches a threshold, if environmental changes occur or if the tree is attacked by other pathogens or pests, this juglone pathway may then be activated.

4.5. *Conclusions*

Walnut trees introduced into the UK are a habitat for a wide variety of bacterial and fungal endophytes. Differences in endophyte richness and diversity are shown here between the two species of walnut: *Juglans regia* and *Juglans nigra*, between tissue types (leaves and twigs), between trees from different provenances and between trees showing different vigour. Intergeneric differences in fungal and bacterial endophyte composition also existed between *Juglans* and *Quercus*. Some of the endophytes found here pose a potential threat to plant health and must be considered if *Juglans* species are to be cultivated more widely for timber production in the UK. More research should be allocated to understanding the effect of tree provenance on endophyte community as endophytes have the potential to determine the success of *Juglans* species in the UK, tree provenance should be carefully selected.

CHAPTER 5 - *Evaluating the interactions between leaf endophytic and insect herbivore communities in UK native Quercus species*

5.1. *Introduction*

Trees are not discrete units but are host to a wide array of endophytic fungi and bacteria (Wilson, 1993). Endophytes exist, for at least part of their lifecycle, within internal tissues of their host without causing symptoms of disease (Wilson, 1995a) and they have been isolated from virtually every tree species studied to date (Strobel, 2018). Endophytes have been shown to confer benefits to the host such as heat (Redman et al., 2002) and drought tolerance (Waller et al., 2005, Khan et al., 2016) and resistance against pathogens (Arnold et al., 2003, Waller et al., 2005, Ganley et al., 2008, Ren et al., 2013). These endophytic microbes are also likely to influence the interactions between insect herbivores and their shared host plant.

Due to the agronomic importance of grass species, the endophytes associated with this group have been intensively studied (Johnson et al., 2013, Kuldau and Bacon, 2008, Vikuk et al., 2019) and have been found to provide protection against insect herbivores (Saikkonen et al., 2010). Herbivore resistance results mainly from the production of secondary metabolites such as toxic alkaloids by the endophyte (Clay, 1988). These defensive mutualistic relationships are thought to be most common in systemic, vertically transmitted endophytes, such as those dominant in grass species (Saikkonen et al., 2010). These seed transmitted endophytic species are more likely to have reduced virulence and therefore a higher propensity towards a mutualistic relationship (Saikkonen et al., 2004). On the other hand, the endophytes associated with woody plants contain many unspecialised endophytes, such as latent pathogens, saprophytes and entomopathogens (Schulz and Boyle, 2005). Mutualistic relationships are predicted to be less common in these horizontally transmitted endophytes,

that have limited growth within the plant and a broad host range (Saikkonen et al., 2010, Faeth, 2002). Compared to the graminaceous endophytes, the tripartite relationship between endophyte, insect and host is less well characterised in woody plants, especially in broad leaved trees (Eberl et al., 2019). However, since herbivorous insects are certain to encounter one (if not many) endophytic species when feeding on tree tissues, these three entities are likely to interact. Results from studies that have addressed the relationship are, however, inconsistent (Suryanarayanan, 2013), with some reporting negative interactions (Butin, 1992, Faeth and Hammon, 1997a, Wilson, 1995b, Gaylord et al., 1996, van Bael et al., 2009, Gange et al., 2012, Gange et al., 2019), others neutral (Faeth and Hammon, 1997a, Lappalainen and Helander, 1997, Saikkonen et al., 2006) and others positive (Gange, 1996, Gaylord et al., 1996, Preszler et al., 1996, Gange et al., 2012).

Endophytes can affect insects directly through the production of alkaloids (Miller, 1986, Zhang et al., 2006, Nisa et al., 2015, Gurulingappa et al., 2011, Schulz et al., 2002) or indirectly i.e. by altering the host plant. The fungal leaf endophyte, *Discula quercina*, is thought to grow into the galls produced by a cynipid wasp from the surrounding leaf of *Quercus garryana*. The endophyte subsequently cuts off the nutrient supply from the leaf to the gall, resulting in insect starvation (Wilson, 1995b). Endophytes may slow the development of sedentary insects, like leaf miners, so that they are unable to complete development before leaf abscission at the end of the season (Faeth and Hammon, 1997a). Other studies have shown endophytes to act as nutrient absorbing sinks, drawing nutrients away from insect herbivores (Smith, 1985) or by changing the leaf nutrient quality (Hatcher, 1995). Endophytes can also induce host plant chemical defences (Karban et al., 1987) or alter the production of volatiles that influence the behaviour of herbivore natural enemies, such as parasitoids (Preszler et al., 1996).

Hammon and Faeth (1992) suggest that endophyte colonisation may influence the timing of insect herbivore feeding. As the richness and diversity of endophytes (Scholtysik et al., 2012)

and the toxins they produce (Wilson and Carroll, 1994) build up during the growing season, early season feeding may be an advantage. Over evolutionary time, late season specialist feeders may adapt to the toxic compounds produced by the endophytes and therefore may prefer endophyte infected leaves (Hammon and Faeth, 1992).

It is equally possible that insect herbivores may facilitate endophyte infection of a plant host by creating wounds in the plant tissue (Faeth and Hammon, 1996). It has also been suggested that insect herbivores may be involved in the dissemination of endophytic species and therefore a defensive mutualistic relationship would unlikely be selected for (Faeth, 2002, Devarajan and Suryanarayanan, 2006, Raman et al., 2012).

The inconsistencies in the literature exploring the effects of endophyte presence on insect herbivore success may be due to varying experimental design (Gange et al., 2019) but may also be due to environmental conditions. The richness and diversity of endophyte communities are known to be affected by biotic and abiotic conditions such as: host species (Morrica et al., 2012, Redford et al., 2010, Whipps et al., 2008, Lambais et al., 2006), host genotype (Balint et al., 2013, Todd, 1988, Cordier et al., 2012a, Bodenhausen et al., 2014), plant organ (Sieber, 1989, Ragazzi et al., 2001), tree or tissue age (Hata et al., 2011), tree health (Giordano et al., 2009, Morrica et al., 2012), climate (Terhonen et al., 2011, Millberg et al., 2015, U'Ren et al., 2012, Zimmerman and Vitousek, 2012, Cordier et al., 2012b) and season (Shen and Fulthorpe, 2015, Borruso et al., 2018). It is therefore difficult to say with certainty that the endophyte presence alone affects the success of insect herbivores, if these confounding factors are not also considered. The nature of the interaction between endophyte and insect is also likely to differ based on the feeding behaviour of the insect (Gange et al., 2019). Generalist insects often respond negatively to changes in host chemistry, whilst specialist insects react positively (Schoonhoven et al., 2005). Sedentary insects that feed internally, such as leaf miners and

gallers, are likely to be more sensitive to endophyte presence as they are less able to avoid the direct or indirect effects of the endophyte (Butin, 1992, Faeth and Hammon, 1996).

The majority of the literature reviewed above concerns the interactions between fungal endophytes and their host plant, research regarding the influence of bacterial endophytes is severely lacking. In addition, most studies to date have considered the effect of a single species of endophyte on a single insect species; however, in the natural environment insects are likely to ingest plant material containing an array of endophytic species. The recent development of culture independent techniques should allow for a more in-depth study of the endophytes associated with tree species, in particular for bacterial species. However, no known studies have as yet used culture-independent metabarcoding to establish the endophyte community of tree species compared to the insect herbivore community. Using an oak provenance trial, this study considers the influence of the fungal and bacterial endophytic community composition of UK native *Quercus* species, on the abundance of insect herbivores that share the plant host. Plant traits such as tree species, tree vigour and budburst phenology will also be considered as contributing factors to the endophyte-host-insect relationships. The following hypotheses will be tested:

Hypothesis 1: trees with a similar insect community are likely also to have a similar endophytic community i.e. the same aspects of tree phenology act as ecological filters for insect herbivores as they do for endophytes

Hypothesis 2: the galling and mining insect guilds would correlate more strongly with differences in endophyte communities due to their sedentary nature compared to free feeders

Hypothesis 3: there would be few mutualistic relationships between endophytes and *Quercus* trees due to the lifestyles of tree endophytes i.e. horizontally transmitted, limited growth within the tree and a broad host range

5.2. *Materials and methods*

5.2.1. *Site description and tree selection*

The oak provenance trial (BSO trial) in Paradise Wood, Oxfordshire was selected for insect and endophyte analysis. For full details of the trial and trial design see *Chapters 2 & 3*. Nineteen oak families belonging to seven provenances were selected for analysis across three blocks (replicates). *Quercus* species was determined using leaf morphometric measurements, the full details of which are described in *Chapter 2*. Of the 19 families selected, 10 were assigned to *Quercus robur* and nine to *Q. petraea*. Two trees (22_18_HAM012 and 35_56_HAM013) were removed from analysis as their species assignment was not consisted with the rest of their family; 55 trees remain for analysis.

5.2.2. *Measured tree traits*

The timing of budburst of the *Quercus* trees was recorded in spring 2016 (see *Chapter 2, Section 2.2.4.1*) and leaf retention (also known as leaf marcescence) in autumn 2016 (see *Chapter 2, Section 2.2.4.2*). DBH and average shoot length of 10 shoots per tree were used to gauge tree vigour (see *Chapter 2, Section 2.2.3*).

5.2.3. *Insect assessment*

Insect herbivores were recorded from 10 shoots, randomly selected from each of the 55 trees in spring and autumn 2016 (see *Chapter 2, Section 2.2.5*). To find correlations between the endophyte community and the insect community all insect species and groups were

considered (see *Chapter 2, Section 2.3.4).* For pairwise interactions between individual endophyte species and insect herbivores the insects were grouped into the following feeding guilds: spring gallers, autumn gallers, leaf webbers, leaf rollers, leaf chewers and leaf miners. The gallers, miners and leaf rollers were expressed as abundance per 10 shoots per tree. Leaf webbers and chewers were presented as average percentage damage per leaf. Eight of the most abundant insect species (or genera) were also included separately in the analysis: *Neuroterus albipes* (agamic generation), *N. anthracinus* (agamic generation), *N. quercusbaccarum* (agamic generation), *Macrodiplosis pustularis*, *N. numismalis* (agamic generation), *Trioza remota*, *Phyllonorycter* species and *Coleophora* species.

5.2.4. *Endophyte assessment*

The endophytes and insects recorded here were not from the same leaves, which allowed for the study of correlations between the endophytic and insect communities that trees select for but reduces the likelihood for studying direct interactions. Four insect and disease-free leaves were removed from each tree, they were surface sterilised, pooled together and the total DNA was extracted (see *Chapter 3, Section 3.3.1*). The Illumina Miseq platform was used to sequence endophytic DNA, using the V5-V6 16S region for bacterial species and the ITS2 region for fungal species (see *Chapter 3, Section 3.3.2*). Bioinformatic analysis was carried out by Dr Soon Gweon, University of Reading using the PIPITS pipeline (Gweon et al., 2015) for fungi and for 16S the pipeline outlined in *Chapter 3*, *Section 3.3.3.* The original OTU table contained twig samples used in another part of this study (see *Chapter 3*), the OTU table was therefore subsampled to only include the leaf samples for this Chapter. The OTU table was rarefied to the lowest sequencing depth of 33126 for ITS and 147 for 16S using the vegan package (version 2.5-3, Oksanen et al. (2018)) in R to avoid biases resulting from differences in sample size (See *Chapter 3, Section 3.3.5* for more details on rarefaction).

5.2.5. *Statistical analysis*

Species richness of the insects, bacterial endophytes and fungal endophytes were recorded as counts i.e. the total number of different species present in each tree. Species diversity was calculated using the Shannon-Wiener index in the vegan package (version 2.5-3, Oksanen et al. (2018)). Pairwise correlations between insect richness and bacterial endophyte richness and between insect richness and fungal endophyte richness were calculated using Pearson product-moment correlations. The same correlations were computed for the Shannon-Wiener diversity indices. The dissimilarities in the OTU composition between the leaf samples were calculated using the Bray-Curtis dissimilarity index for bacterial and fungal endophytes separately, and also for insect herbivore species. The fungal dissimilarity matrix was compared to the insect dissimilarity matrix using a *mantel* test in the *vegan* package. The same was computed for bacterial endophytes and insect herbivores.

The correlation between endophyte community and insect abundance was calculated using the *Adonis* function in the *vegan* package, with OTU dissimilarity as the response variable and each insect guild or dominant insect species abundance as the explanatory variable with 999 permutations. Measures of tree vigour (DBH and shoot length), tree species (*Quercus robur* or *Q. petraea*), tree provenance (latitude and longitude), tree phenology (budburst date) and marcescence habit (leaves retained or abscised in autumn) were also included in the model. Block number was also included as a strata.

The abundance of the 10 most dominant fungal and bacterial OTUs were tested for their correlation with the abundance of the most dominant insect groups/taxa. Generalised linear mixed effect models were computed using the *glmmTMB* package with negative binomial error distributions. Fixed variables were the same as above. To account for similarities

between families within each provenance, family was included as a random factor nested within provenance. Trial block number was also included as a random factor.

The rarefied fungal OTU table contained members of the Hypocreales order, these are of interest here as they are potentially entomopathogenic fungi (Vega, 2018). Using the same GLMM models as above, the three most dominant entomopathogenic fungal endophytes were also tested against the most dominant insect groups/taxa.

Table 5.1 - (1) 6 insect groupings and the 7 most abundant insect taxa (2) 10 most dominant bacterial leaf endophytes and (3) 10 most dominant fungal leaf endophytes and (4) 3 most abundant potential entomopathogens found in the *Quercus* trees

Insect guild or species	Bacterial OTUs	Fungal OTUs	Entomopathogens
Spring gallers	**OTU_B682** Rhizobiales	**OTU_F890** *Erysiphe* sp.	**OTU_F923** *Trichoderma viride*
Autumn gallers	**OTU_B467** Rhizobiales	**OTU_F2984** *Ramularia endophylla*	**OTU_F1265** *Fusarium* sp.
Leaf miners	**OTU_B1684** Rhizobiales	**OTU_F3047** *Ramularia endophylla*	**OTU_F985** *Trichoderma* sp.
Leaf chewers	**OTU_B2259** Rhizobiales	**OTU_F2902** *Ramularia endophylla*	
Leaf webbers	**OTU_B522** *Ralstonia* sp.	**OTU_F528** *Vishniacozyma* sp.	
Leaf rollers	**OTU_B402** Actinomycetales	**OTU_F2406** *Cladosporium* sp.	
Neuroterus albipes (agamic)	**OTU_B675** Myxococcales	**OTU_F577** *Exobasidium bisporum*	
Neuroterus anthracinus (agamic)	**OTU_B2439** *Hymenobacter* sp.	**OTU_F1760** Unknown fungus	
Neuroterus quercusbaccarum (agamic)	**OTU_B2550** *Hymenobacter* sp.	**OTU_F2725** Unknown fungus	
Macrodiplosis pustularis	**OTU_B2738** Unknown bacterium	**OTU_F1433** Unknown fungus	
Neuroterus numismalis (agamic)			
Trioza remota			
***Phyllonorycter* spp.**			
***Coleophora* spp.**			

5.3. *Results*

There was no significant positive or negative correlation between insect species richness and bacterial or fungal OTU richness among the *Quercus* trees and neither was there any correlation between insect diversity and bacterial or fungal OTU diversity.

A total of 1976 OTUs for fungi (ITS) and 516 OTUs for bacteria (16S) were used, after rarefaction, to generate the Bray-Curtis dissimilarity indices. Results from the *mantel* test found no positive or negative correlation between the bacterial endophyte community and insect herbivore community and neither was there any correlation between the fungal endophyte community and insect herbivore community.

31 fungal OTUs were assigned to the order Hypocreales, which contains the entomopathogenic fungi. The top three most dominant of these were tested for the effects of fungal abundance on the abundance of insect herbivores using a GLMM, the results of which are found in *Table 5.2* and *Figure 5.1*. Similarly, the correlations of the ten individually most dominant bacterial and fungal endophytes with insect herbivore abundance were tested (*Table 5.2*, *Figure 5.1 & 5.2*).

Table 5.2 – results of the *Adonis* model comparing the fungal or bacterial endophyte Bray-Curtis dissimilarity matrix with the abundance of insect groups and taxa. Other tree variables were also included in the model.

Insect guild or species	Significant variables	F	p
Spring gallers	Fungal community	1.84	0.06
	Species	2.66	<0.01
	Latitude	0.95	<0.05
Autumn gallers	No significant variables		
Miners	No significant variables		
Leaf rollers	**Fungal community**	**2.23**	**<0.05**
	Species	2.33	<0.01
	Latitude	1.80	<0.05
Leaf chewers	No significant variables		
Leaf webbers	**Bacterial community**	**2.36**	**<0.01**
	Species	2.99	<0.001
Neuroterus albipes (agamic) Galling insect	**Bacterial community**	1.62	<0.05
	Species	2.83	<0.001
Neuroterus anthracinus (agamic) Galling insect	**Fungal community**	**2.13**	**<0.01**
	Species	2.62	<0.01
	Latitude	1.84	<0.05
Neuroterus quercusbaccarum (agamic) Galling insect	No significant variables		
Macrodiplosis pustularis Galling insect	No significant variables		
Neuroterus numismalis (agamic) Galling insect	No significant variables		
Trioza remota Galling insect	No significant variables		
Phyllonorycter **spp.** *Mining insect*	No significant variables		
Coleophora **spp.** *Mining insect*	No significant variables		

Table 5.3 – results of the GLMM model assessing the influence of bacterial and fungal OTUs (and other tree variables) on the abundance of the most dominant insect guilds and species.

Insect group/taxa	Significant variables	df	Wald X^2	p	Direction of effect
Spring gallers	**OTU_F2984**	**5**	**4.69**	**<0.05**	**Positive**
	OTU_F1265	**6**	**4.52**	**<0.05**	**Negative**
	DBH	6	4.43	<0.05	Negative
Autumn gallers	**OTU_F577**	**5**	**3.96**	**<0.05**	**Negative**
Miners	**OTU_F577**	**6**	**6.12**	**<0.05**	**Positive**
	Latitude	6	6.24	<0.05	Negative
Leaf rollers	**OTU_B2439**	**5**	**4.34**	**<0.05**	**Positive**
	OTU_B2738	**5**	**4.47**	**<0.05**	**Positive**
Leaf chewers	No significant OTUs				
Leaf webbers	No significant OTUs				
Neuroterus albipes (agamic)	**OTU_F3047**	**6**	**5.14**	**<0.05**	**Positive**
	Oak species	6	13.68	<0.001	*Quercus robur* > *Q. petraea*
Neuroterus anthracinus (agamic) Galling insect	**OTU_F528**	**5**	**4.82**	**<0.05**	**Positive**
	OTU_F890	**5**	**3.90**	**<0.05**	**Negative**
	OTU_F2984	**5**	**6.10**	**<0.05**	**Negative**
Neuroterus quercusbaccarum (agamic) Galling insect	**OTU_B2259**	**5**	**4.79**	**<0.05**	**Negative**
Macrodiplosis pustularis Galling insect	**OTU_B2259**	**5**	**3.82**	**<0.05**	**Positive**
Neuroterus numismalis (agamic) Galling insect	**OTU_F577**	**7**	**5.81**	**<0.05**	**Negative**
	Oak species	7	7.24	<0.01	*Quercus robur* > *Q. petraea*
	Budburst	7	14.45	<0.001	Positive
Trioza remota Galling insect	No significant OTUs				
***Phyllonorycter* spp.** Mining insect	**OTU_F577**	**5**	**7.30**	**<0.01**	**Positive**
***Coleophora* spp.** Mining insect	No significant OTUs				

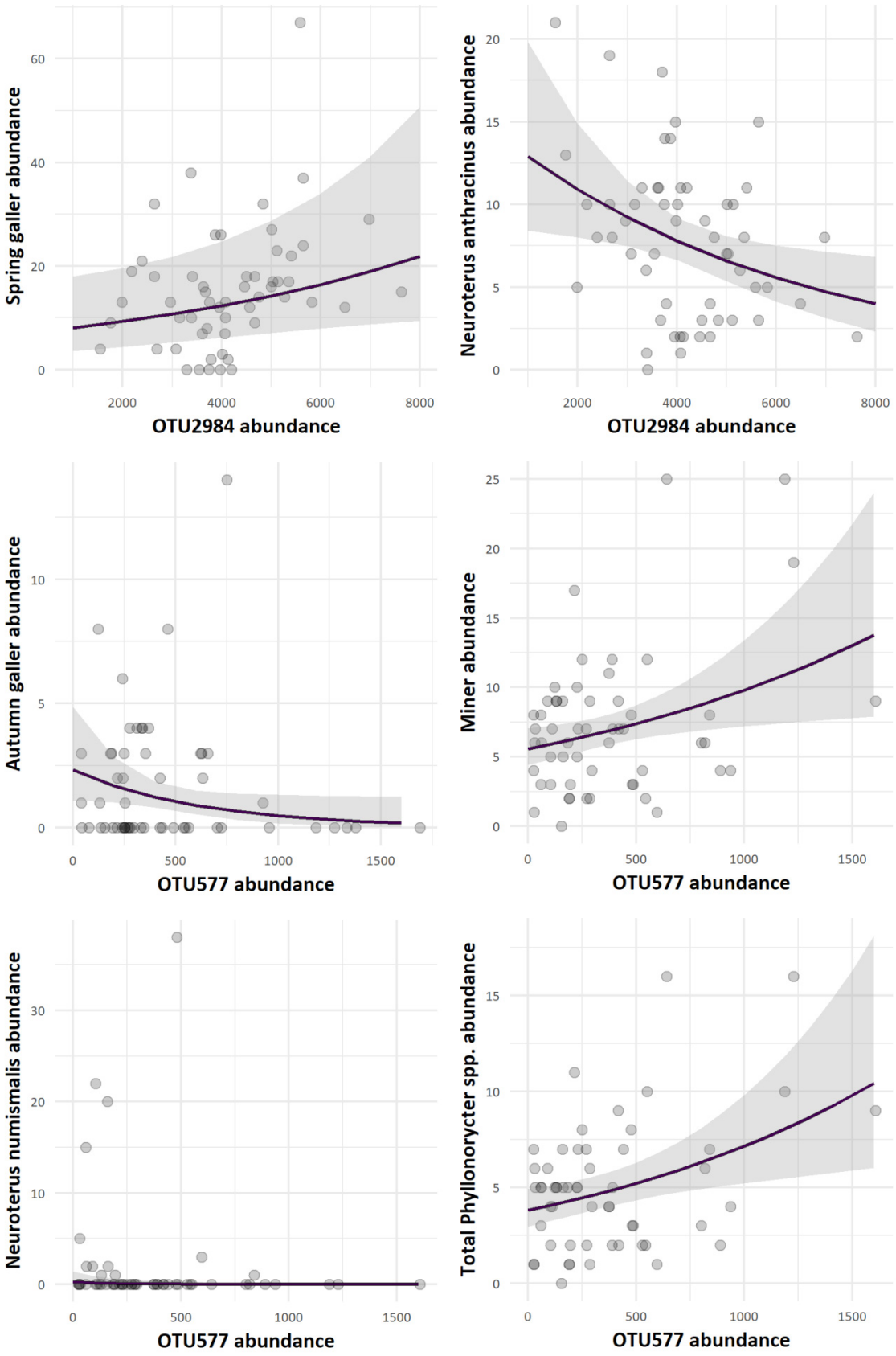

Figure 5.1 – results of the GLMM models, showing the insect herbivores that were influenced by the presence of fungal endophytes

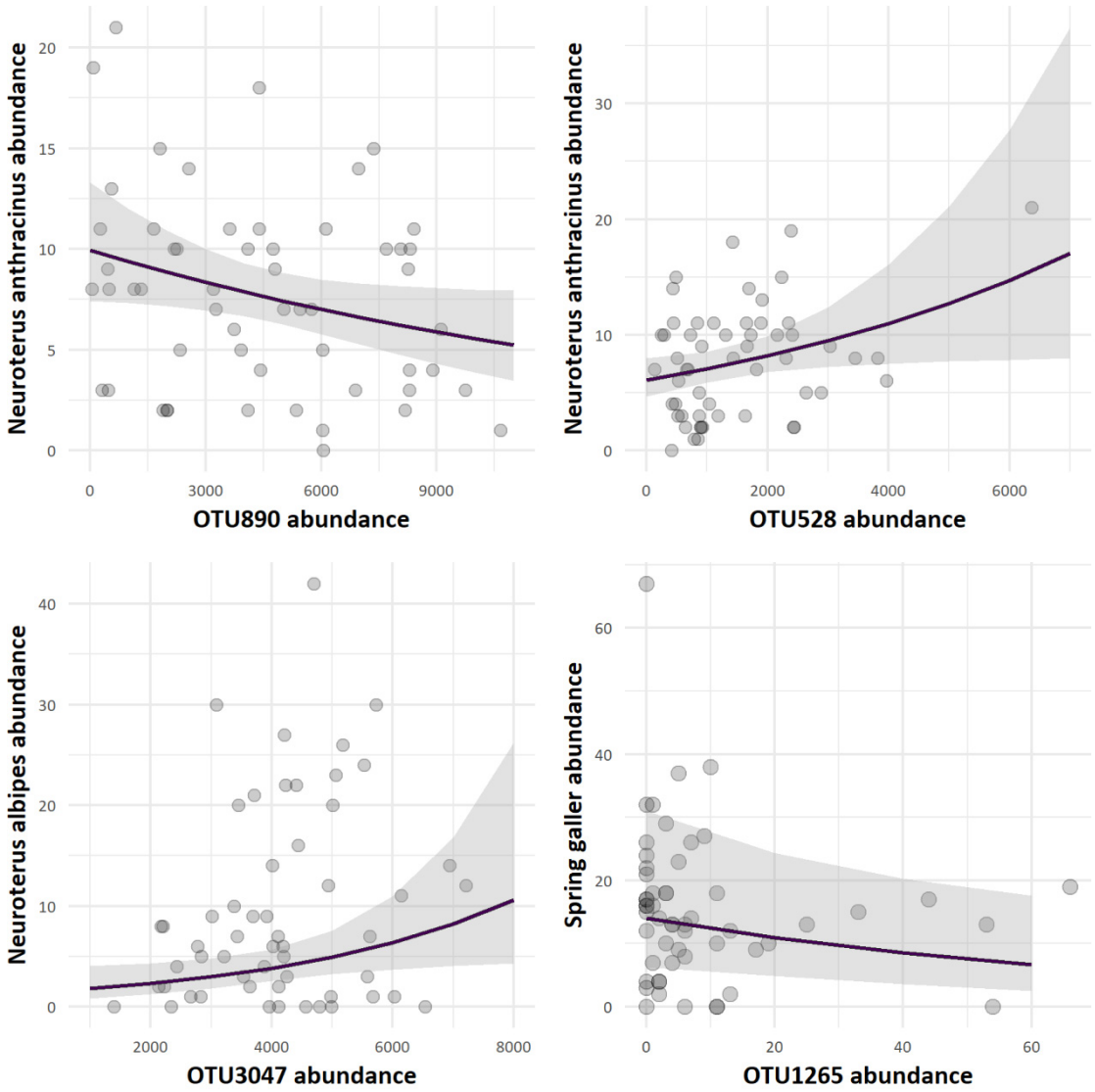

Figure 5.1 cont. – results of the GLMM models, showing the insect herbivores that were influenced by the presence of fungal endophytes

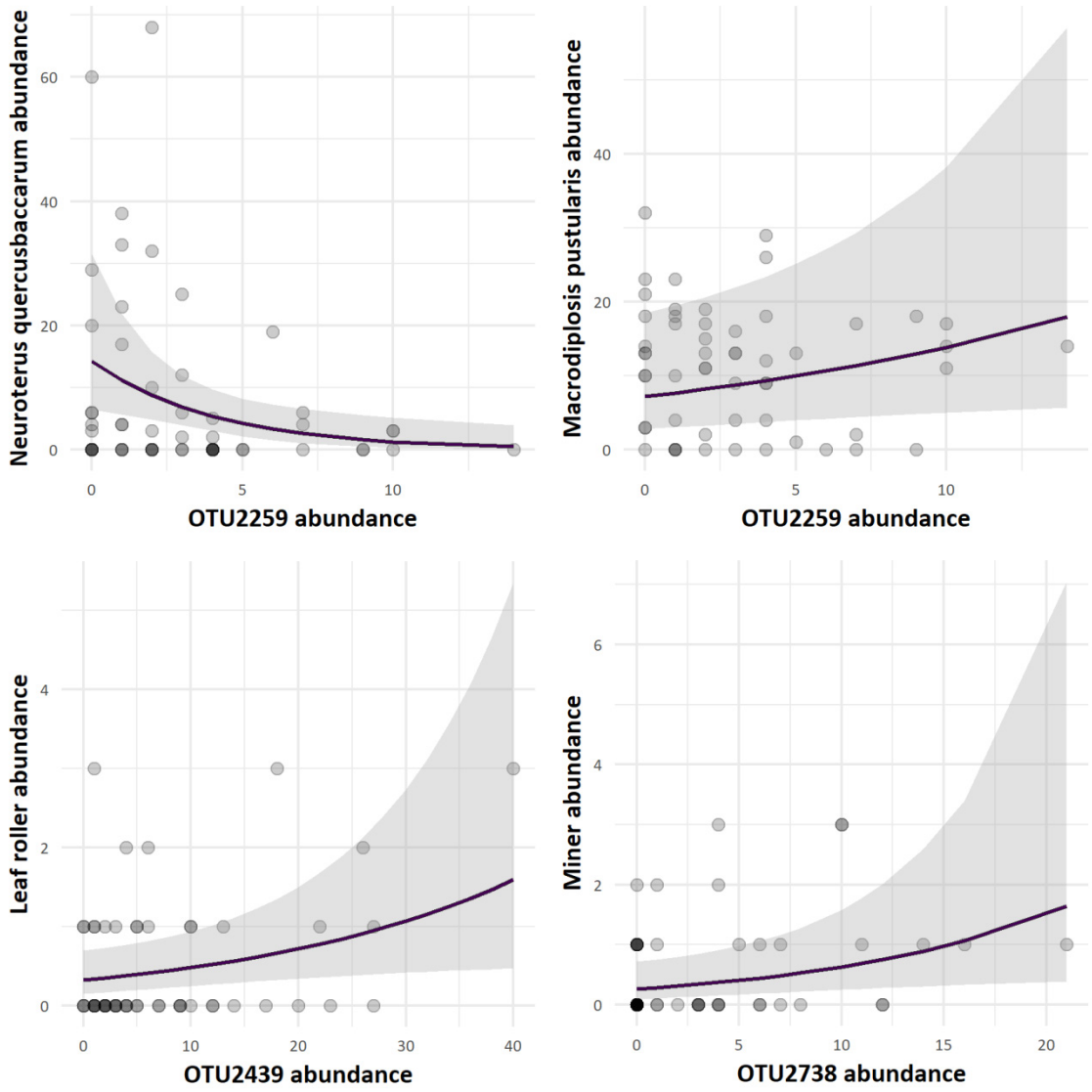

Figure 5.2 - results of the GLMM models, showing the insect herbivores that were influenced by the presence of bacterial endophytes

5.4. *Discussion*

Endophytes have long been considered plant mutualists that potentially play a role in mediating plant-insect interactions. However, most studies have considered the so called 'true endophytes' that are vertically transmitted in grass species (Clay, 1990, Clay, 1988, Saikkonen et al., 2010). Studies of endophyte-insect relationships in woody plants have shown a continuum of responses from mutualistic to antagonistic (Gange et al., 2019, Faeth and Hammon, 1997a, Gange et al., 2012), and this study is no exception. Endophytes associated

with *Quercus* species in this study correlated with the abundance of many insect herbivores, but the observed insect response to individual endophytic taxa was varied.

It might be expected that aspects of the tree phenotype, for example vigour and phenology, that act as ecological filters for insect herbivores may also affect the endophytic community in similar ways. In this study, this is termed *passive interaction,* where host plant characteristics (phenotype) that make a tree suitable to the insect herbivores also favour endophytes (positive correlation) or make the internal environment unsuitable for endophytes (negative correlation). For *passive interactions* to occur it could be the same phenotypic traits acting on the different communities or different traits that correlate with each other. The second mechanism is termed *active interaction* where either the endophyte or insect affects the abundance of the other. This can be further classed into *direct interactions* where one organism effects the other without any influence from the plant itself, or *indirect interactions* where either herbivore attack or the presence of the endophyte changes tree phenotypic traits that effect the other group e.g. through induced defences.

Results from this study showed that *Quercus* trees with a similar fungal or bacterial endophytic community did not share a similar insect herbivore community. Richness and diversity of endophytic communities also did not correlate with the richness and diversity of insect herbivores. This suggests that tree phenotypic traits that act as ecological filters for insect herbivores do not act on endophytic species in the same way.

Galling and mining insects, due to their sedentary nature, are unable to avoid direct or indirect interactions with endophytic organisms (Faeth and Hammon, 1996). Therefore, it was predicted that differences in the endophytic community would correlate strongly with galler abundance. The abundance of the agamic generation of *Neuroterus albipes* and *N. anthracinus* were correlated with differences in the bacterial endophytic community and fungal endophytic

community respectively. However, these correlations were not shown at the guild level or with any of the other galling insects. The abundance of leaf mining species was also not correlated with either bacterial endophyte composition or fungal endophyte composition. Free feeding insects are thought to be less affected by endophyte presence as they are able to move freely from tree to tree, actively selecting feeding sites. However, the abundance of leaf rollers was also correlated with differences in the fungal endophyte community and the abundance of leaf webbers by the bacterial endophyte community. Although, these two groups are classed as free feeders they form structures in which they feed and are therefore considered somewhat sedentary and may therefore be unable to avoid the direct or indirect effects of endophytes.

To better understand which of these endophytic species in the community might be contributing to the observed differences in herbivore preference, the relative abundances of the most dominant endophytic species were considered individually. 60% of the endophyte-insect relationships of galling insects recorded on the *Quercus* trees in the BSO trial were negative. Most other studies concerning the effects of endophyte presence on galling insects have also shown a negative relationship (Wilson, 1995b, Butin, 1992, Wilson and Carroll, 1994). The sedentary and persistent nature of galling insects makes them particularly susceptible to endophytes as they are unable to avoid the direct or indirect endophyte effects (Faeth and Hammon, 1996). Characteristically, endophytes associated with woody plants are unlikely to grow systemically in the host plant (Yan et al., 2015, Saikkonen et al., 1996), this reduces the chances of '*direct interactions*' of fungal hyphae or bacterial cells with insect herbivores. Secondary metabolites produced by endophytes are known to be involved in insect antagonism in grass species (Clay, 1991) but are less well characterised for those residing in woody plants. Several toxins have been isolated from tree endophytes that show herbivore antagonistic potential (Calhoun et al., 1992, Schulz et al., 2002) but these have only been

tested *in vitro*. It is unknown whether these secondary metabolites are produced in sufficient quantity *in planta* to control insect herbivores.

Just as plant pathogens elicit changes in the chemistry of plant tissues (Levin, 1976), endophyte infection may also evoke a chemical response in the host i.e. *'indirect interactions*'. The use of metabolomic techniques have recently been used to show that several plant metabolites involved in the jasmonate signalling pathway were produced in response to endophyte colonisation of *Cirsium arvense* (Hartley et al., 2015). The metabolites produced were similar to those produced following wounding by herbivores or attack by pathogens (Hartley et al., 2015). It is thought, therefore, that certain endophytes play a role in the 'priming' of the host plant against subsequent attack by insect herbivores or pathogens through induced systemic resistance (Biere et al., 2013, Thaler et al., 2012).

Other studies have shown that endophyte presence may play an indirect role in mortality of sedentary insects through early leaf abscission (Faeth and Hammon, 1997a), through leaf necrosis around the gall (Butin, 1992) or by restricting nutrients to the developing gall (Wilson, 1995b). Increased mortality of these larvae may reduce the population of insects the following year but at a cost to the host plant through reductions in photosynthesis (Faeth and Hammon, 1997a). The endophyte-host relationship is only mutualistic in relation to herbivore resilience if the benefits outweigh the cost of necrosis.

The galls formed by many invertebrates, contain highly differentiated nutritive tissues that have concentrated nutrients high in lipids and oligosaccharides and reduced defensive compounds e.g. phenolic compounds, compared to non-galled tissues (Stone and Schönrogge, 2003, Schönrogge et al., 2000, Diamond et al., 2008, Stone et al., 2002, Nyman and Julkunen-Tiitto, 2000). Gall tissue may therefore be a better food source for endophytic species than ungalled plant tissue, this may be especially true for copiotrophic yeast species (Glushakova

and Kachalkin, 2017). Glushakova and Kachalkin (2017) found that the diversity and abundance of yeast species found in galled tissue was considerably different from the yeast species living endo-and epiphytically. In the current study the abundance of the yeast, *Vishniacozyma* sp. was positively correlated with galls of *Neuroterus anthracinus*. It is possible, therefore, that this *Vishniacozyma* sp. is utilising the higher nutritive cells and reduced defences of the galled tissues created by *Neuroterus anthracinus*. Although much simpler in tissue organisation, composition of endophytic species from gall tissues of aphid galls on poplar trees (*Populus* sp.) were distinctly different from the endophytic community in non-galled tissue (Lawson et al., 2013).

The intimate and persistent interaction of galling insects with their host plant may mean that they are more sensitive to the distribution of endophytic species within the host, especially if the endophyte inhibits or promotes herbivory. If endophytes negatively affect fitness, it has been shown that galling insects may even avoid areas within a leaf with high endophyte infection (Wilson and Carroll, 1997). It is thought that ovipositing females may be able to avoid high 'endophyte space' by choosing tissues with high levels of tannins, as endophyte fungal growth is inhibited by high tannin levels (Taper et al., 1986). Hammon and Faeth (1992) argued that endophyte activity may determine when and where an insect feeds on the host plant. However, even if galling sites are relatively free from endophytes at oviposition, the galler itself may stimulate endophyte growth (Faeth and Hammon, 1997a, Butin, 1992).

For an endophyte-host relationship to be stated as truly mutualistic, it has been suggested that agreement with modified Koch's postulates should be tested: (1) endophyte occurrence must be associated with benefits to the host plant; (2) the endophyte must be isolated from the host showing benefits; (3) the cultured endophyte must show the same benefits when re-inoculated into an endophyte-free plant and (4) the endophyte must be re-isolated from the inoculated plant (Sieber, 2007). Therefore, more study is needed to test if the relationship

shown between endophytes OTU_F2047, OTU_B2259 and OTU_F2984 and galling species are truly mutualistic.

The leaf miners in this study were positively associated with higher levels of infection by OTU_F577 and OTU_B2738. Faeth and Hammon (1997b) also found a lack of antagonistic interaction between endophytes and leaf mining species in *Quercus emoryi*. It has been shown, at least for one species of leaf miner, that leaf damage created by leaf miners facilitates the entry of endophytes into the leaf (Faeth and Hammon, 1996). Another study shows that spores of the endophytic fungi *Colletotrichum gloeospoides* passed though the gut of an insect herbivore (*Poekilocerus pictus*) retain viability; these herbivores may therefore act as dispersal agents (Devarajan and Suryanarayanan, 2006). Different feeding habits may also result in differing amounts of tissue damage, wounds created by leaf chewers or leaf webbers will be much greater than the small oviposition wounds created by leaf miners and gallers. If insect herbivores are aiding dispersal and colonisation, a defensive mutualism is unlikely to be selected (Faeth, 2002). However, the leaves that were analysed for endophyte composition and abundance in this study were free from insect damage, therefore any positive relationship between insect and endophyte abundance shown here is unlikely to be due to increased facilitation.

Three of the endophytes analysed here are considered pathogens. OTU_F2984 and OTU_F3047 were assigned to the species *Ramularia endophylla* known to cause leaf spots on *Quercus* species, OTU_F890 is thought to belong to the genus *Erysiphe* that causes powdery mildew and OTU_F577 was assigned to the species *Exobasidium bisporum* thought to be pathogenic against *Quercus* species. The correlation between the abundance of these potentially latent pathogens and the abundance of insect herbivores was variable, from negative to positive. Negative responses of insect herbivores to endophyte presence may be a result of: (1) direct competition for nutrients and/or space; (2) direct antagonism via endophytic production of

metabolites; (3) indirect plant mediated chemical response by the host induced by the endophyte or (4) mediated by natural enemies (Tack and Dicke, 2013). Positive responses could be a result of direct feeding on endophyte tissue, for example, the larvae of the moth *Lobesia botrana* showed higher survival, faster development and increased fecundity when fed on the nutritious fungus *Botrytis cinerea* (Mondy and Corio-Costet, 2004). However, horizontally transmitted endophytes have relatively little biomass so are unlikely to be a substantial food source for insect herbivores (Carroll, 1988). Although, the pathogens isolated here were from asymptomatic trees it is possible that they were attracted to stressed trees with reduced defences and potentially increased levels of nitrogen, insect herbivores may also be attracted to these declining trees and may explain their increased abundance (White, 1969).

The impact of oak powdery mildew on different insect herbivores has been well studied in the *Quercus* host system (Tack et al., 2012, Field et al., 2019) but results have proved to be inconsistent. Tack et al. (2012) found oak powdery mildew reduced the growth rate of a free-feeding caterpillar (*Acronicta psi*) but increased the growth rate of a leaf miner (*Tischeria ekebladella*). Field et al. (2019) found no clear association between insect herbivores and oak powdery mildew. In the present study, presence of *Erysiphe* (OTU_F890) had a negative impact on the galling insect *Neuroterus anthracinus*. It is unlikely that the endophyte would have a direct impact on the galling insect as the fungus is known to only penetrate the epidermis with its feeding organs (Braun, 1987) and the galling insects resides in the parenchyma tissue (Stone et al., 2002). Antagonism is therefore likely due to '*indirect interactions'* via plant-mediated chemical changes induced by endophyte colonisation, as explained above.

Entomopathogenic fungi, in the order Hypocreales, have been shown to asymptomatically colonise internal plant tissues (Vidal and Jaber, 2015). Of the three most dominant entomopathogenic fungi noted in the study, only OTU_F1265 assigned to a *Fusarium* species

negatively influenced the spring gallers. Entomopathogenic fungi have been shown to affect insects through both direct and indirect pathways (Gurulingappa et al., 2011). However, as the fungal endophytes of woody plants show limited systemic growth it is unlikely that the fungus would come into direct contact with the insect herbivore. Insect death by mycosis is rarely reported in the literature (Vega, 2018). Disruption to insect fitness is likely due to systemic chemical changes in the host plant (Gibson et al., 2014).

OTU_B2259 was assigned to the Rhizobiales order, one of the orders that contain genera of the nitrogen fixing bacteria. Although more commonly associated with the roots of leguminous plants, some nitrogen fixing bacteria have been shown to colonise the phyllosphere of plants endophytically (Doty, 2011). It is thought that these endophytic bacterial strains may also be able to fix nitrogen for the host plant (Doty et al., 2016, Tashi-Oshnoei et al., 2017) and in turn elevated nitrogen may lead to an increase in herbivory (Li et al., 2016, Athey and Connor, 1989). In this study the potential nitrogen fixing bacteria (OTU_B2259) correlates positively with the abundance of the galling insect *Macrodiplosis pustularis*, as might be expected if this bacterium is able to fix nitrogen in the oak tree. On the other hand, presence of OTU_B 2259 negatively influenced another galler, *Neuroterus quercusbaccarum*. Further research on the actual gall tissues and on OTU_B2259 physiology would be required to resolve any interactions.

It is generally accepted that some endophytes contribute positively to the phenotype of their plant host. Endophytes may protect the plant against abiotic or biotic stresses (Redman et al., 2002, Rodriguez et al., 2008, Waller et al., 2005, Khan et al., 2016, Arnold et al., 2003, Ren et al., 2013, Ganley et al., 2008) and it may be expected, therefore, that plants infected by certain endophytes would be healthier and more vigorous than endophyte-free hosts. In accordance with the plant vigour hypothesis (Price, 1991), the endophyte may be providing additional biomass for the insect herbivores by stimulating plant growth.

5.5. *Conclusions*

Studies of the interaction between endophytes and insect herbivores in the literature show a wide range of responses, these inconsistencies are ascribed partly to differences in the species involved, host genotype, endophyte genotype and environment. Studies, like this one, that consider one host-endophyte system may be a better approach to understand the influence of endophytes on different insect species/guilds. However, the response of insect herbivores to fungal and bacterial endophytes is variable even within a single host-endophyte system.

Results from this study have shown that trees that share a similar assemblage of endophytes do not share a similar insect herbivore community. Some insect feeding guilds correlated positively with endophyte presence, others negatively and others were unaffected. Two mechanisms are discussed here: (1) *passive interaction,* where tree traits that make the internal environment suitable for insect herbivores also favour endophytes (positive correlation) or disfavour endophytes (negative correlation) and (2) *active interaction* where the presence of one party affects the other negatively or positively, this could be through '*direct interactions*' where one organism affects the other without any influence from the plant itself or '*indirect interactions*' where one organism affects the other through plant mediated effects. Based on the results of this study it would be problematic to make generalisations of herbivore-endophyte correlations in relation to insect feeding guilds.

The results of this study have shown that the abundance of some insect herbivores correlates with the presence of endophytic species. However, it is not possible to determine whether the observed results are solely in response to endophyte presence or absence. Manipulative experiments, in which trees are artificially infected with endophytes and concurrently exposed to herbivore attack should be used to establish cause and effect. This would be challenging in

the field however, in part because it is impossible to produce endophyte-free trees in order to start these types of experiment (Sieber, 2007).

Future research should also consider the effect of these interactions on higher trophic levels such as the natural enemies of galling or mining insects e.g. parasitoids (Omacini et al., 2001, Tack et al., 2012).

CHAPTER 6 - *Comparing the endophytic communities of Quercus trees in the UK symptomatic and asymptomatic for Acute Oak Decline*

6.1. *Introduction*

Since the 1920s an increasing number of serious decline episodes have been recorded in *Quercus* trees around the world (Gibbs and Greig, 1997, Leininger, 1998, Thomas et al., 2002, Biosca et al., 2003, Brady et al., 2014a, Moradi-Amirabad et al., 2019). Tree decline can be defined as a progressive deterioration in health and vigour, the aetiology is often complex involving multiple interacting biotic and abiotic forces (Manion, 1981, Haavik et al., 2015). One such decline episode currently affecting oak trees in the UK, referred to as Acute Oak Decline or AOD, shows characteristic 'bleed' symptoms on the bark.

In the UK, incidence of AOD is highest in south east England extending into the midlands, south west England and into Wales (Denman et al., 2014, Brown et al., 2016). AOD affects both UK native oak species, *Quercus robur* and *Q. petraea*. *Quercus robur* seems to be affected to a greater extent, but this may be due to the current geographic range of the decline syndrome coinciding with the predominance of *Q. robur* in southern England (Denman and Webber, 2009). AOD seems to only affect mature trees over 50 years old (Brady et al., 2010) and it is thought that host genetics and site conditions are important predisposing factors in syndrome development (Denman et al., 2014). In spring and autumn, dark, sticky 'bleeds' can be observed originating from longitudinal splits in the bark, beneath which necrotic lesions develop (*Figure 6.1*). It is thought that these necrotic lesions may eventually grow large enough to prevent vascular flow, reducing tree vigour and typically resulting in mortality (Denman et al., 2014, Brady et al., 2017). Forest research have differentiated AOD from

Chronic Oak Decline (another oak decline syndrome in the UK) based on the speed at which decline, and mortality occurs (Denman and Webber, 2009). Trees with AOD typically decline rapidly and die within 5-10 years after the onset of symptoms, although some trees are known to partially recover, forming callus tissue over necrotic lesions (Brown et al., 2014).

Three novel gram-negative bacterial species: *Gibbsiella quercinecans* (*Gq*) (Brady et al., 2010), *Brenneria goodwinii* (*Bg*) (Brady et al., 2012) and *Rahnella victoriana* (*Rv*) (Brady et al., 2014b) are consistently isolated from these necrotic lesions. All three bacteria are thought to play an active role in tissue degradation as genomic analysis has shown they possess necrogenic enzymes and virulence factors (Denman et al., 2017, Doonan et al., 2019). *In vitro* inoculation of oak logs with *Bg* and *Gq* produced tissue necrosis confirming necrogenic ability (Denman et al., 2017). It is thought that these bacteria, together with others found in the AOD lesion microbiota, operate in synergy to cause necrosis, in what has been referred to as a pathobiome (Vayssier-Taussat et al., 2014). *Gq*, for example, produces an enzyme that digests the oak defence compound tannin, therefore aiding bacteria such as *Bg* that have a higher pathogenic potential (Doonan et al., 2019). It is these complex interactions between the biotic components of AOD that make the outcome of host-microbe interactions unpredictable.

Figure 6.1 – bleeding bark lesion characteristic of AOD. Wimbledon Common, London, photo taken by S. Roy

It is believed that the native two spotted oak buprestid beetle, *Agrilus biguttatus*, is associated with AOD in the UK, however its precise role is still under scrutiny (Brown et al., 2014, Vuts et al., 2016, Brown et al., 2017, Reed et al., 2018). The larval galleries of the beetle are not always found on AOD infected trees (Denman et al., 2014), suggesting that the beetle is not necessary

for the necrotic lesions to form and may simply be taking advantage of weakened trees. However, studies have shown that *Agrilus* larvae may aid the spread of the necrogenic bacteria within the tree tissue leading to the formation of multiple bleed sites on the same tree, hastening decline symptoms (Denman et al., 2017).

Forest conditions such as edaphic or climatic factors usually dictate which sites are more severely affected by tree decline than others (Haavik et al., 2015). Water stress is considered to be one of the most significant predisposing factors in oak decline (Haavik et al., 2015). Although oak trees are generally thought to possess multiple physiological adaptations to tolerate water stress e.g. deep rooting structure (Abrams, 1990), they are still likely to endure stress-induced changes in physiology and growth which results in a depleted carbon store (Bréda et al., 2006). 'Carbon starvation' may occur if carbon stores are sufficiently depleted that the tree is unable to invest in the necessary defences against biotic agents (McDowell et al., 2008). Droughts can also interrupt the uptake of nitrogen leading to depleted nitrogen stores (Millard and Grelet, 2010). Climate change predictions indicate that forests in the UK are likely to experience drier summers and wetter winters (Broadmeadow et al., 2005, Lowe et al., 2019). With the intensity, duration and frequency of water stress expected to increase, oak trees are likely to become more vulnerable to biotic agents (Desprez-Loustau et al., 2006). Climate change is also likely to alter the geographic range and/or phenology of pests, pathogens, vectors and hosts, that may introduce new inciting agents e.g. defoliating insects, or intensify damage by existing agents (Sturrock et al., 2011, Jactel et al., 2019). As a result, the incidence and severity of AOD is likely to increase with climate change if a suitable control measure is not implemented.

Host susceptibility to disease is reliant on genetic, ecological and environmental factors. One aspect of host susceptibility that is gaining attention in the literature is the potential role of host associated endophytic species in plant defence (Busby et al., 2016, Rodriguez et al., 2009).

Endophytes are organisms, most commonly bacteria or fungi, that live for at least part of their lifecycle within plant tissues without inducing symptoms of disease (Schulz and Boyle, 2006). The interactions between endophytes and their hosts are poorly understood; most endophytes have been classed as commensalistic with as yet unknown functions within plants, others have been shown to have a mutualistic nature and others may have detrimental effects on plants as latent pathogens or parasites (Hallmann et al., 1997, Sieber, 2007, Schulz and Boyle, 2006, Porras-Alfaro and Bayman, 2011). Tree endophytes are horizontally transmitted in the environment (Frank et al., 2017) and are shaped by host-related factors (i.e. genotype, organ, species, health status etc.) and environmental factors (Schulz and Boyle, 2006) (see also *Chapter 3 and 4*).

Some endophytes are thought to play a role in plant health and productivity (Turner et al., 2013). These beneficial endophytes may directly compete with pathogens for space and nutrients within the endosphere (Mejía et al., 2008), others may inhibit pathogens through the production of antimicrobial compounds (Martín et al., 2015, Mousa and Raizada, 2013) or by inducing plant defences that indirectly influence pathogen recognition and growth (Shoresh et al., 2010, Mejia et al., 2014). Others have been shown to have plant growth-promoting activity (Doty, 2011) or to protect their hosts against abiotic stress (Rodriguez and Redman, 2008), giving these trees a competitive advantage for resource acquisition. Given the potential for endophytes to protect hosts against pathogens, interest is growing in the use of endophytes as possible biocontrol agents in forestry (Rabiey et al., 2019, Witzell and Martin, 2018).

On the other hand, there have been limited studies about how tree disease may affect the establishment of endophytes that share the same ecological niche. Trees that show resistance to AOD may also limit colonisation by endophytic organisms. For example, ash trees that are highly susceptible to *Hymenoscyphus fraxineus*, the causal agent of ash dieback, have a greater abundance of iridoid glycosides (Sollars et al., 2017) that acts against herbivores but can also

enhance fungal growth (Marak et al., 2002). Also, elm trees with genetic resistance against Dutch Elm Disease (DED) showed a reduction in endophytic fungal density in their xylem tissue as a result of increased secondary metabolites (Martín et al., 2013). However, the findings presented in this latter study do not exclude the possibility that elm tree resistance is a result of the direct or indirect antagonism of endophytes on the pathogens causing DED.

Denman et al. (2016) studied the cultivable fungal and bacterial endophytes from the bark of symptomatic (AOD) and asymptomatic oak bark. They found no differences between the fungal community in symptomatic or asymptomatic trees, but they did find a difference in bacterial community (Denman et al., 2016). The development of metabarcoding techniques has allowed for a much deeper analysis of the plant microbiome and understanding how these microbes contribute to plant health (Knief, 2014). Using high throughput sequencing techniques one study considered the bacterial microbiome associated with oak bark in healthy trees and those displaying symptoms of AOD. In contrast to the previous study they found only a very small effect of health status on the general bark microbiome, suggesting that there is not a shift towards a signature AOD indicator microbiome (Sapp et al., 2016). No studies to date have characterised the fungal microbiome associated with symptomatic and asymptomatic oak trees using culture-independent methods, and no studies have considered the potential wider impact of AOD on microbial communities inhabiting other tree tissues such as leaf endophytes.

Comparing the endophytes associated with symptomatic trees to those associated with asymptomatic trees is the first step towards understanding the interactions between plant pathogens and the endophytic community of the host. Further research can then target the mechanisms of endophytic function in plant health and to improve biological control methods. The aim of this study was (1) to examine the geographic (landscape scale) variability of *Quercus* bacterial and fungal endophytic communities in leaves and inner bark (2) to evaluate

the effect of AOD on the bacterial and fungal endophytes associated with the leaves and inner bark of *Quercus* trees (3) to analyse the influence of AOD on carbon and nitrogen reserves in the leaves, and to determine the effect these concentrations may have on bacterial and fungal endophytes. Culture-independent methods will be used in order to perform a deeper analysis of endophytic species including rare and uncultivable species.

6.2. *Materials and Methods*

6.2.1. *Writtle Forest, Stratfield Brake and Monks Wood*

Three oak woodlands were chosen spanning the known distribution of acute oak decline in the UK: Writtle Forest in Essex, Monks Wood in Cambridgeshire and Stratfield Brake in Oxfordshire (*Figure 6.2*). The trees used in this study were the same trees selected for a BBSRC funded project: 'PuRpOsE: PRotecting Oak Ecosystems: understanding and forecasting causes and consequences, management for future climates' (https://protectouroaks.wordpress.com/).

Writtle Forest, a privately-owned woodland located in Essex, UK (lat: 51.691, long: 0.367), is an ancient semi-natural oak woodland, approximately 600 hectares in size (*Figure 6.3*). The woodland was historically managed as 'coppice with standards', with sweet chestnut and hornbeam coppice and native oak standards. At an altitude of around 80m, the mean temperature is around 11°C and an average monthly rainfall of 90mm (Met Office, 2019). The soil in Writtle Forest is slightly acidic (pH5-6.5) with a clayey loam to silty loam texture and is described as mostly planosol, the subsurface clay content leads to both seasonal waterlogging and drought stress. Some areas of the forest are described as stagnosol, where soils have perched water (UK Soil Observatory, 2019, IUSS Working Group WRB, 2015). Bleeding cankers, characteristic of AOD, were first noted in 2006 and the number of trees in decline has increased steadily over the years (Booth, 2019 unpublished).

Stratfield Brake Nature Reserve located in Oxfordshire, UK (lat: 51.804, long: -1.282), has been owned by the Woodland Trust since 1997, it includes both young and mature woodland, meadow and wetland areas in approximately 18 hectares (*Figure 6.4*). The region of interest here is the 2.5 hectare mature (100+ years) secondary woodland which is dominated by oak (The Woodland Trust, 2014). Access to the mature woodland has recently been restricted to the public in response to the presence of AOD. The woodland is located at an altitude of around 60m, the mean temperature here 11.5°C and the average monthly rainfall is approximately 100mm. The soil in this woodland is clayey loam to silty loam in texture, it is slightly acidic (pH 6.5) and is described as planosol; stagnating water is therefore common.

Monks Wood National Nature Reserve (NNR) located in Cambridgeshire (lat: 52.404, long: -0.242), it is one of Britain's best-known lowland woods measuring approximately 150 hectares (*Figure 6.5*). It is designated a Site of Special Scientific Interest (SSSI) and a Nature Conservation Review (NCR) site, as a consequence of being an important place for nature conservation. For centuries it was managed as a traditional coppice-with-standards woodland system, until the end of the First World War when the wood was clear-felled, since then the wood has been left somewhat unmanaged (English Nature, 1993). It is predominantly ash high canopy together with oak. At an altitude of 50m, the mean temperature is 11°C and the average rainfall is 90mm per month. The soil texture is clayey loam to silty loam and described as cambisol, with weak horizon differentiation in the soil profile. The northern region of the woodland has slightly alkaline soils (pH 7.5-8) and the southern slightly acidic at pH5-6 (UK Soil Observatory, 2019, IUSS Working Group WRB, 2015).

Symptomatic trees were identified by the presence of bleeding cankers and were selected from across the entire area of each woodland (*Figures 6.3, 6.4 & 6.5*). Asymptomatic trees (no lesions and no obvious dieback) were selected within a 25m range of the sampled

symptomatic trees with similar physical characteristics e.g. DBH, height. A total of 10 symptomatic and 10 asymptomatic trees were selected from each woodland.

6.2.2. *Tissue sampling*

Samples were collected together with Dr Mateo San José Garcia, University of Reading (PuRpOsE project) from June to September 2016. Writtle Forest was sampled first, then Monks Wood and finally Stratfield Brake. Gloves were used at all times to avoid human contact with the samples, all waste was collected in autoclave bags and taken back to the laboratory for autoclaving to avoid the potential spread of the bacterial pathogens within and between woodlands. Boots and equipment were also sprayed with 5% Biocleanse (Teknon) on leaving the woodland to avoid the spread of the potential pathogens from the woodland site.

6.2.2.1. *Bark samples*

For symptomatic trees, bark was extracted from the most prominent (and most active) bleed site. An area of approximately 40mm x 30mm was cut into the bark using a chisel to a depth of around 20mm. The outer bark was removed from the inner bark using the chisel and the inner bark was placed into a falcon tube stored on ice. The chisel was sterilised using 100% ethanol and flamed in between each sample. For symptomatic trees, inner bark was also sampled in the same way from the opposite side of the tree to the bleed, where no bleed was present. For asymptomatic trees, bark was sampled in the same way at approximately the same height as the bleed sites, usually around 1.5m from ground level. Samples were returned to the laboratory on ice and stored at -80°C until processing. The bark samples from the healthy tissues of symptomatic trees were misplaced during storage and were stored at 4°C, this is taken into consideration.

6.2.2.2. *Leaf samples*

Five branches (approximately 30-55mm in diameter) were cut at random from the main canopy of each tree. Three leaves were collected from each branch; one for endophyte analysis, one for carbon and nitrogen analysis and one for species allocation. For relatively short trees a telescopic tree pruner was used to cut each branch. For taller trees, an arborist throw-line launcher was used to throw a rope over the branch, after securing the line, the branch was then pulled downwards (Youngentob et al., 2016). For difficult branches a flexible saw was attached to the rope and used to cut the branch down using a sawing action (Youngentob et al., 2016). For very tall trees, with minimal lower branches a tree climber (Oliver Booth, University of Reading) climbed the trees and cut branches using a pruning saw. Samples were returned to the laboratory on ice and stored at -80°C until processing.

6.2.2.3. *Bark swab samples*

To determine whether the potential pathogens involved in AOD (*Bg*, *Gq*, *Rv* and *Rp*) were present on the outside of tree bark, a targeted search was carried out. Swabs were taken from asymptomatic and symptomatic *Quercus* trees in Stratfield Brake only in July 2019. For asymptomatic trees, one swab stored in 2mL of phosphate buffered saline (PBS) was used to swab an area of the outer bark at approximately 1.5m from ground level. For symptomatic trees, the bleed area was swabbed, 10cm away from the bleed and 20cm away from the bleed was also swabbed. All swab samples were stored on ice blocks to transport back to the laboratory. Samples were stored at 4°C until processing, which took place within 24 hours of the samples being taken.

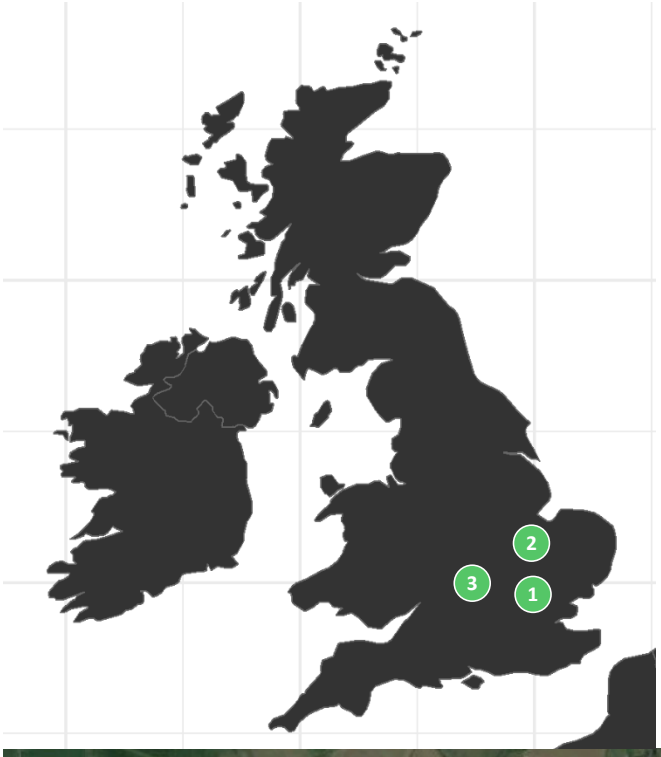

Figure 6.2 – locations of the three woodlands sampled for the study spanning the current distribution of AOD, (1) Writtle Forest, (2) Monks Wood and (3) Stratfield Brake

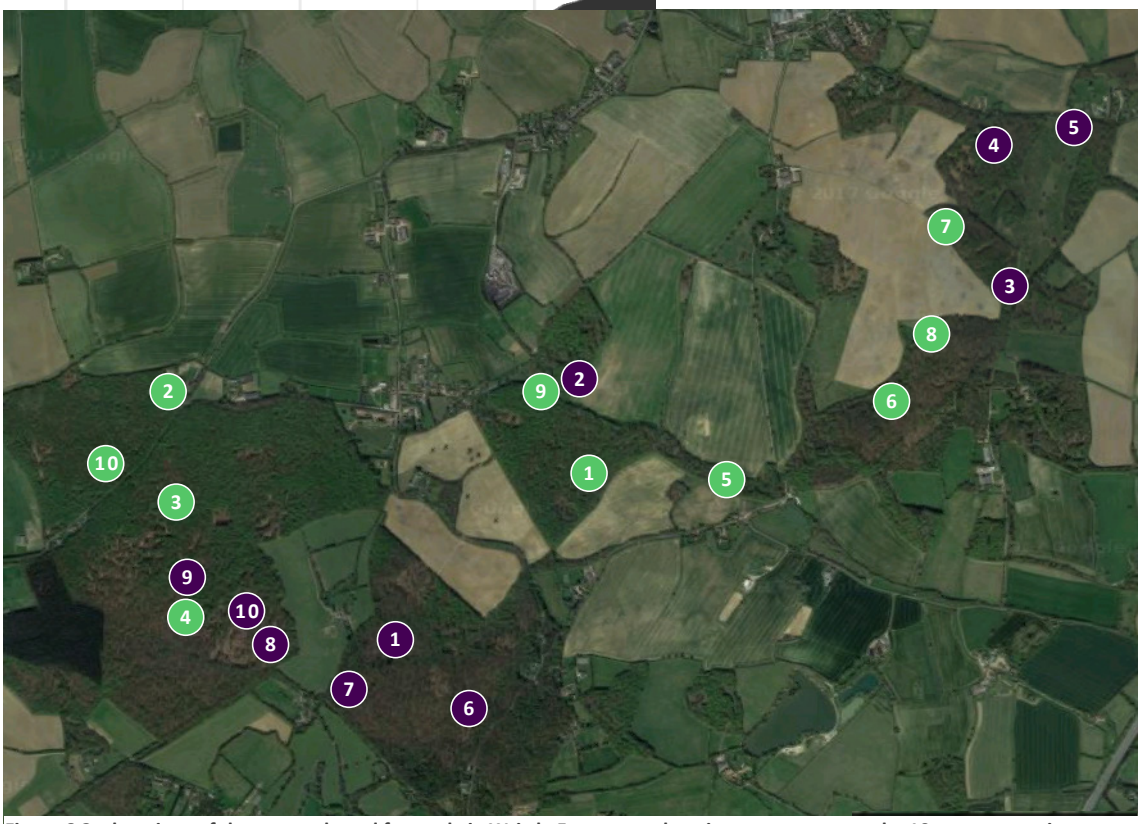

Figure 6.3 – locations of the trees selected for study in Writtle Forest, numbers in green represent the 10 asymptomatic trees and numbers in purple represent the 10 symptomatic trees. Photo taken from Google maps.

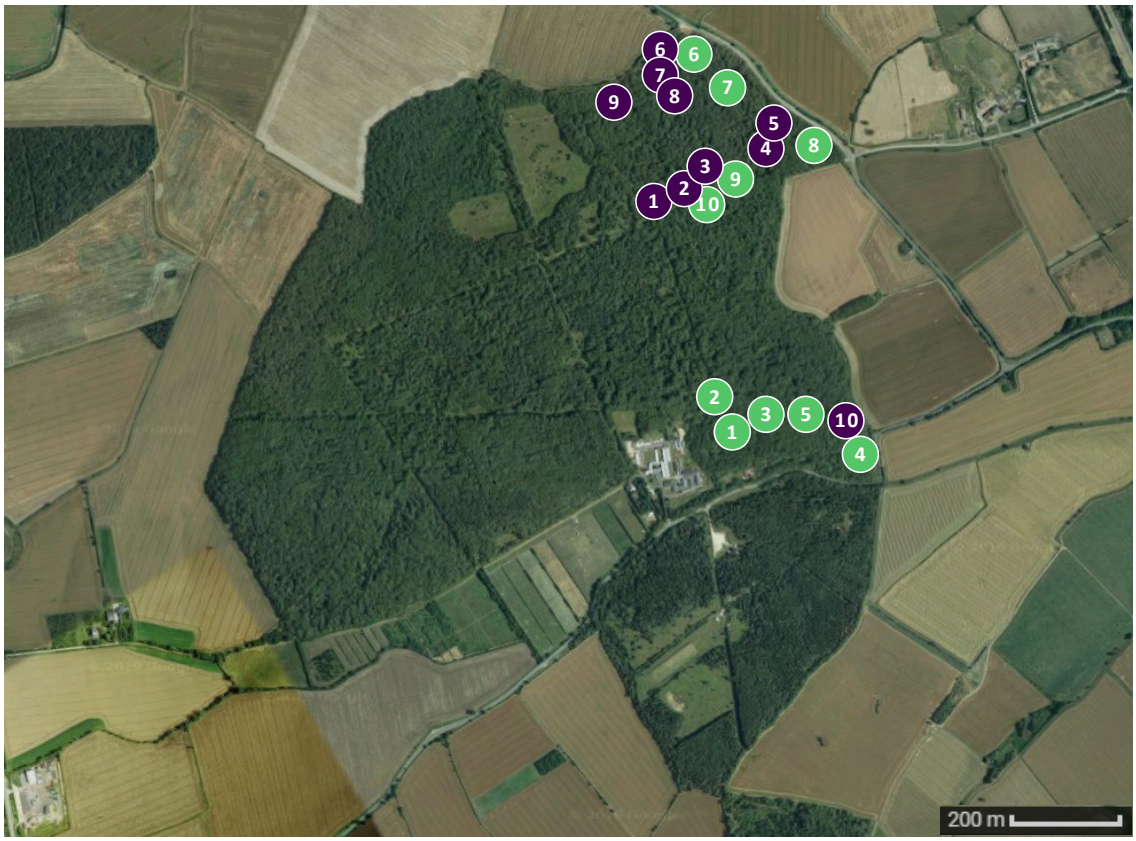

Figure 6.4 - locations of the trees selected for study in Monks Wood, numbers in green represent the 10 asymptomatic trees and numbers in purple represent the 10 symptomatic trees. Photo taken from Google Maps.

Figure 6.5 - locations of the trees selected for study in Stratfield Brake, numbers in green represent the 10 asymptomatic trees and numbers in purple represent the 10 symptomatic trees. Photo taken from Google Maps.

6.2.3. *Determining Quercus species*

Trees were assigned to either *Quercus robur* or *Q. petraea* using leaf morphometric measurements, a method developed by (Kremer et al., 2002). Five leaves from each tree were measured as described in *Chapter 2, Section 2.2.2*. The *MASS* package (version 7.3-51.3) (Venables and Ripley, 2002) in R was used to perform a principal component analysis (PCA) as described in *Chapter 2, Section 2.2.2 & 2.3.1*.

6.2.4. *Chlorophyll fluorescence*

A continuous excitation chlorophyll fluorometer (Hansatech Handy PEA+) was used to take measurements of leaves evenly distributed over each tree. Care was taken to only select fully expanded, similar sized leaves from the first flush with no visible pest or disease damage. As soon as the branches were cut 30 leaves were dark adapted using leaf clips 20 minutes prior to measurement. The Fv/Fm value was used for further analysis; this measurement indicates the maximum efficiency of photosystem II chemistry if all capable reaction centres are functional, the most common technique for measuring plant stress (Murchie and Lawson, 2013).

As Fv/Fm output is a proportional value within a standard unit interval (0, 1) a beta regression model was used to analyse the effect of health status (symptomatic or asymptomatic) nested within woodland (Writtle Forest, Monks Wood or Stratfield Brake) on tree stress (Fv/Fm). The *betareg* package (Version 3.1-2) (Cribari-Neto and Zeileis, 2010) was used.

6.2.5. *Leaf carbon and nitrogen content*

Five leaves from each tree were oven dried in paper envelopes at 70°C for 48 hours, and then stored in a desiccator until processing. Samples were ground using a planetary mill (Fritsch Pulverisette 5). All equipment was cleaned with acetate before use and between every sample.

Samples were placed in 80mL agate bowls with five 20mm agate balls and run at a rotational speed of 380rpm for 15 minutes. 5±10%mg of each ground sample was transferred into tin capsules (CE Instruments) and folded close, removing all air pockets. Each sample was repeated twice. An aspartic acid sample, a plant standard and blank tins were included as quality checks. Samples were run on a FLASH 2000 Elemental Analyzer (Therma Scientific) by Fengjuan Xiao, University of Reading.

The percentage of nitrogen and carbon in each sample was calculated based on the weight of the material of each sample before analysis and an average was taken between the two reads. The output is a proportional value within a standard unit interval, so a beta regression model was used, with %C and %N as response variables and health status (symptomatic or asymptomatic) nested within woodland (Writtle Forest, Monks Wood or Stratfield Brake) as explanatory terms. The *betareg* package (Version 3.1-2) (Cribari-Neto and Zeileis, 2010) was used.

6.2.6. *Endophyte analysis*

6.2.6.1. *Surface sterilisation and DNA extraction*

To remove epiphytic fungal or bacterial DNA from the plant surfaces, all plant material was surface sterilised before the DNA was extracted for sequencing analysis. As samples were stored at -80°C, it was important to test whether the surface sterilisation techniques used in previous chapters would be too harsh on the potentially weakened frozen plant tissue. Results from a pilot experiment (*Appendix B*) showed no effect of storage temperature (-80°C compared to 4°C) on the amount of endophytic DNA recovered. Therefore, all samples were surface sterilised using method A (*Appendix B*). For bark samples they were shaken in the bleach for 8 minutes, all other steps remain the same. A 5mmx5mm square of each leaf was cut using a sterile scalpel and pooled (5 leaves per tree) into a 2mL microcentrifuge tube and

were immediately DNA extracted using method A (*Appendix C*). A 10mmx10mm section was cut from the centre of the piece of inner bark, and DNA extracted using method C (*Appendix C*). DNA quantity and quality were assessed using a spectrophotometer (NanoDrop™ ND-1000, Thermo Fisher Scientific).

Also included were four known isolates of: *Brenneria goodwinii*, *Gibbsiella quercinecans*, *Rahnella victoriana* and *Raoultella planticola* all isolated from tree WD01195 previously (see *Chapter 3*). *Bg*, *Gq* and *Rv* are thought to be involved in the AOD decline syndrome. *Rp* was repeatedly isolated from diseased tissue in another study (Booth, 2019 unpublished), so was also included here as a control, however the role of *Rp* in AOD is unknown.

6.2.6.2. *Illumina Miseq analysis*

All samples were prepared for Illumina Miseq sequencing in the same way as described in *Chapter 3*, *Section 3.3.2*. In total, 4 plates were sequenced; 16SA, 16SB, ITSC and ITSD all combined into one library. This library was run on one flow cell with the addition of 10% PhiX on the Illumina Miseq platform using V3 chemistry by Dr Melanie Gibbs and Dr Anna Oliver, CEH. Unfortunately, the quality output from the Illumina Miseq suggested low cluster density (below the recommended 80%). This could be as a result of contaminants in the libraries such as adapter dimers, primer dimers or partial library constructs that may have affected library quantification and subsequent clustering efficiency (Illumina, 2019). Each step of the process, from DNA extraction, both PCR steps and library preparation were quality checked again by running each of the products on an agarose gel. It was thought that there was potentially too much DNA in the first step PCR that may have inhibited the binding of the Illumina tag primers in the 2nd step PCR. Therefore, a qPCR method was used to confirm that the Illumina primers had successfully bound to the PCR products in the second round. To compensate for potential

low base diversity in these samples, the sequencing run was repeated using a higher concentration of PhiX but sequencing results were still not of adequate quality.

Bioinformatics analysis (performed by Dr Soon Gweon, University of Reading and described in *Chapter 3, Section 3.3.3*) showed that only 285,577 reads remained for 16S after quality filtering (compared to 6,113,724 in samples from Paradise Wood, *Chapter 3*) and 92,097 quality filtered reads for ITS (compared to 8,034,269 in *Chapter 3*). After rarefaction, it was thought that an insufficient number of samples or insufficient sampling depth would result, and any analysis would not be justifiable. Other methods could be employed to improve sequencing efficiency such as designing different primers, using blocking primers etc. but time and experience did not allow for this within this study. The decision was made, therefore, to perform the same analysis using terminal restriction fragment length polymorphism (TRFLP) analysis instead.

6.2.6.3. *Terminal restriction fragment length polymorphism (TRFLP) analysis*

Community fingerprinting was performed using terminal restriction fragment length polymorphism (TRFP) analysis on the same samples as above. For studying the influence of environmental variables on microbial community structure, TRFLP analysis is still regarded as a valid approach that is relatively inexpensive and rapid compared to next generation sequencing methods (van Dorst et al., 2014).

DNA was diluted to 20ng/µL and arranged in 96-well plates as above. The 16S and ITS regions were targeted using the primers listed in *Table 6.1*. The forward primers were labelled at the 5' end with 6FAM fluorescent dye. PCR reactions were set up with the following reaction mix: 5µL 10X PCR buffer (standard reaction buffer, New England Biolabs), 0.25µL each of forward and reverse primers (50µM, Sigma-Aldrich, *Table 6.1*), 0.5µL bovine serum albumin (BSA,

molecular biology grade, New England Biolabs), 0.5µL dNTP mix (each 10mM), 0.35µL *Taq* DNA polymerase (5U/µL, New England Biolabs) and made up to 50µL with molecular grade water. PCR conditions consisted of an initial denaturation at 94°C for 4 minutes, followed by 35 cycles of: 94°C for 45 seconds, 55°C for 1 minute and 72°C for 90 seconds and a final elongation step at 72°C for 10 minutes (PCR conditions provided by Dr Anna Oliver and Dr Lindsey Newbold, CEH). PCR products were cleaned using Monarch® PCR & DNA Clean-up Kit (New England Biolabs), following manufacturer's instructions and eluted in 40µL of molecular grade water.

Amplicons were digested using restriction endonuclease *MspI* for bacteria (Thomson et al., 2010) and *TaqI* for fungi (Jasalavich et al., 2000). For the enzyme digest, 5µL of purified PCR product was added to 1µL 10X buffer (buffer B for 16S and buffer E for ITS, Promega), 0.1µL BSA (molecular biology grade, New England Biolabs), 0.25µL restriction enzyme (*msp*I for 16S and *taqI* for ITS, 10U/µL, Promega) and made up to 10µL with molecular grade water. Samples were incubated on a PCR block at 37°C for *msp*I and 65°C for *taq*I for 4 hours. To prepare samples for the ABI 3730 DNA analyzer (Applied Biosystems™), 1µL of each digest was transferred to the corresponding well of a 96-well plate. 1µL GeneScan™ 600 LIZ™ (Applied Biosystems™) and 9µL of Hi-Di™ formamide (Applied Biosystems™) was made up for each sample, heated at 95°C for 3 minutes and then cooled on ice. 9µL of this Hi-Di/Liz mix was transferred to each sample in the 96-well plates. Samples were run on the ABI 3730 immediately.

Table 6.1 - primers used to amplify DNA from symptomatic and asymptomatic *Quercus* samples for TRFLP analysis

Primer name	Primer sequence	Target region	Reference
63F **530R**	5'- (6FAM) CAGGCCTAACACATGCAAGTC -3' 5'- GTATTACCGCGGCTGCTG -3'	16S	Thomson et al. (2010)
ITS1 **ITS4**	5'- (6FAM) CTTGGTCATTTAGAGGAAGTAA -3' 5'- TCCTCCGCTTATTGATATGC -3'	ITS	Klamer et al. (2002) Klamer and Hedlund (2004)

6.2.6.4. *Statistical analysis*

Individual terminal restriction fragments (TRFs) were binned manually using GeneMarker software (SoftGenetics, version 3.0.1). All TRFs were selected that were above a peak detection threshold of 100. The intensity of each of the TRFs was converted to relative abundance based on the total intensity of all detected TRFs within each of the groups analysed.

Each endophyte kingdom (fungi or bacteria) and each tissue type (leaf or twig) was analysed separately. Leaf samples from all three woods were analysed in the first instance, to determine whether woodland location had an influence on the bacterial and fungal endophytes present. Secondly, leaf samples were separated into woodlands and the influence of health status (asymptomatic or symptomatic), leaf carbon content, leaf nitrogen content, tree stress (measured using Fv/Fm values) and for Writtle Forest only, tree species (*Quercus robur* or *Q. petraea*) were analysed. Inner bark samples were analysed in the same groupings as above but also included 'sampling location' in the models (asymptomatic, symptomatic from bleed site, symptomatic from non-bleed site). Tissue location was nested within health status.

TRF richness (the total number of observed peaks in each sample) and Shannon-Wiener diversity, calculated using the vegan package (version 2.5-3, Oksanen et al. (2018)) were square root transformed before using general linear models with Gaussian error distributions with the explanatory terms described above. The residuals were tested for normality using a Shapiro-Wilk normality test and for heteroscedasticity by plotting the residuals versus the fitted values for each model (Crawley, 2007).

Dissimilarities in TRF composition between samples (beta diversity) was calculated based on Bray-Curtis dissimilarity index. Sample dissimilarities were visualised using an NMDS plot, ensuring a stress value close to 0.2. PERMANOVA models were used to test for differences

between samples using the *adonis* function in the vegan package, with 999 permutations and the explanatory terms described above.

The positive bacterial samples (*Bg*, *Gq*, *Rv* and *Rp*) were run through the TRFLP analysis with the other samples and the TRF peaks were matched to those in the samples. The relative abundance of the four bacteria in each of the samples was calculated based on the intensity of the peak in the control samples. A negative binomial glm model was run in the *MASS* package (version 7.3-51.3, Venables and Ripley (2002)) in R with tissue type nested within health status and woodland as explanatory variables. Models were tested for heteroscedasticity by plotting the residuals versus the fitted values for each model (Crawley, 2007).

6.2.7. *Processing bark swab samples*

Swab samples (containing the 2mL of PBS) were vortexed for 1 minute at top speed and then 100µL was transferred to a MacConkey agar (MAC) plate and spread using an ethanol-flamed glass spreader. Plates were incubated at 27°C for 24 hours. MAC agar contains neutral red, enteric bacteria that are able to ferment lactose are detected by pink/red colonies. Representative photos of the colonies of interest are shown in *Chapter 3, Section 3.4.4*. All pink/red colonies were sub-cultured onto MAC agar. These bacteria were identified using ITS ribotyping and gyrB sequencing as described in full in *Chapter 3*. Forward and reverse sequences were trimmed and aligned and a consensus sequence was assembled using BioEdit Sequence Alignment Editor (version 7.2.6, Hall (1999)). These sequences were matched against the GenBank database using BLAST (Basic Local Alignment Search Tool, https://blast.ncbi.nlm.nih.gov/Blast.cgi). The top 20 BLAST hits for each of the samples were included in the creation of the phylogenetic tree together with all representative gyrB sequences from the most recent paper of the taxonomy of the AOD pathogens (Brady et al., 2017) (*Appendix I*). All gyrB sequences were 742bp in length. MrModeltest 2.3 was executed in

PAUP (version 4.0b10, Swofford (2002)) and found SYM+G to be the best-fit evolutionary model to apply, based on Akaike information criteria. Bayesian phylogenetic analysis was performed using MrBayes (version 3.2, Ronquist et al. (2012)), with 5000000 generations, saving a tree every 1000 steps. Burn-in was determined to be 4000, phylogenetic trees were visualised and edited in FigTree (version 1.4.2, Rambaut (2014)).

6.3. *Results*

6.3.1. *Determining Quercus species*

The principal component analysis (described in *Chapter 2*) divided the 60 trees from Writtle Forest, Monks Wood and Stratfield Brake into two species groups based on their leaf morphology. 55 individuals were assigned to *Q. robur*, and 5 individuals to *Q. petraea* (*Figure 6.6*). A discriminant function analysis was used to determine that an individual of unknown species had a 100% chance of being assigned to the correct group based on this principal component analysis. The five *Q. petraea* trees were located in Writtle Forest.

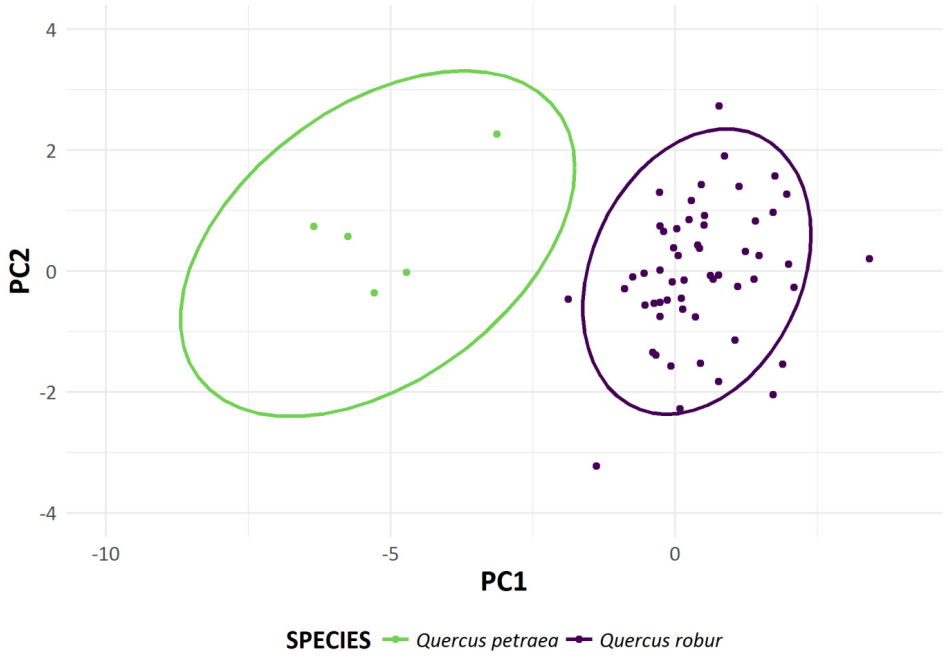

Figure 6.6 – results of the principal component analysis used to assign trees from the three woodlands to a species based on their leaf morphology. The first two components grouped the trees into two groups, on the left *Quercus petraea* and on the right *Quercus robur*. Each point represents one tree. Ovals represent 95% confidence intervals.

6.3.2. *Chlorophyll fluorescence*

Fv/Fm readings did not differ significantly between symptomatic and asymptomatic trees in any of the woodlands (*Figure 6.7*).

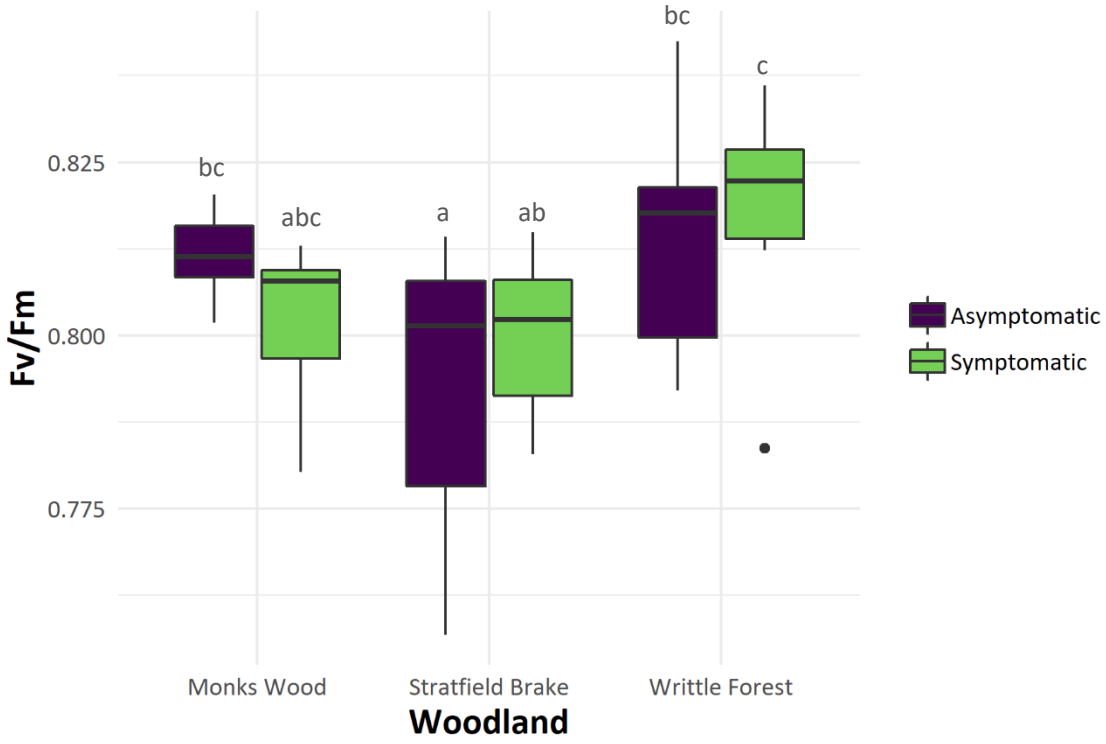

Figure 6.7 – differences in Fv/Fm values, an indication of tree stress, in trees with different health statuses from each of the woodlands. Low values of Fv/Fm represents high stress. Letters represent statistical differences calculated using a GLM with beta regression.

6.3.3. *Leaf carbon and nitrogen content*

Tree health status affected the carbon content of the leaves (df=4, χ^2=37.18, p<0.001) but only in Stratfield Brake and Monks Wood. In Stratfield Brake asymptomatic trees had a higher carbon content and in Monks Wood symptomatic trees had a higher carbon content (*Figure 6.8*). There were no differences in nitrogen content between symptomatic and asymptomatic trees (*Figure 6.9*).

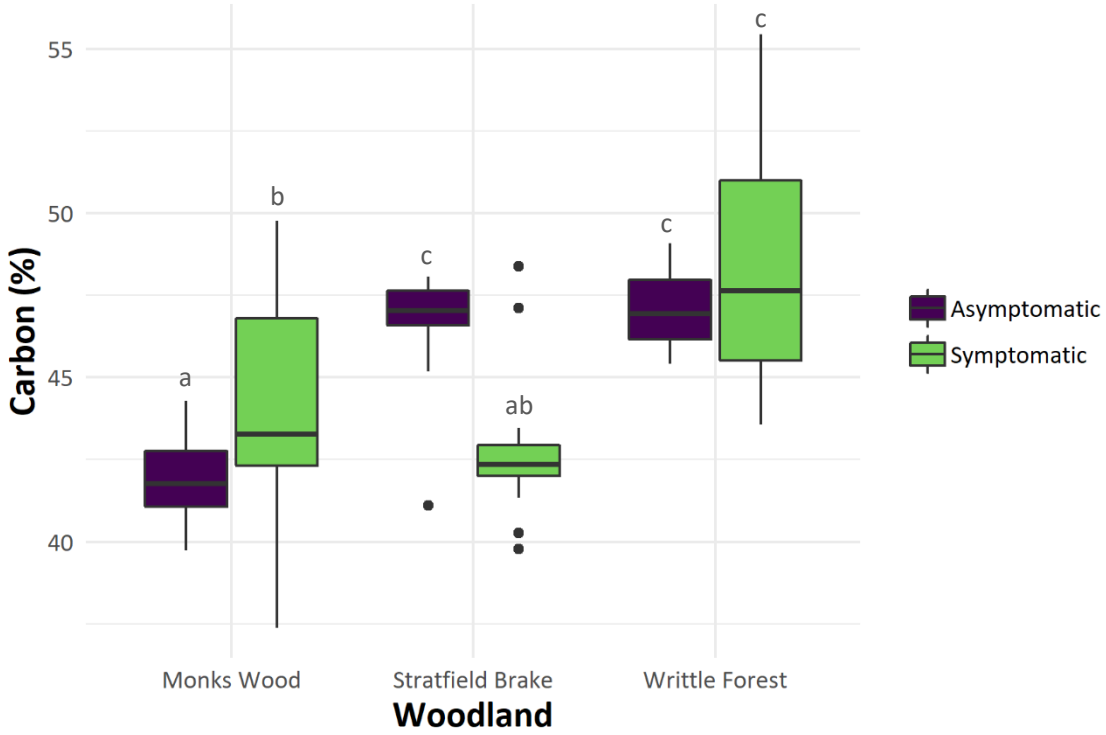

Figure 6.8 – differences in percentage carbon in the leaves of the trees of different health status and in different woodlands. Letters represent statistical differences calculated using a GLM with beta regression.

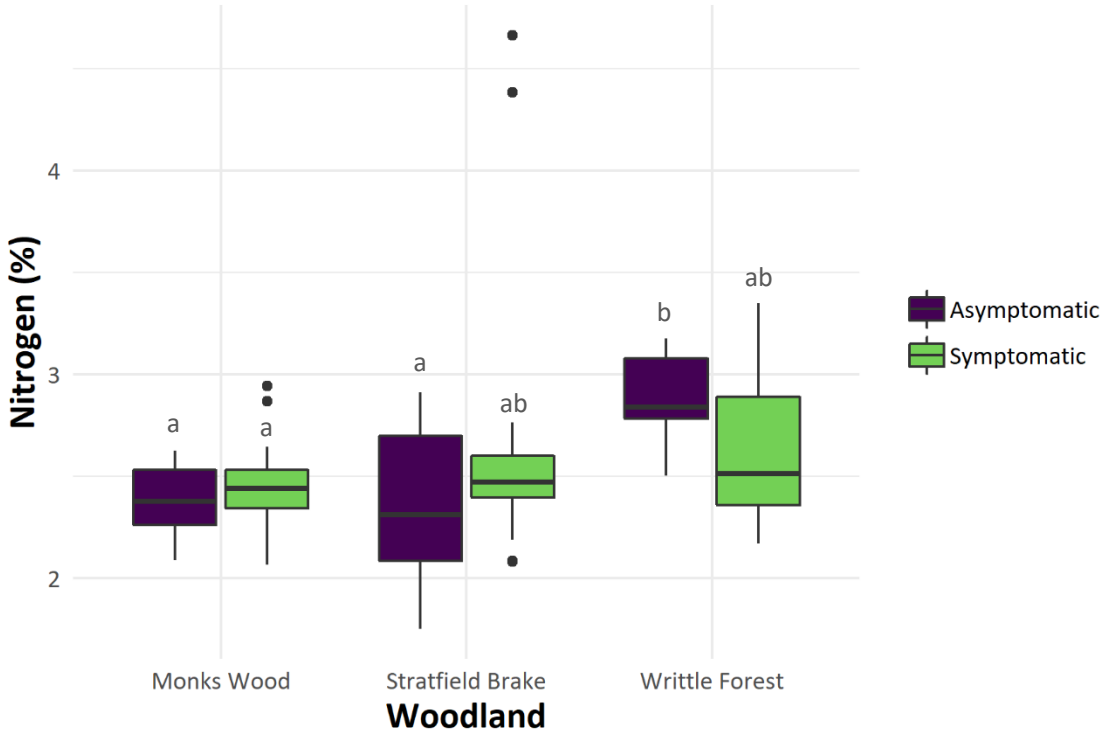

Figure 6.9 – differences in percentage nitrogen in the leaves of the trees of different health status and in different woodlands. Letters represent statistical differences calculated using a GLM with beta regression.

6.3.4. *Endophyte analysis – TRFLP*

A total of 1303 TRFs were recorded for 16S and 1310 TRFs for ITS. Bacterial and fungal endophyte richness and diversity was affected by several variables, as shown in *Table 6.2* and *Table 6.3*. PERMANOVA tests also revealed significant differences between *Quercus* samples, the results of which are summarised in *Table 6.4* & *6.5* and *Figures 6.11 & 6.12*.

As the PERMANOVA test showed considerable differences in fungal endophyte composition between symptomatic and asymptomatic bark samples, a Venn diagram was produced to show how many of the TRFs were shared between the trees with different health statuses for all the woodlands (*Figure 6.10*).

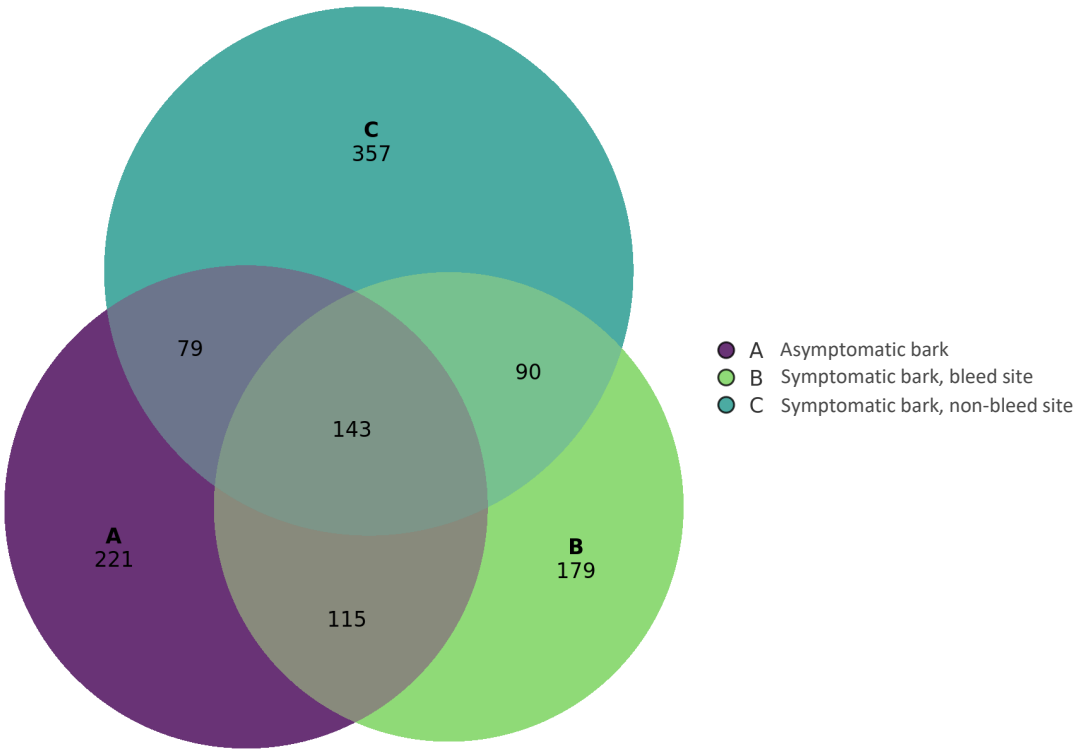

Figure 6.10 – number of fungal TRFs shared between symptomatic and asymptomatic bark and the location of bark sampling for all woodlands.

Table 6.2 – results from the linear model for species richness and diversity of fungal endophytes associated with the *Quercus* trees in each of the woodlands. SB = Stratfield Brake, WF = Writtle Forest and MW = Monks Wood

		RICHNESS					DIVERSITY				
Tissue	**Samples**	**Signif. variables**	**df**	**F**	**p**	**Direction of effect**	**Signif. variables**	**df**	**F**	**p**	**Direction of effect**
Leaf	All woodlands	**Woodland**	**2, 46**	**9.65**	**<0.001**	**SB > WF SB = MW WF = MW**	**Woodland**	**2, 46**	**7.33**	**<0.01**	**SB > WF SB > MW WF = MW**
	Writtle Forest	No significant variables					No significant variables				
	Monks Wood	No significant variables					No significant variables				
	Stratfield Brake	No significant variables					No significant variables				
Inner bark	All woodlands	No significant variables					No significant variables				
	Writtle Forest	**Sampling location**	**2, 24**	**4.58**	**<0.05**	**Non-bleed > bleed site**	No significant variables				
	Monks Wood	No significant variables					**Sampling location**	**2, 25**	**4.88**	**<0.05**	**Bleed > non-bleed site**
	Stratfield Brake	No significant variables					**Health status Sampling location**	**2, 23 2, 23**	**6.23 7.70**	**<0.05 <0.05**	**Asymptomatic > Symptomatic Bleed site > non-bleed site**

Table 6.3 – results from the linear model for species richness and diversity of bacterial endophytes associated with the *Quercus* trees in each of the woodlands

		RICHNESS					DIVERSITY				
Tissue	**Samples**	**Signif. variables**	**df**	**F**	**p**	**Direction of effect**	**Signif. variables**	**df**	**F**	**p**	**Direction of effect**
Leaf	All woodlands	No significant variables					No significant variables				
	Writtle Forest	No significant variables					No significant variables				
	Monks Wood	No significant variables					No significant variables				
	Stratfield Brake	**% carbon** **% nitrogen**	**2, 17** **2, 17**	**8.11** **4.53**	**<0.05** **<0.05**	**Positive** **Positive**	**% carbon** **% nitrogen**	**2, 17** **2, 17**	**4.24** **4.78**	**0.055** **<0.05**	**Positive** **Positive**
Inner bark	All woodlands	No significant variables					No significant variables				
	Writtle Forest	**Species**	**1, 24**	**6.79**	**<0.05**	***Q. robur* > *Q. petraea***	**Health status** **Fv/Fm**	**2, 23** **2, 23**	**7.69** **5.18**	**<0.05** **<0.05**	**Symptomatic > Asymptomatic** **Negative**
	Monks Wood	**Sampling location**	**2, 26**	**4.64**	**<0.05**	**Non bleed > bleed site**	**Sampling location**	**2, 26**	**5.72**	**<0.05**	**Non bleed site > bleed site**
	Stratfield Brake	No significant variables					Health status	1, 28	3.37	0.07	Symptomatic > Asymptomatic

Table 6.4 - results from the PERMANOVA for fungal endophyte beta diversity of *Quercus* trees in the three woodlands.

Tissue	Samples	Significant variables	Stress value	F	p
Leaf	All woodlands	**Woodland**	**0.23**	**1.64**	**<0.05**
	Writtle Forest	**Species**	**0.12**	**1.55**	**<0.05**
	Monks Wood	**No significant variables**			
	Stratfield Brake	**No significant variables**			
Inner bark	All woodlands	**Woodland**	**0.28**	**1.21**	**<0.05**
	Writtle Forest	**Health status**	**0.22**	**1.63**	**<0.001**
		Sampling location		**1.82**	**<0.001**
		Fv/Fm		**1.43**	**<0.05**
	Monks Wood	**Health status**	**0.24**	**2.21**	**<0.001**
		Sampling location		**1.47**	**<0.01**

Table 6.5 – results from the PERMANOVA for bacterial endophyte beta diversity of *Quercus* trees in the three woodlands.

Tissue	Samples	Significant variables	Stress value	F	p
Leaf	All woodlands	No significant variables			
	Writtle Forest	No significant variables			
	Monks Wood	No significant variables			
	Stratfield Brake	No significant variables			
Inner bark	All woodlands	No significant variables			
	Writtle Forest	**Sampling location**	**0.20**	**1.87**	**<0.001**
	Monks Wood	**Sampling location**	**0.19**	**1.69**	**<0.05**
	Stratfield Brake	No significant variables			

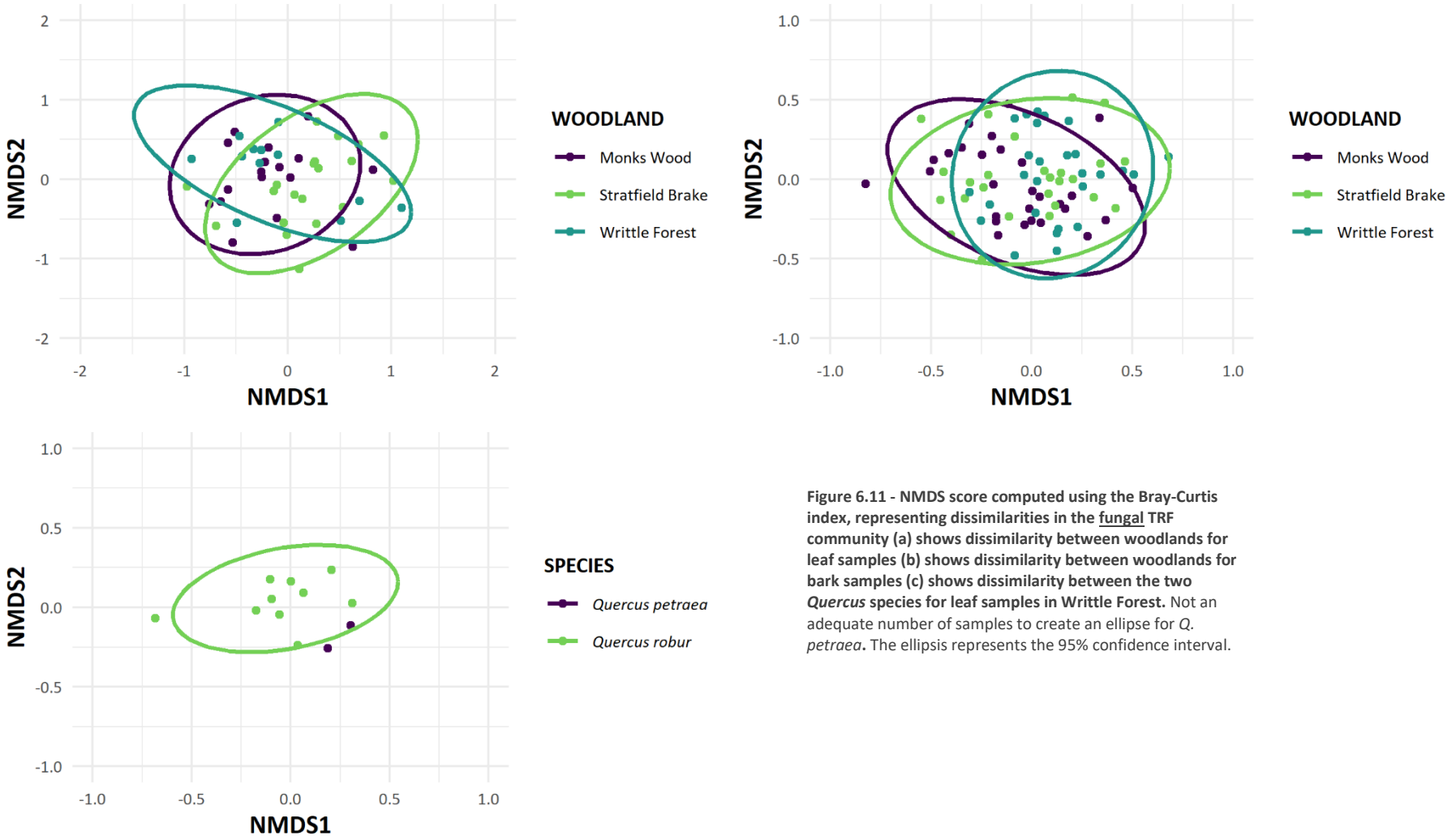

Figure 6.11 - NMDS score computed using the Bray-Curtis index, representing dissimilarities in the fungal TRF community (a) shows dissimilarity between woodlands for leaf samples (b) shows dissimilarity between woodlands for bark samples (c) shows dissimilarity between the two *Quercus* species for leaf samples in Writtle Forest. Not an adequate number of samples to create an ellipse for *Q. petraea*. The ellipsis represents the 95% confidence interval.

Figure 6.12 - NMDS score computed using the Bray-Curtis index, representing dissimilarities in the fungal TRF community between asymptomatic and symptomatic trees and for the different bark sampling locations (a) Writtle Forest (b) Monks Wood (c) Stratfield Brake. The ellipsis represents the 95% confidence interval.

6.3.4.1. *AOD pathogens*

Results from the TRFLP analysis of the four positive control samples suggest that the *Enterobacteriaceae* family potentially lie between TRF 450 and 460 base pairs with: *Brenneria goodwinii* around 455.7, *Gibbsiella quercinecans* 454.1, *Rahnella victoriana* 454.6 and *Raoultella planticola* around 452.9. All four bacterial species were found at a higher abundance in symptomatic compared to asymptomatic trees. Differences in abundance were also found between the tissue types (*Table 6.6*, *Figures 6.13*).

Table 6.6 – results from the generalised linear model comparing the abundance of bacterial species of interest with the health status, woodland and tissue type of the samples with negative binomial errors. BNB = Inner bark, non-bleed site, BB = inner bark, bleed site and L = leaf.

Bacteria	Significant variables	df	χ^2	p	Direction of effects
Brenneria goodwinii	Health status	138	7.1	<0.02	Symptom. > asymptomatic
	Tissue	138	15.2	<0.01	BNB > BB BNB > L BB > L
Gibbsiella quercinecans	Health status	138	13.5	<0.01	Symptom. > asymptomatic
	Tissue	138	11.1	<0.001	BNB > BB BNB > L BB > L
Rahnella victoriana	Health status	138	20.0	<0.001	Symptom. > asymptomatic
	Tissue	138	23.5	<0.001	BNB > BB BNB > L BB > L
Raoultella planticola	Health status	139	6.1	<0.05	Symptom. > asymptomatic
	Woodland	139	8.3	<0.05	SB > MW WF > MW WF = SB

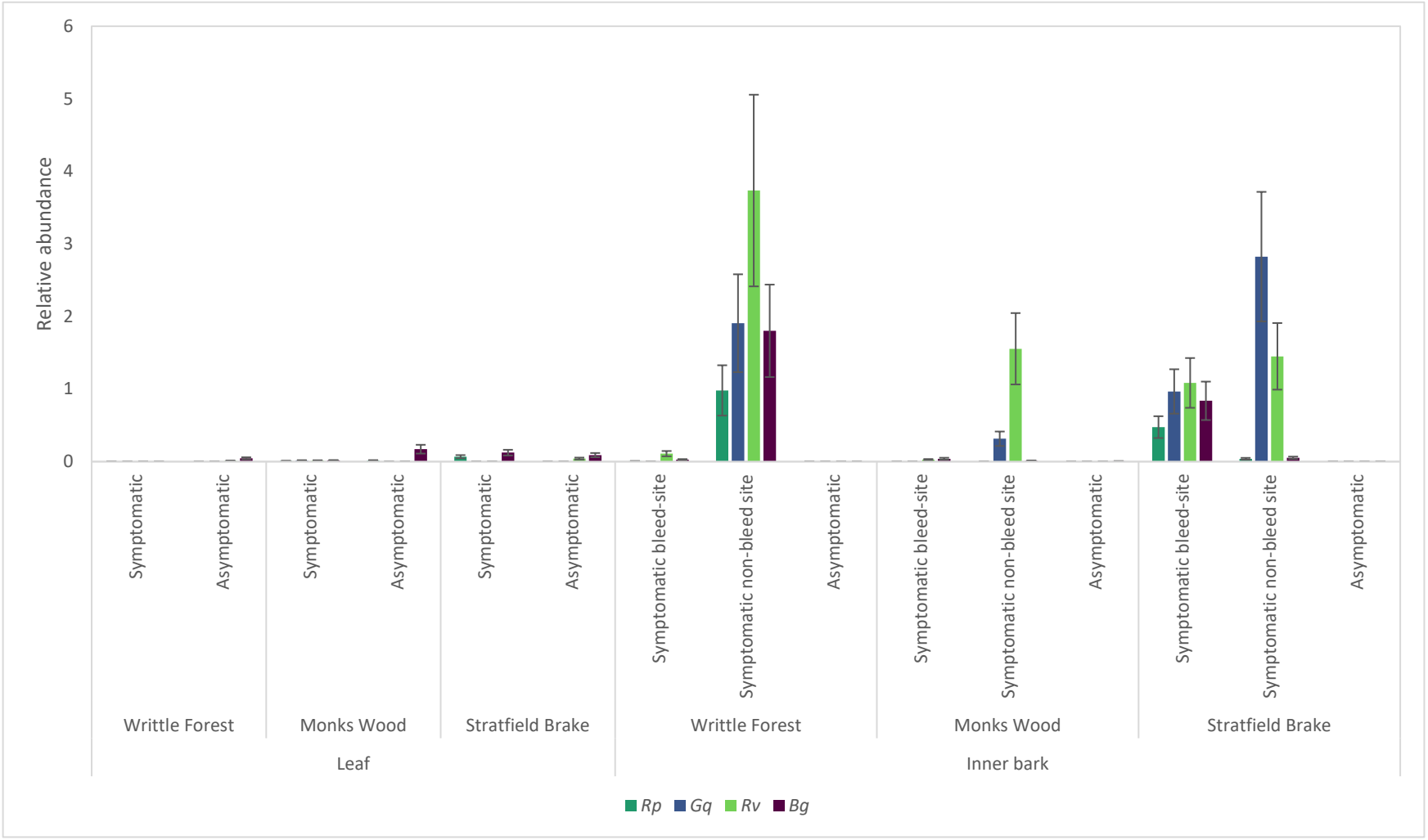

Figure 6.13 – relative abundance of the four bacterial species of interest in the *Quercus* leaf and inner bark samples. Error bars represent standard error

6.3.5. *Bark swab samples*

After using selective media and narrowing the search for the four bacterial species of interest (*Bg*, *Gq*, *Rv* and *Rp*) using ITS ribotyping, 17 colonies were sequenced using the gyrB region (*Table 6.6*). *Bq* and *Gq* were not found from any of the trees, not even from the bleed site of symptomatic trees. *Rahnella* species were frequently isolated from the bleed site but also away from the bleed site in symptomatic trees and on asymptomatic trees. *Rp* was isolated from one bleed site.

Table 6.7 – results of gyrB sequencing of the bacteria isolated from bark swab samples of symptomatic and asymptomatic trees in Stratfield Brake. The closest match here is from the NCBI GenBank database with the highest identity match.

Tree ID	Health status	Distance from bleed	Closest match	Identity match (%)
SD0054	Symptomatic	Bleed site	*Rahnella victoriana*	99.34
		Bleed site	*Raoultella planticola*	99.64
SD0056	Symptomatic	Bleed site	*Raoultella* sp.	98.35
		Bleed site	*Rahnella bruchi*	99.35
SD00736	Symptomatic	Bleed site	*Rahnella victoriana*	99.67
SD00737	Symptomatic	Bleed site	*Rahnella aquatilis*	94.40
		10cm from bleed	*Serratia* sp.	88.67
SD00739	Symptomatic	Bleed site	*Rahnella variigena*	99.84
SD00813	Symptomatic	Bleed site	*Rahnella victoriana*	100.00
		Bleed site	*Rahnella victoriana*	100.00
SD00816	Symptomatic	Bleed site	*Pseudomonas* sp.	92.49
		20cm from bleed	*Rahnella variigena*	99.84
SDX	Symptomatic	Bleed site	*Rahnella victoriana*	100.00
SH05	Asymptomatic		*Pseudomonas* sp.	89.99
			Rahnella victoriana	100.00
SH07	Asymptomatic		*Pseudomonas* sp.	88.78
SH09	Asymptomatic		*Rahnella victoriana*	100.00

6.4. *Discussion*

Distinctive fungal endophytic inner bark communities were associated with *Quercus* trees dependent on their health status. No differences in bacterial endophyte composition were recorded between asymptomatic *Quercus* trees and those displaying the characteristic stem bleeds associated with AOD for leaf and inner bark samples, although some differences in richness and diversity were recorded. While leaf fungal endophyte communities differed in richness, diversity and composition by woodland, bacterial endophyte communities were largely unstructured and only correlated with tissue carbon and nitrogen content at one site. Based on the results from this study there is no indication for systemic differences correlated with presence of bleeding lesions. However, it is not clear how such differences would affect the leaf internal environment in ways that are relevant to the endophytes.

6.4.1. *Effect of AOD on the endophytic community*

The fungal endophytic composition of asymptomatic *Quercus* inner bark was considerably different from those associated with trees showing symptoms of AOD. This directly contrasts with the study by Denman et al. (2016) that found no differences in fungal endophytic community associated with the bark of AOD symptomatic and healthy trees. However, Denman et al. (2016) only considered the cultivable fungal species, which is likely to have underestimated fungal diversity. Results here are more in line with those reported by Koskella et al. (2017) who also found a strong difference in endophytic species in bark from asymptomatic horse chestnut trees and those with symptoms of horse chestnut bleeding canker.

Given the differences in fungal endophytes between asymptomatic and symptomatic *Quercus* trees there may be an overlooked fungal component or components inciting the AOD syndrome. Disease complexes that involve the synergistic interactions of several plant

pathogens are now being recognised in the literature, for example brown apical necrosis of walnut is thought to involve a bacterial agent (*Xanthomonas arboricola* pv. *juglandis*) and several potentially opportunistic pathogens (*Fusarium* spp. and *Alternaria* spp.) (Belisario et al., 2002). More research is needed to determine if fungal pathogens or opportunistic pathogens are involved in the AOD decline syndrome.

Another explanation for the differences in fungal endophyte composition of asymptomatic trees is that these trees contain one (or more) fungal species that confers protective benefits to their host against AOD. As the fungal endophytes and the bacterial pathogens share the same ecological niche they may directly compete for space and nutrients (Arnold and Herre, 2003). Some fungal endophytes can produce antimicrobial metabolites such as terpenoids, alkaloids or extracellular enzymes such as cellulases and proteases that directly inhibit phytopathogens (Mousa and Raizada, 2013). Fungal endophytes may also protect the host from pathogen attack through induced systemic resistance i.e. the presence of the endophyte primes the host for pathogen attack which results in increased physical or chemical barriers of resistance (Ganley et al., 2008, Shoresh et al., 2010). The fungal endophytes present in asymptomatic trees may also possess plant-growth promoting activity (Doty, 2011) which could enable the tree to better defend against pathogen attack.

Further research is necessary to determine whether these fungal endophytes have the potential to inhibit disease progression or to promote plant growth. The next step would be to sequence the TRFs that are present in asymptomatic trees and not in symptomatic, and to determine whether they produce antibiotic compounds *in vitro* in response to the pathogens of interest. *In planta*, analyses would be necessary to determine if endophytes control pathogens through niche competition or through induced systemic resistance. It is possible, as with the pathogenic agents of AOD, that biological control would require a consortium of

endophytes for effective biological control i.e. a probiotic application of microbial endophytes (Podolich et al., 2014, Sarma et al., 2015).

It is possible that host genetic background may be a determining factor of tolerance to AOD which may also influence the composition of endophytes. Ash and elm trees that show resistance to a particular disease have been found to produce secondary metabolites that not only impact the pathogen but also endophytic fungi sharing the same ecological niche (Sollars et al., 2017, Marak et al., 2002, Martín et al., 2013). It is possible that fungal endophytes in asymptomatic trees were influenced by secondary metabolite production by the host. These secondary metabolites could also be produced by fungal endophytes or by the tree in response to infection by the AOD pathogens. For example, grapevines (*Vitis* spp.) infected by phytoplasma diseases (Bulgari et al., 2011) and citrus trees infected with citrus greening disease (or Huanglongbing) (Trivedi et al., 2010) have higher levels of reactive oxygen species (ROS), essential components of signal transduction cascades that lead to plant defences, such as the hypersensitive response and the salicylic acid pathway. Therefore, only endophytes that can overcome ROS stress can succeed in the endosphere (Kniskern et al., 2007).

In this study, no differences in bacterial or fungal endophyte community were recorded in the leaves of trees affect with AOD. As the AOD pathogens cause necrosis in the inner bark of trees, they share the same ecological niche as endophytes in the inner bark and are likely therefore to interact. Unless, AOD leads to systemic changes in the host plant such as increased chemical defences, the endophytes in the leaf are likely to remain unaffected. Martín et al. (2013) reported that changes in endophyte richness in response to Dutch elm disease was restricted to xylem endophytes where the pathogen attacks and did not extend to other plant parts such as the bark and leaves.

The composition of bacterial endophytes in the bark of trees with AOD did not differ significantly from asymptomatic trees. This is in accordance with a previous study of the effects of AOD on bacterial endophytes that showed little difference between healthy and symptomatic trees (Sapp et al., 2016) and between asymptomatic trees and trees affected by ash dieback (Schlegel et al., 2018). However, the alpha diversity of symptomatic trees was higher in the bark of trees in Writtle Forest and Monks Wood compared to asymptomatic trees. This contrasts with other studies that show that diversity of endophytic bacteria drops drastically in response to pathogen infection (Purahong et al., 2018, Koskella et al., 2017). Higher endophyte diversity in symptomatic trees may be as a result of compromised tree tissues which may in turn increase endophyte colonization from the surrounding environment. During the formation of necrotic lesions by the AOD pathogens the tree cell walls are broken down potentially facilitating the entry of endophytes (Trivedi et al., 2010)

There were considerable differences in composition, richness and diversity of fungal and bacterial microbial communities of bark samples from the bleed site and of the non-bleed site of a symptomatic tree, although it is unclear whether this is due to the temperature in which these samples were mistakenly stored. Further study is needed to determine if attack by AOD pathogens not only influences the site of bacterial infection but also directly or indirectly influences non-symptomatic tissue of the same tree as has been shown in horse chestnut bleeding canker (Koskella et al., 2017).

This study represents a one-time snapshot of the communities observed and it is not possible to determine whether the differences observed are due to endophytic composition shifts after infection by the disease/decline or whether these differences existed before pathogen colonisation. Future studies should take repeated samples from individual trees as the decline syndrome progresses to fully understand the changes in endophyte composition as a result of

AOD and how endophytes may be involved in facilitation or antagonism of the biotic factors involved.

6.4.2. *Effect of geographic location on the endophyte community*

Geographic location had a strong impact on the composition, richness and diversity of fungal endophytes in leaves and inner bark of *Quercus*, with marked differences between woodland sites. This suggests that fungal endophytes are locally adapted to their environment, which has also been shown in other tree species (Finkel et al., 2011, Sapp et al., 2016, Denman et al., 2016). These differences could reflect differing climatic conditions (Hashizume et al., 2008, Terhonen et al., 2011) but also through physiological, phenological or chemical changes in the host plant. Geographic location has been shown to influence the concentrations of polyphenolic compounds in *Quercus* trees as a result of differences in soil type, rainfall etc. (Prida and Puech, 2006, Zhang et al., 2015). As phenolics are known to have antimicrobial properties (Nagle et al., 2011, Karioti et al., 2011), these variations in concentrations are likely to impact the endophytic population. In future studies, the phenolic compounds of the trees should be considered. Differences in fungal endophyte composition between the tree sites could also be attributed to tree age, or the interaction between tree age and environmental factors (Khorsandy et al., 2015, Bernstein and Carroll, 1977), and should also be included in future studies.

On the other hand, geographic location had no effect on the bacterial endophytic communities of *Quercus* trees in this study. Another study found few differences in bacterial phylloplane communities of Ponderosa pine trees across geographic location with more variation found within individuals of the same site (Redford et al., 2010). This supports the theory that microbial species are seldom restricted by geographical barriers (Fenchel, 2003). However, the

geographic distances covered in this study were small (approximately 140km between woodlands). It is possible that with greater distance the endophytic communities would vary more significantly (Martiny et al., 2006). Understanding the differences in endophyte composition across geographic regions may help to explain the spread of AOD and requires further study.

6.4.3. *Effect of carbon and nitrogen leaf content on endophyte community*

The necrotic lesions within the bark of trees affected with AOD are thought to eventually effect the vascular system of the tree, leading to dieback in the canopy (Denman et al., 2014). Tree dieback has been linked with reduced carbon stores due to reductions in photosynthesis (Jordan, 2015, McDowell et al., 2008). Tree disease has been shown to reduce net CO_2 assimilation and alter stomatal conductance leading to reduced photosynthetic capacity (Hajji et al., 2009, Pinkard and Mohammed, 2006), although these changes have only been assessed in depth in foliar pathogens. Studies have shown that plant growth is directly related to the internal nitrogen content of a tree (Dickson, 1989). However, the effects of tree decline on nitrogen assimilation potential has not been studied in sufficient depth, nor has the effect of nitrogen on foliar communities. In this study, no differences were reported here in nitrogen content between symptomatic and asymptomatic leaves. For carbon, there were significant differences in amount dependent on health status but in Monks Wood carbon content was higher in symptomatic trees and in Stratfield Brake carbon content was higher in asymptomatic trees.

For epiphytic bacteria, the availability of carbon and, to a lesser extent, nitrogen in the leaves are major determinants of epiphyte colonisation (Lindow and Brandl, 2003), but very little research of this context has studied this effect in endophytic species. In this study, carbon and

nitrogen content had a limited effect on the endophytic species associated with *Quercus* trees. Carbon and nitrogen content did positively affect bacterial endophytes, but this effect was only recorded in one woodland, Stratfield Brake and may reflect the differences in sampling time of the three woodland sites.

A recent study observed differences in leaf reflectance of AOD symptomatic trees and asymptomatic (Gerard et al., 2018). It was therefore expected that stress caused by AOD would affect photosystem II efficiency and therefore result in decreased Fv/Fm values. However, there were no differences in Fv/Fm values between symptomatic and asymptomatic trees in any of the woodlands or between woodlands. The trees chosen for this study showed the 'bleed' symptoms but the extent to which the necrotic lesions had spread was not recorded. It is possible that the trees selected were in the early stages of decline and therefore, the vascular systems were not yet compromised. As with carbon and nitrogen availability, Fv/Fm values had limited impact on the composition of bacterial and fungal endophytes.

6.4.4. *Presence of AOD pathogens*

TRFs closely matching the size of the *Bg, Gq* and *Rv* control samples, were all isolated at high abundance from symptomatic bark, in accordance with other studies (Sapp et al., 2016, Denman et al., 2017, Denman et al., 2016) and consistent with the theory that these pathogens are involved in the formation of the bleeding canker symptom of AOD. A TRF matching the size of the *Raoultella planticola* control was also isolated from disease lesions. *Raoultella planticola* is known to be associated with soil, plant and aquatic environments (Drancourt et al., 2001). It has been known to cause rare cases of infection in humans (Skelton et al., 2017) but has not been associated with plant disease. However, *Raoultella* species have commonly been isolated from diseased *Quercus* bark (Booth, 2019, unpublished) and

potentially from asymptomatic *Quercus* trees (*Chapter 3*). The species would deserve further study as a possible inciting agent of AOD.

TRFs matching all four species (*Bg, Gq, Rv* and *Rp*) were also found in asymptomatic bark samples, although to a much lesser extent. *Bg*, *Gq* and *Rv* have been found in low abundance in healthy bark tissues in other studies (Sapp et al., 2016, Denman et al., 2017, Denman et al., 2016) and also in healthy leaf and twig tissues in woodlands with no recorded incidence of AOD (*Chapter 3*). In this study all four bacteria were believed also to be associated with symptomatic leaf tissue and *Bg* and *Rv* from asymptomatic leaves using TRFLP. *Rv* was also found associated with the outside of tree bark in asymptomatic trees and healthy bark of a symptomatic trees using selective sequencing. This raises questions about the existence of these bacteria as potential endophytes or epiphytes of asymptomatic tissue. As an endophyte, growth of these bacteria may be restricted by plant defences and/or antagonism by other endophytes, this may explain why detection in asymptomatic tissue is low and inconsistent. Evidence suggests that the nature of tree-endophyte relationships is relatively plastic and can change as a result of environmental or host factors (Moricca and Ragazzi, 2008, Kuo et al., 2014). It is possible that these four bacteria live as endophytes in a balanced interaction with the host, when this balance is disturbed by a predisposing factor of decline e.g. drought stress or by tissue necrosis caused by another inciting organism then they may switch to a pathogenic lifestyle taking advantage of their impaired host. Although these bacteria have been shown to form lesions in log inoculation and tree trials (Denman et al., 2017, Booth, 2019, unpublished), results have been unpredictable. It is possible that the successful formation of a lesion is dependent on the interactions between pathogens and associated microorganisms, in what has been recently termed the 'pathobiome' concept (Vayssier-Taussat et al., 2014, Jakuschkin et al., 2016).

Closely related to *Brenneria goodwinii*, *B. salicis*, the causal agent of willow watermark disease, has also been shown to be an endophyte in asymptomatic tissues of willow trees (Maes et al., 2009). *Brenneria salicis* is associated with a disease of the wood vessels but the pathogen was found to spread throughout the entire tree, including the leaves (Maes et al., 2009). Maes et al. (2009) proposed that leaf-to-leaf spread is a possible mechanism for *Brenneria salicis* dispersal (Maes et al., 2009), more study is needed to determine if this is the mode of dispersal of *Brenneria goodwinii*.

The role that *Rahnella victoriana* plays in AOD requires further consideration. In this study, the TRF matching *Rahnella victoriana* was recorded at a relatively high abundance in all the plant tissues studied, symptomatic and asymptomatic. *Rahnella victoriana* and other species of *Rahnella* have been recorded, also in high abundance, in other studies of *Quercus* (Denman et al., 2017, Booth, 2019, unpublished, Moradi-Amirabad et al., 2019) and as an endophyte in willow (Maes et al., 2009). *Rahnella victoriana* has been shown to possess the genomic capability to form tissue necrosis but *Quercus* log inoculations have proven to be unreliable (Denman et al., 2017). *Rahnella aquatilis* has shown promise as a biological control agent of grape crown gall (Chen et al., 2007) and fire blight (Laux et al., 2002) and as a plant growth promoter in *Picea abies* seeds (Cankar et al., 2005). More study is required to establish the role of *Rahnella* species in the tree ecosystem, are they simply ubiquitous commensalistic organisms, do they have a role in plant protection, or do they play a role in diseases such as AOD?

It must be noted that the use of TRFLP is not always accurate to species level identification and like next-generation sequencing is hindered by limited taxonomic resolution of the 16S rRNA gene for the *Enterobacteriaceae* family (Janda and Abbott, 2007, Naum et al., 2008). Species-specific primers should be developed for *Bg*, *Gq*, *Rv* and *Rp* to determine whether these

species are present as endophytes in asymptomatic tissues and to accurately determine the spread of these pathogens within the tree and the woodland environment.

6.5. *Conclusions*

The inner bark of symptomatic *Quercus* trees and those displaying symptoms of AOD have a different assemblage of fungal endophytes. At this stage it is unclear whether the differences observed are due to community shifts after infection by AOD or whether these differences existed pre-infection. These differences in endophyte fungal community were not reflected in the leaf environment. Geographic location of the woodland did however influence the fungal communities in both the leaf and inner bark. No differences in bacterial endophytic communities were noted between symptomatic and asymptomatic trees and no differences between woodland location.

TRFLP is an effective method to study the influence of abiotic and biotic conditions on microbial community structure, but further research is needed to allocate TRFs to species or genus level. This would allow for greater understanding of the roles that these endophytes play in the plant ecosystem. Identifying fungal endophytes in the inner bark of asymptomatic trees that are not present or at a lower abundance in symptomatic trees would allow for further *in vitro* and *in planta* investigation into potentially plant growth promoting or antagonistic endophytes that could be used as biological agents against AOD.

CHAPTER 7 - *Conclusions and future work*

This study has revealed how phenotypic variation of a tree host can play a defining role in the structuring of associated insect herbivore and microbial endophytic species, forming extended community phenotypes. Tripartite interactions between microbial endophytic species and insect herbivores within a *Quercus* study system were shown, including mutualistic, antagonistic and neutral relationships. The effect of an oak decline syndrome on microbial endophytes revealed a shift in fungal bark communities with implications for further research into biological control avenues.

7.1. *Bipartite interactions - host tree and insect herbivores*

This study demonstrated, for the first time, differences in the composition of insect taxa (species, genus or guild) between the two native oak species: *Quercus robur* and *Quercus petraea*. Interspecific differences in insect herbivores was particularly apparent for galling species, which may reflect the intimate relationship these insects share with their host plant. *Quercus robur* was also shown to support a higher richness and diversity of insect herbivores. This analysis provides evidence for oak species acting as ecological filters. There are morphological traits and chemical defence differences between the two species (Mosedale and Savill, 1996, Popovic et al., 2013) that may affect the selection and performance of insect herbivores. Metabolomic studies could be used to discern possible differences in plant defence metabolites between oak species and how these bioactive compounds are involved in the interaction between oak trees and their herbivores (Jansen et al., 2008).

This study used leaf morphometric analysis to differentiate between the two oak species revealing two distinct groups (Kremer et al., 2002). However, this method was unable to

determine if hybrid individuals were present in the BSO trial. As hybrid trees are likely to differ in their susceptibility to insect herbivory compared to either parent (Fritz et al., 1994, Boecklen and Spellenberg, 1990, Pearse and Baty, 2012), future research should use molecular methods to discern hybrid individuals and to confirm the accuracy of species assignment based on leaf morphometric analysis used in this study (Petit et al., 2002, Dumolin et al., 1995, Muir et al., 2000, Guichoux et al., 2011).

This study did not find evidence to support the local adaptation hypothesis that herbivores perform better on trees from local provenances (Egan and Ott, 2007). Few effects of tree provenances were recorded, and the greatest abundance of insects was not recorded on local provenance trees. The historical origin of the trees used in the BSO trial were not controlled, given the widespread historical movement of oak around Europe and the UK (Worrell, 1992, Petit et al., 2002), their provenance may not be a good indicator of shared co-evolutionary history. Sinclair et al. (2015) reported that studies that treat provenances as statistically independent entities are likely to underestimate the effect of tree provenance on insect abundance. Future studies should consider the genetic differentiation among populations, this could be achieved using pairwise F_{ST} values (Weir and Cockerham, 1984, Sinclair et al., 2015).

Oak provenances were shown here to vary significantly in phenotypic traits that are likely to influence insect herbivores. Consistent with other studies, herbivory by early season free feeders correlated strongly with phenotypic differences. The survival of these free feeding insects is reliant on synchrony between their emergence and budburst in the spring when leaves have a high nitrogen content and reduced tannin concentration (Feeny, 1970, Forkner et al., 2004). In general, mining insects preferred trees that budburst early and both agamic and sexual generations of galling insects preferred later bursting trees. Climate matching is likely to negatively affect galling insects and positively affect mining insects, as trees from further south are likely to burst earlier in the season (Ducousso et al., 1996, Deans and Harvey,

1995). Results from this study suggest that leaf marcescence may be an adaptation to late or deep frosts (Nilsson, 1983), as trees that retained their senescent leaves were later to burst in the spring, potentially protected by the senescent leaves. Marcescence may also be an adaptation to insect herbivory as it was shown to influence feeding by two non-cynipid gallers. Future research should consider the marcescence habit across latitudinal and altitudinal gradients to determine if leaf retention is correlated with incidence and timing of frost. Experimentally manipulating leaf retention could be used to further test the influence of marcescence on non-cynipid galling insects (Karban, 2007). Tree vigour, measured using DBH and shoot length, were consistent predictors of insect herbivory, with more vigorous trees supported a higher richness and diversity of insect herbivores. This was particularly evident in the sessile feeding guilds i.e. galling and mining insects.

The oak breeding seedling orchard (BSO) in Paradise Wood is replicated in seven other sites in the UK. This study should be repeated in these other sites to confirm the results shown here but also to investigate the effect of genotype-environment interactions on the insect herbivore communities across a broad geographic range. This would allow for the identification of tree populations that will perform well under future climates (Aitken et al., 2007) and also to determine the impacts of these non-local provenance trees on insect herbivore communities.

In conclusion, results show that climate matching would have variable impacts on oak herbivore communities. To minimise the risks of biodiversity loss as a result of climate matching, matched provenances could be mixed with local provenance trees to increase the niche space available for insect herbivores that are less able to interact with non-local provenance trees.

7.2. *Bipartite interactions - host tree and microbial endophytes*

Using culture independent techniques, the bacterial and endophytic species associated with the leaves and twigs of two *Quercus* species and two *Juglans* species were determined and the influence of tree provenance and tree phenotypic traits on these communities were recorded. Intergeneric differences in the composition of bacterial and fungal endophytes were recorded for the first time between *Quercus* trees and *Juglans* trees growing in the same locality. Around 60% of bacterial and fungal taxa (OTUs) were shared between the two genera. Interspecific differences in bacterial and fungal endophytes were also recorded for *Quercus robur* and *Q. petraea* and between *Juglans regia* and *J. nigra*. Other studies have also shown differences to occur between different tree species growing in the same location (Morrica et al., 2012, Redford et al., 2010, Whipps et al., 2008, Lambais et al., 2006), suggesting that tree species act as ecological filters i.e. selective forces influence the colonisation of endophytes from the environment. Differences in endophyte species composition was also dependent on the tissue that was sampled, thought to be due to the different biotic and abiotic characteristics of leaves and twigs (Leff et al., 2015). Tree provenance, tree phenology and tree vigour showed variable effects on endophytic species and few generalisations can be made.

A number of latent pathogens and saprophytes were found in the endosphere of asymptomatic *Quercus* and *Juglans* trees. Tree stress as a result of climate change may make these trees vulnerable to attack from these symbionts. More research is needed to understand the aetiology of these bacteria and fungi to understand what triggers an endophyte to become pathogenic. Of most interest here were species of *Brenneria* that were tentatively found as endophytes in *Quercus* and *Juglans* species. OTUs matching *Brenneria rubrifaciens*, the causal agent of deep bark canker of *Juglans* (McClean et al., 2008), were recorded in some *Juglans* trees and *Brenneria goodwinii*, a pathogen involved in Acute Oak Decline (Brady et al., 2017)

was recorded in asymptomatic *Quercus* trees. It should be noted, however, that the 16S sequencing used in this study is not considered a reliable method of determining members of the Enterobacteriaceae family due to the conserved nature of this genomic region. Using species specific primers for *Brenneria rubrifaciens* (McClean et al., 2008) did not detect this species in the *Juglans* tissue here, although more work is needed to develop this protocol for detecting the potentially low levels of bacteria. The development of species-specific primers for *Brenneria goodwinii* and the other pathogens involved in AOD is crucial if we are to understand whether these species are able to live as endophytes and to further understand the factors involved in disease progression.

7.3. *Tripartite interactions - host tree, insect herbivores and microbial endophytes*

Tripartite interactions between host, insect herbivores and microbial endophytes have rarely been studied in tree ecosystems (Eberl et al., 2019). The majority of studies consider the effect of a single endophyte on a single insect species, and the results have been varied from negative (Butin, 1992) to neutral (Faeth and Hammon, 1997) to positive (Gange, 1996). This is the first known study to consider the effects of the endophytic bacterial and fungal community isolated from a tree using culture independent methods on the community of insect herbivores. Results were characterised into two categories with evidence to support each theory: (1) *passive interaction*, where tree traits that influence insect herbivores also positively or negatively influence endophytes and (2) *active interaction*, where the presence of one organism affects the other negatively or positively, this could be through *direct interactions* where one organism affects the other without any influence from the plant or *indirect interactions*, through plant mediated effects. Consistent with previous studies, results from this study showed positive, negative and neutral interactions between endophytes and insects. Results could not be generalised for each insect feeding guild, and neither guild was more or

less affected by the presence of endophytes in general. Manipulative experiments are required to determine the cause and effect of endophyte presence on herbivore abundance, but this is likely to be difficult in the field as endophyte-free trees are almost impossible to produce (Sieber, 2007).

This study has produced a vast amount of data particularly for two UK native oak species in a provenance trial in the UK which includes: (1) a record of the major insect herbivore species, (2) a record of the endophytic microbes associated with the leaves and twigs and (3) a record of tree phenotypic traits such as phenology and vigour of the same trees. This database is a useful resource for further analysis. For example, the results presented here consider bacterial and fungal endophytic populations separately, the interactions between both groups inside the tree and their effects on insect abundance is an area of research that has not yet been studied in detail but holds great potential. The data presented here should be assembled into a database where species of interest could be searched and interactions between species of interest could be analysed in the future.

7.4. *Tripartite interactions - host tree, microbial endophytes and pathogens*

Trees were chosen for this study that displayed the characteristic bleed symptoms of AOD. The necrotic lesions within the bark of trees are thought to eventually effect the vascular system of the tree leading to dieback in the canopy (Denman et al., 2014). However, the photosynthetic potential and carbon and nitrogen content of the leaves was not consistently reduced in symptomatic trees compared to asymptomatic trees. Bacterial and fungal endophytes associated with the leaves did not differ between symptomatic and asymptomatic trees, which may be expected if the effects of the decline syndrome have not yet reached the leaves.

Differences in fungal endophyte community were, however, recorded in the inner bark of symptomatic trees compared to asymptomatic trees. It is difficult to establish if these differences are due to community shifts after infection by AOD or whether these differences existed pre-infection. It is possible that asymptomatic trees harbour endophytes that provide plant-growth promoting properties which allow them to adequately defend against pathogen infection or they have endophytes that can actively defend against AOD pathogens i.e. through competition or antagonism. Species identification and culturing of these fungi that are not present or are at a lower abundance in symptomatic trees are required. *In vitro* and *in planta* study would allow for the identification of potential biological control agents for AOD.

Geographic location of the woodlands was a major determining factor in the composition of fungal endophytes associated with *Quercus* trees. Therefore, the fungal endophytes associated with the inner bark of symptomatic and asymptomatic trees should be sampled from other woodlands in the UK. If asymptomatic trees are host to fungal endophytes that protect the tree from AOD, trees outside the current range of AOD should also be sampled. It would be of interest also, to consider oak trees in Europe and the US where similar decline syndromes have been recorded (Thomas et al., 2002, Biosca et al., 2003, Moradi-Amirabad et al., 2019, Brady et al., 2014).

Special attention should be given to *Rahnella* species, that were consistently isolated in high abundance in this study as a leaf and twig endophyte of asymptomatic trees in AOD-free woodlands, as an epiphyte on the bark of symptomatic and asymptomatic trees and as an endophyte in symptomatic and asymptomatic inner bark. The role that *Rahnella victoriana* plays in the pathobiome of AOD is not clear (Brady et al., 2017), and requires further investigation.

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REVIEWS

Endophytes vs tree pathogens and pests: can they be used as biological control agents to improve tree health?

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Abstract Like all other plants, trees are vulnerable to attack by a multitude of pests and pathogens. Current control measures for many of these diseases are limited and relatively ineffective. Several methods, including the use of conventional synthetic agro-chemicals, are employed to reduce the impact of pests and diseases. However, because of mounting concerns about adverse effects on the environment and a variety of economic reasons, this limited management of tree diseases by chemical methods is losing ground. The use of biological control, as a more environmentally friendly alternative, is becoming increasingly popular in plant protection. This can include the deployment of soil inoculants and foliar sprays, but the increased knowledge of microbial ecology in the phytosphere, in particular phylloplane microbes and endophytes, has stimulated new thinking for biocontrol approaches. Endophytes are microbes that live within plant tissues. As such, they hold potential as biocontrol agents against plant diseases because they are able to colonize the same ecological niche favoured by many invading pathogens. However, the development and exploitation of endophytes as biocontrol agents will have to overcome numerous challenges. The optimization and improvement of strategies employed in endophyte research can contribute towards discovering effective and competent biocontrol agents. The impact of environment and plant genotype on selecting potentially beneficial and exploitable endophytes for biocontrol is poorly understood. How endophytes synergise or antagonise one another is also an important factor. This review focusses on recent research addressing the biocontrol of plant diseases and pests using endophytic fungi and bacteria, alongside the challenges and limitations encountered and how these can be overcome. We frame this review in the context of tree pests and diseases, since trees are arguably the most difficult plant species to study, work on and manage, yet they represent one of the most important organisms on Earth.

Keywords Endophytes · Biological control · Trees · Pathogen · Pest · Disease

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Introduction

Importance of trees and their diseases

Being some of the largest organisms on Earth, trees in forest and woodland settings cover 40% of the Earth's terrestrial surface (Fao 2010). This forms a major part of the global biomass and provides habitat for large numbers of animal and plant species with varying levels of association. To humans, the importance of trees for food, timber and non-timber resources has been historically and widely identified (Cazorla and Mercado-Blanco 2016). Carbon sequestration is one of the most significant ecosystem services provided by trees, with

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total carbon stocks in UK forests (including soil) alone calculated at 800 megatons (Quine et al. 2011).

The economic value of trees and shrubs in urban landscapes has been increasingly recognised since the turn of the millennium, with the popularisation of electronic tools for estimating this value, e.g. i-Tree software, and research revealing a suite of functional benefits; providing habitat to urban wildlife, reducing air pollution, intercepting rainfall, shading and reducing heat absorption by man-made surfaces (Binner et al. 2017; Nowak 2004; Tyrväinen et al. 2005; Xiao and Mcpherson 2002). The cultural services provided by trees are also significant, offering both physical and mental health benefits. The capitalised value of the social and environmental benefits provided by woodlands and forests in the UK alone was estimated to be over £29 billion (Lawrence et al. 2009; Willis et al. 2003).

However, trees in particular can be susceptible to attack from pests and diseases (Table 1), especially if invading from other geographical locations (Hansbrough 1965; Tubby and Webber 2010). Most diseases are caused by microbial pathogens (fungi, bacteria and viruses), the effects of which are amplified during periods of unfavourable environmental conditions including unseasonal temperature shifts and extremes in rainfall patterns (Cazorla and Mercado-Blanco 2016) and anthropogenic climate change (Dukes et al. 2009; La Porta et al. 2008; Sturrock 2012; Sturrock et al. 2011; Tubby and Webber 2010).

Tree pest invasions are also increasing alongside climate change and expanded global trade and may act in tandem with native or invasive diseases, as vectors or co-occurring on hosts, to greatly reduce the populations of particular tree species (Brasier 2008; Tubby and Webber 2010), with the potential to ultimately cause their local extinction.

Plant susceptibility to pests and diseases is often related to the stress level of the individual. Unfortunately, trees in urban areas, which have a particularly high value to humans, often face high stress levels. In urban areas, stress can arise from mismatching of the planting stock's ecological traits to the planting site, root deformities, damage and desiccation, planting at improper depths in unsuitable soils, poor nutrient and water availability, and increased exposure to pollutants, xenobiotics and contaminants (Aldhous and Mason 1994; Ferrini and Nicese 2002; Gilman et al. 2015; Grossnickle 2005; Pauleit 2003; Percival et al. 2006; Pfeiffer et al. 2014; Sjöman and Busse Nielsen 2010). Monocultures also pose a specific problem, as plants grown in monoculture are more susceptible to pest and disease outbreaks and are sensitive to changes in climate, which are less likely with polycultures (Sjöman et al. 2012). Lax biosecurity, including the importation of planting stock and tree products, can also drive biological invasions by tree pests and diseases, as has been demonstrated in Europe (Brasier 2008; Epanchin-Niell 2017; Potter et al. 2011). Some non-native pests are highly destructive and can cause substantial damage to forests and urban/suburban trees (Aukema et al. 2011). Such invasions often lead to significant changes in forest structure and species composition, which in turn lead to changes in ecosystem functions (Lovett et al. 2016). Given the range of pests and diseases that trees are facing, the long generation time of trees, the practical difficulty of working with many of them, and also the speed with which the environment is changing, we are faced with a very difficult challenge – how do we improve our disease and pest management to help trees survive?

Classical control approaches for tree pests and diseases

The application of plant protection products (PPPs) for the control of tree pests and diseases is already often limited by ecological concerns and modulated by the particular local context, as exemplified by the varied management of oak processionary moth, *Thaumetopoea processionea*., in Europe (Tomlinson et al. 2015). However, PPPs are well accepted within commercial tree fruit production and the tree care industry of North America.

Presently, PPPs are generally synthetic chemicals that disrupt the cellular function, or life cycle of the target organism. Other PPPs work on a physical basis e.g. killing insect or acarid targets on contact via suffocation, or abrasion of the exoskeleton and subsequent desiccation. These products are typically those formulated for use in agriculture. Aboveground and external tree pests and diseases are often controlled with aqueous sprays of PPPs to the foliage and bark. Specialised high-pressure spray systems can be used for such applications to large trees (Hirons and Thomas 2018).

Internalised pests and diseases, such as nematodes, are more difficult to reach due to their physical concealment within the host; adjuvants (additives) may improve the penetration of externally applied PPPs for such targets e.g. through bark (Garbelotto et al. 2007),

Table 1 Examples of some current major pathogens and pests of trees

Pathogen	Host	Symptoms	Reference
Oomycete *Phytophthora ramorum*	*Larix* spp. and *Quercus* spp. (sudden death)	Shoots and foliage can be affected. Visible as wilted, withered shoot tips with blackened needles. Trees with branch dieback can have numerous resinous cankers on the branches and upper trunk. It has killed millions of native oak and tan-oak trees in the USA	Davidson et al. (2003)
Fungus *Ceratocystis fagacearum*	*Quercus* spp. (wilt)	Symptoms vary between oak species. White oaks may suffer from scattered dieback in the crown to the death of a single limb of major fork. Red oak succomb to the disease usually within a month. Early foliar symptoms start as vein banding whch later develop to foliar necrosis. Thus far only recorded cases in the USA.	Juzwik et al. (2008) Yang and Juzwik (2017)
Fungus *Ceratocystis platani*	*Platanus* spp.	Wound coloniser causing cankers, xylem staining and restriction of water flow throughout the tree resulting in eventual death of the tree. In oriental plane, *Platanus orientalis*, parts of the crown can suddenly die. Can be identified by cankers on the trunk, defined by bluish-black to reddish-brown discolouration of sap-wood and necrosis of the inner bark. Found in the United states and across Europe, such as in Greece, France and Turkey.	Ocasio-Morales et al. (2007) Lehtijarvi et al. (2018)
Fungus *Hymenoscyphus fraxineus*	*Fraxinus* spp. (Chalara ash dieback)	*Dark brown/orange lesions on leaves, diamond-shaped lesions may occur on stems which, if girdled, can cause wilting. The wood beneath lesions usually is strongly stained. Dieback can be seen throughout the crown, with dieback shoots and twigs at the edges of crowns. Originating in Asia but a serious pathogen across Europe.*	*Landolt* et al. *(*2016*)* *McMullan* et al. *(*2018*)*
Fungus *Cryphonectria parasitica*	*Castanea* spp. (blight)	Attacks the bark by entering cracks or wounds which may lead to crown dieback. Discolouration of the bark and dead bark forms sunken cankers. Pin-head sized, yellow-orange pustules develop on the infected bark and exude long, orange-yellow tendrils of spores in moist conditions. Pale brown mycelial fans may be found in the inner bark. Discolouration of the bark may be more visible in younger trees. Widespread thoughout the eastern US, China, Japan and many European countries with an abundance of sweet chestnut.	Rigling and Prospero (2017)
Fungus *Ophiostoma ulmi* and *O. novo-ulmi*	*Ulmus* spp. (Dutch Elm Disease)	Symptoms emerge in early summer as clusters of wilting/yellowing leaves that turn brown and fall. A mixture of healthy and suffering foliage may be seen as the disease progresses throughout the tree. Affected shoots die back from the tip and twigs may turn down-wards. Exposing the outer wood on symptomatic twigs should reveal dark brown or purple streaks. When cut across, a dark brown stain may be present in the outer wood. Common across Europe, North America and Western Asia.	Brasier and Buck (2001)
Fungus *Rigidoporus microporus*	*Hevea* spp. (white root rot)	Fungal mycelium can be found on the tree collar. Multi-layered fruiting bodies form at the base of the tree and white/white-brown rhizomorphs can be seen on the root surface. Off season flowering may occur as well as yellow-brown discolouration of the foliage. Significant funal pathogen to timber and rubber industry in Indonesia, Malaysia, Sri Lanka and the Ivory coast.	Siri-udom et al. (2017) Khairuzzaman et al. (2017) Hamidson and Naito (2004)
Fungus *Colletotrichum acutatum*	*Olea* spp. (anthracnose)	Fruit rot. Soft to dark brown rot that produces an orange, gelatinous matrix in moist conditions and	Talhinhas et al. (2011)

Table 1 (continued)

Pathogen	Host	Symptoms	Reference
		mummification in dry as the fruit loses moisture. In spring there may be extensive yellowing of the leaf blade which in summer leads to premature fall of infected leaves. Found in the majority of olive growing countries.	Cacciola et al. (2012)
Fungus *Cytospora chrysosperma, Phomopsis macrospora,* and *Fusicoccum aesculi*	*Populus* spp. (canker)	Young twigs form brown, sunken, rough circle areas in the bark which may spread to the larger branches. Large cankers may form on the branches an trunk. Orange/orange-brown discolouration of bark is often seen exuding orange-brown viscouse liquid. Fruiting bodies in the bark make the canker appear pimpled. In later stages of infection, perithecial stroma form in the dead cankered areas. Widespread across North America, Europe and China.	Ren et al. (2013)
Fungus *Heterobasidion spp.*	Conifers and some deciduous trees	Symptoms may vary depending on the pathogen involved and host plant. White root rot fungus that in early stages of growth causes staining and discolouration of the host wood. Initial decay is usually pale yellow, developing to light brown and resulting in a white pocket rot with black flecks. Eventually results in tree death. Widespread across the Northern Hemisphere and cases in Australia.	Asiegbu et al. (2005) Garbelotto and Gonthier (2013)
Fungus *Dothistroma septosporum* and *Dothistroma pini*	Conifers (Dothistroma (red band) needle blight)	Yellow bands on needles develop into red bands, where small, black fruiting bodies can occur. Can cause needle dieback, defoliation and eventual tree death. Occurs worldwide. Severe cases in Southern hemisphere plantations of New Zealand, Australia, Chile and Kenya. Also found in North America, Canada and Europe.	Schneider et al. (2019) Barnes et al. (2004) Bradshaw (2004)
Bacterium *Xylella fastidiosa*	*Vitis* spp., *Citrus* spp., *Olea* spp. and several species of broadleaf trees	Leaf scorch/browning, wilting foliage and withering of branches. In extreme cases can result in dieback and stunted growth. Cases found in the Americas, Taiwan, Italy, France and Spain.	Simpson et al. (2000) Araújo et al. (2002) Almeida et al. (2019)
Most likely a decline syndrome with possible Bacterial pathogen components: *Brenneria goodwinii*, *Gibbsiella quercinecans, Rahnella victoriana*	*Quercus* spp. (Acute oak decline)	Stem bleeds occur on the trunk, weeping dark, translucent liquid. Bark cracks, which may reveal underlying dark, necrotic tissue. Lesions and 'D' shaped exit holes of *Agrilus biguttatus* may be present in the bark.	Denman et al. (2014)
Chronic oak dieback – Complex disorder or syndrome (also referred to as oak decline, dieback-decline)	*Quercus* spp. (particularly *Q. robur*)	Results from a combination of abiotic and biotic factors. Early foliage deterioration, gradual branch death and dieback in the crown. Abiotic stressors and weakening of trees allows for opportunistic attack from insects and disease which can result in tree death. Seen in the UK and across Europe.	Thomas et al. (2002) Gagen et al. (2019) Mitchell et al. (2019)
Bacterium *Xanthomonas citri* subsp. *citri*	Citrus cultivars (canker)	Distinct raised, necrotic lesions on fruits, stems and leaves. As the disease progresses, lesions on the stem can appear as corky, rough, dead tissue with a yellow halo. Present in South America, Africa, Middle East, India, Asia and South Pacific.	Graham and Leite (2004) Ference et al. (2018)
Bacterium *Erwinia amylovora*	Pome trees and rosaceous plants (fireblight)	Affects all above ground parts of the plant. The floral recepticle, ovary and peduncles turn a greyish green, eventually whithering to black. Creamy white bacterial droplets may emerge from affected tissues in humid conditions. Shoots wilt rapidly, forming 'Shepard's crooks', that turn necrotic. In later stages, bark becomes	Mohan and Thomson (1996) Johnson (2015) Schropfer et al. (2018)

Table 1 (continued)

Pathogen	Host	Symptoms	Reference
		cracked, sunken and may leak amber bacterial ooze. Found across North America, Central Europe, Israel, Turkey Lebanon and Iran.	
Bacterium *Candidatus Liberibacter* spp.	Citrus trees (Huanglongbing disease)	Blotchy, asymmetric mottling of newly mature leaf blades. Fruit may exhibit stunted growth, premature drop and low soluble acid content. Found across Asia, America and Africa.	Kalyebi et al. (2015)
Bacterium *Pseudomonas syringae* pv *aesculi* (*Phytophthora cactorum* and *Ph. plurivora*)	*Aesculus hippocastanum* (Bleeding canker of Horse Chestnut)	Rusty-red/brown/black gummy ooze found on the bark. Dead phloem under the bleeds which may appear mottled orange-brown. In extensive cases where affected areas encircle the trunk or branch, leaf yellowing and defoliation may occur and eventual crown death. Fungal bodies may also be seen in areas of dead bark. Found across the UK and Europe, including France, Netherlands, Belgium and Germany.	Webber et al. (2008) Green et al. (2009) Green et al. (2010)
Asian longhorn beetle *Anoplophora glabripennis*	Wide range of broadleaved trees	Adults are about 20-40mm long, black with white makings and long, black and white antennae. Oval shaped pits on scraoed into the bark where eggs are laid, occasional sap may be visible bleeding from the damaged areas. Galleries in bark may be up to 10mm in diamteter and several cm long. Wood shavings may be found in distinctive chambers where pupation occurs. Large, circular exit holes from emerging adult beetles in the upper trunk and branches, usually 10mm in diameter. Piles of sawdust may be found at the base of infested trees. Originally from China and the Korean peninsula, now found in the USA, Italy and across the EU.	McKenna et al. (2016) Haack et al. (2010)
Beetle *Dendroctonus micans*	*Picea* spp.	Resin bleeding on stems with resin tubes coloured purple-brown with bark particles where the female enters the trunk. Attacks may occur anywhere on tree. Found across Europe.	Yaman et al. (2010) Wainhouse et al. (1990)
Leaf miner *Cameraria ohridella*	*Aesculus* spp.	In early summer, elongated blotches appear white at first but turn brown throughout the foliage. Caterpillars or pupal cocoons may be seen within mined areas. Heavily infested trees may drop their leaves prematurely. Spread throughout central and eastern Europe.	Pocock and Evans (2014) Gilbert and Tekauz (2011)
Lappet moth *Dendrolimus pini*	*Pinus* spp.	Needle defoliation and subsequent tree death. Prescence of cocoons on trunk. Native to Europe, Russia and Asia.	Ray et al. (2016)
Oak processionary moth *Thaumetopoea processionea*	*Quercus* spp.	Voraciously feed on the foliage of oak trees. Large populations lead to significant defoliation, making the tree susceptible to other threats. Found in Central and Southern Europe. In the UK, outbreaks are localised to London and a few neighbouring counties.	Freer-Smith et al. (2017) Quero et al. (2003)
Ambrosia beetle (Black timber bark beetle), *Xylosandrus germanus*	Wide range of hardwood host species	Infestations can be indentified by entry holes into the bark and distinctive, compact cylindrical frass about 3-4cm in length. Other indications of their presence include wilting and yellowing of the leaves, defensive sap production and dieback. Native to East Asia but has spread across North America, Europe and the Caucasus region.	Agnello et al. (2015)
Citrus longhorn beetle *Anoplophora chinensis*	Deciduous and shrub species	Adult males are about 21mm long, females 37mm. They are black with white markings, with distinctive, long antennae. Symptoms include feeding damage from adult beetles on bark and twigs, circular exit holes in bark and 'T' shaped oviposition slits where eggs are laid within the bark tissue. Tunnelling in bark and larval galleries may cause structural weaknesses, disrupt the	Eschen et al. (2015) Eyre et al. (2010) Haack et al. (2010)

Table 1 (continued)

Pathogen	Host	Symptoms	Reference
		vascular system and result in eventual plant death. Native ranges of China, Japan and Sout East Asia although incidences have occurred in Europe, such as in the Netherlands, Germany, France, Italy, Croatia, Switzerland and the UK.	
Oak Pinhole Borer *Platypus cylindrus*	*Quercus* and other hardwood species	Adult beetles are blackish in colour, 5-7mm long. Usually establishing in stressed trees, galleries about 1.6mm wide are made in the bark with bore dust appearing pale and fibrous. The beetles introduce ambrosia fungi for their nourishment, principly *Raffaelea* spp., which stains the surrounding wood blacky-brown. Found across Europe and North Africa with some incidences occuring in healthy Portuguese trees.	Belhoucine et al. (2011) Bellahirech et al. (2016) Inácio et al. (2011)
Pine wood nematode *Bursaphelenchus xylophilus*	*Pinus* spp. (wilt disease)	Discolouration of some/many branches from green to yellow. Rapid loss of resin flow occurs in 48 hours. Found in Japan, China, Korea, Taiwan, Portugal and Spain.	Futai (2013) Odani et al. (1985)

although many products will still not be transported significantly within the tree. Some PPPs can be injected into the vascular system at the base of the tree and transported upwards e.g. emamectin benzoate used in the control of Emerald Ash Borer larvae in North America (Smitley et al. 2010). The neonicotinoid compound imidacloprid is a soil applied insecticide that is taken up through roots and into the whole plant. However, neonicotinoids face severe restrictions on their use in many countries due to associations with negative impacts on bees (Goulson 2013). Control of fully internalised diseases of trees are also a particular issue, for instance, one of the difficulties in controlling *Verticillium dahliae* and *Xylella fastidiosa* in olive (*Olea europaea*) and grapevine (Baccari and Lindow 2011) is due to the inaccessible location of the pathogen within the vascular system (Cazorla and Mercado-Blanco 2016). Similar difficulties are faced in the control of Huanglongbing disease, *Candidatus liberibacter* spp., which causes citrus greening and is a phloem-limited phytoplasma spread by insect vectors (Abdullah et al. 2009).

Root and soil-borne pathogens have been treated by injections into the soil of PPPs or sterilizing agents such as phenolic compounds or methyl bromide gas (Martin 2003; West and Fox 2002). While many synthetic PPPs break down quickly when exposed on stems or foliage, soil applied compounds may persist for extended periods once bound to soil particles (Edwards 1975).

Stump treatments, e.g. urea, sodium borate, or the saprobic fungus *Phlebiopsis gigantea*, have also been applied to exclude and reduce the build-up of fungal pathogens that can also utilize buried dead wood saprobically, often *Heterobasidion* spp., but may also exclude *Armillaria* spp. and other basidiomycetes, while allowing non-pathogenic species to proliferate (Nicolotti and Gonthier 2005; Nicolotti et al. 1994; Vasiliauskas et al. 2004).

In Europe, and elsewhere, environmental concerns have fuelled a movement away from synthetic "chemical" PPPs or those based on toxic heavy metals e.g. copper (Lamichhane et al. 2018). In the absence of other effective controls this reduction in authorised pesticides may conflict with protecting vital resources such as food and timber.

Biocontrol agents (BCAs)

An area that is gaining much more attention in recent years is biological control (or biocontrol) – the use of biological agents to counter a pest or disease. The desired outcome of a biological control application is to reduce the pathogen or pest population below a threshold of ecological and economic impact, ideally enabling the host to regain health and eventually restoring the invaded community to the pre-invaded state (Bale et al. 2008). This approach is highly favourable because most BCA source species are already present in the host's environment, and in some cases provide a narrow range

of target specificity, so are less likely to be harmful to non-target organisms. BCAs can come in many forms, from viruses or bacteriophage, to bacteria or fungi, and even higher organisms like nematodes, mites or insects (Lenteren et al. 2018).

As PPPs, BCAs are generally applied in similar ways to synthetic compounds and the selected application method typically aims to maximise contact with the target organism. *Bacillus subtilis* QST 713 is a commercialized bacterial strain used in biocontrol programmes around the world (Abbasi and Weselowski 2014). For foliar pathogens like *Botrytis* of fruit or nut trees, *B. subtilis* QST 713 is applied as an aqueous spray, whereas for protection against *Phytophthora* root rots it is applied as an aqueous drench, e.g. via pressurized soil injection systems or irrigation. BCAs may also be physically incorporated into soils (Abbasi and Weselowski 2015). For example, *Trichoderma* strains, often grown on a solid food source such as grain, but also as spore powders, are variously mixed into the soil around roots or placed in cores in close proximity to roots for the treatment of root diseases (Srivastava et al. 2016). One study demonstrated that trunk injections of various *Bacillus* strains into the vascular system of Avocado trees, *Persea americana*, reduced the disease severity of *Phytophthora cinnamomi* infections (Darvas and Bezuidenhout 1987). However, as with the majority of studies discussed in this review, this control method does not appear to have been commercialized or widely utilized to date.

Nematodes, which are used against slugs and snails or insect larvae feeding on roots within the soil, may be dispersed in water and applied to the target area as a drench. Although relatively understudied, nematodes and other soil microfauna e.g. springtails, also have potential in the integrated control of soil borne fungal plant pathogens (McGonigle and Hyakumachi 2001, Riffle 1973, Tomalak 2017). Control of stem boring *Zeuzera pyrina* larvae has been demonstrated by injecting nematode suspensions into the stem cavities created by the larvae (Ashtari et al. 2011). The spores of *Verticillium* strain WCS850 have been applied to Elm trees (*Ulmus* spp.) via punctures in the bark of the tree to induce host resistance to Dutch Elm Disease, caused by the pathogens *Ophiostoma ulmi* and *O. novo ulmi*. The BCA itself does not move far from these sites and the disease is controlled via plant-mediated effects (Scheffer et al. 2008).

Natural enemies are also a popular option for biological control of insect pests in agroforestry settings (Dix et al. 1995). Insects as BCAs have shown great applicability for controlling pests of woody plants, forming around 55% of such introductions up until 2010. The establishment rates of natural enemies and success rates were higher when targeting pests of woody plants than other pests (Kenis et al. 2017). Aphids cause extensive economic losses around the world, as one of the major pest groups of crops plants but are also problematic for trees. To control and counter this, aphid predators, including ladybird larvae, lacewings and gall midges as well as adult spiders, carabids (Carabidae) and rove beetles (Staphylinidae) are used in integrated pest management strategies (Evans 2009; Gardiner & Landis 2007; Messelink et al. 2013; Schmidt et al. 2004; Snyder & Ives 2003). However, the efficiency of control is limited due to insufficient post-application persistence, slow kill rate and high host specificity, in combination with generally high production and maintenance costs, and thus contribute to restricted use in pest control.

The greatest challenges of using BCAs with trees, however, relate to the scales associated with trees – many are very large, thus restricting access to the whole tree and canopy, and woodlands can occupy great areas. Arguably, there is also a dearth of information on many tree diseases and pests, especially for newly emergent outbreaks where monitoring endeavours are struggling to keep up with the incidence and speed of outbreaks (Boyd et al. 2013).

Other major challenges in developing BCAs are the identification, characterisation, formulation and application of the agents. Laboratory analyses may not be reliable predictors of the protective capability of biocontrol agents. For example, the modes of action for most BCAs are still not fully understood, and there is no efficient and effective screening method for identifying field-competent BCAs by laboratory tests (Parnell et al. 2016). The development of appropriate screening methods for BCAs may therefore rely on studies of their interaction with plants, which would slow the screening process. Factors affecting production and delivery of a BCA from laboratory to field include loss of viability, storage stability, environmental conditions, compatibility with other microorganisms, and consistent efficacy over multiple time periods including seasonal variations (Bashan et al. 2013; Slininger et al. 2003). In comparison to synthetic PPPs, storage requirements for preserving BCA product efficacy can be far more varied and

particular, which has been a major issue historically (Bashan et al. 2013; Corkidi et al. 2005).

In this review, we will examine the options for using BCAs for tree diseases and consider how they might be used. In particular, we will focus on endophytes, which are a relatively understudied group. Presented here are examples of endophytes reported as biological control agents in the literature, and most have not been commercialized, but have proven effects under laboratory conditions.

Endophytes as BCAs

Endophytes are defined as microorganisms that accomplish part of their life cycle within living host tissues without causing apparent damage to the plant (Schulz and Boyle 2005; Sun et al. 2014). In all ecosystems, many plant parts are colonized by endophytes (Brundrett 2002; Mandyam and Jumpponen 2005). Depending on the species and the interaction, endophytes may be located in roots, leaves or needles, shoots, or adapted to growth within the bark (Grünig et al. 2008; Rodriguez-Cabal et al. 2013; Sokolski et al. 2007; Verma et al. 2007). Endophytes may grow inter– and intra– cellularly as well as endo– and epi– phytically (Schulz and Boyle 2005; Zhang et al. 2006). However, endophytes can switch their behaviour depending on a set of abiotic and biotic factors, including the genotypes of plants and microbes, environmental conditions, and the dynamic network of interactions within the plant biome (Hardoim et al. 2015; Schulz and Boyle 2005).

Plant ecosystems rely heavily on their microbial communities to optimise health (Pfeiffer et al. 2014), though this intimate association can be a fine balance between mutualism and disease (Knief 2014). Microbes (as epiphytes) can colonise the surfaces of roots (rhizoplane) and leaf/shoot (phylloplane) as well as the internal spaces of plants (as endophytes), with overall abundance being higher for epiphytes compared to endophytes, and rhizosphere compared to phyllosphere (Lindow and Brandl 2003). These differences may reflect the short life span of leaves, nutrient richness in the rhizosphere, and the ability of microorganisms to survive in soil in a dormant state for long periods of time (Vorholt 2012) or due to the physiochemical variations between these two respective environments (Lindow and Brandl 2003).

Endophytes can act in defence against pathogens and disease (Ownley et al. 2004), as well as provide protection or act as deterrents to insect herbivores and nematodes (Breen 1994; Slippers and Wingfield 2007; Vega et al. 2008). However, these defensive properties may not be unanimous to every endophyte-host-pathogen interaction, as shown by Gonthier et al. (2019) where investigations into the protective benefits of ectomycorrhizal fungus *Suillus luteus* in Scots pine (*Pinus sylvestris*) against the fungal pathogens *Heterobasidion irregular* and *Heterobasidion annosum* found that it only reduced host tree susceptibility to *H. annosum*, not both pathogens.

The roles of endophytes in disease and pest resistance are comparatively understudied, but recent work has started to highlight the importance of endophytes, in particular, as an increasingly popular biological control option (Dutta et al. 2014; Gao et al. 2010). Endophytes are also being increasingly recognised as potential controls of significant economic threats such as the invasive spotted lanternfly in North America (Eric et al. 2019).

Isolation and identification of endophytes

Traditionally, the research of endophytes has focussed on identification of culturable fungi and bacteria from plants has involved culturing them from plant tissue on different media. Although successful, it is apparent from the use of culture independent approaches (e.g. metagenomics), that the true diversity and abundance of the endophytic community has not been fully represented or utilized (Bisseling et al. 2009). As a result, it is highly likely that a range of potential candidate organisms with beneficial and exploitable biocontrol capabilities are being overlooked (Moricca et al. 2012; Ragazzi et al. 2001). Slower growing endophytic species are likely to be outcompeted or inhibited in the medium by more rapidly growing species. Other species may be as yet unculturable due to lack of a key growth component, because of an obligate relationship with their host plant for survival or due to a range of environmental parameters. Culture-dependent methods tend to favour the dominant endophytic species, so rarer species that have an irregular existence, are likely to be missed in any sampling effort (Moricca and Ragazzi 2008). However, methods used to isolate, and study endophytes have continued to be improved in light of developments in genetics and genomics. The advancements in next generation sequencing (NGS) has greatly improved the study of endophytes by allowing enormous amounts of genetic sequence data to be processed in parallel at a

fraction of the cost of traditional methods (Knief 2014; Rastogi et al. 2013). Metagenomic analysis employing NGS of whole microbial communities allows much deeper and more accurate DNA sequencing, thus providing insight into the composition and physiological potential of plant-associated microorganisms. NGS reveals both culturable and unculturable endophytes that may be beneficial microbes and appropriate isolation media can then be developed to further study these species of interest (Akinsanya et al. 2015). For example, the presence of endophytic fungi in roots of different plant species in a temperate forest in Japan were identified using NGS (Toju et al. 2013), while whole genome analysis of endophytic microbes has revealed the genetic features that directly or indirectly influence the various bioactivities and colonisation preferences (Kaul et al. 2016). Identification, isolation and characterisation of genes involved in beneficial endophyte-host interactions is critically important for the effective manipulation of the mutualistic association between the two. Endophyte genomic analysis has provided a new tool to pick apart the mechanisms of endophytic associations and to reveal the requisite features needed to inhabit plants. Studies have revealed a wide range of specific genes commonly found across genomes that are important for endophytic lifestyles and symbioses. These include genes coding for nitrogen fixation, phytohormone production, mineral acquisition, stress tolerance, adhesion and other colonization related genes (Firrincieli et al. 2015, Fouts et al. 2008, Kaul et al. 2016, Martínez-García et al. 2015).

Examples of tree endophytes as BCAs

As BCAs, endophytes have diverse mechanisms of action, categorised into direct, indirect or ecological effects (Gao et al. 2010). Endophytes may possess the ability to directly inhibit pathogens by producing antifungal or antibacterial compounds. For example, the endophytic bacterium *Bacillus pumilus* (JK-SX001) is particularly efficient at reducing the infection rate and severity of canker caused by three pathogens (*Cytospora chrysosperma*, *Phomopsis macrospora* and *Fusicoccum aesculi*) in Poplar cuttings. This *Bacillus* strain produces a combination of extracellular enzymes (including cellulases and proteases) and other secondary metabolites that are thought to inhibit the mycelial growth of the pathogen (Ren et al. 2013). When *B. pumilus* (JK-SX001) was applied as a root drench, the bacterial cells migrated from the roots up to the leaves and were reported to also increase host photosynthetic activity and ultimately increase biomass production in the saplings, while suppressing pathogenic activities. These results were promising, but the experiments were performed under greenhouse conditions using fast growing, young cuttings that were sensitive to the canker pathogens. These young trees were easier to inoculate and probably more likely to respond to pathogens and colonisation by endophytes than mature trees.

In another study, the pathogen *Phytophthora meadii*, which causes abnormal leaf fall of rubber trees (*Hevea brasiliensis*), was suppressed using the endophytic bacterium *Alcaligenes* sp. (EIL-2) isolated from healthy rubber tree leaves. In dual cultures, *Alcaligenes* sp. (EIL-2) produced a substance that inhibited hyphal growth of the pathogen. When the endophyte was applied as a foliar and soil drench to one-year old greenhouse plants prior to infection by the pathogen, infection rates were reduced by more than 50% (Abraham et al. 2013). Whilst promising in scope, trials need to be conducted in natural systems to ascertain effectiveness *in situ*.

Species of *Pseudomonas* were the most commonly isolated endophytes antagonistic to the oak wilt fungus, *Ceratocystis fagacearum* (Brooks et al. 1994). When grown in dual culture, these endophytes produced siderophores and/or antibiotic compounds in response to the pathogen. Injecting trees with *Pseudomonas denitrificans* reduced crown loss to *C. fagacearum*, but the response varied depending on what time of year the inoculum was introduced. Seasonal changes affect the physiological state of trees and therefore the availability of nutrients in their vascular system to the introduced endophytes (Brooks et al. 1994), which is likely to influence efficacy of the endophytic BCA.

Berger et al. (2015) compared foliar applications of phosphite, and the endophytes *Trichoderma aureoviride* UASWS and *T. harzianum* B100 on reducing the necrotic area of *Phytophthora plurivora* lesions on oak leaves (*Quercus robur)*. Results showed that given the diffusable nature of phosphite it was able to reduce necrosis on both treated and untreated leaves. However, with UASWS and B100, only untreated leaves showed reduced necrosis suggesting that the interaction was affected by a number of fungal secondary metabolites. However, when applied via trunk injections (endotherapy) a similar endophyte,

T. atroviride ITEC was able to significantly reduce the necrosis size, compared to the control and the phosphite treatment, on 30-year-old beech trees (*Fagus sylvatica*) artificially inoculated with *P. plurivora*. It is clear from this example that the effectiveness of an endophytic BCA is likely to be influenced by the mode of application.

Endophytes may induce such delocalized plant defence reactions, called induced systemic resistance, leading to a higher level of host tolerance toward pathogens (Robert-Seilaniantz et al. 2011; Zamioudis and Pieterse 2012). There is increasing evidence that in the initial stages of endophyte colonisation, interactions between beneficial microorganisms and plants trigger an immune response in plants similar to that against pathogens, but that, later on in the plant growth stage and/or interaction stage, mutualists escape host defence responses and are able to successfully colonize plants (Zamioudis and Pieterse 2012). The shoot endophyte *Methylobacterium* sp. strain IMBG290 was observed to induce resistance against the pathogen *Pectobacterium atrosepticum* in potato, in an inoculum density-dependent manner (Pavlo et al. 2011). The observed resistance was accompanied by changes in the structure of the innate endophytic community. Endophytic community changes were shown to correlate with disease resistance, indicating that the endophytic community as a whole can play a role in disease suppression (Pavlo et al. 2011). Inoculation of white pine (*Pinus monticola*) seedlings with native fungal endophytes reduced disease severity caused by *Cronartium ribicola,* the causal agent of white pine blister rust. The results were temporally persistent suggesting a form of induced resistance. However, the authors did not measure any gene expression or defence pathways to confirm this hypothesis (Ganley et al. 2008).

Inoculation of part of a plant with an endophyte may benefit plants via the production or suppression of phytohormones; for example, genes encoding proteins for biosynthesis of indole acetic acid (IAA) (Zúñiga et al. 2013), cytokinins (CKs) (Bhore et al. 2010) and gibberellins (GAs) (Shahzad et al. 2016) are often present in the metagenome of plant endophytic bacterial communities (Liu et al. 2017). Induction of jasmonic acid biosynthesis enhances localized resistance to biotic agents such as *Hylobius abietis* (large pine weevil) (Heijari et al. 2005), *Ceratocystis polonica* (bluestain fungus) (Krokene et al. 2008; Zeneli et al. 2006) and *Pythium ultimum* (white root rot) (Kozlowski et al. 1999). Mycorrhizae can influence tree susceptibility and tolerance to economically important root pathogens such as *Heterobasidion* spp. and *Armillaria mellea*, even in the absence of direct antagonism of the pathogen by the endophyte (Gonthier et al. 2019; Nogales et al. 2010). Mycorrhizae are well recognized for their positive influence on tree growth and health so may antagonise pathogens via plant-mediated responses or ecologically through inhabiting the same niche, as is seen in other endophytes. The economically important tropical tree, *Theobroma cacao,* is a natural host to endophytes that can significantly reduce the foliar damage caused by a *Phytophthora* species (Arnold et al. 2003). Leaves inoculated with endophytes showed reduced leaf necrosis and mortality when exposed to the foliar pathogen compared to endophyte-free leaf controls. The method of defence appears to be either direct or ecological and not one of induced plant resistance. Only leaves inoculated with the endophytes were resistant to *Phytophthora* infection. This may pose a problem for feasible endophyte application as a BCA if effective disease control is dependent on each individual leaf being sprayed with the endophyte inoculum.

Host-associated microbes can colonize the host horizontally via the environment, vertically from within the parent to the offspring, or by mixed transmission modes (Bright and Bulgheresi 2010). Ecological and evolutionary relationships affect transmission mode and vice versa (Frank et al. 2017). Theory predicts that vertical transmission evolves when symbiotic partners are mutualistic, as a way to ensure faithful transmission of the beneficial symbiont from one generation to the next (Herre et al. 1999). Vertical transmission of bacterial symbionts from parent to offspring is, indeed, common in systems where the symbiont provides an indispensable function, as in the extensively studied nutritional symbioses between bacteria and insects (Moran 2006). Vertical transmission via seeds is also well documented for certain groups of fungal endophytes, e.g., the well-studied *Epichloë* fungal endophytes of grasses (Schardl 2001).

Entomopathogens including fungi, nematodes and bacteria, naturally play important roles in regulating insect populations and are being exploited in biocontrol strategies (Lacey et al. 2015). Miller et al. (2002) investigated the effects of endophytic organisms in white spruce trees (*Picea glauca*) on the pest spruce budworm. They observed that larval growth was significantly

affected by the presence of the endophytes, with some strains proving toxic to the insects. Production of endophytic metabolites is thought to have been the antagonistic factor and in a follow-up study conducted by Miller (2008), the presence of rugulosin toxin produced by the needle endophyte in nursery grown *P. glauca* significantly reduced budworm (*Choristoneura fumiferana*) growth. Decreased palatability for insects and antagonism towards pathogens of needles might be possible benefits for the host trees. Sieber (2007) also found that the colonization of elm bark by *Phomopsis velata* had significant effects on two beetle pests of bark, *Scoltus scolytus* and *Scolytus multistriatus*. These beetles are known vectors of the Dutch Elm disease pathogen *Ophiostoma ulmi* and on introduction of *P. oblonga*, there was a noticeable reduction both in beetle galleries as well as larval success rate thus providing evidence in support of an effective biocontrol agent. In addition to the discovery of more effective isolates and toxins, an increase in the use of entomopathogens will rely on innovations in formulation and better delivery systems.

Challenges in biocontrol of tree pathogens and pests with endophytes

Climate change has and will continue to alter the ranges of pests and diseases and aid their establishment by subjecting plants to stress (Shaw and Osborne 2011). Occurrence of extreme temperatures and weather events, such as heatwaves and flooding, are increasing in frequency worldwide as CO_2 levels increase and thus our natural capital may require active management to protect its current condition (Fischer and Knutti 2015; Hailey and Percival 2015).

Climate change is predicted to have a profound impact on the distribution, abundance, physiology, productivity, phenology, behaviour and ecology of all plant species (Hughes 2000; Nooten et al. 2014). Forest species are particularly susceptible to climate change as the higher longevity of trees hinders rapid adaptation (Broadmeadow et al. 2005; Lindner et al. 2010). Moreover, climate change is known to impact plant-associated microbes some of which play critical, mutualistic roles in maintaining healthy environments. For example, climate change is likely to impact the dispersal of mycorrhizal fungi, key symbionts of trees, which may in turn limit tree migration and colonisation of novel habitats (Pickles et al. 2015). Warming may induce a decrease in arbuscular mycorrhizal fungal colonisation, as has been demonstrated in a manipulated Mediterranean climate, with a likelihood of significant impacts on plant communities and ecosystem function (Wilson et al. 2016).

It has also been recognised that changes in the environment or host can alter the nature of the host-endophyte interaction (Schulz and Boyle 2005). When a tree is subjected to physiological or environmental stress the intimate plant-endophyte relationship is altered and the endophyte may become pathogenic. For example, the fungal endophyte *Discula quercina,* which inhabits healthy *Quercus cerris* trees, causes damage to host structure and function when the tree experiences drought stress (Moricca and Ragazzi 2008; Ragazzi et al. 2001). *Picea abies* (Norway spruce) and other conifers are predicted to become unsuitable for forestry in the central regions of Europe due to rises in temperature (Breymeyer 1996; Fanta 1992), especially at lower elevations (Lexer et al. 2002), which may subject the present large spruce forests to severe stress in the future. Similarly, altered climates may affect BCA function and efficacy. Climate change may also change the lifecycles and feeding behaviour of phytophagous insects, with vector-mediated impacts on tree disease spread (Battisti 2008). It is therefore possible that we will observe an increasing incidence of disease in trees caused by endemic endophytic species, in addition to and potentially interacting with highly destructive pest invasions.

Numerous factors play a role in the under implementation of BCAs for control of diseases in trees and woody plants. These include the size, area, complex root system, inoculum size, and impact of release on the associated ecological system. Many of the difficulties are shared with conventional PPPs, such as reaching internal pests and diseases. Arthropod pests of crops and trees are extremely diverse like their hosts and thus can be notoriously hard to control. Rapid reproduction rates of some of these species (e.g. aphids, gypsy moth, and spruce budworm) mean dense infestations can arise rapidly, reaching levels damaging the plants that then leads to losses that impact both the environment and local and regional economies. Furthermore, different life cycle stages mean that one method of control may not be adequate to manage a pest population effectively, such as in the case of scale insects (Mansour et al. 2017). There is a general consensus that the detrimental effects of insect pests on crops and woody plants are set to worsen with accelerated climate change and control of these will be required for agriculture to keep up with the demands of a growing world population (Dukes et al. 2009).

Effects of introduction of 'alien' species. What are the consequences?

It is as yet unclear as to whether endophytes introduced as BCAs on plants may be effective in reducing disease, but another important aspect is to understand if they have adverse effects on the natural microbial community of the host when the plant is under environmental stress. The introduction of endophytes that have not co-evolved with the host plant may result in the loss of beneficial organisms and so negatively impact the host plant (Whipps 2001). Furthermore, it is important to consider whether the gains provided by the endophyte outweigh the costs associated with it. For example, gall wasps are a problematic species to trees. However, *Apiognomonia errabunda*, the dominant endophyte in beech leaves, has been found to cause abscission of galls by forming necrotic tissue around the affected area, but this may, in time, prove to be more harmful to the host than the gall would have been (Sieber 2007). Furthermore, there is also a risk that some endophytes may not be as useful as hoped in integrated pest management systems as they may affect the efficacy of other BCAs employed to combat pest species. Bultman et al. (2017) found that although endophytes proved effective against plant herbivores, they had repercussions higher up the trophic chain, significantly affecting the performance of parasitoids by reducing pupal mass, which would reduce the parasitoids' success as a BCA.

Pros of using endophytes as BCAs

1. No known adverse environmental effects of native endophytic biological control agents as they are already present in the plant ecosystem.
2. Ability to colonize internal host tissues, the same ecological niche as pathogens, allowing persistence and competition for resources in addition to antagonism. The internal tissue also protects the biological control agent from dangerous UV rays and temperature fluctuations therefore they can persist for longer periods of time.
3. As well as controlling infection, endophytes may simultaneously promote plant growth, for example by increasing photosynthetic activity (Ren et al. 2013).
4. Narrow range of target specificity, less likely to be harmful to non-target organisms.
5. Endophytes may induce systemic resistance in the host and may consequently induce resistance against other pathogens and/or pests (Zamioudis and Pieterse 2012).
6. Pathogens may be less likely to acquire resistance to endophytic BCAs than they are to pesticides due to dynamic interactions.

Cons of using endophytes as BCAs

1. Most research to date has taken place in laboratory conditions, but it is unknown how the endophyte-pathogen interaction will alter in the presence of changing environmental conditions and competition with other organisms in the tree ecosystem.
2. More research must be conducted to find the optimum time for delivery of biocontrol agent inoculum, as seasonal changes in weather and tree physiology could alter efficacy (Brooks et al. 1994).
3. In some cases, resistance to pathogens is isolated only to the plant part that is inoculated with endophytic control. Delivery, and systemic transmission, of BCA to whole tree is likely to be difficult in many cases.
4. Possible changes in host-endophyte-pathogen interaction with climate change, could the endophyte itself become a pathogen? (Moricca and Ragazzi 2008; Ragazzi et al. 2001).
5. Endophytic BCA may alter the microbial community of the host tree, which may adversely affect the host or may have consequences at higher trophic levels.

Conclusion

With growing concern about environmental pollution and the harmful effects of chemicals, the use of biological control as an alternative environmentally friendly option is becoming necessary. The traditional breeding of trees for resistance remains one potential route, but it is a strategy that might be outpaced by the spread and introduction of pests and diseases, as well as being a time consuming and sometimes difficult task. Despite the challenges confronting biocontrol of tree diseases and

pests, research shows that endophyte treatments can be successfully implemented and there is clear potential for endophytes to be applied to trees as BCA in the future. However, it is unclear how the endophyte enters the plant tissues and disperses throughout the plant. The efficacy of the biocontrol method can be enhanced by integrating it with complimentary cultural and environmental conditions to stimulate plant health and enhance inhibition of the pathogen or pest, but this still requires more attention in the future. Advancements in molecular techniques, such as NGS, are revealing more accurate community structures and, as new environments are studied, it is very likely that new bacterial and fungal species will be discovered and enable the dissection of community effects of individual organisms. Application of community analysis and metagenomics technologies in future studies will advance understanding in both plant-microbe associations and biological control science, with endophytes being prime candidates for use as BCAs.

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Compliance with Ethical Standards

Conflict of interest The authors confirm no conflict of interest.

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Appendix

APPENDIX A - *Media, buffer and gel preparation*

A.1 *Media preparation*

The following media were prepared in one litre of deionised water (Avidity Science, Duo™) and sterilised by autoclaving at 121°C (15psi) for 20 minutes. Cooled to around 50°C, 20mL of medium was poured into 90mm triple vented Petri dishes (SLS Select). Once solidified, agar was stored inverted at 4°C until use, agar was warmed to room temperature prior to inoculation. For broths, 10mL was transferred to 25mL glass universal bottles before autoclaving as above.

Nutrient agar (NA) - a general purpose medium used to culture a wide variety of microorganisms. 28g/L dehydrated culture media (Oxoid Ltd) boiled to dissolve.

Potato dextrose agar (PDA) – a general purpose medium used to culture fungi. 39g/L dehydrated culture media (Neogen) boiled to dissolve.

Luria Bertani agar (LA) – nutrient rich medium used for the cultivation of members of the Enterobacteriaceae family (Lennox, 1955). 10g/L tryptone, 5g/L yeast extract, 5g/L sodium chloride, 15g/L agar, boiled to dissolve, pH adjusted to 7.5.

Luria Bertani broth (LB) – nutrient rich broth used for the cultivation of members of the Enterobacteriaceae family (Lennox, 1955). 10g/L tryptone, 5g/L yeast extract, 5g/L sodium chloride, 1g/L glucose, boiled to dissolve, pH adjusted to 7.5.

MacConkey agar with crystal violet, sodium chloride and 0.15% bile salts (MAC) – a selective and indicator medium for isolating Gram-negative bacteria (MacConkey, 1905). Enteric

bacteria that are able to ferment lactose are detected using the pH indicator, neutral red. 51.55g/L dehydrated culture media (Sigma-Aldrich) boiled to dissolve.

A.2 *Buffer and solution preparation*

Phosphate buffered saline (PBS) – a salt solution that helps to maintain a constant pH, it is isotonic and non-toxic to cells. 8g/L sodium chloride, 0.2g/L potassium chloride, 1.15g/L di-sodium hydrogen orthophosphate and 0.2g/L potassium di-hydrogen orthophosphate dissolved in one litre of deionised water and pH adjusted to 7.3. Sterilised by autoclaving at 121°C (15psi) for 20 minutes. Stored at room temperature.

1M Tris solution – 121g/L tris base dissolved in 800mL deionised water, adjusted to pH7.5 with concentrated hydrochloric acid, and made up to 1L with deionised water. Sterilised by autoclaving at 121°C (15psi) for 20 minutes. Stored at room temperature.

0.5M EDTA solution – 186.1g/L ethylenediaminetetraacetic acid dissolved in 800mL deionised water, adjusted to pH7.5 with concentrated sodium hydroxide, and made up to 1L with deionised water. Sterilised by autoclaving at 121°C (15psi) for 20 minutes. Stored at room temperature.

Tris-borate-EDTA buffer (TBE) 5X – used in preparation of agarose gels and for running electrophoresis. 54g/L tris base and 27.5g/L boric acid dissolved in 800mL of deionised water, pH adjusted to 8.0. 20mL 0.5M EDTA solution (above) added and made up to 1L with deionised water. 100mL of 5X buffer diluted with 900mL deionised water to a working concentration of 0.5X. Stored at room temperature.

Tris-EDTA buffer – solubilises DNA and protects it from degradation. 10mL of 1M Tris pH7.5 (above) and 0.2mL made up to 1L with deionised water. Sterilised by autoclaving at 121°C (15psi) for 20 minutes. Stored at room temperature.

5M sodium chloride solution – 292g/L sodium chloride dissolved in 800mL of deionised water, made up to 1L with deionised water. Sterilised by autoclaving at 121°C (15psi) for 20 minutes. Stored at room temperature.

5M lithium chloride solution – 211.95g/L dissolved in 800mL deionised water, made up to 1L with deionised water. Sterilised by autoclaving at 121°C (15psi) for 20 minutes. Stored at 4°C.

3M sodium acetate solution – 246g/L dissolved in 800mL deionised water, pH adjusted to 5.2 with glacial acetic acid and made up to 1L with deionised water. Filter sterilised though 0.2µM filter membrane. Stored at room temperature.

A.3 *Agarose gel electrophoresis*

Gels were self-cast using molecular grade agarose (Bioline) heated until dissolved in 0.5X TBE buffer (Appendix A2). The concentration of agarose ranged from 1%-3%, depending on requirements. GelRed® (10000X in water, Biotium) was added at a final concentration of 1X. Loading buffer (5X, Bioline) was mixed with DNA samples to a final concentration of 1X and samples were run in a horizontal gel tank (Alpha Laboratories) typically at 90V for 40 minutes in 0.5X TBE buffer. HyperLadder™ 1kb (Bioline) was run in tandem with samples, and DNA bands visualised and photographed using a UV light box (G:BOX, Syngene).

APPENDIX B - *Surface sterilisation preliminary study*

The effectiveness of two surface sterilisation methods were tested using leaves of the genus *Quercus*. Thirty leaves were collected at random from multiple branches of a mature *Quercus robur* tree on the University of Reading, Whiteknights Campus, Berkshire. Transferred to sterile plastic zip-lock bags, half the leaves were stored at -80°C and the other half at 4°C to test whether storage temperature affected the effectiveness of the surface sterilisation method. After seven days, five leaves from -80°C and five leaves from 4°C were washed with tap water to remove debris and surface sterilised using one of two methods (*Supplementary table B.1*). The same number of leaves were also treated with a simple sterile ultrapure (Avidity Science, Duo™) water wash as the control treatment. All transfers took place under a laminar flow cabinet and all tools were sterilised with ethanol and flamed. Leaves were air-dried in a laminar flow cabinet and the final wash solution from each method was retained for analysis. The effectiveness of these surface sterilisation methods was tested using both culture dependent and independent techniques.

Supplementary table B.1 - two surface sterilisation methods tested using *Quercus robur* leaves. [a] Dilutions by volume from household bleach with a sodium chloride concentration of 4.6% [b] Sonics & Materials, Inc., VCX130. All solutions were made in 50mL falcon tubes and leaves were transferred between tubes using sterilised forceps in a laminar flow. Tubes were shaken by hand.

Method name	Steps
Method A modified from Schulz et al. (1993)	96% ethanol, shaken for 30 seconds Sterile ultrapure water, shaken for 30 seconds 33% sodium hypochlorite solution[a], shaken for 5 minutes 96% ethanol, shaken for 30 seconds 4 washes with sterile ultrapure water, shaken for 1 minute
Method B modified from Gweon et al. (2015)	70% ethanol, shaken for 1 minute 33% sodium hypochlorite solution[a], shaken for 5 minutes 70% ethanol, shaken for 30 seconds 0.05% Tween 20 solution, ultrasonic processor probe[b] 40% amp for 3 minutes 4 washes with sterile ultrapure water, shaken for 1 minute

Culture dependent analysis

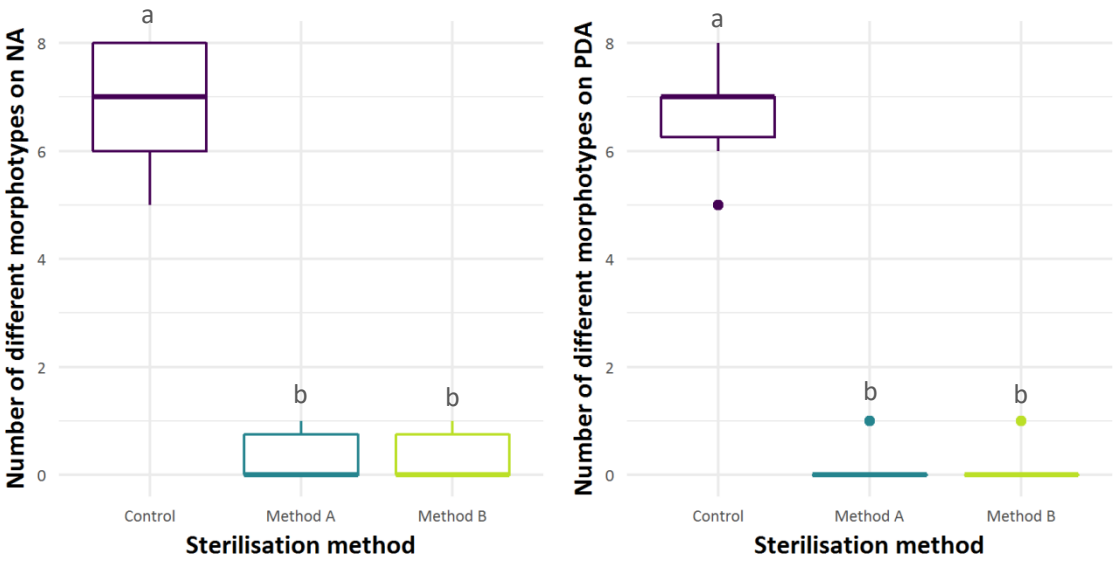
To optimise growth of all bacterial species, nutrient agar (NA) was used and for fungi, potato dextrose agar (PDA) (Basu et al., 2015). Four 20mm squares were cut from each sterilised leaf using a sterile scalpel, two were pressed onto nutrient agar and two onto PDA; leaf pieces were left for one minute before removing the leaf and discarding. 100µL of the final wash solution from each sample was also spread onto both nutrient agar and PDA using a sterilised glass spreader. All plates were incubated at 27°C for four days. After four days the bacterial and fungal endophytes were grouped into morphotaxa based on shared morphological traits, such as colour, colony shape and texture. The number of different morphotypes was recorded for each treatment.

As the response variable was in the form of counts a generalised linear model with Poisson errors was computed using the *MASS* package (Version 7.3-51.3) (Venables and Ripley, 2002). The explanatory terms included in the model were: sterilisation method, storage temperature and an interaction factor between the two. Terms were tested for significance using Chi-squared tests and non-significant terms were removed to achieve the minimal adequate model. Models were tested for overdispersion of the residuals using the *sjstats* package (Version 2.7.9) (Lüdecke, 2018) and for heteroscedasticity using residual plots (Crawley, 2007).

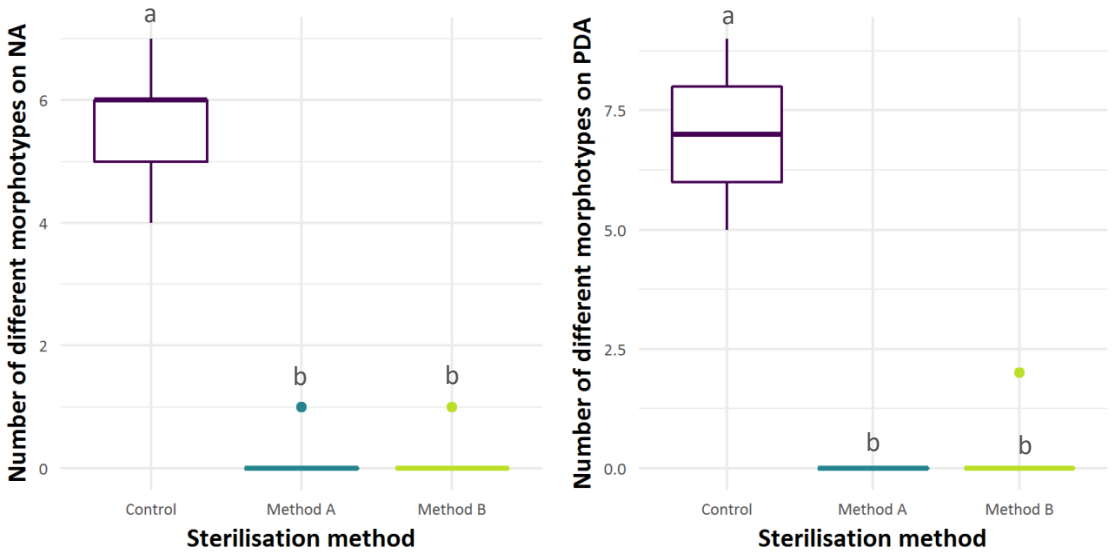
Both methods were effective at removing culturable epiphytic bacteria and fungi from the leaf surface (*Supplementary table B.2*, *Supplementary figure B.1 & B.2*). Storage temperature and the interaction between storage temperature and sterilisation method were not significant. There were no differences between the two surface sterilisation techniques.

Supplementary table B.2 – results from the GLM Poisson model, comparing the effectiveness of two surface sterilisation methods for removing bacterial and fungal epiphytes to a control treatment for both culture dependent methods.

Method	Kingdom	Signif. variables	df	χ^2	p
Leaf imprint	Bacteria	Sterilisation method	27	-114.7	<0.001
	Fungi	Sterilisation method	27	-125.2	<0.001
Final wash	Bacteria	Sterilisation method	27	-96.9	<0.001
	Fungi	Sterilisation method	27	-139.9	<0.001



Supplementary figure B.1 – number of different morphotypes growing on the two agars, NA for bacterial species (left) and PDA for fungal species (right), after imprinting with surface sterilised leaves. The two sterilisation techniques are compared here to the control treatment. Box plot shows minimum, first quartile, median, third quartile, maximum and outliers.



Supplementary figure B.2 – number of different morphotypes growing on the two agars, NA for bacterial species (left) and PDA for fungal species (right), after spreading the final wash solution on the agar. The two sterilisation techniques are compared here to the control treatment. Box plot shows minimum, first quartile, median, third quartile, maximum and outliers.

Culture independent analysis

To test whether the sterilising agent had penetrated the leaf surface and denatured the endophytic DNA, DNA was extracted from the surface sterilised leaves and the amount recovered was compared to the control leaf. Six 5x5mm squares were cut from each leaf and placed in a microcentrifuge tube. Using 50mg of sterile acid washed sand and a micropestle, the leaf samples were ground to a fine powder. DNA was extracted using Qiagen DNeasy plant mini kit following kit instructions.

To test if the sterilising agent had successfully removed all epiphytic DNA, DNA was extracted from 200µL of the final wash solution using the same extraction kit. A spectrophotometer (NanoDrop™ ND-1000, Thermo Fisher Scientific) was used to test quality and quantity of DNA. PCR's were performed using generic bacterial and fungal primers. PCR reactions were performed in a volume of 20µL containing 10µL 2X PCR buffer (MyTaq™ Mix, Bioline), 1.6µL each of forward and reverse primers (10µM, Eurofins Genomics, *Supplementary table B.3*) and made up with molecular grade water. Extracted leaf DNA was diluted to 20ng/µL and 0.8µL was used in the PCR. 0.8µL of the extracted wash DNA was used in the PCR. PCR conditions for ITS were: 95°C for 10 minutes; 35 cycles of 94°C for 20 seconds, 47°C for 30 seconds, 72°C for 40 seconds and a final extension at 72°C for 7 minutes (Toju et al., 2012). For 16S primers: 95°C for 3 minutes; 30 cycles of 95°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute and a final extension of 72°C for 10 minutes (Coenye et al., 1999).

Supplementary table B.3 - primers used for the amplification of DNA from (a) oak leaves to test that the surface sterilisation method had not negatively affected endophytic DNA and (b) the final wash solution to test the effectiveness of the surface sterilisation method to remove epiphytic DNA

Name	Primer sequence	Target region	Source
ITS-1F-KY02	5'- TAGAGGAAGTAAAAGTCGTAA-3'	Entire ITS region	Toju et al. (2012)
ITS4	5'- TCCTCCGCTTATTGATATGC-3'		
16S 8F	5'- AGAGTTTGATCCTGGCTGAG-3'	16S region (8-1541 in *Escherichia coli*)	Coenye et al. (1999)
16S 1541R	5'- AAGGAGGTGATCCAGCCGCA -3'		

PCR products were run on a 1% w/v agarose gel at 90V for 40 minutes, bands were expected at approximately 1347bp for 16S and approximately 586bp for ITS. Gel band intensity was estimated using ImageJ software (Schneider et al., 2012). ImageJ software produces a peak for each band on the gel; the area of this peak is recorded considering any background signal from the gel image, with higher areas indicating higher intensity and therefore more PCR product present.

A linear model was used with sterilisation method, storage temperature and an interaction factor between the two as explanatory variables. Terms were tested for significance using F tests and non-significant terms were removed to achieve the minimal adequate model. Models were tested for normality using the Shapiro Wilk test and for heteroscedasticity using residual plots (Crawley, 2007).

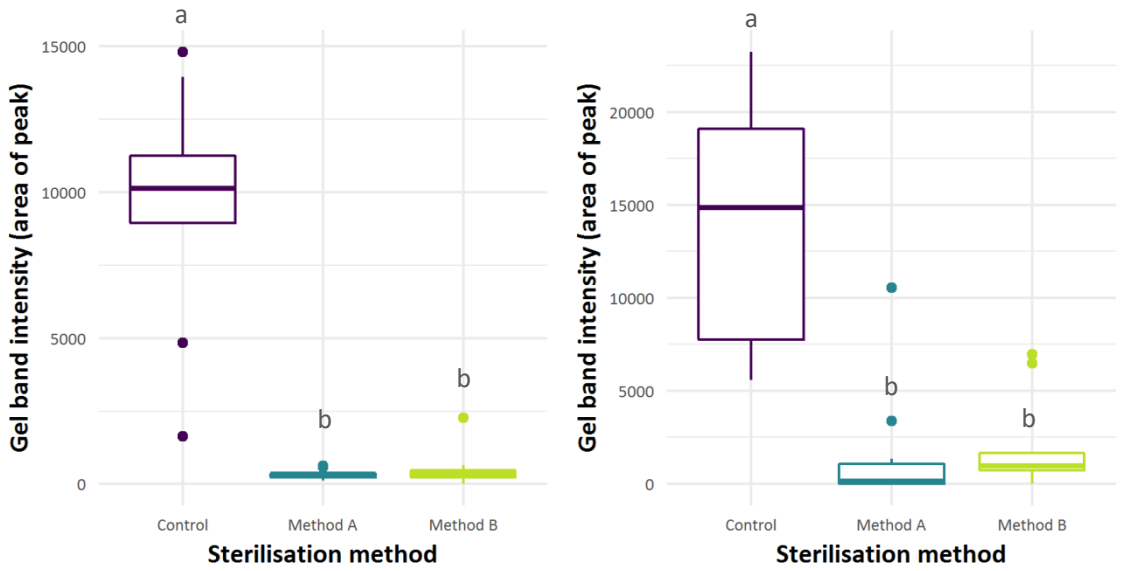
Both surface sterilisation methods were effective at removing epiphytic DNA from the *Quercus* leaves (*Supplementary table B.4* and *Supplementary figure B.3*). Both sterilisation methods also did not affect the amount of endophytic DNA recoverable from the leaf (*Supplementary table B.5* and *Supplementary figure B.4*). There was no effect of storage temperature and no difference between the two sterilisation methods.

Implications

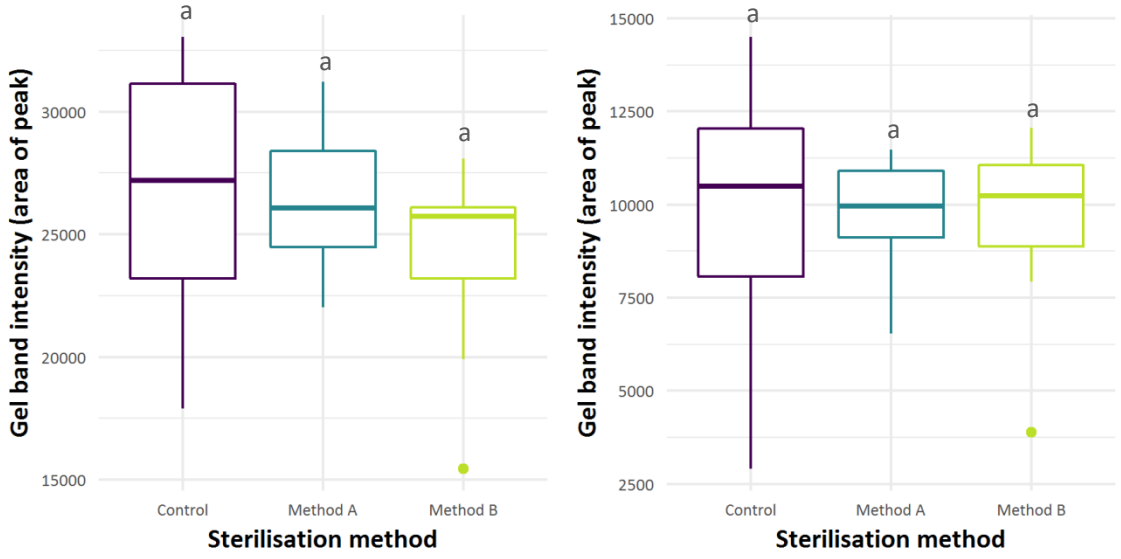
Both methods were effective at removing culturable epiphytic bacteria and fungi and also at removing the DNA of epiphytic organisms. The sterilising agents used did not penetrate the leaf tissue and therefore were unlikely to damage the DNA of the endophytic species of interest. Although both methods were equally effective, it was decided that method A be used in this project as results were more consistent. Method A was also considerably quicker for processing the samples and removes the unnecessary sonication step that may introduce the chance of contamination when inserting the probe into the sample.

Supplementary table B.4 - results from the linear model, comparing the effectiveness of two surface sterilisation methods for removing bacterial and fungal epiphytic DNA. Table also shows the total DNA extracted from surface sterilised leaves to ensure the surface sterilising agent did not penetrate the leaf surface

Method	Kingdom	Signif. variables	df	F	p
Final wash	Bacteria	Sterilisation method	2, 26	44.6	<0.001
	Fungi	Sterilisation method	2, 25	26.3	<0.001
Leaf extraction	Bacteria	No significant variables			
	Fungi	No significant variables			



Supplementary figure B.3 – gel band intensity, as calculated using ImageJ, as an estimate of the amount of DNA remaining in the final wash solution from epiphytic species, bacterial species on the left, fungal species on the right. The two sterilisation techniques are compared here to the control treatment. Box plot shows minimum, first quartile, median, third quartile, maximum and outliers.



Supplementary figure B.4 – gel band intensity, as calculated using ImageJ, as an estimate of the amount of DNA remaining in the surface sterilised leaf from endophytic species, bacterial species on the left, fungal species on the right. The two sterilisation techniques are compared here to the control treatment. Box plot shows minimum, first quartile, median, third quartile, maximum and outliers.

APPENDIX C - *DNA extraction preliminary study*

The two plant genera used in this study are known for possessing high levels of secondary metabolites such as polyphenols and polysaccharides in their tissues. *Juglans* produces the characteristic phenolic compound juglone (Cosmulescu et al., 2014) and *Quercus* possesses high levels of tannins amongst other polyphenols (Forkner et al., 2004, Pearse and Hipp, 2012). These secondary chemicals make traditional DNA extraction methods unsuitable. Polyphenolic contaminants oxidise when released from plant tissue, bind irreversibly with the DNA, imparting a brown discoloration to the extract which is extremely resistant to further purification (Katterman and Shattuck, 1983). PVP (polyvinylpyrrolidone) and 2-mercaptoethanol are typically added to avoid these insoluble bonds forming between polyphenolic substances and the nucleic acids (Khanuja et al., 1999, Lefort and Douglas, 1999). Polysaccharides will co-precipitate with the DNA forming a highly viscous solution, which hinders further processing (Rether et al., 1993, Porebski et al., 1997). Polysaccharide residues may also make DNA unsuitable for further applications by inhibiting enzyme activity, such as *Taq* polymerase (Fang et al., 1992) and therefore inhibit NGS library preparation (Healey et al., 2014). Higher concentrations of sodium chloride in the extraction buffer (Sahu et al., 2012) or including a high concentration of salt prior to DNA precipitation (Fang et al., 1992) is typically used to remove polysaccharides.

A number of DNA extraction methods were tested for their suitability, described here are three methods that proved the most reliable for the plant species and tissues of interest. Leaf, twig and bark samples were collected from mature *Quercus* and *Juglans* trees on the University of Reading, Whiteknights Campus, Berkshire. Samples were surface sterilised using method A (*Supplementary table B.1*). At the bleach step, the leaves were immersed for 5 minutes and the twig and bark samples for 8 minutes. Five 5x5mm squares of each leaf, one 10mm section of twig and one 5x5mm section of bark (oak only) were cut with a sterile

scalpel; this was repeated 9 times per tree species. All samples were stored in individual 2mL microcentrifuge tubes at -80°C.

Tubes containing the leaf sections were removed from -80°C and immediately placed into liquid nitrogen. The first step in the extraction process was to homogenise the plant host, the use of liquid nitrogen at this stage allowed for effective lysis of the tough cell walls and the cold temperature inhibited endogenous nucleases (Sharma et al., 2010). 50mg of autoclaved acid washed sand was added to each tube and the leaves were ground using a micro-pestle until powdered. Twig and bark samples were ground to a fine powder using a sterile cooled mortar and pestle (sterilised in 100°C oven for 16 hours), with 50mg autoclaved acid washed sand and liquid nitrogen. 100mg of twig or bark powder was transferred to a 2mL microcentrifuge tube. Three repeats of each sample were extracted using each of the following three methods:

1. **Method A**: DNeasy® plant mini kit (QIAGEN) following kit instructions but with a longer 15-minute incubation step with solution AP1. DNA pellets were re-suspended in 50µL molecular grade water.

2. **Method B:** this is a modified method from Lefort and Douglas (1999), a CTAB method designed for tree species with high levels of polysaccharides, phenolics and endonucleases. 1mL of CTAB buffer [50mM tris-hydrochloride pH 8.0, 20mM ethylenediaminetetraacetic acid disodium salt dihydrate pH 8.0, 0.7M sodium chloride, 0.4M lithium chloride, 1% w/v hexadecyltrimethylammonium bromide, 1% w/v polyvinylpyrrolidone 40, 2% sodium dodecyl sulfate] pre-warmed to 65°C, was added to each tube, together with 10µL 2-mercaptoethanol. Samples were vortexed for 5 seconds and then incubated for 15 minutes at 65°C, with mixing every 5 minutes during incubation. 0.5mL of chloroform/isoamylalcohol (24:1) was added to each

sample and inverted slowly for 5 minutes, before centrifuging at 13000rpm for 5 minutes. The top aqueous phase (approximately 0.8mL) was transferred to a new 1.5mL microcentrifuge tube and centrifuged for 1 minute at 13000rpm. Again, the top aqueous phase (approx. 0.7mL) was transferred to a new 1.5mL microcentrifuge tube and an equal volume of ice-cold isopropanol was added. Tubes were inverted gently and centrifuged at 13000rpm for 3 minutes. Carefully the supernatant was discarded leaving a DNA pellet, this pellet was washed with 0.5mL 70% ethanol and centrifuged for 1 minute at 13000rpm. Ethanol was removed and the pellet was dried in a heat block set at 37°C. DNA pellets were re-suspended in 50µL molecular grade water and to remove RNA, 1µL RNase A (1mg/mL) was added and incubated for 30 minutes at 37°C.

3. **Method C:** this is a modified method from Healey et al. (2014). 1mL CTAB buffer [100mM tris-hydrochloride pH 7.5, 25mM ethylenediaminetetraacetic acid disodium salt dihydrate pH 8.0, 1.5M sodium chloride, 2% w/v hexadecyltrimethylammonium bromide] pre-warmed to 60°C, was added to each tube together with 10µL 2-mercaptoethanol. Samples were vortexed before incubating at 60°C in a heat block for 60 minutes with inversion every 10 minutes during incubation. Samples were centrifuged at 8000rpm for 5 minutes, the supernatant (approx. 0.9mL) was transferred to 2mL microcentrifuge tubes and an equal volume of chloroform/isoamylalcohol (24:1) was added to each. Samples were slowly inverted continuously for 5 minutes and then centrifuged at 10000rpm for 10 minutes. The aqueous phase (approx. 0.8mL) was transferred carefully to another 2mL microcentrifuge tube without disturbing the interphase layer. 1µL RNase A (1mg/mL) was added to the sample and incubated at 37°C for 15 minutes, with gentle mixing occasionally during incubation. Another equal volume of chloroform/isoamylalcohol

(24:1) was added to each sample and again inverted continuously for 5 minutes. Samples were centrifuged for 10000rpm for 10 minutes before transferring the supernatant (approx. 0.75mL) to a final 1.5mL microcentrifuge tube. An equal volume of ice cold 100% isopropanol and 1/10 volume 3M sodium acetate was added and inverted gently. Samples were incubated at -20°C for 60 minutes (no longer). Samples were centrifuged at 10000rpm for 10 minutes before the supernatant was carefully discarded leaving the white DNA pellet. The DNA pellet was washed with 0.5mL 70% ethanol; the tube was swirled to dislodge the pellet and then centrifuged at 10000rpm for another 10 minutes. Pellets were washed again in the same way. Ethanol was carefully removed, and pellets were air dried at 37°C for 5 minutes before re-suspending in 50µL molecular grade water.

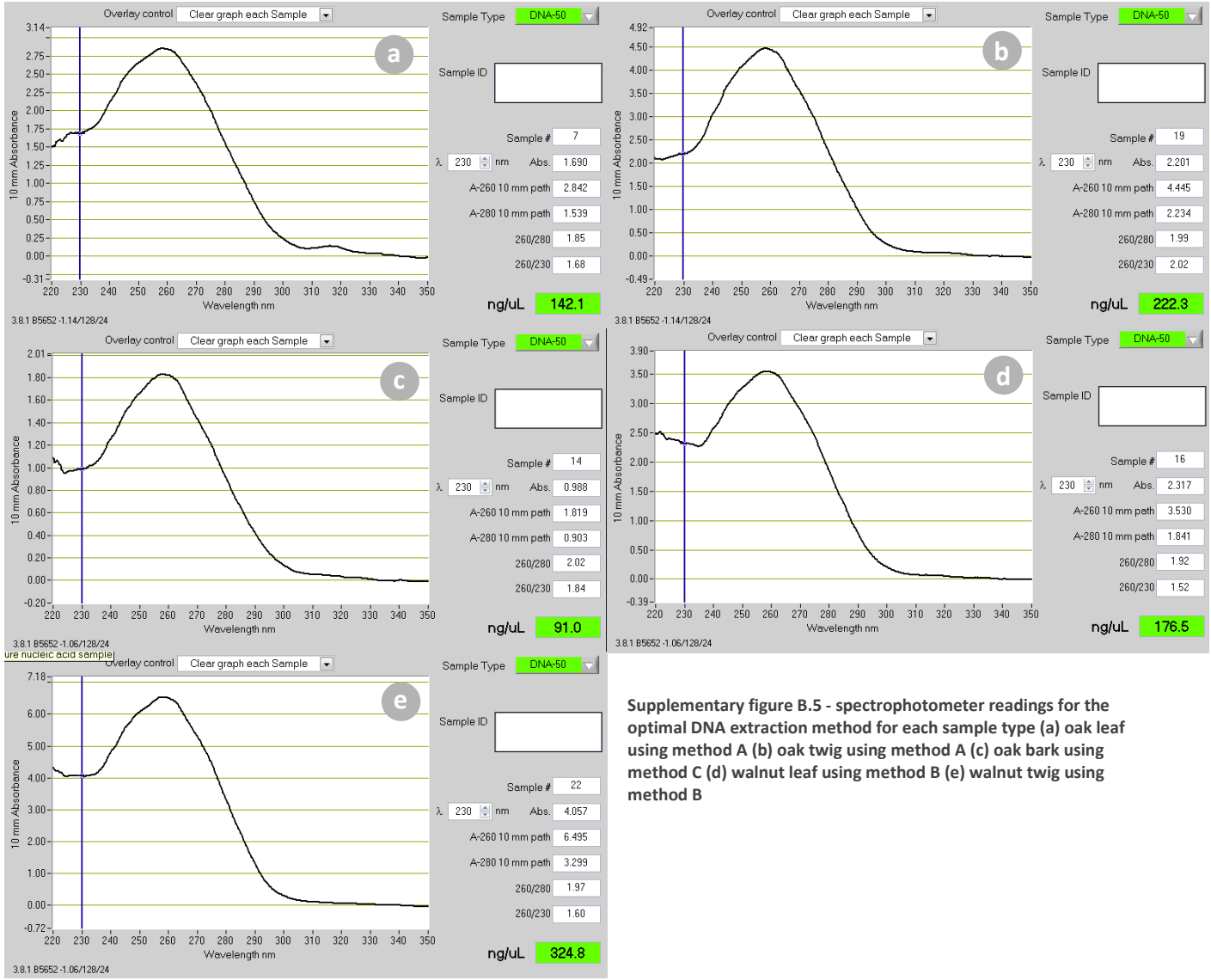
DNA quantity and quality were examined with the use of a spectrophotometer (NanoDrop™ ND-1000, Thermo Fisher Scientific). A single absorbance peak at 260nm, a 260/280 absorbance ratio between 1.8 and 2.0 and a 260/230 absorbance ratio between 2.0 and 2.2 indicated high quality DNA with minimal contamination from proteins, polyphenols, RNA or polysaccharides (Thermo Scientific, 2008). DNA quality was also determined using electrophoresis. Samples were run on a 1% w/v agarose gel at 90V for 40 minutes, a tight single band of at least 10kb with light or no smearing indicated good quality DNA.

For NGS analysis at least 20ng/ µL of DNA is required, using all three methods the quantity of DNA extracted was sufficient for all tissue types. However, the quality of the extracted DNA varied considerably between methods (*Supplementary table B.6* and *Supplementary figure B.5*). For oak leaves and twigs, all methods were equally efficient at extracting high quality DNA. DNA pellets were clear, suggesting that polyphenols were removed, and were not viscous, signifying that no polysaccharide residues remained. The commercial kit (method A) was deemed preferable due to the ease and speed of processing the large number of samples

needed for NGS analysis. Method A was, however, unsuitable for the extraction of *Quercus* bark and all *Juglans* tissue types, yielding considerably lower quality DNA that in several instances was brown in colour. Method C proved optimal for extracting DNA from the more tannin and polysaccharide rich *Quercus* bark, and method B for all *Juglans* tissue types.

Implications

The commercial kit (QIAGEN DNeasy® plant mini kit) extracted high quality DNA from leaves and twigs of the genus *Quercus*. Modified method C was successfully used to extract DNA from the bark of *Quercus* and modified method B for extracting high quality DNA from the leaves and twigs of *Juglans* species. The DNA methods chosen here for each plant species and tissue type were able to extract the quantity and quality of DNA required for analysing endophyte assemblages of *Quercus* and *Juglans* species using NGS systems.



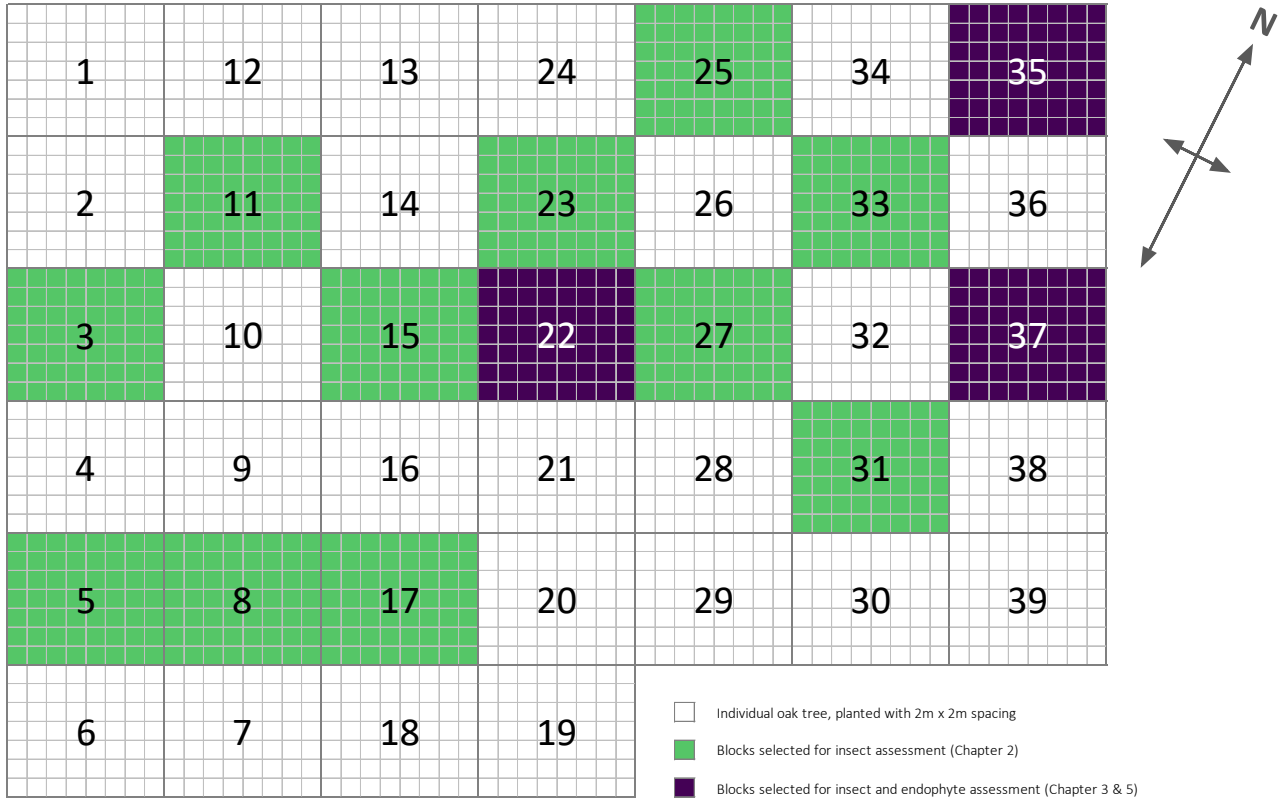
Supplementary figure B.5 - spectrophotometer readings for the optimal DNA extraction method for each sample type (a) oak leaf using method A (b) oak twig using method A (c) oak bark using method C (d) walnut leaf using method B (e) walnut twig using method B

Supplementary table B.5 – results from the spectrophotometer readings for each of the three DNA extraction methods, showing quantity and quality of DNA. The method deemed the most suitable for each plant species and tissue type, based on these readings, are highlighted in purple.

Tree genus	Sample type	Method	DNA concentration (ng/µL)	260/280	260/230
			120.3	**1.91**	**1.64**
Quercus	**Leaf**	**Method A**	**94.4**	**1.92**	**1.43**
			142.1	**1.85**	**1.68**
			471.5	1.96	1.40
Quercus	Leaf	Method B	164.4	1.92	1.02
			201.4	1.85	1.19
			107.1	1.98	1.95
Quercus	Leaf	Method C	185.3	2.06	2.13
			247.6	2.09	1.98
			77.7	**1.64**	**0.85**
Quercus	**Twig**	**Method A**	**97.3**	**1.67**	**0.96**
			222.3	**1.99**	**2.02**
			138.5	1.46	0.72
Quercus	Twig	Method B	204.6	1.66	1.01
			273.3	1.83	1.13
			496.5	1.91	1.53
Quercus	Twig	Method C	205.1	1.74	1.18
			310.8	1.83	1.28
			104.4	1.44	0.49
Quercus	Bark	Method A	73.7	1.36	0.49
			96.6	1.82	0.77
			64.0	1.92	1.58
Quercus	Bark	Method B	123.9	1.80	1.12
			114.2	1.93	1.04
			65.0	**2.08**	**1.88**
Quercus	**Bark**	**Method C**	**251.4**	**2.08**	**1.72**
			91.0	**2.02**	**1.84**
			57.4	1.79	0.93
Juglans	Leaf	Method A	148.9	1.97	0.92
			156.2	1.96	0.85
			176.5	**1.92**	**1.52**
Juglans	**Leaf**	**Method B**	**277.8**	**1.89**	**1.29**
			214.6	**1.98**	**1.22**
			69.1	1.62	1.22
Juglans	Leaf	Method C	269.5	1.66	0.99
			46.4	1.73	1.46
			113.5	1.71	0.82
Juglans	Twig	Method A	212.6	1.29	0.77
			122.5	1.32	0.92
			324.8	**1.97**	**1.60**
Juglans	**Twig**	**Method B**	**201.6**	**1.86**	**1.45**
			307.8	**1.87**	**1.47**
			129.3	2.16	0.89
Juglans	Twig	Method C	388.9	2.31	0.71
			196.7	1.87	1.32

APPENDIX D - *Paradise Wood trial layouts*

D.1 *Oak breeding seedling orchard (BSO) trial*



Supplementary figure D.1 – layout of the oak trees in the BSO trial in Paradise Wood, block numbers are shown in bold. Each square represents an individual oak tree, planted with 2m by 2m spacing. Blocks in **green** represent blocks selected for insect assessment, blocks in **purple** represent blocks selected for both insect and endophyte assessment.

BLOCK 3

1	**14** NETHERLANDS NORTH BRABANT ZE46-1	**15** NETHERLANDS NORTH BRABANT ZE58-2	**28** FRANCE NORMANDY REN001	**29** ENGLAND NORTHAMPTONSHIRE NHP008	**42**	**43** ENGLAND HAMPSHIRE HAM006	**56**
2 ENGLAND SURREY ALICE HOLT	**13** ENGLAND NORTHUMBERLAND NMB001	**16**	**27** SCOTLAND BORDERS BOR001	**30** NETHERLANDS GELDERLAND ZE23-2	**41** FRANCE NORMANDY REN003	**44** ENGLAND GLOUCESTERSHIRE GLS022	**55** NETHERLANDS NORTH BRABANT ZE64-1
3	**12** NETHERLANDS MID BRABANT ZE80-1	**17** ENGLAND HAMPSHIRE HAM013	**26**	**31**	**40** SCOTLAND EAST LOTHIAN ELT004	**45** ENGLAND GLOUCESTERSHIRE GLS014	**54** SCOTLAND BORDERS BOR002
4 ENGLAND LINCOLNSHIRE LNC001	**11**	**18** ENGLAND HEREFORDSHIRE HRF004	**25** NETHERLANDS BRABANT ZE82-1	**32** ENGLAND NORFOLK NOR007	**39** FRANCE SARTHE BRC001	**46** NETHERLANDS NORTH BRABANT ZE142-1	**53** ENGLAND LEICESTERSHIRE LEI001
5 ENGLAND HAMPSHIRE HAM004	**10** ENGLAND HEREFORDSHIRE HRF017	**19** NETHERLANDS GELDERLAND ZE34-1	**24** ENGLAND GLOUCESTERSHIRE GLS012	**33**	**38** SCOTLAND HIGHLANDS HGH001	**47** ENGLAND SOMERSET SOM002	**52** ENGLAND NORTHAMPTONSHIRE NHP002
6 ENGLAND HAMPSHIRE HAM007	**9** ENGLAND NORTHAMPTONSHIRE NHP006	**20** ENGLAND SUFFOLK SUF004	**23** NETHERLANDS NORTH BRABANT ZE47-2	**34**	**37**	**48** FRANCE CHARTRES SEN002	**51** ENGLAND WORCESTERSHIRE WOR007
7 ENGLAND GLOUCESTERSHIRE GLS025	**8** ENGLAND HEREFORDSHIRE HRF013	**21** NETHERLANDS GELDERLAND ZE11-1	**22** ENGLAND HAMPSHIRE HAM012	**35** ENGLAND SUFFOLK SUF001	**36** IRELAND WICKLOW WIK003	**49** ENGLAND SUFFOLK SUF003	**50** ENGLAND NORFOLK NOR005

BLOCK 5

1 ENGLAND GLOUCESTERSHIRE GLS016	**14** ENGLAND HEREFORDSHIRE HRF017	**15**	**28** SCOTLAND EAST LOTHIAN ELT005	**29**	**42**	**43** ENGLAND NORTHUMBERLAND NMB001	**56** ENGLAND NORTHAMPTONSHIRE NHP008
2	**13**	**16** ENGLAND HAMPSHIRE HAM006	**27**	**30** NETHERLANDS GELDERLAND ZE34-1	**41** ENGLAND HEREFORDSHIRE HRF004	**44** SCOTLAND BORDERS BOR002	**55** ENGLAND SOMERSET SOM002
3 NETHERLANDS NORTH BRABANT ZE142-1	**12**	**17** ENGLAND HAMPSHIRE HAM004	**26** ENGLAND NORFOLK NOR005	**31** ENGLAND HEREFORDSHIRE HRF013	**40** FRANCE SARTHE BRC002	**45**	**54** NETHERLANDS BRABANT ZE82-1
4 ENGLAND CUMBRIA CUM001	**11** IRELAND OFFALY OFL002	**18**	**25** NETHERLANDS NORTH BRABANT ZE64-1	**32** ENGLAND SUFFOLK SUF001	**39**	**46** ENGLAND GLOUCESTERSHIRE GLS022	**53** SCOTLAND HIGHLANDS HGH001
5 ENGLAND HEREFORDSHIRE HRF008	**10** ENGLAND HAMPSHIRE HAM013	**19** ENGLAND LEICESTERSHIRE LEI001	**24**	**33** NETHERLANDS MID BRABANT ZE80-1	**38** SCOTLAND BORDERS BOR001	**47** ENGLAND NORTHAMPTONSHIRE NHP002	**52**
6 NETHERLANDS GELDERLAND ZE23-2	**9**	**20** ENGLAND HEREFORDSHIRE HRF006	**23**	**34** FRANCE SARTHE BRC001	**37** FRANCE NORMANDY REN001	**48** ENGLAND GLOUCESTERSHIRE GLS014	**51** ENGLAND NORFOLK NOR007
7	**8** NETHERLANDS GELDERLAND ZE11-1	**21** ENGLAND SUFFOLK SUF003	**22**	**35**	**36**	**49**	**50** ENGLAND SOMERSET SOM003

Supplementary figure D.2 – tree layout within the BSO trial, each rectangle represents each tree. Blank rectangles represent dead trees and trees excluded from analysis. Only the 14 blocks selected for analysis are shown, all trees shown were analysed for insect herbivores, trees shaded purple were selected for endophyte analysis also.

BLOCK 8

1 NETHERLANDS NORTH BRABANT ZE47-2	**14**	**15**	**28**	**29** ENGLAND NORTHUMBERLAND NMB001	**42** ENGLAND NORTHAMPTONSHIRE NHP002	**43**	**56** ENGLAND CUMBRIA CUM001
2 ENGLAND GLOUCESTERSHIRE GLS014	**13** FRANCE CHARTRES SEN002	**16**	**27** ENGLAND HEREFORDSHIRE HRF008	**30** NETHERLANDS NORTH BRABANT ZE64-1	**41** SCOTLAND EAST LOTHIAN ELT004	**44** NETHERLANDS GELDERLAND ZE11-1	**55** ENGLAND NORTHAMTONSHIRE NHP006
3 ENGLAND HAMPSHIRE HAM013	**12** ENGLAND LEICESTERSHIRE LEI001	**17** ENGLAND NORTHAMPTONSHIRE NHP009	**26** ENGLAND NORFOLK NOR007	**31**	**40** FRANCE NORMANDY REN003	**45**	**54** NETHERLANDS MID BRABANT ZE80-1
4 ENGLAND HAMPSHIRE HAM013	**11** NETHERLANDS BRABANT ZE82-1	**18**	**25**	**32** FRANCE SARTHE BRC001	**39** ENGLAND SURREY ALICE HOLT	**46**	**53** ENGLAND LINCOLNSHIRE LNC001
5	**10** NETHERLANDS NORTH BRABANT ZE58-2	**19**	**24**	**33** ENGLAND HAMPSHIRE HAM012	**38**	**47** ENGLAND HEREFORDSHIRE HRF006	**52** NETHERLANDS NORTH BRABANT ZE46-1
6 NETHERLANDS GELDERLAND ZE34-1	**9** NETHERLANDS NORTH BRABANT ZE142-1	**20** ENGLAND GLOUCESTERSHIRE GLS016	**23** FRANCE SARTHE BRC002	**34** ENGLAND HAMPSHIRE HAM006	**37**	**48**	**51** ENGLAND SUFFOLK SUF001
7 SCOTLAND HIGHLANDS HGH001	**8** FRANCE NORMANDY REN001	**21** ENGLAND GLOUCESTERSHIRE GLS012	**22** ENGLAND HEREFORDSHIRE HRF004	**35** ENGLAND NORTHAMPTONSHIRE NHP008	**36**	**49**	**50**

BLOCK 11

1	**14** ENGLAND SUFFOLK SUF004	**15** ENGLAND NORFOLK NOR005	**28** NETHERLANDS GELDERLAND ZE11-1	**29** ENGLAND HAMPSHIRE HAM012	**42** ENGLAND GLOUCESTERSHIRE GLS016	**43** NETHERLANDS GELDERLAND ZE34-1	**56**
2 ENGLAND SUFFOLK SUF001	**13** ENGLAND HEREFORDSHIRE HRF004	**16** NETHERLANDS NORTH BRABANT ZE47-2	**27** ENGLAND NORTHAMPTONSHIRE NHP006	**30** ENGLAND GLOUCESTERSHIRE GLS022	**41** FRANCE NORMANDY REN003	**44** NETHERLANDS NORTH BRABANT ZE46-1	**55** SCOTLAND DUMFRIES & GALLOWAY D&G003
3	**12**	**17** ENGLAND HAMPSHIRE HAM006	**26** FRANCE NORMANDY REN001	**31**	**40** NETHERLANDS BRABANT ZE82-1	**45** ENGLAND LEICESTERSHIRE LEI001	**54** NETHERLANDS MID BRABANT ZE80-1
4 SCOTLAND HIGHLANDS HGH001	**11** ENGLAND NORTHUMBERLAND NMB001	**18**	**25**	**32** FRANCE CHARTRES SEN002	**39** IRELAND OFFALY OFL002	**46** ENGLAND HAMPSHIRE HAM013	**53** NETHERLANDS GELDERLAND ZE23-2
5 ENGLAND GLOUCESTERSHIRE GLS012	**10**	**19** ENGLAND NORTHAMPTONSHIRE NHP008	**24**	**33**	**38**	**47** FRANCE SARTHE BRC001	**52** ENGLAND HEREFORDSHIRE HRF013
6 ENGLAND HAMPSHIRE HAM007	**9** NETHERLANDS NORTH BRABANT ZE142-1	**20** NETHERLANDS NORTH BRABANT ZE64-1	**23**	**34** ENGLAND NORTHAMPTONSHIRE NHP002	**37**	**48** ENGLAND SOMERSET SOM003	**51**
7 SCOTLAND EAST LOTHIAN ELT004	**8**	**21**	**22**	**35**	**36**	**49** ENGLAND NORTHAMPTONSHIRE NHP009	**50**

Supplementary figure D.2 cont. – tree layout within the BSO trial, each rectangle represents each tree. Blank rectangles represent dead trees and trees excluded from analysis. Only the 14 blocks selected for analysis are shown, all trees shown were analysed for insect herbivores, trees shaded purple were selected for endophyte analysis also.

BLOCK 15

1 ENGLAND HEREFORDSHIRE HRF006	**14** ENGLAND NORFOLK NOR005	**15** ENGLAND SUFFOLK SUF004	**28** NETHERLANDS GELDERLAND ZE11-1	**29**	**42**	**43** IRELAND OFFALY OFL002	**56** NETHERLANDS MID BRABANT ZE80-1
2 NETHERLANDS NORTH BRABANT ZE47-2	**13** FRANCE SARTHE BRC002	**16** ENGLAND SUFFOLK SUF003	**27** ENGLAND SUFFOLK SUF001	**30**	**41** SCOTLAND BORDERS BOR002	**44**	**55** NETHERLANDS NORTH BRABANT ZE64-1
3	**12** ENGLAND LINCOLNSHIRE LNC001	**17** FRANCE NORMANDY REN001	**26** ENGLAND NORTHAMTONSHIRE NHP008	**31** ENGLAND HEREFORDSHIRE HRF008	**40**	**45** ENGLAND NORFOLK NOR007	**54** SCOTLAND EAST LOTHIAN ELT004
4 ENGLAND SOMERSET SOM002	**11** NETHERLANDS NORTH BRABANT ZE58-2	**18** ENGLAND HAMPSHIRE HAM006	**25** ENGLAND WORCESTERSHIRE WOR007	**32**	**39** NETHERLANDS GELDERLAND ZE23-2	**46** NETHERLANDS BRABANT ZE82-1	**53** ENGLAND LEICESTERSHIRE LEI001
5 SCOTLAND EAST LOTHIAN ELT005	**10** ENGLAND GLOUCESTERSHIRE GLS012	**19** ENGLAND HEREFORDSHIRE HRF017	**24**	**33**	**38** ENGLAND SOMERSET SOM003	**47** ENGLAND NORMANDY REN003	**52** NETHERLANDS NORTH BRABANT ZE46-1
6	**9** ENGLAND CUMBRIA CUM001	**20**	**23** ENGLAND NORTHUMBERLAND NMB001	**34** ENGLAND GLOUCESTERSHIRE GLS025	**37** SCOTLAND HIGHLANDS HGH001	**48**	**51** ENGLAND GLOUCESTERSHIRE GLS022
7 IRELAND WICKLOW WIK003	**8** ENGLAND HEREFORDSHIRE HRF004	**21**	**22** NETHERLANDS NORTH BRABANT ZE142-1	**35** ENGLAND HAMPSHIRE HAM013	**36** ENGLAND HAMPSHIRE HAM012	**49** SCOTLAND DUMFRIES & GALLOWAY D&G003	**50**

BLOCK 17

1	**14** NETHERLANDS NORTH BRABANT ZE47-2	**15** NETHERLANDS MID BRABANT ZE80-1	**28**	**29**	**42** ENGLAND SUFFOLK SUF001	**43** ENGLAND NORFOLK NOR005	**56** SCOTLAND DUMFRIES & GALLOWAY D&G003
2 ENGLAND HAMPSHIRE HAM013	**13** NETHERLANDS GELDERLAND ZE34-1	**16** SCOTLAND BORDERS BOR001	**27** ENGLAND GLOUCESTERSHIRE GLS014	**30** ENGLAND LEICESTERSHIRE LEI001	**41** ENGLAND LINCOLNSHIRE LNC001	**44**	**55** ENGLAND NORTHAMPTONSHIRE NHP006
3 ENGLAND NORTHUMBERLAND NMB001	**12** NETHERLANDS GELDERLAND ZE23-2	**17** ENGLAND HEREFORDSHIRE HRF006	**26** ENGLAND SUFFOLK SUF003	**31** ENGLAND HAMPSHIRE HAM006	**40** FRANCE SARTHE BRC002	**45** ENGLAND HEREFORDSHIRE HRF004	**54** FRANCE SARTHE BRC001
4	**11** SCOTLAND BORDERS BOR002	**18** SCOTLAND EAST LOTHIAN ELT005	**25** IRELAND WICKLOW WIK003	**32** ENGLAND NORTHAMPTONSHIRE NHP002	**39** ENGLAND HAMPSHIRE HAM014	**46** NETHERLANDS NORTH BRABANT ZE64-1	**53**
5 ENGLAND HEREFORDSHIRE HRF013	**10** FRANCE NORMANDY REN003	**19** ENGLAND HAMPSHIRE HAM007	**24**	**33** ENGLAND NORTHAMPTONSHIRE NHP009	**38** ENGLAND HAMPSHIRE HAM012	**47**	**52** ENGLAND GLOUCESTERSHIRE GLS016
6 ENGLAND SOMERSET SOM002	**9** ENGLAND HEREFORDSHIRE HRF008	**20** IRELAND OFFALY OFL002	**23** ENGLAND CUMBRIA CUM001	**34** NETHERLANDS NORTH BRABANT ZE142-1	**37** ENGLAND WORCESTERSHIRE WOR007	**48** ENGLAND NORTHAMPTONSHIRE NHP008	**51**
7	**8** ENGLAND HAMPSHIRE HAM004	**21**	**22**	**35** ENGLAND GLOUCESTERSHIRE GLS022	**36** SCOTLAND EAST LOTHIAN ELT004	**49** ENGLAND HEREFORDSHIRE HRF017	**50** NETHERLANDS NORTH BRABANT ZE46-1

Supplementary figure D.2 cont. – tree layout within the BSO trial, each rectangle represents each tree. Blank rectangles represent dead trees and trees excluded from analysis. Only the 14 blocks selected for analysis are shown, all trees shown were analysed for insect herbivores, trees shaded purple were selected for endophyte analysis also.

BLOCK 22

1 ENGLAND HEREFORDSHIRE HRF008	**14** ENGLAND WORCESTERSHIRE WOR007	**15** NETHERLANDS NORTH BRABANT ZE142-1	**28** ENGLAND GLOUCESTERSHIRE GLS025	**29**	**42**	**43**	**56**
2 ENGLAND HEREFORDSHIRE HRF013	**13** NETHERLANDS NORTH BRABANT ZE46-1	**16** ENGLAND SUFFOLK SUF001	**27** NETHERLANDS NORTH BRABANT ZE58-2	**30** ENGLAND NORTHAMPTONSHIRE NHP008	**41**	**44**	**55** ENGLAND LEICESTERSHIRE LEI001
3 ENGLAND HEREFORDSHIRE HRF017	**12** ENGLAND GLOUCESTERSHIRE GLS022	**17**	**26** IRELAND OFFALY OFL002	**31**	**40** SCOTLAND EAST LOTHIAN ELT005	**45**	**54**
4 FRANCE NORMANDY REN001	**11** NETHERLANDS GELDERLAND ZE11-1	**18**	**25** ENGLAND HAMPSHIRE HAM004	**32** ENGLAND NORTHAMPTONSHIRE NHP009	**39** NETHERLANDS GELDERLAND ZE23-2	**46**	**53**
5 ENGLAND NORTHAMPTONSHIRE NHP006	**10** ENGLAND HAMPSHIRE HAM007	**19**	**24** FRANCE SARTHER BRC002	**33** ENGLAND HAMPSHIRE HAM013	**38** FRANCE CHARTRES SEN002	**47** ENGLAND HAMPSHIRE HAM014	**52** NETHERLANDS MID BRABANT ZE80-1
6 ENGLAND HEREFORDSHIRE HRF004	**9**	**20** SCOTLAND BORDERS BOR002	**23**	**34** SCOTLAND EAST LOTHIAN ELT004	**37** ENGLAND NORFOLK NOR007	**48**	**51** FRANCE SARTHE BRC001
7 ENGLAND GLOUCESTERSHIRE GLS016	**8** ENGLAND HAMPSHIRE HAM006	**21** ENGLAND SUFFOLK SUF003	**22** ENGLAND SUFFOLK SUF004	**35** ENGLAND GLOUCESTERSHIRE GLS012	**36** NETHERLANDS NORTH BRABANT ZE47-2	**49** ENGLAND LINCOLNSHIRE LNC001	**50** FRANCE NORMANDY REN003

BLOCK 23

1 ENGLAND NORTHUMBERLAND NMB001	**14** ENGLAND SUFFOLK SUF004	**15**	**28** NETHERLANDS NORTH BRABANT ZE47-2	**29** ENGLAND GLOUCESTERSHIRE GLS012	**42**	**43** FRANCE NORMANDY REN001	**56** ENGLAND CUMBRIA CUM001
2 ENGLAND LEICESTERSHIRE LEI001	**13** NETHERLANDS GELDERLAND ZE11-1	**16** ENGLAND NORTHAMPTONSHIRE NHP006	**27**	**30** SCOTLAND HIGHLANDS HGH001	**41** SCOTLAND EAST LOTHIAN ELT005	**44** ENGLAND NORTHAMPTONSHIRE NHP008	**55** ENGLAND SURREY ALICE HOLT
3 NETHERLANDS NORTH BRABANT ZE64-1	**12** ENGLAND GLOUCESTERSHIRE GLS014	**17** FRANCE CHARTRES SEN002	**26**	**31** NETHERLANDS NORTH BRABANT ZE142-1	**40**	**45**	**54** ENGLAND HAMPSHIRE HAM007
4	**11** ENGLAND NORFOLK NOR005	**18**	**25** NETHERLANDS BRABANT ZE82-1	**32**	**39** ENGLAND HAMPSHIRE HAM006	**46** ENGLAND GLOUCESTERSHIRE GLS025	**53**
5 ENGLAND HEREFORDSHIRE HRF013	**10** NETHERLANDS NORTH BRABANT ZE46-1	**19** SCOTLAND DUMFRIES & GALLOWAY D&G003	**24** FRANCE SARTHES BRC001	**33** ENGLAND HAMPSHIRE HAM012	**38** NETHERLANDS GELDERLAND ZE34-1	**47**	**52** SCOTLAND BORDERS BOR002
6 SCOTLAND BORDERS BOR001	**9** ENGLAND SOMERSET SOM003	**20** ENGLAND SUFFOLK SUF003	**23** SCOTLAND EAST LOTHIAN ELT004	**34**	**37** IRELAND OFFALY OFL002	**48** NETHERLANDS GELDERLAND ZE23-2	**51** ENGLAND LINCOLNSHIRE LNC001
7	**8** ENGLAND GLOUCESTERSHIRE GLS002	**21**	**22**	**35** ENGLAND NORTHAMPTONSHIRE NHP009	**36** ENGLAND HAMPSHIRE HAM013	**49** NETHERLANDS MID BRABANT ZE80-1	**50** ENGLAND HEREFORDSHIRE HRF008

Supplementary figure D.2 cont. – tree layout within the BSO trial, each rectangle represents each tree. Blank rectangles represent dead trees and trees excluded from analysis. Only the 14 blocks selected for analysis are shown, all trees shown were analysed for insect herbivores, trees shaded purple were selected for endophyte analysis also.

BLOCK 25

1	**14** NETHERLANDS NORTH BRABANT ZE64-1	**15** ENGLAND SUFFOLK SUF004	**28**	**29** ENGLAND GLOUCESTERSHIRE GLS016	**42** NETHERLANDS NORTH BRABANT ZE47-2	**43** FRANCE NORMANDY REN001	**56** ENGLAND HEREFORDSHIRE HRF017
2 ENGLAND NORTHUMBERLAND NMB001	**13** FRANCE NORMANDY REN003	**16** ENGLAND HEREFORDSHIRE HRF006	**27**	**30** SCOTLAND BORDERS BOR001	**41** ENGLAND SUFFOLK SUF003	**44** ENGLAND HEREFORDSHIRE HRF004	**55** ENGLAND SURREY ALICE HOLT
3	**12**	**17** IRELAND WICKLOW WIK003	**26** ENGLAND HEREFORDSHIRE HRF013	**31**	**40** FRANCE SARTHE BRC002	**45** ENGLAND WORCESTERSHIRE WOR007	**54** SCOTLAND EAST LOTHIAN ELT005
4 SCOTLAND DUMFRIES & GALLOWAY D&G003	**11** ENGLAND HAMPSHIRE HAM013	**18** ENGLAND HAMPSHIRE HAM014	**25** NETHERLANDS BRABANT ZE82-1	**32** ENGLAND SOMERSET SOM002	**39** NETHERLANDS NORTH BRABANT ZE58-2	**46** ENGLAND NORTHAMPTONSHIRE NHP008	**53** ENGLAND GLOUCESTERSHIRE GLS025
5 ENGLAND NORTHAMPTONSHIRE NHP002	**10** NETHERLANDS NORTH BRABANT ZE46-1	**19**	**24**	**33** ENGLAND GLOUCESTERSHIRE GLS012	**38** NETHERLANDS GELDERLAND ZE34-1	**47** ENGLAND SUFFOLK SUF001	**52**
6 ENGLAND GLOUCESTERSHIRE GLS014	**9**	**20** ENGLAND NORFOLK NOR007	**23** ENGLAND GLOUCESTERSHIRE GLS022	**34** ENGLAND CUMBRIA CUM001	**37** FRANCE CHARTRES SEN002	**48** SCOTLAND EAST LOTHIAN ELT004	**51** ENGLAND NORFOLK NOR005
7 NETHERLANDS GELDERLAND ZE11-1	**8** ENGLAND NORTHAMPTONSHIRE NHP006	**21** NETHERLANDS MID BRABANT ZE80-1	**22** ENGLAND NORTHAMPTONSHIRE NHP009	**35** ENGLAND LINCOLNSHIRE LNC001	**36** ENGLAND HAMPSHIRE HAM006	**49** NETHERLANDS NORTH BRABANT ZE142-1	**50** SCOTLAND HIGHLANDS HGH001

BLOCK 27

1 ENGLAND NORFOLK NOR007	**14** ENGLAND SUFFOLK SUF004	**15** ENGLAND SUFFOLK SUF003	**28** ENGLAND HAMPSHIRE HAM006	**29** IRELAND OFFALY OFL002	**42** NETHERLANDS GELDERLAND ZE23-2	**43** ENGLAND LEICESTERSHIRE LEI001	**56** ENGLAND NORTHAMPTONSHIRE NHP009
2 ENGLAND SOMERSET SOM002	**13** NETHERLANDS GELDERLAND ZE11-1	**16** ENGLAND NORFOLK NOR005	**27**	**30** FRANCE NORMANDY REN003	**41** NETHERLANDS MID BRABANT ZE80-1	**44** ENGLAND HEREFORDSHIRE HRF017	**55** NETHERLANDS NORTH BRABANT
3 NETHERLANDS GELDERLAND ZE34-1	**12** ENGLAND GLOUCESTERSHIRE GLS016	**17**	**26** ENGLAND LINCOLNSHIRE LNC001	**31** ENGLAND SURREY ALICE HOLT	**40**	**45**	**54** NETHERLANDS NORTH BRABANT ZE58-2
4	**11**	**18** NETHERLANDS NORTH BRABANT ZE46-1	**25** NETHERLANDS NORTH BRABANT ZE142-1	**32**	**39** ENGLAND HAMPSHIRE HAM013	**46** FRANCE NORMANDY REN001	**53** NETHERLANDS NORTHUMBERLAND NMB001
5 ENGLAND SUFFOLK SUF001	**10** ENGLAND HEREFORDSHIRE HRF013	**19** ENGLAND WORCESTERSHIRE WOR007	**24** NETHERLANDS BRABANT ZE82-1	**33**	**38**	**47** ENGLAND CUMBRIA CUM001	**52**
6	**9** ENGLAND HEREFORDSHIRE HRF006	**20** ENGLAND SOMERSET SOM003	**23**	**34**	**37** ENGLAND NORTHAMPTONSHIRE NHP008	**48** ENGLAND GLOUCESTERSHIRE GLS014	**51** SCOTLAND HIGHLANDS HGH001
7 SCOTLAND DUMFRIES & GALLOWAY D&G003	**8** FRANCE SARTHE BRC001	**21** NETHERLANDS NORTH BRABANT ZE47-2	**22** ENGLAND NORTHAMPTONSHIRE NHP006	**35**	**36** ENGLAND NORTHAMPTONSHIRE NHP002	**49**	**50** SCOTLAND EAST LOTHIAN ELT004

Supplementary figure D.2 cont. – tree layout within the BSO trial, each rectangle represents each tree. Blank rectangles represent dead trees and trees excluded from analysis. Only the 14 blocks selected for analysis are shown, all trees shown were analysed for insect herbivores, trees shaded purple were selected for endophyte analysis also.

BLOCK 31

1 ENGLAND NORTHAMPTONSHIRE NHP008	**14**	**15** ENGLAND GLOUCESTERSHIRE GLS014	**28** ENGLAND GLOUCESTERSHIRE GLS022	**29**	**42**	**43** ENGLAND HAMPSHIRE HAM014	**56** NETHERLANDS NORTH BRABANT ZE58-2
2 SCOTLAND DUMFRIES & GALLOWAY D&G003	**13** FRANCE SARTHE BRC002	**16**	**27** FRANCE NORMANDY REN001	**30** ENGLAND NORFOLK NOR005	**41** ENGLAND HAMPSHIRE HAM013	**44** ENGLAND SUFFOLK SUF003	**55** NETHERLANDS NORTH BRABANT ZE47-2
3 ENGLAND NORTHUMBERLAND NMB001	**12** IRELAND OFFALY OFL002	**17** ENGLAND HEREFORDSHIRE HRF008	**26** ENGLAND GLOUCESTERSHIRE GLS025	**31**	**40** NETHERLANDS BRABANT ZE82-1	**45**	**54** NETHERLANDS NORTH BRABANT ZE46-1
4 IRELAND WICKLOW WIK003	**11** SCOTLAND BORDERS BOR001	**18** SCOTLAND EAST LOTHIAN ELT004	**25** ENGLAND CUMBRIA CUM001	**32**	**39** ENGLAND SUFFOLK SUF001	**46** ENGLAND LINCOLNSHIRE LNC001	**53**
5 NETHERLANDS NORTH BRABANT ZE64-1	**10**	**19** ENGLAND NORTHAMPTONSHIRE NHP002	**24** SCOTLAND BORDERS BOR002	**33** ENGLAND NORTHAMPTONSHIRE NHP006	**38** ENGLAND SOMERSET SOM003	**47** NETHERLANDS NORTH BRABANT ZE142-1	**52** ENGLAND HEREFORDSHIRE HRF006
6	**9** ENGLAND SURREY ALICE HOLT	**20** ENGLAND NORFOLK NOR007	**23** ENGLAND NORTHAMPTONSHIRE NHP009	**34** ENGLAND GLOUCESTERSHIRE GLS016	**37** NETHERLANDS GELDERLAND ZE34-1	**48** FRANCE NORMANDY REN003	**51** ENGLAND LEICESTERSHIRE LEI001
7 ENGLAND HAMPSHIRE HAM007	**8** ENGLAND GLOUCESTERSHIRE GLS012	**21** NETHERLANDS GELDERLAND ZE11-1	**22**	**35**	**36** FRANCE SARTHE BRC001	**49** FRANCE CHARTRES SEN002	**50**

BLOCK 33

1	**14** ENGLAND NORTHUMBERLAND NMB001	**15**	**28** IRELAND WICKLOW WIK003	**29**	**42** NETHERLANDS GELDERLAND ZE11-1	**43**	**56**
2 NETHERLANDS MID BRABANT ZE80-1	**13** ENGLAND SUFFOLK SUF003	**16** FRANCE SARTHE BRC001	**27** NETHERLANDS NORTH BRABANT ZE46-1	**30**	**41**	**44** ENGLAND GLOUCESTERSHIRE GLS025	**55** ENGLAND NORTHAMPTONSHIRE NHP008
3 FRANCE NORMANDY REN003	**12** ENGLAND GLOUCESTERSHIRE GLS022	**17** SCOTLAND EAST LOTHIAN ELT004	**26**	**31** SCOTLAND EAST LOTHIAN ELT005	**40** ENGLAND HEREFORDSHIRE HRF017	**45** ENGLAND SURREY ALICE HOLT	**54** NETHERLANDS NORTH BRABANT ZE142-1
4 ENGLAND HEREFORDSHIRE HRF008	**11** NETHERLANDS NORTH BRABANT ZE64-1	**18**	**25** ENGLAND CUMBRIA CUM001	**32** ENGLAND HAMPSHIRE HAM006	**39** ENGLAND SUFFOLK SUF001	**46** SCOTLAND HIGHLANDS HGH001	**53**
5 ENGLAND NORTHAMPTONSHIRE NHP002	**10** ENGLAND LEICESTERSHIRE LEI001	**19** ENGLAND NORFOLK NOR007	**24** ENGLAND NORTHAMPTONSHIRE NHP009	**33** NETHERLANDS NORTH BRABANT ZE58-2	**38** ENGLAND NORFOLK NOR005	**47**	**52** ENGLAND SUFFOLK SUF004
6 ENGLAND HAMPSHIRE HAM013	**9**	**20** NETHERLANDS GELDERLAND ZE23-2	**23** ENGLAND HEREFORDSHIRE HRF006	**34** ENGLAND HEREFORDSHIRE HRF004	**37** SCOTLAND BORDERS BOR002	**48** NETHERLANDS GELDERLAND ZE34-1	**51** ENGLAND GLOUCESTERSHIRE GLS016
7 ENGLAND HAMPSHIRE HAM004	**8** ENGLAND HEREFORDSHIRE HRF013	**21** NETHERLANDS BRABANT ZE82-1	**22** ENGLAND HAMPSHIRE HAM012	**35**	**36** ENGLAND LINCOLNSHIRE LNC001	**49** NETHERLANDS NORTH BRABANT ZE47-2	**50** ENGLAND NORTHAMPTONSHIRE NHP006

Supplementary figure D.2 cont. – tree layout within the BSO trial, each rectangle represents each tree. Blank rectangles represent dead trees and trees excluded from analysis. Only the 14 blocks selected for analysis are shown, all trees shown were analysed for insect herbivores, trees shaded purple were selected for endophyte analysis also.

BLOCK 35

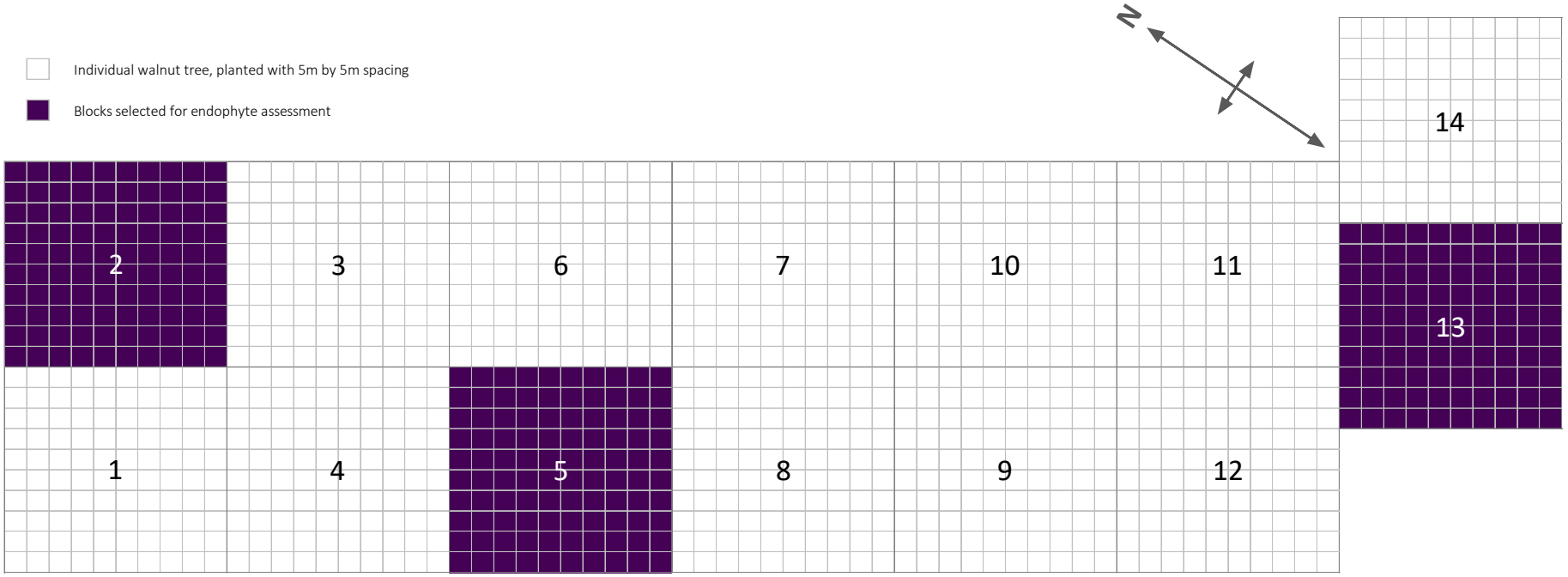
1 ENGLAND HAMPSHIRE HAM007	**14** ENGLAND SOMERSET SOM003	**15** ENGLAND LINCOLNSHIRE LNC001	**28** ENGLAND GLOUCESTERSHIRE GLS022	**29** SCOTLAND BORDERS BOR002	**42** ENGLAND HEREFORDSHIRE HRF004	**43** NETHERLANDS GELDERLAND ZE23-2	**56**
2 ENGLAND NORFOLK NOR007	**13** ENGLAND NORTHAMPTONSHIRE NHP009	**16** NETHERLANDS MID BRABANT ZE80-10	**27**	**30** NETHERLANDS NORTH BRABANT ZE46-1	**41** ENGLAND HEREFORDSHIRE HRF006	**44** ENGLAND LEICESTERSHIRE LEI001	**55**
3 ENGLAND GLOUCESTERSHIRE GLS014	**12** FRANCE NORMANDY REN003	**17** FRANCE OFFALY OFL002	**26** FRANCE CHARTRES SEN002	**31**	**40** ENGLAND NORTHAMPTONSHIRE NHP008	**45** ENGLAND GLOUCESTERSHIRE GLS012	**54** ENGLAND HEREFORDSHIRE HRF017
4 ENGLAND HEREFORDSHIRE HRF008	**11** ENGLAND HAMPSHIRE HAM014	**18** IRELAND WICKLOW WIK003	**25** NETHERLANDS NORTH BRABANT ZE64-1	**32** SCOTLAND EAST LOTHIAN ELT005	**39** ENGLAND GLOUCESTERSHIRE GLS016	**46** NETHERLANDS BRABANT ZE82-1	**53** FRANCE NORMANDY REN001
5 ENGLAND NORTHUMBERLAND NMB001	**10** ENGLAND CUMBRIA CUM001	**19** ENGLAND NORTHAMPTONSHIRE NHP002	**24** SCOTLAND EAST LOTHIAN ELT004	**33** ENGLAND SUFFOLK SUF001	**38** ENGLAND SURREY ALICE HOLT	**47** ENGLAND GLOUCESTERSHIRE GLS025	**52** ENGLAND HEREFORDSHIRE HRF013
6 SCOTLAND BORDERS BOR001	**9** NETHERLANDS NORTH BRABANT ZE47-2	**20** ENGLAND SUFFOLK SUF003	**23** NETHERLANDS GELDERLAND ZE11-1	**34** ENGLAND HAMPSHIRE HAM006	**37** ENGLAND SUFFOLK SUF004	**48** NETHERLANDS NORTH BRABANT ZE142-1	**51**
7	**8** ENGLAND NORFOLK NOR005	**21**	**22** SCOTLAND DUMFRIES & GALLOWAY D&G003	**35** ENGLAND HAMPSHIRE HAM012	**36** NETHERLANDS GELDERLAND ZE34-1	**49** NETHERLANDS NORTH BRABANT ZE58-2	**50**

BLOCK 37

1 ENGLAND HAMPSHIRE HAM014	**14** NETHERLANDS NORTH BRABANT ZE64-1	**15** ENGLAND GLOUCESTERSHIRE GLS014	**28** ENGLAND HEREFORDSHIRE HRF008	**29** ENGLAND NORFOLK NOR007	**42**	**43** FRANCE SARTHE BRC001	**56** ENGLAND HEREFORDSHIRE HRF006
2 ENGLAND HEREFORDSHIRE HRF017	**13** ENGLAND NORTHAMTONSHIRE NHP002	**16** ENGLAND GLOUCESTERSHIRE GLS022	**27** ENGLAND CUMBRIA CUM001	**30** ENGLAND NORTHAMPTONSHIRE NHP006	**41**	**44** ENGLAND NORFOLK NOR005	**55**
3 IRELAND OFFALY OFL002	**12** ENGLAND NORTHUMBERLAND NMB001	**17** SCOTLAND BORDERS BOR001	**26** SCOTLAND EAST LOTHIAN ELT004	**31** NETHERLANDS MID BRABANT ZE80-1	**40** NETHERLANDS GELDERLAND ZE23-2	**45** ENGLAND GLOUCESTERSHIRE GLS016	**54** ENGLAND LINCOLNSHIRE LNC001
4 NETHERLANDS GELDERLAND ZE34-1	**11** SCOTLAND BORDERS BOR002	**18** ENGLAND SUFFOLK SUF001	**25** ENGLAND NORTHAMPTONSHIRE NHP008	**32** ENGLAND SUFFOLK SUF003	**39** FRANCE NORMANDY REN003	**46** ENGLAND LEICESTERSHIRE LEI001	**53** NETHERLANDS NORTH BRABANT ZE46-1
5 ENGLAND GLOUCESTERSHIRE GLS025	**10**	**19**	**24** FRANCE NORMANDY REN001	**33** NETHERLANDS GELDERLAND ZE11-1	**38** SCOTLAND DUMFRIES & GALLOWAY D&G003	**47** NETHERLANDS BRABANT ZE82-1	**52** ENGLAND HAMPSHIRE HAM013
6	**9** ENGLAND HAMPSHIRE HAM006	**20**	**23** NETHERLANDS NORTH BRABANT ZE142-1	**34** ENGLAND SOMERSET SOM003	**37** ENGLAND HEREFORDSHIRE HRF013	**48** ENGLAND HAMPSHIRE HAM012	**51** NETHERLANDS NORTH BRABANT ZE47-2
7 SCOTLAND EAST LOTHIAN ELT005	**8** ENGLAND SOMERSET SOM002	**21** ENGLAND HAMPSHIRE HAM007	**22** ENGLAND GLOUCESTERSHIRE GLS012	**35**	**36** ENGLAND HEREFORDSHIRE HERF004	**49** ENGLAND SUFFOLK SUF004	**50** ENGLAND HAMPSHIRE HAM004

Supplementary figure D.2 cont. – tree layout within the BSO trial, each rectangle represents each tree. Blank rectangles represent dead trees and trees excluded from analysis. Only the 14 blocks selected for analysis are shown, all trees shown were analysed for insect herbivores, trees shaded purple were selected for endophyte analysis also.

D.2 *Common walnut provenance trial*



Supplementary figure D.3 – layout of the 'common walnut provenance trial' in Paradise Wood, block numbers are shown in bold. Each square represents an individual walnut tree, planted with 5m by 5m spacing. Blocks in **purple** represent blocks selected for endophyte assessment.

BLOCK 2

100	99	98	97	96	95	94	93	92	91
81	82	83	84	85	86	87	88	89	90
80	79	78	77	76	75	74	73	72	71
61	62	63	64	65	66	67	68	69	70
60	59	58	57	56	55	54	53	52	51
41	42	43	44	45	46	47	48	49	50
40	39	38	37	36	35	34	33	32	31
21	22	23	24	25	26	27	28	29	30
20	19	18	17 KYRGYZSTAN K11.16	16	15	14	13	12	11
1	2 KYRGYZSTAN K11.1	3	4	5	6	7	8	9	10

BLOCK 5

100	99	98	97	96	95	94	93	92	91
81	82	83	84	85 KYRGYZSTAN K11.16	86	87	88	89	90
80	79	78	77	76	75	74	73	72	71
61	62	63	64 SPAIN E1.2	65	66	67	68	69	70
60	59	58	57	56	55	54	53	52	51
41	42	43	44	45	46	47	48	49	50
40	39	38	37	36	35	34	33	32	31
21	22	23	24	25	26	27	28	29	30
20	19	18	17	16	15	14	13	12	11
1	2	3 KYRGYZSTAN K11.1	4	5	6	7	8	9	10

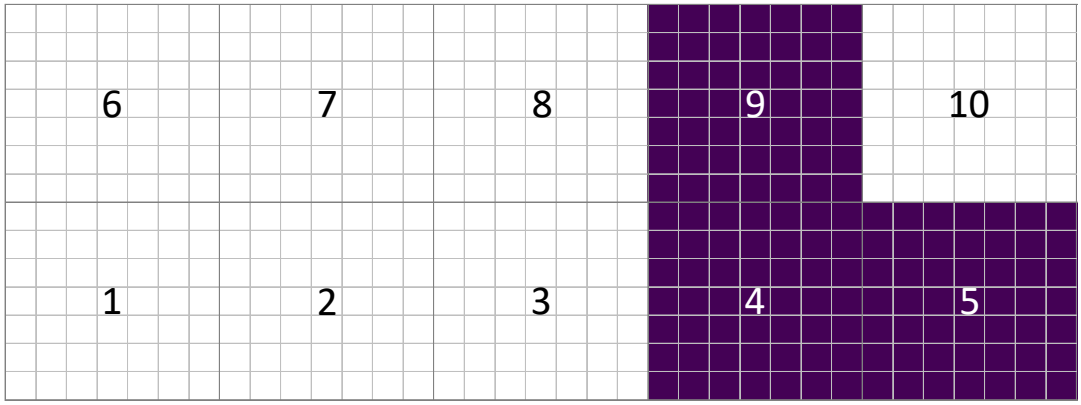
Supplementary figure D.4 – tree layout within the 'common walnut provenance trial', each rectangle represents each tree. Only the 3 blocks selected for analysis are shown, trees shaded purple were selected for endophyte analysis.

BLOCK 18

100	99	98	97	96	95	94	93 KYRGYZSTAN K11.16	92	91
81	82	83	84	85	86	87	88	89	90
80	79	78	77	76	75	74	73	72	71
61	62	63	64	65	66	67	68	69	70
60	59 SPAIN E1.2	58	57	56	55	54	53	52	51
41	42 KYRGYZSTAN K11.11	43	44	45	46	47	48	49	50
40	39	38	37	36	35	34	33	32	31
21	22	23	24	25	26	27 SPAIN E1.4	28	29	30
20	19	18	17	16	15	14	13	12	11
1	2	3	4	5	6	7	8	9	10

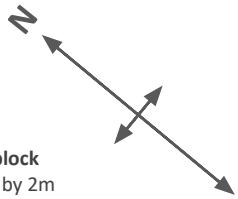
Supplementary figure D.4 cont. – tree layout within the 'common walnut provenance trial', each rectangle represents each tree. Only the 3 blocks selected for analysis are shown, trees shaded purple were selected for endophyte analysis.

D.3 *Black walnut provenance trial*



Individual walnut tree, planted with 2m by 2m spacing

Blocks selected for endophyte assessment



Supplementary figured D.5 – layout of the 'black walnut provenance trial' in Paradise Wood, block numbers are shown in bold. Each square represents an individual walnut tree, planted with 2m by 2m spacing. Blocks in **purple** represent blocks selected for endophyte assessment.

BLOCK 4

1	14	15	28	29	**42** CZECH REPUBLIC CR11	43
2 AUSTRIA AU09	13	16	27	30	41	**44** CZECH REPUBLIC CR04
3	12	17	26	31	**40** AUSTRIA AU11	45
4	11	18	25	32	39	46
5	10	19	24	33	38	47
6	9	20	23	34	37	48
7	8	21	22	35	36	49

Supplementary figure D.6 - tree layout within the 'black walnut provenance trial', each rectangle represents each tree. Only the 3 blocks selected for analysis are shown, trees shaded purple were selected for endophyte analysis.

BLOCK 5

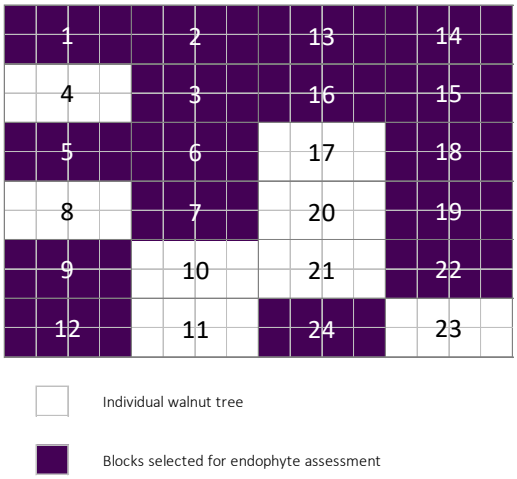
1	14	15	28	29	42	43
2	13	16	**27** CZECH REPUBLIC CR04	30	41	44
3	12	17	26	31	40	45
4	11	18	**25** AUSTRIA AU09	32	39	46
5 AUSTRIA AU11	10	19	24	33	38	47
6	9	20	23	**34** CZECH REPUBLIC CR11	37	48
7	8	21	22	35	36	49

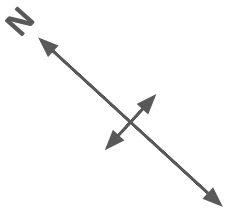
BLOCK 9

1	14	15	28	29	42	43
2	13	16	27	30	41	44
3 AUSTRIA AU09	12	17	26	31	40	45
4	11	18	25	32	39	46
5	10	**19** CZECH REPUBLICH CR11	24	33	38	47
6	9	**20** CZECH REPUBLIC CR04	**23** AUSTRIA AU11	34	37	48
7	8	21	22	35	36	49

Supplementary figure D.6 cont. - tree layout within the 'black walnut provenance trial', each rectangle represents each tree. Only the 3 blocks selected for analysis are shown, trees shaded purple were selected for endophyte analysis.

D.4 *Walnut nitrogen trial*





Supplementary figure D.7 – layout of the 'nitrogen trial' in Paradise Wood, block numbers are shown in bold. Each square represents an individual walnut tree. Blocks in **purple** represent blocks selected for endophyte assessment.

BLOCK 1

1 *JUGLANS NIGRA* FRANCE CONTROL	**2** *JUGLANS NIGRA* FRANCE CONTROL	**3** *JUGLANS NIGRA* FRANCE CONTROL	**4** *JUGLANS NIGRA* FRANCE CONTROL
5 *JUGLANS NIGRA* FRANCE CONTROL	**6** *JUGLANS NIGRA* FRANCE CONTROL	**7** *JUGLANS NIGRA* FRANCE CONTROL	**8** *JUGLANS NIGRA* FRANCE CONTROL

BLOCK 2

1 *JUGLANS REGIA* FRANCE 200 N kg/ha	**2** *JUGLANS REGIA* FRANCE 200 N kg/ha	**3** *JUGLANS REGIA* FRANCE 200 N kg/ha	**4** *JUGLANS REGIA* FRANCE 200 N kg/ha
5 *JUGLANS REGIA* FRANCE 200 N kg/ha	**6** *JUGLANS REGIA* FRANCE 200 N kg/ha	**7** *JUGLANS REGIA* FRANCE 200 N kg/ha	**8** *JUGLANS REGIA* FRANCE 200 N kg/ha

BLOCK 3

1 *JUGLANS NIGRA* FRANCE 400 N kg/ha	**2** *JUGLANS NIGRA* FRANCE 400 N kg/ha	**3** *JUGLANS NIGRA* FRANCE 400 N kg/ha	**4** *JUGLANS NIGRA* FRANCE 400 N kg/ha
5 *JUGLANS NIGRA* FRANCE 400 N kg/ha	**6** *JUGLANS NIGRA* FRANCE 400 N kg/ha	**7** *JUGLANS NIGRA* FRANCE 400 N kg/ha	**8** *JUGLANS NIGRA* FRANCE 400 N kg/ha

BLOCK 5

1 *JUGLANS REGIA* FRANCE CONTROL	**2** *JUGLANS REGIA* FRANCE CONTROL	**3** *JUGLANS REGIA* FRANCE CONTROL	**4** *JUGLANS REGIA* FRANCE CONTROL
5 *JUGLANS REGIA* FRANCE CONTROL	**6** *JUGLANS REGIA* FRANCE CONTROL	**7** *JUGLANS REGIA* FRANCE CONTROL	**8** *JUGLANS REGIA* FRANCE CONTROL

BLOCK 6

1 *JUGLANS NIGRA* FRANCE 100 N kg/ha	**2** *JUGLANS NIGRA* FRANCE 100 N kg/ha	**3** *JUGLANS NIGRA* FRANCE 100 N kg/ha	**4** *JUGLANS NIGRA* FRANCE 100 N kg/ha
5 *JUGLANS NIGRA* FRANCE 100 N kg/ha	**6** *JUGLANS NIGRA* FRANCE 100 N kg/ha	**7** *JUGLANS NIGRA* FRANCE 100 N kg/ha	**8** *JUGLANS NIGRA* FRANCE 100 N kg/ha

BLOCK 7

1 *JUGLANS REGIA* FRANCE 400 N kg/ha	**2** *JUGLANS REGIA* FRANCE 400 N kg/ha	**3** *JUGLANS REGIA* FRANCE 400 N kg/ha	**4** *JUGLANS REGIA* FRANCE 400 N kg/ha
5 *JUGLANS REGIA* FRANCE 400 N kg/ha	**6** *JUGLANS REGIA* FRANCE 400 N kg/ha	**7** *JUGLANS REGIA* FRANCE 400 N kg/ha	**8** *JUGLANS REGIA* FRANCE 400 N kg/ha

Supplementary figure D.8 – tree layout within the 'nitrogen trial', each rectangle represents each tree. Only the 16 blocks selected for analysis are shown.

BLOCK 9

1 *JUGLANS REGIA* FRANCE 100 N kg/ha	**2** *JUGLANS REGIA* FRANCE 100 N kg/ha	**3** *JUGLANS REGIA* FRANCE 100 N kg/ha	**4** *JUGLANS REGIA* FRANCE 100 N kg/ha
5 *JUGLANS REGIA* FRANCE 100 N kg/ha	**6** *JUGLANS REGIA* FRANCE 100 N kg/ha	**7** *JUGLANS REGIA* FRANCE 100 N kg/ha	**8** *JUGLANS REGIA* FRANCE 100 N kg/ha

BLOCK 12

1 *JUGLANS NIGRA* FRANCE 200 N kg/ha	**2** *JUGLANS NIGRA* FRANCE 200 N kg/ha	**3** *JUGLANS NIGRA* FRANCE 200 N kg/ha	**4** *JUGLANS NIGRA* FRANCE 200 N kg/ha
5 *JUGLANS NIGRA* FRANCE 200 N kg/ha	**6** *JUGLANS NIGRA* FRANCE 200 N kg/ha	**7** *JUGLANS NIGRA* FRANCE 200 N kg/ha	**8** *JUGLANS NIGRA* FRANCE 200 N kg/ha

BLOCK 13

1 *JUGLANS REGIA* FRANCE 200 N kg/ha	**2** *JUGLANS REGIA* FRANCE 200 N kg/ha	**3** *JUGLANS REGIA* FRANCE 200 N kg/ha	**4** *JUGLANS REGIA* FRANCE 200 N kg/ha
5 *JUGLANS REGIA* FRANCE 200 N kg/ha	**6** *JUGLANS REGIA* FRANCE 200 N kg/ha	**7** *JUGLANS REGIA* FRANCE 200 N kg/ha	**8** *JUGLANS REGIA* FRANCE 200 N kg/ha

BLOCK 14

1 *JUGLANS NIGRA* FRANCE 400 N kg/ha	**2** *JUGLANS NIGRA* FRANCE 400 N kg/ha	**3** *JUGLANS NIGRA* FRANCE 400 N kg/ha	**4** *JUGLANS NIGRA* FRANCE 400 N kg/ha
5 *JUGLANS NIGRA* FRANCE 400 N kg/ha	**6** *JUGLANS NIGRA* FRANCE 400 N kg/ha	**7** *JUGLANS NIGRA* FRANCE 400 N kg/ha	**8** *JUGLANS NIGRA* FRANCE 400 N kg/ha

BLOCK 15

1 *JUGLANS NIGRA* FRANCE 100 N kg/ha	**2** *JUGLANS NIGRA* FRANCE 100 N kg/ha	**3** *JUGLANS NIGRA* FRANCE 100 N kg/ha	**4** *JUGLANS NIGRA* FRANCE 100 N kg/ha
5 *JUGLANS NIGRA* FRANCE 100 N kg/ha	**6** *JUGLANS NIGRA* FRANCE 100 N kg/ha	**7** *JUGLANS NIGRA* FRANCE 100 N kg/ha	**8** *JUGLANS NIGRA* FRANCE 100 N kg/ha

BLOCK 16

1 *JUGLANS NIGRA* FRANCE 200 N kg/ha	**2** *JUGLANS NIGRA* FRANCE 200 N kg/ha	**3** *JUGLANS NIGRA* FRANCE 200 N kg/ha	**4** *JUGLANS NIGRA* FRANCE 200 N kg/ha
5 *JUGLANS NIGRA* FRANCE 200 N kg/ha	**6** *JUGLANS NIGRA* FRANCE 200 N kg/ha	**7** *JUGLANS NIGRA* FRANCE 200 N kg/ha	**8** *JUGLANS NIGRA* FRANCE 200 N kg/ha

BLOCK 18

1 *JUGLANS REGIA* FRANCE CONTROL	**2** *JUGLANS REGIA* FRANCE CONTROL	**3** *JUGLANS REGIA* FRANCE CONTROL	**4** *JUGLANS REGIA* FRANCE CONTROL
5 *JUGLANS REGIA* FRANCE CONTROL	**6** *JUGLANS REGIA* FRANCE CONTROL	**7** *JUGLANS REGIA* FRANCE CONTROL	**8** *JUGLANS REGIA* FRANCE CONTROL

BLOCK 19

1 *JUGLANS REGIA* FRANCE 100 N kg/ha	**2** *JUGLANS REGIA* FRANCE 100 N kg/ha	**3** *JUGLANS REGIA* FRANCE 100 N kg/ha	**4** *JUGLANS REGIA* FRANCE 100 N kg/ha
5 *JUGLANS REGIA* FRANCE 100 N kg/ha	**6** *JUGLANS REGIA* FRANCE 100 N kg/ha	**7** *JUGLANS REGIA* FRANCE 100 N kg/ha	**8** *JUGLANS REGIA* FRANCE 100 N kg/ha

BLOCK 22

1 *JUGLANS NIGRA* FRANCE CONTROL	**2** *JUGLANS NIGRA* FRANCE CONTROL	**3** *JUGLANS NIGRA* FRANCE CONTROL	**4** *JUGLANS NIGRA* FRANCE CONTROL
5 *JUGLANS NIGRA* FRANCE CONTROL	**6** *JUGLANS NIGRA* FRANCE CONTROL	**7** *JUGLANS NIGRA* FRANCE CONTROL	**8** *JUGLANS NIGRA* FRANCE CONTROL

BLOCK 24

1 *JUGLANS REGIA* FRANCE 400 N kg/ha	**2** *JUGLANS REGIA* FRANCE 400 N kg/ha	**3** *JUGLANS REGIA* FRANCE 400 N kg/ha	**4** *JUGLANS REGIA* FRANCE 400 N kg/ha
5 *JUGLANS REGIA* FRANCE 400 N kg/ha	**6** *JUGLANS REGIA* FRANCE 400 N kg/ha	**7** *JUGLANS REGIA* FRANCE 400 N kg/ha	**8** *JUGLANS REGIA* FRANCE 400 N kg/ha

Supplementary figure D.8 cont. – tree layout within the 'nitrogen trial', each rectangle represents each tree. Only the 16 blocks selected for analysis are shown.

APPENDIX E - *Supplementary information for Illumina Miseq sequencing*

E.1 *Second step PCR primers*

Supplementary table E.1 – primer sequences used to make the primer arrays (*Appendix* E.2-E.5) for the second step PCR

Primer name	Primer sequence
ITF.SA501	AATGATACGGCGACCACCGAGATCTACACATCGTACGACACTCTTTCCCTACACGACG
ITF.SA502	AATGATACGGCGACCACCGAGATCTACACACTATCTGACACTCTTTCCCTACACGACG
ITF.SA503	AATGATACGGCGACCACCGAGATCTACACTAGCGAGTACACTCTTTCCCTACACGACG
ITF.SA504	AATGATACGGCGACCACCGAGATCTACACCTGCGTGTACACTCTTTCCCTACACGACG
ITF.SB505	AATGATACGGCGACCACCGAGATCTACACACGTCTCGACACTCTTTCCCTACACGACG
ITF.SB506	AATGATACGGCGACCACCGAGATCTACACTCGACGAGACACTCTTTCCCTACACGACG
ITF.SB507	AATGATACGGCGACCACCGAGATCTACACGATCGTGTACACTCTTTCCCTACACGACG
ITF.SB508	AATGATACGGCGACCACCGAGATCTACACGTCAGATAACACTCTTTCCCTACACGACG
ITR.SA701	CAAGCAGAAGACGGCATACGAGATAACTCTCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SA702	CAAGCAGAAGACGGCATACGAGATACTATGTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SA703	CAAGCAGAAGACGGCATACGAGATAGTAGCGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SA704	CAAGCAGAAGACGGCATACGAGATCAGTGAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SA705	CAAGCAGAAGACGGCATACGAGATCGTACTCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SA706	CAAGCAGAAGACGGCATACGAGATCTACGCAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SA707	CAAGCAGAAGACGGCATACGAGATGGAGACTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SA708	CAAGCAGAAGACGGCATACGAGATGTCGCTCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SA709	CAAGCAGAAGACGGCATACGAGATGTCGTAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SA710	CAAGCAGAAGACGGCATACGAGATTAGCAGACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SA711	CAAGCAGAAGACGGCATACGAGATTCATAGACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SA712	CAAGCAGAAGACGGCATACGAGATTCGCTATAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SB701	CAAGCAGAAGACGGCATACGAGATAAGTCGAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SB702	CAAGCAGAAGACGGCATACGAGATATACTTCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SB703	CAAGCAGAAGACGGCATACGAGATAGCTGCTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SB704	CAAGCAGAAGACGGCATACGAGATCATAGAGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SB705	CAAGCAGAAGACGGCATACGAGATCGTAGATCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SB706	CAAGCAGAAGACGGCATACGAGATCTCGTTACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SB707	CAAGCAGAAGACGGCATACGAGATGCGCACGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SB708	CAAGCAGAAGACGGCATACGAGATGGTACTATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SB709	CAAGCAGAAGACGGCATACGAGATGTATACGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SB710	CAAGCAGAAGACGGCATACGAGATTACGAGCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SB711	CAAGCAGAAGACGGCATACGAGATTCAGCGTTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
ITR.SB712	CAAGCAGAAGACGGCATACGAGATTCGCTACGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

E.2 *Array A*

	1	2	3	4	5	6	7	8	9	10	11	12
A	A701 A501	A702 A501	A703 A501	A704 A501	A705 A501	A706 A501	A707 A501	A708 A501	A709 A501	A710 A501	A711 A501	A712 A501
B	A701 A502	A702 A502	A703 A502	A704 A502	A705 A502	A706 A502	A707 A502	A708 A502	A709 A502	A710 A502	A711 A502	A712 A502
C	A701 A503	A702 A503	A703 A503	A704 A503	A705 A503	A706 A503	A707 A503	A708 A503	A709 A503	A710 A503	A711 A503	A712 A503
D	A701 A504	A702 A504	A703 A504	A704 A504	A705 A504	A706 A504	A707 A504	A708 A504	A709 A504	A710 A504	A711 A504	A712 A504
E	A701 A505	A702 A505	A703 A505	A704 A505	A705 A505	A706 A505	A707 A505	A708 A505	A709 A505	A710 A505	A711 A505	A712 A505
F	A701 A506	A702 A506	A703 A506	A704 A506	A705 A506	A706 A506	A707 A506	A708 A506	A709 A506	A710 A506	A711 A506	A712 A506
G	A701 A507	A702 A507	A703 A507	A704 A507	A705 A507	A706 A507	A707 A507	A708 A507	A709 A507	A710 A507	A711 A507	A712 A507
H	A701 A508	A702 A508	A703 A508	A704 A508	A705 A508	A706 A508	A707 A508	A708 A508	A709 A508	A710 A508	A711 A508	A712 A508

Supplementary figure E.1 – primer array layout A used in the second step PCR for Illumina Miseq sequencing

E.3 *Array B*

	1	2	3	4	5	6	7	8	9	10	11	12
A	A701 B501	A702 B501	A703 B501	A704 B501	A705 B501	A706 B501	A707 B501	A708 B501	A709 B501	A710 B501	A711 B501	A712 B501
B	A701 B502	A702 B502	A703 B502	A704 B502	A705 B502	A706 B502	A707 B502	A708 B502	A709 B502	A710 B502	A711 B502	A712 B502
C	A701 B503	A702 B503	A703 B503	A704 B503	A705 B503	A706 B503	A707 B503	A708 B503	A709 B503	A710 B503	A711 B503	A712 B503
D	A701 B504	A702 B504	A703 B504	A704 B504	A705 B504	A706 B504	A707 B504	A708 B504	A709 B504	A710 B504	A711 B504	A712 B504
E	A701 B505	A702 B505	A703 B505	A704 B505	A705 B505	A706 B505	A707 B505	A708 B505	A709 B505	A710 B505	A711 B505	A712 B505
F	A701 B506	A702 B506	A703 B506	A704 B506	A705 B506	A706 B506	A707 B506	A708 B506	A709 B506	A710 B506	A711 B506	A712 B506
G	A701 B507	A702 B507	A703 B507	A704 B507	A705 B507	A706 B507	A707 B507	A708 B507	A709 B507	A710 B507	A711 B507	A712 B507
H	A701 B508	A702 B508	A703 B508	A704 B508	A705 B508	A706 B508	A707 B508	A708 B508	A709 B508	A710 B508	A711 B508	A712 B508

Supplementary figure E.2 – primer array layout A used in the second step PCR for Illumina Miseq sequencing

E.4 *Array C*

	1	2	3	4	5	6	7	8	9	10	11	12
A	B701 A501	B702 A501	B703 A501	B704 A501	B705 A501	B706 A501	B707 A501	B708 A501	B709 A501	B710 A501	B711 A501	B712 A501
B	B701 A502	B702 A502	B703 A502	B704 A502	B705 A502	B706 A502	B707 A502	B708 A502	B709 A502	B710 A502	B711 A502	B712 A502
C	B701 A503	B702 A503	B703 A503	B704 A503	B705 A503	B706 A503	B707 A503	B708 A503	B709 A503	B710 A503	B711 A503	B712 A503
D	B701 A504	B702 A504	B703 A504	B704 A504	B705 A504	B706 A504	B707 A504	B708 A504	B709 A504	B710 A504	B711 A504	B712 A504
E	B701 A505	B702 A505	B703 A505	B704 A505	B705 A505	B706 A505	B707 A505	B708 A505	B709 A505	B710 A505	B711 A505	B712 A505
F	B701 A506	B702 A506	B703 A506	B704 A506	B705 A506	B706 A506	B707 A506	B708 A506	B709 A506	B710 A506	B711 A506	B712 A506
G	B701 A507	B702 A507	B703 A507	B704 A507	B705 A507	B706 A507	B707 A507	B708 A507	B709 A507	B710 A507	B711 A507	B712 A507
H	B701 A508	B702 A508	B703 A508	B704 A508	B705 A508	B706 A508	B707 A508	B708 A508	B709 A508	B710 A508	B711 A508	B712 A508

Supplementary figure E.3 – primer array layout A used in the second step PCR for Illumina Miseq sequencing

E.5 *Array D*

	1	2	3	4	5	6	7	8	9	10	11	12
A	B701 B501	B702 B501	B703 B501	B704 B501	B705 B501	B706 B501	B707 B501	B708 B501	B709 B501	B710 B501	B711 B501	B712 B501
B	B701 B502	B702 B502	B703 B502	B704 B502	B705 B502	B706 B502	B707 B502	B708 B502	B709 B502	B710 B502	B711 B502	B712 B502
C	B701 B503	B702 B503	B703 B503	B704 B503	B705 B503	B706 B503	B707 B503	B708 B503	B709 B503	B710 B503	B711 B503	B712 B503
D	B701 B504	B702 B504	B703 B504	B704 B504	B705 B504	B706 B504	B707 B504	B708 B504	B709 B504	B710 B504	B711 B504	B712 B504
E	B701 B505	B702 B505	B703 B505	B704 B505	B705 B505	B706 B505	B707 B505	B708 B505	B709 B505	B710 B505	B711 B505	B712 B505
F	B701 B506	B702 B506	B703 B506	B704 B506	B705 B506	B706 B506	B707 B506	B708 B506	B709 B506	B710 B506	B711 B506	B712 B506
G	B701 B507	B702 B507	B703 B507	B704 B507	B705 B507	B706 B507	B707 B507	B708 B507	B709 B507	B710 B507	B711 B507	B712 B507
H	B701 B508	B702 B508	B703 B508	B704 B508	B705 B508	B706 B508	B707 B508	B708 B508	B709 B508	B710 B508	B711 B508	B712 B508

Supplementary figure E.4 – primer array layout A used in the second step PCR for Illumina Miseq sequencing

E.6 *Sequencing plate layout*

	1	2	3	4	5	6	7	8	9	10	11	12
A	22 ELT004 LEAF	22 ELT005 LEAF	22 HAM007 LEAF	22 HAM013 LEAF	22 HAM014 LEAF	22 HRF004 LEAF	22 HRF013 LEAF	22 HRF017 LEAF	22 REN001 LEAF	22 REN003 LEAF	22 SUF001 LEAF	22 SUF003 LEAF
B	22 SUF004 LEAF	22 ZE11-1 LEAF	22 ZE142-1 LEAF	22 ZE23-2 LEAF	22 ZE46-1 LEAF	22 ZE47-2 LEAF	35 ELT004 LEAF	35 ELT005 LEAF	35 HAM007 LEAF	35 HAM012 LEAF	35 HAM014 LEAF	35 HRF004 LEAF
C	35 HRF013 LEAF	35 HRF017 LEAF	35 REN001 LEAF	35 REN003 LEAF	35 SUF001 LEAF	35 SUF003 LEAF	35 SUF004 LEAF	35 ZE11-1 LEAF	35 ZE142-1 LEAF	35 ZE23-2 LEAF	35 ZE46-1 LEAF	35 ZE47-2 LEAF
D	37 ELT004 LEAF	37 ELT005 LEAF	37 HAM007 LEAF	37 HAM012 LEAF	37 HAM013 LEAF	37 HAM014 LEAF	37 HRF004 LEAF	37 HRF013 LEAF	37 HRF017 LEAF	37 REN001 LEAF	37 REN003 LEAF	37 SUF001 LEAF
E	37 SUF003 LEAF	37 SUF004 LEAF	37 ZE11-1 LEAF	37 ZE142-1 LEAF	37 ZE23-2 LEAF	37 ZE46-1 LEAF	37 ZE47-2 LEAF	22 ELT004 TWIG	22 ELT005 TWIG	22 HAM007 TWIG	22 HAM013 TWIG	22 HAM014 TWIG
F	22 HRF004 TWIG	22 HRF013 TWIG	22 HRF017 TWIG	22 REN001 TWIG	22 REN003 TWIG	22 SUF001 TWIG	22 SUF003 TWIG	22 SUF004 TWIG	22 ZE11-1 TWIG	22 ZE142-1 TWIG	22 ZE23-2 TWIG	22 ZE46-1 TWIG
G	22 ZE47-2 TWIG	35 ELT004 TWIG	35 ELT005 TWIG	35 HAM007 TWIG	35 HAM012 TWIG	35 HAM014 TWIG	35 HRF004 TWIG	35 HRF013 TWIG	35 HRF017 TWIG	35 REN001 TWIG	35 REN003 TWIG	35 SUF001 TWIG
H	35 SUF003 TWIG	35 SUF004 TWIG	35 ZE11-1 TWIG	35 ZE142-1 TWIG	35 ZE23-2 TWIG	35 ZE46-1 TWIG	35 ZE47-2 TWIG	*Brenneria goodwinii*	*Gibbsiella quercinecans*	Qiagen extraction blank	Negative control	*Rahnella victoriana*

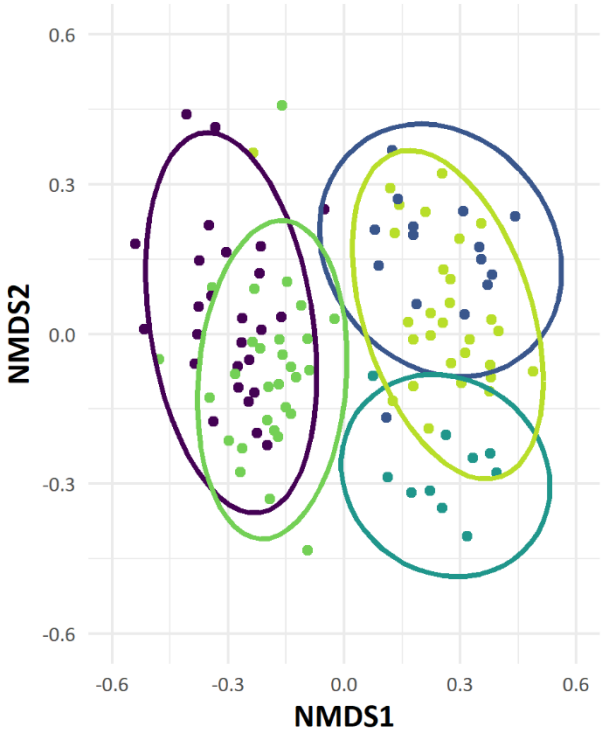
Supplementary figure E.5 – sequencing plant layout 1, repeated for 16S and ITS

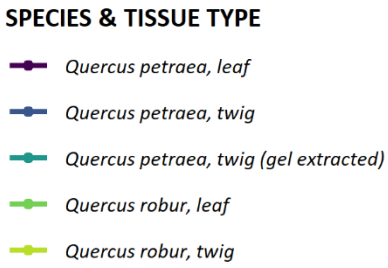
	1	2	3	4	5	6	7	8	9	10	11	12
A	37 ELT004 TWIG	37 ELT005 TWIG	37 HAM007 TWIG	37 HAM012 TWIG	37 HAM013 TWIG	37 HAM014 TWIG	37 HRF004 TWIG	37 HRF013 TWIG	37 HRF017 TWIG	37 REN001 TWIG	37 REN003 TWIG	37 SUF001 TWIG
B	37 SUF003 TWIG	37 SUF004 TWIG	37 ZE11-1 TWIG	37 ZE142-1 TWIG	37 ZE23-2 TWIG	37 ZE46-1 TWIG	37 ZE47-2 TWIG	2 K11.1 LEAF	2 K11.16 LEAF	5 E1.2 LEAF	5 K11.1 LEAF	5 K11.16 LEAF
C	13 E1.2 LEAF	13 E1.4 LEAF	13 K11.1 LEAF	13 K11.16 LEAF	2 K11.1 TWIG	2 K11.16 TWIG	5 E1.2 TWIG	5 K11.1 TWIG	5 K11.16 TWIG	13 E1.2 TWIG	13 E1.4 TWIG	13 K11.1 TWIG
D	13 K11.16 TWIG	4 AU09 LEAF	4 AU11 LEAF	4 CR04 LEAF	5 AU09 LEAF	5 AU11 LEAF	5 CR11 LEAF	9 AU09 LEAF	9 AU11 LEAF	9 CR04 LEAF	9 CR11 LEAF	4 AU09 TWIG
E	4 AU11 TWIG	4 CR04 TWIG	4 CR11 TWIG	5 AU09 TWIG	5 AU11 TWIG	5 CR04 TWIG	5 CR11 TWIG	9 AU09 TWIG	9 AU11 TWIG	9 CR04 TWIG	9 CR11 TWIG	4 COMMON 0 LEAF
F	21 COMMON 0 LEAF	6 BLACK 0 LEAF	23 BLACK 0 LEAF	2 COMMON 100 LEAF	22 COMMON 100 LEAF	9 BLACK 100 LEAF	20 BLACK 100 LEAF	7 COMMON 200 LEAF	18 COMMON 200 LEAF	1 BLACK 200 LEAF	17 BLACK 200 LEAF	10 COMMON 400 LEAF
G	13 COMMON 400 LEAF	8 BLACK 400 LEAF	19 BLACK 400 LEAF	4 COMMON 0 TWIG	21 COMMON 0 TWIG	6 BLACK 0 TWIG	23 BLACK 0 TWIG	2 COMMON 100 TWIG	22 COMMON 100 TWIG	9 BLACK 100 TWIG	20 BLACK 100 TWIG	7 COMMON 200 TWIG
H	18 COMMON 200 TWIG	1 BLACK 200 TWIG	17 BLACK 200 TWIG	10 COMMON 400 TWIG	13 COMMON 400 TWIG	8 BLACK 400 TWIG	19 BLACK 400 TWIG	*Brenneria nigrifluens*	*Brenneria rubrifaciens*	Li-CTAB ext. blank	Negative control	*Raoultella planticola*

Supplementary figure E.6 – sequencing plate layout 2, repeated for 16S and ITS

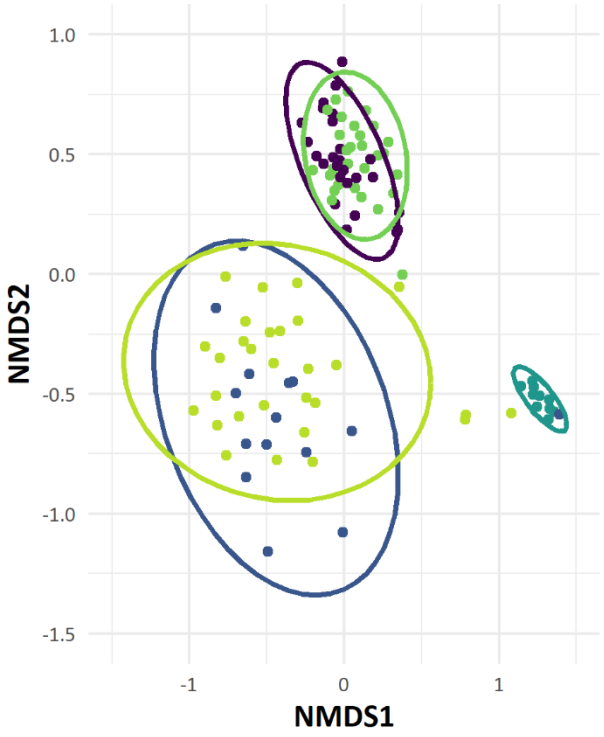
APPENDIX F - *Supplementary oak endophyte results*

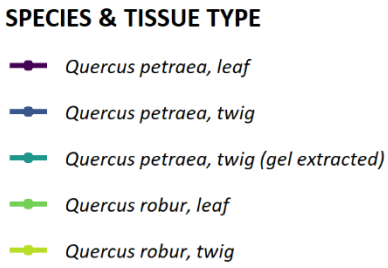
F.1 *NMDS plots with gel extracted samples*





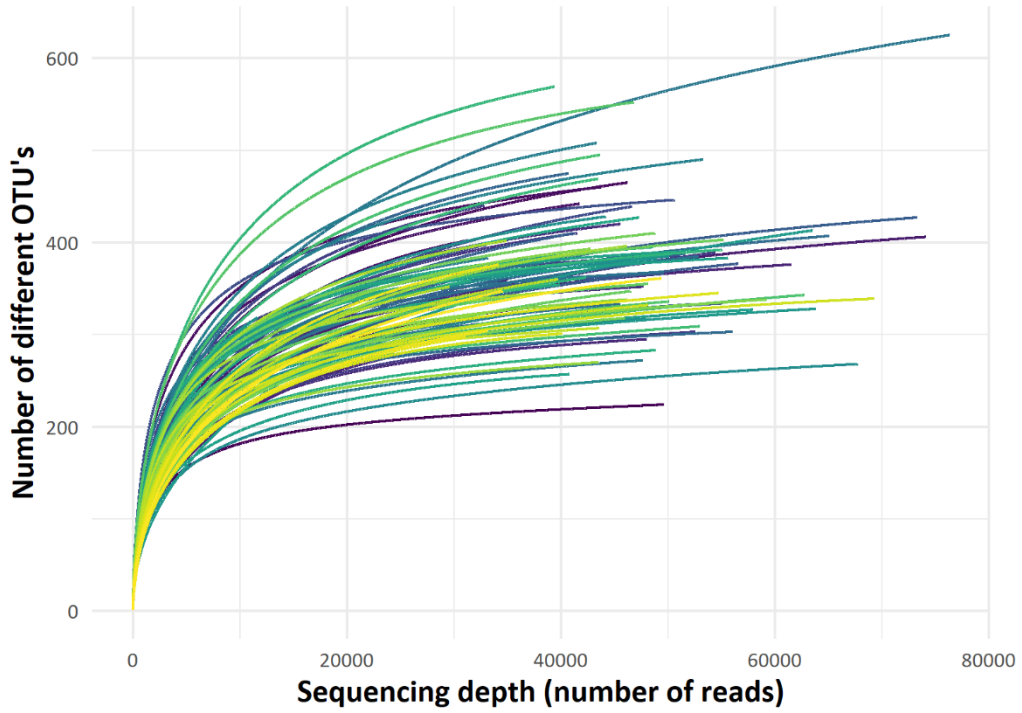
Supplementary figure F.1 – NMDS plot for the 16S OTU matrix with the gel extracted twig samples included. NMDS values were calculated using Bray-Curtis similarity, with a final stress of 0.1729. These gel extracted samples were removed from subsequent analysis.



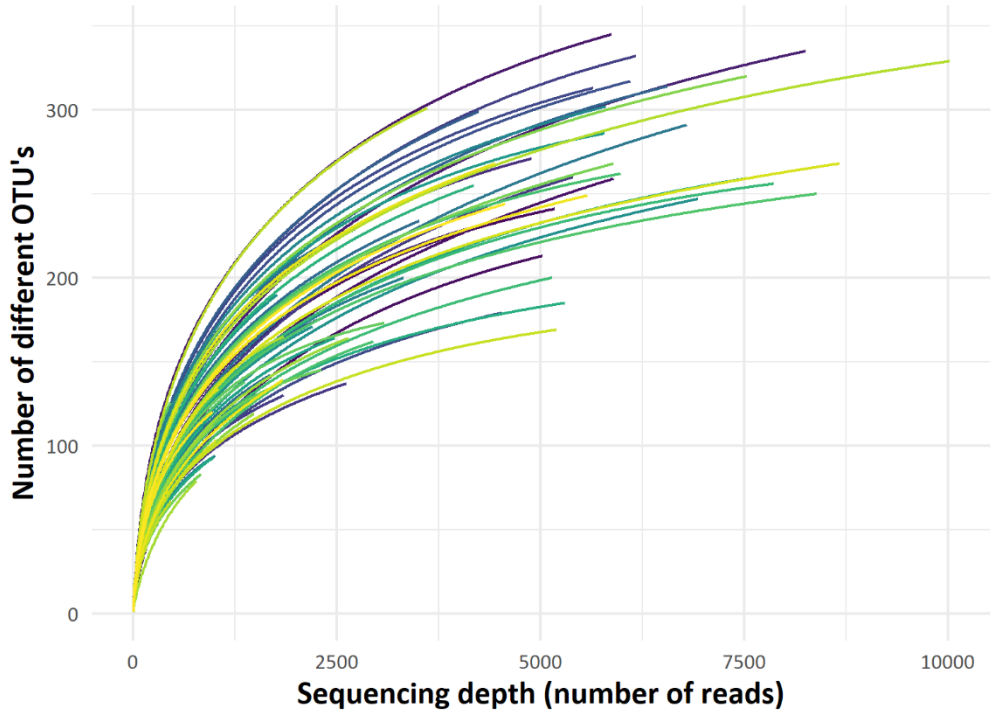


Supplementary figure F.2 – NMDS plot for the ITS OTU matrix with the gel extracted twig samples included. NMDS values were calculated using Bray-Curtis similarity, with a final stress of 0.2079. These gel extracted samples were removed from subsequent analysis.

F.2 *Rarefaction curves*



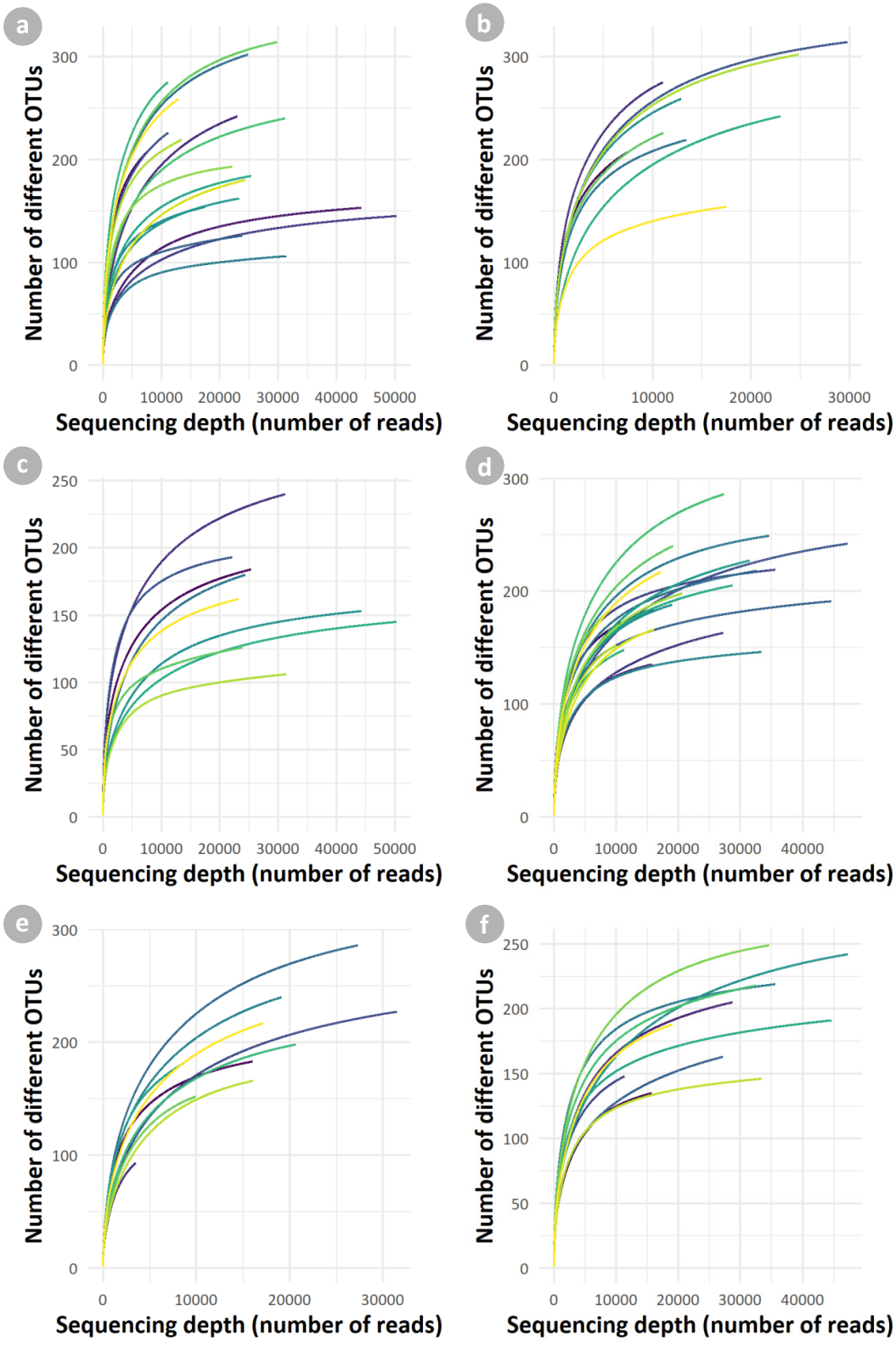
Supplementary figure F.3 – ITS rarefaction curve. Each colour represents a different sample. Plots were produced using the vegan package (version 2.5-3, Oksanen et al. (2018))



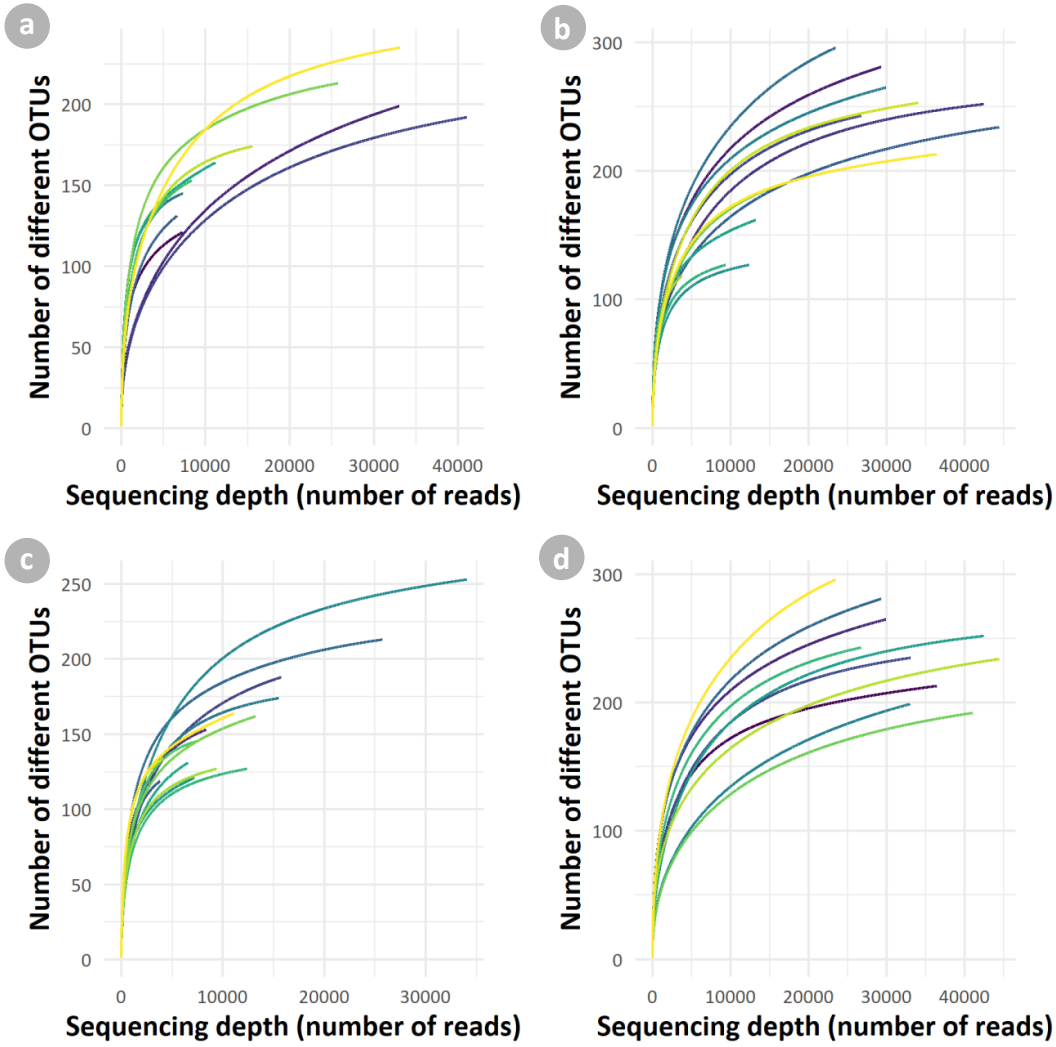
Supplementary figure F.3 – 16S rarefaction curve. Each colour represents a different sample. Plots were produced using the vegan package (version 2.5-3, Oksanen et al. (2018))

APPENDIX G - *Supplementary walnut endophyte results*

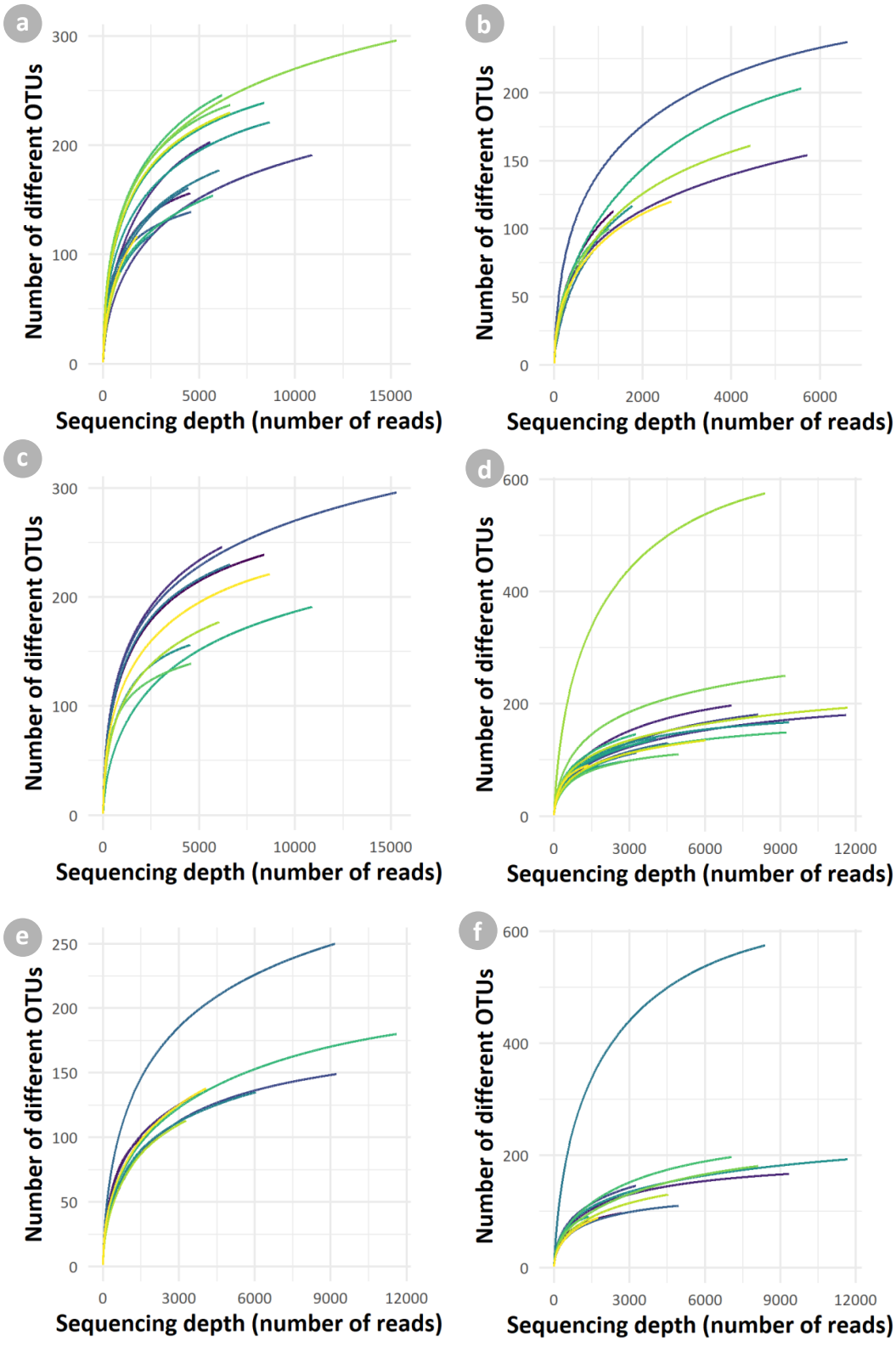
G.7 *Rarefaction curves*



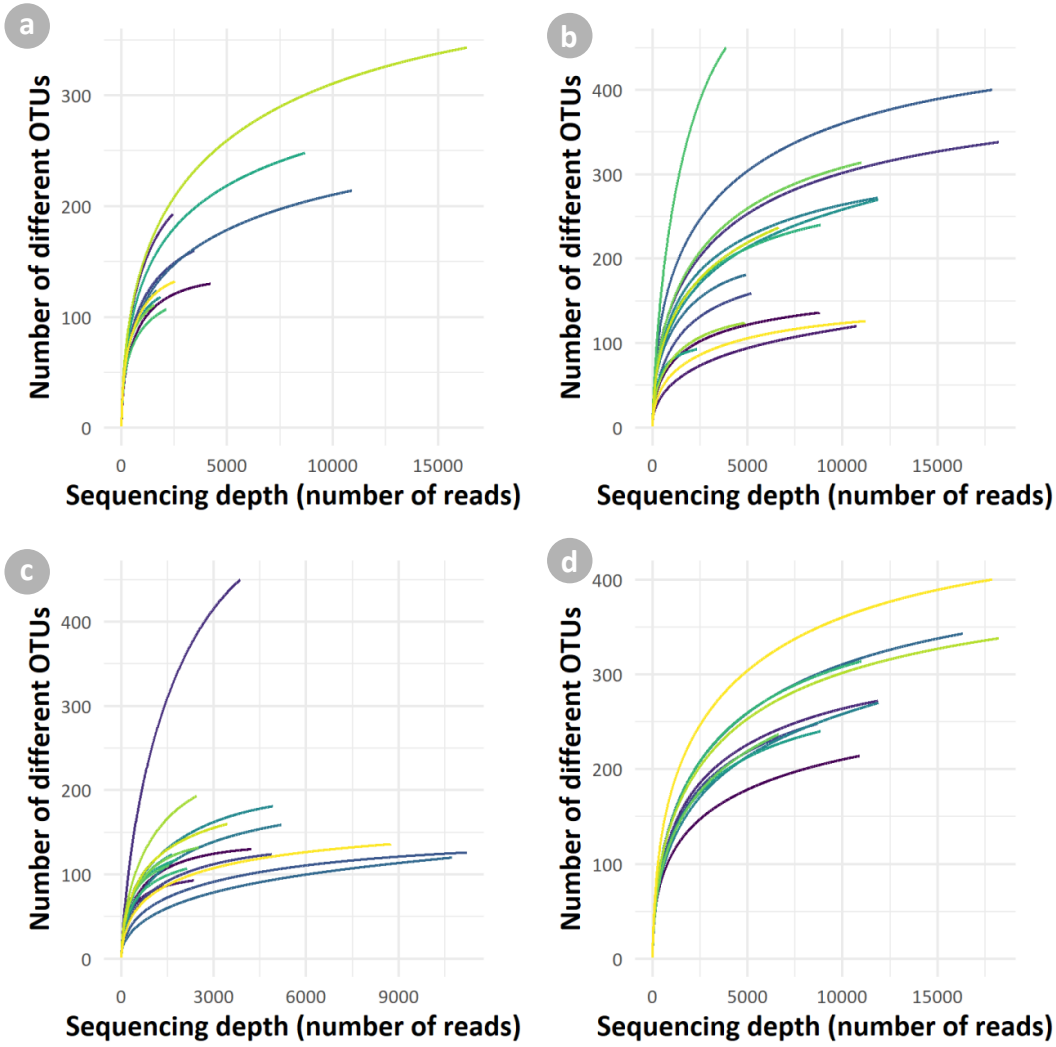
Supplementary figure G.1 – fungal OTU rarefaction curve. Each colour represents a different sample. From 'common walnut provenance trial': (a) all tissues (b) leaf (c) twig. From 'black walnut provenance trial': (d) all tissues (e) leaf (f) twig Plots were produced using the vegan package (version 2.5-3, Oksanen et al. (2018))



Supplementary figure G.2 – <u>fungal</u> OTU rarefaction curve. Each colour represents a different sample. From 'nitrogen trial': (a) walnut common, (b) walnut black (c) walnut leaf (d) walnut twig. Plots were produced using the vegan package (version 2.5-3, Oksanen et al. (2018))



Supplementary figure G.3 – <u>bacterial</u> OTU rarefaction curve. Each colour represents a different sample. From 'common walnut provenance trial': (a) all tissues (b) leaf (c) twig. From 'black walnut provenance trial': (d) all tissues (e) leaf (f) twig Plots were produced using the vegan package (version 2.5-3, Oksanen et al. (2018))



Supplementary figure G.4 – bacterial OTU rarefaction curve. Each colour represents a different sample. From 'walnut nitrogen trial: (a) *Juglans regia* (b) *Juglans nigra* (c) leaf, both species (d) twig, both species. Plots were produced using the vegan package (version 2.5-3, Oksanen et al. (2018))

G.8 *Results before rarefaction*

Supplementary table G.1 – results from the PERMANOVA for fungal endophyte beta diversity of walnut trees in the tree trials. OTU tables were not rarefied before analysis.

Trial	Samples included in analysis	Stress value	Significant variables	F	p
Common walnut provenance trial	**Leaf** + **twig** *J. regia*	0.118	**Tissue**	**5.81**	**<0.001**
	Leaf only *J. regia*	0.126	No significant variables		
	Twig only *J. regia*	0.129	No significant variables		
Black walnut provenance trial	**Leaf** + **twig** *J. nigra*	0.141	**Tissue** **DBH**	**5.92** **3.67**	**<0.001** **<0.001**
	Leaf only *J. nigra*	0.077	**DBH**	**4.35**	**<0.01**
	Twig only *J. nigra*	0.290	**DBH**	**1.63**	**<0.05**
Walnut nitrogen trial	***J. regia*** only Leaf + twig	0.044	**Tissue**	**9.65**	**<0.01**
	J. nigra only Leaf + twig	0.050	**Tissue**	**6.02**	**<0.001**
	Leaf only *J. regia* + *J. nigra*	0.184	**Species**	**2.17**	**<0.05**
	Twig only *J. regia* + *J. nigra*	0.081	**Species**	**2.52**	**<0.01**

Supplementary table G.2 – results from the GLMM for species richness and diversity of fungal endophytes associated with the walnut trees in the different trials. OTU tables were not rarefied before analysis.

Trial	Samples included	RICHNESS Signif. variables	df	F	p	Direction of effect	DIVERSITY Signif. variables	df	F	p	Direction of effect
Common walnut provenance trial	**Leaf** + **twig** *J. regia*	**Tissue** **Provenance**	**4** **4**	**13.39** **5.24**	**<0.001** **<0.05**	**Leaf > Twig** **Kyrgyzstan > Spain**	**Tissue** **Budburst**	**4** **4**	**14.52** **8.06**	**<0.001** **<0.01**	**Leaf > Twig** **Positive**
	Leaf only *J. regia*	No significant variables					No significant variables				
	Twig only *J. regia*	**Budburst** **Provenance**	**4** **4**	**4.56** **8.46**	**<0.05** **<0.01**	**Positive** **Kyrgyzstan > Spain**	**Budburst**	**3**	**8.10**	**<0.01**	**Positive**
Black walnut provenance trial	**Leaf** + **twig** *J. nigra*	**DBH**	**3**	**15.64**	**<0.001**	**Negative**	**DBH**	**3**	**8.90**	**<0.01**	**Negative**
	Leaf only *J. nigra*	**DBH**	**3**	**12.00**	**<0.001**	**Negative**	No significant variables				
	Twig only *J. nigra*	**DBH** **Provenance**	**4** **4**	**11.70** **10.60**	**<0.001** **<0.01**	**Negative** **Czech Rep. > Austria**	**DBH** **Budburst** **Provenance**	**5** **5** **5**	**30.56** **8.81** **17.47**	**<0.001** **<0.01** **<0.001**	**Negative** **Negative** **Czech Rep. > Austria**
Walnut nitrogen trial	***J. regia*** only Leaf + twig	**Tissue**	**9**	**7.55**	**<0.05**	**Twig > Leaf**	No significant variables				
	J. nigra only Leaf + twig	**Tissue**	**13**	**14.16**	**<0.001**	**Twig > Leaf**	**Tissue**	**13**	**9.16**	**<0.01**	**Twig > Leaf**
	Leaf only *J. regia* + *J. nigra*	No significant variables					No significant variables				
	Twig only *J. regia* + *J. nigra*	**Species** **DBH**	**7** **7**	**8.66** **13.01**	**<0.05** **<0.01**	***J. nigra* > *J. regia*** **Positive**	**Species** **DBH**	**7** **7**	**6.27** **7.56**	**<0.05** **<0.05**	***J. nigra* > *J. regia*** **Positive**

Supplementary table G.3 – results from the PERMANOVA for bacterial endophyte beta diversity of walnut trees in the tree trials. OTU tables were not rarefied before analysis.

Trial	Samples included in analysis	Stress value	Significant variables	F	p
Common walnut provenance and progeny trial	**Leaf** + **twig** *J. regia*	0.100	**Tissue**	4.90	<0.01
	Leaf only *J. regia*	0.326	No significant variables		
	Twig only *J. regia*	0.048	No significant variables		
Black walnut provenance and progeny trial	**Leaf** + **twig** *J. nigra*	0.119	**Tissue** **DBH** **Provenance**	2.70 2.40 1.80	<0.01 <0.01 <0.01
	Leaf only *J. nigra*	0.110	**DBH**	2.22	<0.01
	Twig only *J. nigra*	0.108	No significant variables		
Common and black walnut nitrogen trial	***J. regia*** only Leaf + twig	0.035	**Tissue**	7.31	<0.05
	J. nigra only Leaf + twig	0.045	**Tissue**	9.21	<0.01
	Leaf only *J. regia* + *J. nigra*	0.212	**Species**	3.81	<0.01
	Twig only *J. regia* + *J. nigra*	0.056	No significant variables		

Supplementary table G.4 - results from the GLMM for species richness and diversity of bacterial endophytes associated with the walnut trees in the different trials. OTU tables were not rarefied before analysis.

Trial	Samples included	RICHNESS Signif. variables	df	F	p	Direction of effect	DIVERSITY Signif. variables	df	F	p	Direction of effect
Common walnut provenance trial	**Leaf** + **twig** *J. regia*	**Tissue** **Provenance**	4 4	11.07 4.30	<0.001 <0.05	Twig > Leaf Kyrgyzstan > Spain	No significant variables				
	Leaf only *J. regia*	**Provenance**	3	4.42	<0.05	Kyrgyzstan > Spain	No significant variables				
	Twig only *J. regia*	No significant variables					No significant variables				
Black walnut provenance trial	**Leaf** + **twig** *J. nigra*	**Provenance**	3	19.14	<0.001	Czech Rep. > Austria	**Provenance**	3	14.68	<0.001	Czech Rep. > Austria
	Leaf only *J. nigra*	**Provenance**	3	5.81	<0.05	Czech Rep. > Austria	**Provenance**	3	4.87	<0.05	Czech Rep. > Austria
	Twig only *J. nigra*	**Provenance**	3	17.26	<0.001	Czech Rep. > Austria	**Provenance**	3	10.48	<0.01	Czech Rep. > Austria
Walnut nitrogen trial	***J. regia*** only Leaf + twig	**Tissue** **Budburst**	8 8	41.59 5.90	<0.001 <0.05	Twig > Leaf Negative	No significant variables				
	J. nigra only Leaf + twig	**Tissue**	12	53.89	<0.001	Twig > Leaf	**Tissue**	12	23.39	<0.001	Twig > Leaf
	Leaf only *J. regia* + *J. nigra*	No significant variables					**Species** **Nitrogen**	12 12	20.19 6.70	<0.001 <0.05	*J. regia* > *J. nigra* Positive
	Twig only *J. regia* + *J. nigra*	No significant variables					No significant variables				

APPENDIX H - *Supplementary information for TRFLP*

	1	2	3	4	5	6	7	8	9	10	11	12
A	WD01187 LEAF	WD01191 LEAF	WD01192 LEAF	WD01193 LEAF	WD01194 LEAF	WD01195 LEAF	WD01196 LEAF	WD01197 LEAF	WD01198 LEAF	WD01199 LEAF	WH01183 LEAF	WH01184 LEAF
B	WH01185 LEAF	WH01188 LEAF	WH01200 LEAF	WH01872 LEAF	WH01874 LEAF	WH01875 LEAF	WH01876 LEAF	WH01879 LEAF	MD00991 LEAF	MD00992 LEAF	MD00993 LEAF	MD00994 LEAF
C	MD00995 LEAF	MD00996 LEAF	MD00997 LEAF	MD00998 LEAF	MD00999 LEAF	MD01000 LEAF	MH00812 LEAF	MH00891 LEAF	MH00892 LEAF	MH00893 LEAF	MH00894 LEAF	MH00895 LEAF
D	MH00896 LEAF	MH00897 LEAF	MH00898 LEAF	MH00899 LEAF	SDX LEAF	SD0054 LEAF	SD0056 LEAF	SD00736 LEAF	SD00737 LEAF	SD00738 LEAF	SD00739 LEAF	SD00813 LEAF
E	SD00816 LEAF	SD00970 LEAF	SH01 LEAF	SH02 LEAF	SH03 LEAF	SH04 LEAF	SH05 LEAF	SH06 LEAF	SH07 LEAF	SH08 LEAF	SH09 LEAF	SH10 LEAF
F	WD01187 BARK BLEED	WD01191 BARK BLEED	WD01192 BARK BLEED	WD01193 BARK BLEED	WD01194 BARK BLEED	WD01195 BARK BLEED	WD01196 BARK BLEED	WD01197 BARK BLEED	WD01198 BARK BLEED	WD01199 BARK BLEED	WH01183 BARK	WH01184 BARK
G	WH01185 BARK	WH01188 BARK	WH01200 BARK	WH01872 BARK	WH01874 BARK	WH01875 BARK	WH01876 BARK	WH01879 BARK	MD00991 BARK BLEED	MD00992 BARK BLEED	MD00993 BARK BLEED	MD00994 BARK BLEED
H	MD00995 BARK BLEED	MD00996 BARK BLEED	MD00997 BARK BLEED	MD00998 BARK BLEED	MD00999 BARK BLEED	MD01000 BARK BLEED	MH00812 BARK	MH00891 BARK	MH00892 BARK	MH00893 BARK	MH00894 BARK	Negative Control

Supplementary figure H.1 - TRFLP plate layout 1, repeated for 16S and ITS

	1	2	3	4	5	6	7	8	9	10	11	12
A	MH00895 BARK	MH00896 BARK	MH00897 BARK	MH00898 BARK	MH00899 BARK	SDX BARK BLEED	SD0054 BARK BLEED	SD0056 BARK BLEED	SD00736 BARK BLEED	SD00737 BARK BLEED	SD00738 BARK BLEED	SD00739 BARK BLEED
B	SD00813 BARK BLEED	SD00816 BARK BLEED	SD00970 BARK BLEED	SH01 BARK	SH02 BARK	SH03 BARK	SH04 BARK	SH05 BARK	SH06 BARK	SH07 BARK	SH08 BARK	SH09 BARK
C	SH10 BARK	WD01187 BARK NON-BLEED	WD01191 BARK NON-BLEED	WD01192 BARK NON-BLEED	WD01193 BARK NON-BLEED	WD01194 BARK NON-BLEED	WD01195 BARK NON-BLEED	WD01196 BARK NON-BLEED	WD01197 BARK NON-BLEED	WD01198 BARK NON-BLEED	WD01199 BARK NON-BLEED	MD00991 BARK NON-BLEED
D	MD00992 BARK NON-BLEED	MD00993 BARK NON-BLEED	MD00994 BARK NON-BLEED	MD00995 BARK NON-BLEED	MD00996 BARK NON-BLEED	MD00997 BARK NON-BLEED	MD00998 BARK NON-BLEED	MD00999 BARK NON-BLEED	MD01000 BARK NON-BLEED	SDX BARK NON-BLEED	SD0054 BARK NON-BLEED	SD0056 BARK NON-BLEED
E	SD00736 BARK NON-BLEED	SD00737 BARK NON-BLEED	SD00738 BARK NON-BLEED	SD00739 BARK NON-BLEED	SD00813 BARK NON-BLEED	SD00816 BARK NON-BLEED	SD00970 BARK NON-BLEED					
F												
G												
H												Negative Control

Supplementary figure H.2 - TRFLP plate layout 2, repeated for 16S and ITS

APPENDIX I - *Phylogenetic analysis of AOD pathogens*

Supplementary table I.1 – representative *gyrB* sequences from GenBank used in the construction of a phylogenetic tree for *Brenneria goodwinii*

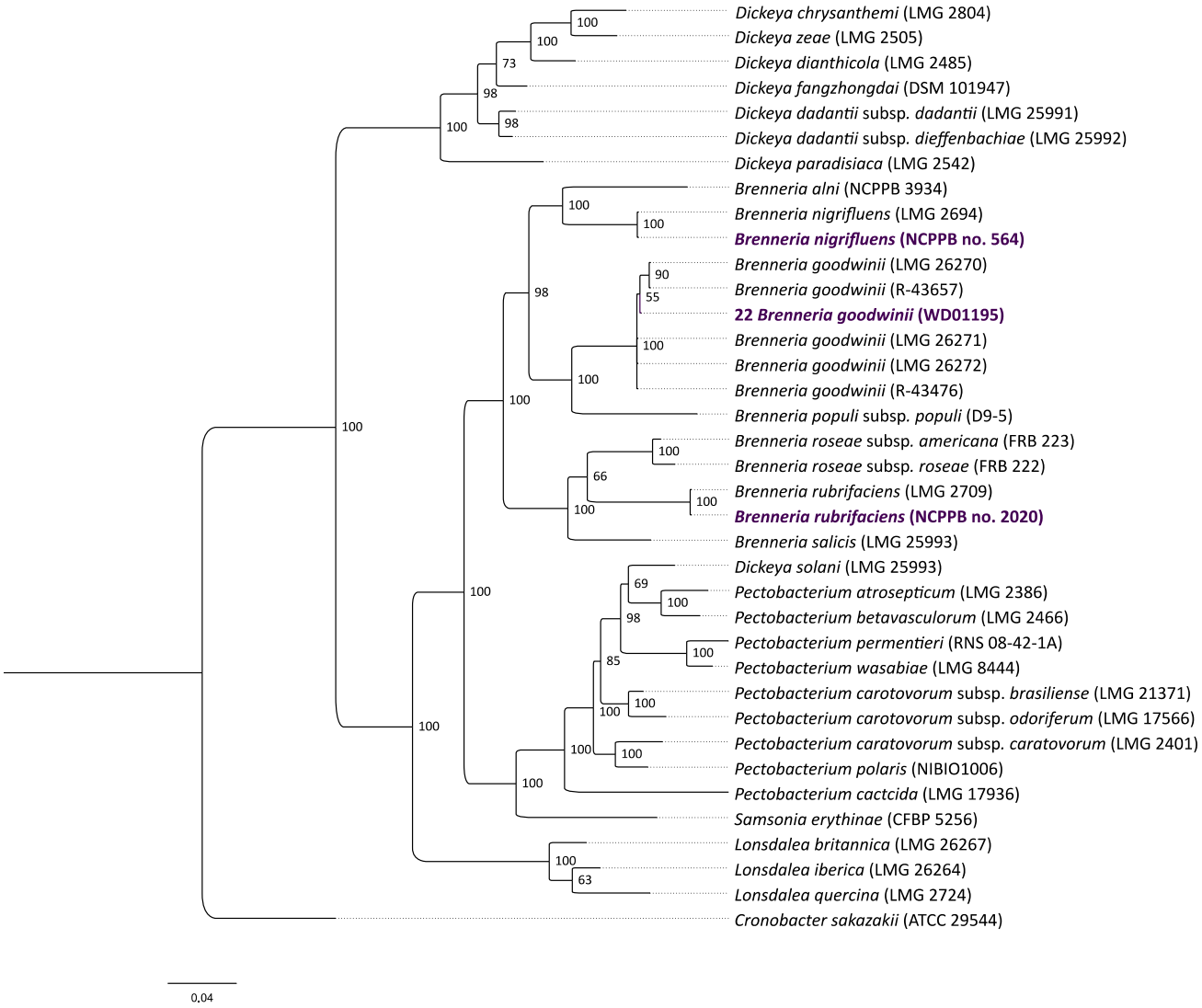
Species name	Strain number	Accession number:
***Cronobacter sakazakii* (Outgroup)**	ATCC 29544^T	CP011047.1
Brenneria alni	NCPPB 3934^T	JF311627
Brenneria goodwinii	LMG 26270^T	JN544220
Brenneria goodwinii	LMG 26271	JN544216
Brenneria goodwinii	LMG 26272	JN544222
Brenneria goodwinii	R-43657	JN544221
Brenneria goodwinii	R-43476	JN544214
Brenneria nigrifluens	LMG 2694^T	JF311612
Brenneria populi* subsp. *populi	D9-5^T	KJ672083
Brenneria roseae* subsp. *americana	FRB 223^T	KF308310
Brenneria roseae* subsp. *roseae	FRB 222^T	KF308303
Brenneria rubrifaciens	LMG 2709^T	JF311617
Brenneria salicis	LMG 2698^T	JF311622
Dickeya chrysanthemi	LMG 2804^T	JF311636
Dickeya dadantii* subsp. *dadantii	LMG 15991^T	JF311644
Dickeya dadantii* subsp. *dieffenbachiae	LMG 25882^T	JF311652
Dickeya dianthicola	LMG 2485^T	JF311648
Dickeya fangzhongdai	DSM 101947^T	CP025003
Dickeya paradisiaca	LMG 2542^T	JF311640
Dickeya solani	LMG 25993^T	KC238453
Dickeya zeae	LMG 2505^T	JF311632
Lonsdalea britannica	FRB 18^T	JF311666
Lonsdalea iberica	1915-14^T	JF311665
Lonsdalea quercina	LMG 2724^T	JF311656
Pectobacterium atrosepticum	LMG 2386^T	JF311589
Pectobacterium betavasculorum	LMG 2466^T	JF311593
Pectobacterium cacticida	LMG 17936^T	JF311597
Pectobacterium carotovorum* subsp. *brasiliense	LMG 21371^T	JF311605
Pectobacterium carotovorum* subsp. *carotovorum	LMG 2404^T	JF311602
Pectobacterium carotovorum* subsp. *oderiferum	LMG 17566^T	JF311607
Pectobacterium parmentieri	RNS 08-42-1A^T	CP015749
Pectobacterium polaris	NIBIO1006^T	CP017481
Pectobacterium wasabiae	LMG 8444^T	JF311608
Samsonia erythinae	CFBP 5256^T	JF419469

Supplementary table I.2 – representative *gyrB* sequences from GenBank used in the construction of a phylogenetic tree for *Gibbsiella quercinecans, Rahnella victoriana* and *Raoultella planticola*

Species name	Strain number	Accession number
Xenorhabdus nematophila	ATCC 19061^T	FN667742
Ewingella americana	LMG 7869^T	KF308476
Gibbsiella dentisursi	DSM 23818^T	KF308367
Gibbsiella greigii	FRB 224^T	KF308356
Gibbsiella quercinecans	LMG 25500^T	JX425084
Gibbsiella quercinecans	LMG 25501	JX425085
Gibbsiella quercinecans	LMG 25502	JX425086
Gibbsiella quercinecans	N78	GU562335
Gibbsiella papilionis	JCM 18389^T	KF308368
Klebsiella aerogenes	JCM 1235^T	JX425098
Kluyvera ascorbata	LMG 7871^T	JX425103
Kluyvera cryocrescens	LMG 7859^T	JX425104
Kluyvera georgiana	ATCC 51603^T	CP022114
Kluyvera intermedia	LMG 2785^T	JX425105
Rahnella aquatilis	LMG 2794^T	KF387630
Rahnella bruchi	FRB 226^T	KF308467
Rahnella inusitata	DSM 30078^T	KF308464
Rahnella inusitata	FOD 9/5a	KF308465
Rahnella inusitata	FOD 9/21	KF308466
***Rahnella* sp.**	Y9602	CP002505
Rahnella variigena	CIP 105588^T	KF308456
Rahnella variigena	PFK 1/1C2a	KF308458
Rahnella variigena	SOT 2/10	KF308457
Rahnella variigena	SOT 2/16	KF308461
Rahnella variigena	GC 165b	KF308459
Rahnella victoriana	AT 15Ab	KF308454
Rahnella victoriana	BRK 3	KF308452
Rahnella victoriana	BRK 18a	KF308447
Rahnella victoriana	FRB 225^T	KF308446
Rahnella victoriana	GC 176	KF308453
Rahnella victoriana	USA 3	KF308448
Rahnella victoriana	USA 13	KF308449
Rahnella victoriana	USA 39	KF308450
Rahnella victoriana	USA 47	KF308451
Rahnella victoriana	WAL 23a	KF308455
Rahnella woolbedingensis	FRB 227^T	KF308474
Raoultella ornithinolytica	FDAARGOS_431	CP023888
Raoultella planticola	FDAARGOS_64	CP026047
Raoultella planticola	FDAARGOS_429	CP023874
Raoultella planticola	FDAARGOS_430	CP023877
Raoultella planticola	GODA	CP019899
Raoultella planticola	LMG 7870^T	JX425101

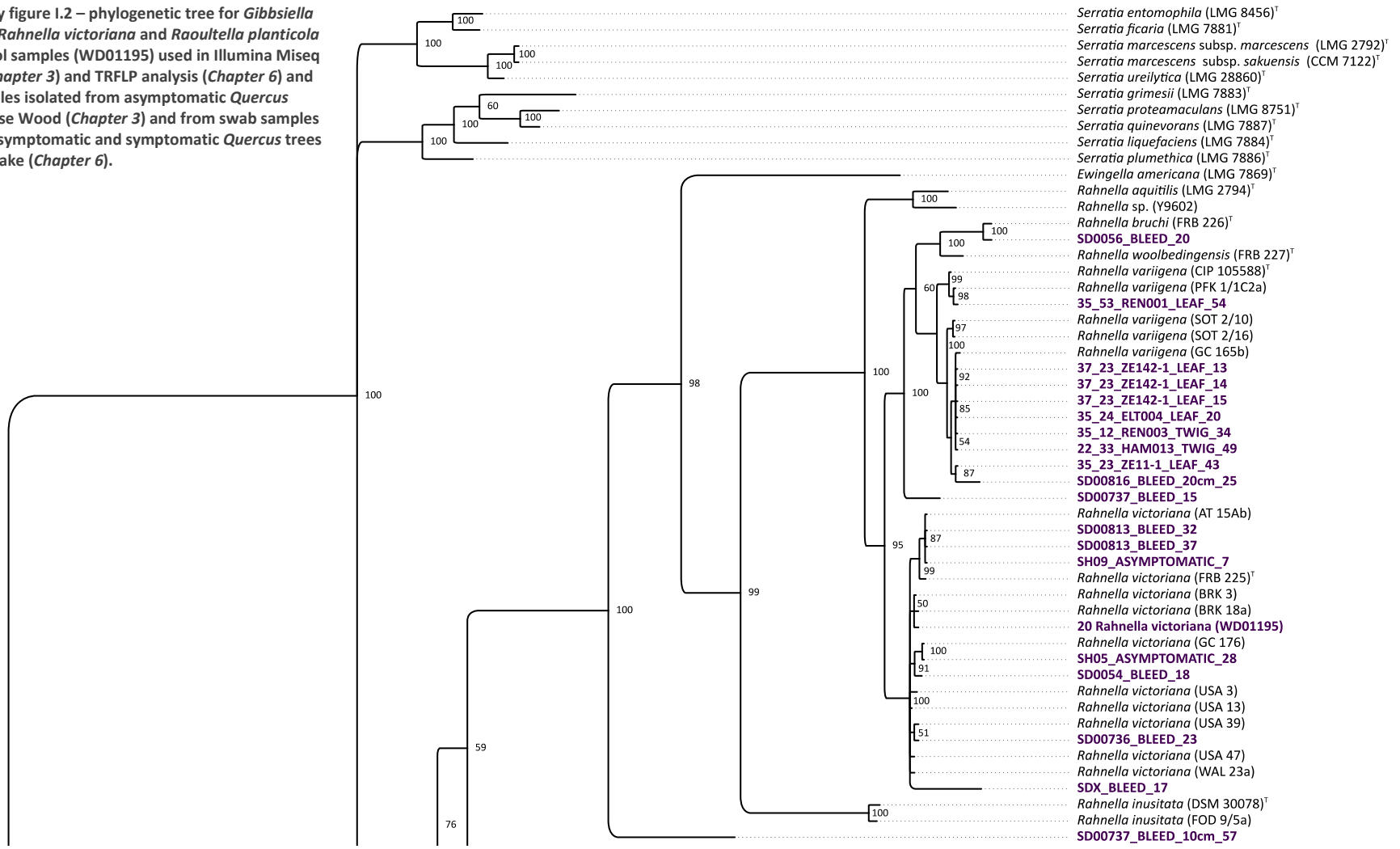
Supplementary table I.2 cont. – representative *gyrB* sequences from GenBank used in the construction of a phylogenetic tree for *Gibbsiella quercinecans, Rahnella victoriana* and *Raoultella planticola*

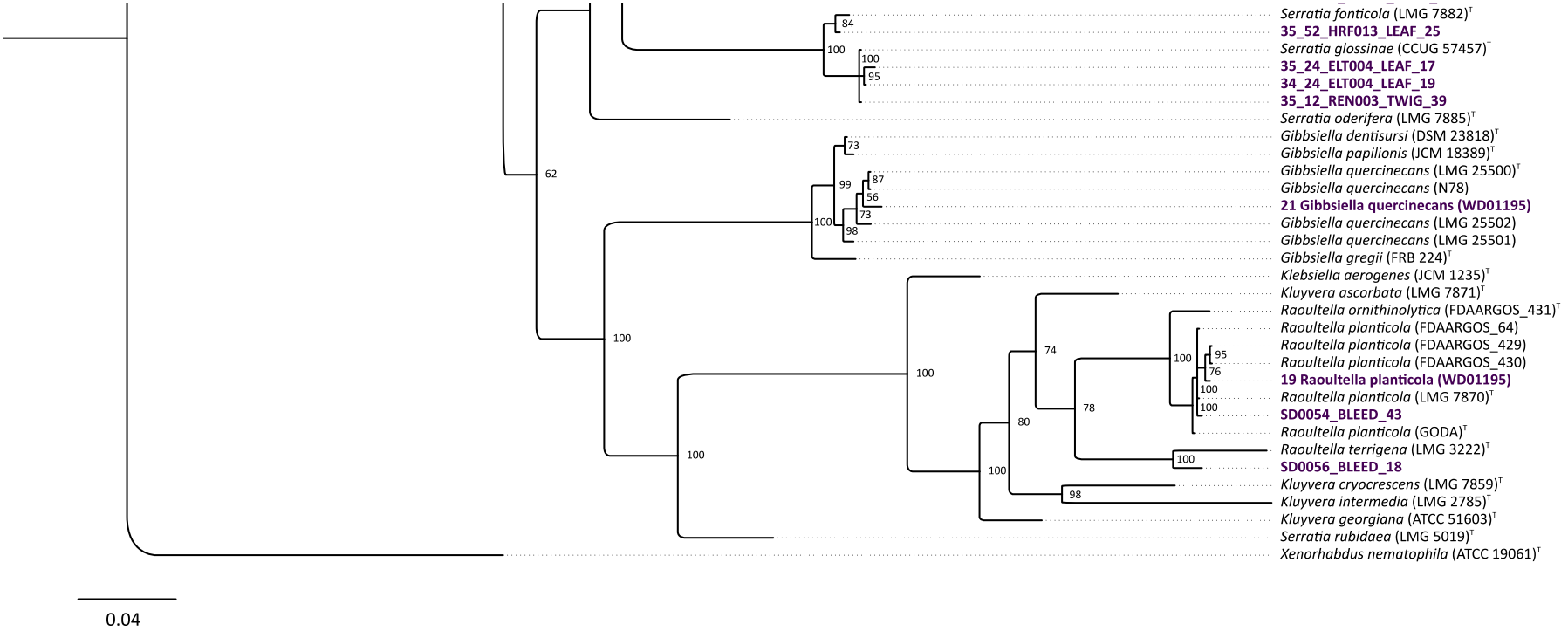
Species name	Strain number	Accession number
Raoultella terrigena	LMG 3222^T	JX425102
Serratia entomophila	LMG 8456^T	JX425056
Serratia ficaria	LMG 7881^T	JX425044
Serratia fonticola	LMG 7882^T	JX425048
Serratia glossinae	CCUG 57457^T	JX425078
Serratia grimesii	LMG 7883^T	JX425051
Serratia liquefaciens	LMG 7884^T	JX425079
Serratia marcescens* subsp. *marcescens	LMG 2792^T	JX425060
Serratia marcescens* subsp. *sakuensis	CCM 7122^T	JX425062
Serratia odorifera	LMG 7885^T	JX425065
Serratia plymuthica	LMG 7886^T	JX425074
Serratia proteamaculans	LMG 8751^T	JX425063
Serratia quinivorans	LMG 7887^T	JX425053
Serratia rubidaea	LMG 5019^T	JX425069
Serratia ureilytica	LMG 22860^T	JX425073



Supplementary figure I.1 – phylogenetic tree for *Brenneria goodwinii* positive control sample (WD01195) used in Illumina Miseq sequencing (*Chapter 3*) and TRFLP analysis (*Chapter 6*) and *Brenneria nigrifluens* and *B. rubrifaciens* used as positive controls in the walnut study (*Chapter 4*)

Supplementary figure I.2 – phylogenetic tree for *Gibbsiella quercinecans, Rahnella victoriana* and *Raoultella planticola* positive control samples (WD01195) used in Illumina Miseq sequencing (*Chapter 3*) and TRFLP analysis (*Chapter 6*) and bacterial samples isolated from asymptomatic *Quercus* trees in Paradise Wood (*Chapter 3*) and from swab samples of bark from asymptomatic and symptomatic *Quercus* trees in Stratfield Brake (*Chapter 6*).





Supplementary figure I.2 cont. – phylogenetic tree for *Gibbsiella quercinecans, Rahnella victoriana* and *Raoultella planticola* positive control samples (WD01195) used in Illumina Miseq sequencing (*Chapter 3*) and TRFLP analysis (*Chapter 6*) and bacterial samples isolated from asymptomatic *Quercus* trees in Paradise Wood (*Chapter 3*) and from swab samples of bark from asymptomatic and symptomatic *Quercus* trees in Stratfield Brake (*Chapter 6*).