DOCTOR OF PHILOSOPHY

Understanding the role of plant-microbe symbiosis in the cycling of carbon in temperate forest ecosystems

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Award date:
2020

Awarding institution:
Bangor University

Understanding the role of plant-microbe symbiosis in the cycling of carbon in temperate forest ecosystems

A thesis submitted to Bangor University by

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In candidature for the degree

Philosophiae Doctor

January 2020

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“I may not have gone where I intended to go, but I think I have ended up where I needed to be.”

Douglas Adams

“It is a miracle that curiosity survives formal education.”

Albert Einstein

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Abbreviations

SOM	Soil Organic Matter
SOC	Soil Organic Carbon
TON	Total Organic Nitrogen
DOC	Dissolved Organic Carbon
MB	Microbial Biomass
CMN	Common Mycorrhizal Network
EMF	Ectomycorrhizal Fungi
AMF	Arbuscular Mycorrhizal Fungi
NSC	Non-structural carbohydrates

Abstract

Soil microorganisms and their symbiotic relationships with plants are fundamental to nutrient cycling in temperate forest ecosystems. This highly diverse microbiome contains up to a quarter of Earth's biodiversity, but our understanding of how this affects the function of forests is not well understood. This thesis investigated the role of plant symbionts on the allocation of C to belowground microbial symbionts and to ground vegetation via microbial symbionts. Radio-isotope pulse labelling was used to determine the belowground C dynamics of these highly complex systems by allowing us to quantify pools and fluxes within the plant-microbe-soil continuum. In Chapter 3, the role of arbuscular and ecto-mycorrhizal fungi in belowground allocation of C in three temperate tree species was investigated by destructive harvesting of trees 336 days after a pulse label had been applied. The results suggested that *Alnus glutinosa* and *Betula pendula* allocated C belowground to microbes, whereas *Castanea sativa* transferred the C to the soil where it was sequestered. In Chapter 4, inter- and intra-specific C transfer was studied using trees connected via a common mycorrhizal network (CMN), the results suggested that more C was transferred between inter- than intra-specific species combinations. In Chapter 5, C transferred via three "donor" tree species to the root nodules of *A. glutinosa* "receiver" tree connected with a CMN was investigated using the methodology pioneered in Chapter 3. The plant: fungal amalgam preferentially allocated C from the donor trees to the root nodules of the receiver *A. glutinosa* tree. We postulated that this was due to the considerable energetic demands of nitrogen-fixation by *Frankia alni* in the root nodule creating a strong C sink. In Chapter 6, the transfer of C from 13-year-old coppiced *A. glutinosa* and *C. sativa* trees to ground vegetation via CMN was investigated. ^{14}C activity in the ground vegetation under the *A. glutinosa* trees was expected to be greatest, as *A. glutinosa* share arbuscular mycorrhizal partnerships with the ground vegetation. No difference in ^{14}C activity was found in the hyphae, soil solution or ground vegetation under *A. glutinosa*. We postulated that this could be due to root grafting, mycorrhizal types exchanging nutrients, or reabsorption of tree rhizodeposits. Overall this study suggests that the plant: microbe symbiosis that is ubiquitous across the temperate biome is both important for nutrient cycling and C storage, but also that the sharing of resources via CMNs could be altering plant competition dynamics that have previously been based on the assumption that plants are not physically connected and actively sharing resources. Further work to determine how plants or mycorrhizae control belowground resource sharing could lead to a paradigm shift in our understanding of competition and facilitation in plant community dynamics.

Acknowledgements

I would like to acknowledge the funding for this PhD from the Natural Environment Research Council's (NERC) (part of RCUK), funding through ENVISION DTP. The ENVISION doctoral training provision is an environmentally focussed initiative which brings together Lancaster University (lead organisation), with the Universities of Nottingham and Bangor, the Centre for Ecology and Hydrology, the British Geological Survey and Rothamsted Research.

This project was supervised by Dr. Andrew R. Smith and Prof. Davey L. Jones, from Bangor University. Thanks especially to Andy for putting up with my constant bothering and tangential conversations. Thanks also go to Dr. Gareth Griffiths of Aberystwyth University for taking the time to advise me regarding DNA extraction techniques.

I particularly enjoyed the glasshouse and fieldwork component of my research particularly at Henfaes, the University farm and would like to thank all the technical staff who assisted both at the farm; Llinos Hughes and Mark Hughes, and in ECW; Sarah Chesworth and Jonathan Roberts. I have not been alone in my PhD adventure, (although it has sometimes felt that way) and the support, both academically and morally, from fellow PhD students has been at times, what has kept me going. Thanks especially to Clo Ward for the chats over tea, Luke Tyler for assisting me with laboratory techniques, Bid Webb for soil core technique discussions and my helpful intern Léa Sgro, for all the assistance and support in the field and laboratory.

Finally, thanks to my parents for the inherited curiosity, to my partner Melissa for the endless patience and to my children Thorin, Indica and Ashanti for being understanding beyond their years.

Chapter 1: Introduction

1.1. General Introduction and Rationale

Since Darwin first described the evolution of species, the focus of ecological research has been on competition or survival of the fittest as the dominant driver of biological diversity (Ryan, 2002). This term is widely misinterpreted and often symbiosis is seen as incommensurable with the competitive model (Ryan, 2002). However, others disagree and suggest that the co-operation enables the organism to do this better and thus increases its fitness (Axelrod and Hamilton, 1981). The model of evolution described by Darwin is typified by gradual changes though natural competitive advantage gained by multiple diminutive mutations (Roossinck, 2005). Darwin (1859) stated "Nature ... can never take a leap but must advance by the slowest and shortest steps" and yet fossil records and phylogenetic analyses show that leaps forward have historically occurred (Ryan, 2002). It is now generally accepted amongst evolutionary theorists that competition alone cannot account for the speed of evolution and vast diversity of life on our planet (Tilman, 1994). One possible explanation, known as the symbiosis model, suggests that two or more previously symbiotic individuals merge and become new taxonomic groups (Ryan, 2002). For example, the discovery that mitochondria and chloroplasts contain both DNA and ribosomes reminiscent of those located in bacteria has led to a distillation of these ideas, that the importance of facilitation and symbiosis may have been underestimated (Sagan 1967; Gaia et al., 2018). In 1975, analysis of rDNA confirmed that a chloroplast's 16S RNA oligonucleotide was more closely related to that of cyanobacteria than to the 18S RNA of the chloroplast containing plant (Zablen et al., 1975). Plant mitochondrial rDNA was finally linked to the organelles of alphaproteobacteria (Yang et al., 1985). A recent hypothesis known as the eocyte hypothesis suggests that the nucleus and cytoplasm of eukaryotic cells originate from an ancient archaeon, which enveloped a bacterial mitochondrial ancestor at some point in evolutionary history (Archibald, 2008).

The natural world is full of co-operative partnerships that do not obviously sit comfortably within the competitive framework proposed by Darwinian Theory. For example, the lichens that cover the rocks and exposed surfaces of our ecosystems are complex symbiotic relationships between cyanobacteria and often multiple species of fungi (Margulis and Barreno, 2003), in some cases totalling four partners in stable equilibrium (Spribille et al., 2016; Tuovinen, 2019). Traditionally, it was thought that coral reefs were comprised of microbial algae called *Symbiodinium* and a basic animal from the *Anthozoa* group. However, DNA

analysis now suggests that coral reefs that inhabit the World's seas include a third player, a group of animals normally associated with the *Apicomplexa* (Richards and McCutcheon, 2019). Normally these organisms are thought to be associated with parasitic diseases, such as malaria (Kwong et al., 2019). Clements as far back as 1916 suggested that facilitation or mutualistic symbiosis was more fundamental to ecological theory and, to plant succession particularly. Unfortunately, since then this has been largely overlooked by ecologists due to the popularity of the alternatives; the competition (Tilman, 1982; Grime 1974) and individualistic theories (Gleason 1926; Whittaker 1956). In the mid-nineties interest in facilitation was renewed after a series of experiments in extreme conditions suggested facilitation was more fundamental to ecosystem stability (Michalet and Pugnaire, 2016). In 1994, Bertness and Callaway hypothesised a new ecological model whereby the importance of competition gives way to facilitation, under periods of ecological stress. Since this theory was proposed, the number of experiments only concerned with competitive drivers of ecosystems has dropped from 90 % to 76 % (Michalet and Pugnaire, 2016). Facilitative symbiosis is also prevalent in the belowground component of our ecosystems and recent research is suggesting that in some cases mitigates aboveground competitive pressures between plants (Fonseca, 2017). It would now seem that facilitative relationships are more widespread and particularly during periods of environmental stress (McIntire and Fajardo, 2014). Ecological theory probably needs to be modified to include relationships which undermine competitive pressures amongst biological communities and more research needs to be conducted to see if their importance increases under stress (Bruno et al., 2003).

In temperate forest ecosystems, ectomycorrhizal (ECM) fungi dominate the belowground mutualistic symbiotic landscape with over 7000 known isolated species forming relationships with a range of trees species (Tedersoo et al., 2012). Although the presence of these complex interactions has been known since the 1850s, facilitative symbioses were seen as exceptional and as a result were largely overlooked as a selective pressure by Darwinian Theory (Ryan, 2002). The Earth's biosphere is a fundamental component of nutrient and water cycling and as a result global carbon (C) budgeting (Fahey et al., 2010). Although most studies have investigated the aboveground C stored in forest ecosystems as either live biomass, woody debris and detritus or as soil organic matter. However, the belowground component (i.e. soil) represents the largest reservoir of organic C within terrestrial ecosystems and the world's forests are of particular importance as C storage pools. The belowground processes of forests mediate many key biogeochemical processes, which have an important role in regulating

climate, and are major hotspots of biodiversity. Understanding the mechanisms controlling soil C sequestration and stability are essential in maximizing soil C sequestration potential. Knowledge gaps still exist in quantifying the contribution of plant: microbe symbiosis, specifically; mycorrhizal hyphal turnover, hyphal biomass, root exudation, rhizodeposition, and the contribution of microbial derived C to soil C stocks (Clemmensen et al., 2013; Gougoulias et al., 2014; Averill et al., 2014). Within the heterogeneous soil matrix microbial hotspots, have been identified that preferentially sequester C (Vogel et al., 2014), and show disproportionately high reactions rates relative to the surrounding soil matrix (Johnson et al., 2014), but the mechanisms surrounding their formation, function, and ecological relevance remains poorly understood. To maximize the provision of ecosystem services from our forests and to inform policy makers there is a need to improve our understanding of belowground processes influencing the C cycle (Hulvey et al., 2013; Cotrufo et al., 2013). Increasing forest diversity and exploiting species' functional traits offers the potential to enhance ecosystem resilience and resistance to our changing climate (Lavorel, 2013; Forrester, 2014), but will also likely modify belowground microbial transformations and ecological interactions altering C cycling and long-term C storage.

This thesis investigates the role and contribution of belowground plant-microbe symbiosis to C storage and cycling, by utilising novel radio-isotopic pulse chase experimental set-ups. A greater understanding of the belowground microbial components of temperate forest ecosystems will enable us to choose appropriate species and silvicultural management techniques to further increase terrestrial C storage.

1.2. Carbon Dynamics Terminology

Studies that have investigated C dynamics in forest ecosystems have often used inconsistent terminology (Litton et al., 2007), and, as a result, the terminology used in this thesis may benefit from clarification with reference to the terminology used in the scientific literature. Studies frequently refer to different nouns e.g., apportionment, allocation, distribution, partition, transportation, translocation, that are frequently used interchangeably causing widespread confusion (Gower et al., 1995). The term allocation is used with reference to everything from the growth of biomass, to the flux of C to a specific plant part to the distribution of fractions of the net primary production (Ryan et al., 1996; Giardina et al., 2003). During this thesis, I propose to accept the standardised definitions proposed by Litton et al., (2007), which are as follows:

Allocation: a wide-reaching term that can be referring to a diverse range of the physiology of both plants, fungi and ecosystems, including a flux of C to a tree partition, an allometry of live biomass or the delivery of a portion of the products of photosynthesis to a particular plant organ (Litton et al., 2007; Epron et al., 2012). C allocation is the result of many complex mechanisms (Cannell and Dewar, 1994).

Biomass: the mass of any or all organic components of an ecosystem (Odum et al., 2005) and therefore biomass allocation refers to the distribution to different plant structure, for example root: shoot (Litton et al., 2007).

Compartment: there is no unambiguous definition for the word compartment within the biological literature. Nevertheless, for the purpose of this thesis a compartment is a subdivision of a larger biological unit based on unique or characteristic function or physiological structure (Hauri and Schweizer, 1992).

Flux: the rate at which C is moved or moves from one compartment to another and the processes or mechanism underpinning this transfer.

Partition: is the division of photosynthetically-fixed C into subdivisions (to particular forest ecosystem compartments) and this is a fraction of total (gross) primary production (GPP) (Odum et al., 2005) and usually expressed a percentage or a proportion (0–1, no units). C partitioning- is an overarching definition that can cover all three of the above (Litto et al., 2007).

Pool: a compartment where in this case, C is stored.

1.3. Plan of Thesis

This thesis was divided into six chapters commencing with the literature review, which critically evaluates the role that plant microbial symbiosis has in the cycling and storage of C belowground in temperate forest ecosystems. It was used to identify the knowledge gaps addressed in this thesis.

The experimental work was divided into four chapters. As each is intended to be a stand-alone research article, there is inevitably some repetition of introductory material, methodological techniques and the use of references. The first experimental chapter quantifies the relative importance of the soil microbial community to the three tree species studied, by tracing recently fixed C to quantify the allocation of C to different belowground biomass partitions.

The second and third experimental chapters build on the first, by investigating the role of common mycorrhizal networks to C exchange in both intra- and inter-specific species combinations. The third chapter more specifically looks at the importance of the presence of nitrogen-fixing bacteria, which are located in actinorhizal nodules in the tree roots of the species *A. glutinosa* used in this study. The fourth and final experimental chapter investigated the spatiotemporal transfer of C in field conditions using *A. glutinosa* and *C. sativa* but not *B. pendula*.

Finally, the last chapter includes a synthesis of the experimental chapters and a general discussion of the aims and objectives of the thesis and of the results from each experimental chapter. Overall conclusions are drawn, and areas of future research priorities identified. Appendices include a selection of photographs of the experimental work.

1.4. Aims and Objectives

The aims and objectives as framed by the context of the above were as follows:

1) To investigate the differences in belowground C allocation between early and late-successional temperate tree species (Chapter 3 and Chapter 6)

2) To investigate the intra and interspecific transfer of C via common mycorrhizal networks (CMNs) in temperate forest ecosystems (Chapter 4)

3) To investigate the effect of trees with tripartite mutualistic symbiotic relationships on the magnitude and allocation of C transferred via CMN (Chapter 5)

4) To investigate the differences in belowground allocation between glasshouse-based and field-based experiments (Chapter 6)

1.5. Hypotheses

H_1: Belowground allocation of C will be greater in species with the largest diversity of microbial symbiotic associations (Chapter 3).

H_2: The transfer of recently assimilated C to the soil microbial community will be greater in early (*B. pendula* and *A. glutinosa*), than late successional trees (*C. sativa*) (Chapter 3).

H_3: Transfer of C by CMNs is greater inter-specifically than intra-specifically, as a result of differences in C demand between species. (Chapter 4).

H_4: Greater mycorrhizal diversity per unit of rhizosphere soil will result in more C transfer between trees via CMN (Chapter 4).

H_5: The abundance of mycorrhizal hyphae is positively correlated with C transfer between trees (Chapter 4).

H_6: Belowground allocation of C via inter- and intra-specific CMNs will be greatest in "receiver" trees with actinorhizal associations, due to an increased C sink strength generated by the metabolic processes of the root nodule (Chapter 5).

H_7: Transfer of C to ground vegetation will be greatest via the CMN of *A. glutinosa* that shares an arbuscular mycorrhizal symbiosis than via the obligate ectomycorrhizal species *C. sativa* (Chapter 6).

H_8: The percentage transfer of recently fixed C to mycorrhizal fungal hyphae in the field will be similar to those calculated in laboratory experiments (Chapter 6).

1.6. Methods Overview

C flows through the air-plant-soil continuum by first being photoassimilated by the plant, allocated to plant organs whereby a proportion is autotrophically respired and returns to the atmosphere. The remainder is either released into the rhizosphere or mycorrhizosphere where it is rapidly consumed by soil micro-organisms and either respired heterotrophically or retained as quasi-inert compounds which are stored in the soil. These processes are highly complex and understudied but represent a fundamental component of forest C budgeting (Epron et al., 2012). Further, C allocation to the plant organs and microbial component of the air-plant-soil continuum is of particular interest as this can adjust the trees growth pattern and allometry by mediating competition for C between the above- belowground sinks (Litton et al., 2007). The change is C partition size is vital, as in the case of the leaf biomass/area, as this obviously alters the light intercepted by the tree and the resulting primary production of the tree (Gower et al., 2001; Sinsabaugh et al., 2017). C partitioning is also essential for facilitating, the acquisition of nutrients and trace elements required for healthy tree growth through modifying the belowground biomass such as root volume and which effects C sequestration as both soil organic matter and plant material (above and belowground standing biomass) (Litton et al., 2007; McCormack et al., 2015).

C allocation was historically estimated using mass balance calculations, which traditionally are the addition of measured aboveground biomass and respiration measurements. The belowground component including the importance of fine-root turnover, mycorrhizal hyphal growth and root exudation is then estimated by subtracting the C inputs from aboveground (litter and woody debris inputs) from the total cumulative soil CO_2 efflux (Giargdina and Ryan, 2002). In some cases, measurements of annual fluctuations in aboveground respiration from tree organs and allometric relationships are used to calculate aboveground C flux (Ryan et al., 1996). Eddy covariance can also be used to measure net CO_2 flux and belowground C fluxes, which are then subtracted from gross primary production to estimate total aboveground C flux (Navarro et al., 2008). Although these techniques have been useful in the quantification of C budgets for an entire ecosystem or area it is near impossible to disentangle the autotrophic and heterotrophic respiration components (Carbone et al., 2016). Mass balance approaches whilst useful, do not elucidate the short-term C dynamics essential to understanding the annual allocation dynamics of temperate deciduous tree species or explain the relative roles of phenology climatic drivers of seasonal changes in allocation or how these factors could change in importance in changing climatic conditions (Epron et al., 2012). Mycorrhizal plants present

their own additional methodological challenges especially when attempting to quantify C fluxes and the concealed molecular mechanisms that underpin these processes (Slavíková et al., 2017). Mycorrhizal fungi obtain the majority of their C from plant photosynthesis, alongside a small proportion derived from decomposing plant matter (Olsson and Johnson, 2005; Hobbie et al., 2014; Lindahl and Tunlid, 2015). The colonisation of roots by mycorrhizal fungi can also alter the relative allocation to plant organs, particularly the roots (Wright et al., 1998; Slavíková et al., 2017), as well as altering the root architecture in some cases (Chen et al., 2000; Eissenstat et al., 2015; Chen et al., 2016). The presence of mycorrhizal fungi has also been found to increase photosynthetic activity through providing the tree with nutrients directly or by increasingly the belowground sink strength (Douds et al., 2000; Kaschuk et al., 2009; Valentine et al., 2013). The flux of C from the plant can only be measured directly by employing the use of C isotopes and a percentage of total C budget diverted to the symbiosis calculated (Řezáčová et al., 2017). Studies have shown that the cost of mycorrhizal associations to photosynthetically fixed C ranges from 4 – 20 % (Smith and Read, 2010). The highest value of 20 % has only been observed in one mesocosm, containing young cucumber (*Cucumis sativus*) plants by Jakobsen and Rosendahl (1990) and has unfortunately been extrapolated up to a total global figure (Brzostek et al., 2014). Contemporary research suggests that the actual figure is far lower than even the lowest 4 % suggested previously; both Tomè et al., (2015) and Slavíková et al., (2017) found only a few percent of the plants total C budget was expended on mycorrhizae. The investigation of the true cost of forming a mycorrhizal symbiosis to the plant have only been made possible by the increase of availability of isotopic methodologies and a reduction in the cost of equipment used to analyse them (Řezáčová et al., 2017).

Isotopic C can be used to trace the source and fate of C recently fixed by photosynthesis through the plant partitions, into metabolites and respiration and onto the mycorrhizosphere (Dawson et al., 2002), but the sometimes-low signal strength (especially with ^{13}C) can make resolving lag times and rates of transfer problematic (Epron et al., 2012). However, that said, these techniques have been employed successfully to study C allocation in trees and forest ecosystems (Knohl et al., 2005; Brandes et al., 2006; Keitel et al., 2006; Kodama et al., 2008; Marron et al., 2009; Wingate et al., 2010). Isotopes of C that have been successfully deployed in this type of allocation experiments include stable isotopes (^{13}C) and radio-isotopes (^{14}C and ^{11}C), these have been used for short-pulses or over extended periods, by artificially altering the isotopic C before photo assimilation (Epron et al., 2012). During pulse-chase experiments, the isotope is used as a proxy for recently fixed C and the relative allocation can be estimated by

the amount of isotope retained in the plant organs or lost to the system by exudation, respiration and emissions of volatile organic compounds and calculated as a percentage of the total fixed C. Isotopes can therefore also determine if the C has been allocated to structural or non-structural components of any plant organ (Kagawa, 2006).

Radioisotopes have been used since the 1950s to study the fate of recently fixed C, although their use remains controlled by both Health and Safety and radioisotope safety regulations (Schuur et al., 2016). Initially, radioisotopes were favoured due to the availability of analytical instruments to trace the radioactivity through the air-plant-soil continuum, namely, biological oxidation, autoradiography, liquid scintillation (LS) spectrometry and accelerator mass spectrometry (AMS). These have been used for pot-based experiments, individual branches of field-grown trees and *in-situ* whole tree labelling experiments (Epron et al., 2012). One famous example of this methodology being used includes the experiments conducted by Calvin and colleagues, which led to the discovery of the photosynthetic reduction cycle (Calvin, 1949). Because recent photosynthates are very rapidly transferred to the soil microbial community (Kaiser et al., 2015), either directly or via mycorrhizae, labelled C can quickly be recaptured in root exudates, soil pore water, heterotrophic respiration and microbial biomass (Epron et al., 2012). Soluble C can also be extracted from the soil using a suitable solvent (usually K_2SO_4). The isotopic C transferred to the mycorrhizal hyphae can also be detected through the use of soil cores, hyphal in growth bags of fractions of a mesocosm by excluding roots with a suitably sized nylon mesh (Johnson et al., 2002; Epron et al., 2011). Novel molecular techniques are also now allowing us to trace the isotope into nucleic acids (Ostle et al., 2003), fatty acids and amino acids (Högberg et al., 2010).

Due to availability of analytical facilities at the School of Natural Sciences, Bangor University, in agreement with my supervisors I decided to use a ^{14}C pulse-chase experiment throughout this thesis. In all experimental data chapters, a radioisotope of C ($^{14}CO_2$ with a half-life of 5730 years) was utilised to produce a C pulse-chase label, which was traced through the plant soil continuum to identify the pathway of recently fixed C from the photobiont to the soil microbial community. The advantage of using ^{14}C over other isotopes is that in a field experiment tiny amounts of isotopic enrichment can be detected partly due to the low detection rates of the analytic devices and also because the atmospheric background readings of ^{14}C are relatively low (Epron et al., 2012). Rhizon suction samplers were used to capture rhizospheric soil pore water that was analysed by liquid scintillation spectrometry after biological oxidisation, which bubbled the resulting gas through liquid scintillation fluid to capture the ^{14}C, and allow

quantification of C inputs from the roots and hyphae by liquid scintillation spectrometry (Plate 2). All chapters with the exception of the final experimental chapter were mesocosm experiments that used the liquid scintillation technique as the ß-radiation remained detectable whilst remaining under safe working limits set by legislation. The heterotrophic and autotrophic soil respiration was captured using sodium hydroxide respiration traps suspended above the soil. After destructive harvesting, the 2-year-old trees (see section 1.7 for species description and provenance information) of the three species studied were separated into plant tissue types (leaves, stem and branches, and roots). These were then analysed for ^{14}C activity by combusting the organic material in the sample with a Harvey Instruments Biological Oxidiser OX400 (R.J. Harvey Instrument Corp., Hillsdale, N.J., U.S.A) (Plate 1). The $^{14}CO_2$ bubbled through and dissolved into Oxysolve C-400 (Zinsser Analytic GmbH, Frankfurt, Germany) scintillation fluid prior to determination of ^{14}C content using a Wallac 1409 liquid scintillation counter (Wallac EGandG, Milton Keynes, UK) (Plate 2).

Plate 1: The Harvey Instruments Biological Oxidiser OX400 located in the fume hood located, in the radiation laboratory, which was used to combust the vegetation, soil and hyphae in sand, for all experimental chapters.

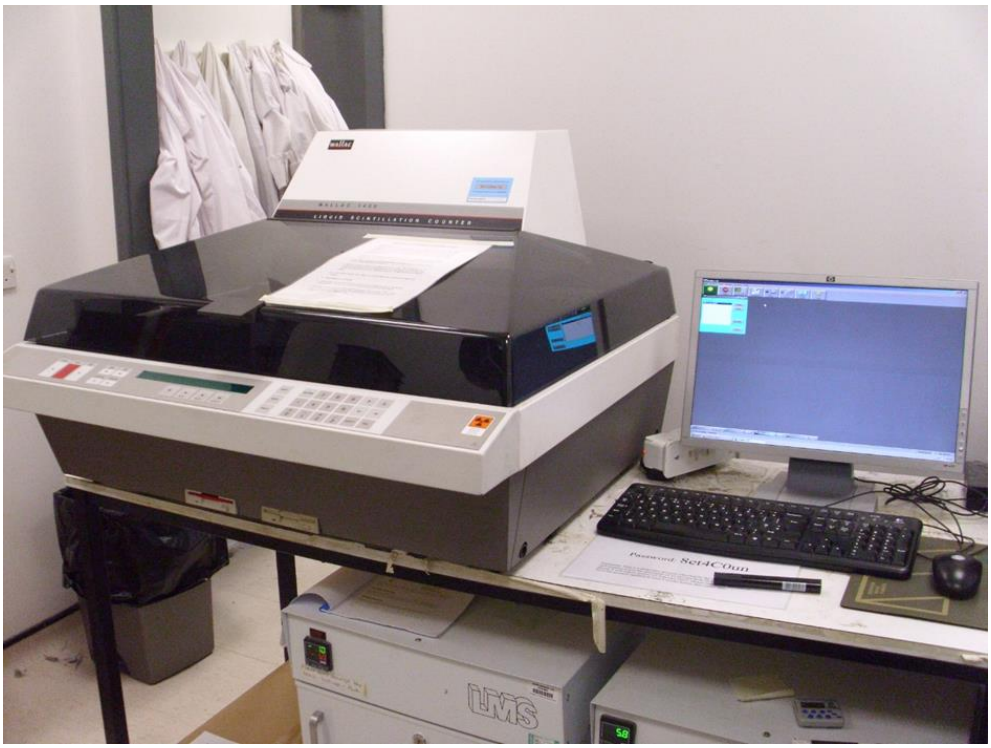

Plate 2: Wallac 1409 liquid scintillation counter located in the counting room and utilised for all ^{14}C analysis of vegetative, soil sand and liquid samples (Wallac EGandG, Milton Keynes, UK).

1.7. Facilities and Field Site Description

All experiments were conducted in either in the glasshouse, located on the roof of Memorial Building in Bangor, or at Bangor University's Henfaes Research Farm. These facilities will be described in this section. A total of 44 x *A. glutinosa* and 36 x *B. pendula* and 36 x *C. sativa* trees were used in the experimental chapters.

Glasshouse experiments

Chapters 3, 4 and 5 were all conducted *ex-situ* in a glasshouse located on the roof of the Memorial Building, Deiniol Road, Bangor, LL57 2UR (53°13'32.0"N 4°07'55.8"W) (Plate 3). All soil used in the glasshouse experiments was collected from beneath established (15 years old) trees of the same species in the BangorDIVERSE experiment located at Henfaes Experimental Farm, Abergwyngregan (Plate 4). The glacial till derived nature of the soil in this area means that it was extremely stony, so stones were removed on site with a homemade wheelbarrow mounted riddle (Plate 4). After the majority of large stones (cobbles) had been removed, the soil was barrowed to a glasshouse where it was air-dried (Plate 5), before sieving to pass through a 2 mm sieve and utilised in the mesocosm experiments (Chapter 3, 4 and 5).

Plate 3: Memorial Building, Deiniol Road, Bangor with the experimental glasshouses on the roof. The lower level glasshouse on the left of the plate was the glasshouse that experimental chapter 3, 4 and 5 were conducted in.

Plate 4: All soil used in the pot experiments was dug from BangorDIVERSE, due to the glacial till nature of the soil we constructed a homemade, wheelbarrow mounted riddle to remove the majority of large stones before air drying the soil in a glasshouse.

Plate 5: Soil removed from BangorDIVERSE was air dried before being passed through a 2 mm. sieve and utilised in the mesocosm experiments (Chapter3, 4 and 5).

The mesocosms used in the three mesocosm experiments (Chapters 3, 4 and 5) were located in the glasshouses (Plate 4) and laid out on the benches in a randomised block design and rotated around the glasshouse to homogenise conditions and compensate for uneven growth conditions (hotspots, shaded areas, etc.).

Plate 6: Inside the glasshouse during the experimental establishment period of chapters 3 and 4 during the summer of 2016.

Field based experiments

Field based research was conducted at Bangor University's tree diversity experiment (BangorDIVERSE), established in 2004 on two fields totalling approximately 2.36 ha at Bangor University's Henfaes Research Farm, Abergwyngregan, Llanfairfechan, North Wales, LL33 0LB (53°14 N, 4°01 W), located 12 km east of Bangor city centre (Ahmed et al., 2016). The BangorDIVERSE forest experiment is part of a global tree diversity network (http://www.treedivnet.ugent.be/) which has already collected data that is of significance to global forest policy and management (Verheyen et al., 2016). BangorDIVERSE was planted on land that had previously been pasture, since the 1980s; one of the fields had been used for small-scale forestry experimentation; the other was ploughed for oil seed rape (*Brassica napus* subsp. *napus)* in 2003. The 60 cm trees were planted in four replicated blocks of one, two and three species mixtures at spacing of 1 m (10,000 stems/ha). The species planted (*Alnus glutinosa* L., *Betula pendula* Roth., *Fagus sylvatica* L., *Fraxinus excelsior*, *Acer pseudoplatanus* L., *Castanea sativa* Mill. and *Quercus robur* L.) were chosen to exhibit contrasting functional traits and successional stages.

The plots are gently sloping (between 1 – 2 º) and approximately 13 – 18 m above sea level with a north-westerly aspect. The underlying geology is alluvial gravel and glacial till with a Eutric Cambisol soil type (Rheidol series) (Smith et al., 2013). Soil has a fine loamy texture (48.2 ± 1.3 % sand, 33.6 ± 0.9 % silt and 18.2 ± 2.1 % clay) as determined by laser diffraction granulometry (Coulter LS particle size analyser) (Gunina et al., 2017). The soil has an Ah horizon (0-40 cm which grades into a Bh horizon with the C horizon apparent at ca. 100 cm. The climate is hyperoceanic, the mean annual precipitation of 1034 mm and mean annual temperature of 11.5 ºC (Campbell Scientific Ltd, Shepshed, UK).

1.8. Autecology of the Tree Species Studied

Tree species were selected from the existing species within the BangorDIVERSE plots. The study will determine the C balance of three species possessing contrasting functional traits. The species studied were as follows:

Common alder (*Alnus glutinosa* [L.] Gaertn.; A) (also commonly known as black alder) is a native broadleaf, deciduous, fast growing but short-lived actinorhizal pioneer species. This tress was of particular interest as not only does it have a symbiotic relationship with nitrogen fixing bacteria (*Frankia alni*), but also possesses both arbuscular and ecto-mycorrhizal associations (tripartite symbiosis). *A. glutinosa* tolerates waterlogged soil and in fact needs high water availability to thrive, ideally with annual rainfall above 1500 mm or accessibility to groundwater (Claessens et al., 2010). As such, it is most commonly found in riparian zones and swamps, the seed is cone-like and floats in water allowing for dispersal along watercourses (McVean, 1956). *A. glutinosa* has adaptations which allow the trees survival in the wettest of conditions, the roots are supplied air from above the water level through the xylem, and this supplies the roots with oxygen (Dittert, 2006). Where watercourses are not present, the rate of spread is generally slower than that of other pioneer species such as *Betula pendula* (silver birch) and *Fraxinus excelsior* (common ash). *A. glutinosa* has one of most northerly ranges of a deciduous tree in the UK, only outdone by *Betula pubescens* and *Alnus incana* (Iverson, 1944) and is found at altitudes of 500 m in Scottish hills (Savill, 2019). To regenerate from seed *A. glutinosa* demand both moisture and high light levels and therefore flourish on disturbed sites. Initially, growth rates are high in years 7-10 after which they slow dramatically; *A. glutinosa* prefer sites with a pH above 6 and are tolerant of salt spray (Savill, 2019).

Timber from *A. glutinosa* is not that dense (530 kg m^{-3} at 15 % moisture) and is used as a source of energy, for paper and to make chipboard, as well as for joinery veneers and traditionally for clogs and charcoal (Savill, 2019; Claessens et al., 2010). A useful characteristic is that the wood is decay resistant when submerged in water and as a result is used for sluice gates, jetties, underwater supports and small boats (Savill, 2019; Hines et al., 2016). Rotation length is normally seventy years if heart rot is to be avoided and mean average growth rate is between 4 to 14 m^3 ha^{-1} $year^{-1}$ (Claessens et al., 2010). Average lifespan is reported to be only 20- 25 years with a maximum height of 22 m and diameter at breast height (1.3 m) of 0.65 m (Mitchell et al., 1974; Savill, 2019). The commonest pathogen of *A. glutinosa*, *Phytophora alni* has been

identified in many European countries and is expected to increase in frequency in the near future (Hines et al., 2016).

Silver birch (*Betula pendula* Roth; B) is an early successional, pioneer, broadleaved deciduous native tree species with ectomycorrhizal associations and labile leaf litter, which occurs across the whole of Europe (Hines et al., 2016). *B. pendula* is quite short-lived with lifespans typically between 35 and 100 years. It has an average maximum height of 30 m and its diameter at breast height (1.3 m) tends not to exceed 0.95 m (Mitchell et al., 1974). *B. pendula* trees are extremely hardy and are distributed and flourish at altitudes where only *Sorbus aucuparia* (rowan) will survive (Savill, 2019). *B. pendula* require full sun to thrive but can cope with soils that possess poor nutritional status (Hines et al., 2016), due to their ability to form mycorrhizal associations with a large range of fungal species (Atkinson, 1992). Due to their production of highly labile litter they are generally considered to be soil improvers (Miles, 1986). The flowers are monoecious with both sexes appearing at different times as unisex catkins. Males develop in summer and disperse their seed a few days following the female flowers in spring (Hines et al., 2016). *B. pendula* produce seeds prolifically (between 1,650,000 – 2 million seeds kg^{-1}), which are very small (2 – 3 mm across) and have small wings to facilitate their dispersal by wind (Hines et al., 2016).

Growth rates are 7 $m^3 ha^{-1} year^{-1}$ on the best sites and *B. pendula* has a characteristic smooth white bark and is used for high value veneers and furniture as well as firewood. It is not however a durable wood (Lorrain-Smith and Worrell, 1992), with an average density of 670 kg m^{-3} (at 15 % moisture content) (Savill, 2019). *B. pendula* has few diseases, although it can be parasitised by *Amillaria* spp. (honey fungus) and can commonly be observed with 'Witches brooms' a distorted growth pattern caused by a fungus *Taphrina betulina* (Savill, 2019). Birch is considered to have high wildlife value, supporting a large number of both generalist and specialist phytophagous insects, birds and mammals (Patterson, 1993).

Sweet chestnut (*Castanea sativa* Mill.; C) is a late successional non-native but naturalised tree species with solely ectomycorrhizal associations and recalcitrant leaf litter. *C. sativa* occurs naturally across the Mediterranean region but because of widespread cultivation for its nuts and durable wood now has a wide-ranging distribution (Hemery and Simblet, 2014). It is believed that *C. sativa* was first bought to the UK by the Romans and is relatively long-lived, living up to several thousand years old and has a maximum height of 35 m and a diameter at breast height (1.3 m) of 3.2 m (Mitchell et al., 1974). The species is considered reasonably

shade-tolerant, grows fast and coppices well on a rotation of 12 – 16 years (Savill, 2019). *C. sativa* prefers deep, fertile, light soils with moderate moisture (minimum rainfall required is between 600 and 800 mm) and flowers in late June (Savill, 2019). Large nuts (average weight 239 nuts kg^{-1}) develop during summer and ripen in autumn if the summer has been warm and dry although heavy crops are rare in the UK and trees need to at least 30 years old for good production (Savill, 2019).

C. sativa is one of the most productive deciduous broadleaved species, with mean annual yield reported up to 8 m^3 ha^{-1} $year^{-1}$ (Locke, 1978). The timber is naturally durable, but not dense (560 kg m^{-3} at 15% moisture) and is frequently used for green woodwork and furniture, due to its ease of splitting and was traditionally used for pit props and coppices extremely well (Savill, 2019). It is affected by chestnut blight, *Endothia parasitica* (Savill, 2019), ink disease (*Phythophtora* spp.), and the newly introduced chestnut blight (*Cryphonectria parasitica*). Insect pests of *C. sativa* include Chinese gall wasp (*Dryocosmus kuriphilus*), chestnut weevil (*Curculio elephas*) and tortrices (*Cydia splendana*; *Cydia fagglandana*; *Pammene fasciana*) (Hines et al., 2016).

The three trees were chosen as they exhibit both types of mycorrhizal symbiosis and *A. glutinosa* is the UK's only native tree which forms an association with nitrogen-fixing bacteria. In addition, the trees differ in their typical successional stages with *B. pendula* considered to be an early successional stage tree whereas *C. sativa* is most typically a late-successional stage tree. The trees were all purchased as 1+1 saplings (20- 40 cm height) from Maelor Forest Nurseries Ltd., Bronington, UK, in November 2015. At the time of planting, the height of all tree saplings was between 0.2 – 0.4 m. The provenance details of the trees purchased and those already established in the BangorDIVERSE plots (used in Chapter 6) are shown in Table 1. Of the three species studied experimentally, two are considered UK native species (*A. glutinosa* and *B. pendula*) and the other studied species is known as a naturalised species. Tree species are considered UK natives if they colonised the UK between the end of the last ice-age (10,000 years ago) and the formation of the English Channel, separating us from Europe several thousand years later. Species that have reached the UK since the formation of the Channel are considered naturalised (e.g., *C. sativa*). All forestry tree species are categorised by origin and provenance, for native species these two terms are synonymous, however for naturalised species the origin would be the origin that is considered the natural range and the provenance would be the location of the tree from which the seed was collected (Hubert and Cundall, 2006). Plate 7 is the UK Forestry Commissions provenance and seed zone map showing the lines of

demarcation, Table 1 is a list of the provenance of the trees used in the mesocosm experiments (Chapter 3, 4 and 5) and the provenance of the trees planted in BangorDiverse experiment in 2004.

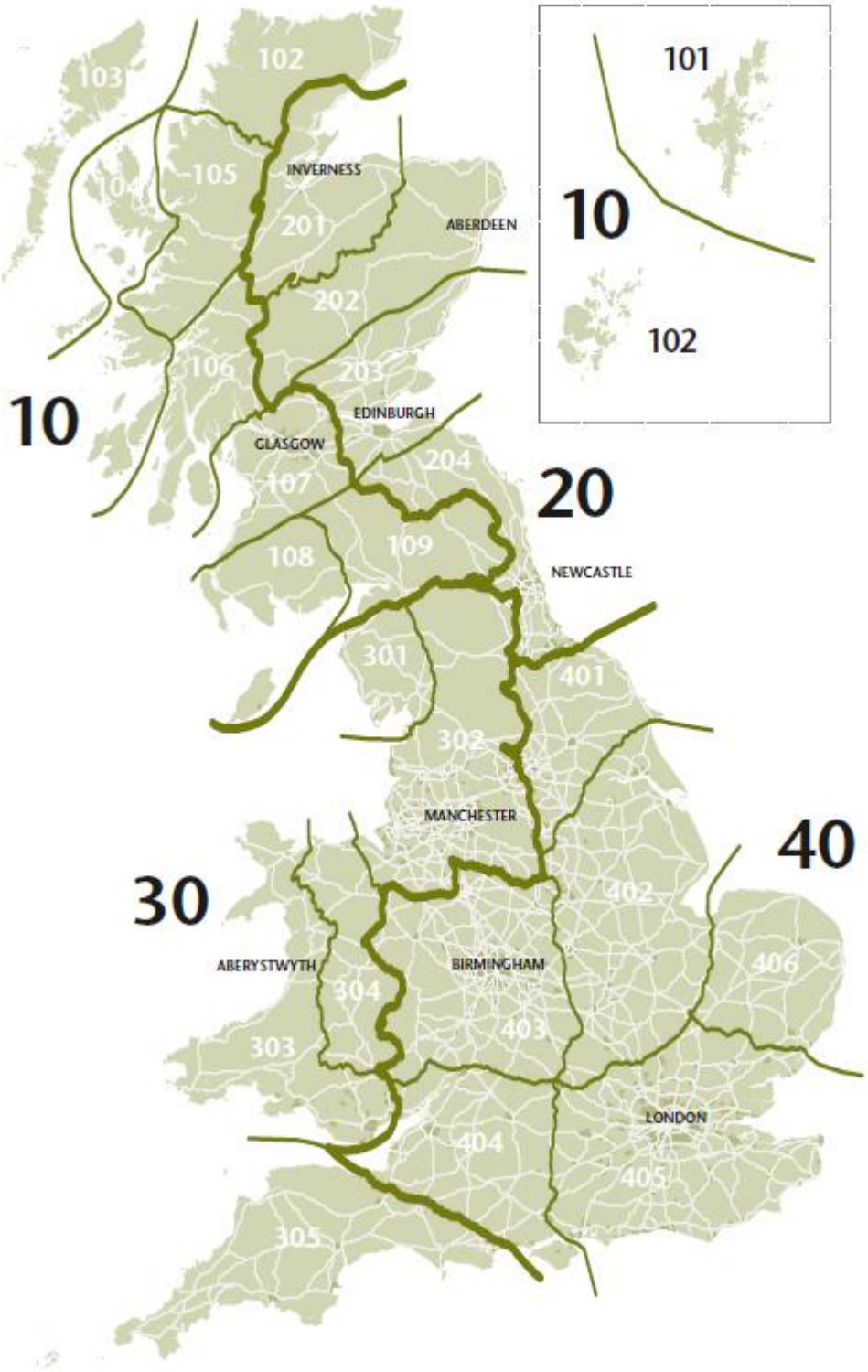

Plate 7: Regions of provenance and seed zones of the UK. Source: Hubert and Cundall (2006)

Table 1: The provenances of the three-tree species studied in both the glasshouse experiments (Chapters 3, 4 and 5) and the field experiment (Chapter 6).

Species **Common name** **(*Latin name*)**	**Juvenile 1+1 (20-40 cm) trees ordered from Maelor nurseries, Whitchurch (403)**	**Mature trees within Bangor DIVERSE plots planted 2004**
Silver birch **(*Betula pendula*)**	204 (Northumberland)	204 (Northumberland)
Common alder **(*Alnus glutinosa*)**	204 (Northumberland)	403 (Shropshire)
Sweet chestnut **(*Castanea sativa*)**	404 (South-west England)	303/304 (Wales)

1.9. References

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Chapter 2

Literature review: Understanding the role of plant-microbe symbiosis in the cycling of carbon in temperate forest ecosystems

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Unpublished manuscript

Author contributions:
TDP wrote the first draft of the manuscript with all authors contributing to the final version.

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1. Introduction

Since Darwin first proposed the theory of evolution it has been widely understood that intra- and interspecific competition is the driving force in structuring the world's complex and diverse plant communities (Tilmam et al., 1982), whereas co-operation and facilitation has been seen to be of lesser importance (Bruno et al., 2003). The traditional view of inter- and intra-specific competition was that the availability of resources is a major factor in speciation and ultimately contributes to the vast biodiversity observed in the natural world (Wright et al., 2017). Ecological theory over the last few centuries has been dominated by the belief in the importance of competition, predation and perturbation as the primary drivers of community structure (Boucher, 1988). However more recent empirical data has elucidated to the importance of positive interactions between species in both terrestrial and marine ecosystems (Boucher, 1985; Callaway, 1995; Bertness and Leonard, 1997; Jones et al., 1997, Selesse, 2006; Van der Heijden and Horton, 2009). Facilitation appears to be more widespread and important than

originally thought and it has been widely reported that facilitative relationships become more importance at times of stress (McIntire and Fajardo, 2014). Therefore, more studies are required to inform ecological theory and to improve predictions of how biological communities will respond to the climatic changes the world is currently experiencing (Bruno et al., 2003).

In this paper we review the current knowledge and recent advances in the field of plant: microbe symbiosis, focusing particularly on how they mediate and facilitate biogeochemical cycling of carbon (C). In the process of reviewing the literature we will also identify understudied areas which need elucidation by future research.

2. What is symbiosis?

The term symbiosis was first used by A Frank, in 1877 when describing the mutualistic relationship observed between algae and fungi in lichen. However, over time, symbiosis has been used in a broader context to describe any long-term relationship between two unalike species (Tariq et al., 2017). Symbiosis is generally considered to include three separate types of relationship known as mutualism, commensalism and parasitism, although these categories are not always mutually exclusive (Martin and Schwab, 2012; Saffo, 1992). Rather than thinking of these types of symbioses as distinct categories they are a continuous spectrum of symbiosis which exhibits plasticity depending on environmental pressures (Leung and Poulin, 2008).

2.1. Mutualism

Mutualism is a symbiotic relationship when both organisms involved benefit, this can also be termed as reciprocal altruism (Paracer and Ahmadjian, 2000). Mutualisms may be far more important than current ecological theory suggests as mutualism facilitates or mediates a vast array of ecosystem processes that include symbiosis in corals, lichens, plants, and the evolutionary development of eukaryotic cells (Margulis, 1981). Small-scale effects of mutually positive biological relationships have been studied and their importance is widely accepted. However, the feedbacks from small to large scale positive relationships and how these processes impact on species richness, diversity, ecology and evolution of communities, ecosystems and populations remain poorly understood (Stachowicz, 2001).

2.1.2. Commensalism

Commensalism is a relationship between two living organisms where one is benefitted and the other is not significantly affected either positively or negatively (Nair, 2004). In practice these types of relationship are extremely hard to distinguish from parasitic relationships that cause the host very low levels of harm (which may not yet be detectable by current methods) or relationships where the host has developed adaptive strategies to minimise the damage caused by the microorganism (Leung and Poulin, 2008).

2.1.3. Parasitism

Parasitism is where one of the organisms involved benefits and to the detriment of the other. The benefitting organism (or parasite) can either keep the other alive, in this case the relationship is a biotrophic parasitism, or can result in its death, known as necrotic parasitism (Paracer and Ahmadjian, 2000).

Including these three types of biological relationships into a broader modern definition of symbiosis includes many more relationships than the original meaning of the term (Wilkinson, 2001). In 1994, Douglas redefined symbiosis as associations which persist for long periods of time, the time period being relative to the lifespan of the interacting organisms. This definition therefore excludes most parasitic relationships as over longer time periods one of the partners is affected detrimentally and will eventually die, ending the relationship (Ogle and Brown, 1997). It is Douglas's definition which we will be referring to throughout this thesis.

3. What are soil microbes?

During this review we will refer to microbes or micro-organisms, but what are microbes? Microbes or micro-organisms are general terms that describe a diverse range of organisms with a vast range in sizes. Microbes are organisms that are too small to see without the use of a microscope during one or more of their lifecycle stages although they can often exist as single cells or in clusters or colonies (Jenkinson and Lamont, 2005). Currently the most studied and important plant: microbe symbioses include both bacterial and fungal microbionts, although Protista, methanogenic archaea and ammonia-oxidizing archaea that have symbiotic relationships with plants do also exist (Rai, 2018; Minamisawa et al., 2016; Moissl-Eichinger et al., 2018). Soils are known to contain the largest abundance of terrestrial biodiversity which is responsible for providing a significant proportion of ecosystem function (Baldrian et al.,

2012). Terrestrial microorganisms are thought to exceed 10^{29} and regulate C storage and respiration (Flemming and Wuertz, 2019) as well as providing essential macronutrients which ensure continued plant productivity (Bardgett and Van Der Putten, 2014).

4. Temperate forests and how they differ from other forests

The Earth's temperate zone is defined as occupying the land area between the polar and tropical regions (i.e. latitudes of 25 degrees north or 50 degrees south of the Equator) (Kottek et al., 2006). The Köppen climate classification system describes the temperate climatic zone as when the coldest month has a mean temperature above −3 °C but below 18 °C (Köppen, 1884). Temperate forests cover 1038 Mha of land and are occupied by both deciduous and coniferous tree species that contain between 60 to 130 Mg C ha^{-1} in aboveground biomass. The total C stock estimated to be stored in temperate forest ecosystems is between 119 ± 6 Pg C (14 % of estimated current C stock in the world's forests), of which approximately one-third is found in aboveground biomass and two-thirds belowground (Dixon et al., 1994; Lal, 2005; Pan et al., 2011; Bolin, 1977). The extent of temperate forests has increased in recent years with an increase in tree canopy cover estimated to have gone from 4681 km^2 in 1982 to 5540 km^2 in 2016 (Song et al., 2018). The has led to temperate forests being a net sink of C with net emissions between 2006 and 2015 being estimated at -0.3 Pg C/year (Houghton and Nassikas, 2018). Temperate regions experience seasonal variations normally with a hot dry season or summer followed by a colder wetter season, known as winter. Trees in this region have developed strategies to increase the chances of them surviving these prolonged cold spells including leaf abscission known as deciduousness. In the UK there are 32 native tree species of which 29 are broadleaf, although currently only accounts for 49 % of the UK's total forest cover (Forestry Commission, 2018).

Temperate forests are characterised by these seasonal variations in climate and vegetation dynamics as due to the deciduous nature of some of the trees in the temperate biome seasonal variations in C sequestration occurs with changes in photosynthesis linked to leaf phenology (Gond et al., 1999). Photosynthesis ultimately drives C assimilation and sequestration in temperate deciduous forests and the timing of budburst and leaf senescence and abscission is therefore critical in determining the photosynthetically active months of the year (Chen et al., 1999; Richardson et al., 2009; Stoy et al., 2014). The environmental factors which affect temperate phenology are temperature and photoperiod and vernalisation (Hodges, 1990).

5. Belowground C in temperate forests

Soil C accounts for the largest pool of terrestrial C, with more C stored below-ground than can be found in terrestrial plants and the atmospheric system combined (Jobbágy and Jackson, 2000; Schelesinger, 1977). The soil C content is determined by the processes of litter fall, exudation and decomposition rates (Jandl et al., 2007). Seasonal variations also occur in the soil microbial community as the microbial biomass is dependent on root turnover, exudation and their own temperature dependence (Voříšková et al., 2014). Winter abundance of soil microbes has been reported to be 26- 33 % lower than that of summer microbe abundance, whilst soil winter fungal abundance was 15 % lower than summer abundance (Žifčáková et al., 2016). The addition to the rhizosphere of C from fine roots and mycorrhizae is estimated to be from 2 to 5 times more than the C derived from above-ground biomass (Fogel and Hunt, 1983). Understanding the dynamic processes of the soil organic C balance for climate change mitigation is an area of increasing interest and importance to humanity (McGuire et al., 2001). However, uncertainty about the precise amounts of belowground C allocation by plants is still preventing accurate model development (Reich et al., 2014). Soil organic matter was historically thought of as either labile or recalcitrant, distinguished by different residency times and turnover rates within the soil (Ahmed et al., 2015).

5.1. Fast pool or labile C

This C within the soil structure is readily available and therefore easily degradable by soil organisms and consists of exudates such as carbohydrates, amino acids, monosaccharides and soluble sugars which can be cycled between eight to ten times per annum (Coleman et al., 1983). Wilkinson et al (2014), estimate that amino acid and peptide turnover rates in productive grassland soils could be as fast as 20 times a minute. Due to the fast turnover times of these nutrients they are far more easily utilised for plant growth (Lee and Pankhurst, 1992) and decomposition (Belay-Tedla et al., 2009).

5.2. Recalcitrant C

More recalcitrant forms of soil C such as humic materials are much slower to turnover (Ahmed et al., 2015) and estimates for this suggests that the cycling takes places every decade to every century. In the case of C molecules in most protected, lignified, forms it is thought they remain in the soil for up to thousands of years (Qiao et al., 2014; Dungait et al., 2012; Kellner et al., 2014).

However more recently doubt has been cast on this concept as being over simplistic and is being replaced by new ideas that have led to an emerging new perspective of soil organic carbon dynamics (Schmidt et al., 2011). The molecular structure which was previously thought to account for the persistence of C in soil has been shown to have a minimal effect of the longevity of C in the soil system (Marschner et al., 2008). Keiluweit et al., (2017) postulated that the unexplained persistence of soil C was as a result of anaerobic microsites. The suggestion is that even in freely drained soils anaerobic conditions exist in microsites, confirmed by the presence of anaerobic soil processes such as reduction of Fe or methanogenesis (Nico and Fendorf, 2016). These anaerobic microsites provide refuge for highly reduced, fine particle sized organic compounds such as lipids, cutin and waxes (Mikutta et al., 2006) allowing accumulation due to oxygen limitation reducing decomposition processes (Koven et al., 2013). This is a rapidly developing area of soil science which will no doubt continue to be developed into more pragmatic modelling of soil C (Luo et al., 2016).

A large range of other factors can influence the total belowground C stocks, these include climatic factors (particularly temperature and moisture), soil and, landscape factors (Lange et al., 2015). The site location, climate and vegetation of each location can drastically alter the ability of the soil to sequester C; this is known as the C carrying capacity (Liang et al., 2017). The site conditions (soil type, pH, and nutrient availability, water content and organic matter) can also have a huge influence on the microbial diversity and activity (Pickles and Anderson, 2016). The forest soil C stock is also affected by anthropogenic factors such as forest management regimes and disturbance perturbations (Seidl et al., 2014; Triviño et al., 2015).

Precipitation can play a major role in determining the C storage capacity of soils. Meta-analysis of soil C content in different land-use management scenarios revealed that areas with annual rainfall of 2000- 3000 mm sequestered 24 % more C than land with either higher or lower rainfall rates (Guo and Gifford, 2002). It has been postulated that although higher rainfall theoretically increases decomposition that this is offset by increased C leaching into the deep soil profiles with less microbial activity (Jenny 2012; Post et al., 1982). Water availability to plants is a major abiotic factor that influences net primary productivity and therefore the relative partitioning of C under all land-uses including forests. Water stress, however, can be difficult to evaluate at a stand scale due to the spatial heterogeneity of moisture within forest soils and differences in water use efficiency of tree species (Anderegg et al., 2015).

6. Plant-microbe symbiosis

Plant: microbe symbiotic relationships are a vital component of plant nutrient acquisition by mediating soil organic matter decomposition and nutrient cycles in forest soils (Van De Heijden et al., 2008). As microbial communities are generally limited by C (Oren et al., 2001) they are reliant on and stimulated by exudates within the rhizosphere (Van De Heijden et al., 2008). Microorganisms within the rhizosphere rapidly consume C-base exudates leading to expeditious belowground C turnover and respiration (Helal and Sauerbeck, 1989). The presence of symbiotic microbial communities within the rhizosphere has been found to increase the plants allocation of C to the root structures (Reid et al., 1983; Harris et al., 1985; Koch and Johnston, 1984) and to increase root exudation (Smith and Read, 2010). The mechanics of these processes are not fully understood, but microorganisms could be increasing plant exudation by producing root hormones that increase the roots leakiness (Bowen, 1994) or by physically damaging roots to facilitate leakage (Grayston et al., 1998). However, this can be confused by the fact that micro-organisms can also exude similar substances (Rambelli, 1973) and that plants respond to symbiosis by increasing photosynthesis (Rygiewicz and Anderson, 1994). Exudation is also extremely important in acquisition of nutrition by stimulating microbial activity through increasing availability of cations (Grayston et al., 1997) and in some cases through mineral weathering (Linehan et al., 1985). Root exudation is not uniformly dispersed over the root surface and generally the exudation activity is clustered towards the growing tip of the roots (Bowen and Rovira, 1991; Farrar et al., 2003). Experiments with stable C isotopes to map the roots regions of exudation revealed that the longitudinal cell junctions at the apical regions were the most important for amino and organic acid exudation (Bowen, 1979; Jones and Durrah, 1994; Hoffland, 1992).

Plant microbe symbiosis occurs as a range of intimacies from extremely loose to intercellular (Mendes et al., 2013). In order for a plant-microbe symbiosis to occur the two partners have to locate each other this involves a series of recently discovered signalling stages (Venturi and Keel, 2016). The mycorrrhizosphere is awash with a mixture of high- and low-molecular weight compounds derived from root border cells shed from the root tip, dead root cap cells during decomposition processes. Additionally, primary metabolites, organic and amino acids, polymerised sugars (Holmer et al., 2017) and secondary metabolites such as phenols, terpinoids and alkaloids (Venturi and Keel, 2016) are also present. These compounds provide a food source not only for those microbes that are beneficial, but also those that could be potentially harmful to the plants (Jones et al., 2009). Beneficial microbes can promote plant health by

increasing nutrient availability or by increasing the plants resistance to biotic and abiotic perturbations (Coleman-Derr and Tringe, 2014). The parasitic or pathogenic components of the rhizosphere's microbiome will, however, have the opposite effect on the plant's health and in some cases eventually lead to death (Mendes et al., 2013). The plants have therefore evolved strategies to distinguish between those microbes that will harm and those that will facilitate good health in order to develop relationships with those which will be beneficial (Holmer et al., 2017). It has also been hypothesised that root exudation is used as a form of messaging, whereby plants can stimulate physical and biological responses from other plants and soil micro-organisms (Walker et al., 2003). Interactions can be either symbiotic or defensive depending on the organism encountered and communication can be root-to-root or root-to-microbe. This process starts with the plant signalling the microbes with attractant chemicals, mostly flavonoids but also some strigolactanes followed by the micro-organism signalling to the plant to notify it of its proximity (Holmer et al., 2017). Recent advances in genomics has revealed the mechanisms in plants for regulating and signalling the microbial symbionts are similar in both mycorrhizal associations and nitrogen fixing symbiosis (Delaux et al., 2015; Bravo et al., 2016; Kamel et al., 2017). Modern high throughput genetics has revealed a vast diversity of micro-organisms within the rhizosphere soil microbiome, which can be both attracted and repelled by complex chemical signals released by the plant root into the rhizosphere (Holmer et al., 2017). Without an understanding of how root exudation is regulated within the plant it is difficult to fully appreciate the significance of this process to plants or the rhizosphere (Jones et al., 2004).

The purpose and impact that plant and microbial exudates have in shaping the rhizomicrobiome is the focus of current research. The hope is that we can begin to understand more about these positive relationships and promote them, thus reducing the need for artificial fertilisers and pesticides (Bai et al., 2015; Bulgarelli et al., 2012; Lundberg et al., 2012). What is known is that signalling chemicals stimulate morphological changes in both the mycosymbiont and the plant that are essential for the formation of the symbiotic relationship (Martin et al., 2007; Kohler et al., 2015). Through novel techniques utilising cDNA arrays, signature proteins specific to mycorrhizal fungi and nitrogen-fixing bacteria have been isolated and are believed to be involved in stimulating the required morphological modifications required to allow successful plant- microbe symbiosis to occur (Plett et al., 2014; Kohler et al., 2015; Martin et al., 2016). Plant: microbe symbiosis is increasingly being found to be fundamental to many ecosystem functions and processes including the acquisition of nutrients by plants, the

formation of soils and biogeochemical cycling (Wagg et al., 2014; Van Elsa et al., 2012; Van der Heijden et al., 2015). Many of these symbionts are presently unculturable (Newman, 2016), which means that they remain unclassified and their functions are yet undiscovered (Van der Heijden, 2008; Drigo et al., 2010; Mendes et al., 2011; Bulgarelli et al., 2012; Sessitsch et al., 2012).

It is almost impossible to separate root exudates from root secretions and therefore all organic substances released by healthy plant roots are considered to be exudates (Uren and Reisenauer, 1988). Quantifying exudates is difficult and as a result estimates vary considerably. What is generally accepted is that perennial plant species exude more assimilated C than annuals (Mooney, 1972). The reason for this is unclear, but it has been hypothesised that perennials need to divert more assimilate into the rhizosphere in order to increase survival all-year around (Harris et al., 1980), or that annuals have less C to allocate as they need to invest more into the woody growth of stems (Mooney, 1972). Total exuded C as a percentage of total C photo-assimilated is frequently estimated as 40 % (Van Venn et al., 1991). However, experiments with some tree species have revealed much higher percentages. Scots pine (*Pinus sylvestris*) transferred 60 % of total C assimilated below-ground (Ågren et al., 1980; Persson, 1978) and 73 % for Douglas fir (*Pseudotsuga menziesii*) (Fogel and Hunt, 1983; Santantonio, 1979). Jones and Durrah, (1995) have shown that the flow of exudates is not just one way and that significant amounts of C-based exudate can be reabsorbed by the plant roots partially explaining why the quantification of exudation rates is difficult.

6.1. Endophytes

Endophytes are microbes which live inside a plant for at least part of their lifecycle without causing disease symptoms (Wallace and May, 2018). Endophytes can be either bacteria, fungi, Protista or Achaea and it is believed that they are ubiquitous across all plant groups and climatic zones (Hardoim et al., 2015). Endophytes were first described by the German botanist, Johann Heinrich Friedrich Link in 1809 and are sometimes referred to as endosymbionts. Plant endophytes are extremely diverse and only very few have been classified or studied but can include fungi, bacteria and viruses (Rodriguez et al., 2009). Bacterial endophytes belong to a broad range of taxa, including α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, Firmicutes, Actinobacteria (Miliute et al., 2015). It is believed that every plant contains an endophyte and some may contain more as a result is it estimated that the most endophyte diversity occurs in the tropics (Deshmukh et al., 2015). Discovery of this diverse range of micro-organisms has

led to an understanding that the plant biome is as important to the survival of a plant as the bacteria which mammals rely on to aid digestion (Hardoim et al., 2015). Genetic links have been found which suggests that bacterial and fungal endophytes evolved alongside each other and endophytes such as arbuscular mycorrhizal fungi (Gherbi et al., 2008). Although this group of micro-organisms have been known about and studied for over a century there is still some work to be done, in categorising them (Rodriguez et al., 2009). What is clearly understood is that endophytes have played a crucial role in the development, health, evolution and ecology of land plants and have been present within plants since they became terrestrial (Brundrett et al., 2006). Endophytic fungi will be discussed in more detail in section 3.2.4.1.

6.2. Plant-bacterial symbiosis

Plants normally defend themselves against bacterial infection by producing defence chemicals (Jones and Dangl, 2006), however in the case of plant: microbe symbiosis this does not occur. Our mechanistic understanding of plant: microbe symbiotic chemical signalling is in its infancy and it has been proposed that these plants have developed mechanisms to distinguish between pathogenic and mutualistic bacteria (Hirsch, 2004; Tellström et al., 2007). Alternatively, bacteria may suppress normal defence mechanisms (Bueno et al., 2001; Shaw and Long, 2003). In the upper litter layer of forest soils fungi dominates as the major decomposer, but in the mineral soil layer bacteria and fungi are more comparable in numbers (Baldrian, 2012). The importance of the bacterial component of soil generally increases with soil depth (Bååth and Anderson 2003). In the soils surface the relative bacterial to fungal composition is determined by pH, C: N ratio, climatic conditions and the identity of the plants located in the region (Högberg et al., 2007; Lladó, 2018; Van Der Linde, 2018). However, bacteria are less important for C storage than fungi within the soil as they are less efficient at C assimilation (Adu and Oades, 1978). The C that is stored by bacteria tends to be more labile and therefore shorter lived than that stored by fungi, this is because, in contrast to the soil fungi, the bacterial cell walls are comprised of phosolipids, which are rich in energy and can be readily degraded by other soil micro-organisms (Bailey et al., 2002). Plant-bacterial symbiosis is extremely species diverse and the development of modern genetic techniques has enabled the identification of previously unknown species involved in symbiosis. It is likely that our knowledge of the number of species involved in symbiosis will increase in future (Tilak et al., 2005). Some bacterial species are known to inhabit fungal fruiting bodies of both basidiomycetes (Dahm et al.,2005; Timonen and Hurek 2006; Pent et al.,2017) and ascomycetes (Barbieri et al.,2007, Quandt et al.,2015). The bacterial: fungal partnership may in some cases supply the fruiting

bodies with fixed atmospheric nitrogen (Barbieri et al.,2010) and in some fungal species may even be required for fungal fruiting to occur (Cho et al.,2003).

Generally, in plant: microbe symbiosis the fungal component microbial component utilises C which has been photosynthetically fixed by the photobiont and in return is provided with either nutrition, water or both which has been collected from the soil or bedrock by the mycorrhizal hyphae (Van der Heijden et al., 2015). The obvious exceptions to this rule are the mycoheterotrophic plants although some of these plants only require C from the mycobiont for the initial stages of germination and development (Dearnaley et al., 2016), after which they can photosynthesise and repay the debt to the fungi, known as the 'take now and pay later hypothesis' (Field et al., 2015). Normal plant: microbe resource exchange however has a cost to both partners and as a result both partners have evolved systems to withdraw rewards for partners not providing a service in return (Werner and Kiers, 2015). As a result of this partners that can provide more benefits are favoured over those that cannot (Selosse et al., 2016) and overtime this has led to the diversity of symbioses that currently exist. Upscaling to largescale from individual experimental data to model the importance of the C flow mediated by plant: microbe symbiosis is fraught with issues as many plant: microbe symbioses exhibit mixotrophy or plasticity of the partnership (Selosse et al., 2016).

6.2.1 Nitrogen-fixation

Although nitrogen constitutes over three-quarters of the Earth's atmosphere, it is inaccessible to plants in gaseous form (Geddes et al., 2015). In many terrestrial ecosystems plant productivity is limited by available nitrogen as it is one of the ineluctable building blocks of life, essential for the biosynthesis of amino acids, proteins and nucleic acids (Olge and Brown, 1997). The natural world has overcome this limitation with the process known as nitrogen fixation. The process of biological nitrogen fixation (discovered in the 19th century) is the conversion of atmospheric nitrogen gas to ammonia by an enzyme nitrogenase by the following reaction (Postgate, 1998):

$$\mathbf{N_2 + 16ATP + 8e^- + 8H^+ \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi.}$$

Nitrogen-fixation has been described as the second most important biological process after photosynthesis (Gruber and Galloway, 2008). Specialist bacterium have evolved to populate the roots surface or/ and rhizosphere soil, or to pass through the cortex and into the plant as an endophyte to populate either the plants organs or specialised root nodules (Gray and Smith, 2005). Plants have reciprocated by simultaneously evolving strategies to; encourage these

symbiotic relationships and to become more hospitable hosts (Holmer et al., 2017). The genes required for the process are widespread in microbes inhabiting all environments (Gaby and Buckley, 2011). The diazotrophic bacteria responsible for biological; nitrogen fixation can be divided into two distinct categories; Rhizobia, a group of paraphyletic bacteria and the diverse actinobacterial genus Frankia (Holmer et al., 2017). By far the most extensively studied plant: microbe symbiosis, is the relationship between plants and rhizobacteria (Tariq et al., 2017). The most important plant group to known contain nitrogen fixing bacteria are legumes, that fix 128 Tg. of N annually, globally (Cleveland et al., 1999; Galloway et al., 2004). Legumes fix nitrogen in a range of climatic vegetation zones from arid to tropical, as well as being extremely important in global agricultural systems (Van der Heijden, 2016).

Nitrogen- fixing plants have been found in an agricultural field experiments to increase the soils C storage by reducing the C dioxide emissions from the plants (Drinkwater et al., 1998). This is not unexpected as there is a well-documented link between the C and nitrogen biogeochemical cycles (Gruber and Galloway, 2008). However, the majority of these experiments have measured the effect of annual leguminous crops such as peas. Data from actinorhizal plants is sparse but research suggests that planting N-fixing trees has no direct significant effect on soil C (Guo and Gifford, 2002), although it has been found that as nitrogen fixers have a greater soil organic matter input that this results in faster decomposition rates (Conteh et al., 1997) and increases soil organic matter (Kasel et al., 2011; Nilsson and Schopfhauser, 1995). Nitrogen fixing endophytes (acetic acid bacteria) have recently been found on the needles of limber pine (*Pinus flexilis)* and form symbiotic relationships with the trees, exchanging carbohydrates for fixed nitrogen then made available to the plant as foliar feed (Moyes et al., 2016). Since this discovery it has been hypothesised that many more plant: bacterial symbioses may yet be undiscovered in the many more nitrogen limited biomes of the world (Wurzburger, 2016).

6.3. Other microbes forming symbioses with plants

Abundance in soils of archaea and viruses varies with soil temperature and moisture regime can range from levels below detection limits in arid ecosystems to over 1 billion per gram in soils with high moisture contents (Williamson et al., 2017). However, the frequency of these microbes within the soil is underestimated and the function still poorly understood.

6.3.1. Archaea

Until relatively recently archaea were still thought of as a type of bacteria, until with the advent of genomic classification the scientific community realised that these microbes although similar, have some different characteristics and were first reclassified a separate group in 1977 (Schleifer, 2009). Since then scientists have discovered that some archaea are linked to C assimilation through the oxidation of ammonia, although the precise role of these microbes in this process is still not completely understood (Pratscher et al., 2011). DNA analysis of soil micro-organisms suggests that archaea frequency in agricultural soils is higher than that of bacteria and that they are active at greater soil depths than bacteria (Leininger, 2006). Archaea found specifically in temperate forest soils been understudied but there appears to be a link between archaea diversity and low pH soils (Bates et al., 2011). It is also known that Achaea colonise fungal fruiting bodies and tree root which have symbiotic mycorrhizal associations, with populations found to be both more diverse and frequent than on non-mycorrhizal tree roots (Bomberg and Timonen 2009).

6.3.2. Protista

Recent advances in protistology have discovered that protists are far more frequent and diverse in soils than thought (Adl and Gupta, 2006). Many members of these group were previously classified as either protozoa or algae. It is thought that this group through its various functions within the soil are important for nutrient cycling particularly the bacterivores although many other functional traits are present and not fully understood or quantified. Forest soils contain 10^4-10^7active protists per gram but these numbers have been found to fluctuate rapidly due to changes in environmental conditions such as availability of food, moisture and temperature (Adl and Coleman, 2005). During periods of less favourable conditions the protists form cysts which remain dormant until conditions alter but at any time both forms of protists will be present in the soil (Bamforth, 2001). The importance of protest biodiversity to nutrient cycling and decomposition is an area which will need further experimental investigation before being fully understood.

6.3.3. Viruses

Viruses are extremely abundant within soil (10^8 virus particles/g of soil) and are very poorly studied and therefore classified (Reavy et al., 2014). The diversity of soil viruses is now understood to be far higher than previously thought and virus species are thought to exceed all other cellular organisms by at least an order of magnitude (Casas and Rohwer, 2007). This amount of viral diversity within the soil is likely to affect plant growth and exudation in a number of unknown ways (Reavy et al., 2014). Plant viruses will directly affect the plants processes, but viruses also alter the lifecycle of other soil micro-organisms including bacteria and fungi which facilitate nutrients cycling including C.

6.4. Plant-fungal symbiosis

Plant: fungal symbiosis is now widely accepted to be a fundamental process which enabled the earliest rootless plants to adapt to the terrestrial environment (Simon et al., 1993; Heckman et al., 2001; Wang et al., 2010). Early terrestrial pioneers would have faced inhospitable conditions when they first left the water, with little organic matter, no soil formation and rocky mineral substrate. Fossilised plant remains have recently given us conclusive evidence that these early plants did benefit from fungal symbiosis (Remy et al., 1994; Redecker et al., 2000). Primitive plants alive today such as liverworts and ferns have recently been shown through novel genetic analysis to have symbiosis, not with arbuscular mycorrhizal fungi of the Glomeromycota, but with Endogone-like fungi known as Mucoromycotina and it seems likely that members of this group are responsible for colonisation of the land by plants (Bidartondo et al., 2011).

Fungi comprise a large percentage of soils biomass and are the most important microbial group in regulating biochemical cycles (Bailey et al., 2002; Buée et al., 2009). Some research suggests that fungal C storage is up to 26 times more than that of soil bacteria (Suberkropp and Weyers, 1996). However only a very small percentage of the estimated 1.5 million species of fungi have been described (Hawksworth, 2001) and the functional roles they play within nutrient cycles is still not resolved (McGuire and Treseder, 2010). Fungi have hyphal networks which enable them to colonise the upper leaf litter layers to which other forms of soil micro-organisms have little access (Holland and Coleman, 1987). The composition of fungal cell walls is comprised of chitin and melanin polymers, which are resistant to decomposition (Guggenberger et al., 1999), this means C stored by fungi tends to be more recalcitrant (Bailey et al., 2002) than forms sequestered by other microbial groups.

Fungal symbiotic associations are essential components of healthy forest ecosystems (Stamets, 2005). Symbiotic associations have long been known to increase plant growth by allowing the exchange of nutrients between the plants and fungal hyphae (Durrell et al., 1994). The photosynthetic rates of plants with mycorrhizal associations is increased to compensate for the loss of C to the rhizosphere (Dosskey et al., 1990; Rousseau and Reid, 1990). The carbohydrates are transported from the organs of photosynthesis (leaves) through branches and stems, then to root tissue where they are traded with fungi. In return the plants benefit from the far higher surface area and absorbance capacity for both essential nutrients, trace elements and water (Harrison, 2005). Forest belowground micro-organisms are often C limited (Grayston et al., 1998), whereas tree growth is often limited by nutrient availability (Krause et al., 1982). This is due in part to a lack of nutrient heterogeneity in uncultivated forest soils (Millard, 1996). Mycorrhizal fungal mycelium improves both nutrient solubility and spatial accessibility through their extensive hyphal networks (Sparling, 1994, Dighton and Mason, 1985). The fungal networks promote favourable conditions for beneficial bacteria to help the trees regulate nitrogen (Tornberg et al., 2003) but the bacteria are phosphorus limited (Sundareshwar et al., 2003) and rely on the fungus to supply them with that phosphorus (Stamets, 2005). Many plant species are unable to uptake phosphorus directly, but the mycorrhizal fungal mycelium can and supplies it to the plants (Li et al., 2006).

The interactions between the fungal networks and the plant roots within the rhizosphere are extremely complicated and involve transport through the soil as well as across root and hyphal interfaces (Leadley et al., 1997; Tinker and Nye, 2000). It has also been found that trees with mycorrhizal associations are more resistant to drought (Lehto, 1992), salt damage (Porcel et al., 2012), infectious diseases (Pozo, 2007) and other forms of toxicity (Tam, 1995). Within the forestry sector it is common practice for nursery stock to be inoculated with generic soil micro-organisms, known as bio-inoculants, although this may be beneficial, more research is needed to inoculate with the correct fungal species for each tree species, suitable for the site conditions and location (Trappe, 1977). Research suggests that mycorrhizae are significant drivers of mineral weathering (Thorley et al., 2015). Both ectomycorrhizal and arbuscular mycorrhizal have been found to increase soil rock weathering rates (Koele et al., 2014), with particular rock types being favoured by the fungus (Leake et al., 2008; Quirk et al., 2012). In order to capitalise on this beneficial function of mycorrhizal symbiosis a newly emerging science known as mycoforestry is attempting to utilise beneficial associations to maximise forest productivity and assist with habitat restoration projects (Stamets, 2005). Traditional

forestry ground preparation techniques such as tillage are far more destructive to the soil fungal communities than other groups of soil micro-organisms (Frey et al., 1999) and mycoforestry management techniques would avoid some traditional soil preparation techniques (Philips, 2017).

6.4.1 Hyphal turnover

Hyphal shedding and turnover are processes which result in major belowground C fluxes (Simard et al., 2015), in fact belowground inputs are adding more C to the soil than leaf litter (Godbold et al., 2006; Clemmensen et al., 2013) at least in part due to the dominance of saprophytic fungi in the upper soil profile (Hatton et al., 2015). Turnover rates are estimated to be 46 days for hyphae, 11 months for rhizomorphs and between 1 and 6 years for the plant-mycorrhizal interface at hyphal tips (Bledsoe et al., 2014). Experimentally hyphal turnover has been measured using mini-rhizotrons, in-growth mesh bags and cores (Wallander et al., 2013). In growth bag experiments seem to suggest a turnover rate of 0.1 year which equates to a 10-year residency (Wallender et al., 2004). When compared to the root turnover rates this seems a long turnover time suggesting the methodology may be underestimating these rates (Hendricks et al., 2006). The fact that differing parts of the mycelium might turnover at differing rates confounds accurate quantification (Ekblad et al., 2013). Mini-rhizotron investigations have shown that rhizomorphs can persist in the soil for up to 22 months (Vargas and Allen, 2008), whereas it is thought that single hyphae turnover is far quicker (Pritchard et al., 2008). We know little about fungal decomposition, but it is likely to take place via three pathways; autolysis whereby the material is reused, through normal saprophytic decomposition and soil faunal grazing (Pass and Szucsich, 2011). There is an increasing amount of evidence that hyphal turnover is an important player in the forest belowground soil C budget, but limited field experiments have been conducted to confirm this (Ekblad et al., 2013).

6.4.2. Plant: fungal symbiosis temporal dynamics

Succession is defined as `a directional change in the composition, relative abundance and spatial pattern of species comprising communities' (Frankland, 1992). This process occurs within fungal communities as well as plants and fungi replace each other as the environmental conditions alter over time (Frankland, 1998). Fungal succession can be divided into two types, seral succession and substratum succession.

6.4.2.1. Seral Succession

Seral succession is driven by the dominant species of plant and as the plants move through the successional process so then do the fungal associations. This has been observed in field experiments in temperate forests and as the trees develop in age so then do the mycorrhizal fungal communities (Dighton and Mason, 1985).

6.4.2.2. Substratum succession

Substratum succession is the process of succession when driven by changes in the substrate. Initially pathogenic fungi will colonise leaf litter, followed by primary saprophytes and finally decomposers of the most recalcitrant material within the leaf litter (Frankland, 1998).

In reality all these fungal succession processes are occurring simultaneously, and generally fungal species diversity increases until canopy closure and then decreases as litter increases in nitrogen content (Fleming, 1984). The presence or absence of the mycelium can in some cases only be determined non-destructively by identifying the fruiting bodies and as many species fruit irregularly, this becomes difficult to quantify (Frankland, 1998). It appears that in early forest successional stages generalist pioneer mycorrhizae that have associations with more than one tree species are present. Overtime and into the later successional stages more specialised species-specific mycorrhizae colonise and dominate the rhizosphere (Twieg et al., 2007) and are more diverse in number than the pioneer fungi (Hart et al., 2014). It has been found that the practice of clear-cutting alters the soil condition sufficiently to require re-colonisation by species of mycorrhizae not present before harvesting (Jones et al., 2003). Understanding this and alleviating the problem by inoculation after clearcutting could help to reduce some of the post-harvest soil C loss (Heinonsalo and Sen, 2007).

6.4.3. Non-mycorrhizal fungal symbiosis

Approximately 10 % of plants are non-mycorrhizal, given the importance of mycorrhizal associations for plant nutrition, this poses the question; how do these non-mycorrhizal plants supply themselves with sufficient nutrients without them? Brassicaceae have lost the ability to form meaningful relationships with mycorrhizae, however some members of his plant group successfully survive in extremely nutrient limited soils. Almario et al., (2017), analysed the root microbiome of *Arabis alpine* (Brassicaceae), a non-mycorrhizal alpine herb, that grows in extremely impoverished rocky mountain soil, in an attempt to answer this question. The results consistently showed 15 fungal taxa, including Helotiales taxon. These were found to penetrate

the roots of the plants and supply them with phosphorus, in a very similar way to mycorrhizae (Almario et al., 2017). The Helotiades order of fungi are usually associated with ericoid symbiosis (Van der Heijden et al.,2017).

6.4.4. Mycorrhizal symbiosis

The term mycorrhizae was first coined in 1885 by the German plant pathologist A. B. Frank after he had been commissioned by the then King of Prussia (Frank, 2005 (an English translation of AB Frank's classic paper of 1885)) to investigate the possibility of commercial truffle cultivation. He discovered that the truffles formed symbiotic associations with certain tree species. Mycorrhizae literally translated means "fungus root" and is now a term used to describe the symbiotic, usually mutualistic association between plant and fungi (Frank, 2005). Since the late 19th century we have discovered many more types of mycorrhizae and they are now generally accepted as being an important component of all global ecosystems with approximately 95 % of all known plants benefitting from this symbiotic relationship (Mehrotra, 2005).

Recent intergenomic pylogenetic studies have suggested that mycorrhizal fungal species have evolved polyphyletically from a range of saprophytes at several different times (Venturini and Delledonne, 2015; Hibbett and Matheny, 2009). The first wave is thought to have developed alongside primitive terrestrial plants over 450 million years ago (Brundrett and Tedersoo, 2018; Redecker et al., 2013) these early mycorrhizae evolved to become arbuscular mycorrhizal fungi. Due to limited specimens and major gaps in fossil records it remains unclear if the first plants on land developed mycorrhizal associations or non-mycorrhizal associations, although it is known that plant genes associated with fungal symbiosis pre-date the first land plants (Delaux et al., 2015; Martin et al., 2017).

Mycorrhizal symbiotic relationships are based on mutual trading of resources where-by the tree (photobiont) provides the fungus (mycobionts) with C and the fungal hyphae provide nutrients in return (Buscot, 2015) normally nitrogen and phosphorus (Smith and Read, 2010). The mycobiant has access to a greater volume of soil due to their far greater surface area and smaller diameter allowing them to can gain access to a larger area of soil from which to acquire nutrients (Tinker et al., 1994; Philips, 2017). The fungal hyphae in some cases are involved in the mobilization of nutrients that would otherwise be locked into soil organic complexes (Read and Perez-Moreno, 2004) and also by direct mineral weathering (Landeweert et al., 2001). The nutrients obtained are significant, with some plants obtaining up to 80 % of their nitrogen and

phosphorus from their mycorrhizal symbionts (Van der Heijden et al., 2015). Mycorrhizal networks can also move water bi-directionally, this type of transport tends to be diurnal. During the day the water is moved from the soil to the transpiring plants and then during the night moved to the driest parts of the soil matrix (Egerton-Warburton et al., 2007; Querejeta et al., 2003), thus buffering mycorrhizal plants from the effects of drought.

The two main types of mycorrhizae are characterised and differentiated by the type of plant-fungal interface present, the endomycorrhizae penetrate the roots cell wall (Allen, 1991) whereas ectomycorrhizal hyphae do not (Smith and Read, 2010). Although mycorrhizal networks occur most frequently in temperate and boreal ecosystems (Roy et al., 2008; Beiler et al., 2010), they are also found in tropical forests (Onguene and Kuyper, 2002; Mangan et al., 2010) Mediterranean forests and chaparral (Richard et al., 2005; Tedersoo et al., 2008), arctic tundra (Deslippe and Simard, 2011), grasslands (Gai et al., 2009) and woodland savannah (Dickie et al., 2004). Tedersoo et al., (2014) found that the diversity of fungi belowground in forests was inversely proportional to the aboveground diversity. In the tropics where, aboveground plant diversity is high, belowground fungal diversity is relatively low and generally as one moves away from the equator the diversity of mycorrhizal fungi increases (Wardle and Lindahl, 2014). During the early stages of plant root fungal colonisation, metabolic changes in the plant, which allow successful colonisation, are triggered by the presence of the fungi (Harrison, 2005). By priming a plants defence hormone, mycorrhizas can increase a plants resistance to a range of perturbation including nematodes (De La Peña et al., 2006), pathogens (Pozo et al., 2009; Whipps, 2004) as well as abiotic disturbances (Smith and Read, 2010). Mycorrhizal fungi can also attract beneficial insects by stimulating the production of volatiles (Babikova et al., 2014) which then predate on pests, both herbivores (Schausberger et al., 2012) and parasites (Guerrieri et al., 2004).

Dependent on the species of plant involved between 10 and 15 % of the below-ground C is respired by roots and 15- 25 % is exuded from the roots to the soil (Kuzyakov, 2002). Estimates of the total C transferred belowground to mycorrhizal fungi varies greatly. Wallander et al., (2001), estimated that between 700 to 900 kg of C per hectare of forest is transferred to ectomycorrhizae in the Silvåkra forest, in south-west Sweden, a Scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* (L.) Karst.) forest. Whereas studies that investigated the extent of C transfer to arbuscular mycorrhizae in laboratory conditions estimate that anything from 4-14 % of the C fixed by the plant is diverted to the fungi symbiont (Durall et al., 1994, Harris and Paul, 1987, Lambers 1987; Smith and Gianinazzi-Pearson, 1988). Ectomycorrhizae

are also understood to be a major contributor to soil C and are mediators of C fluxes within forest soils (Hogberg and Hogberg, 2002; Godbold et al., 2006; Hobbie, 2006). Ectomycorrhizal studies report anywhere from 6- 36 % of fixed C being allocated to the rhizosphere in laboratory experiments (Reid, Kidd and Ekwebelum, 1983) and in field studies estimates suggest that the ectomycorrhizal fungi consume in the region of 15 % of the photosynthetically fixed C (Vogt et al., 1982; Fogel and Hunt, 1983). Mycelial fins of mycorrhizae can develop and die within weeks (Finlay and Read, 1986; Bending and Read, 1995), this suggests a rapid mycorrhizal mycelium turnover rate (Smith and Read, 2010).

In short fungi are the most important group of micro-organisms for mediating C cycling and for long term belowground C sequestration (Treseder and Holden, 2013). Mycorrhizal C trading is now considered to be of greater importance than that of the saprophytic influence in the breakdown of litter (Clemmensen et al., 2013) and the fungi aid C sequestration by stimulating plant growth, decomposition and deposition. Managing a forest to be favourable to the colonisation of fungal diversity will be beneficial to both the productivity of the forest stand and its belowground C storage (Bailey et al., 2002; Stamets, 2005).

The following section will try to describe the phylogenetics of mycorrhizal fungi, this is an area of taxonomy currently in development aided by the advent of DNA sequencing technology (Shenoy et al., 2007). Recent developments such as Loron et al.'s (2019) discovery of fungal microfossils which appear to indicate that fungi evolved more than half a billion years before previously thought, suggesting that our phylogenetic fungal trees are not yet legitimate.

6.4.4.1. Endomycorrhizae

Endomycorrhizae are a large group of mycorrhizal fungi that have evolved the ability to penetrate the root cells which allows for a less inhibited flow of nutrients and C between the two organisms (Bonfante and Genre, 2010). Types of endomycorrhizae include: arbuscular, vesicular arbuscular mycorrhiza (VAM), ericoid, arbutoid, monotropoid, and orchid ectendomycorrhizas.

6.4.4.1.1. Vesicular arbuscular mycorrhizae

The term vesicular arbuscular mycorrhizal (VAM) associations was used to describe mycorrhizal fungi Glomeromycota. It was then discovered that many members of this taxonomic group do not actually have vesicles and the term was modified to become arbuscular

mycorrhizae (AM). Although arbuscular mycorrhizae are now the accepted term for this group of fungi, some members of the group do not form arbuscles either (Smith and Read, 2010).

6.4.4.1.2. Arbuscular mycorrhizae

The most frequent form of mycorrhizal association is arbuscular, this category accounts for 72 % of all mycorrhizal associations (Brundrett and Tedersoo, 2018). These associations are formed by a single group of fungi known as Glomeromycota (Holmer et al., 2017), which contains an estimated 1000 species (Redecker et al., 2013). Arbuscular mycorrhizae colonise the roots by penetrating the root with their hyphae and producing structures known as vesicles or arbuscles within the root cortex cells (Anderson and Cairney, 2007). These structures increase the contact surface area between the hyphae and the cell cytoplasm allowing efficient nutrient transfer (Peterson et al., 2004). Some have even postured that this symbiosis is so integrated that it should be considered a superorganism (Azco´n-Aguilar and Bago, 1994) with the whole being greater than the sum of the parts (Jakobsen, 1995).

It has been hypothesised that an arbuscular mycorrhiza produces a glycoprotein referred to as Glomalin, which was discovered in 1996 (Wright and Upadhyaya, 1996.). It was believed that this sticky substance, changes the soil structural properties by binding particles together. It was widely accepted to be a major driver of long-term C storage in soils containing arbuscular fungi as it consists of between 30- 40 % C and persists in the soils for an estimated 7- 42 years, depending on soil temperature and moisture (Wright and Nichols, 2002). As a result of recalcitrance of the molecule it is estimated that it accounts for up to 27 % of stored soil C (Wright and Nichols, 2002). It could be of particular interest to modelling future C storage under increasing atmospheric C dioxide concentrations as experimentation has shown hyphae are longer and produce up to five times more glomalin that during current C dioxide concentrations (Rillig et al., 1999). However more recent studies (Rillig et al., 2004) have doubted the reliability of glomalin assays and glomalin has never been positively isolated, leading to the now less specific term glomalin-related soil protein (GRSP) (Singh et al., 2013). Although it was accepted that glomalin was an arbuscular mycorrhizal metabolite no conclusive evidence to support this has yet been published (Vlček and Pohanka, 2019).

6.4.4.1.3. Ericoid

Ericoid mycorrhizal symbiosis is thought to have evolved 140 million years ago (Cullings, 1996) and facilitated the *Ericaceae* colonising the acidic impoverished soil, that they are normally associated with, such as heathlands, bogs and boreal forests (Cairney and Meharg,

2003). The fungi involved in this form of mycorrhizal association are the Ascomycota and they have evolved the ability to supply their photobiont with both phosphorous and nitrogen despite the harsh acidic nutritionally poor soil they inhabit (Smith and Read, 2010). These specialised fungi can mediate the acquisition by the plant of metal such as iron, aluminium and manganese which are often occur in their highly plant-available forms in acidic soils (Meharg and Cairney, 1999). Rather than forming arbuscules, ericoid mycorrhizae colonise root hair epidermal cells using coil-like hyphal structures that can penetrate the walls of cortical cells, but not the plasma membranes (Smith and Read, 2010). The coil acts as the plant-microbe interface allowing nutrient transfer from one to the other. A colonised Ericaceae root can be up to 80 % fungi by volume and ericoid mycorrhizal hyphae have been found to release enzymes that allow the uptake of, otherwise unavailable, complex organic molecules, a semi-saprophytic characteristic (Cairney and Burke, 1998; Read et al., 2004). The importance and extent of this form of mycorrhizal symbiosis is not fully established as the identification is problematic (Smith and Read, 2010) although novel genetic analysis of the ericoid rhizosphere microbiome is beginning to suggest host specificity and geographical range of ericoid mycorrhizae (Walker et al., 2011).

6.4.4.1.4. Arbutoid

Plants that associate with arbutoid fungi are from both the Arbutus (twelve species of flowering Ericaceae plants) and Arctostaphylos (sixty species of small flowering shrubs from the genera Ericaceae), which includes evergreen shrub species such as strawberry tree (*Arbutus unedo*), madrone (*Arbutus menziesii),* bearberry (Arctostaphylos sp.) and manzanita (Arctostaphylos sp.) (Moore et al., 2011). Arbutoid fungi are a type of ectendomycorrhizae, more closely related to ectomycorrhizal, as they are also basidiomycetes and both share the feature of possessing a mantle, or sheath, which covers the host plant root (Smith and Read, 2010). The feature that distinguishes them from the first type of ectendomycorrhizae is that they penetrate the epidermal and not the cortical cells. The fungi also form a structure similar to a Hartig net and can use its sheath to store nutrients for release to the plant during lean times (Smith and Read, 2010). The most significant difference between ecto and arbutoid mycorrhizae are that the arbutoid hyphae enter the plants roots cortical cells.

6.4.4.1.5. Orchid mycorrhizas

Orchid mycorrhizal associations are found on plants of the *orchidaceae* which contains over 22,000 species, making it the largest group of flowering plants, although most are found in

tropical or sub-tropical environments rather than temperate forests (Moore et al., 2011). Unusually all orchids have a stage of their lifecycle where they cannot photosynthesise and as a result during this protocorm phase rely solely on their mycorrhizal association to supply them with C the association is therefore an obligate (for the plant but not the fungi) and without a suitable fungal partner is available seed germination and subsequent growth during the achlorophyllous stage will not occur (McCormick et al., 2018). We know from over a century's research that orchids can form relationships with an ecologically and taxonomically large range of fungi, which can include ectomycorrhizae, saprophytic fungi, root endophytes and fungi normally classified as pathogenic (Dearnaley et al., 2012).

6.4.5. Ectendomycorrhizas

Mycorrhizas which exhibit many of the features associated with ectomycorrhizal fungi but also show high levels of intracellular hyphal penetration of the epidermal and cortical cells of the plant roots (Smith and Read, 2010) and have dark septate hyphae which are mildly pathogenic to their plant hosts. Fungi that are ectendomycorrhizas are from the basidiomycetes the fungi normally associated with trees in ectomycorrhizal symbiosis. Although the ectendomycorrhizas are classified as endomycorrhizas, in evolutionary terms they are somewhere between endo and ectomycorrhizal (Moore et al., 2011).

6.4.6. Endophytic fungi

Endophytic fungi are fungi which are specialised in growing within the leaves, meristems, roots and reproductive tissues of plants (Rodriguez et al., 2009)**.** The colonisation of the plants tissues by these fungi has been found to dramatically improve the plants chances of survival as a result of the secondary metabolites which the fungi produce which can protect the host from herbivory and aid nutrient acquisition particularly under poor light regimes (Davitt et al., 2010). Endophytic fungi are members of a broad range of fungal taxa including both Ascomycota and Basidiomycota (Unterseher, 2011) and are reliant on the photobiont to supply them with C (Miliute, 2015). They colonise there hosts in two distinctly difference routes, the first is known as vertical transmission whereby they pass a clone of themselves from parent to offspring when a hypha penetrates the embryo in the seed (Suryanarayanan, 2013). The second horizontal transmission takes place when sexually reproduced spores are passed from one individual to another (Tadych, 2014). In temperate forest ecosystems approximately one hundred of over 1000 tree species have been investigated to establish if they had endophytic symbiosis all trees studied were found to have endophytes and many had multiple simultaneous colonisations

(Unterseher, 2011). Estimates of the amount of the total global species range from 500,000 (Sieber, 2007) or 1 million **(**Deshmukh et al., 2015) to 1.3 Mio endophytic fungi species (Dreyfuss and Chapela, 1994). Endophytic fungal hosts demonstrate no ill-health symptoms, however on a molecular level the plants are responding to the colonisation (Johnson et al., 2006) an assumption of mutualism prevails as non-endophytic trees are non-existent, so a controlled experiment is impossible (Sieber, 2007). The extent of the importance of this type of symbiotic relationship on C cycling is far from clear although it is known that many species will switch to saprophytic after the host plant senesces (Porras-Alfaro and Bayman, 2011). Their ability to prime the decomposition of recalcitrant C within the soil matrix means they are significant in belowground C cycling (Dai et al., 2010).

6.4.7 Epiphytes

Epiphytic fungi are those species that live on the surface of (in this case) a tree or plant these tend to be primitive fungi such as yeasts and are unlikely to have a significant role in temperate forest ecosystems. Lichens will not be considered in this review as although they are symbioses they are not plant: microbe, rather microbe: microbe symbiosis.

6.4.8. Ectomycorrhizae

Ectomycorrhizal fungi (EMF) are characterised by the presence of a Hartig net and a mycelial mantle which forms around the host's lateral roots and occupy a space between the epidermal and cortical cells (Peterson et al., 2004). In evolutionary terms ectomycorrhizal fungi are the most advanced and have subsequently been around for the least amount of time (Brundrett and Tedersoo, 2018). Ectomycorrhizae are however the least abundant of all mycorrhizal fungi with only 2 % being part of the ectomycorrhizal category (Brundrett and Tedersoo, 2018). The majority of ectomycorrhizal associations are between a mycobiant and a tree species that are found in temperate, boreal and Mediterranean forests rather than tropical (Gerz et al., 2016; Soudzilovskaia et al., 2017; Brundrett and Tedersoo, 2018). Ectomycorrhizae are economically important in terms of supplying many important timber tree species with additional nutrition as well as some of the fungi themselves being valuable such as the truffles and porcini (Mello et al., 2015). Ectomycorrhizal fungi secrete a cocktail of enzymes which can mobilise nitrogen from the soil organic matter, AM fungi do not. As a result of this the EM fungi are able to acquire more nitrogen per surface area than the AM fungi (Averill et al., 2014). A theoretical model has predicted that soil with ectomycorrhizal fungi can store more C due to the uptake of

N decreasing rates of decomposition by limiting free-living decomposers, (Orwin et al., 2011) although no experimental evidence for this currently exists (Averill et al., 2014).

EMF consist of a large range of taxonomic groups the majority are basidiomycetes with fewer being part of the ascomycetes (Smith and Read, 2010), this variety leads to diverse morphological characteristics in colonisation techniques (Agerer, 1987-2002). There are over 20,000 known fungal ectomycorrhizal partners that form relationships with 6,000 trees including pines, beeches, oaks, eucalypts, dipterocarps and poplars (Brundrett, 2002; Van der Heijden, 2015; Martin et al., 2016). Studies comparing nutrient cycling in ectomycorrhizal fungi suggests that these fungi per unit of soil divert significantly more C below-ground than arbuscular mycorrhizae (Averill et al., 2014) particular in temperate forest ecosystems.

In addition to the structures that form in the rhizosphere during colonisation, the ectomycorrhizal fungi also form mycelia which grow into the bulk soil, the structure of these extramatrical mycelium also differs greater between the different taxonomic groups (Agerer, 2001). It is through these extrametrical mycelia that the fungal symbiont is able to forage for and transfer extra nutrients and water than the tree root alone would have access to and also in the cases of common mycorrhizal networks to transfer carbohydrates between trees (Selosse et al., 2006; Simard and Durall, 2004).

6.4.9. Monotropoid

Monotropoid mycorrhizal associations are unusual as they associate with plants with 10 genera of plants which are entirely achlorophyllous and are therefore unable to photosynthesise and produce their own carbohydrates. These plants not only rely on the mycorrhizas for minerals and nutrients as with other mycorrhizal plants, but use common mycorrhizal networks to utilise C supplies from nearby plants. This is therefore considered a parasitic symbiotic relationship and the plant is referred to as mycoheterotrophs (Moore et al., 2011). This type of mycorrhizal association is native in temperate conifer forests and are using common mycorrhizal networks to gain C photosynthesised by common temperate trees such as pine, spruce and fir species. Monotropoid mycorrhizae have many similar features to the arbutoid and until recently were considered part of the same group, when it was realised that there some fundamental morphological differences i.e. that the hyphae do not penetrate the cells (Smith and Read, 2010).

7. Common mycorrhizal networks (CMN)

Mycorrhizal infections are thought to take place when an uninfected root meets a mycelial hypha of a fungi which is already connected to a plant (Read et al., 1976) the mycorrhizae is therefore not reliant on a single plant species or individual to supply them with carbohydrates (Bücking, et al., 2016). Generally, mycorrhizae are not species specific and as a result this mycelial inter-tree connection can be either inter or intraspecific (Selosse et al., 2006) and are commonly referred to as the Wood Wide Web (Beiler et al., 2010). The mycorrhizae by connecting to the plants roots directly can guarantee a share of the photosynthetically fixed C which gives them a competitive advantage over those microbes that are free-living in the soil (Simard et al., 2015). The networks can include many plants with differing traits or various ages the magnitude and direction of the resource fluxes are mediated by the plant and fungal network (Simard et al., 2015).

7.1. Transfer between mycorrhizal plants via CMN

In the real world common mycorrhizal networks tend to be highly complex, often with more than one fungal species as well as more than one tree species (Beiler et al., 2010; Bahram et al., 2011; Horton and Bruns, 2001; Taylor, 2002; Dickie et al., 2004; Nara, 2006; Tweig et al., 2007). This makes the job of disentangling what has been transferred by whom an insurmountable problem with current methodologies (Simard et al., 2015). However, it is accepted that transfer of elements and molecules between multiple plants through mycorrhizal networks does occur and is distinct from transport through rhizomes and root grafts (Fraser et al., 2006; Philip et al., 2010). Indirect uptake of root exudates from the soil water and subsequent transfer through the hyphae is also taking place simultaneously (Perry et al., 1989; Rillig and Mummery; 2006; Philip et al., 2010; Jones et al., 2009).The elements and molecules that we know can and are transported via CMN are currently limited, but include lipids (Bago et al., 2002), amino acids (Jin et al., 2005), C (Francis and Read, 1984; Brownlee et al., 1983; Finlay and Read, 1986), nitrogen (Arnebrant et al., 1993; Bethlenfalvay et al., 1991), phosphorus (Newman and Eason, 1993; Whittingham and Read, 1982), water (Egerton-Warburton et al., 2007; Plamboeck et al., 2007; Querejeta et al., 2003) and other more nutrient analogues (rubidium, arsenic and caesium) (Meding and Zasoski, 2008; Gyuricza et al., 2010) as well as genetic material (Giovannetti et al., 2004; Giovannetti et al., 2006). Phosphorus transfer studies have been less frequent than nitrogen with only a small number looking at

either arbuscular mycorrhizae (Eason and Newman, 1990; Eissenstat, 1990; Wilson et al, 2006) or ectomycorrhizal networks (Finlay and Read, 1986; Perry et al., 1989).

Studies have shown that transfer of C between plants can take place via arbuscular (Hirrel, 1979; Francis and Read, 1984; Grime et al., 1987) or ectomycorrhizal (Brownlee et al., 1983; Simard et al., 1997) fungal symbioses. In arbuscular mycorrhizae however, C is not transferred from one plant to another the C remains in the roots and is not redistributed to the growing aboveground tips (Robinson and Fitter, 1999). The discovery that transfer of C, water and nutrients can be transferred between plants via CMN has been known for over five decades, but it has been found to be hard to quantify (Simard et al., 2015). Initially studies showed that plant seedlings could transfer C via common mycorrhizal networks (CMN) (Simard et al., 1997), but since then transfer has been shown to take place from mature trees to seedlings (Beiler et al., 2010) and between mature trees (Klein et al., 2016). Controversy remains however as experimentation has failed to date top prove net transfer via common arbuscular mycorrhizal networks (Francis and Read, 1984). Klein et al., estimate that transfer between trees via CMN can account for 280 kg of C per hectare, approximately equivalent to 4 % of the forests net primary production, although some of this may be C recaptured from exudation or root and hyphal turnover (Jones et al., 2009). C fluxes via CMN can account for up to 10 % for autotrophic and up to 85 % partially myco-heterotrophic and 100 % for fully myco-heterotrophic plants (Simard et al., 2015).

7.2. Pathways for C transfer

The precise mechanisms by which the resource transfer can take place are not understood (Simard et al., 2015), this problem is compounded by the fact that the mechanisms are likely to be different between plant species, type (deciduous or evergreen/ broadleaf or confer) and mycobiont specific (Agerer, 2001). The literature does suggest that transfer generally takes place along source-sink, concentration gradients or energy potential gradients and the resource is then transported to the area in the plant where growth is occurring (Simard et al., 2015).

7.3. Signalling via CMN

Communication between plants through volatile compounds beings released into the air and then “smelt” by their neighbours is now well established (Balwin and Schultz, 1983; Rhodes, 1983) and the ecological implications are well understood (Heil and Karban, 2010). Since the discovery of common mycorrhizal networks there has been interest in the possibility that CMN are also being used for communication between plants (Johnson and Gilbert, 2015), but the

extent and ecological implications of this is not yet clear. Many volatiles that are released during aerial communication are systemic and therefore found throughout the plant and potentially released belowground simultaneously (Chamberlain et al., 2001). More recently experimentation has revealed that these neighbouring plants produced a defence compound when connected via CMN to a plant under attack by herbivores (Babikova et al., 2013; Song et al., 2014) and pathogenic fungi (Song et al., 2010). We currently do not know if these chemicals are fungal species or stimuli specific (Johnson and Gilbert, 2015).

7.3.1. Allelopathy

The transfer of communication chemicals which have a positive outcome for both plants are in the majority however, allelopathic chemicals are also transferred through common networks (Barto et al., 2011). Allelopathic chemicals are released by some plants into the rhizosphere and give the releasing plant a competitive advantage by altering the soil chemistry to favour itself or negatively impact the competitors (Cipollini et al., 2012). One much studied temperate forest example of this is black walnut (*Juglans nigra* L.) (Willis, 2000), which famously produces the allelopathic aromatic phytotoxic compound juglone (5-hydroxy-1, 4 naphthoquinone) (Rice, 1974), the result of which is reduced plant competition around the walnut tree (Jose, 2002). It has been understood for some time that common mycorrhizal networks can act like 'superhighways' extending the region of influence of these allelopathic compounds in the soil (Barto et al., 2012). Arbuscular common mycorrhizal networks have experimentally been shown to not only increase the bioactive zone but also to increase the effectiveness of the compound by mediating the chemical interaction processes (Achatz et al., 2014). The subject is highly complex with research reporting that allelopathic compounds inhibit (Souto et al., 2000) or stimulate mycorrhizal hyphal growth (Rose et al., 1983). Some species of ectomycorrhizal fungi are intolerant to allelopathic compounds, a phenomenon in the UK known as 'heather check' is well documented and occurs as a result of allelopathic compounds exuded by heather roots. These compounds severely inhibit the fungi normally associated with Sitka spruce (*Picea stichensis*), leading in some cases severe chlorosis of the spruce trees (Taylor and Tabbush, 1990), It has also been reported that arbuscular mycorrhizae can protect native tree species from the effect of the allelopathic chemical released by invasive non-native plant species (Barto et al., 2010). This area of science would benefit from more research being undertaken to but what is clear is that these plants: fungal symbioses have significant implications for plant communities by altering interspecies chemical interactions (Barto et al., 2011).

7.4. Potential pathways for signalling chemicals

The physiological mechanisms and pathways of many of the signalling compounds are still not well understood. It is hypothesised that C and nutrients are transferred both apoplastically and symplastically across a source-sink gradient (Simard et al., 2015) and there are the following speculations as to the potential pathways that these chemicals could take

a) Hyphae have the ability to modify the rhizosphere and it is possible that this modification could increase the soils potential as a transport pathway by either altering aggregation, electrical conductivity or by manipulating the microbial community. Signally speed through this potential pathway is likely to be slow however due to the increased path length and resistance (Hillel, 1998).

b) The chemical could be transported either passively or actively through hyphal cell membranes and into the cytoplasm. This pathway seems improbable as the cell membranes are impermeable and hydrophobic. It transports through the membrane were to occur it would need transport mechanism, such as a membrane transporter chemical and these have not yet been observed in hyphae (Barto et al., 2012).

c) Chemical transport could be apoplastic, although due to the hydrophobic nature of the hyphal cell walls this would likely only be possible for nonpolar chemicals (Allen, 2007).

d) Chemical signals could be transported through the water found on the hyphal surface either by microbes or capillary action, however this would only be possible in those that are water soluble and is unlikely over long distances (Johnson and Gilbert, 2015; Barto et al., 2012; Allen, 1996).

e) In some cases, mycorrhizal hyphae will curl around each other to from a rope-like structure. This structure provides the opportunity for small pockets of air or water to be trapped between the hyphal strands and allow the transport of water-soluble compounds or hydrophobic volatile chemicals respectively (Freise and Allen, 1991). The rope-like structures are not observed frequently and therefore the contribution of this pathway is unlikely to be substantial.

f) Electrical signal may also be conducted through their CMN to enable plant to plant communication (Johnson and Gilbert, 2015), this has been experimentally observed in

both artificial leaf damage (Mousavi et al., 2013) and invertebrate herbivory (Salvador-Recatala, 2014).

Potential pathway a, d and e have the problem of coming into contact with the vast array of microbial inhabitants of the soil and therefore contamination in the form biofilm growth is more likely (Toljander, et al., 2005).

8. Plants with both ecto and arbuscular mycorrhizal associations

Some species of tree can form symbiotic relationship with multiple types of symbiosis, namely both ecto and arbuscular mycorrhizal species simultaneously. These trees known by horticulturalists as bridging trees and are added to species mixtures in the hopes that the nutritional benefits of the foraging of both fungal types can be utilised (Philips, 2017). Trees with AM and EM fungi have different functional traits to those without such as root architecture related to acquisition of nutrients (Comas and Eissenstat, 2004; Guoet al., 2008; Valverde-Barrantes et al., 2015).and leaf litter chemistry (Cornelissen et al., 2001; Midgley et al., 2015; Lin et al., 2016) Temperate trees which exhibit this characteristic include alder (*Alnus* sp.), aspen (*Populus tremula*), willow (*Salex* sp.) and poplar (*Populus* sp.). Although Jack and Lindsey Hartley (1987) list more temperate species as sharing AM and EM symbiosis, including hawthorn (*Crateagus monogyna*), crab-apple (*Malus sylvestris*), wild cherry (*Prunus avium*), wild pear (*Pyrus pycaster*) and rowan (*Sorbus aucuparia*). Studies have noted that in some trees the seedlings seem to develop AM symbiosis before switching to EM partners later in their lifecycle (Adjoud-Sadadou and Halli-Hargas, 2017).

9. Potential effect of increased atmospheric C dioxide on plant microbe symbiosis

As atmospheric C dioxide concentration increases so does productivity (Bazzaz, 1990), therefore we would expect root exudation to increase. Experiments have shown an increase in allocation of C into the roots and an increase in exudation in *Pinus* seedlings (Norby et al., 1987). An increase in exudation has been shown to increase mycorrhizal biomass (Lewis et al., 1994) and density (O'Neill et al., 1987).

Increased atmospheric C dioxide concentrations and their effect on plant physiology has been studied globally using a network of FACE experiments (Norby and Zak, 2011). Increases in C dioxide has been to increase tree water use efficiency by inducing stomatal closure (Field et al., 1995; Picon et al., 1996). Plants also respond to elevated C dioxide by allocating more C to root production and turnover, which allows the roots to penetrate further into the soil and access water located deeper in the soil (Wullschleger, 2002) and can increase above ground biomass (Hoosbeek et al., 2011).

6.1. Stoichiometry

Atmospheric C dioxide levels have increased over the last century (Schimel et al., 2015) and as a result rates of photosynthesis have increased with CO_2 following a Michaelis−Menton curve (Zaehle et al., 2014) however this may become limited by nitrogen availability, due to an imbalance in stoichiometric C:N ratio (Inselsbacher and Näsholm, 2012). Most of the nitrogen found in the soil is there as a result of decomposition (Aber and Melillo, 1980) and if this process does not increase as quickly as photosynthesis, the lack of nitrogen becomes the factor which can limit the plants rate of C sequestration (Chaplin et al., 2009; Inselsbacher and Näsholm, 2012). The N availability in the soil also has a direct impact on its ability to store C (Bonan, 2008; Janssens et al., 2010). As a result of this fact C models that account for nitrogen availability often differ greatly from those that do not (Wieder, et al., 2015). Other nutrients such as potassium, tend not to be so limiting, although may become more so in future this is at least in part due to the fact that for healthy plant growth a plant will need ten times as much nitrogen as potassium (Reed et al., 2015). Nutrient deficiency can be assessed either in terms of a single species or as plant communities and in mixed species stands tree species may coexist that are limited by nitrogen and potassium differentially (DiTomasso and Aarssen, 1989; Koerselman and Meuleman, 1996), however this is not yet fully understood (Aerts and Chapin, 2000).

10. Effect of tree/ plant species on C cycling

The tree species located in a particular area of forest affects the soil organic content and therefore the C in soil due to exudation, root turnover and litter fall (Vesterdal et al., 2013). It has been found that the dominant tree species within the forest also determines the species and composition of fungal and bacterial communities within the soil (Urbanová, 2015). The effect

of tree species on microbial communities is generally accepted and experimentally supported (Grayston et al., 1998, Hackl et al., 2004, Hobbie et al., 2006, Ushio et al., 2008, Aponte et al., 2013), but not quantified (Urbanová, 2015). The dominant tree species will also determine the storage and dynamics of C in the soil, as a result of litter inputs, but also differences in exudates (Leuschner et al., 2013; Jandl et al., 2007; Hagen-Thorn et al., 2004). Differences in NPP requirements (Hansen et al., 2009), varying quality of organic matter addition to detritus (Hagen-Thorn et al., 2004), differences in depth of root penetration into the soil (Lai et al., 2016) and by influencing populations of soil fauna (Hobbie et al., 2006; Lynch et al., 2012) will all change with dominant species and result in a change in belowground C dynamics.

The tree species present within a stand can significantly alter the soil C storage capacity, yet the mechanisms involved in this are not fully understood (Vesterdal et al., 2013). It has been observed that the quality of litter can determine decomposition rates and therefore effect soil C storage (Vesterdal et al., 2008; Berg et al., 2010), however the link remains uncertain (Prescott, 2010). This will be important as tree plantations globally are increasing in area (Paquette and Messier, 2009) and species choices are changing, especially in temperate and boreal forests (Iverson et al., 2008; Garbelotto and Pautasso, 2012). Mixed species stands are increasingly being favoured by European temperate foresters (Mason and Connolly, 2013), as both climatic change risk reduction and adaptation strategies (Forrester, 2014). The driver for this has been to ensure that forests continue to supply society with ecosystems services under the pressures of changing climate and non-native pests and diseases (Quine et al., 2011). In the UK, mixed species stand tend to be more common in England than either Wales or Scotland (Smith and Gilbert, 2003). In some cases, mixed species forests will produce more biomass than the average for those species as single species stands, this is known as over-yielding. In others the mixed stand will produce more biomass than the highest yield of that species alone, this is known as transgressive over-yielding (Harper, 1977; Pretzsch, 2009).

The ability to produce far more as mixtures is dependent on the species having differing ecological niches or functional traits thus reducing interspecies competition (Loreau and Hector, 2001; Kelty, 2006; Richards and Schmidt, 2010) and on the relative growth rates and lifespan of the species differing from each other (Pretzsch, 2009). Soil organic matter also accumulates at a different rate in mixed species stands when compared to that of monocultures (Six *et al.*, 2002; Steinbeiss *et al.*, 2008). This in turn will alter the rate of turnover and detrital decomposition (King *et al.*, 2002) as well as the composition of the microbial communities (Pollierer, 2007). Increased species diversity within an area of forest increases that forest's

resilience to perturbations (Mason, 2007: Bolte *et al.*, 2009). Forests need to increase diversity in all forms; species choices, genetic diversity and provenance diversity will all improve a forests resistance and resilience to perturbations (Noss, 2001; Doim et al., 2010). Diversity within forest plant communities will determine the microbial community's productivity and diversity (Kowalchuk et al., 2002; Wardle et al., 2003).

Research suggests a relationship between plant diversity and ecosystem function (Tilman et al., 1997; Loreau, 2002). This relationship is not a linear relationship, however as some so-called keystone species have more important roles within the ecosystem and over soil functioning (Tilman et al., 1997; Leps et al., 2001, Huston 1997). Experiments using $^{13}CO_2$ can determine how much a species alters the C allocation (Ladygina and Hedlund, 2010). The differences in rates of C sequestration between species can be attributed to differences in rates of growth (Pérez-Cruzado et al., 2012), litter quality and quantity (Berg, 2000; Vesterdal et al., 2008) and type of ground cover (Lemma et al., 2006; Kasel et al., 2011) and other functional traits.

10.1. Functional traits

The functional trait of a plant is the particular ecological niche which it fulfils within an ecosystem (Mayfield et al., 2010). The range of ecosystem services a plant community provides is believed to be based on the number of functional traits within that community rather than the number of species (Díaz and Cabido, 2001; Prinzing et al., 2008) although the relationship is complicated (Naeem and Wright, 2003). Plants functional traits are a controlling factor in below-ground C storage, by controlling assimilation and transfer as well a residency time (De Deyn et al., 2008). Due to the fact that the majority of research in this field has been conducted on conifer species (Berthrong et al., 2009) the true significance of functional traits in forest trees C sequestration is not fully understood (Pérez-Cruzado et al., 2012). Species composition is more important than total species number in determining the size and residency time of the belowground C pools (Hulvey et al., 2013). Plant traits which regulate C sequestration and storage can thought of as two distinct trait types. This is either by increased primary production or allocating more C resources below-ground or by reducing C loss from processes such as respiration and leaching (De Deyn et al., 2008).

11. Conclusions

Knowledge gaps certainly exist in this field and the disconnect between those doing mycorrhizal ecological forest research and those who actually manage forests needs to be addressed in order to maximise the mitigation ability of temperate forests (Lindner et al., 2014). CMN may be far more important to the understanding of forest ecology than previous understood and is likely to determine seedling recruitment, survival and growth, mediate inter and intra specific facilitation and competition and species composition and succession (Fraser al, 2006; Teste et al., 2009; Deslippe and Simard, 2011).Threats to fungal diversity from modern agricultural techniques, habitat fragmentation and nitrogen deposition are growing and have led to a series of academic discussions on how best to manage these micro-organisms. Given the fundamental importance of plant: microbe symbiosis humanity should be concerned by the decrease in abundance and diversity of these essential components of all ecosystems and the underlying services that they provide humanity. The challenge for scientists will be to be honest about the many uncertainties whilst communicating the need for planning for possible future scenarios (Millar et al., 2007; Yousefpour et al., 2012; Spittlehouse and Stewart, 2003). The importance of rhizodeposition of recently captured C into the rhizosphere for uptake by soil microbes has historically been underestimated (Högberg and Read, 2006), as it was always assumed that the main driver of soil microbial activity was litter decomposition (Wardle et al., 2004). It is widely accepted therefore that understanding the role of temperate forests in C cycling and sequestration is essential in the science of climatic change mitigation (Mason et al., 2013). The current models of C cycling and dynamics fail to take full account of plant: microbe symbiosis (Brzostek et al., 2016). This is as a result of a lack of understanding of the mechanisms behind these processes as well as a lack of reliable quantification and data to support the addition of this to models (Cheeke et al., 2017). Only by the development of a mycorrhizal driven, trait-based model, will we begin to reliably predict how climatic changes and shifts in the processes underlying biogeochemical cycles and subsequent shifts in species range.

12. Acknowledgements

This work was funded by NERC through Envision DTP.

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Chapter 3

Carbon partitioning and temporal dynamics of three temperate tree species and associated microbial symbioses, estimated by ^{14}C pulse-labelling.

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Unpublished manuscript

Author contributions:
TDP conducted all empirical work with guidance on design, execution and data analysis from ARS and DLJ. TDP wrote the manuscript with all authors contributing to the final version.

1. Introduction

Forest ecosystems have a fundamental role in the global carbon (C) cycle and remove 30% of global anthropogenic CO_2 emissions annually (Le Quéré et al., 2016). The world's forests are not only absorbing atmospheric CO_2, but also adding C into the soil though a series of processes which can include decomposition of litter and woody debris, root turnover and rhizodeposition (Pausch and Kuzyakov, 2018). Rhizodeposition is the process by which, organic compounds (rhizodeposits) are released or shed from live roots into the rhizosphere or as a consequence of root and mycorrhizal death (Jones et al., 2009). The combined effect of rhizodeposition and litter-fall accounts for half the atmospheric C incorporated into terrestrial plants by photosynthesis (Lal, 2008; Paterson et al., 2009).

European forests are estimated to store 6.1 t C ha^{-1} in litter and 113 t C ha^{-1} in mineral soil (Sommer et al., 2017) and their responsiveness to management make them attractive candidates for C storage mitigation strategies (Nave et al., 2018). Despite developments in the quantification of C sinks and fluxes, many uncertainties in the role of forests to terrestrial C budgets persist (Tian et al., 2016; Pan et al., 2011), due to the relative difficulty in quantification of C allocation belowground (Leake et al., 2001). Specifically, C allocation into the

rhizospheric components of the C cycle (e.g. soils, micro-organisms and especially mycorrhizal hyphae) has often been underestimated or entirely overlooked in trees (Sommer et al., 2016; Sileshi, 2014) and when studied has largely focussed on arbuscular rather than ectomycorrhizae (Ekblad et al., 2013).

Within both coniferous and deciduous temperate forest trees, ectomycorrhizal associations are ubiquitous, and their biomass represents an important belowground C pool (Finlay and Söderström, 1992). Ectomycorrhizal hyphal networks in temperate soils are known to be extensive (Cairney, 2012), in some cases covering many square kilometres (Anderson and Cairney, 2007; Philips, 2017). Estimates of the extent of hyphae in forest soils range from 3–600 m g^{-1} soil (Leake et al., 2004) or 30–8000 m hyphae m^{-1} root (Smith and Read, 2010). Colonisation of roots by ectomycorrhizal fungi can positively influence tree root architecture (Ditengou et al., 2015), plant nutrient acquisition (Smith and Read, 2010) and water transport dynamics and repellency (Carminati et al., 2010; Moradi et al., 2012). These complex fungal symbionts not only benefit their tree hosts, but also perform fundamental ecosystem functions (Powell and Rillig, 2018) such as mediating the rate of decomposition within forest soils (Smith and Read, 2010), improving soil aggregation with fungal exudates (Wu et al., 2014) and increasing the abundance, diversity and activity of microbes in the rhizosphere (Paterson et al., 2009; Jones et al., 2009).

The largest flux of C from terrestrial ecosystems to the atmosphere is belowground autotrophic (e.g. root metabolism) and heterotrophic respiration (e.g. mineralisation of rhizodeposition) (Dilkes et al., 2004; Hill et al., 2007; Glanville et al., 2012). The soils capacity to store C through the process of rhizodeposition is therefore directly linked to the percentage of photosynthetically fixed C allocated belowground that is not rapidly respired (Hill et al., 2007) and which becomes stored in persistent, recalcitrant compounds in the soil. These compounds are typically associated with C which has been processed by the microbial biomass and is therefore secondary processed C. Predicting how increasing atmospheric CO_2 concentrations, temperatures, N deposition and changing precipitation patterns might alter the belowground C allocation and storage will be key to predicting future ecological C fluxes and feedbacks (Pausch and Kuzyakov, 2018).

The quantitative and qualitative investigation of rhizosphere importance to biogeochemical cycling is methodologically challenging (Oburger and Jones, 2018), due to a vast array of confounding issues. These include the fact that: (i) nutrients are simultaneously exuded from roots, hyphae whilst also being liberated from soil organic matter by microbes; (ii) nutrients

are continually removed from the rhizosphere by the microbial community; and (iii) sorption/desorption of nutrients and C by the soil's solid matrix (Oburger and Jones, 2018). Novel methodological developments, particularly in the use of isotopes are extending our understanding of rhizosphere processes (Oburger et al., 2013; Oburger and Schmidt, 2016). For example, the use of continuous or short-term labelling techniques (with stable and/or radioisotopes), are providing exciting opportunities to quantify forest C partitioning within the plant-microbe-soil compartments (Epron et al., 2012; Kuzyakov and Domanski, 2000). The short-term single $^{14}CO_2$ pulse technique allow us to distinguish between C that has been recently fixed from native C already present in the soil (Hill et al., 2007), as well as the separation of plant from soil organic matter derived C (Nguyen, 2003; Kuzyakov and Schneckenberger, 2004; Werth and Kuzyakov, 2008).

The aim of this study was to examine the spatiotemporal dynamics of recently plant captured C in three temperate tree species and to determine the relative allocation of C to belowground pools, using ^{14}C as a proxy. The mechanisms involved in determining C allocation to plant tissues are thought to be controlled by both root and shoot and these can differ between individuals, tree species, tree developmental stage (Farrar and Jones, 2000; Weiner, 2004), in response to climatic conditions (Sanaullah et al., 2012), or as a result of contrasting microbial symbiotic relationships (Brzostek et al., 2015). Here we used a ^{14}C pulse-tracing technique to estimate the relative importance of C pools and fluxes within the plant-fungal-soil system of three contrasting tree species.

Our hypotheses for this experiment were:

H_1: Belowground allocation of C will be greater in species with the largest diversity of microbial symbiotic associations.

H_2: The transfer of recently assimilated C to the soil microbial community will be greater in early (*B. pendula* and *A. glutinosa*), than late successional trees (*C. sativa*), because early successional stage trees need symbiotic partners to help with the poor nutritional status of the soil they colonise

2. Materials and methods

2.1. Experimental establishment

Twelve 2-year-old (1 + 1) bare rooted saplings of two temperate tree species namely; common alder (*Alnus glutinosa* (L.) Gaertn; Tree A (UK provenance zone 204)) and silver birch (*Betula pendula* Roth.; Tree B (UK provenance zone 204)) and twelve 1-year-old cell-grown sweet chestnut (*Castanea sativa* Mill.; Tree C (UK provenance zone 404)) were purchased from local forest nursery stock suppliers (Maelor Forest Nurseries Ltd., Bronington, UK) in November 2015. At the time of planting, the height of all tree saplings was between 0.2 – 0.4 m. The tree species were selected based on their contrasting traits, which was anticipated to maximise the positive effect on net productivity. Specifically, *B. pendula* is a relatively short-lived, light-demanding, early successional pioneer species, *C. sativa* is a late successional, shade tolerant, long lived species and *A. glutinosa* is intermediate in shade tolerance, tolerates waterlogged soils and is nitrogen fixing through an actinorhizal symbiotic association with *Frankia alni*. The saplings were planted into 10 L mesocosms (Ø 250 mm × 200 mm) that had been bisected into two equal-sized compartments using a 40 µm mesh (Normesh Limited, Lancashire, UK) (Plate 1) to allow the growth of root and hyphae in one compartment and the ingrowth of hyphae in the second compartment. Soil was collected from 0-10 cm depth plots under the trees species used in this experiment to avoid the common soil mishandling technique and potential subsequent incorrect estimation of soil biota (Reinhart and Rinella, 2016) and to ensure species-specific symbiotic soil microbes were present. Prior to planting, soil was air-dried, homogenised and sieved to pass 2 mm.

The soil was collected from the Henfaes experimental farm, Abergwyngregan, Gwynedd, Wales, (53°23 N, 4°01 W) (Ahmed et al., 2016) and is classified as a fine loamy textured Eutric Cambisol (Rheidol series) over alluvial gravel (Smith et al., 2013). The saplings were grown in a glasshouse at ambient conditions for 9 months to allow for the development of a root and hyphal network. A growth rate for the experimental establishment period was calculated by dividing the difference between the starting biomass (g) and the biomass prior to pulse-labelling and dividing this by the number of hours that the plants had to establish (274 days).

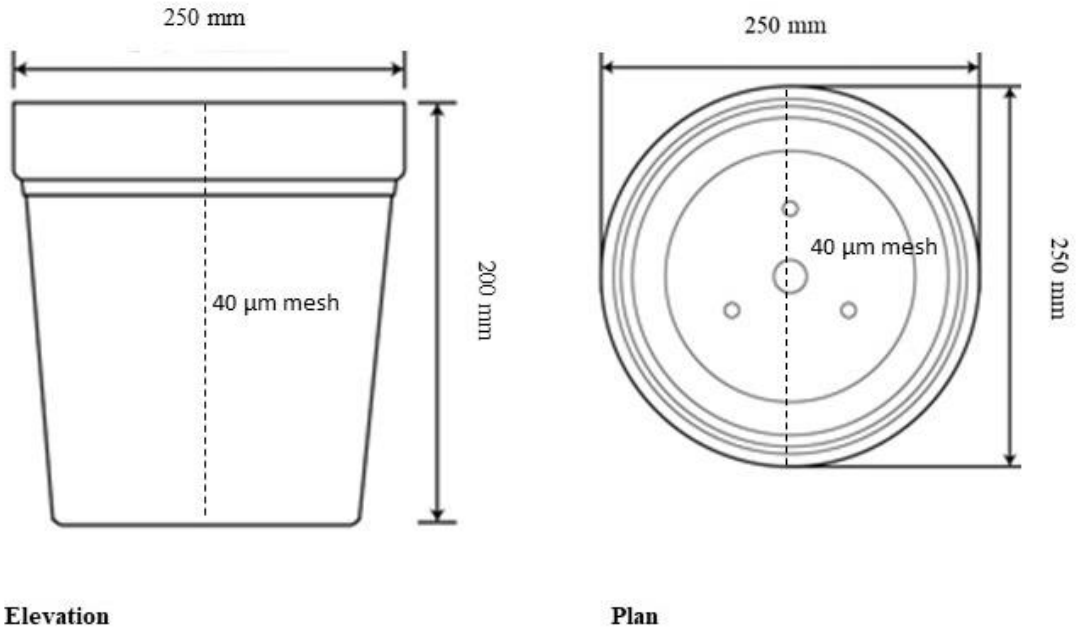

Figure 1: Diagram showing the experimental mesocosm set-up

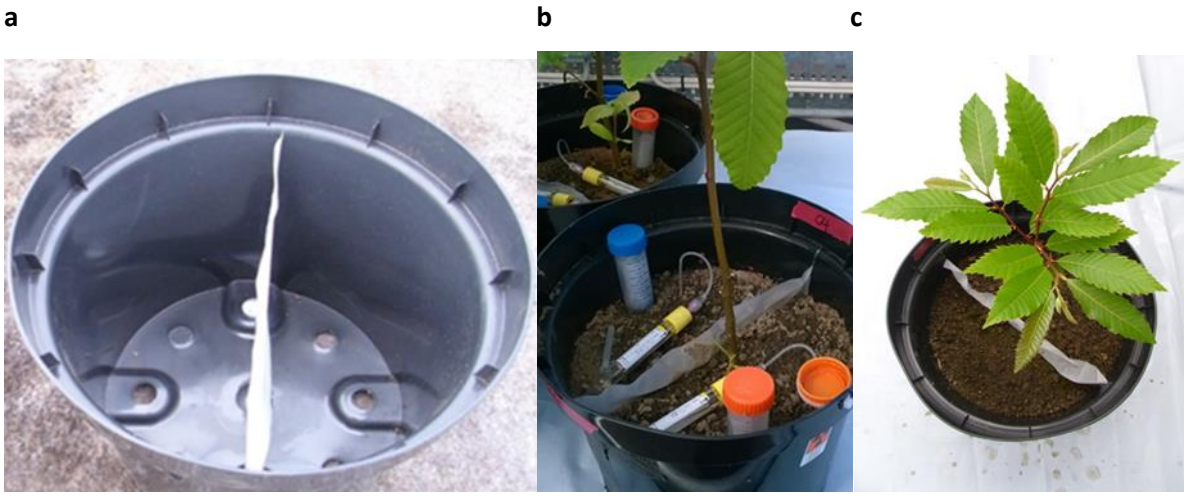

Plate 1: Photographs showing the experimental set-up (a) plant pot bisected with a nylon mesh with a 40-µm pore size to allow hyphal penetration but exclude the trees roots, (b) pot showing the soil solution collection tubes, and (c) showing C (*Castanea sativa*) tree established in the mesocosm.

2.2. Sampling procedure and isotope analysis

On 19th June 2016 each sapling was enclosed by 610 × 920 mm gas-proof bag (CP lab safety, Novato, CA, USA) and exposed to $^{14}CO_2$ generated by the addition of 200 µl of 2 MBq $NaH^{14}CO_3$ (Amersham International, Amersham, UK) to 2 ml of 3 M HCl, in a 30 mm diameter vial fixed inside the plant mesocosm. Each sapling was exposed to the same activity (2 MBq)

for 2 h to allow for photo assimilation in full ambient light with a minimum photosynthetically active radiation level of 800 μmol m^{-1} s^{-1}.

Different approaches were used to trace the ^{14}C pulse within the tree-soil system. Firstly, $^{14}CO_2$ evolved from the soil was collected by suspending two identical 3 M NaOH traps above the soil in both the plant and non-plant fraction. The traps allowed free passage of gases from the soil surface into a headspace chamber containing the NaOH traps. The surface area of the traps was calculated and scaled-up to the total area of half the pot. The traps were replaced at regular intervals (0, 24, 72, 168 and 336 h) after the end of the ^{14}C labelling period. Simultaneously, dissolved organic ^{14}C in soil solution was sampled following at 0, 24, 72, 168 and 336 h after the pulse-label was applied using Rhizon-MOM soil water samplers (Rhizosphere Research Products B.V., Wageningen, The Netherlands) (Jones et al., 2016). The ^{14}C content was determined by liquid scintillation counting, using a Wallac 1409 scintillation counter (Wallac EG and G, Milton Keynes, UK) with automatic quench correction and Optiphase Hisafe 3® (Perkin Elmer, UK) alkali-compatible scintillation fluid. After the defined time-periods (0, 24, 72, 168 and 336 hours after pulse labelling) the trees were destructively harvested and separated into old (present prior to pulse) and new foliage (unfurled during chase period), branch and stem, roots and soil samples. The plant material and soil were immediately oven dried at 105 ºC to prevent loss of ^{14}C to microbial respiration. The roots were then carefully washed in sieves to remove the soil and stone before being re-oven dried at 105 ºC. The plant biomass components (namely leaves since pulse, old leaves, branches and stem and roots) were weighed and recorded (Table 1) before being ground to a fine powder and homogenised using a MM200 laboratory mixer ball mill (Retsch GmbH, Hann, Germany). A subsample of 0.1 g was combusted using a Harvey Instruments Biological Oxidiser OX400 (R.J. Harvey Instrument Corp., Hillsdale, NJ, USA) and evolved $^{14}CO_2$ was dissolved into Oxysolve C-400 (Zinsser Analytic GmbH, Frankfurt, Germany) prior to quantification of ^{14}C using a Wallac 1409 liquid scintillation counter (Wallac EG and G, Milton Keynes, UK).

2.3. Soil Chemical Properties

To determine if the establishment of trees prior to pulse-labelling had modified the soil chemical properties, soil samples were collected using a 4 cm diameter stainless steel soil corer from the centre of each of the two mesocosm fractions immediately following the ^{14}C pulse-label. Soil was placed directly into gas-permeable polythene bags and stored at 5 °C in a cool box. For total elemental C and N measurement, a subsample of soil from each mesocosm was

dried at 105 °C and ball milled before analysis by dry combustion using a CN-2000 analyser (Leco Corp, St Joseph, MI, USA).

Soil ammonium (NH_4^+) and nitrate (NO_3^-) concentrations were determined following extraction from soil in 0.5 M K_2SO_4 in a 1:5 (w/v) slurry and horizontal shaking for 1 hour at 200 rpm, followed by centrifugation at 4000 rpm for 10 min. Nitrate was determined colourimetrically by the vanadium chloride reduction method (Miranda et al., 2001). Ammonium was determined by the salicylate-hypochlorite photometric method of Mulvaney (1996). Plant-available phosphorus was extracted by horizontal shaking from fresh soil in 0.5 M acetic acid (CH_3CO_2H) at a ratio of 1:5 (w/v) and determined colourimetrically as molybdate-reactive P (Murphy and Riley, 1962). Readings obtained from fresh soil samples were adjusted for moisture content after oven drying at 105°C which was determined gravimetrically (Rowell, 1994). Soil pH and EC were measured in 1:2.5 v/v slurry of distilled water according to Smith and Doran (1996). Soil dissolved organic C (DOC) and total dissolved N (TDN) were determined using a Shimadzu TOC-V-TN analyser (Shimadzu Corp., Kyoto, Japan) following extraction (1:5 w/v) in 0.5 M K_2SO_4 (Jones and Willett, 2006). Soil chemical properties are shown in Table 2.

2.4. Root-to-shoot ratio

To assess differences in the source sink relationships between tree species we calculated the ^{14}C activity specific root: shoot ratio (Durall et al., 1994) and adjusted for fundamental differences in above/ belowground biomass using the following equation:

$$\frac{\text{kBq in root g}^{-1}\text{ root dry weight}}{\text{kBq in shoot tissue g}^{-1}\text{ shoot dry weight}}$$

2.5. Statistical analysis

Prior to analysis all data were tested for homogeneity of variance using Levene's test. Data was structured with two independent groups (factors): (i) species treatments comprised of the *A. glutinosa*, *B. pendula* and *C. sativa* as variables; and (ii) time. The dependant data (total plant biomass and partition biomass, soil chemical properties and ^{14}C activity of biomass pools, ^{14}C labelled respiration efflux) were tested for normality prior to analysis using the Shapiro-Wilk Test. The EC data were $\log_{10}$ transformed to satisfy the assumption of normality. The statistical analysis was conducted using a two-way analysis of variance (ANOVA) and Tukey's post-hoc

test with SPSS® Statistics version 25.0 (IBM Corp, Chicago, 2017). Main and interactive effects were considered significant at $P \leq 0.05$.

3. Results

3.1. Experimental establishment

At the start of the pulse label, the total biomass of the three trees species were 57.4 g, 28.6 g and 26.9 g for *A. glutinosa*, *B. pendula* and *C. sativa* respectively (Table 1). At each of the sampling points, the *A. glutinosa* was consistently larger and by the end of the experiment its total biomass was twice that of the other two species. There was a statistically significant difference between the total biomass of the three tree species at the final harvesting time [$F_{(2, 9)} = 9.422$, $P = 0.01$] (Table 1). A post-hoc Tukey test also showed that the significant differences between the biomass partitions of the three species. Key findings included that all the biomass partitions of *A. glutinosa* were significantly larger than those of both *B. pendula* and *C. sativa*, with the exception of the woody branch and stem partitions. *A. glutinosa* was shown to have both larger leaves and both fine and coarse roots but had relatively less branches and stems. In contrast, the plant biomass partitioning for *B. pendula* and *C. sativa* were not significantly different from each other.

The growth rates during the establishment period of the experiment for the three species studied were 7.67×10^{-3} ($\pm$ 1.61×10^{-3}) g/h, 5.87×10^{-3} g/h ($\pm$ 0.20×10^{-3}), 4.08×10^{-3} g/h ($\pm$ 0.43×10^{-3}) dried weight for *A. glutinosa*, *B. pendula* and *C. sativa*, respectively. Each of the four biomass partitions measured for each tree species at each pulse-chase sampling time is shown in Table 1. The growth rates were found to be significantly different from each other [$F_{(2, 9)} = 7.25$, $P = 0.01$]. Overall, *A. glutinosa* had a growth rate that was significantly different to those of both *B. pendula* and *C. sativa*, whereas *B. pendula* and *C. sativa* had growth rates that were not significantly different from each other.

Table 1: Mean (g dwt) ± SE biomass of four biomass partitions and total biomass of *A. glutinosa*, *B. pendula* and *C. sativa* determined by destructive harvesting at 0, 24, 72, 168 and 336 hours after the $^{14}CO_2$ pulse was applied after destructive harvesting (n = 4). Statistically significant results are given in bold and denoted by asterisks (*, $P < 0.1$; **, $P < 0.05$; ***, $P < 0.001$).

		Hours after pulse					*P-value*	
Tree species	**Tree Partition**	**0**	**24**	**72**	**168**	**336**	**336 hours after pulse label completed**	
	New leaf	0	0	0	0.95 ± 0.12	1.75 ± 0.47	***B. pendula***	***C. sativa***
A.	Old leaf	30.60 ± 6.23	30.15 ± 0.89	27.39 ± 5.16	30.37 ± 6.73	34.34 ± 6.52	**0.01***	**0.02***
glutinosa	Branch	12.05 ± 1.41	13.30 ± 0.30	11.09 ± 2.11	15.45 ± 2.36	22.02 ± 3.49	0.09	0.21
	Root	14.76 ± 3.03	14.67 ± 1.59	16.19 ± 2.59	13.43 ± 3.12	18.41 ± 5.63	**0.00****	**0.00****
	Total	57.41 ± 10.58	58.11 ± 10.58	54.67 ± 9.04	60.20 ± 11.45	76.52 ± 15.0	**0.01***	**0.02***
	New leaf	0	0	0	1.28 ± 0.15	1.15 ± 0.48	***A. glutinosa***	***C. sativa***
B. pendula	Old leaf	15.20 ± 0.74	15.24 ± 0.74	12.18 ± 1.58	11.61 ± 1.41	11.04 ± 1.55	**0.01***	0.68
	Branch	6.08 ± 0.54	8.34 ± 0.43	2.96 ± 0.61	5.71 ± 0.15	7.32 ± 0.41	0.09	0.83
	Root	7.34 ± 0.62	7.30 ± 0.66	3.64 ± 0.62	6.97 ± 1.79	6.69 ± 0.80	**0.00****	0.96
	Total	28.61 ± 1.28	30.88 ± 1.82	18.77 ± 2.56	25.57 ± 2.90	26.20 ± 2.47	**0.01***	0.84
	New leaf	0	0	0	0.88 ± 0.19	1.4 ± 0.42	***A. glutinosa***	***B. pendula***
C. sativa	Old leaf	13.18 ± 1.60	13.87 ± 1.63	12.83 ± 0.99	13.43 ± 1.53	15.81 ± 1.36	**0.02***	0.68
	Branch	5.67 ± 0.58	6.50 ± 0.18	4.79 ± 0.60	4.89 ± 0.71	6.55 ± 0.61	0.21	0.83
	Root	8.00 ± 0.90	7.32 ± 0.17	7.99 ± 0.59	8.95 ± 0.55	9.55 ± 1.38	**0.00****	0.96
	Total	26.85 ± 2.85	27.69 ± 1.88	25.60 ± 0.95	28.15 ± 2.45	33.3 ± 1.73	**0.02***	0.84

3.2. Soil chemical properties

Soil chemical properties for each of the species studied determined at the start of the experiment are shown in Table 2. Overall, there were few differences between treatments with the exception of the higher soil NO_3^- in the *A. glutinosa* treatments and lower pH under *C. sativa*.

Table 2: Soil chemical properties for soil collected from the mesocosms 0 hours after the ^{14}C pulse was complete for all three species treatments (*A. glutinosa*, *B. pendula* and *C. sativa)*. Data are mean ± SE (n = 4). Significant differences are highlighted in bold and the level of significance is denoted by $^*P < 0.05$.

	Soil chemical properties							
Tree species	**NO_3^- (mg/kg)**	**NH_4^+ (mg/kg)**	**P (mg/kg)**	**pH**	**EC (µS/ cm)**	**C:N ratio**	**DON (mg/kg)**	**Total C (mg/kg)**
A. glutinosa	12.52 ± 4.38 **a**	3.15 ± 0.95	6.94 ± 0.60	5.99 ± 0.23 **b**	112 ± 27	9.42 ± 0.70	6.57 ± 0.28	26.88 ± 0.98
B. pendula	3.73 ± 0.61 **b**	3.37 ± 0.25	7.88 ± 0.54	6.14 ± 0.10 **b**	124 ± 36	10.01 ± 0.23	5.92 ± 0.38	29.21 ± 1.39
C. sativa	4.61 ± 0.73 **ab**	3.00 ± 0.40	6.74 ± 1.52	5.65 ± 0.04 **a**	76 ± 4	9.48 ± 0.51	6.60 ± 0.88	35.29 ± 8.14
Probability	**0.04***	0.42	0.68	**0.01***	0.45	0.68	0.42	0.50

(EC: electrical conductivity**, TC:** total soluble C)

3.3. Allocation of ^{14}C to biomass compartments

^{14}C allocation to the different plant tissues (as a percentage of total ^{14}C detected in the entire tree), was significantly different between species (see Table 3) [$F_{(2, 537)} = 0.002$, $P = 0.027$]. A Tukey post-hoc test to see which plant partitions had significantly different ^{14}C allocation, revealed that ^{14}C allocation of *A. glutinosa* to leaves was significantly different to that of both *B. pendula* ($P = 0.01$) and *C. sativa* ($P = 0.02$). In contrast, *B. pendula* and *C. sativa* were found to not have differences in ^{14}C allocation to the leaf partition. The allocation of ^{14}C to the branch showed the same trend with *A. glutinosa* allocation being different to both *B. pendula* ($P < 0.001$) and *C. sativa* ($P < 0.001$). The belowground allocation of ^{14}C to the root partition contrarily did not show any differences between the results of the three species treatments.

Table 3: ^{14}C activity of each biomass plant partition determined after destructive harvesting expressed as a percentage of the total ^{14}C activity photo assimilated ^{14}C during the pulse labelling. Data are mean ± SE ($n = 4$)

		Hours after $^{14}CO_2$ pulse to the tree canopy				
Tree species	**Tree partition**	**0**	**24**	**72**	**168**	**336**
	New leaf	0	0	0	2.53 ± 0.28	1.05 ± 0.17
A. glutinosa	Old leaf	93.11 ± 1.49	71.39 ± 3.20	44.29 ± 12.32	50.05 ± 3.99	38.75 ± 6.57
	Branch	3.43 ± 1.77	14.73 ± 1.49	12.32 ± 2.48	15.35 ± 3.65	8.49 ± 1.56
	Root	0.24 ± 0.06	4.40 ± 0.58	12.77 ± 0.66	9.85 ± 2.44	6.01 ± 2.13
	Root-free	2.30 ± 0.88	9.24 ± 2.09	28.54 ± 5.09	21.47 ± 7.29	19.61 ± 8.42
	Soil H_2O	< 0.01 ± 0.00	< 0.01 ± 0.00	< 0.01 ± 0.00	< 0.01 ± 0.00	< 0.01 ± 0.00
	New leaf	0	0	0	7.14 ± 1.26	1.13 ± 0.28
B. pendula	Old leaf	92.75 ± 0.78	47.22 ± 7.05	45.17 ± 3.55	24.87 ± 1.36	16.42 ± 3.69
	Branch	3.09 ± 0.19	10.92 ± 2.30	8.06 ± 1.56	15.50 ± 3.10	7.48 ± 0.74
	Root	0.96 ± 0.41	5.40 ± 1.87	9.89 ± 1.10	14.36 ± 2.88	8.35 ± 0.50
	Root-free	2.69 ± 0.74	36.33 ± 11.61	36.03 ± 11.26	37.71 ± 7.75	26.42 ± 5.40
	Soil H_2O	< 0.01 ± 0.00	< 0.01 ± 0.00	< 0.01 ± 0.00	< 0.01 ± 0.00	< 0.01 ± 0.00
	New leaf	0	0	0	5.16 ± 1.50	
C. sativa	Old leaf	88.27 ± 1.03	55.90 ± 11.27	46.85 ± 7.02	25.29 ± 4.70	
	Branch	5.28 ± 0.96	9.62 ± 2.32	11.03 ± 1.84	8.37 ± 1.97	
	Root	2.43 ± 0.39	5.46 ± 2.23	19.36 ± 2.39	13.20 ± 3.34	
	Root-free	3.63 ± 1.12	28.65 ± 12.37	21.10 ± 3.60	47.42 ± 12.85	
	Soil H_2O	< 0.01 ± 0.00	< 0.01 ± 0.00	< 0.01 ± 0.00	< 0.01 ± 0.00	

The initial harvesting interval directly after the pulse-labelling event showed that the pulse label technique resulted in consistent ^{14}C photo-assimilation across the replicates and the three-tree species. This experiment utilises ^{14}C as a surrogate for recently fixed C thus allowing estimations of the allocation of this C to each of the plant-fungal soil partitions and a comparison amongst the three studied species. This is summarised for the three species as schematic diagrams (Figure 2). The total plant ^{14}C activity determined at 0 hours (directly after the pulse labelling was complete) is assumed to be the total fixed ^{14}C for all the subsequent time treatments. The pools sizes shown in Figure 2, are shown as a percentage of the assumed ^{14}C fixed during the pulse that remained in each of the plant-soil partitions. C allocation as a percentage of the total C within the system is tabulated in Table 4. The ^{14}C remaining in the soil after the roots and soil solution had been recovered is assumed to be largely microbial biomass. The ^{14}C not recovered in any of the plant or soil partitions is therefore assumed to have been either respired by the tree roots or the soil microbial community. An estimation of aboveground respiration was calculated by using the ^{14}C recovered by destructively harvesting the mesocosm immediately after the pulse event as a proxy for the total amount fixed in all time treatments. Once the mass balance calculations for all the plant tissues, soil solution, soil and belowground respiration were combined and subtracted from the proxy for total ^{14}C fixed, then the resulting percentage was assumed to be aboveground respiration from leaves, stem and branch (Figure 2). Key findings include that on average 74.2 ± 18.9 %, 59.9 ± 10.6 % and 83.8 ± 18.0 % in *A. glutinosa*, *B. pendula* and *C. sativa* respectively, of the total pulse labelled ^{14}C was recovered as plant or microbial biomass. We estimate therefore that respiration was on average 25.8 ± 9.6 % from the *A. glutinosa* treatments, 40.1 ± 5.6 % from the *B. pendula* treatments and 15.7 ± 11.1 % from the *C. sativa* microcosms. This was calculated by using the surface area of the respiration trap and scaling this up to the whole pots soil surface. However due to the sampling technique we were unable to definitively separate autotrophically or heterotrophically derived $^{14}CO_2$.

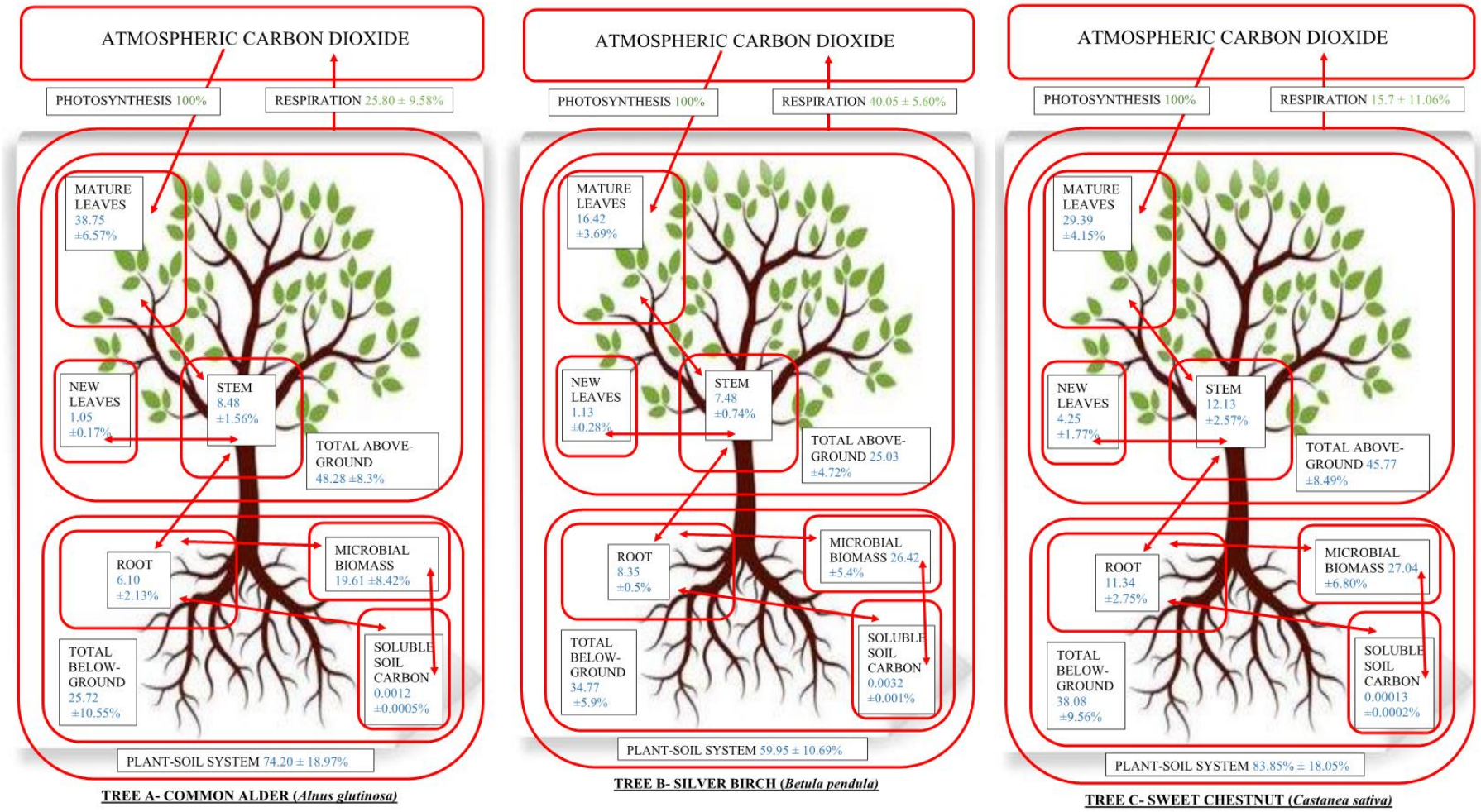

Figure 2: Systematic schematic diagrams of three tree species showing the ^{14}C fluxes and pools as a percentage of the activity assumed to have been fixed during the $^{14}CO_2$ pulse label applied to the tree canopy.

3.4. ^{14}C activity specific root-to-shoot ratio

The root-to-shoot specific activity can be seen to increase initially after the pulse event (Figure 3). Subsequently, as the newly photosynthesised ^{14}C is translocated into the tree roots it is either exuded or respired by the roots or mineralised by the soil microbes and therefore the root-to-shoot specific ^{14}C activity is seen to decrease. Allocation of assimilated ^{14}C was similar between all species for the first 72 hours with a root-to-shoot ratio ranging from 0.54 for *A. glutinosa* to 0.87 for *B. pendula*. After 72 hours, the allocation of ^{14}C between species diverged with the root-to-shoot ratio of *B. pendula* continuing to increase, whilst the root-to-shoot ratio of both *A. glutinosa* and *C. sativa* decreased by 19 and 23 % respectively. 360 hours after the pulse-labelling, the root-to-shoot ratio of *B. pendula* was 1.04, which was different to *A. glutinosa* ($F_{(2, 9)}$ = 7.12, P = 0.01), but not to *C. sativa* ($F_{(2, 9)}$ = 7.12, P = 0.06) (Figure 3).

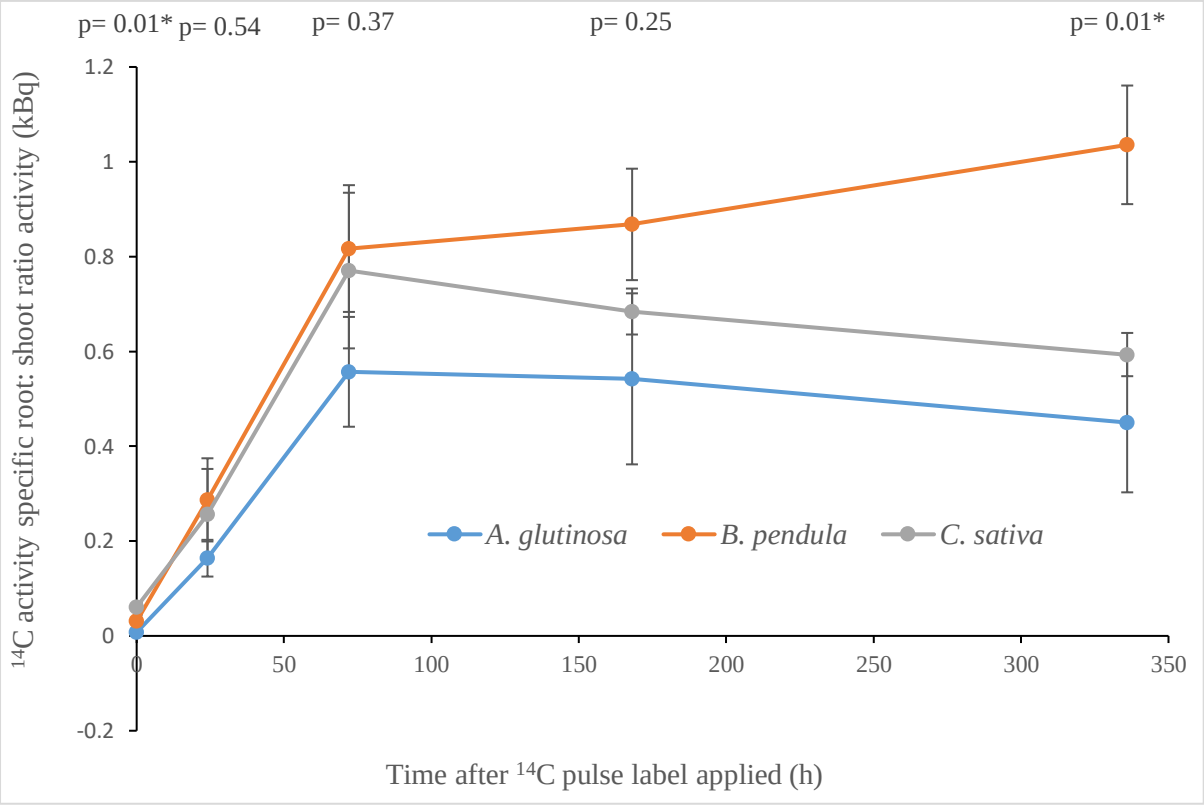

Figure 3: Root-to-shoot specific ^{14}C activity ratio over time of all three-tree species studied values represent mean ± SE (n = 4).

3.5. Allocation of ^{14}C belowground

The percentage of ^{14}C detected in the belowground component of *A. glutinosa* was about ten percent less than that of the other two species although it does appear that the transfer takes place quicker in the case of *A. glutinosa* with the harvesting period 24 hours after the pulse was completed showing maximum belowground C (before respiration has started to reduce this) (Figure 4). In contrast, in *C. sativa* and *B. pendula* the mean maximum belowground allocation was delayed until after 168 and 336 hours after the pulse label was completed, respectively. The differences in timing of maximal belowground allocation was not found to be significant [$F_{(2, 9)} = 0.87$, $P = 0.45$].

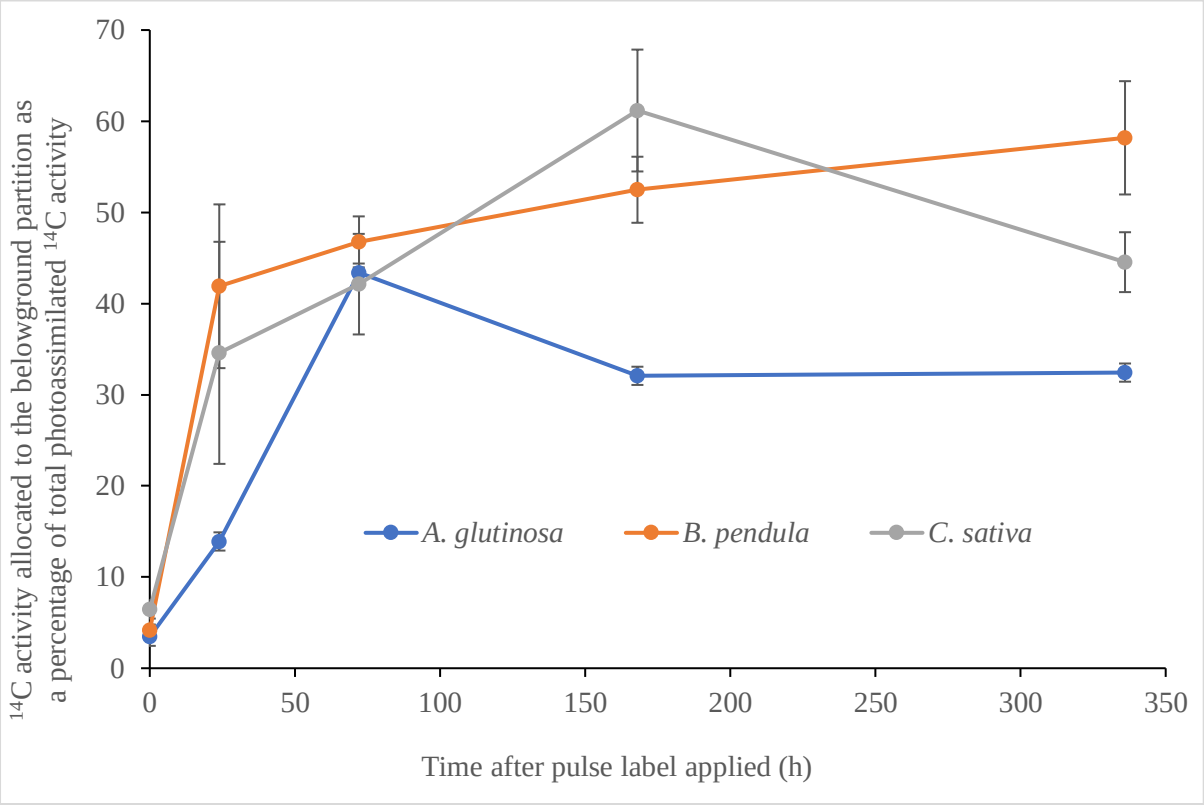

Figure 4: Mean total ^{14}C activity allocated to belowground plant partition determined after destructive harvesting expressed as a percentage of the total ^{14}C activity photo-assimilated during the pulse label. Data are mean ± SE ($n = 4$).

Overall, there was a significant difference between the allocation of ^{14}C belowground as a percentage of total photoassimilated ^{14}C of the three species [$F_{(2,57)} = 3.32$, $P = 0.04$]. Examination of the differences at the end of the experimernt (336 hours after the pulse was completed) (Figure 5) between species showed that belowground allocation of C in *A. glutinosa* was 25.8 % less than *B. pendula* [$F_{(2, 57)} = 3.32$, $P = 0.05$], but not different to *C. sativa* (P =

0.13), and that there was no difference in belowground C allocation between *B. pendula* and *C. sativa* [$F_{(2, 57)} = 3.32$, $P = 0.89$].

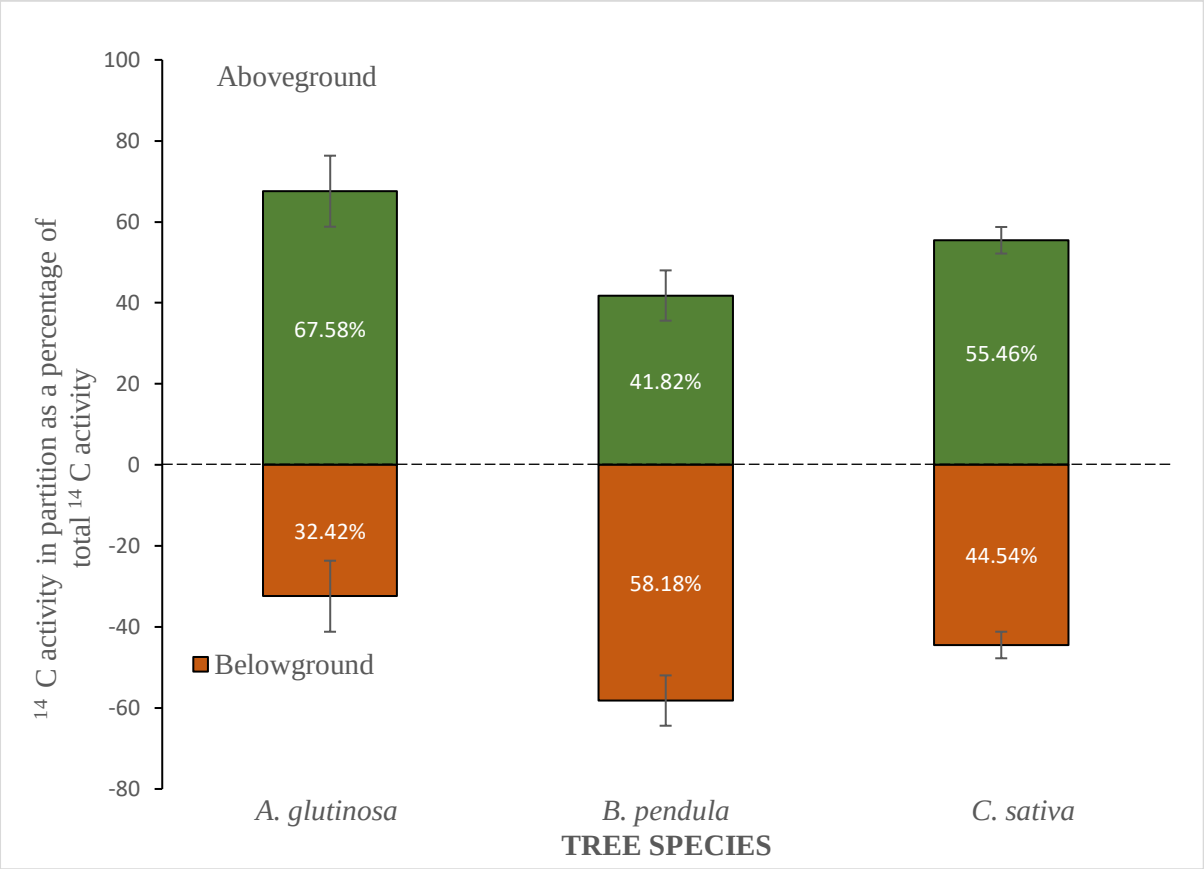

Figure 5: ^{14}C as a percentage of the total amount of ^{14}C fixed allocated to the above and belowground partitions in the three species (*A. glutinosa*, *B. pendula*, *C. sativa*). Data are mean ± SE ($n = 4$).

3.6. Transfer of ^{14}C to microbial community (via mycorrhizal fungi)

The transfer of ^{14}C to the belowground partitions revealed some interesting differences between species. For example, the mean ^{14}C as a percentage of total fixed ^{14}C detected under *B. pendula* was approximately 10 times higher than under *A. glutinosa*. The ^{14}C activity located in the soil was shown to be significantly different under *A. glutinosa* when compared with *B. pendula* and *C. sativa* ($P = 0.04$).

The soil respiration from under the *C. sativa* was approximately four times higher than the $^{14}CO_2$ respired from under the other two species and was found to be significantly different to the soil respiration under *B. pendula* and *A. glutinosa* ($P = 0.02$). The ^{14}C activity in the soil solution was not found to be significantly different in any of the three species treatments. Total estimated transfer of C to micro-organisms, as a percentage of total fixed ^{14}C was 10 times less in *A. glutinosa* than the other two species and all three species were significantly different from each other as shown in Table 7 ($P < 0.001$). The ^{14}C activity measured in the soil solution was very low and therefore unlikely to be driving differences in ^{14}C transferred through the mesh suggesting that these results can largely be explained by differences in movement of ^{14}C via mycorrhizal symbionts.

Table 4: Estimated total ^{14}C activity transferred to microbial community including mycorrhizal fungi expressed as a percentage of total ^{14}C fixed (estimated by total ^{14}C measured immediately post-pulse (time 0 h). Data is a mean ± SE ($n = 4$).

	Mean accumulated ^{14}C activity as % of total fixed ^{14}C			
Tree Species	**^{14}C activity in soil**	**^{14}C activity in soil pore H_2O**	**$^{14}CO_2$ respired**	**Estimated total transfer to microbial biomass**
A. glutinosa	2.99 ± 0.66 **a**	$2.75 \times 10^{-4} \pm 9.87 \times 10^{-5}$	$2.00 \times 10^{-2} \pm 0.724 \times 10^{-2}$ **a**	3.01 ± 0.66 **a**
B. pendula	32.83 ± 10.29 **b**	$2.03 \times 10^{-4} \pm 4.18 \times 10^{-5}$	$2.77 \times 10^{-2} \pm 1.18 \times 10^{-2}$ **a**	32.9 ± 10.3 **b**
C. sativa	22.05 ± 6.44 **b**	$1.85 \times 10^{-4} \pm 4.65 \times 10^{-5}$	$7.78 \times 10^{-2} \pm 3.54 \times 10^{-2}$ **b**	22.1 ± 6.5 **c**
P-value	0.04	0.42	0.02	0.00

4. Discussion

^{14}C activity was detected in both the root biomass partition and Rhizon-extracted soil solution, immediately after the 2 h pulse label suggesting that ^{14}C was almost immediately exuded from the roots of all three species studied and that the $^{14}CO_2$ had been successfully absorbed during the pulse labelling event. Consequently, the ^{14}C activity measured in the destructively

harvested trees of all three species tree partitions was used as a proxy for the total ^{14}C activity assimilated for all subsequent percentage calculations.

4.1. Effect of establishment period on biomass and soil chemistry

To allow the tree saplings to establish and the roots to become colonised by mycorrhizal fungi, the trees were planted into mesocosms and grown for nine months in an unheated glasshouse in a randomised block design. As growth rates are species-specific, differences in plant biomass were apparent between the studied species at the start of the experiment. However, there was only a minimal amount of variation between the biomass compartments of each species studied therefore allowing comparison of C partitioning between and within species. Previous studies have shown that colonisation of roots by both AM and EM fungi can be suppressed in highly fertile soils, especially in the presence of high amounts of available N (Smith and Read, 2010; Kobae et al., 2016) and by long-term N deposition (Lilleskov et al., 2018). Fungal spore germination and viability can also be significantly reduced under high phosphorus conditions (Xavier and Germida, 1999). Suppression of colonisation has been explained by reduced requirement for nutrient acquisition via symbiosis i.e. that a tree that can support itself with its required nutrition will not need to invest photosynthetically derived carbohydrates into the development of microbial symbiosis (Johnson, 2010; Wagg et al., 2011; Johnson et al., 2015). The soils used in this work were Eutric Cambisols collected from a former agricultural field, however, our analysis only showed moderate amounts of nutrient enrichment (N, 6.95 ± 1.91 mg/kg; P, 7.19 ± 0.89 mg/kg P; mean of all species treatments ± SE), and therefore it is possible that mycorrhizal colonisation was not greatly affected. Limited mycorrhizal colonisation due to high soil nutrient status would influence the translocation of C belowground and any attempt at extrapolation should be undertaken with caution. Oburger and Jones, (2018) highlighted that mesocosm based experiments are unlikely to accurately model real-life C dynamics in the field, due in part to faster microbial turnover and decomposition rates resulting from artificially increased temperatures and extreme variation in diurnal temperatures that can be a feature of glasshouse growing conditions.

At the start of the experiment prior to pulse labelling the soil chemistry was tested to determine if the species had significantly altered the belowground nutrient availability. It was found that the nitrate was significantly higher under *A. glutinosa* (see Table 2) than either of the other species studied. This is not surprising as *A. glutinosa* is actinorhizal, however, the biomass of *A. glutinosa* were significantly larger than the other species treatments and the nitrate levels

were still high, suggesting that if plant growth limitation occurred in *A. glutinosa* that nitrate was not the factor causing limitation. The only other chemical property which showed a significant difference between species treatments was pH where *C. sativa* was found to be significantly different to the other two and was slightly more acidic. This could be as a result of differences in chemical exudates released from the roots into the soil during the establishment period.

After the 9-month establishment period the *A. glutinosa* trees were larger, as can be seen in Table 2, and had a significantly larger total biomass. The growth rate of the *A. glutinosa, B. pendula* and *C. sativa* during the establishment period were 7.20, 3.07 and 2.84 g/h for, respectively. This was not unexpected as the literature suggests that *A. glutinosa* is a species with a growth rate which is fast relative to the other two species studied (Kupper et al., 2018). Differences in relative growth rates between tree species have been linked to the trees successional stage, with early-successional pioneer trees typically possessing small wind distributed seeds and high growth rates (Baltzer and Thomas, 2007; Modrzyński et al., 2015). In contrast, late-successional stage trees trade-off high growth rates in lighter conditions against persistence under low light levels (Walters and Reich, 2000, Modrzyński et al., 2015). *A. glutinosa* is actinorhizal and as the trees were potted up without additional fertilisation. It was anticipated that during the establishment period nutrients would become a limiting factor and that the *A. glutinosa* would invest carbohydrates into the developing a relationship with *Frankia alni* to negate this and after harvesting root nodules were observed. In the *A. glutinosa* treatments, this could explain the faster growth rate during the duration of the experiment (Sroka et al., 2018). *B. pendula* had the second largest biomass of three species, which again was expected as *B. pendula* is an early successional stage pioneer species which is short lived, and wind dispersed. As a result, *B. pendula* was expected to be faster growing than *C. sativa*. The growth period occurred in a glasshouse during an unexpectedly hot summer in north Wales and the *B. pendula* treatments seemed to suffer more than the other two species. This could explain why the biomass measured in the *B. pendula* was not as much as expected. *C. sativa*, however, is a Mediterranean species (Krebs et al., 2019) which is better adapted to cope with the elevated temperatures created by the glasshouse growing conditions described earlier. Although it is known that *B. pendula* has a large genotypic variation both within and among populations when compared to other temperate trees (Kasurinen et al., 2016), the *B. pendula* biomass data showed less variation (see Table 1) than *A. glutinosa* and slightly more than *C. sativa*.

4.2. Allocation of ^{14}C activity to biomass partitions

Allocation of ^{14}C to the plant partitions showed that *B. pendula* and *C. sativa* allocate a similar amount of C belowground, however, the *C. sativa* treatments retained C in their biomass and in the soil. In contrast, in the *B. pendula* treatments the C is returned to the atmosphere, as either autotrophic respiration by the plants or by heterotrophic respiration from the soil microbial community. This is consistent with the generally held view that plants at different successional stages adopt different nutrient acquisition strategies. Early successional species have higher growth rates, a higher demand for nutrients, are light demanding and have abundant fine rooting systems with long root hairs to facilitate microbial symbiosis (Rondina et al., 2014; Zangaro et al., 2013). Whereas later successional trees are tolerant of shade, have a slower growth rate, a lower nutrient demand and lower branching of fine roots with shorter root hairs (Rodina et al., 2019). In earlier successional stage trees a larger percentage of fixed C is invested belowground in the fine root system for utilisation by the roots for growth, repair or respiration, to facilitate root exudation and therefore initiate and maintain microbial and more specifically mycorrhizal symbiosis (Rodina et al., 2019). The soil micro-organisms are fundamental in mediating belowground biogeochemical cycles, in the transformation of organic matter and consequently plant nutritional acquisition (Blagodatskaya, 2014), which is a prerequisite of early successional tree growth and survival, but not late-stage trees as they tend to inhabit better formed, relatively fertile soils with higher organic matter.

When understanding the differences in allocation to partitions and belowground of ^{14}C activity as a proxy for C it is necessary to consider the differences in source (such as leaves) sink (parts of tree unable to photosynthesise but which demand carbohydrates) strength between species (Lacointe, 2000). The literature suggests that limited physical sink size may reduce photosynthesis as leaves accumulate leaf starch (Mahmud et al., 2018; Campany et al., 2017). It is possible therefore that if the plants were root-bound during the establishment period that photosynthesis was limited by a lack of sink/ storage space for the carbohydrates. If this were the case, we would expect to see the tree growth rates declining and root biomass occupying the majority of soil volume. The mean root biomass for the three species studied measured after the final 336-hour pulse label was completed were 18.4 ± 5.6 g, 6.7 ± 0.8 g, 9.6 ± 1.4 g for *A. glutinosa*, *B. pendula* and *C. sativa*, respectively. Visual inspection of the root ball when removed from the 10-litre volume mesocosm suggested that the three species were not root-bound. The most productive species, *A. glutinosa*, had a mean root biomass 2- 3 times greater

than that of the other species and retained ^{14}C in old-leaves rather than translocating C to other plant partitions.

4.3. ^{14}C activity specific root-to-shoot ratio

The ^{14}C activity specific root-to-shoot ratio confirmed what had already been established by the allocation of ^{14}C belowground data. Specifically, it showed that the ^{14}C activity that was translocated from shoot to root was largest and quickest in *B. pendula* and least and slowest in *A. glutinosa* with *C. sativa* somewhere between the two. This is as we would expect as previously discussed early successional trees would inhabit poorer soils are subsequently invest more C in facilitating symbiotic relationships with soil micro-organisms and would have to do this promptly in poor soil types to gain the nutrition needed for growth. Previous studies have shown that *B. pendula* has large fine root biomass and abundant short root tips relative to other temperate tree species (Priha et al., 1999). This will result in a stronger sink strength comparatively to *A. glutinosa* and *C. sativa* and therefore may go some way to explaining the speedier transfer belowground that is clearly demonstrated by these results. A larger number of shoot root tips are normally a functional trait associated with plants that need to ensure they develop mycorrhizal associations to fulfil their nutritional requirements (Kleinert et al., 2018).

C allocation belowground has also been shown to be affected by both biotic (e.g. herbivory) and abiotic (e.g. climate) variables and it is estimated that root production accounts for between 33–67 % of global terrestrial net primary productivity (Abramoff and Finzi, 2015). In particular, the storage of non-structural carbohydrates (NSC) belowground at the end of the temperate growing season is determined by climatic conditions and photoperiod, with some species reported as storing NSC belowground for more than ten years (Richardson et al., 2013). The ability of particular tree species in storing starch and soluble sugars belowground varies greatly (Richardson et al., 2015) and studies suggest that belowground allocation is phenologically asynchronous with aboveground allocation (Abramoff and Finzi, 2015). As air temperature warms faster than the soil temperature in the spring some studies report root growth lagging shoot growth by several weeks the reverse could be true in autumn (Steinaker and Wilson, 2008). The current model of plant allocation to partition is known as the Muench mass flow model (Thoms et al., 2017). It suggests a mass-flow system driven by continuous pressure allowing transport of carbohydrates along source sink gradients. This way plants maintain a concentration gradient that drives flows through osmoregulation (Ainsworth and Bush, 2011; Minchin and Thorpe, 1996).

Although we are beginning to understand the mechanisms which mediate the shoot to root allocation, our understanding is far from exhaustive at least in part to the multiple complex factors that can affect it including biotic (species, functional trait, successional stage, microbial symbiosis) and abiotic (soil chemical and physical properties, climate, elevation) (Qi et al., 2019). The optimal allocation hypothesis theorises that plants can modify they allocation strategies according to environmental conditions but that generally speaking those trees with greater belowground biomass will be better suited to sites with high competition or less than optimal water and nutrient regimes (Eziz et al., 2017; Ledo et al., 2018).

4.4. Allocation of ^{14}C activity belowground

In most of the tree species treatments, less than half of the total ^{14}C found in the mesocosm was allocated to the belowground partitions, with a mean of 32.4 % and 44.5 % for *A. glutinosa* and *C. sativa*, respectively. In the case of *B. pendula,* the transfer of C belowground was higher than the other two species with an average of 58.18 % of the ^{14}C activity located belowground at the last destructive harvest (360 hours after pulse-label was complete). It is known that mycorrhizal colonisation of tree roots plays an important role in facilitating primary succession (Brundrett, 2002) and the subsequent soil formation processes (Del Moral and Rozzell, 2005). Pioneer species involved in the initial colonisation of substrates will experience not only unfavourable nutrient regimes, but also extreme microclimatic conditions (e.g. drought), due to the lack of buffering capacity of young mineral soils with a low organic matter content. In these instances, mycorrhizal symbiotic relationships may have an important role in ensuring the persistence of early successional species by increasing water availability and therefore drought tolerance (Kikvidze et al., 2010).

The estimation of total belowground respiration revealed that the highest mean ^{14}C activity was respired from under the *B. pendula* treatments suggesting faster throughput of recently fixed C to soil solution where it was likely utilised by the microbial community as non-structural (not taken up by microbial biomass) and resulted in higher heterotrophic respiration. This could be as a result of the availability of the form of carbohydrate exuded by the roots of this particular species and relates to the early successional stage of *B. pendula*. *B. pendula* is known to contain more sugar in the sap than most UK native tree species (Ozolinčius et al., 2016) and in many European countries the sap was traditionally used to make *B. pendula* syrup (Enescu, 2017). *B. pendula* sap is approximately 98 % water, the other 2 % is fructose, glucose, acids, such as ascorbic acid (vitamin C), calcium, phosphorus, potassium, magnesium, manganese, zinc

sodium and iron (Korhonen and Maaranen, 2018). The rhizodeposits of *B. pendula* have also been found in studies to be of better nutritional value than those of other tree species such as conifers. The extra input of substrate resulting from the qualitative nature of the *B. pendula* rhizodeposits could have stimulated soil microbial growth, thus leading to the increased heterotrophic respiration in these treatments (Priha et al., 1999). Contrastingly *C. sativa* soil respiration ^{14}C activity and soil solution ^{14}C activity had very low readings, although the transfer of C to microbial biomass is similar to that seen in *B. pendula*. Under the *C. sativa* treatments, more ^{14}C activity was retained in the soil partitions, probably as structural microbial biomass then by the other two species in the following order *A. glutinosa* and *B. pendula*.

As *B. pendula* is an early successional pioneer species, exudation could be a mechanism to promote decomposition, to mineralise nutrients (i.e. organic matter priming) and to develop soil via accelerated weathering of primary minerals. *A. glutinosa* overcomes N being the limiting element in primary succession by N-fixation processes that reduce the need to allocate resources to soil mineralisation processes, whereas allocation of C to leaves maximises photo-assimilation and net primary productivity. Despite both *A. glutinosa* and *B. pendula* being early successional species *A. glutinosa* retained ~15 % more C in the plant-soil system with the majority being retained in aboveground biomass partitions with 26 % respired back to the atmosphere. Whereas in *C. sativa*, the partitioning of C above- and belowground was 46 % and 38 % respectively with only 16 % of the ^{14}C activity being respired.

It has been reported that as *A. glutinosa* is a species which tolerates waterlogged soil has the functional trait of depositing gaseous bubbles into its roots as it grows by a thermo-osmotic gas transport system (Schröder, 1989). These root bubbles are a morphological adaption that has evolved in response to extended periods of waterlogging and as *A. glutinosa* shares this trait with many aquatic plants, it has been suggested that it results in the species tolerance to waterlogging (Gill, 1975). The mechanism by which this is achieved is not entirely understood with Schröder suggesting that *A. glutinosa* can transport O_2 through its stems and therefore improve the supply to respiring roots under waterlogging (Schröder, 1989). However, a study that analysed the composition of the root gas bubbles and found the root gas was slightly lower in O_2 and richer in CO_2 than that of the atmospheric air and hypothesised that the root bubbles are enriched in CO_2 by root respirations (McVean, 1956). As these results were found in well-drained soil (below the soil water table) it is possible that the root bubbles are occupied by either O_2 absorbed by the stem and translocated to the roots or CO_2 respired by the root depending on the history of previous waterlogging and if the soil is anoxic or aerobic (McVean,

1956). Dittert et al., (2006) found that O_2 net flow was inversely correlated to O_2 concentration in the rooting media, suggesting that when root and microbial respiration increased the flux of O_2 from the stems into the root. In contrast to *A. glutinosa*, *B. pendula* allocates C belowground, but it would appear that more C is exuded into the soil solution and that a combination of microbial turnover and plant metabolism results in 40 % being respired back to the atmosphere. *B. pendula* as a species is not well suited to waterlogged soils and has likely contrasting root mechanisms as a result.

Methodological issues, such as the location of respiration traps could have compromised the reliability of both the microbial or tree root respiration data, particularly that heterogeneity may not have been captured by the use of one small respiration trap (which was 2.96 % of the total area of each half mesocosm) being used in each half of the bisected mesocosm. Differences in root architecture, as a result of different techniques used during propagation in the tree nursery, meant that the *A. glutinosa* and *B. pendula* seedlings were undercut whilst the *C. sativa* were cell-grown, which could have affected subsequent comparative root development. It is also interesting to note that although *B. pendula* is known to have more genetic diversity than most temperate tree both (Eriksson et al., 2003; Rusanen et al., 2003; Hynynen et al., 2009; Possen et al., 2014) that the standard error observed was smaller in this treatment than the other two.

The biomass of the three root partitions were 18.4 ± 5.6 g, 6.7 ± 0.8 g and 9.6 ± 1.4 g, for *A. glutinosa*, *B. pendula* and *C. sativa*, respectively. Of the three species, *B. pendula* is known to have the largest proportion of fine roots. Priha et al., (1998) found that *B. pendula* seedlings had a mean of 11,450 short root tips. However, root washing has been reported by others to be a potential source of inaccuracy for this experiment type because due to time constraints roots are often under sampled (Addo-Danso et al., 2016; Berhongaray et al., 2013; Levillain et al., 2011; Taylor et al., 2013). It is known that during washing procedures, fine roots and root hairs have the potential to be damaged and lost (Judd et al., 2015) even when utilising the small sieve size (0.2 mm^2). Estimates of dry biomass losses from the standard root washing, drying and storage technique range from 20 % to 40 % (Aung, 1974; Maria do Rosário et al., 2000). Due to the fact that the *B pendula* had most likely the highest proportion of fine to coarse roots when compared to the other two species, it seems likely that the quantification of belowground biomass is likely to be least accurate in *B. pendula.*

4.5. Transfer of ^{14}C activity to soil microbial community

The ^{14}C measured on the non-plant fraction is referred to as C transferred to the soil microbial community. Presumably, the majority of this has been transferred through the mesh by either the mycorrhizal hyphae or as exudates transported by the soil water solution before being utilised by the soil microbial community. Reported values for total C exuded belowground as a percentage of total fixed C from the gross primary production are variable and generally around 25– 63 % (Litton et al., 2007). However, the flow of rhizodeposition can be bi-directional and significant amounts of C-based exudate can be reabsorbed by the roots and (Jones and Darrah, 1994) potentially altering the net C flux to soil via exudation.

The results of this analysis were surprising as the total transfer was estimated to be in 3.01 ± 0.66 %, in *A. glutinosa*, 32.86 ± 10.30 % in *B. pendula* and 22.13 ± 6.47 % in *C. sativa*. It is known that the presence of mycorrhizal symbionts can increase the trees allocation of C belowground and the cost of photosynthetically fixed C of a mycorrhizal symbiosis has been reported to be between 4- 20 % for arbuscular mycorrhizal fungi (Řezáčová et al., 2017). Our estimate therefore seems plausible although it is interesting that the species show such a range of C allocated to mycobionts. A previous study quantified the number of viable N-fixing actinomycete *Frankia alni* by collecting a range of soils from under *B. pendula* and *A. glutinosa* and comparing the nodulation capacity by adding diluted soil solutions to potted *Alnus* plants, leaving for a predetermined time and then counting the root nodules (Smolander, 1990). The results found that the nodulation capacity of the soil under birch varied between 490- 6500 nodulation units (nu) cm^{-3} soil and was consistently as high as or higher than of *A. glutinosa*. This is surprising because if the *B. pendula* is using readily accessible carbohydrates as a medium for encouraging the development of free-living N fixing bacteria and as a strategy to overcome nitrogen limitation, then this may go some way to explaining the results we observed.

The total transfer of ^{14}C was problematic to quantify as a significant proportion of it will have been rapidly mineralised and then became difficult to disentangle from the autotrophic respiration of the tree roots. We can assume that the ^{14}C that was collected in the exclusively mycorrhizal partition originated as rhizodeposited C which was then assimilated into microbial biomass before being mineralised as $^{14}CO_2$. However, some of the respiration trapped on the plant half will have originated from the microbial community inhabiting the rhizosphere. To estimate the total transfer to soil microbes we will assume that the same amount of microbial

respiration occurs in both sides of the mesocosm, although there will likely be more microbial activity in closest proximity to the source of carbohydrates i.e. rhizosphere.

It is known from previous experimental results that rhizodeposition and subsequent transfer to soil micro-organisms and more specifically mycorrhizal fungi is ecologically significant (Godbold et al., 2006). Disentangling the importance of hyphal C from that of the roots they are connected in the field, is problematic. In this laboratory-based experiment we use a divided mesocosm to begin to understand the relative contribution of roots and hyphae to belowground C dynamics. The source of the ^{14}C measured in the mycorrhizal compartment can reasonably be assumed to have originated from roots via the process of exudation either as microbial or hyphal biomass or in soil solution. Visually the mycorrhizal fraction was not heavily colonised by mycelium of hyphae and this is supported by other fungal studies conducted using the same soils (Gunina et al., 2017). The nutrient regime of the soil used is known to have relatively high nitrogen status (Table 2) that will affect mycorrhizal development which could result in an underestimate of the importance of mycorrhizae to soil C storage.

4.6. Hypothesis discussion

Our first hypothesis was that the belowground allocation of C will be greater in species with the largest diversity of microbial symbiotic associations. The results of the comparison of the belowground allocation of ^{14}C activity, 336 hours after the pulse label was completed was 26 %, 35 % and 38 % for *A. glutinosa*, *B. pendula* and *C. sativa*, respectively. The lowest transfer was found in *A. glutinosa* which is actinorhizal, has arbuscular and ectomycorrhizal associations, whereas both *B. pendula* and *C. sativa* possess only ectomycorrhizal associations. Belowground allocation of C in *A. glutinosa* was 25.75 % less than *B. pendula* [$F(2, 57) = 3.32$, $P = 0.05$], but not different to *C. sativa* ($P = 0.13$), and that there was no difference in belowground C allocation between *B. pendula* and *C. sativa* [$F(2, 57) = 3.32$, $P = 0.89$]. The differences between the allocation of ^{14}C belowground as a percentage of total photo-assimilated ^{14}C, 336 hours after the pulse was completed, of the three species treatments was found to be significantly different [$F(2, 57) = 3.32$, $P = 0.04$] specifically and H_1 can therefore be accepted.

The second hypothesis was that the transfer of recently assimilated C to the soil microbial community will be greater in the early successional *B. pendula* and *A. glutinosa* than the late successional tree species *C. sativa.* However, the estimated total transfer to the microbial community (including the mycorrhizal component) using ^{14}C activity as a proxy for recently

fixed C was 3.01 ± 0.66 %, in *A. glutinosa*, 32.86 ± 10.30 % in *B. pendula* and 22.13 ± 6.47 % in *C. sativa*. Therefore although *A. glutinosa* is an early successional species it allocated the least ^{14}C activity to the microbial community, although as the *Frankia alni* are located in the root nodule and were homogenised with the roots some C transferred to internal microbes will not be included in this result. H_2 was therefore rejected.

5. Conclusions

C allocation in three temperate tree species was determined by the use of ^{14}C as a surrogate for recently fixed photosynthates to enable the approximate quantification of C allocated to different plant structures and soil under the three-tree species. The evidence from this study suggests that in the case of C offsetting or storage plantings it seems it would be best to plant *C. sativa* of the three species studied, to maximise belowground C storage This study highlights the importance of the continuing focus on identifying and understanding the factors involved in belowground competition and facilitation in a range of ecological settings. In particular the fact that many current C accountancy models do not fully quantify the C sequestered belowground to fine roots and subsequently microbial symbionts. Anthropogenic modification of global C cycles is further increasing the need for more accurate C accountancy and global circulatory models. This will enable humanity to identify land-management changes that can best drawn-down previously released anthropogenic greenhouse gas emissions and increase C sequestration rates.

6. Acknowledgements

This work was funded by NERC through Envision DTP. The authors would also like to acknowledge the role that technicians Jonathon Roberts and Sarah Chesworth had in producing this research and all the help and support I received in the field and laboratory from Léa Sgro.

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8. Appendices/ Supplementary

Table S1: Root-to-shoot ratios with significance at each experimental time point. Values represent means ± SE (n = 4).

Tree species	Hours after pulse				
	0	**24**	**72**	**168**	**336**
A. glutinosa	0.01 ± 0.00**b**	0.16 ± 0.04	0.56 ± 0.12	0.54 ± 0.18	0.45 ± 0.15**b**
B. pendula	0.03 ± 0.01**a**	0.29 ± 0.09	0.82 ± 0.13	0.87 ± 0.12	1.04 ± 0.13**a**
C. sativa	0.06 ± 0.01**b**	0.26 ± 0.10	0.77 ± 0.16	0.68 ± 0.05	0.59 ± 0.05**b**
Probability	0.01	0.54	0.37	0.25	0.01

Table S2: mean total ^{14}C activity allocated to belowground plant partition determined after destructive harvesting expressed as a percentage of the total ^{14}C activity photo-assimilated during the pulse label. Data are mean ± SE (n = 4)

Tree species	% of total ^{14}C assimilated/ Hours after pulse				
	0	**24**	**72**	**168**	**336**
A. glutinosa	3.46 ± 0.93	13.91 ± 1.89	43.39 ± 4.84	32.07 ± 4.13	32.43 ± 8.77
B. pendula	4.16 ± 0.63	41.90 ± 8.98	46.77 ± 2.80	52.48 ± 3.62	58.18 ± 6.21
C. sativa	6.46 ± 1.01	34.60 ± 12.18	42.13 ± 5.52	61.18 ± 6.68	44.54 ± 3.28

Chapter 4

Investigating inter- and intra-specific carbon transfer between three temperate tree species via common mycorrhizal networks

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Unpublished manuscript

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TDP conducted all empirical work with guidance on design, execution and data analysis from ARS, GWG and DLJ. TDP wrote the manuscript with all authors contributing to the final version.

1. Introduction

Plants act as a conduit between the abiotic and biotic fractions of the carbon (C) cycle by transforming atmospheric carbon dioxide (CO_2) to soil C through the process of photosynthesis (Pausch and Kuzyakov, 2018). Addition of photosynthetically derived carbohydrates to the soil comes in many forms including roots, root turnover, hyphal turnover, leaf and litter decomposition, mucilage and sloughed off cells, which then promotes microbial diversity and proliferation (Dennis et al., 2010; Neumann and Römheld, 2007). The soil micro-organisms present in the soil can be either free-living species that tend to concentrate within hot spots in the rhizosphere or those that form a more intimate relationships with plants and colonise large volumes of bulk soil. Mycorrhizal symbiotic relationships between a plant (photobiont) and mycorrhizal fungus (mycobiont) are ubiquitous amongst nearly all known terrestrial plant groups (Smith and Read, 2010; Van der Heijden, 2016). Mycorrhizal fungi can simultaneously colonise multiple plants at different developmental stages, intra- and inter-specifically (Selosse et al., 2006), and therefore are not exclusively reliant on a single partner for C or nutrient

acquisition (Bücking et al., 2016). This means that within forest ecosystems many trees can be connected underground by vast common mycorrhizal networks (CMNs) commonly referred to as the Wood Wide Web (Beiler et al., 2010).

These ecologically important plant-microbe partnerships allow the photobiont access to fungal-derived water and nutrients (C, nitrogen, phosphate or micronutrients) acquired from their extensive hyphal network, which they can trade for C with the plants (Smith and Read, 2010; Van der Heijden, 2009). The importance of mycorrhizal fungi as a pathway for photosynthetically derived C to be transferred belowground is considerable. Wallander et al., (2001) estimated that between 700 to 900 kg of C ha^{-1} of net primary production in forests is assimilated by the mycorrhizal network, equivalent to 15 to 28% of net fixation (Vogt et al., 1982; Finlay 2008). Klein et al., (2016) estimated that transfer between trees via CMNs can account for 280 kg of C ha^{-1}, roughly equivalent to 4 % of the forests net primary production, although some of this may be C recaptured from exudation or root and hyphal turnover (Jones et al., 2009). It is also known that CMNs can connect plant species mediating the transport of plant signalling chemicals, stress chemicals, and allelochemicals over large distances, allowing multiple fungal and plant species to 'communicate' thus affecting the behaviour, fitness and survival of both partners by increasing the relative competitiveness of plants and fungi linked via these networks (Bücking, et al., 2016).

Mycorrhizal associations are classified into distinct groupings based on their morphology (Lukac and Godbold, 2011). In temperate forests, the commonest of these groupings are arbuscular mycorrhizal (AM) and ectomycorrhizal (EM) associations. Studies have demonstrated that transfer of C occurs between plants via both AM (Hirrel, 1979; Francis and Read, 1984; Grime et al., 1987) and EM networks (Brownlee et al., 1983; Simard et al., 1997). Although it seems that the evidence of transfer between plants connected by EM fungi is more compelling in comparison to AM networks (Selosse, et al., 2006). Averill et al., (2014) found that EM networks can store up to 1.7 times more C than AM networks. Field experiments have observed transfer between seedlings (Simard et al., 1997), to seedlings from mature trees (Beiler et al., 2010) and between mature trees (Klein et al., 2016). AM transfer seems less clear, however, with some believing that the C remains in the hyphae that have colonised the plants root cell walls (Robinson and Fitter, 1999), and that the C is not nutritionally available to the plant (Pfeffer et al., 2004). The difficulty in quantifying this surreptitious belowground C transfer between mycorrhizal plants has kept the mechanics of CMN equivocal (Fellbaum et al., 2014), however, there is a growing consensus of the ecological importance of mycorrhizal

symbiosis (Johnson and Gilbert, 2015). The concept of a group of trees (often referred to as a guild), sharing resources mediated by CMNs is an ecological paradigm shift (Simard et al., 1997), which undermines our understanding the dynamics of plant competition. The importance of CMNs in the dynamics and community structure of plants may vary depending on host (Weremijewitz et al., 2016), mycorrhizal species (Hoeksema et al., 2018; Walder et al., 2012), soil nutritional status (Elumeeva et al., 2018) and climatic conditions (Bennett et al., 2018). It has been hypothesised that the trading of C and nutrients between mycorrhizae and plants is based on reciprocal rewards system, whereby those plants that can supply the most C to their fungal symbiont are supplied with the most nutrients (Lekberg et al., 2010; Hammer et al., 2011; Kiers et al., 2011; Fellbaum et al., 2014), although this has been disputed (Walder and Van der Heijden, 2015) who argue that mycorrhizal fungi use CMNs to redistribute carbohydrates amongst plants thus lessening competition (Shi et al., 2017; Van Der Heijden, 2009).

Soils comprise the largest pool of terrestrial organic C (Jobbágy and Jackson, 2000; Tarnocai et al., 2009), however, there is still much uncertainty in our understanding of the mechanisms of C storage in soils and their relative importance to C cycling (Arora et al., 2013; Todd-Brown et al., 2013). This sizable knowledge-gap is currently limiting both the reliability and robustness of the climatic change predictions, and C cycling and global circulatory models (Luo et al., 2016).

It is now generally accepted that the soil microbial biomass plays a crucial role in biogeochemical cycling as the majority of stable soil C is formed by micro-organisms oxidising belowground inputs (Schmidt et al., 2011; Luo et al., 2016). Historically competition between plants has been viewed as the primary driver of evolution and community structure, however, increasingly the relative importance of facilitative and mutualistic interactions is being appreciated (Tilman, 1988; Bruno et al., 2003; Stachowicz, 2001). This is at least in part due to the fact that in forest ecosystems most of the facilitative relationships are microscopic and therefore were not immediately obvious to those Victorians who were crucial in developing the ideas of ecological theory (Ryan, 2002). Under changing climatic conditions there is an obvious need to better understand these complex belowground symbiotic interactions to increase the accuracy of models used to predict the impact of future land-use on C cycling (Kleber et al., 2007; Schmidt et al., 2011; Davidson et al., 2014).

Radio-isotopic experimentation has allowed us to improve our understanding of C dynamics and C allocation along the plant-fungal-soil continuum. Due to the relative longevity of the photobionts involved in ectomycorrhizal symbiosis and the fact that the plants involved are generally not agricultural, this type of facilitative relationship has been relatively neglected in C flow studies (Molina et al., 1992; Simard et al., 2015). A short-term ^{14}C (half-life of 5730 years) pulse-chase was employed to quantify inter- and intra-specific C transfer occurring between two trees linked by a CMN. We used short chase times as previous studies suggest that belowground ^{14}C dilution starts to occur 48 hours post-pulse labelling event (Wu et al., 2002). An ecological understanding of the importance of ectomycorrhizal fungi to the competition dynamics of forests is needed to elucidate the black box of belowground biogeochemical cycling. In addition, this has implications for our understanding of competition and succession within forest ecosystem associated with Common Ectomycorrhizal Networks (CectoMN) could fundamentally alter our understanding of plant community dynamics (Robinson and Fitter, 1999).

Our hypotheses for this experiment were-

H_1: Transfer of C by CMNs is greater inter-specifically than intra-specifically, as a result of differences in C demand between species.

H_2: Greater mycorrhizal diversity per unit of rhizosphere soil will result in more C transfer between trees via the CMN.

2. Materials and methods

2.1. Plant and mycorrhizal establishment

Sixteen saplings of three temperate tree species were purchased from local forest nursery stock suppliers (Maelor Nursery Bronington SY13 3HZ) in November 2015. Namely, 2-year-old bare-rooted; alder (*Alnus glutinosa* (L.) Gaertn; Tree A) and silver birch (*Betula pendula* Roth; Tree B) and 1-year old cell-grown sweet chestnut (*Castanea sativa* Mill.; Tree C). The three temperate forest tree species were selected from trees already planted as part of BangorDIVERSE, an established 15-year old tree diversity experiment located at Henfaes experimental farm, Abergwyngregyn, Gwynedd, Wales, (53°14 N, 4°01 W) (Ahmed et al., 2016) on the basis of their divergent symbiotic relationships and contrasting ecological niches.

Specifically, *Betula pendula* is a wind dispersed pioneer species whereas *Castanea sativa* is an animal dispersed late successional species, also with ectomycorrhizal associations and both form ectomycorrhizal symbiotic relationships. Contrastingly, *Alnus glutinosa*, typically a riparian species with seeds dispersed by watercourses, is actinorhizal and forms symbioses with both arbuscular and ectomycorrhizal fungi. The UK provenance zones of the three species were; zone 204 (Northumberland, England) for both *A. glutinosa* and *B. pendula* and zone 404 for *C. sativa*.

The saplings were planted into pairs of 10 L mesocosms (Ø 250 mm × 200 mm) joined via a 10 cm diameter Perspex tube filled with 2 kg of horticultural grade sharp sand mixed with 20 g of bone meal and covered at both ends with a 40 µm mesh (Normesh Limited, Lancaster, UK; Figure 1). The mesh allowed the hyphae to access and utilise the sand and bone meal but excluded the tree roots. Both mesocosms were potted using species-specific soil collected from the 0-10 cm layer of BangorDIVERSE, which had been air dried, homogenised and then sieved to pass through a 2 mm mesh. Soil is classified as a fine loamy textured Eutric Cambisol (Rheidol series) with underlying alluvial gravel (Smith et al., 2013). The trees were planted in pairs that established intra- or inter-specific combinations (A-A, A-B, A-C, B-B, B-C, and C-C) with four replicates. The tree mesocosms were then grown in a glasshouse in ambient conditions for 9 months to allow for the establishment and development of the root system and associated CMN. To apply a ^{14}C pulse-label, one tree in each pair was sealed inside 610 × 920 mm gas-proof bags (CP lab safety, Novato, CA, USA) before being labelled with 2 MBq of $NaH^{14}CO_3$ (Amersham International, Amersham, UK) reacted in a vial containing 3 M HCl inside the bag to form $^{14}CO_2$. Each tree was exposed to the same radio-isotopic activity for 2 hours in full ambient light with a minimum photosynthetically active radiation level of 800 µmol m^{-1} s^{-1}. The trees were then returned to the unheated glasshouse for the post pulse-chase period (336 hours). During the chase period the donor trees were placed with a control tree equidistant to the receiver tree (see Figure 1) to determine how much $^{14}CO_2$ was respired by the donor tree into the greenhouse and then re-fixed in photosynthesis by the control tree.

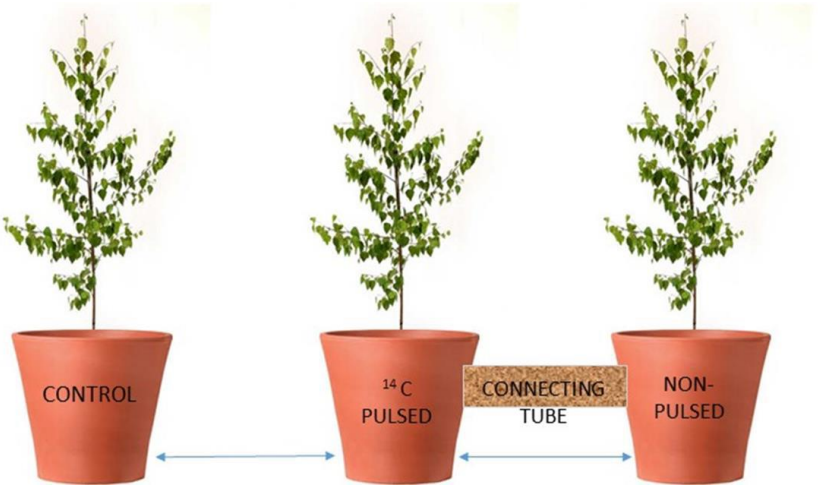

Figure 1: Experimental design with two potted trees connected by a sand and bone-meal filled Perspex tube and the control plant the same distance from the donor tree as the receiver tree. The control tree absorbed the $^{14}CO_2$ respired from the donor tree and was then subtracted from the activity found in the receiver treatment.

2.2. Sampling procedure and isotope analysis

To establish the fate of the recently fixed C, a ^{14}C pulse was used as a proxy. In the case of the ^{14}C evolved as $^{14}CO_2$ from soil respiration, a 28 mm diameter CO_2 trap that contained 3 M NaOH was suspended above the soil in both pots to allow free passage from the soil surface and replaced following 0, 24, 72, 168 and 336 hours of incubation at room temperature (21 ºC). Simultaneously, dissolved ^{14}C in soil solution was sampled following 0, 24, 72, 168 and 336 hours after the pulse-label was completed, using Rhizon-MOM soil water samplers (Rhizosphere Research Products B.V., Wageningen, The Netherlands) (Jones et al., 2016). The $H^{14}CO_3$ content was determined by liquid scintillation spectrometry. Counts were standardised using a quench curve and regularly calibrated using standard samples and expressed in disintegrations per minute, using a Wallac 1409 scintillation counter (Wallac E G and G, Milton Keynes, UK) with automatic quench correction and Optiphase 3® alkali compatible scintillation fluid (Wallac E G and G). After the defined chase period (336 hours) the trees were destructively harvested and separated into foliage, branch and stem, fine and coarse roots, soil and sand from the interconnecting tube for analysis. The plant material (including the control trees), soil and sand were immediately dried at 105° C to prevent loss of isotope to microbial respiration, after which they were homogenised using a laboratory mixer ball mill (Retsch MM 200) then combusted using a Harvey Instruments Biological Oxidiser OX400

followed by trapping of the evolved $^{14}CO_2$ in Oxysolve which was then analysed as described above.

2.3. Analysis of soil chemical properties

At time point T0 (immediately post pulse-label), soil was sampled from each mesocosm using a 4 cm soil corer. The soils were then analysed to determine if the trees presence during the tree and mycorrhizal establishment period had significantly altered the soil chemical properties.

Soil samples were dried at 105 °C and ball milled before C and N analysis was measured by dry combustion using a CN Analyser (Leco Corp., St Joseph, MI, USA) and soil moisture by the gravimetric method (Reynolds, 1970). Nitrate was determined colourimetrically by photometric method (λ=540 nm) with vanadium chloride (Miranda et al., 2001) and ammonium by the salicylate-hypochlorite method of Mulvaney (1996). Soil pH was measured in 1:5 soil: water slurry according to Smith and Doran (1996). Plant available phosphorus was extracted in 0.5 M sodium bicarbonate ($NaHCO_3$) pH 8.5, at a ratio of 1:5 (w/v) (Olsen et al., 1954) and determined colourimetrically as molybdate-reactive P (Murphy and Riley, 1962). Soil dissolved organic C (DOC) and total dissolved N (TDN) was determined using a Shimadzu TOC-V-TN analyser (Shimadzu Corp., Kyoto, Japan) following extraction (1:5 w/v) in 0.5 M K_2SO_4 (Jones and Willett, 2006). The resultant soil chemical and properties are shown in Table 1.

2.4. Fungal DNA analysis

A subsample of soil collected at T0 was collected from the two most contrasting treatments namely the tree combination with the most transfer (C-A) and the least (A-A). The samples were freeze dried using a Lyovac GT2, benchtop freeze drier (Finn-Aqua, Huerth, Germany) and finely ground before being sieved to 500 μm and thoroughly homogenised. A representative subsample of 0.25 g of each soil sample was then weighed and added to the power bead tube for DNA extraction using the Qiagen DNeasy kit (Qiagen DNeasy, Valencia, CA, USA), closely following the manufacturers protocol. Novel fungal specific primers, ITS3 (GCATCGATGAAGAACGCAGC) and the tagged ITS4 reverse primer (CAGACTT[G/A]TA[C/T]ATGGTCCAG), were then utilised to isolate the ITS2 region of the large sub unit (LSU) of ribosomal DNA (rDNA). Subsequently, the DNA was amplified in a 25 μl Polymerase Chain Reaction (PCR) (Saiki et al., 1985), using Promega GoTaq G2 DNA polymerase (Promega, Madison USA). Each reaction contained 7.83 μl of each primer, 39.13

µl BSA, 78.26 µl dNTPs and 1.96 µl of DNA polymerase in the supplied buffer. The PCR conditions were 94 °C for 5 min (initial denaturation) followed by 30 cycles at 94 °C, 30 s (denaturation); 52 °C, 30 s (annealing); 72 °C, 30 s (extension) and a final 5 min extension at 72 °C. PCR reactions were cleaned using spin columns (NBS Biological, Huntingdon UK) and amplified DNA were quantified using NanoDrop (NanoDrop Products, Wilmington USA). DNA yield was then checked using gel photophoresis and quality assessed using Qubex device before being exposed to an ION torrent. Primer ITS2 was linked at the 5' end to the IonTorrent A-adapter sequence (pending), the TCAG key and an IonXpress Barcode. Primer ITS4 was linked at the 5' end to Ion Torrent B adapter sequence (unpublished).

2.5. Statistical analysis

Prior to analysis all data were tested for homogeneity of variance using Levene's test. Data was structured as an independent variable group of species treatments that was comprised of *A. glutinosa, B. pendula* and *C. sativa*, whilst the dependant variables were plant and partition biomass, soil chemical properties, ^{14}C activity of biomass pools, ^{14}C labelled respiration efflux). Data were tested for normality prior to analysis using the Shapiro-Wilk Test. The EC data were $\log_{10}$ transformed to satisfy the assumption of normality. The statistical analysis was conducted using a one-way ANOVA and Tukey's post-hoc test with SPSS® Statistics version 25.0 (IBM Corp, Chicago, 2017). Results of the analysis were considered significant at $P \leq 0.05$.

3. Results

3.1. Soil chemical properties

Growth of tree species during the 9-month establishment period did not affect soil NO_3^-, NH_4^+, P, pH, EC and TDN. Soil C: N ratio was found to be significantly higher under *C. sativa* compared to *A. glutinosa* and *B. pendula* ($P = 0.03$), Dissolved organic carbon (DOC) was also found to be significantly altered by tree species ($P < 0.001$) with soil under *A. glutinosa* soil containing almost 2-fold the DOC of *C. sativa* ($P < 0.001$) and significantly more than the *B pendula* treatments ($P = 0.01$). The DOC of *C. sativa* was not found to be significantly different from the other treatments ($P = 0.86$). Soil chemical properties are shown in Table 1.

Table 1: Soil chemical properties for soil collected from the mesocosms 0 hours after the pulse completed and after a 9-month period of establishment for all three species treatments (*A. glutinosa, B. pendula* and *C. sativa)*. Data are mean ± SE (n = 4). Significant differences are highlighted in bold and the level of significance is denoted by $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

	Soil chemical properties							
Tree species	**NO_3^- (mg/kg)**	**NH_4^+ (mg/kg)**	**Available P (mg/kg)**	**pH**	**EC (µS/cm)**	**C:N ratio**	**TDN (mg/kg)**	**DOC (mg/kg)**
A. glutinosa	4.81 ± 2.74	8.54 ± 2.21	20.4 ± 7.3	5.54 ± 0.06	197.0 ± 24.1	8.30 ± 0.13	7.43 ± 0.78	34.5 ± 0.7 a
B. pendula	2.22 ± 0.71	7.28 ± 1.66	13.4 ± 1.9	5.54 ± 0.05	247.3 ± 35.4	8.51 ± 0.14	8.42 ± 2.87	24.0 ± 2.0
C. sativa	7.56 ± 3.23	5.73 ± 2.34	14.5 ± 4.6	5.46 ± 0.07	251.6 ± 36.3	9.50 ± 0.52 a	5.03 ± 0.39	19.7 ± 1.6
P value	0.54	0.62	0.57	0.50	0.39	**0.01***	0.33	**0.001****

3.2 ^{14}C transfer via the CMN

3.2.1 Total ^{14}C activity in receiver trees Intra- vs Inter-specific

The destructive harvesting of the receiver plants tissues allowed the quantification of ^{14}C that had been transferred through the Perspex sand-filled tube and into the receiver trees. Figure 2 shows the mean total ^{14}C activity in all tree tissues expressed as a percentage of the total ^{14}C activity found in the entire mesocosm as mean inter- and intra-specific data. The total mean ^{14}C was higher in the inter-specific combinations and the results were found to be significantly different from the total ^{14}C activity counted in the intra-specific species treatment combinations (P = 0.03). The mean ^{14}C activity transferred through the CMN to the receiver plants as a percentage of total mesocosm activity was 0.39 ± 0.15 % for intra-specific treatments and 1.30 ± 0.64 %.

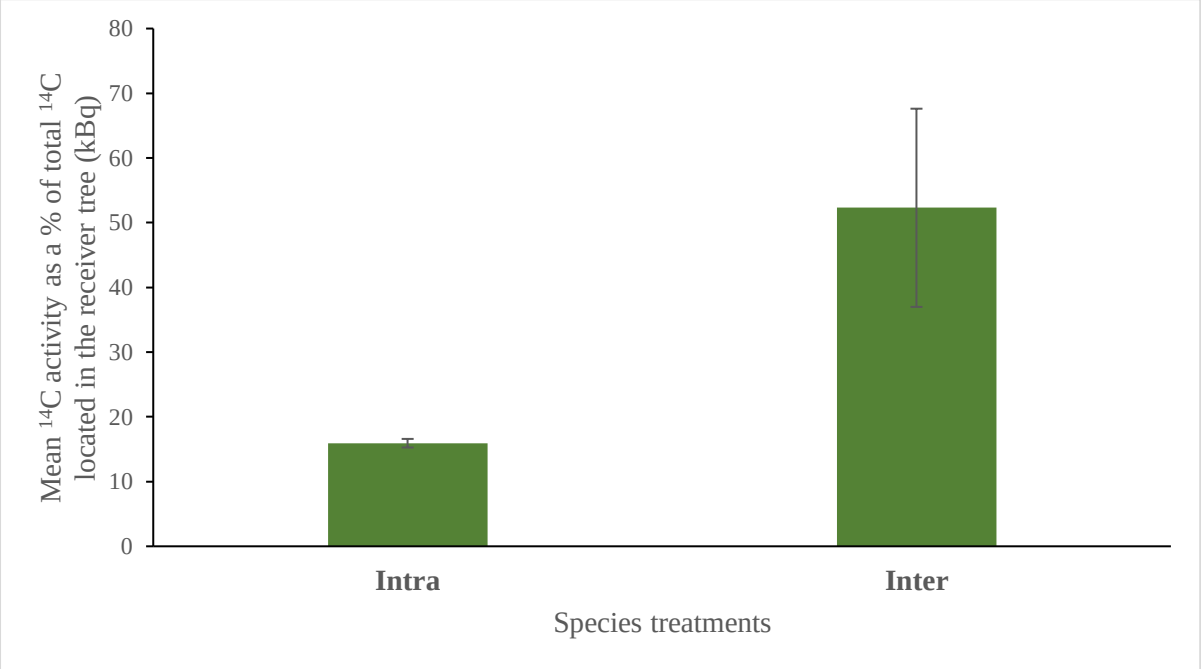

Figure 2: Mean total ^{14}C activity located in the intra- and inter-specific species treatments of the receiver trees in kBq. Data are mean ± SE (n = 12).

3.2.2. ^{14}C activity in receiver tree partitions

Table 2: The activity of the partitions (kBq) divided into intra- and interspecific treatments for comparison. Data are mean ± SE (*n* = 4). Significant differences are highlighted in bold.

	Mesocosm partitions									
	Leaves	**Branch**	**Coarse roots**	**Fine roots**	**Plant total**	**Sand**	**Soil CO_2**	**Soil H_2O**	**Soil**	**Total mesocosm**
Intra	0.63± 0.12	0.73± 0.13	0.57± 0.15	0.37± 0.12	2.31± 0.37	2.91± 0.16	0.14± 0.03	0.00± 0.00	13.48± 0.45	15.9± 0.7
Inter	0.61± 0.10	0.76± 0.18	16.20±10.31	6.96± 3.90	24.53±14.07	4.46± 0.81	1.10± 0.37	0.00± 0.00	26.67± 5.80	52.3± 15.3
P-value	0.92	0.90	0.14	0.11	0.13	0.73	**0.02***	0.57	**0.03***	**0.03***

The interesting results from the analysis of the inter- and intraspecific ^{14}C activity (Table 2) of the partitions included; that the allocation to the tree tissues were similar between the treatments with no significant differences found for leaves, branches and stems, coarse roots, fine roots or total plant activity. The mean ^{14}C activity located in the interspecific soil treatments were found to be twice that of the intraspecific treatment,

(13.5 ± 0.5 kBq and 26.7 ± 5.8 kBq, respectively) and were found to be significantly different (P = 0.03). The soil respiration showed a similar trend with the mean interspecific treatments total soil respiration being almost eight times more than the intraspecific and the results were found to be significant (P = 0.03). Total mean plant ^{14}C activity was three times as much in the interspecific as the intraspecific treatments, this difference was found to be significant (P = 0.03). This data is presented in graphical form for ease of comparison (Figure 3).

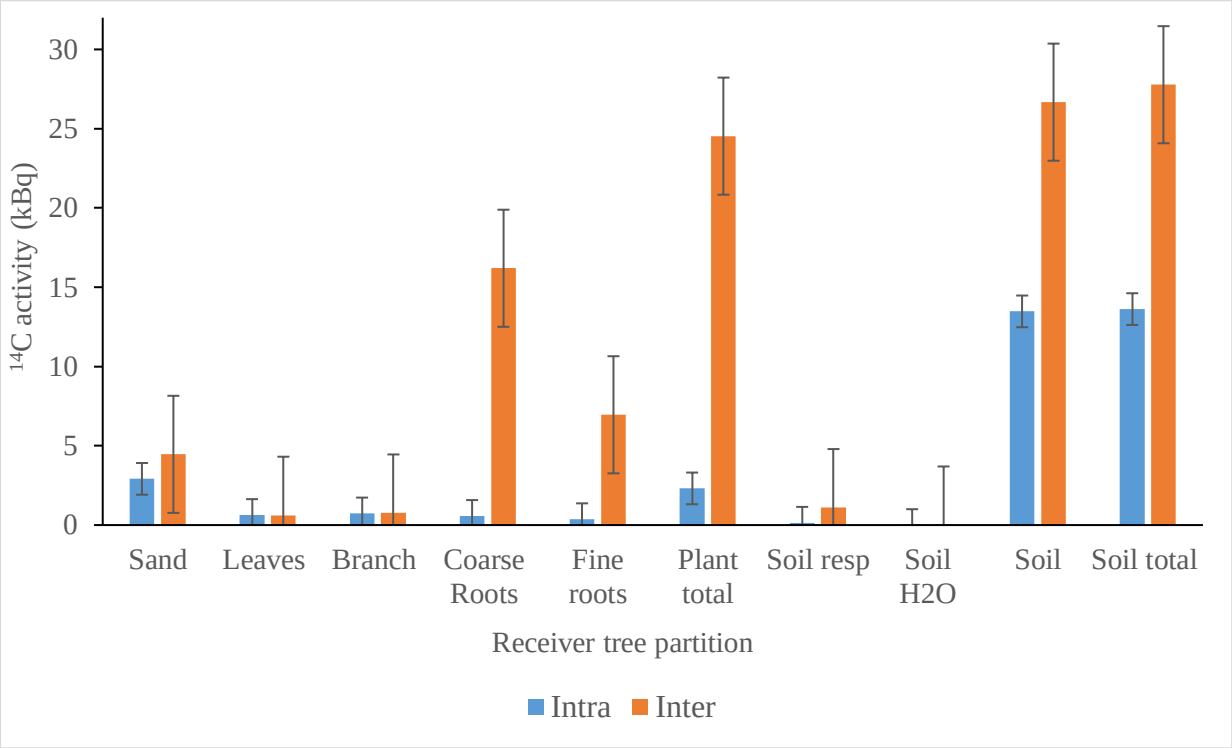

Figure 3: ^{14}C activity of intra- and inter-specific receiver tree partitions (kBq). Data is mean (n = 12 ± SE).

3.5.5. Receiver plant relative ^{14}C partition allocation

The mean ^{14}C activity counted in the receiver tree had been transferred from the donor tree via CMN and this data is tabulated with the standard error in Table 3. The A-A treatments have mean ^{14}C leaf and branch activity (0.25 ± 0.11 % and 0.25 ± 0.18 %) which appears to be four times larger than most other treatments and twice as much as the next largest (Table 3). However, statistical analysis revealed no significant differences between treatments either when considered as individual treatments (6 in total), or when grouped together as two treatments (inter- and intra-specifically).

Table 3: Allocation of ^{14}C activity to the plant's tissues in the receiver trees as a percentage of the total ^{14}C activity counted in the entire mesocosm, for the six species treatment combinations (A- *A. glutinosa*, B- *B. pendula*, and C- *C. sativa*). Intraspecific and interspecific combinations are grouped together for comparison. Data are mean ± SE (n = 4).

	Biomass partition			
Species combination	**Leaves**	**Branch**	**Coarse root**	**Fine root**
A-A	0.25 ± 0.11 %	0.25 ± 0.18 %	0.14 ± 0.09 %	0.11 ± 0.06 %
B-B	0.06 ± 0.01 %	0.08 ± 0.03 %	0.08 ± 0.03 %	0.02 ± 0.01 %
C-C	0.03 ± 0.01 %	0.07 ± 0.01 %	0.04 ± 0.01 %	0.04 ± 0.01 %
B-A	0.04 ± 0.15 %	0.03 ± 0.01 %	0.97 ± 0.93 %	0.49 ± 0.47 %
C-A	0.09 ± 0.02 %	0.10 ± 0.02 %	0.24 ± 0.18 %	0.27 ± 0.15 %
C-B	0.02 ± 0.01 %	0.03 ± 0.01 %	1.22 ± 0.39 %	0.41 ± 0.39 %

To try and visualise the differences between the intra and inter-specific receiver ^{14}C allocation, we expressed the receiver allocation as a percentage of total ^{14}C located in the entire receiver tree (Figure 4). This illustrates how the interspecific combinations appear to have a greater proportion of the ^{14}C activity in the belowground tissues when compared to the intraspecific combinations.

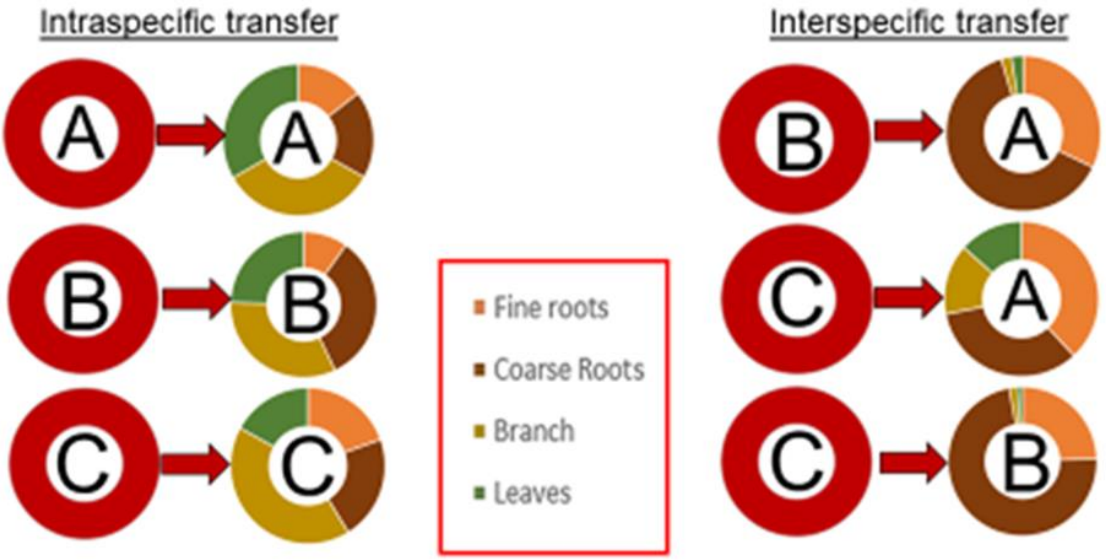

Figure 4: Allocation of ^{14}C activity to the plant's tissues in the receiver trees as a percentage of the total ^{14}C activity counted in the mesocosms, for the six species treatment combinations (A- *A. glutinosa*, B- *B. pendula*, and C- *C. sativa*). Intraspecific and interspecific combinations are grouped together for comparison. Data are means (n = 4).

3.6. Total transfer

The results of the total amount of ^{14}C that was transferred inter and intra-specifically through the interlinking Perspex tube showed that transfer was highest in *A. glutinosa* to *A. glutinosa* treatments (Figure 5). Overall, the treatments that included *A. glutinosa* had slightly higher transfer and those species combinations without *A. glutinosa* showed the least amount of transfer, although overall the results were not shown to be significant ($P = 0.63$).

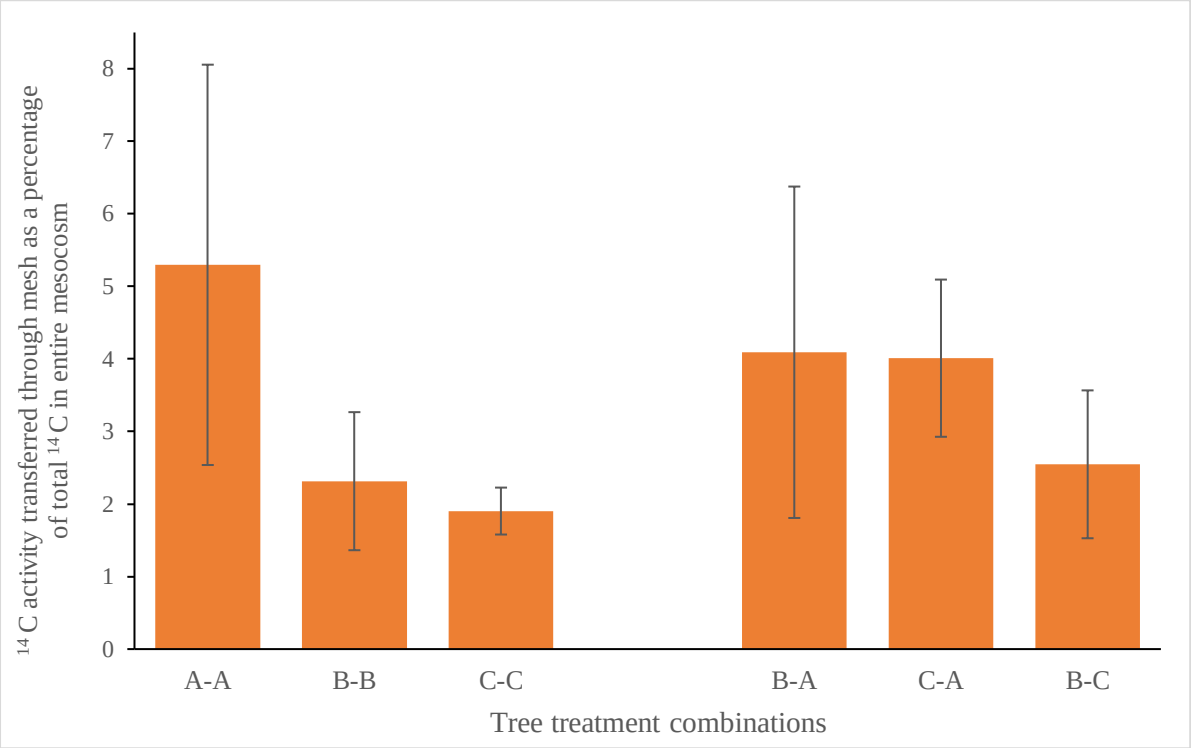

Figure 5: Total ^{14}C activity transferred through the mesh expressed as a percentage of the total ^{14}C activity in the entire mesocosm, in the six species treatment combinations (A- *A. glutinosa*, B- *B. pendula*, and C- *C. sativa*). Intra-specific and inter-specific combinations are grouped together for comparison. Data are mean ± SE ($n = 4$).

3.7. Estimated ^{14}C transfer to micro-organisms via mycorrhizal fungi

The ^{14}C activity that was transferred though the mesh was assumed to be transferred by mycorrhizal fungal hyphae. This includes the ^{14}C activity located in the soil, the soil solution and the total soil respiration from under the receiver tree. This data is plotted in Figure 6.

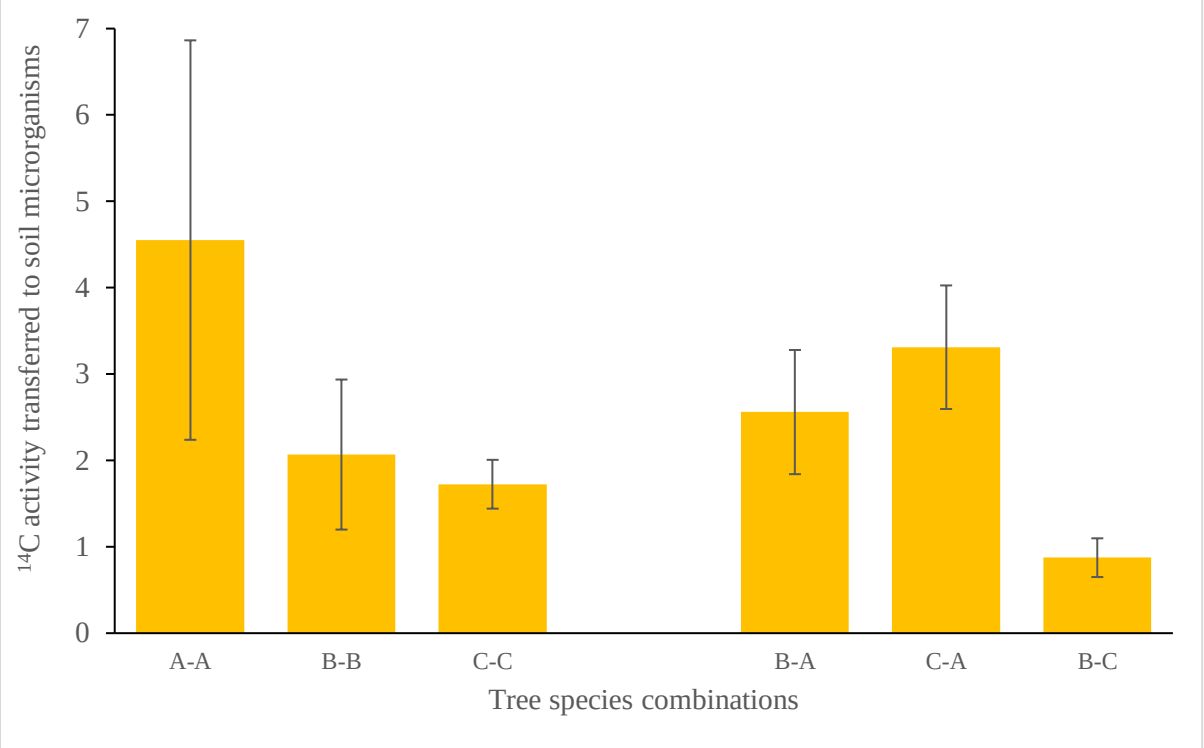

Figure 6: Total ^{14}C activity estimated to have been transferred to the soil micro-organisms in the receiver plant pot, expressed as a percentage of the total ^{14}C activity in the entire mesocosm, in the six species treatment combinations (A- *A. glutinosa*, B- *B. pendula*, and C- *C. sativa*). Intra-specific and inter-specific combinations are grouped together for comparison. Data are mean ± SE (n = 4).

3.8 Fungal DNA analysis

To determine the driver of the interspecific C transfer it was decided to analyse the soil to determine which fungal symbionts were present and therefore distinguish likely conduits for biogeochemical cycling in the soil and subsequent transfer between trees. The percentage DNA is presented in Table 4.

Table 4: DNA species data tabulated as a percentage of total DNA extracted and replicated.

Fungal species	**Tree species**	***C. sativa***		***C. sativa***	***B. pendula***		***C. sativa***
	Ecology	**Donor**	**Sand**	**Receiver**	**Donor**	**Sand**	**Receiver**
Sphaerosporella brunnea	MR EM	69.03%	84.85%	78.55%	95.48%	90.95%	91.72%
Inocybe curvipes	MR EM	23.88%	6.69%	0.29%	0.11%	0.11%	0.23%
Laccaria oblongospora	MR EM	0.11%	0.16%	20.28%	0.03%	0.00%	0.63%
Penicillium abidjanum	SAP	0.28%	7.15%	0.27%	0.25%	1.66%	0.31%
Peziza sp.	MR EM	0.00%	0.00%	0.00%	0.00%	4.70%	0.08%
Fusarium oxysporum	PARA SAP	0.75%	0.35%	1.23%	0.87%	0.30%	1.65%
Inocybe rufoalba	MR EM	0.04%	4.71%	6.27%	0.00%	0.00%	0.03%
Hebeloma sp.	MR EM	1.23%	0.00%	1.18%	1.04%	0.08%	0.00%
Trichothecium ovalisporum	PATH	2.55%	0.00%	0.11%	0.05%	0.00%	1.33%
Thelephora terrestris	MR EM	0.63%	0.29%	1.07%	0.03%	0.00%	0.28%
Capnodiales sp.	SAP ENDO	1.15%	0.16%	0.07%	0.16%	0.06%	0.22%
Trichothecium roseum	SAP	0.00%	0.15%	3.43%	0.02%	0.00%	0.05%
Aureobasidium pullulans	EPI ENDO	0.00%	0.00%	0.03%	0.06%	0.01%	0.42%

We plotted the percentage frequency of the mycorrhizal species against each other (Figure 7). *Sphaerosporella brunnea* was the most abundance in all species treatments with very little difference between species treatments. No significant difference was found between the treatment combinations (P = 0.94).

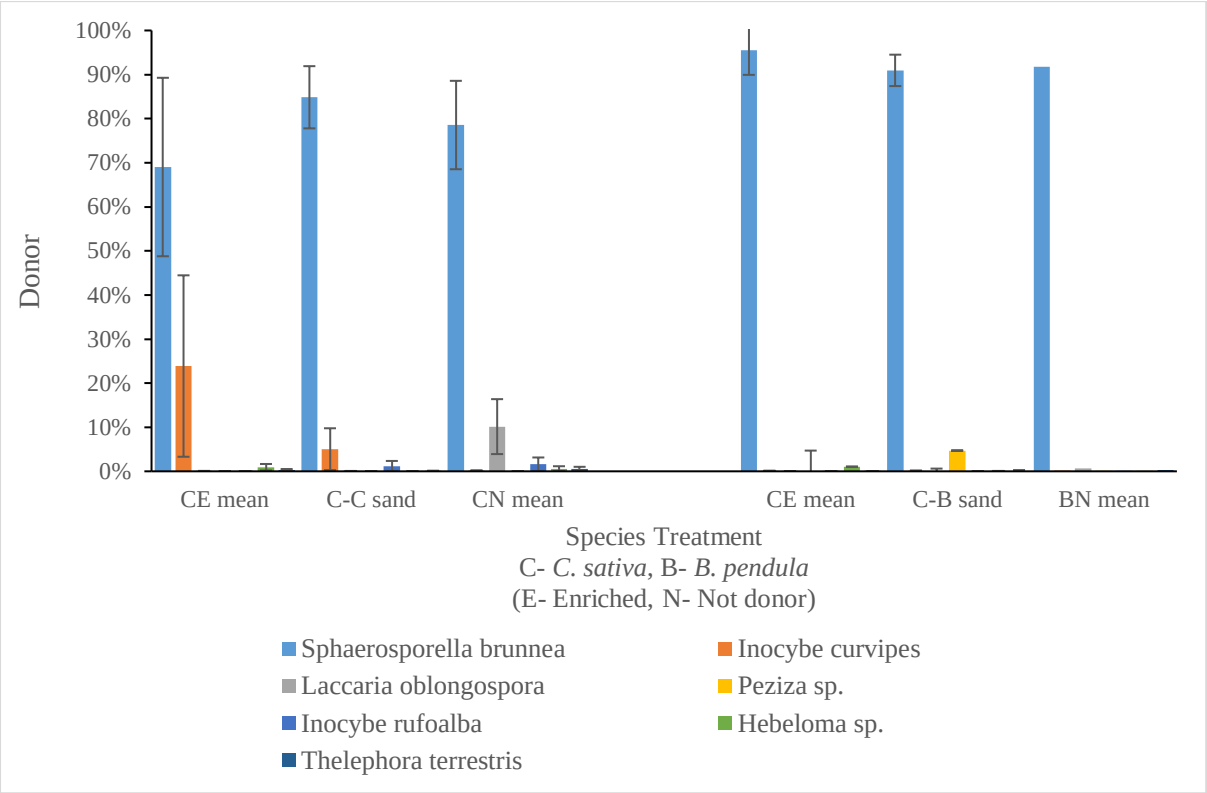

Figure 7: Results from the soil and sand DNA analysis of the two most contrasting treatments (*C. sativa* to *C. sativa* and *C. sativa* to *B. pendula*). Data are mean abundance expressed as a percentage of total of DNA extracted ± SE (n = 4)

We then plotted the percentage frequency of the mycorrhizal species identified in the sand against each other (Figure 8). These data were interesting as the mycorrhizal species located in the sand must be the species responsible for the CMN formation linking the two plants and subsequent belowground ^{14}C activity transferred from the donor to the receiver trees.

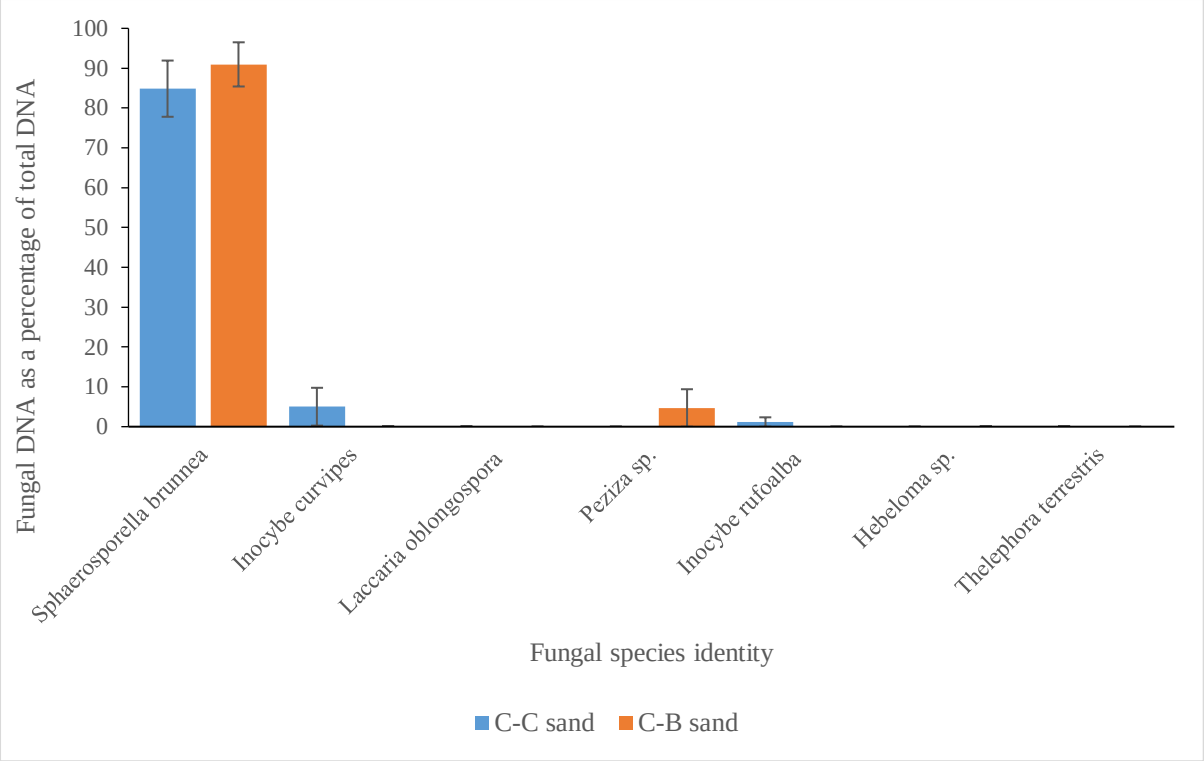

Figure 8: Comparison of the mean DNA abundance of the seven most frequently identified species, in the sand connecting the two fractions of the mesocosm, in the two treatments with the most contrasting ^{14}C transfer results expressed as a percentage of total DNA extracted ± SE (n = 4)

4. Discussion

4.1. Soil chemical properties

The differences in soil conditions under the two species which we would expect after the establishment period were not observed. Therefore, the identity of the tree species made no significant difference to the soil conditions. Overall, the *A. glutinosa* pots had slightly more ammonium than the other species treatments but not nitrate, which is surprising as the *A. glutinosa* treatments are actinorhizal. This could be as a result of the nitrate fixed by the *Frankia alni* in the *A. glutinosa* root nodules being transferred to the non-actinorhizal species, further studies using ^{15}N could confirm this. This transfer of N is highly likely in both common arbuscular mycorrhizal networks (Frey and Schuepp, 1992, 1993; Johansen and Jensen, 1996; Montesinos-Navarro, 2019) and via common ectomycorrhizal networks (Arnebrant et al., 1993; Ekblad and Huss-Danell, 1995; Wipf et al., 2019).

4.2. ^{14}C activity in donor trees

The consistent mean total activity and comparable error bars of the donor trees suggests that the enrichment with $^{14}CO_2$ pulse was successful, was consistent across all species treatment combinations allowing us to compare the ^{14} C activity of the receiver plants with a degree of certainty.

The differences in the allocation of ^{14}C to the donor tree tissues illustrated the differences in relative biomass of the three species. Overall, the mean results showed that the *A. glutinosa* trees had the greatest proportion of leaves (34.9 ± 9.1 %) followed by *B. pendula* (21.5 ± 5.5 %), the *C. sativa* (18.7 ± 1.8 %). The results found to be significant were the ^{14}C allocation to the fine root, soil respiration and soil solution. The mean ^{14}C allocation to fine-roots in the *A. glutinosa* to *A. glutinosa* species treatments was found to be significantly different from the *C. sativa* to *C. sativa* treatments. This may be as a result of the fine roots not being separated from and being homogenised with the root nodules. Later experiments will distinguish between the ^{14}C activity in the root nodules by separating them from the fine roots and analysing the ^{14}C activity independently. Interestingly, the soil solution and soil respiration had the same significant trend, which was that the *A. glutinosa* to *A. glutinosa* treatment had the highest soil respiration (6.39 ± 1.06 %) and was significantly different to the treatment with the lowest soil respiration *B. pendula* to *A. glutinosa* (2.30 ± 1.20 %). This could be a result of the actinorhizal relationship that *A. glutinosa* with *Frankia alni*. In addition, Burton et al., (1998) found that under non-drought conditions there is a direct correlation between increased soil respiration and fine-root N concentration and suggests that root N concentration and temperature can be used a predictor for belowground respiration. As our experiment took place in black coloured pots placed in a glasshouse, soil temperatures are likely to have been elevated and could go some way to explaining this result.

4.3. ^{14}C activity of the connecting sand tube

The ^{14}C activity results for the sand in the connecting Perspex tubes are interesting not least because they do not directly correlate to the total ^{14}C activity that was counted in the receiver trees. This suggests that some of the species treatments have transferred ^{14}C activity through the mesh via mycorrhizal hyphae but not then passed the ^{14}C activity onto the receiver tree. The *A. glutinosa* to *A. glutinosa* treatment showed the most ^{14}C activity in the sand but the least ^{14}C activity in the receiver tree. It could be that the ^{14}C activity is being utilised for hyphal growth by the fungi and not transferred or as there was only a single harvesting event it could

be that the transfer between plants of the same species is delayed and that given a longer chase-period the ^{14}C activity would have been detected in the receiver plant. Although the literature suggests that transfer can occur within 24 hours for plants of the same species in the case of volatiles believed to defend the plant against aphid attack (Babikova et al., 2013). This would be interesting to investigate in future studies.

4.4. ^{14}C activity in receiver mesocosm partitions

The soil solution data was the first data that we collected which confirmed the ^{14}C activity had passed through the sand and both the mesh Perspex tube coverings. The ^{14}C activity in the soil solution was as we would expect from the sand data, with the exception of the *B. pendula* to *A. glutinosa* treatment where the soil solution contained less ^{14}C activity than expected, suggesting that the ^{14}C activity that was transferred through the sand was retained in the soil or respired by the soil microbial community. Although no statistically significant difference in the soil ^{14}C activity was found in the soil respiration traps, the results seem to suggest that the ^{14}C activity found in the respiration traps was slightly lower in all intraspecific treatments when compared to interspecific combinations.

The interesting result from this data was the trend seen in the *B. pendula* to *A. glutinosa* treatments. Namely, the *B. pendula* to *A. glutinosa* treatments have relatively high ^{14}C activity in the soil but lower counts in soil solution and soil respiration than would be expected considering the ^{14}C activity located in the soil. This suggests that the ^{14}C activity is residing in the soil for longer, possibly as hyphal biomass and not being exuded and metabolised by the soil micro-organisms as quickly as the other treatments. It could be that the ^{14}C had been incorporated into the structural component of the fungal mycelium.

4.5. ^{14}C transfer via CMN

The total ^{14}C transfer was expected to be greater in the intra- than inter-specific treatments; this prediction was based on several previous studies which suggested that C transfer is preferential when receiver plants are full-siblings of the donor tree (Pickles et al., 2017). A theory known as the *kin selection* theory is widely supported by previous studies (Graves et al., 1997; Verbruggen et al., 2012; Platt and Bever, 2009; Dudley et al., 2013; Murphy and Dudley, 2009; Asay, 2013). The *mother tree* hypothesis goes further suggesting that recognition can go as far as trees sensing their root exudates of their offspring (Nara, 2006). The likely mechanism for this would mediation by the mycorrhizal fungi's ability to recognise kin root exudates and for this to stimulate increased transfer (Biedrycki et al., 2010). Other studies suggest that kin

recognition could stimulate hyphal growth such as File et al., (2012), that found that the root colonization, size of mycorrhizal network, number of arbuscles were greater when *Ambrosia artemisiifolia* L. was grown with siblings compared to non-siblings but that the root lesions were reduced in number. Many of these studies focussed on the less complex symbiotic relationship with arbuscular mycorrhizae and it is possible that this trend is a result of soil or plant nutritional status and not directly related to plant fungal symbiotic interactions. Contrastingly, however, many other studies confirm the transfer of C between interspecific tree treatments (Simard, 1995; Simard et al., 1997a, Simard et al., 1997b) and a recent meta-analysis suggests that the greater the phylogenetic distance between species the greater the ectomycorrhizal facilitation (Montesinos-Navarro et al., 2019).

4.6. Total transfer

The ^{14}C activity data for the receiver trees suggested that there was more transfer in the interspecific transfer treatments. After a literature search this left us with several possible hypotheses namely;

1) If the mycorrhizal fungi present are species-specific, but connected to each other, then multiple tree species would mean more fungal diversity than single species mesocosms. Increased mycorrhizal diversity could then lead to more ^{14}C activity being transferred between tree species.

2) If the mycorrhizal fungi present are not species-specific and are soil substrate or climate specific then more ^{14}C activity could have been transferred from the donor to three receiver trees as a result these treatments having greater mycorrhizal colonisation.

However, fungal DNA data failed to prove that the treatment exhibiting the most ^{14}C transfer had either a greater fungal diversity or abundance. This led us to postulate that the differences in transfer are due to a source sink relationship, i.e. the plants with the most metabolically active below microbial symbionts will allocate the most photosynthetically fixed C belowground. Partners within the plant microbe symbiosis are able to determine the energetic demands of the partner (Kiers et al., 2011; Fellbaum et al., 2012; Fellbaum et al., 2014). It is also known that trees species can differ in the amount of photosynthetically derived C that they are prepared to invest in the CMN (Walder et al., 2012), potentially through a biological market trading system (Wyatt et al., 2014), also known as the economic game theory (Archetti et al., 2011) and in some cases the presence of the symbiotic partnership magnifies the inequity (Weremijewicz and Janos, 2013; Booth and Hoeksema, 2010). We hypothesised that transfer

would be most in the intraspecific species treatment either as plants can used CMN to transfer resources to their kin (Pickles et al., 2017) possibly mediated by volatile cues (Karban and Shiojiri, 2009; Karban et al., 2013). The results although not statistically significant suggested that the transfer was in fact more in the interspecific treatments. However, Montesinos-Navarro et al., (2019) conducted a meta-analysis of plant facilitation through mycorrhizal networks and suggested that facilitative resource sharing increases with trading plant phylogenetic distance. This could be as the nutritional demands of distantly related plants and their nutrient resource strategies may differ (Cornwell et al., 2014).

4.7. Fungal DNA analysis

Analysis of the fungal DNA in the sand and soil revealed that the communities were dominated by three mycorrhizal species namely *Sphaerosporella brunnea, Inocybe curvipes* and *Laccaria oblongospora* with the mean % of total DNA being 85.1 ± 4.0 %, 5.2 ± 3.9 % and 3.5 ± 3.4 % respectively for the three fungi species, making these three species 93.9 % of the total DNA on average. The fact that the *Laccaria* sp. could be important as Chen et al., (2000) found that in a *Eucalyptus* species which exhibits dual mycorrhizal symbiosis (both arbuscular and ectomycorrhizal), that the presence of *Laccaria* sp. significantly reduces colonisation by arbuscular mycorrhizal symbionts. The mechanism for this is not yet fully understood (Lodge, 2000) but several theories have been postulated including; that as *Laccaria* is an ectomycorrhizal fungi with a mantle, that this may act as a physical barrier to arbuscular mycorrhizal colonisation (Chilvers et al., 1987; Chilvers and Gust, 1982), or the fungi may be exuding chemical deterrents to the colonisation of the root by arbuscular mycorrhizal fungi (Lodge and Wentworth, 1990), or it could just be as a result of greater competition for plant derived carbohydrates (Lodge, 2000). Chen et al., (2000) did find that the presence of *Laccaria* sp. modified the root architecture, specifically by reducing the growth of new root tips and thereby reducing the availability of suitable spots for arbuscular fungal colonisation.

The barcodes used for identifying species are located within ribosomal RNA genes (George et al., 2019). Specifically, for fungi, the internal transcribed spacer (ITS) section is utilised to identify taxonomic groupings (Schoch et al., 2012). This novel methodology has led to advances in our understanding of fungal diversity (Kumar et al., 2019), but it also has been criticised for potential bias based on the ITS region chose and the fungal database used to identify species (Tedersoo et al., 2016; Xue et al., 2019). ITS barcoding is considered particularly limited when concerned with unknown samples as analysis can only identify to

family level, at best (Cavender-Bares et al., 2009). Use of ITS barcodes has also been found to be particular unreliable in identifying the Glomeromycetes (Stockinger et al., 2010). For example, in bulk soil this method has been found to underestimate Glomeromycetes (Berruti et al., 2017), which would in this case be arbuscular mycorrhizal associated with *A. glutinosa*. Future studies could use the 18S region which is better to suited to identifying the presence of Glomeromycetes

5. Conclusions

H_1: Transfer of C by CMNs is greater inter-specifically than intra-specifically.

H_2: Greater mycorrhizal diversity per unit of rhizosphere soil will result in more C transfer between trees via the CMN.

H_1: The transfer appeared to be more in the inter-specific species treatments than the inter-specific although this was not born out statistically. This could be at least in part as a result of multiple biological interactions with potential variations within tree species examples of this are differences in; root architecture leading to differences in belowground sink strength and aboveground biomass differences and C allocation differences. These are then confounded by more potential differences in root colonisation by mycorrhizal fungi. In short, the sample size was probably too small for a complex biological chain gang such as this and as a result very few differences were found to be significant.

H_2: DNA analysis of the soil under the two most contrasting treatments (in terms of the amount of photosynthetically derived C that was transferred via CMN to the receiver tree), failed to find differences in species diversity. This hypothesis was therefore rejected.

The transfer between plants of resources, in this case C, does not appear to be driven by either the diversity of abundance of mycorrhizal fungi in the interconnected sand tube. We postulate therefore that the fungal mediated resource transfer is most likely determined by the supply and demand of the partners participating in the symbiotic relationship. It could be that each species, due to differences in nutrient demand during different developmental stages, has a period of supplying the other with photosynthetically derived, only for this to be reciprocated at a later stage once the other species enters a period of increased growth rate.

6. Acknowledgements

This work was funded by NERC through Envision DTP. The authors would also like to acknowledge the role that technicians Jonathon Roberts and Sarah Chesworth had in producing this research and all the help and support I received in the field and laboratory from Léa Sgro.

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8. Appendices/ Supplementary

8.1. ^{14}C activity in donor trees

8.1.1 Total ^{14}C activity in donor trees

The activity of the donor trees was used as an indication that the pulse labelling event was successful across all species treatment combinations (Figure 8). ^{14}C activity was seen to be consistent across species treatment combinations with no significant differences found between species treatment combinations (P = 0.62) and standard errors were seen to be very similar for all treatments.

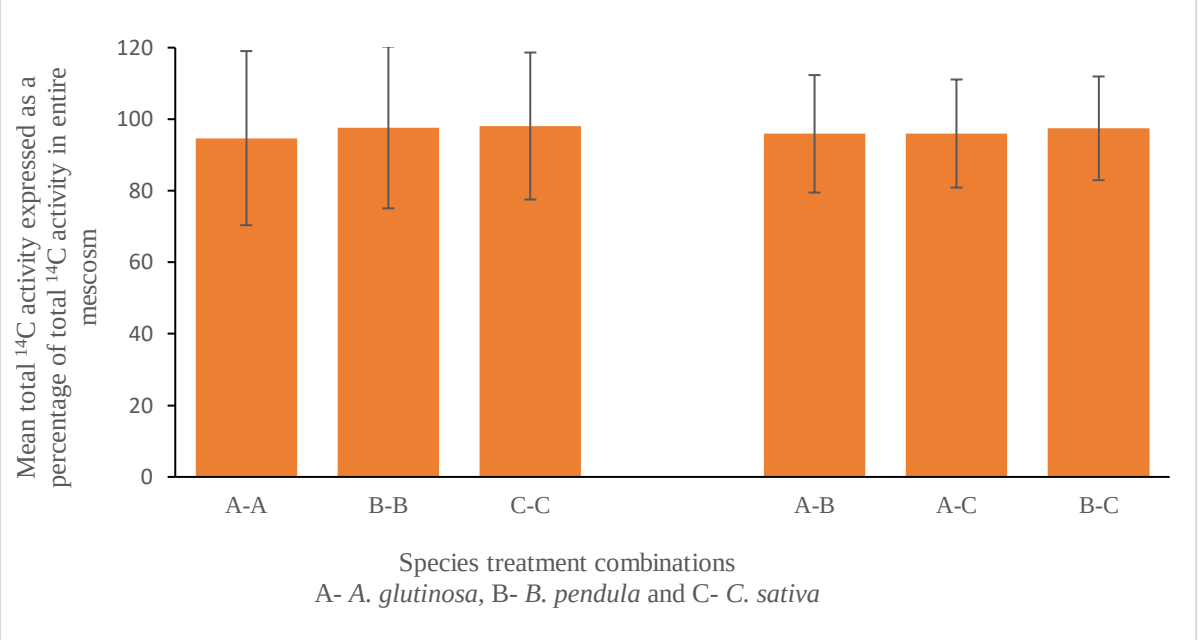

Figure S1: ^{14}C activity measured in donor plant partitions expressed as a percentage of total 14 C activity counted in entire mesocosm in the six species treatment combinations (A- *A. glutinosa*, B- *B. pendula*, and C- *C. sativa*). Data are mean ± SE (n = 4).

8.1.2 Partition allocation in donor plants

To establish if there were differences in belowground C allocation between species the ^{14}C partition allocation of the donor plants was investigated and the results are shown in Table 4.

Table S1: Mean ^{14}C activity expressed as a percentage of the ^{14}C activity in the entire mesocosm ± SE of the six species treatment combinations (A- *A. glutinosa,* B- *B. pendula* and C- *C. sativa*) determined by destructive harvesting 336 hours after the $^{14}CO_2$ pulse (*n*= 4). Statistically significant results are given in bold and denoted by superscript letters and asterisks (*, $P < 0.1$).

	Donor plant partition						
Treatment	**Leaves**	**Branch**	**Coarse root**	**Fine root**	**Soil respiration**	**Soil solution**	**Soil**
A-A	34.98 ±9.31	12.57 ±2.16	7.16 ±1.88	2.60 ±0.50	6.39 ±1.06	<0.01 ±0.00	31.00 ±9.42
B-B	21.15 ±5.35	21.39 ±4.34	5.35 ±1.59	4.88 ±1.67	5.44 ±0.67	<0.01 ±0.00	39.48 ±9.00
C-C	18.67 ±1.76	16.73 ±4.36	9.98 ±2.05	8.28 ±1.33	5.16 ±0.91	<0.01 ±0.00	39.28±10.13
B-A	26.60 ±3.44	23.92 ±4.07	9.55 ±0.49	5.14 ±1.03	2.30 ±1.20	<0.01 ±0.00	28.40 ±6.20
C-A	21.94 ±3.54	13.11 ±2.04	7.50 ±1.83	5.71 ±1.13	3.91 ±0.52	<0.01 ±0.00	43.82 ±6.05
C-B	28.76 ±4.59	27.03 ±4.67	7.37 ±0.63	2.90 ±0.96	3.61 ±0.63	<0.01 ±0.00	27.79 ±3.01
P value	0.30	0.07	0.50	**0.03***	**0.03***	**0.03***	0.59

The allocation of ^{14}C activity results show that the highest percentage of ^{14}C was retained in the leaves in the donor *A. glutinosa,* although this was not found to be significant ($P = 0.30$). In the branch and stem partition the donor *B. pendula* treatments retained more ^{14}C as a percentage of overall ^{14}C activity than the other species, although this was not found to be significant ($P = 0.50$). Coarse roots appeared not to show allocation trends by species. In contrast, the fine root results showed more activity in the *C. sativa* to *C. sativa* species treatment combinations ($P = 0.03$). A post-hoc Tukey test revealed that the mean ^{14}C activity located in the fine-roots of the *A. glutinosa* to *A. glutinosa* species treatment combination was significantly different to the *C. sativa* to *C. sativa* ($P = 0.03$). No other combinations were found to be significantly different. Soil solution and soil respiration were both also found to have results that were significantly different. A post-hoc Tukey test showed in both the soil respiration and soil solution the significant differences in mean ^{14}C activity were between the *A. glutinosa* to *A. glutinosa* species treatments and the *A. glutinosa* to *B. pendula* combinations

(P = 0.03). The soil ^{14}C activity showed no trends and showed no significant differences between treatments.

8.2. Activity of the connecting sand tube

The results of the ^{14}C analysis of the sand enabled us to assess the transfer of ^{14}C from the donor tree to the mycorrhizal hyphae regardless of if the ^{14}C was then passed on to the receiver tree. The sand in the *A. glutinosa* to *A. glutinosa* treatment was seen to have the largest amount ^{14}C activity when expressed as a percentage of the total ^{14}C activity counted in the entire mesocosm (Figure 10). The species treatments were not found to be significantly different from each other (P = 0.43).

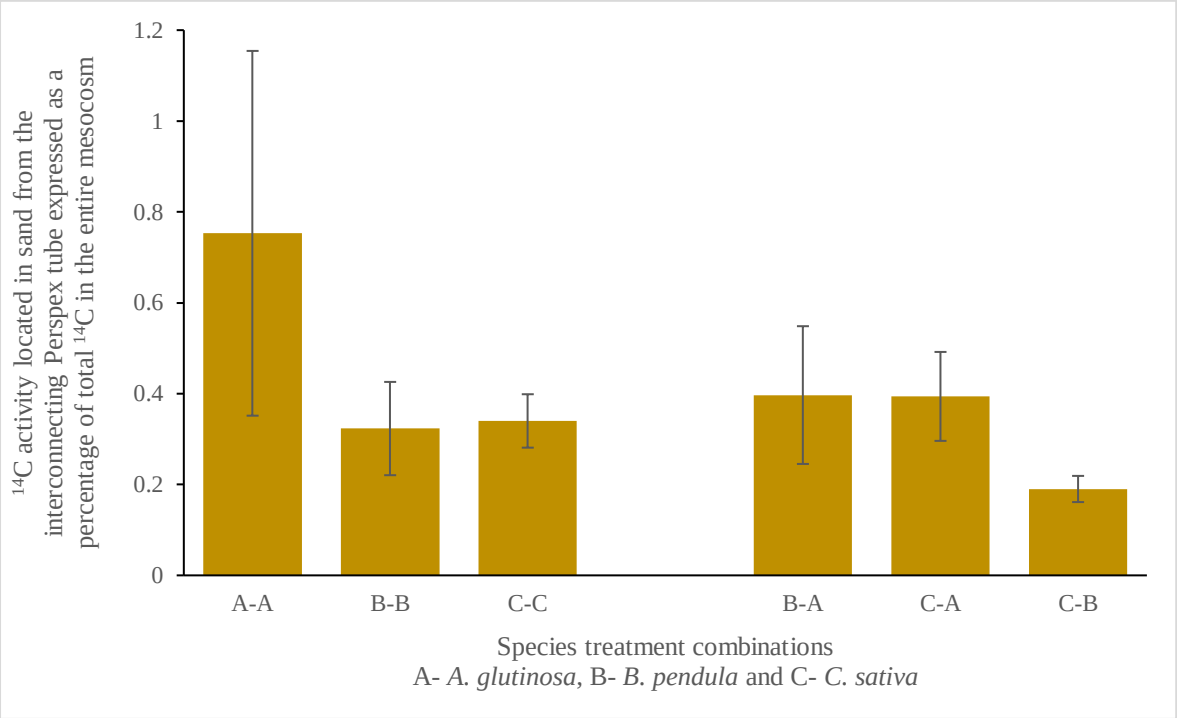

Figure S2: ^{14}C activity counted in the sand from the Perspex interconnecting tube in the six species treatment combinations (A- *A. glutinosa*, B- *B. pendula*, and C- *C. sativa*), post destructive harvesting 336 hours after the $^{14}CO_2$ pulse. Intraspecific and interspecific combinations are grouped together for comparison. Data are mean ± SE (n = 4).

8.3. ^{14}C activity in receiver mesocosm partitions

8.3.1 Soil solution ^{14}C activity

The soil water ^{14}C activity collected by Rhizon suction sampling devices from the receiver fraction showed that the intraspecific combinations seemed to have slightly higher ^{14}C activity counts (Figure 11), however, the results of the intraspecific combination were not found to be significantly different to the interspecific combinations (P = 0.41).

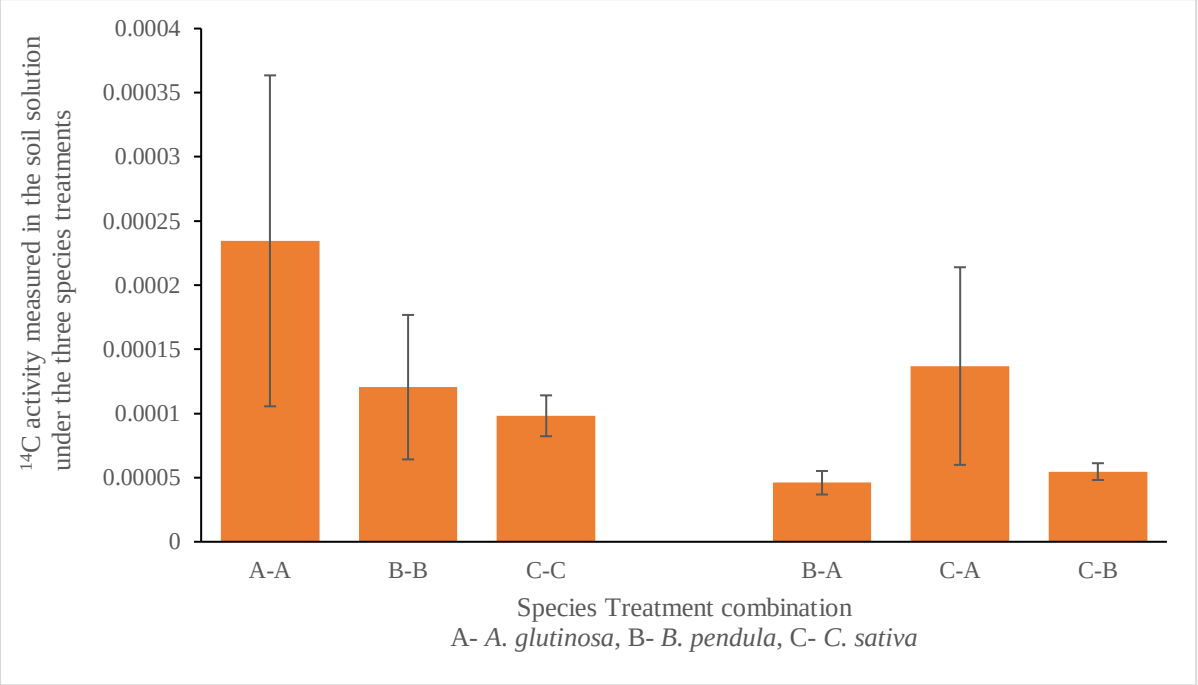

Figure S3: ^{14}C activity measured in soil solution extracted from the soil using a Rhizon suction sampler under the six species treatment combinations (A- *A. glutinosa*, B- *B. pendula*, and C- *C. sativa*). Data are mean ± SE (n = 4).

8.3.2. Soil ^{14}C activity

The ^{14}C activity in the soil under the receiver tree (Figure 12) showed largely similar results to that of the soil solution ^{14}C activity (Figure 11), with the exception of the combination B-A which appeared to have retained the ^{14}C activity in the soil relative to the ^{14}C counted in the soil respiration traps. The species treatments were not found to be significantly different to each other (P = 0.23).

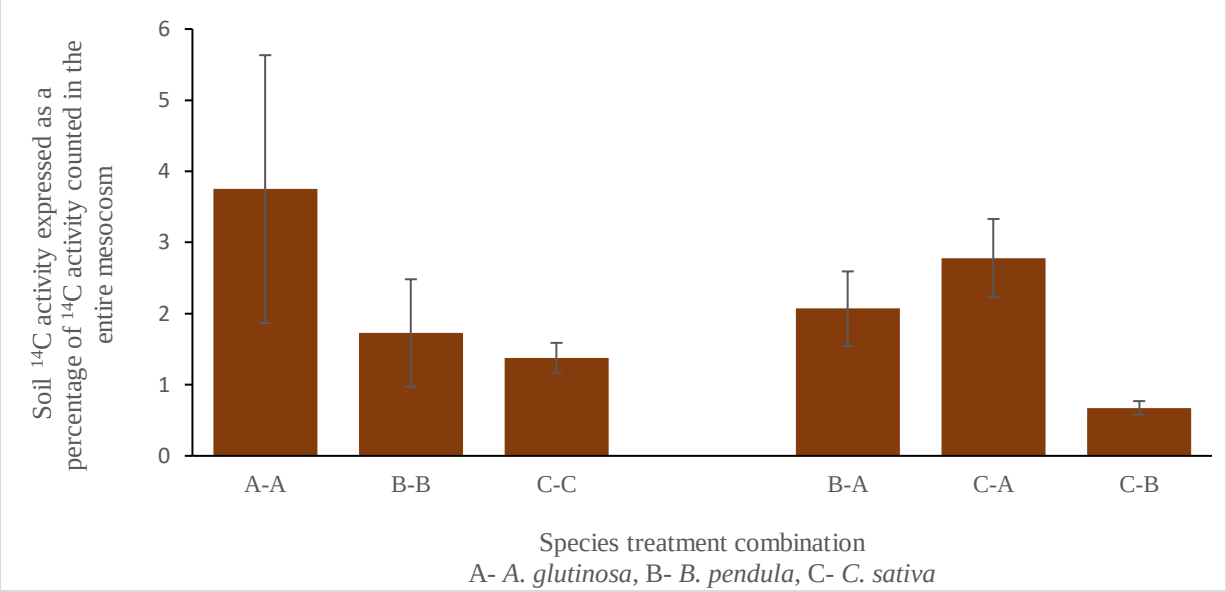

Figure S4: ^{14}C activity measured in soil under the six species treatment combinations and expressed as a percentage of total ^{14}C activity in the entire mesocosm (A- *A. glutinosa*, B- *B. pendula*, and C- *C. sativa*). Data are mean ± SE (n = 4)

8.3.3. Soil respiration

The accumulative soil respiration results (Figure 13) revealed some interesting results as the trend approximately followed those seen in both the soil water ^{14}C activity and soil ^{14}C activity. However, the soil respiration was lower than expected in the intraspecific species combinations based on the soil and soil water ^{14}C activity, although the intra and interspecific soil respiration were not found to be significantly different to each other (P = 0.09).

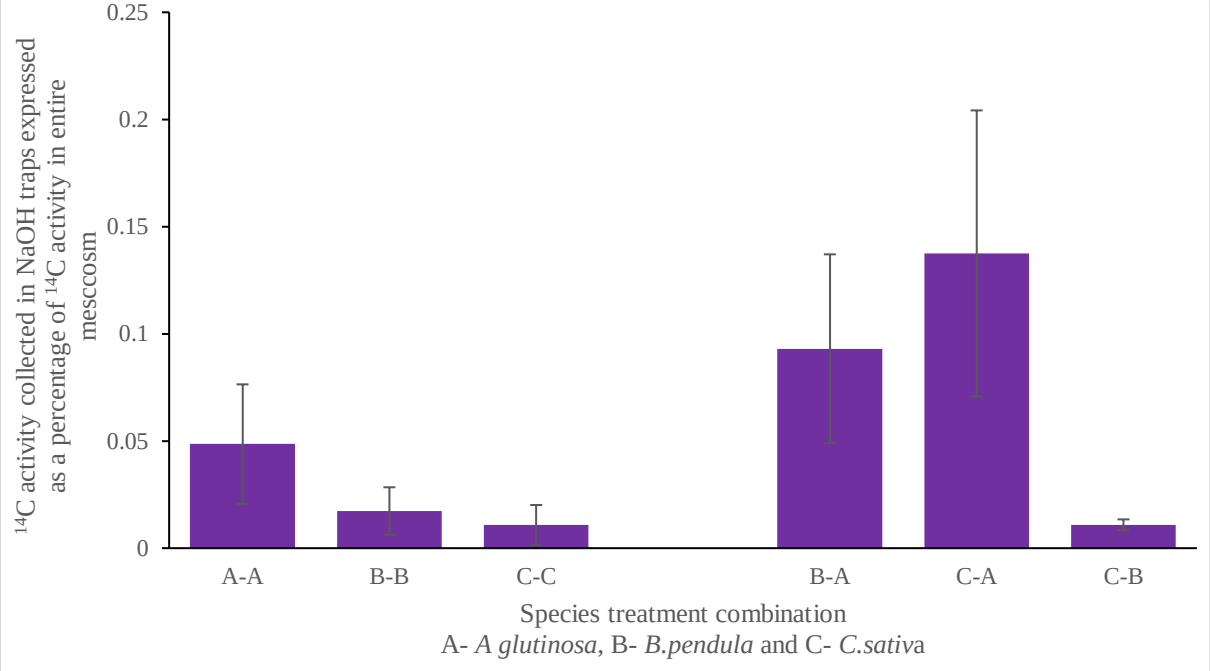

Figure S5: Accumulated soil respiration ^{14}C activity collected in sodium hydroxide soil respiration traps under the receiver tree in the six species treatment combinations (A- *A. glutinosa*, B- *B. pendula*, and C- *C. sativa*). Intraspecific and interspecific combinations are grouped together for comparison. Data are mean ± SE (n = 4).

8.3.4. ^{14}C activity in the receiver trees leaves

To try to further understand the differences in C allocation in the receiver trees, we investigated the ^{14}C in each of the plant tissues. The ^{14}C activity located in the receiver tree leaves is shown in Figure 2. The ^{14}C activity was higher in the *A. glutinosa* to *A. glutinosa* treatments and overall the mean leaf ^{14}C activity were found to be significantly different from each other (P = 0.02). A post-hoc test revealed that the *A. glutinosa* to *A. glutinosa* treatments had a mean leaf ^{14}C activity that was significantly different to the; *C. sativa* to *C. sativa* treatments (P = 0.04), the *A. glutinosa* to *B. pendula* treatments (P = 0.04) and the *B. pendula* to *C. sativa* treatments (P = 0.04).

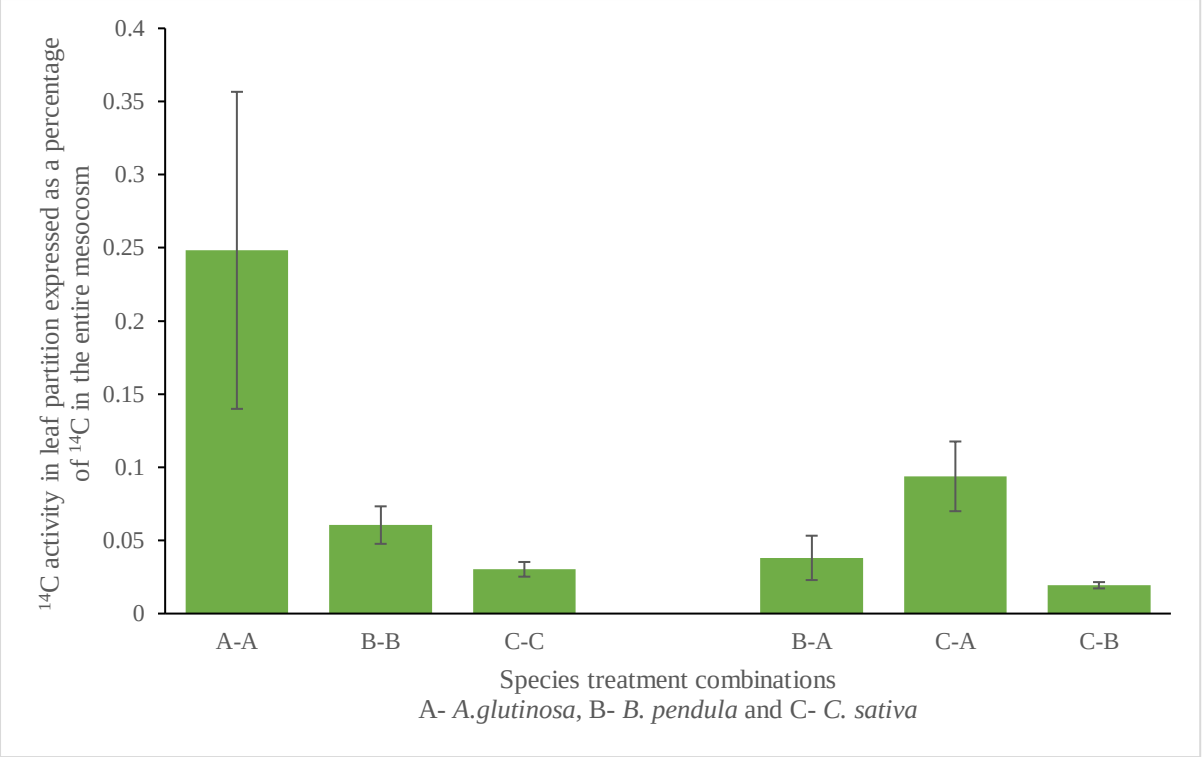

Figure S6: ^{14}C activity counted in the leaf partition of the receiver tree and expressed as a percentage of the total ^{14}C activity in the entire mesocosm, in the six species treatment combinations (A- *A. glutinosa*, B- *B. pendula*, and C- *C. sativa*). Intraspecific and interspecific combinations are grouped together for comparison. Data are mean ± SE (n = 4).

8.3.5. ^{14}C activity in receiver tree branches

The results of the receiver ^{14}C allocation to the branch and stem shows largely the same trend as was seen in the leaf tissue (shown in Figure 14), with a larger percentage of the entire mesocosm ^{14}C activity located in the branch and stem in the *A. glutinosa* to *A. glutinosa* species treatment combination, although again this was not a significant result (P = 0.39).

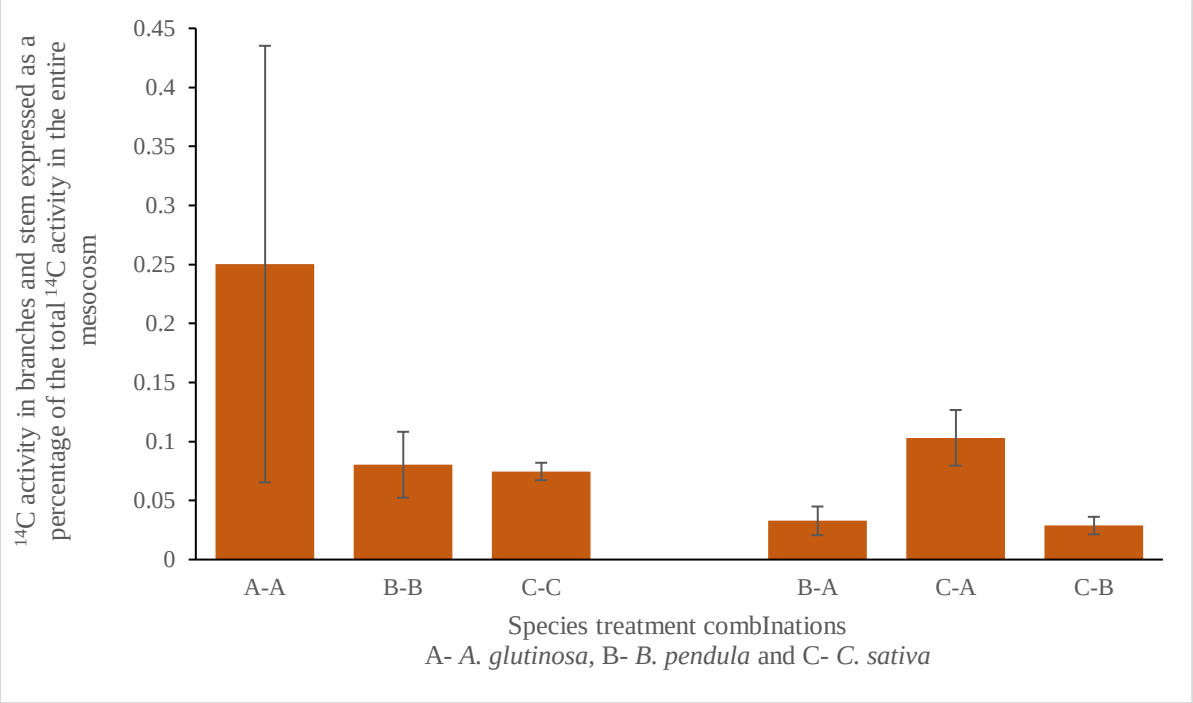

Figure S7: ^{14}C activity counted in the branch and stem partition of the receiver tree and expressed as a percentage of the total ^{14}C activity in the entire mesocosm, in the six species treatment combinations (A- *A. glutinosa*, B- *B. pendula*, and C- *C. sativa*). Intraspecific and interspecific combinations are grouped together for comparison. Data are mean ± SE (n = 4).

8.3.6. ^{14}C activity in receiver tree roots

The results of the analysis of the ^{14}C activity located in the coarse and fine roots are shown in Figure 16. Here the intraspecific treatment combinations appear to have proportionately more ^{14}C activity in them compared to the interspecific combinations. Overall, the total mean ^{14}C activity found in the roots results were not found to be significant (P = 0.66) neither were they significant for the coarse roots alone (P = 0.62) or the fine roots (P = 0.70).

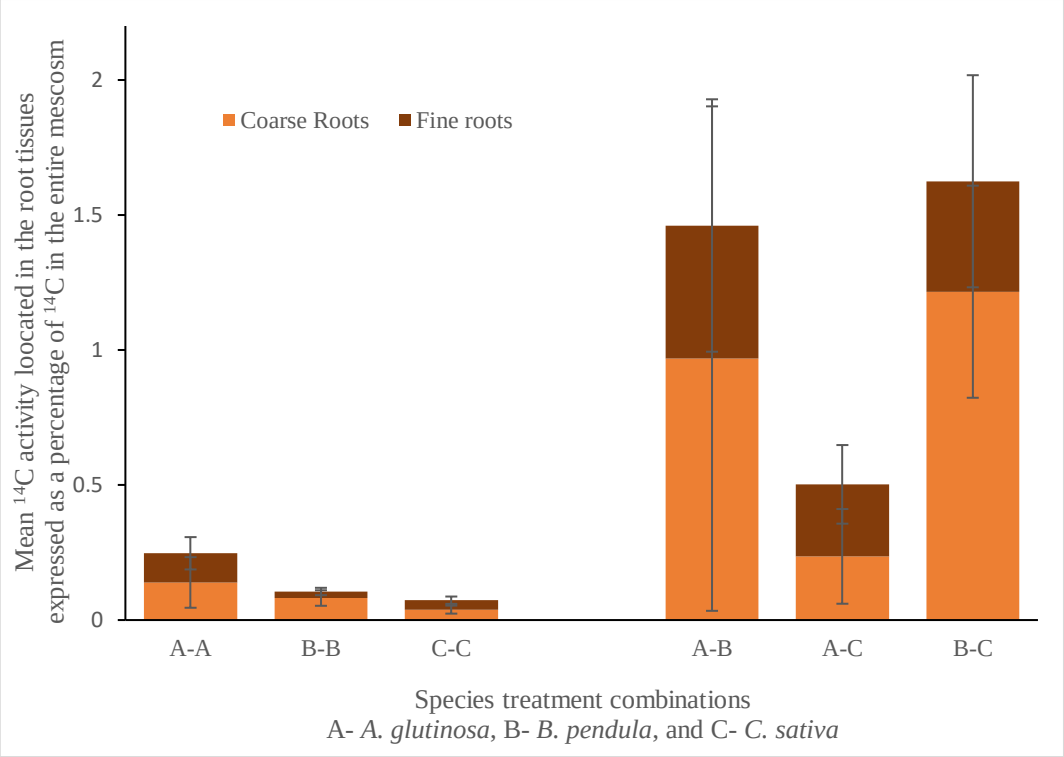

Figure S8: ^{14}C activity counted in the fine and coarse roots of the receiver tree and expressed as a percentage of the total ^{14}C activity in the entire mesocosm, in the six species treatment combinations (A- *A. glutinosa*, B- *B. pendula*, and C- *C. sativa*). Intraspecific and interspecific combinations are grouped together for comparison. Data are mean ± SE (n = 4).

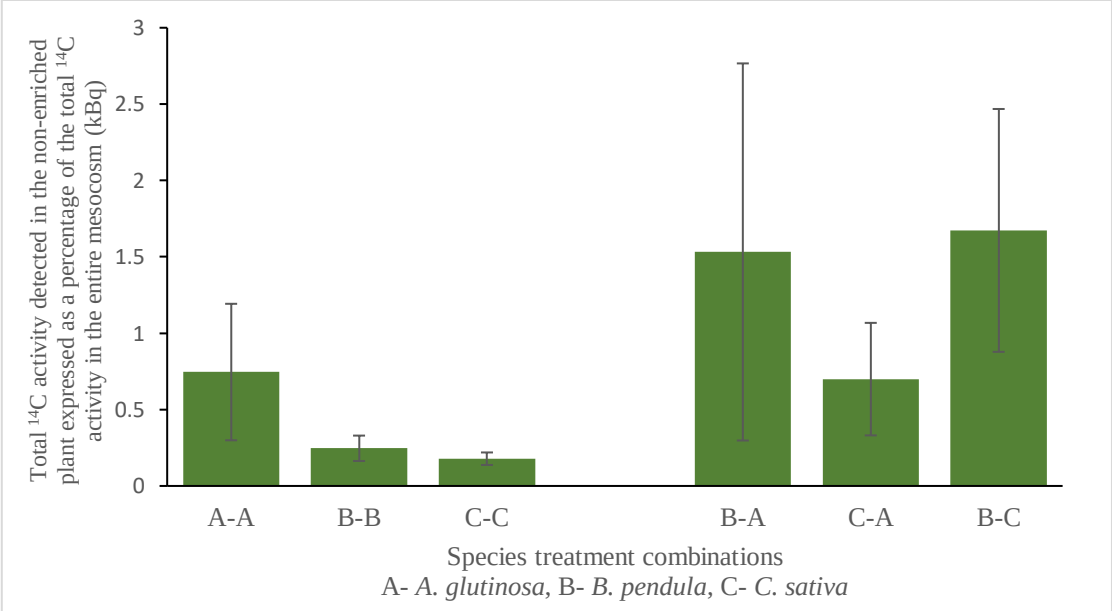

Figure S9: Total ^{14}C activity located in the receiver plant expressed as a percentage of the total ^{14}C activity in the entire mesocosm, in the six species treatment combinations (A- *A. glutinosa*, B- *B. pendula*, and C- *C. sativa*). Intraspecific and interspecific combinations are grouped together for comparison. Data are mean ± SE (n = 4).

Chapter – 5

Preferential allocation of carbon to the root nodules of common alder (*Alnus glutinosa*) via common mycorrhizal networks.

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Unpublished manuscript

Author contributions:

TDP conducted all empirical work with guidance on design, execution and data analysis from ARS and DLJ. TDP wrote the manuscript with all authors contributing to the final version.

1. Introduction

Nitrogen (N) is an essential building block for all life as it is required to produce nucleic acids, amino acids and proteins (Ogle and Brown, 1997). Despite the Earth's atmosphere being 78% di-nitrogen, this gaseous form of N is unavailable to plants and is often a major limitation to plant productivity (Geddes et al., 2015). Plant-bacterial symbiosis has evolved to overcome this limitation through a process known as N-fixation that that is widely accepted to be the second most important biological process in the world, after photosynthesis (Gruber and Galloway, 2008). During the process of N-fixation, the plant host supplies N-fixing bacteria with carbon (C) that is exchanged for N if the form of ammonium following the reduction of di-nitrogen by bacteria that produce the enzyme nitrogenase as shown in Equation 1 (Vessey et al., 2005; Herridge et al., 2008; Postgate, 2012).

$$N_2 + 16ATP + 8e^- + 8H^+ \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi. \qquad \text{(Eq. 1)}$$

As can be seen in the equation, the requirement for 16 ATP molecules shows that the process is extremely metabolically costly (Tjepkema and Winship, 1980).

Plant: bacterial symbiotic N-fixation has evolved in many divergent plant phyla (Mus et al., 2016) and occurs when N-fixing bacteria enter a plant and inhabit root nodules. Plants have

concurrently evolved multiple strategies to facilitate and foster these relationships and become hospitable hosts to the relevant bacterium (Holmer et al., 2017). These diazotrophic bacteria fall broadly into two categories: (i) Rhizobia, a group of paraphyletic bacteria; and (ii) the diverse actinobacterial genus *Frankia* (Holmer et al., 2017).

The most commonly identified N-fixing bacteria are *Rhizobia*, these bacteria represent 15 genera in 8 families of α-, β- and γ-Proteobacteria (Remigi et al., 2016). Globally this form of symbiosis is the most economically important for agricultural production (Desbrosses and Stougaard, 2011) and consequently the most extensively studied (Tariq et al., 2017). The second most frequent N-fixing bacteria is the filamentous sporangia forming *Frankia*, which forms root nodules known as actinorhizas in woody plants known as actinorhizals (Ghedira et al., 2018). *Frankia* was first identified in 1886 by Jørgen Brunchorst but was not successfully isolated until 1978 (Callaham, 1978). Consequently, these actinomycetes are poorly understood in comparison to *Rhizobia*. Indeed, *Frankia* was originally classified as a fungus until Becking (1970) identified *Frankia* as an obligate prokaryotic actinomycete, and created a new family, Frankiaceae, within the family Actinomycetales (Becking, 1970; Benson and Sylvester, 1993). Actinorhizals are a diverse range of 150 species of non-leguminous woody dicotyledonous plants from 17 genera, 8 families and 3 orders (Fagales, Rosales, Cucurbitales), which has led to postulation that they are likely the result of several evolutionary events (Pawlowski and Demchenko, 2012). Many of these specialist plants are pioneers that excel in colonising poor nutrient status soils (Ogle and Brown, 1997; Van Nguyen and Pawlowski, 2017) or land area devoid of soil (del Moral and Wood 1993, Walker et al., 2003).

Since the development of the Haber-Bosch process in the first-half of the twentieth century the production of man-made fertilisers has grown exponentially and currently two-thirds of the world's human population are reliant on this process to fertilise their food-crops (Vicente and Dean, 2017). Although this process has been heralded as one of the most important technological advances of the 20th century, over application of industrially fixed N in agriculture has resulted in myriad of environmental and human-health hazards (Tariq et al., 2017). Among these externalities are eutrophication, enhanced N-deposition, greenhouse gas production, particularly nitrous oxide (N_2O), and in part has facilitated the human population explosion (Vicente and Dean, 2017). Grassland species mixtures which include N-fixing plants have the potential to increase agricultural production by improving resource efficiency, by reducing the need for industrially produced N inputs (Peyraud et al., 2009). It is worth considering that every 1 kg of mineral N requires 55 MJ of energy to produce, transport and

spread, whereas nitrogen-fixation requires only sunlight (Peyraud et al., 2009). A better understanding of N-fixation combined with modern genetic engineering techniques could lead to artificially extending the range of N-fixing plant hosts to reduce reliance on fertiliser use, with its associated fossil-fuel consumption (Mus et al., 2016; Beatty and Good, 2011).

N-fixation is ecologically important in temperate forest ecosystems as non-N-fixing plants can benefit from the presence of N-fixing plants by the releases of N_2 by the root nodules, transfer of N containing molecules via common mycorrhizal networks (CMN), or through the decomposition of above- and below-ground N rich plant biomass (Roggy et al., 2004; Daudin and Sierra, 2008). CMN are fungal networks which connect two or more plants together underground and can be used to transport water, carbon, nitrogen, as well as other nutrients, and signalling chemicals (Simard et al., 2012). It is known that actinorhizal trees can transfer N fixed to the surrounding soil and therefore indirectly to neighbouring non-N-fixing plants (Ekbald and Huss-Danell, 1995). In a study of the indirect translocation of N via CMN Arnebrant et al. (1993) used $^{15}N_2$ gas as a proxy for N_2 to quantify the transfer of atmospheric N fixed by *Alnus glutinosa* to neighbouring *Pinus contorta* trees and found significant transfer occurred. The net benefit of including N-fixing species of tree in temperate forests communities could be equivalent to the annual addition of 150 kg N ha^{-1} (Cleveland et al., 1999). However, temperate tree species that have developed actinorhizal symbiosis are uncommon and consequently have not been widely studied. In this study we investigate the role of *Frankia alni* on the C source-sink dynamics of common mycorrhizal networks, on allocation belowground and subsequent transfer to root nodules, using ^{14}C as a proxy.

Here we investigate the transfer of C between two trees: (i) a ^{14}C pulse-labelled "donor" tree; and (ii) a CMN conjoined "receiver" tree; to assess the importance of actinorhizal N-fixation to belowground C allocation and elucidate the importance of plant: bacterial symbiosis in combination with the ubiquitous plant: fungal, mycorrhizal symbiotic relationships.

Our hypothesis for this experiment was:

H_{null} Belowground allocation of C via inter- and intra-specific CMNs will be greatest in "receiver" trees with actinorhizal associations, due to an increased C sink strength generated by the metabolic processes of the root nodule.

2. Materials and methods

2.1. Plant, root nodule and CMN establishment

1-year old cell-grown sweet chestnut (*Castanea sativa* Mill; Tree C) and bare rooted saplings of two temperate tree species namely; common alder (*Alnus glutinosa* (L.) Gaertn; Tree A) and silver birch (*Betula pendula* Roth; Tree B) were purchased from local forest nursery stock suppliers (Maelor Forest Nurseries Ltd, Bronington, SY13 3HZ) in November 2015. The tree selections were based on their contrasting belowground symbioses and functional traits, which was anticipated to maximise the positive effect on net productivity. Explicitly, *A. glutinosa* is actinorhizal, intermediate in shade tolerance, tolerates waterlogged soils and has seeds generally dispersed by watercourses. In contrast, *B. pendula* is a relatively short-lived, light demanding, early successional pioneer species with wind dispersed seeds and *C. sativa* is a late successional, shade tolerant, long-lived species with seed dispersed by animals. The saplings were planted into two connected 10 L pots (Ø 250 mm × 200 mm) which were coupled via a 10 cm diameter Perspex tube. Soil to backfill the pots was a collected from the 0-10 cm layer of plots from the BangorDiverse experiment (Ahmed et al., 2016) where, since 2004, the Fluventic Dystrochrept soil had developed under the aforementioned species grown in monoculture. Prior to backfilling, the soil was coarse sieved to remove large stones, air dried, homogenised and sieved to pass 2 mm. The connecting tubes were filled with a homogenised mixture of 2 kg horticultural grade sharp sand and 20 g of bone meal (to encourage hyphal colonisation), which was covered at both ends with a 40 µm mesh (Normesh Limited, Lancaster, UK; Figure 1). The mesh pore size excluded trees roots but allowed penetration of the connecting tube by hyphae.

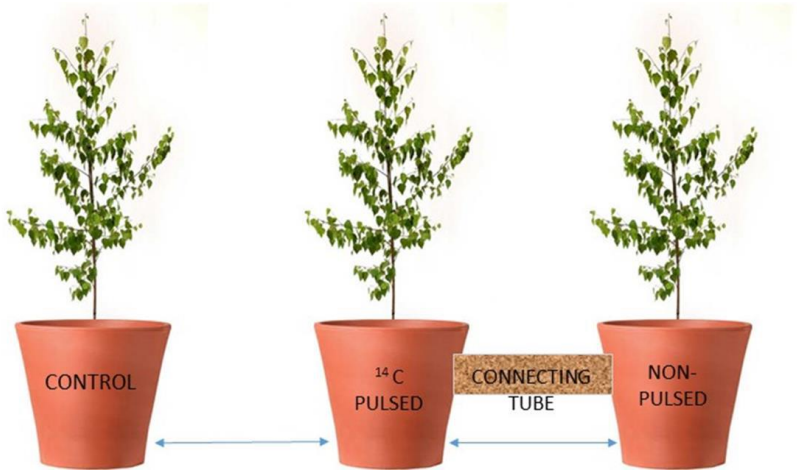


Figure 1: Diagram showing experimental design with two potted trees connected with the sand filled tube and the control plant.

Trees were planted in intra- or inter-specific combinations, creating three treatments (i.e., A-A, B-A, C-A; n = 4) with *A. glutinosa* consistently used as the ^{14}C receiver tree, with *A. glutinosa*, *B. pendula* and *C. sativa* all being ^{14}C donor trees. In all cases, the donor tree was the tree which was enriched in ^{14}C with a $^{14}CO_2$ pulse, whereas the receiver trees had no direct enrichment and therefore it was assumed that all ^{14}C measured in the receiver had been translocated belowground via a CMN or reabsorbed $^{14}CO_2$ that had previously been respired. To account for the possibility of re-photo assimilated $^{14}CO_2$ being counted in the receiver tree, a control tree was used and kept at the same distance from the donor as was the receiver (Figure 1). The conjoined pots were then grown in ambient conditions inside a glasshouse for 9 months to allow for the development of roots, mycorrhizal hyphae and root-nodule development, before being ^{14}C pulse-labelled using $NaH^{14}CO_3$ (Amersham International, Amersham, UK) which was reacted to $^{14}CO_2$ with an excess of 3 M HCl. To achieve ^{14}C enrichment, trees were allowed to photo assimilate the 2 MBq of $^{14}CO_2$ for 2 hours in full sunlight with a minimum photosynthetically active radiation intensity of 800 μmol m^{-2} s^{-1}.

2.2. Sampling procedure and isotope analysis

To trace the ^{14}C through the plant-microbe-soil continuum we measured the ^{14}C content of soil and plant biomass pools using a range of analytical methodologies. To estimate the plant and microbial respiration we measured mineralisation by suspending 28 mm diameter CO_2 traps containing 3 M NaOH above the soil allowing gaseous free passage from the soil surface. These were regularly replaced before saturation of the NaOH occurred and counted by liquid

scintillation spectrometry using a Wallac 1409 scintillation counter (Wallac EG and G, Milton Keynes, UK) with automatic quench correction and Optiphase Hisafe 3® (Perkin Elmer, UK) compatible scintillation fluid. Dissolved ^{14}C in soil pore water was sampled simultaneously using mini-rhizon suction sampling devices (Rhizosphere Research Products B.V., Wageningen, The Netherlands) (Jones et al., 2016) and liquid scintillation counting as above. Disintegrations per minute were counted, standardised against a quench curve, and regularly calibrated using standards. Once the defined pulse chase period had elapsed (336 hours) the trees were harvested destructively and separated into the following partitions: foliage, branch and stem, fine and coarse roots, root nodules soil and sand from the interconnecting tube for ^{14}C quantification (Figure 2). To prevent further plant and microbial losses of $^{14}CO_2$ after harvest, the plant partition, soil and sand were immediately dried at 105 ºC for 24 hours. Subsequently, the partitions were weighed, homogenised and ground using a Retsch MM200 laboratory ball mill (Retsch GmbH, Hann, Germany) before being combusted using a Harvey Instruments Biological Oxidiser OX400 (R.J. Harvey Instrument Corp., Hillsdale, NJ, USA) and mineralised into Oxysolve C-400 (Zinsser Analytic GmbH, Frankfurt, Germany) and counted by liquid scintillation counting as described above.

Plate 1: The root nodules were painstakingly separated from the wet roots (panel A) and then dried (panel B) in an oven 105 ºC for 24 hours, whereby the original orange colouration was lost.

2.3. Soil chemical properties analysis

Immediately post pulse-label, soil was sampled from each mesocosm using a 4 cm soil corer then the chemical properties were analysed to determine if the trees presence during the tree and mycorrhizal establishment period had significantly altered the soil. The soils were weighed, then dried at 105 °C and ball milled before gravimetric determination of moisture content (Rowell, 1994) and C and N analysis, measured by dry combustion (Elementar analyzer). Nitrate was determined colourimetrically by the vanadium chloride method of Miranda et al. (2001), ammonium by the salicylate-hypochlorite method of Mulvaney (1996) both extracted in K_2SO_4, and plant-available phosphate by the molybdate-reactive method of Murphy and Riley (1962) following a 0.5 M sodium bicarbonate ($NaHCO_3$) pH 8.5 (Olsen et al., 1954) extraction. Soil pH and electrical conductivity (EC) were measured in a 1:5 (w/v) slurry of soil and distilled water (Smith and Doran, 1996). Soil dissolved organic carbon (DOC) and total dissolved N (TDN) was determined using a Shimadzu TOC-V-TN analyser (Shimadzu Corp., Kyoto, Japan) following extraction (1:5 w/v) in 0.5 M K_2SO_4 (Jones and Willett, 2006). The resultant soil chemical properties are shown in Table 1.

2.4. Statistical analysis

Prior to analysis all data were tested for homogeneity of variance using Levene's test. The independent variable was the species treatment (A. glutinosa, B. pendula and C. sativa). The dependant data (total plant biomass and partition biomass, soil chemical properties and 14C activity of biomass pools, 14C labelled respiration efflux) were tested for normality prior to analysis using the Shapiro-Wilk Test and no transformations were required. The statistical analysis was conducted using a one-way ANOVA and Tukey's posthoc test with SPSS® Statistics version 25.0 (IBM Corp, Chicago, 2017). Results were considered significant at $P \leq 0.05$.

3. Results

3.1. Soil chemical properties

Table 1 shows the results of the soil chemical analysis. As the soil samples were collected from the mesocosms below the three tree species at time point zero (directly post pulse label) they were interpreted based on the species identity rather than the treatment combination.

Table 1: Soil chemical properties for soil collected from the mesocosms 0 hours after the pulse was complete for all three species treatments (*A. glutinosa*, *B. pendula* and *C. sativa*). Data are mean ± SE (n = 4 for *B. pendula* and *C. sativa*, n = 16 for A. glutinosa). Significant differences are highlighted in bold and the level of significance is denoted by $^{*}P < 0.05$.

Tree species	**NO_3^- (mg/kg)**	**NH_4^+ (mg/kg)**	**Available-P (mg/kg)**	**pH**	**EC (µS/cm)**	**C: N**	**TDN (mg/kg)**	**DOC (mg/kg)**
A. glutinosa	2.06 ± 1.02	6.61 ± 1.38	17.44 ± 4.77	5.49 ± 0.07	190.75 ± 22.36	8.30 ± 0.13 **ab**	7.11 ± 0.91	34.09 ± 4.10
B. pendula	0.84 ± 0.40	6.64 ± 2.01	12.56 ± 1.29	5.63 ± 0.11	141.25 ± 32.61	7.78 ± 0.28 **b**	6.08 ± 0.67	31.62 ± 6.88
C. sativa	1.94 ± 1.37	3.79 ± 1.28	13.55 ± 2.99	5.56 ± 0.14	220.75 ± 78.78	8.95 ± 0.40 **a**	5.54 ± 0.84	20.50 ± 0.85
P-value	0.83	0.59	0.62	0.79	0.82	**0.03***	0.28	0.63

The soil chemical properties did not show major differences between species, meaning that the effect of the trees presence during the period of tree and mycorrhizal establishment of the soil was negligible and as result there were no significant differences in soil properties, except for the soil C: N ratio. The C: N mean data indicated that the C: N ratio was significantly lower (by ca. 15) for *B. pendula* than *C. sativa*, however, there were no statistically significant differences between *A. glutinosa* and *B. pendula* or *A. glutinosa* and *C. sativa*. The total amount of C and N in the soils collected from below the three tree species is presented in Table 2 to investigate which of the two variables was driving the differences between species. As can be seen in Table 2, the variable that differs is the % of C. The C content of soil under *C. sativa* trees is ca.15 % higher when compared with the C content of the soil under *A. glutinosa* and *B. pendula*.

Table 2: Total C (%) and N (%) of the three species (irrespective of the species treatment combination). Data are mean ± SE (n = 4 for *B. pendula* and *C. sativa* and n = 16 for *A. glutinosa*).

Species identity	**% C**	**% N**
A. glutinosa	2.52 ± 0.07	0.30 ± 0.01
B. pendula	2.44 ± 0.02	0.31 ± 0.02
C. sativa	2.82 ± 0.03	0.31 ± 0.02

3.2. Plant biomass

To reduce nutrient availability in the mesocosm soil and to therefore increase the chances of the *A. glutinosa* trees forming root nodules, the trees were grown in 10 litre pots for nine months before pulse-labelling. On visual inspection at the point of destructive harvesting the alder plants exhibited root nodules in all treatments and replicates.

After 336 hours of the ^{14}C pulse being applied, destructive harvest of the plant biomass showed that the total mean (n = 16) dry weight of the three species irrespective of the species treatment combinations was 73.3 ± 4.8 g. At the final harvesting time point (336 hours after pulse completed), the mean (n = 4) plant biomass of *A. glutinosa* was 106.8 ± 7.5 g, for *B. pendula* it was 47.4 ± 4.6 g, and for *C. sativa* it was 65.5 ± 2.4 g. The total biomass of the three species

was found to be different from each other *(P* < 0.001; Table 3). Mean total biomass of *A. glutinosa* was almost twice that of *B. pendula* and half as much as *C. sativa* and the difference between the conjoined *A. glutinosa* and *B. pendula*, and *A. glutinosa* and *C. sativa* were found to be significant, (*P* < 0.001) and (*P* = 0.09), respectively. However, the biomass of *B. pendula* and *C. sativa* were not significantly different to each other (*P* = 0.50). The relative dry weights of the plant partitions were also quantified and a post-hoc Tukey test showed that there were significant differences between the biomass partitions of the three species. All biomass partitions of *A. glutinosa* were larger than those of both *B. pendula* and *C. sativa* (*P* < 0.001). *A. glutinosa* was shown to have twice the mean leaf biomass of the *B. pendula* treatments and half as much as the *C. sativa* (*P* < 0.001), treatments. Total leaf biomass of *B. pendula* and *C. sativa* where not found to be significantly different to each other (*P* = 0.48), whereas branch and stem total mean biomass were significantly different to each other (*P* < 0.001) for all treatment comparisons, with the exception of *B. pendula* and *C. sativa*, which were not significantly different from each other (*P* = 0.80).

Analysis of coarse and fine root biomass data revealed that *A. glutinosa* had a larger mean dry root biomass that was significantly different from that of *B. pendula* and *C. sativa* (*P* = 0.03 and *P* = 0.01) for fine and coarse root, respectively. The root biomass of *B. pendula* and *C. sativa* were not significantly different from each other (*P* = 0.53). As root nodules are only present in *A. glutinosa*, no comparison could be undertaken. Overall, at the time of pulse-labelling, the mean total biomass of the *A. glutinosa* (14.2 ± 0.9 g) was significantly greater than *B. pendula* (5.8 ± 1.2 g; *P* = 0.001) and *C. sativa* (8.4 ± 0.7 g; *P* = 0.01).

Table 3: Mean biomass (g dwt) ± SE of five biomass partitions and total biomass of *A. glutinosa*, *B. pendula* and *C. sativa* determined by destructive harvesting at 336 hours after a $^{14}CO_2$ pulse was applied. (n = 4 for *B. pendula* and *C. sativa*; n = 16 for *A. glutinosa).* Significant differences are highlighted in bold and the level of significance is denoted by asterisks (*, $P < 0.1$; **, $P < 0.05$; ***, $P < 0.001$).

Tree species	**Tree partition**					
	Leaf	**Branch**	**Coarse root**	**Fine root**	**Root nodule**	**Total biomass**
A. glutinosa	27.92 ± 1.88	40.58 ± 1.97	22.52 ± 2.06	14.48 ± 1.44	1.31 ± 0.14	106.8 ± 7.49
B. pendula	13.61 ± 1.67	17.18 ± 1.14	9.99 ± 1.17	6.61 ± 0.58	N/A	47.39 ± 4.56
C. sativa	19.46 ± 1.01	20.31 ± 0.23	15.41 ± 0.43	10.34 ± 0.74	N/A	65.52 ± 2.42
P-value	**<0.001****	**<0.001*****	**0.01****	**0.02****	**<0.001*****	**<0.001*****

3.3. Partitioning of ^{14}C activity within mesocosms C pools

In order to determine the relative concentration of ^{14}C activity between the mesocosm C pools of the different treatments, the ^{14}C activity per gram of dried biomass was calculated (Table 4). The total biomass of *A. glutinosa* was twice that of the *B. pendula* and almost twice that of the mean total *C. sativa* biomass. Table 3 clearly shows that all the mesocosm's C pools were almost twice as large in the *A. glutinosa* trees and all were found to have significant differences except for the soil ^{14}C activity per gram, which was not dependent on the tree species (P = 0.26). A post-hoc Tukey test showed that ^{14}C activity per gram of leaf was significantly different between the *A. glutinosa* and *B. pendula* treatments ($P < 0.001$), between *B. pendula* and *C. sativa* treatments ($P = 0.03$), but not between the *A. glutinosa* and *C. sativa* treatments ($P = 0.67$). The branch data also showed significant differences between the *A. glutinosa* and *B. pendula* treatments ($P < 0.001$), between *B. pendula* and *C. sativa* treatments ($P = 0.03$), but not between the *A. glutinosa* and *C. sativa* treatments ($P = 0.41$). The same trend was apparent from both the coarse and fine root data, with *A. glutinosa* showing significant differences from *B. pendula* ($P < 0.001$ and $P < 0.001$ in coarse and fine root respectively). *B. pendula* showing significant differences from *C. sativa* ($P = 0.01$ and $P = 0.04$, in coarse and fine roots

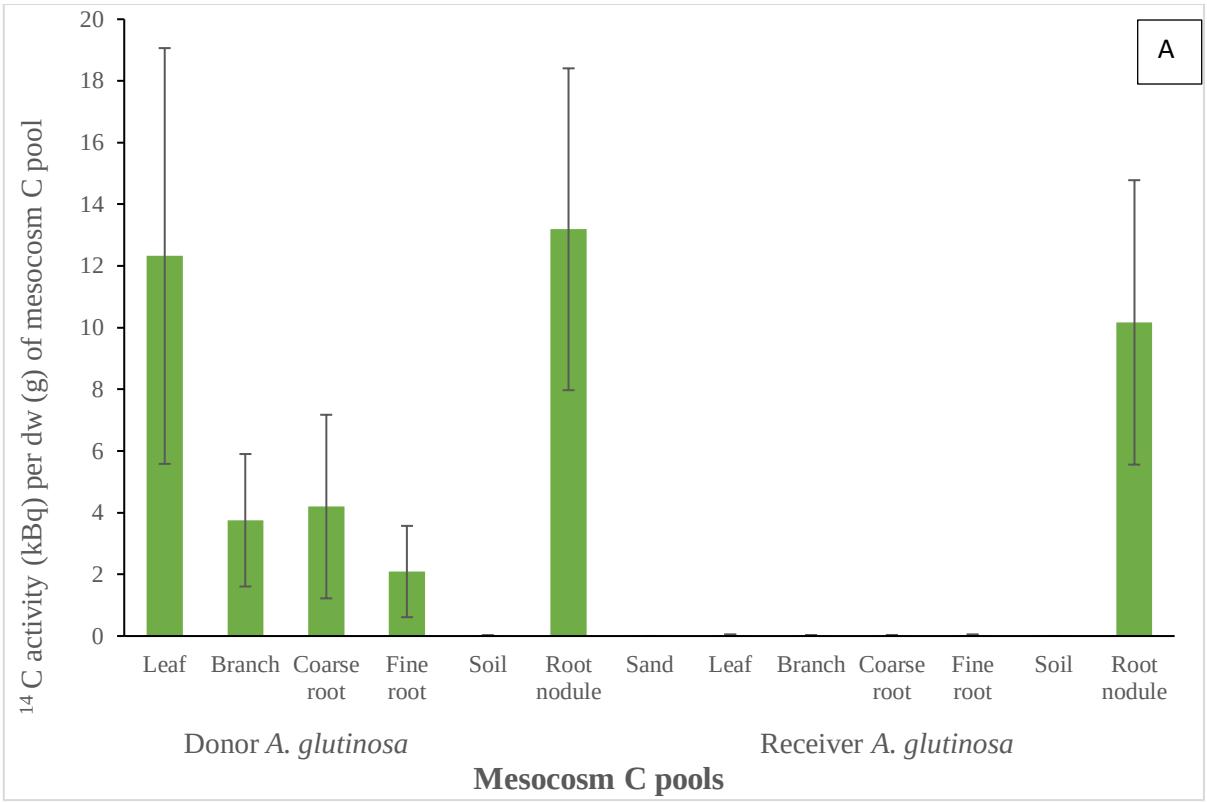
respectively). In contrast, in both root partitions, *B. pendula* was found not to be significantly different to *C. sativa* in coarse ($P = 0.54$) and fine roots ($P = 0.23$).

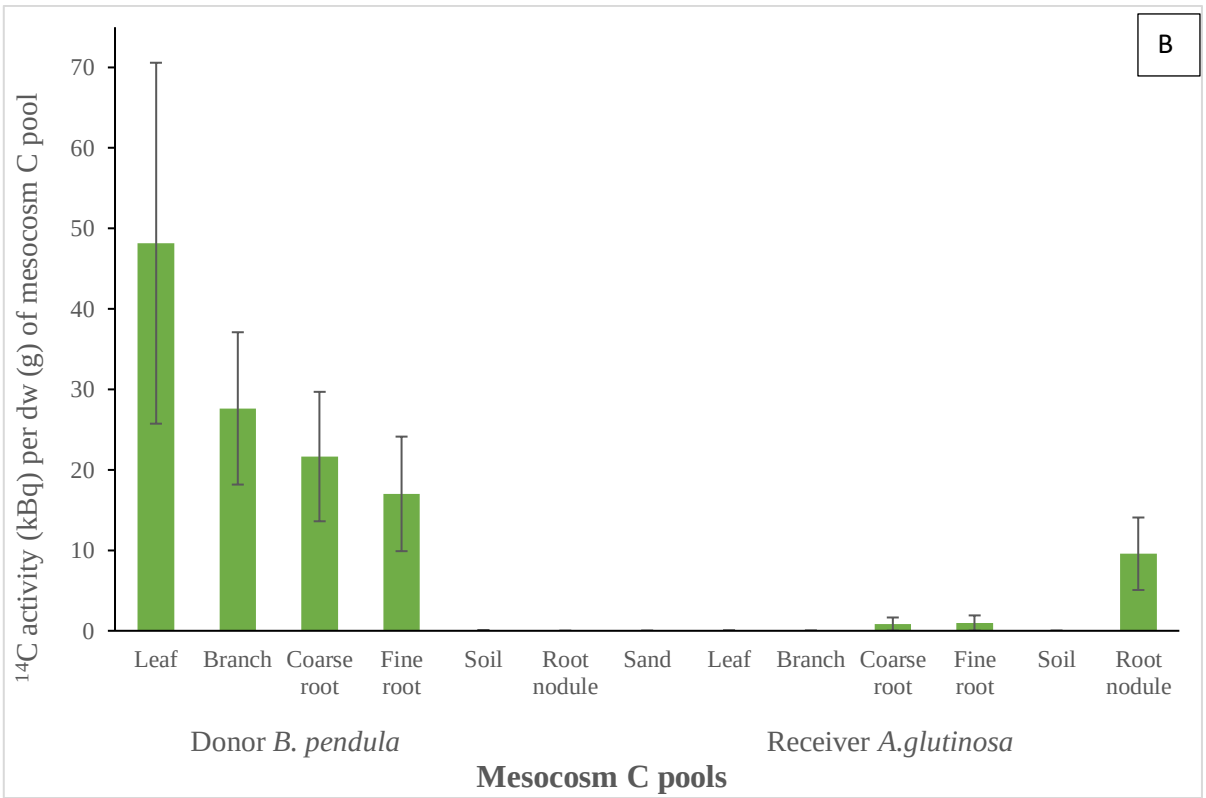
Mean ^{14}C activity was examined when the receiver species was *A. glutinosa* to establish if the identity of the donor tree significantly altered the ^{14}C activity detected in the receiver fraction. Statistical analysis of these data suggests that there were no significant differences between the ^{14}C activity detected in the receiver plant and the species identity of the donor plant.

Table 4: ^{14}C activity of each C pool within the mesocosm determined after destructive harvesting expressed as total ^{14}C activity (kBq) per gram of mean plant biomass for each biomass partition (g dwt) ± SE (n = 4 for A-A, B-A, C-A). Significant differences are highlighted in bold and the level of significance is denoted by asterisks (*, $P < 0.1$; **, $P < 0.05$; ***, $P < 0.001$). The sand C pool was determined from sand collected in the conjoined tube.

	^{14}C activity (kBq) per gram of mesocosm C pools						
Species combination	**A-A**	**A-A**	**B-A**	**B-A**	**C-A**	**C-A**	***P-value***
Tree species	***A. glutinosa***	***A. glutinosa***	***B. pendula***	***A. glutinosa***	***C. sativa***	***A. glutinosa***	
^{14}C status	**Donor**	**Receiver**	**Donor**	**Receiver**	**Donor**	**Receiver**	
Leaf	12.32 ± 6.74	0.04 ± 0.01	48.16 ±22.42	0.02 ± 0.01	11.98 ±2.44	0.03 ± 0.01	0.001**
Branch	3.75 ± 2.15	0.02 ± 0.00	27.63 ± 9.46	0.01 ± 0.00	6.47 ± 1.19	0.03 ± 0.01	0.000***
Fine root	2.09 ± 1.48	0.03 ± 0.02	17.01 ± 7.12	0.97 ± 0.94	6.28 ± 1.57	0.26 ± 0.18	0.000***
Coarse root	4.20 ± 2.98	0.02 ± 0.01	21.65 ± 8.04	0.84 ± 0.81	5.34 ± 1.46	0.13 ± 0.10	0.000***
Root nodule	13.19 ± 5.22	10.17 ± 4.61	19.63 ± 7.56	9.57 ± 4.5	5.70 ± 1.45	8.11 ± 3.02	N/A
Soil $\times 10^{-3}$	16.28 ± 4.32	1.41 ± 0.13	4.75 ± 0.86	3.42 ± 0.73	49.45 ± 16.24	3.37 ± 1.45	0.261
Sand $\times 10^{-3}$	1.35 ± 0.05	1.35 ± 0.05	3.17 ± 1.12	3.17 ± 1.12	1.8 ± 0.23	1.80 ± 0.23	0.000***

Transfer of pulsed-labelled ^{14}C from the donor tree species to the conjoined *A. glutinosa* tree are shown in Figure 1a-c.
N/A denotes not applicable.


A
^{14}C activity (kBq) per dw (g) of mesocosm C pool
0
2
4
6
8
10
12
14
16
18
20
Leaf
Branch
Coarse root
Fine root
Soil
Root nodule
Sand
Leaf
Branch
Coarse root
Fine root
Soil
Root nodule
Donor *A. glutinosa*
Receiver *A. glutinosa*
Mesocosm C pools


B
^{14}C activity (kBq) per dw (g) of mesocosm C pool
0
10
20
30
40
50
60
70
Leaf
Branch
Coarse root
Fine root
Soil
Root nodule
Sand
Leaf
Branch
Coarse root
Fine root
Soil
Root nodule
Donor *B. pendula*
Receiver *A.glutinosa*
Mesocosm C pools

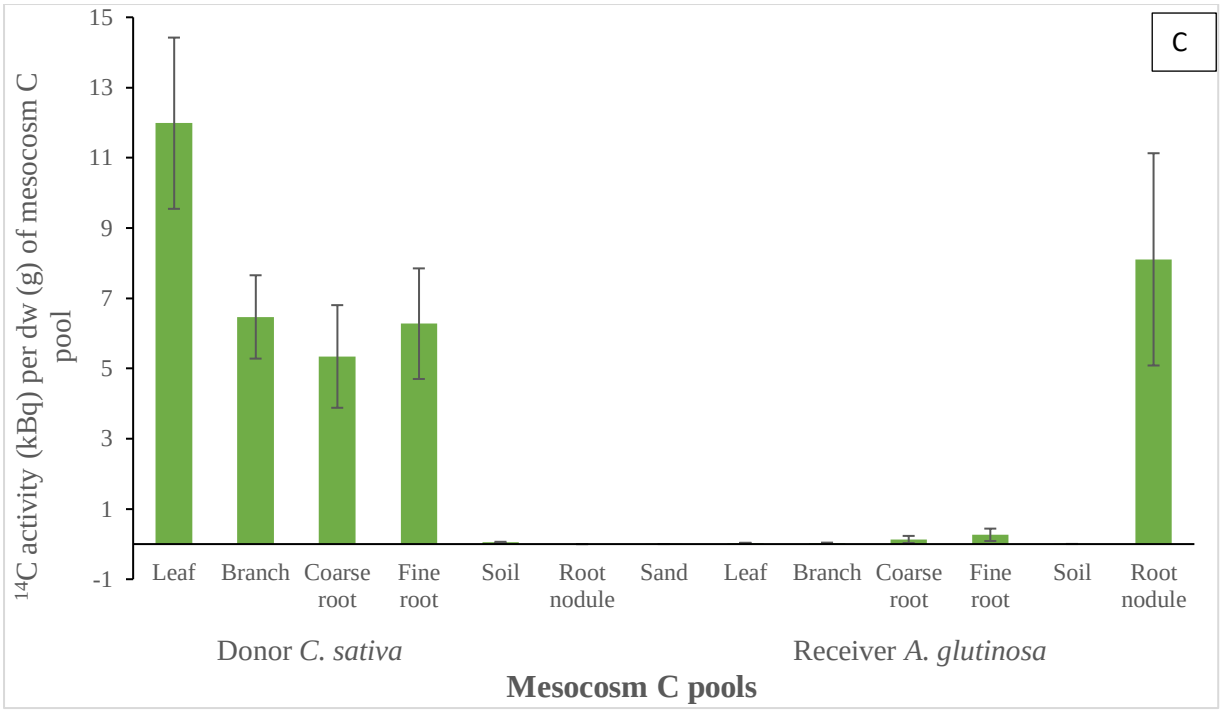


Figure 2: Panel A shows the mean dried weight (gram) of the partitions in both the donor and receiver fractions of the *A. glutinosa* to *A. glutinosa* species treatment combinations and SE (*n* = 4); Panel B shows the mean dried weight (grams) of the partitions in both the donor and receiver fractions of the *B. pendula* to *A. glutinosa* species treatment combinations and SE (*n* = 4); Panel C shows the mean dried weight (grams) of the partitions in both the donor and receiver fractions of the *C. sativa* to *A. glutinosa* species treatment combinations and SE (*n* = 4)

3.4. Effect of treatment (identity of donor plant) on ^{14}C activity in root nodule

The ^{14}C activity per gram of biomass data allowed the differences in size of tree partitions to be accounted for and therefore more direct comparison to be made. The mean root nodule activity per gram (*n* =4) for the donor trees was 13.2 ± 5.2 kBq/g, and for the receiver trees was 10.2 ± 4.6 kBq/g when the identity of the donor was *A. glutinosa*, 9.6 ± 4.5 kBq/g when the donor trees identity was *B. pendula* and 8.1 ± 3.0 kBq/g when the donor trees identity was *C. sativa*. A comparison of the ^{14}C activity per gram of the receiver tree root nodules is shown in Figure 2b and a comparison between ^{14}C activity per gram of the donor root nodules is shown in Figure 2c. There were no significant differences in the ^{14}C activity regardless of the species identity of the donor plant (*P* = 0.72). In the case of the data plotted in Figure 4, no significant difference was observed between the ^{14}C activity of the donor *A. glutinosa* tree root nodules and the ^{14}C activity measured in the receiver trees. For the relative ^{14}C activity of the donor and receiver trees as a percentage of total ^{14}C recovered in all plant partitions see Section 3.5.

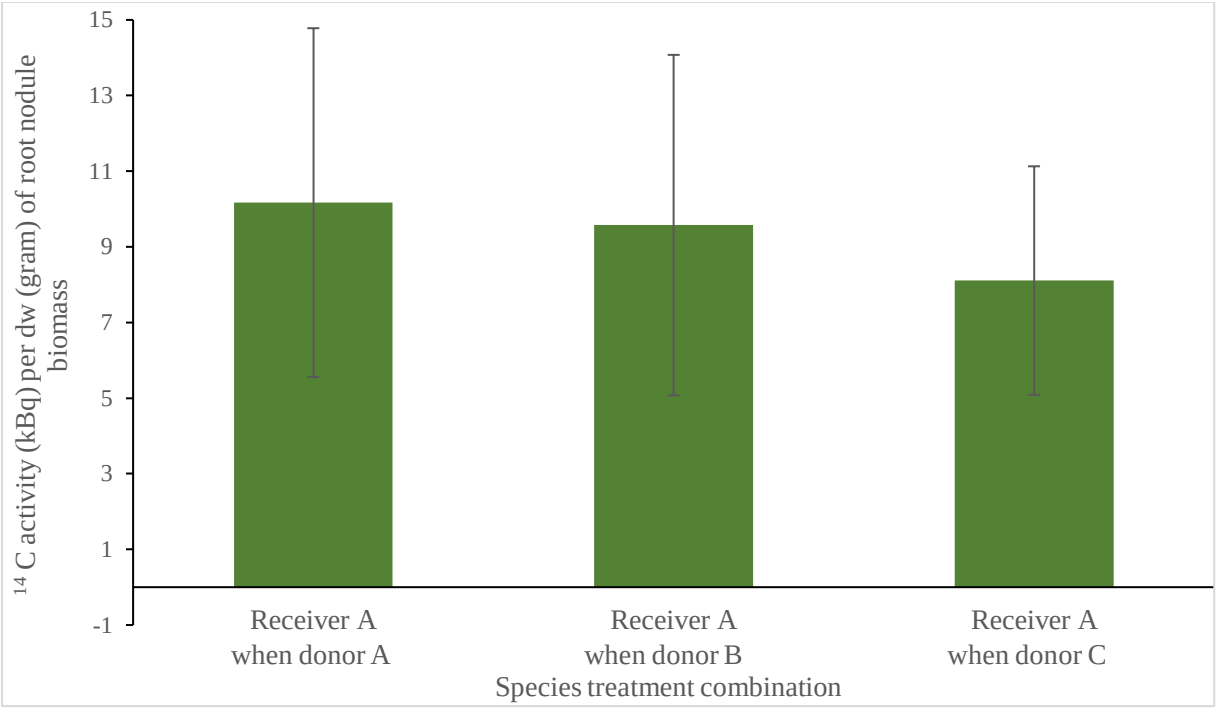


Figure 3: Root nodule ^{14}C activity (kBq) of the three treatments where in all cases the receiver plant is the actinorhizal *A. glutinosa* but the donor tree is of three tree species both intra- and inter-specifically with the identity of the pulse tree also being *A. glutinosa,* and interspecifically *B. pendula* and *C. sativa*. Data is mean (*n* = 4) with SE.

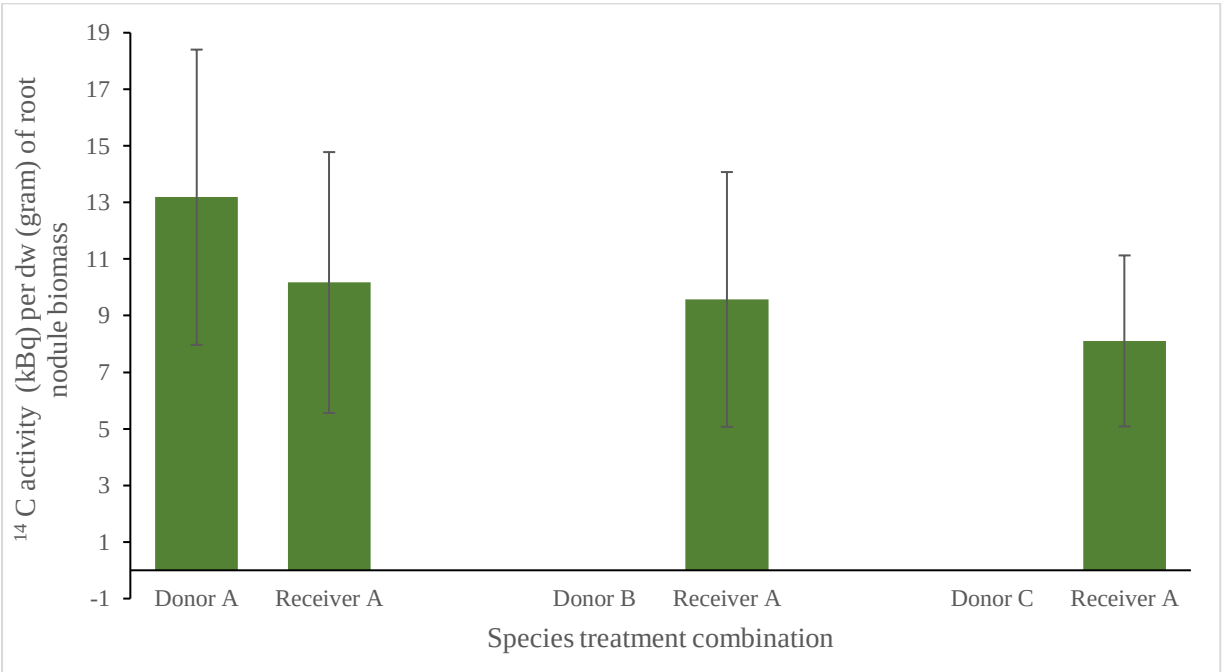


Figure 4: Root nodule ^{14}C activity (kBq) quantified from the nodules separated from the roots of the pulsed (donor) *A. glutinosa*, and the activity of the root nodules for each of the receiver *A. glutinosa* in each species combination. Data is mean ± SE (*n* = 4).

3.5. Root nodule activity as % of total ^{14}C in receiver plants

To normalise the ^{14}C activity of the three species by biomass, the ^{14}C activity detected in the root nodules of the receiver tree as a percentage of the total ^{14}C activity in the receiver tree biomass was calculated. As can be seen in Table 5, in both the *A. glutinosa* to *A. glutinosa* treatments and the *C. sativa* to *A. glutinosa* treatments over 50 % of the total ^{14}C activity in the whole tree was detected in the root nodules (65.8 ± 21.9 % and 56.8 ± 18.0 %, respectively). The *B. pendula* to *A. glutinosa* treatments had slightly less ^{14}C activity as a percentage of the ^{14}C detected in the whole tree on average with just slightly under 50 % of the total (49.8 ± 24 %). A statistical comparison of the ^{14}C activity detected in the root nodule as a percentage of the total ^{14}C activity detected in the entire receiver trees biomass partitions revealed no significant difference between the treatments (Table 5).

Table 5: ^{14}C activity of each biomass partition determined after destructive harvesting expressed as a percentage of the total ^{14}C activity detected in the receiver tree ± SE (*n* = 4*).*

Donor tree identity	**Biomass partitions of receiver tree (*A. glutinosa*)**				
	Leaf	**Branch**	**Coarse root**	**Fine root**	**Root nodule**
A. glutinosa	11.09 ± 4.82	7.31 ± 4.08	6.63 ± 5.29	9.18 ± 8.43	65.79 ± 21.94
B. pendula	7.66 ± 5.40	8.56 ± 7.03	22.99 ± 13.73	11.09 ± 6.60	49.76 ± 23.99
C. sativa	5.99 ± 1.06	8.25 ± 3.21	14.20 ± 10.12	14.73 ± 8.03	56.84 ± 17.99
P-value	0.69	0.98	0.55	0.88	0.87

4. Discussion

Using ^{14}C as a proxy for recently photosynthetically fixed C, this study indicated that C fixed by a conjoined tree was transferred between trees via a CMN and that the C was preferentially allocated to the root nodules of *A. glutinosa.* Figures 2a-c showed that ^{14}C was transferred to the root nodule of *A. glutinosa* (recipient) whatever conjoined species (donor) was ^{14}C pulse labelled. This suggests that the identity of the donor tree species is not important in determining the quantity of ^{14}C activity that is transferred to the root nodules of the receiver *A. glutinosa* tree. This result was unexpected but can be explained by the considerable energetic requirement of nitrogen fixation, as the nitrogen fixing cells require a supply of energy (ATP) and a reductant, commonly $Na_2S_2O_4$ (Huss-Danell and Hahlin, 1988). This huge energy requirement alters the balance between the C source (shoots, leaf) and the C sinks (growing roots,

mycorrhizal fungi, extramatrical mycorrhizal fungi and endophytic nitrogen-fixing bacteria), which could be the mechanism mediating the transfer of C between trees via a CMNs (Simard et al., 1997; Simard and Durall, 2004; Selosse et al., 2006). It would appear that the C demand of the root nodule in the receiver tree is sufficient enough to translocate C from the roots of the donor tree to root nodules in the receiver. Presumably, the same is simultaneously occurring in reverse i.e. the C fixed by the receiver tree is being conveyed across the CMN to the donor root nodules, although this unfortunately cannot be substantiated from the data collected in this study.

Additionally, the transfer of C between the *A. glutinosa* and *A. glutinosa* species combination treatment showed that the receiver plant-microbe soil system allocated the most ^{14}C activity to the root nodules, with very little present in either the fine or coarse root. In contrast, when the *B. pendula* to *A. glutinosa* species combination treatment was examined it appeared that *B. pendula* retained more of the ^{14}C in plant tissues relative to the other donor species (Figure 3).

4.2. Plant biomass

Differences in the biomass of the three species were expected due to the differential growth rates of the tree species. Of the two early successional species *B. pendula* typically yields a net primary production (NPP; gross photosynthesis minus plant respiration) of 8.5 t C ha^{-1} $year^{-1}$ (Varik et al., 2015) or 5.3 m^3 ha^{-1} yr^{-1} to 11.4 m^3 ha^{-1} yr^{-1} (Karlsson et al., 1997), whereas *A. glutinosa* has a typical NPP of 4 to 14 m^3 ha^{-1} $year^{-1}$. (Claessens et al., 2010). In contrast, the late-successional and generally fast-growing species *C. sativa* has a typical mean NPP of 11 m^3 ha^{-1} $year^{-1}$, when grown in the UK (Everard and Christie, 1995). Depending on the growing conditions of the three species, *A. glutinosa* has the potential to have the largest annual yield and has a distinct advantage being actinorhizal species in nutritionally deficient soil conditions (Isopi et al., 1994). However, Orfanoudaki et al., (2010) found that even when *A. glutinosa* trees were supplied with an excess of nitrogen and phosphorus, (phosphorus at 240 mg kg^{-1} and nitrogen 83 mg kg^{-1}) that those inoculated with mycorrhizal fungi and nitrogen fixing bacteria still outperformed those without. When the plant tissues were analysed for nitrogen and phosphorus content, it was found that they were the same in both cases, suggesting that the growth promotion properties of *Frankia* and mycorrhizal fungi are not confined to purely nutritional benefits. It has been suggested that other benefits of the symbiosis include growth assistance through modification to the root system (Hooker and Atkinson, 1996), more specifically through increased root branching (Berta et al., 1990).

It seems logical that the greater biomass of *A. glutinosa* could be accounted for by the N status of the soil. However, the only soil chemical property that was different between the species treatments was the C: N ratios of *B. pendula* and *C. sativa* whereas, the C: N ratio of soil under *A. glutinosa* was similar to both *B. pendula* and *C. sativa*. This was quite surprising as *A. glutinosa* is actinorhizal and we would therefore expect the levels of nitrate to be slightly higher under this species than the other two. A more detailed investigation of what was driving the differences between the C: N ratio of the *C. sativa* soil and the other two species shows the mean C % under *C. sativa* was higher than the other two species. Whereas in contrast, the N % was almost identical under the three species (Table 2). The differences could be explained by the trees species having slightly different rates of growth and nutritional requirements during the periods of growth and therefore as they use resources from the soil at different rates the stoichiometry of the remaining nutrients would be altered by the identity of the tree species potted into the mesocosm or most likely to be a result of organic N and C inputs (i.e. root turnover, litter inputs, hyphal turnover, microbial [fungi/bacterial turnover]). The biomass inputs will eventually change the soil C: N ratio. An explanation for the lack of differences in N % under the three species could be that because the mycorrhizal hyphae are spanning both pots through the sand that the fungi may be redistributing the N fixed by the *Frankia* in the root nodules to the non-actinorhizal species in the case of the interspecific species combination. It well-known that the presence of nitrogen-fixing nodules not only improves the N status of the host plant but can also benefit plants in the vicinity through litter inputs either above or belowground, root exudation and leaching from leaves (He et al., 2009). This has been experimentally substantiated as when leguminous plants are planted in mixtures with non-leguminous plants, a significant increase in growth and yield in the non-leguminous plants is often observed (Herridge et al., 2008; Li et al., 2007; Malezieux et al., 2009). In a study of nitrogen fixing *Canavalia ensiformis* (jackbean), Sierra and Defontaines (2009) showed that N transferred to *Musa acuminata* (banana) 6% had originated from exudates, 32 % from root turnover, and 62 % from soil N. However, common genes are needed for the colonisation of roots by both nitrogen-fixing bacteria and mycorrhizal fungi (Ané et al., 2004; Sprent and James, 2007) and therefore most nitrogen fixing tree species display dual symbiosis (Teste et al., 2019). The effect of dual symbiosis could further increase the transfer to non-leguminous plants not only by direct release from nitrogen-fixing plants (Ledgard, 2001; Paynel et al., 2001; Shen and Chu, 2004; Sierra and Desfontaines, 2009; Sierra and Nygren, 2006), but by the following potential mechanisms; mycorrhizal N uptake and transfer (Koltai and Kapulnik, 2010; Smith and Read, 2010); through CMNs (Koltai and Kapulnik, 2010; Smith and Read,

2010; Moyer-Henry et al., 2006). Arbuscular mycorrhizae in CMN are known to mediate this (Johansen and Jensen, 1996; Moyer-Henry et al., 2006); as well as ectomycorrhizal networks where N-transfer through ecto-CMN has been detected between *Alnus* spp. (*A. glutinosa* and *A. incana*) and non-actinorhizal *Pinus contorta* (Lodgepole pine) and *P. sylvestris* (Scots pine) (Arnebrant et al., 1993; Ekblad and Huss-Danell, 1995). Single-directional N transfer by CMN has been reported to range between 0 to 80 % (Newman et al., 1994; He et al., 2009). In N limited conditions in single-species stands, the advantage conferred to *A. glutinosa* through N-fixation has been shown to result in a faster growth rate in the seedling phase than other temperate tree species (Kupper et al., 2018).

4.3. ^{14}C activity of mesocosms C pool

^{14}C activity detected in the soil below the three receiver species was not significantly altered by the species identity that was potted in it suggesting that there was not a measurable difference in the ^{14}C exuded by the roots of the three species, or that any difference in exudation was quickly eliminated due to consumption of exudates by the soil microbiota and eventually respired to the atmosphere. Ryan et al. (2001) added realistic concentrations of soluble C to the rhizosphere to quantify rates of mineralisation and confirmed what others had found, that microbial consumption of root exudates was extremely quick (Jones et al., 2004; Kuzyakov and Cheng, 2001; Nguyen et al., 1999) and that substrate half-lives are between 0.5 and 2 h for the most common exudates such as sugars, amino and organic acids (Ryan et al., 2001). The partition data itself revealed significant differences in ^{14}C activity as a % of total ^{14}C activity in the mesocosm; this is at least in part due to the significant differences between species in the relative sizes of the biomass partitions reported in sections 3.2 and 4.2. The *A. glutinosa* treatments had significantly greater mean rate of growth and resultantly had larger biomass partitions. As the size of the ^{14}C activity was the same for each species treatment a significant difference in biomass will result in a significant difference in % ^{14}C activity in each biomass compartment.

Statistical analysis to establish if the mean ^{14}C activity of the root nodule was determined by the species identity of the donor tree. This could distinguish if the differences in recently fixed C allocation alters the quality or quantity of root exudates, which would then impact the supply of ^{14}C to the mycorrhizal fungi and subsequently to the receiver tree linked to the common mycorrhizal network. It was found that the ^{14}C activity located in the receiver tree was not determined by the species identity of the donor tree. This suggested that the transfer of C to the

root nodules is mediated by the fungal network and not by the tree, as has been previously suggested in several theories such as the Mother tree theory (Simard, 2017) and Kin hypothesis (Pickles et al., 2017). Our results show that although the growth, relative biomass partitions and ensuing ^{14}C activity in the partitions is significantly different, that these differences are mediated by the fungal component of the plant-microbe symbiosis.

4.4. Effect of treatment (identity of donor plant) on ^{14}C activity in root nodule

The results from the *A. glutinosa* to *A. glutinosa* treatment (Figure 4) revealed that the majority of the ^{14}C activity located in the receiver fraction was in the root nodules. This is slightly unexpected as we would expect the ^{14}C to have travelled through the mycorrhizal fungal hyphae through the sand filled Perspex tube where it colonises the receiver tree fine roots before being translocated into the root nodules. The data shows, however, very small ^{14}C activity in both the fine and coarse roots suggesting the possibility that the mycorrhizal fungi are colonising the root in the proximity of the root nodules or possibly even colonising the root nodules themselves. This has been suggested in studies but only confirmed in leguminous *Rhizobium* containing root nodules rather the *Frankia* root nodules that we have on the *A. glutinosa* roots. Scheublin et al. (2004) found that one type of arbuscular mycorrhizal fungi, known as Glo3 was found in the roots of legumes (39 %) and in higher concentrations in the root nodules (63 %), while the control non-leguminous plants had lower results (13 %). This suggestion that some mycorrhizal fungi can link directly into the root nodules may go some way to explaining the very high ^{14}C activity located in the root nodules but not the fine or coarse roots. Scheublin et al. (2004) go on to suggest that the arbuscular mycorrhizal community was significantly different in roots nodules than roots in the leguminous plants, again suggesting more abundant or diverse colonisation of the root nodules when compared to the roots of the same plant. Orfanoudakis et al. (2010) suggested that there are interactions between arbuscular mycorrhizal fungi, *Frankia* and *A. glutinosa* that can alter belowground root architecture. Specifically, the presence of tripartite symbiosis increased the number of lateral roots, root hairs and the amount of branching whilst decreasing root hair density (Orfanoudakis et al., 2010). In this study unfortunately the root architecture was not analysed to confirm this hypothesis.

4.5. Root nodule activity as % of total ^{14}C in receiver plants

The root nodule activity as a percentage of total ^{14}C in receiver plants shows us that they appear to be some differences in the allocation to partitions in the receiver *A. glutinosa* tree depending

on the species of the donor tree. Although the results were not found to be significantly different from each other, they did show some interesting differences. The *A. glutinosa* receiver trees had more ^{14}C activity in the leaves than the other species treatments (11.09 ± 4.82 % for *A. glutinosa* donor), compared with 7.66 ± 5.40 % in treatments when *B. pendula* was the donor tree and 5.99 ± 1.06 % for *C. sativa* treatments). This could be as the *A. glutinosa* had a larger leaf biomass and therefore may have been more efficient at reabsorbing ^{14}C that had been respired from the donor tree from the glasshouse atmosphere, or the ^{14}C that had been transferred via the CMN into the roots and then reallocated by the tree to the leaves. During an earlier experiment we used control trees to quantify the amount of ^{14}C reuptake from the ^{14}C respired by the donor tree. It was found that although respired ^{14}C reuptake did occur it was an extremely small percentage of the ^{14}C in the entire mesocosm and therefore it seems more likely that the leaves reallocation rather than reabsorption was the cause of the higher ^{14}C activity in the leaves.

The percentage of ^{14}C activity in the branches of the receiver trees showed no differences with the *A. glutinosa*, *B. pendula* and *C. sativa* donor treatments all showing very similar results (7.31 ± 4.08 %, 8.56 ± 7.03 % and 8.25 ± 3.21 %, respectively; $P = 0.98$). Coarse and fine roots seemed to show greater differences, although not significantly different. Treatments with *B. pendula* as the donor had more ^{14}C activity in the coarse roots than the other two treatments (22.99 ± 13.73 % compared with 6.63 ± 5.29 % for *A. glutinosa* and 14.20 ± 10.12 % for *C. sativa*). The literature provided no mechanistic explanation for these differences due to the novel nature of the experimental design. Fine roots showed less differences between the species treatments although the highest percentage was found in the treatments with *C. sativa* as the donor tree (14.20 ± 10.12 % compared with 9.18 ± 8.43 % and 11.09 ± 6.60 % for the *A. glutinosa* and *B. pendula* treatments).

The percentage of total ^{14}C in the root nodules appeared to show slight differences depending of the donor tree species identity, although these differences were not found to be significantly different. The most ^{14}C activity in the root nodule was located in the root nodules of the *A. glutinosa* donor treatments (65.79 ± 21.94 % compared with 49.76 ± 23.99 % for *B. pendula* and 56.84 ± 17.99 % for *C. sativa* donor tree treatments).

In a world with increasing atmospheric carbon dioxide concentration, plant primary production will likely be limited by the availability of other essential nutrients, including nitrogen (Norby et al., 2010). It is believed that root nodulation as a functional trait in higher plants evolved

approximately between 56- 50 million years ago, at a time when atmospheric carbon dioxide concentrations, temperature and humidity were much higher than we observe today (Sprent and James, 2007; Bowen et al., 2004; Sprent, 2008). It has been theorised that because establishing N-fixing symbiosis with bacteria has substantial C cost to the plant, that the excess of atmospheric carbon dioxide that was available to plants at this time could have been a driver in the evolution of this fundamental plant-microbe symbiosis (Sprent and James, 2007). We suggest that in future, the primary producers will be limited by nutrients such as N and, that once again, this will give actinorhizal and leguminous plants a competitive advantage over species without N-fixing symbiotic associations.

The dynamics of tripartite mutualistic symbiosis are not yet fully understood, due to difficulties in quantifying nutrient flows and the highly complex nature of these belowground interactions. Transfer of C via CMNs is probably mediated by both the trees and the mycorrhizal fungi, but in our study, we showed that the C demand of N-fixation processes resulted in preferential transfer of ^{14}C to the root nodules of the receiver plant. This could be as a result of a source-sink relationship (Fellbaum, 2014). For example, in experiments where a shaded tree was connected to another via CMN, C was preferentially translocated from the photosynthetically active tree (the source) to the more demanding shaded tree (sink). It could also be explained by the belowground trading market (Wipf et al., 2019), which postulates that the accessibility of nitrogen and phosphorus drives the rates of mycorrhizal colonisation and whether the assistance this provides outweighs the cost to the plant (Johnson et al., 2015). This seems unlikely as Orfanoudaki et al. (2010) discovered that tripartite symbiosis developed successfully and still delivered the plant host growth benefits even when N and P were unlikely to be limiting. The other possibility is that the plants are individuals acting as with firms and co-ops (Noë and Kiers, 2018) operating based on the principles of mutualistic reciprocal rewards (Kiers et al., 2011). In this model, neither partner has overall control of the symbiosis and are therefore both willing participants and the equilibrium is maintained through both rewarding the other (Noë and Hammerstein, 1995; Schwartz and Hoeksema, 1998; Hammerstein, 2003). Further experimental work using N and C isotopes simultaneously could begin to elucidate the dynamics of belowground trading in these tripartite symbioses.

5. Conclusion

Our hypothesis for this experiment was:

H_{null} Belowground allocation of C via inter- and intra-specific CMNs will be greatest in "receiver" trees with actinorhizal associations, due to an increased C sink strength generated by the metabolic processes of microbial symbionts.

It was found that belowground allocation was not greater in the species treatments with actinorhizal symbiosis, however the soil respiration was greater in the species treatments with actinorhizal symbiosis and the C storage was greatest in the *C. sativa*. This suggests that the energetic demand of the nitrogen fixation is utilising the photosynthates as an energy supply and that this is driving the increase in soil respiration. Whereas for the *C. sativa* treatments the belowground biomass compartments are less demanding and implying that unutilised exudates in the soil has led to the increased ^{14}C activity measured in the soil under these trees. The hypothesis was therefore rejected.

The most fascinating result from this research was that the energetic demand for carbohydrates of their root nodules nitrogen-fixation is enough of a sink to expedite the ^{14}C transfer from the donor tree across the CMN and into the root nodule of the receiver plant. It was not possible to determine if the ^{14}C was moving directly from the mycorrhizal hyphae into the root nodule or if it as first passing through the receiver trees fine roots before residing in the nodule biomass.

6. Acknowledgements

This work was funded by NERC through the Envision Doctoral Training Partnership. The authors would also like to acknowledge the role that technicians Jonathon Roberts and Sarah Chesworth had in assisting with the production of this research and all the help and support I received in the field and laboratory from Léa Sgro.

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Chapter 6

Investigating carbon allocation in two temperate tree species to understory vegetation via mycorrhizae.

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Unpublished manuscript

Author contributions:
TDP conducted all empirical work with guidance on design, execution and data analysis from ARS and DLJ. TDP wrote the manuscript with all authors contributing to the final version.

1. Introduction

Mycorrhizal symbiosis is the commonest global plant-fungal relationship that involves a diverse range of fungi from both divergent taxonomic groups and functional guilds colonising over 90 % of known plant species (Nguyen et al., 2016; Prasad et al., 2017). These ecologically fundamental associations are comprised of two or more partners trading nitrogen (N), phosphorus and/or a variety of trace elements for photosynthates (Bever, 2015). Research has often focussed on the improved nutritional status of the host. Plants that possess these relationships imbibe water and nutrients, however, fewer studies have attempted to quantify the carbon (C) fluxes from host plant to mycorrhizal fungi and the mechanisms that support allocation of C belowground (Slavíková et al., 2017). It is well-known that, as heterotrophs, ectomycorrhizal fungi are nearly always reliant on C derived from the photosynthesis of their plant-host (Lindahl and Tunlid, 2015), although unlike arbuscular mycorrhizae in some cases ectomycorrhizal fungi have been found to derive some C from decomposition of non-living organic matter (Talbot et al., 2008). However, this flux has not been reliably quantified, with current estimates for arbuscular symbiosis ranging from 4 to 20 % of gross photosynthetic production (Grimoldi et al., 2006; Jakobsen and Rosendahl 1990; Wright et al., 1998a) and ectomycorrhizal symbiosis is reported to vary from 3 to 36 % of gross primary production

(Bryla and Eissenstat, 2005; Řezáčová et al., 2017). Variation in the estimates of C allocation to mycorrhizal partners is thought to be influenced by the species identity of the fungal partner, nutrient availability and the photosynthetic photon flux density that drives photosynthesis and ultimately the plant-fungi source-sink relationship (Lendenmann et al., 2011; Konvalinková and Jansa, 2016).

When two or more trees of either the same or different species are involved in a relationship with the same fungi, this is known as a common mycorrhizal network (CMN) or colloquially the Wood Wide Web (Simard et al., 1997; Helgason et al., 1998). CMNs have been shown to occur in all global biomes (Francis and Read, 1994; Simard and Durall, 2004; Simard et al., 2012; Molina and Horton, 2015), in both natural and agricultural ecosystems (Gosling et al., 2006). Agricultural soils tend to be dominated by arbuscular CMNs whereas in forest ecosystems ectomycorrhizal species tend to prevail. Arbuscular mycorrhizal fungi are the oldest form of mycorrhizal association within the order Glomales (Zygomycetes), whereas ectomycorrhizal associations are more recently evolved symbioses that consist of a small number of ascomycetes and some *basiomycetes* mycobionts (Brundrett and Tedersoo, 2018). Arbuscular and ecto-mycorrhizae exhibit different physiological characteristics and as a result can benefit the host and ecosystem in different ways (Lodge, 2000). For example, ecto-mycorrhizae are known to protect trees from pathogens by forming a complex branched structure between the cortical and epidermal root cells known as the Hartig net and sheath like mantle around the short lateral root tips. This physically isolates the plants roots from the pathogen whilst simultaneously increasing nutrient update (Smith and Read, 2010). It has been found that the rate of ectomycorrhizal colonisation directly promotes plant disease resistance (Machón et al., 2009), whereas arbuscular mycorrhizal fungi do not have this ability (Johnson et al., 2018); untangling the relative importance of each of the two types of relationship to the tree host is extremely difficult (Jones et al., 1998).

Tripartite mycorrhizal symbiotic relationships, commonly known as “bridge trees” are of particular ecological importance as they are an interface between the arbuscular and ectomycorrhizal hyphal networks found in temperate forest soils (Lodge, 2000). Examples of tree species that form both arbuscular and ectomycorrhizal associations are uncommon, but do occur in some cases in temperate forest ecosystems e.g. aspen (*Populus tremula*) (Brundrett et al., 1998) and common alder (*Alnus glutinosa*) (Brundrett et al., 1990) and in Australia e.g. *Acacia* spp., *Casuarina* (Brundrett et al., 1998) and jarrah (*Eucalyptus marginata*) (Brundrett et al., 1994). There is some evidence that the dominant type of symbiotic association changes

during the lifetime of a tree, with arbuscular relationships being more important at the beginning of a trees life and then later giving way to the ecto-mycorrhizae has been observed in both field experiments (Bellei et al., 1992; Dominik, 1956; Gardner and Malajczuk, 1988; Lodge and Wentworth, 1990; Adjoud-Sadadou and Halli-Hargas, 2017) and glasshouse experiments (Lapyeyrie and Chilvers, 1985; Chen et al., 1998). Multiple mechanistic explanations have been suggested for this replacement of arbuscular with ectomycorrhizal fungal associations in tripartite plant species over time (Lodge, 2000). These include: (i) that initially the ectomycorrhizal fungi are slower to colonise plant roots than arbuscular fungi (Chilvers et al., 1987), but once the ectomycorrhizae have established that the mantle creates a physical barrier preventing arbuscular mycorrhizal colonisation (Chilvers et al., 1987); (ii) that chemicals are released by either the fungi or plant host which allows the dominance of ectomycorrhizal fungi (Lodge, 2000), or (iii) that the ectomycorrhizae can outcompete the arbuscular for plant-derived carbohydrates (Lodge and Wentworth, 1990). Chen et al. (2000) were able to demonstrate that multiple drivers were at play simultaneously, the sheaths were shown to quickly colonise the roots blocking colonisation by competitors (see also Chilvers et al., 1987; Chilvers and Gust, 1982), the presence of ectomycorrhizal fungi was also shown to reduce the growth of fine roots and reduce the ability of new arbuscular colonisation (Chen et al., 2000). Some ectomycorrhizal species were found to reduce arbuscular mycorrhizal success more than others, particularly *Laccaria* spp. suggesting a chemical interaction. Although these studies clearly indicate that mycorrhizal fungi have an effect on succession either by interference and/or competition, they also suggest that rates of arbuscular mycorrhizal fungi decrease over time even without the presence of ectomycorrhizae, suggesting changes in the plants physiology altering the colonisation receptivity (Lodge, 2000). In the case of *A. glutinosa*, the tree's roots are quadripartite, that is, that they not only symbiotic relationships with arbuscular and ecto-mycorrhizae, but also form a symbiotic relationship with the N-fixing bacteria *Frankia alni*.

Complex interactions between multiple symbionts within the rhizosphere are understood to be important: (i) as mediators of plant health by protecting the plant host from antagonists (Berendsen, 2012; Laliberté et al., 2015; Vannette and Rasmann, 2012); (ii) in modifying soil structure (Rillig, 2004); (iii) for nutrient acquisition (Bhandari and Garg, 2017); (iv) biogeochemical cycling (Leake and Read, 2017); (v) population dynamics of forest ecosystem plant communities (Wilson et al., 2009; Bennett et al., 2017); (vi) seedling survival (Wężowic et al., 2017); and (vii) in mediating successional development (Allen et al., 2018). The type of

symbiont present can alter plant successional dynamics as ectomycorrhizal fungi can improve rates of seedling survival more than the presence of arbuscular fungi (Van Der Heijden and Horton, 2009).

Transfer of resources through CMNs is a well-established phenomenon. However, primarily due to differentiation between the type of mycorrhizal associations of species used in agriculture (predominantly arbuscular mycorrhizae) and those associations found in temperate forests (predominantly ectomycorrhizae), arbuscular mycorrhizae have not been widely researched in forest ecosystems. An understanding of belowground C dynamics that details which fungal symbionts mediate biogeochemical cycling are increasingly salient in a world of changing environmental conditions (Pickles et al., 2016; Richter and Billings, 2015). C isotope labelling is a powerful technique that can be used to improve our understanding of the complex role the belowground microbial community has in the C cycle (Körner et al., 2005; Klein et al., 2016). Isotopic pulse techniques can utilise both radio-isotopes such as ^{14}C (half-life of 5730 years) and ^{11}C (half-life of 20.4 min) or stable isotopes like ^{13}C, which have increased in popularity since stable isotope ratio mass spectrometry has increased in availability (Epron et al., 2012). An advantage in using the radioisotope ^{14}C in field experiments over the stable isotope ^{13}C is that relatively small amounts can be detected as the ^{14}C isotope does not exist in any significant amount naturally (Epron et al., 2012).

In this field-based experiment we use ^{14}C as a proxy for recently fixed C to monitor the transfer of C from trees to other plant species in the field. Specifically, a ^{14}C pulse chase was conducted to establish the fate of C fixed by two temperate tree species to establish the relative importance of mycorrhizal type to belowground C allocation and subsequent transfer of C to mycorrhizal hyphal networks. The two temperate tree species studied were *Alnus glutinosa* (commonly known as black alder, common alder or European alder), a species with multiple symbiotic relationships and *Castanea sativa* (known commonly as sweet chestnut), a species with only ectomycorrhizal associations.

Our hypothesis for this experiment was:

H_1Transfer of C to ground vegetation will be greatest via the CMN of *A. glutinosa*, that has a symbiosis with both ecto- and arbuscular mycorrhizae, rather than *C. sativa*, an obligate ectomycorrhizal species.

2. Materials and methods

2.1. Study site

The two-tree species studied were *Castanea sativa* Mill. and *Alnus glutinosa* (L.) Gaertn and were chosen from species that comprised BangorDIVERSE, a tree diversity experiment located at the Henfaes experimental farm, Abergwyngregyn, Gwynedd, Wales, (53°14 N, 4°01 W). The experiment was established in March 2004, on two previously agricultural fields, with a total area of 2.36 ha (Ahmed et al., 2016) and has a fully replicated (n = 4) planting design of one, two and three species mixtures of alder (*Alnus glutinosa* L.), birch (*Betula pendula* Roth.), beech (*Fagus sylvatica* L.) and oak (*Quercus robur* L.) at a density of 10,000 stems ha^{-1} planted in 92 plots (Gunina et al., 2017), and has a fully replicated (n = 4) planting design of one, two and three species mixtures of alder (*Alnus glutinosa* L.), birch (*Betula pendula* Roth.), beech (*Fagus sylvatica* L.) and oak (*Quercus robur* L.) at a density of 10,000 stems ha^{-1}. BangorDIVERSE plots are approximately 13-18 m above sea level and 700 m from the high tide mark, with a relatively exposed aspect (53º24 N, 4º01 W). The soil is a sandy loam and classified as a Eutric Cambisol (FAO) (Rheidol series) found over alluvial gravel (Smith et al., 2013) or Fluventric Dystrocherept and Dystric Eutrudepts (US Soil Taxonomy classification system) (Glanville et al., 2012). Soil texture in the 0-10 cm layer was 48.2 ± 1.3 % sand, 33.6 ± 0.9 % silt and 18.2 ± 2.1 % clay, determined by laser diffraction (Coulter LS particle size analyser) (Ahmed et al., 2016). The climate is classified as hyper-oceanic and average rainfall is 1250 mm annually and the annual mean soil temperature at 10 cm depth is 11 ºC (Campbell Scientific Ltd., Shepshed, UK) (Glanville et al., 2016).

2.2. Trees studied

The two temperate forest tree species were selected from trees existent in the experimental forestry plot, on the basis of their contrasting traits. Explicitly, *A. glutinosa* is actinorhizal, with both arbuscular and ectomycorrhizal fungal association, tolerates waterlogged soils and has intermediate shade tolerance, whereas *C. sativa* is exclusively an ectomycorrhizal, late successional, shade tolerant and relatively long-lived tree species. The trees studied were planted in 2004 and had previously been coppiced (cut at ground level) multiple times, as they were part of the experimental plot which was located below power cables supplying electricity to farm buildings. Three individuals of the two species were selected for being an appropriate and uniform size for $^{14}CO_2$ pulse labelling. In the case of the *A. glutinosa*, the mean height was

175 ± 14 cm and diameter at breast height (DBH; 1.3 m) was 8 ± 1 cm, whilst the mean height of *C. sativa* was 178 ± 6 cm with a DBH of 6 ± 1 cm. Initially a brush cutter was used to clear an area of unwanted and interfering vegetation from a 5 m diameter around each treatment tree. Prior to this intervention,the dominant ground vegetation species had included: stinging nettle (*Urtica dioca*), bramble (*Rubus fruiticosa*), thistle (*Cirsium vulgare*), rosebay willowherb (*Chamaenerion angustifolium*), and grasses including Yorkshire fog (*Holcus lanatus*), dock (*Rumex obtusifolius*), chickweed (*Cerastium fontanum*) and regeneration or regrowth of tree species including: sycamore (*Acer pseudoplatanus*), *A. glutinosa* and *C. sativa*.

2.3. Soil chemical properties

To determine if the basic chemical properties of the soil under the canopy of the two tree species studied exhibited any major differences prior to pulse-labelling the soil chemical properties were analysed. Four soil samples were collected using a 7 cm diameter soil corer from under the tree canopy approximately 25 - 35 cm from the tree stem. The soil samples were placed directly into gas permeable polythene bags and stored at 5 °C in a cool box immediately after returning from the field. The soil samples were then passed through a 2 mm sieve prior to analysis. In order to quantify total elemental C and N, a subsample of the soil from under each tree was dried at 105 °C and ball milled before analysis by dry combustion using a CN-2000 analyser (LECO Corp, St Joseph, MI, USA). Soil ammonium (NH_4^+) and nitrate (NO_3^-) concentrations were determined following extraction from soil in 1 M K_2SO_4 in a 1:5 (w/v) slurry and horizontal shaking for 1 hour at 200 rpm, before centrifugation at 4000 rpm for 10 min. Colourimetric determination of nitrate was undertaken (λ = 540 nm) by the vanadium chloride reduction method (Miranda et al., 2001). Ammonium was determined by the salicylate-hypochlorite photometric method (λ = 667 nm) of Mulvaney (1996). Plant available phosphorus was determined by horizontal shaking of fresh soil in 0.5 M acetic acid (CH_3CO_2H) at a ratio of 1:5 (w/v) and determined colourimetrically as molybdate-reactive P (Murphy and Riley, 1962). Moisture content was determined gravimetrically by oven drying at 105 °C (Rowell, 1994). The Smith and Doran (1996) method was utilised to measure soil pH and electrical conductivity (EC) in distilled water in a 1:2.5 v/v slurry. Soil dissolved organic carbon (DOC) and total dissolved N (TDN) were quantified using a Shimadzu TOC-V-TN analyser (Shimadzu Corp., Kyoto, Japan) following extraction (1:5 w/v) in 0.5 M K_2SO_4 (Jones and Willett, 2006). The soil chemical and physical properties are shown in Table 1.

2.4. Pulse labelling technique

A single ^{14}C radioisotopic pulse-label was applied in late summer (8th August 2017) by covering the tree with a large gas-proof bag and then sealing the stem at the bottom to prevent $^{14}CO_2$ gaseous loss. A reaction vessel containing 2 ml of 3 M HCl (namely a 30 mm diameter centrifuge tube lid), was secured inside the bag into which 200 µl of 3 MBq $NaH^{14}CO_3$ was added (Amersham International, Amersham, UK) leading to the formation of a $^{14}CO_2$ enriched atmosphere inside the bag. Each tree was exposed to the same activity (3 MBq) for 4 hours to allow for photo-assimilation in full ambient light with a minimum photosynthetic photon flux density of 800 µmol m^{-2}, s^{-1}. The day was meticulously chosen to ensure favourable photosynthetically active radiation and ambient temperature conducive to photoassimilation to maximise the efficacy of the ^{14}C pulse.

2.5. Non-plant tissue

2.5.1. ^{14}C activity of fungal hyphae

To determine the transfer of ^{14}C (as a proxy for recently fixed C), to the extramatrical hyphae, hyphal in-growth mesh bags were manufactured with the dimensions 10 × 5 × 2 cm using 40 µm mesh (Normesh Ltd, Lancashire, UK) by melting the edges together using a plastic bag sealer using a modified methodology from Wallander et al. (2001). The pore size in the mesh enabled the fungal hyphae to penetrate the bag but excluded roots of the trees and ground vegetation. The bags were then filled with 120 g of acid-washed horticultural grade sand mixed with 20 g of bone-meal to encourage the hyphal colonisation of the bags. The mesh bags were then placed horizontally into the upper-most 10 cm of soil (Ah Horizon) at specified points in two concentric circles 0.5 m and 1 m from the base of the each of the 6 trees, the experimental design is shown in Figure 1. The mesh bags were placed in the soil 1008 hours (42 days) prior to the pulse label was completed to allow for the penetration and colonisation of the sand medium.

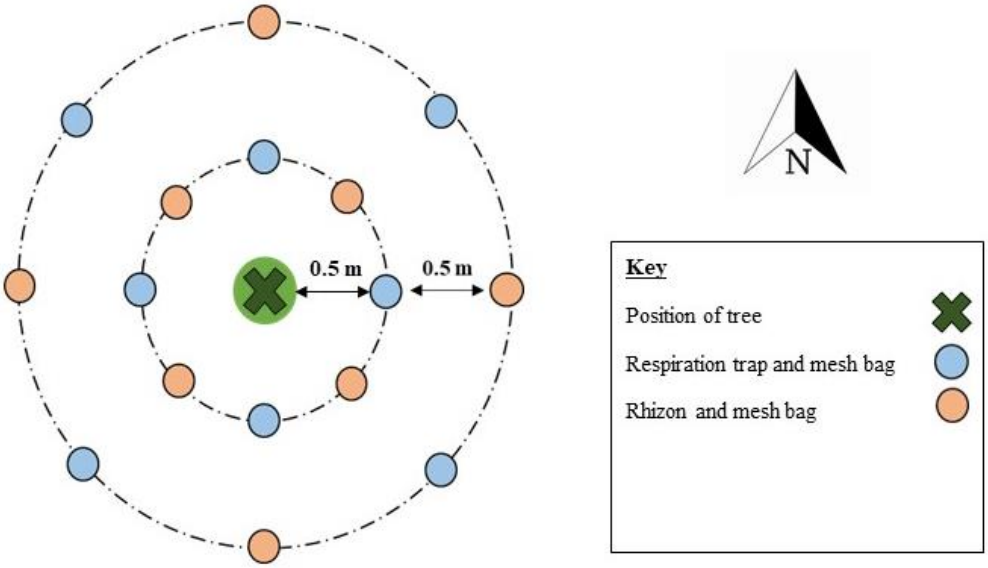

Figure 1: Configuration of hyphal in-growth mesh bags, Rhizons and soil sampling positions relative to each tree, using magnetic North as a datum to locate the first sampling point. Mesh bags were left *in-situ* for a minimum of 42 days to allow for colonisation of mycorrhizal hyphae, then carefully removed at pre-determined time intervals (24, 72, 168, 354 hours after the pulse-label was completed).

Plate 1: *In-situ* and relative positions of the sampling locations in relation to the tree (in centre of plate). Sampling positions for Rhizons, soil sampling, respiration traps and hyphal in-growth mesh bags were protected by plant pots between sampling time intervals (24, 72, 168, 354 hours after the pulse-label was completed).

At the specified time intervals post pulse label, the mesh bags were carefully harvested using a trowel, placed in zip lock bags in a freezer at -20 ºC until analysis could commence. The sand was then were homogenised prior to a 0.4 g sub-sample being analysed for ^{14}C activity by combusting the organic material in the sample with a Harvey Instruments Biological Oxidiser OX400 (R.J. Harvey Instrument Corp., Hillsdale, NJ, USA). The evolved $^{14}CO_2$ was bubbled through and dissolved into Oxysolve C-400 (Zinsser Analytic GmbH, Frankfurt, Germany) scintillation fluid prior to determination of ^{14}C content using a Wallac 1409 liquid scintillation counter (Wallac EG and G, Milton Keynes, UK).

2.5.2. Soil respiration

To trace the ^{14}C pulse throughout the plant-soil continuum, the total soil respiration (heterotrophic and autotrophic) was measured as $^{14}CO_2$ efflux from soil using a 28 mm diameter CO_2 trap containing 1 ml of 3 M NaOH that was suspended above the soil to allow free passage of gases from the soil surface. Each CO_2 trap was collected and replaced at the 0, 24, 72, 168, 354 hours after the initial pulse had been completed.

2.5.3. Soil pore water

^{14}C dissolved in soil solution was sampled following 0, 24, 73, 168, 354 hours after the pulse-label was completed using mini-rhizon suction sampling devices (Rhizosphere Research Products B.V., Wageningen, The Netherlands) (Jones et al., 2016). The $H^{14}CO_3$ content was determined by liquid scintillation counting, in disintegrations per minute, using a Wallac 1409 scintillation counter (Wallac EG and G, Milton Keynes, UK) with automatic quench correction and Optiphase Hisafe 3® (Perkin Elmer, UK) alkali compatible scintillation fluid.

2.5.4. Spatial and temporal dynamics of ^{14}C translocation

Soil respiration and soil pore water was collected at five-time intervals (0, 24, 72, 168, 354 hours) immediately following the ^{14}C pulse-label and hyphal in-growth bags (four time points; 24, 72, 168, and 354), from the sampling positions shown in Figure and Plate 1. All samples were analysed for ^{14}C activity by oxidation and liquid scintillation spectrometry. These sampling positions provided the spatial the dimension to the ^{14}C was translocated through the soil.

2.6. Plant biomass

Trees were destructively harvested after 192 hours and separated into four biomass pools (foliage, branch and stem, roots). Additionally, soils were collected from under each replicate tree using a 4 cm diameter soil auger. The foliage, branch and stem material were immediately oven dried at 105 ºC until constant mass, and soil oven dried at 105 ºC to prevent loss of ^{14}C by microbial respiration. Roots were carefully washed using a sieve stack to remove the adhered soil and stones before being oven-dried at 105 ºC until constant mass. The mass of the plant biomass partitions was determined and then each biomass partition was homogenised, subsampled and ground to a fine powder using a MM200 laboratory mixer ball mill (Retsch GmbH, Hann, Germany). A subsample of each ground and homogenised tree partition was combusted using a Harvey Instruments Biological Oxidiser OX400 as described above.

2.7. Ground vegetation sampling

Twenty-four hours prior to the pulse labelling event the vegetation was removed from a circular area, approximately 5 m radius, from the stem of each of the experimental trees. The ground vegetation that had regrown since the pulse was examined and species present identified and recorded. Ground vegetation species which occurred in all the species replicates were then selected from the species present to allow for comparison between treatments. Then, 192 hours (8 days) following the ^{14}C pulse-label, all ground vegetation within the 5 m radius circle was destructively harvested, placed into paper bags and oven-dried at 105 ºC until constant mass. The ground vegetation biomass was divided into above and belowground biomass and weighed and analysed for ^{14}C activity independently. The biomass of each of the tree's partitions were weighed and recorded before they were then homogenised, ground to a fine powder using a MM200 laboratory mixer ball mill (Retsch GmbH, Hann, Germany). A subsample of each ground and homogenised biomass sample was combusted as described previously to determine their ^{14}C content.

2.8. Statistical analysis

Prior to analysis all data were tested for homogeneity of variance using Levene's test. The independent variable was the species treatment (*A. glutinosa* and *C. sativa*) and time. The dependant data (total plant biomass and partition biomass, soil chemical properties, ^{14}C activity of biomass pools and ^{14}C labelled respiration efflux) were tested for normality prior to analysis using the Shapiro-Wilk Test. The statistical analysis was conducted using ANOVA (non-

parametric) and Tukey's posthoc test with SPSS® Statistics version 25.0 (IBM Corp, Chicago). Main and interactive effects were considered significant at $P \leq 0.05$.

3. Results

3.1. Soil chemical properties

The biogeochemical properties of the soil under the *A. glutinosa* and *C. sativa* treatments were analysed in order to determine if the soil had been differentially altered by the tree species. The results from the laboratory analysis of the soils are shown in Table 1. Overall, the results show that the species had a very small effect on soil quality. Soils under the actinorhizal tree *A. glutinosa* had slightly elevated concentrations of NO_3^- and NH_4^+, but these results were not significantly different from the soils under the *C. sativa* trees ($P = 0.60$ and 0.72 for NO_3^- and NH_4^+ respectively). The only soil property which was found to have significantly different results between the two species was electrical conductivity ($P = 0.03$) with the soil under the *C. sativa* treatments being higher than under *A. glutinosa* (167 ± 14 versus 245 ± 19 µS cm^{-1}, respectively).

Table 1: Soil chemical properties under *A. glutinosa* or *C. sativa* prior to the ^{14}C pulse being initiated. Data are mean ± SE (n = 3). Significant differences are highlighted in bold and the level of significance is denoted by *P < 0.05

	Soil chemical properties								
Tree species	**NO_3^- (mg/kg)**	**NH_4^+ (mg/kg)**	**Available P (mg/kg)**	**pH**	**EC (µS/cm)**	**C:N ratio**	**TDN (mg/kg)**	**TC (mg/kg)**	**Gravimetric H_2O Content (%)**
A. glutinosa	3.71 ± 0.38	3.14 ± 0.64	1.44 ± 0.26	5.86 ± 0.05	167 ± 14	10.02 ± 0.67	8.22 ± 0.91	37.64 ± 3.60	22.8 ± 0.1
C. sativa	3.26 ± 0.48	2.76 ± 0.39	1.89 ± 0.21	5.80 ± 0.03	245 ± 19	9.87 ± 0.84	7.43 ± 0.68	34.81 ± 3.24	24.5 ± 0.7
P-value	0.60	0.72	0.30	0.34	**0.03***	0.89	0.55	0.81	0.09

EC, electrical conductivity; TDN, total dissolved N; TC, total soluble C.

3.2. Hyphal in-growth mesh bags

Twenty-four hours after the initial ^{14}C pulse label, the ^{14}C activity of hyphae within the in-growth bags was 2.53 ± 1.21 Bq g^{-1} and 1.44 ± 0.10 Bq g^{-1} for *A. glutinosa* and *C. sativa*, respectively (Figure 2). Throughout the 348-hour experiment, the ^{14}C activity of hyphae under *C. sativa* remained relatively unchanged. In the case of the hyphae of *A. glutinosa*, the values were not different to *C. sativa* until 168 h, while by 336 h the activity of *A. glutinosa* was 14.3 ± 1.3 Bq g^{-1}, 12-fold more activity than found in *C. sativa* (1.28 ± 0.01 Bq g^{-1}). However, the high degree of variability indicated that the difference was not significant (P = 0.36).

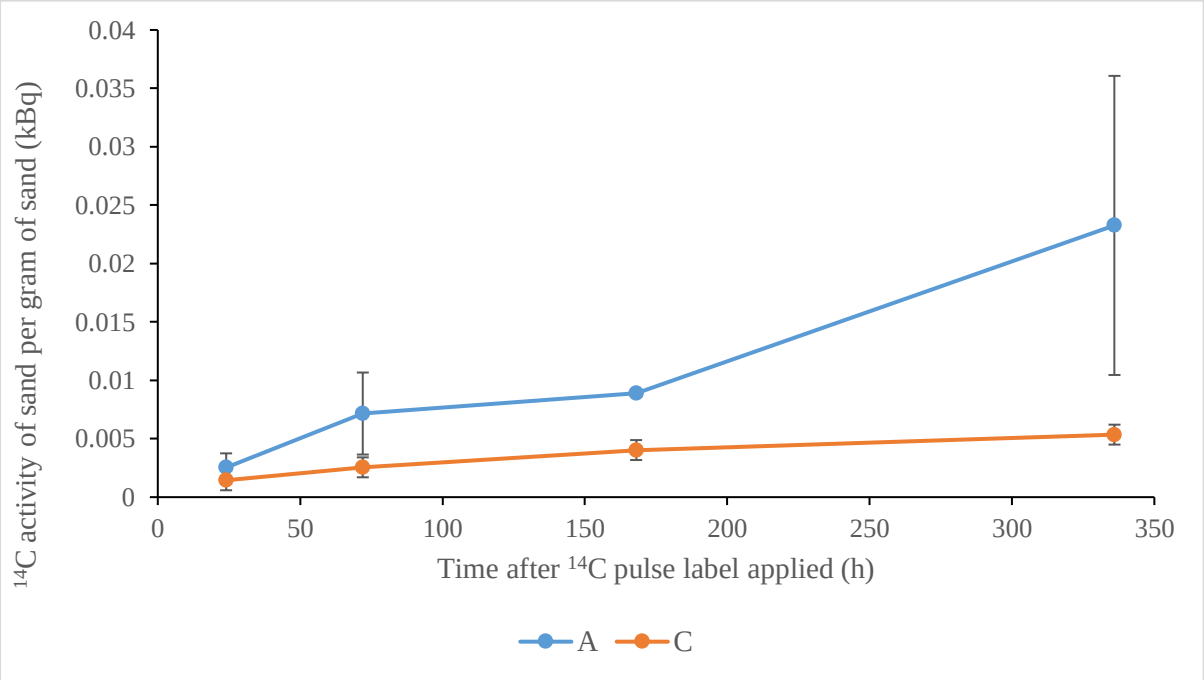

Figure 2- Mean accumulated ^{14}C activity of hyphae collected from hyphal in-growth bags under both species *A. glutinosa* (A) and *C. sativa* (C), during the duration of the experiment. Data is mean (n = 3) ± SE.

The accumulated ^{14}C activity of the sandbags suggests a difference in accumulated ^{14}C activity over the period of the experiment; the differences between species treatments appear to diverge 170 hours after the pulse label was completed. Overall, there were no significant difference between the accumulated ^{14}C activity found in the hyphal in-growth mesh bags under the two species treatments (P = 0.07). When the time points were statistically analysed separately, they were not significantly different from each other (with P = 0.42, 0.37, 0.45 and 0.37 for samples taken at 24, 72, 168 and 336 hours after the pulse label was completed).

3.3. Soil respiration

The cumulative production of $^{14}CO_2$ by the belowground community under the two species is shown in Figure 3. Results show that the mean *C. sativa* treatments had consistently more $^{14}CO_2$ respired immediately post pulse-label event. However, no significant difference between the ^{14}C activity of soil respiration was observed under the two different species treatments (P = 0.55).

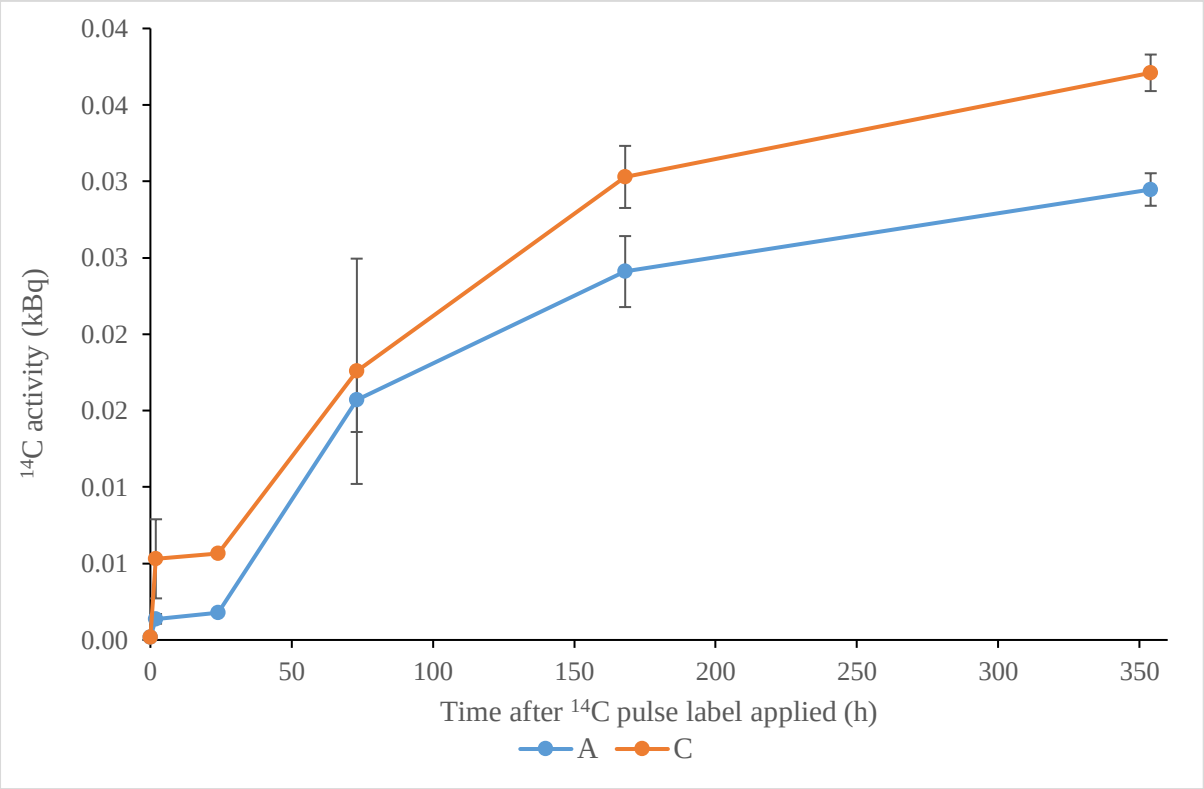

Figure 3- Mean accumulated ^{14}C activity counted in the respiration traps under *A. glutinosa* (A) and *C. sativa* (C) during the duration of the experiment. Data are mean (n = 3) ± SE.

3.4. ^{14}C activity of soil pore water

The ^{14}C activity of the soil pore water was also plotted cumulatively over time (Figure 4) to give an idea of the temporal dynamics of this variable. The results show a similar trend to the soil respiration data plotted in Figure 3, namely that initially the mean ^{14}C activity appears higher in the *A. glutinosa* treatments only to be overtaken by the *C. sativa* treatments approximately 162 hours after the pulse label was completed. Although these trends do suggests slight differences in the speed of transfer or mechanisms underlying transfer to the soil between species, there was no significant difference between the mean ^{14}C activity in the soil solution under the two different tree species [$F_{(1,28)}$= 0.75, P = 0.79].

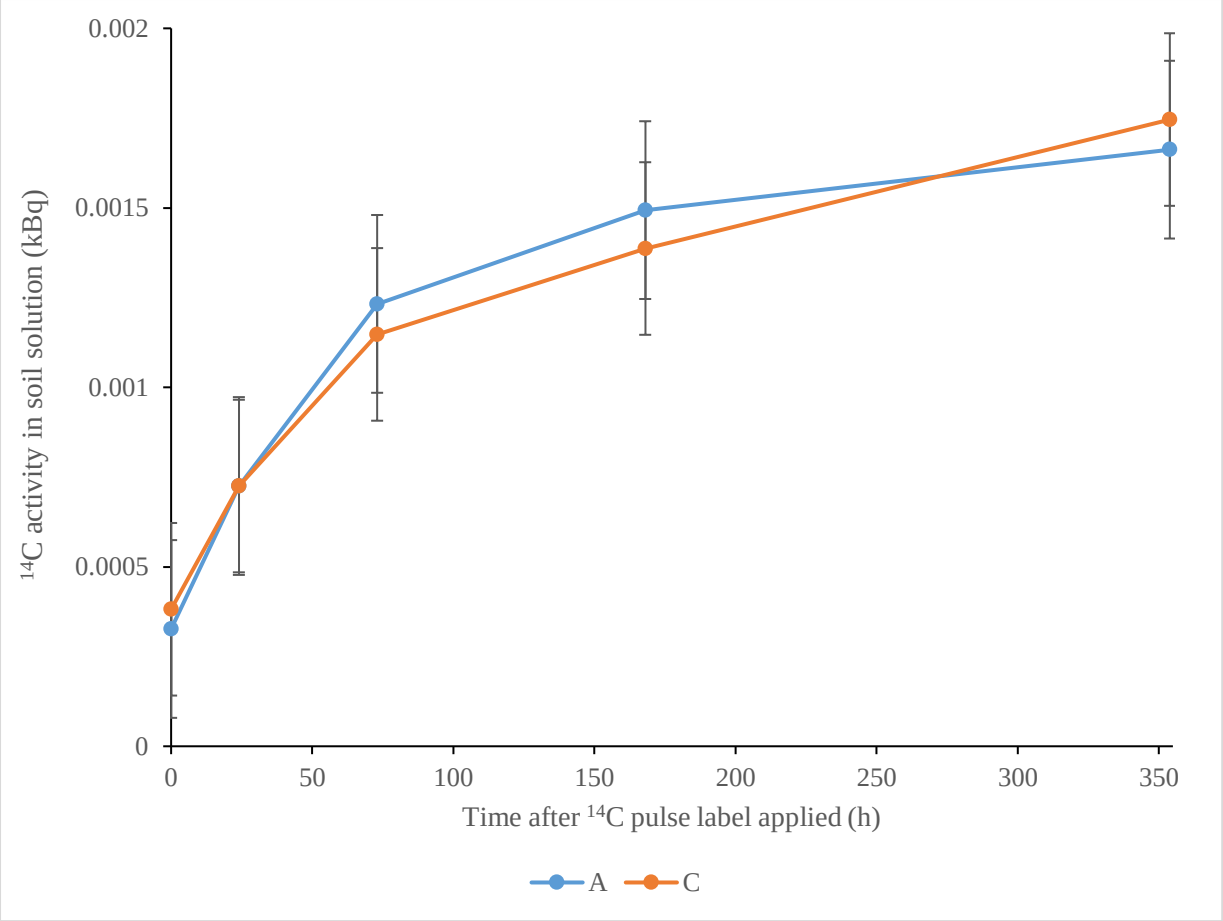

Figure 4- Mean accumulated ^{14}C activity counted in the soil water under *A. glutinosa* (A) and *C. sativa* (C), during the duration of the experiment. Data are mean (n = 3) ± SE.

3.5. Tree partition biomass

Mass of the post-destructively harvested tree partitions are shown in Table 2. Although it appeared that there are differences between the dry weight of the tree partitions between the two species, statistical analysis revealed that the only significant difference between the two species was the coarse root biomass (P = 0.01) (Table 2).

Table 2- Mean dry weight biomass (grams) ± SE (n = 3) of each of the tree species and partitions.

Tree species Tree partition	*A. glutinosa*	*C. sativa*	*P value*
Leaves	85.0 ± 19.5	48.2 ± 16.9	0.49
Stem	57.4 ± 12.1	37.2 ± 8.8	0.23
Branches	324.7 ± 119.6	187.4 ± 80.3	0.25
Total aboveground biomass	467.1 ± 144.5	272.8 ± 104.9	0.39
Coarse roots	112.6 ± 9.3	37.9 ± 16.6	0.01*
Fine roots (E)	1367.9 ± 397.2	1249.2 ± 184.8	0.48
Total belowground biomass	1480.5 ± 406.5	1287.1 ± 201.4	0.53

3.6 ^{14}C activity as a percentage of total ^{14}C activity

Our results show that there are clear differences in ^{14}C allocation between the two species in the field. Aboveground partitions in the *A. glutinosa* treatment (leaf, branch and stem) were larger than the *C. sativa* treatments (Table 3) although the results were not significantly different ($P = 0.63$, 0.35 and 0.11 for leaf, branch and stem, respectively). The total ^{14}C belowground showed the opposite trend with the *C. sativa* treatments having almost twice as much ^{14}C relative to the total activity ($P = 0.02$). This significant difference was driven by the root results which revealed that the relative amount of ^{14}C in *C. sativa* treatment was over twice that of *A. glutinosa* ($P = 0.02$).

Table 3- Mean ^{14}C activity as a percentage of total ^{14}C activity in each tree ± SE ($n = 3$)

Mean % of total ^{14}C	Leaf	Branch	Stem	Total above-ground	Total root	Soil respiration	Soil solution	Soil	Sand	Total below-ground
A. glutinosa	16.76 ± 5.00	6.70 ± 2.92	44.98 ± 9.69	68.44 ± 5.86	30.98 ± 5.85	< 0.01	< 0.01	0.58 ± 0.33	< 0.01	31.56 ± 5.86
C. sativa	14.11 ± 1.09	3.44 ± 0.99	22.73 ± 4.75	40.28 ± 4.28	58.86 ± 4.8	< 0.01	< 0.01	0.86 ± 0.54	< 0.01	59.72 ± 4.28
Probability	0.63	0.35	0.11	0.28	0.02*	0.22	0.29	0.68	0.28	0.02*

The ^{14}C activity when calculated as a percentage of the total ^{14}C activity for each partition, revealed significantly differences in the root activity ($P = 0.02$) and as a result of this significant data the total belowground activity as a percentage of total activity was also significant ($P = 0.02$).

3.6.1. ^{14}C activity above and belowground

A graphical representation of the total above- and below-ground ^{14}C allocation emphasises the differences between the species treatment (Figure 5). The total aboveground ^{14}C activity was not found to be significantly different by treatment ($P = 0.28$), however, the belowground data did show a clear difference ($P = 0.02$).

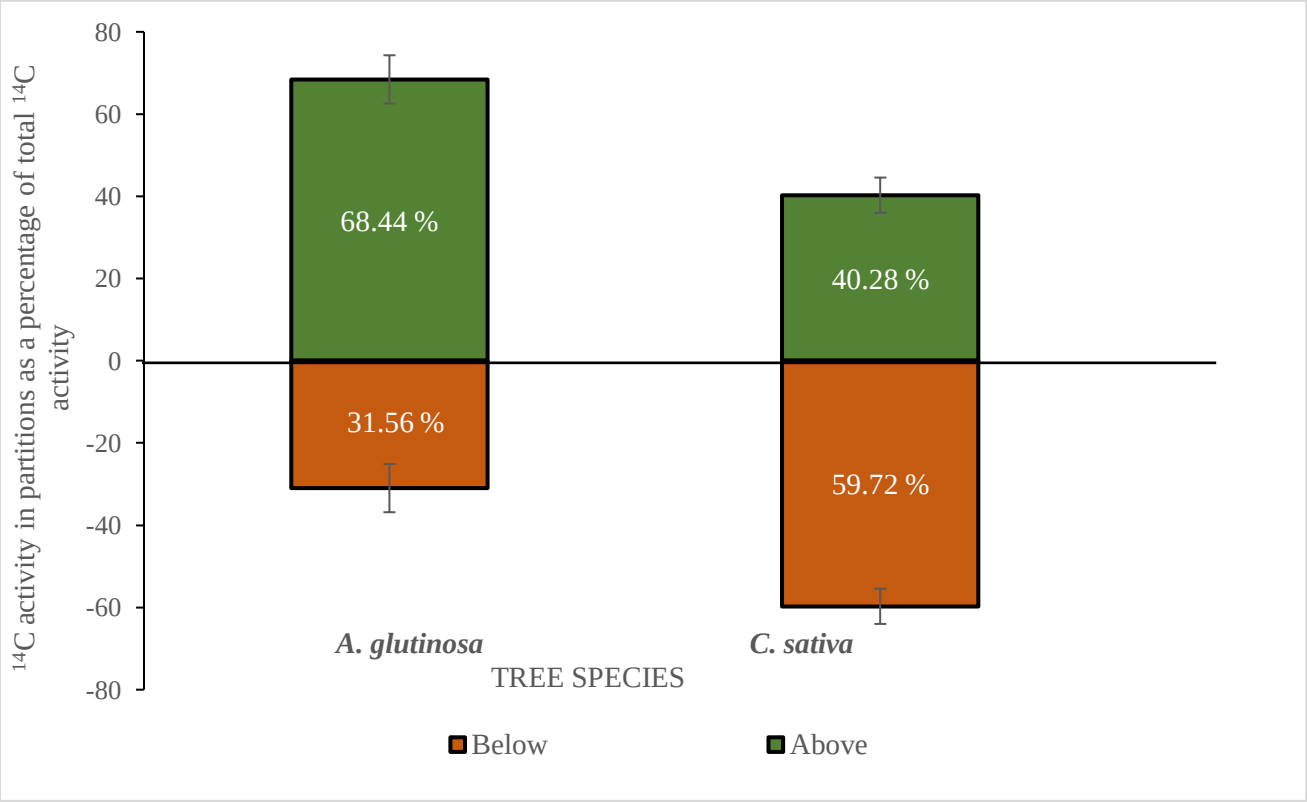

Figure 5- Differences in relative ^{14}C allocation (expressed as a percentage of total ^{14}C activity located in the tree) to both above and belowground tree tissues in the two species treatment (*A. glutinosa* and *C. sativa*). Data is mean ($n = 4$) ± SE.

3.7.1. ^{14}C activity as kBq per gram

The mean ^{14}C activity as kBq per gram was calculated to normalise allocation of ^{14}C activity to the total size of the species' biomass partitions as shown in Table 4. The ^{14}C activity concentration was found to be roughly the same between the two species in the leaves, branch, stem and coarse root (Table 4). However, the mean fine root data showed that the ^{14}C activity concentration was significant different ($P = 0.01$) and four times higher for *A. glutinosa* than *C. sativa*.

Table 4- ^{14}C activity as kBq per gram for five biomass compartments of *A. glutinosa* and *C. sativa* following a ^{14}C pulse label and destructive harvesting after 14 days (336 hours). Data shown are means ± SE ($n = 3$). Statistically significant differences are denoted by $^{*}P < 0.05$.

Species	Leaves	Branch	Stem	Coarse root	Fine root
A. glutinosa	22.98 ± 7.50	18.64 ± 12.90	12.84 ± 8.11	2.64 ± 0.98	47.01 ± 15.93
C. sativa	28.73 ± 10.16	12.90 ± 5.51	8.11 ± 3.44	3.74 ± 0.67	11.19 ± 0.56
P-value	0.67	0.53	0.46	0.41	0.01*

3.7.2. Above and belowground allocation

The mean ^{14}C activity allocated aboveground (as kBq per dried weight gram) was 54.5 ± 9.5 kBq/g for *A. glutinosa* and 49.7 ± 6.4 kBq/g for *C. sativa*, whereas the belowground ^{14}C activity (as kBq per dried weight gram) was 49.7 ± 8.5 kBq/g for *A. glutinosa* and 14.9 ± 0.6 kBq/g for *C. sativa*. Although aboveground allocation was similar in both species' treatments, the belowground allocation was three times the ^{14}C activity per gram of biomass in *A. glutinosa* than *C. sativa* (Figure 6). Statistical analysis revealed that the above and belowground ^{14}C allocation was significantly different between the two tree species ($P = 0.02$).

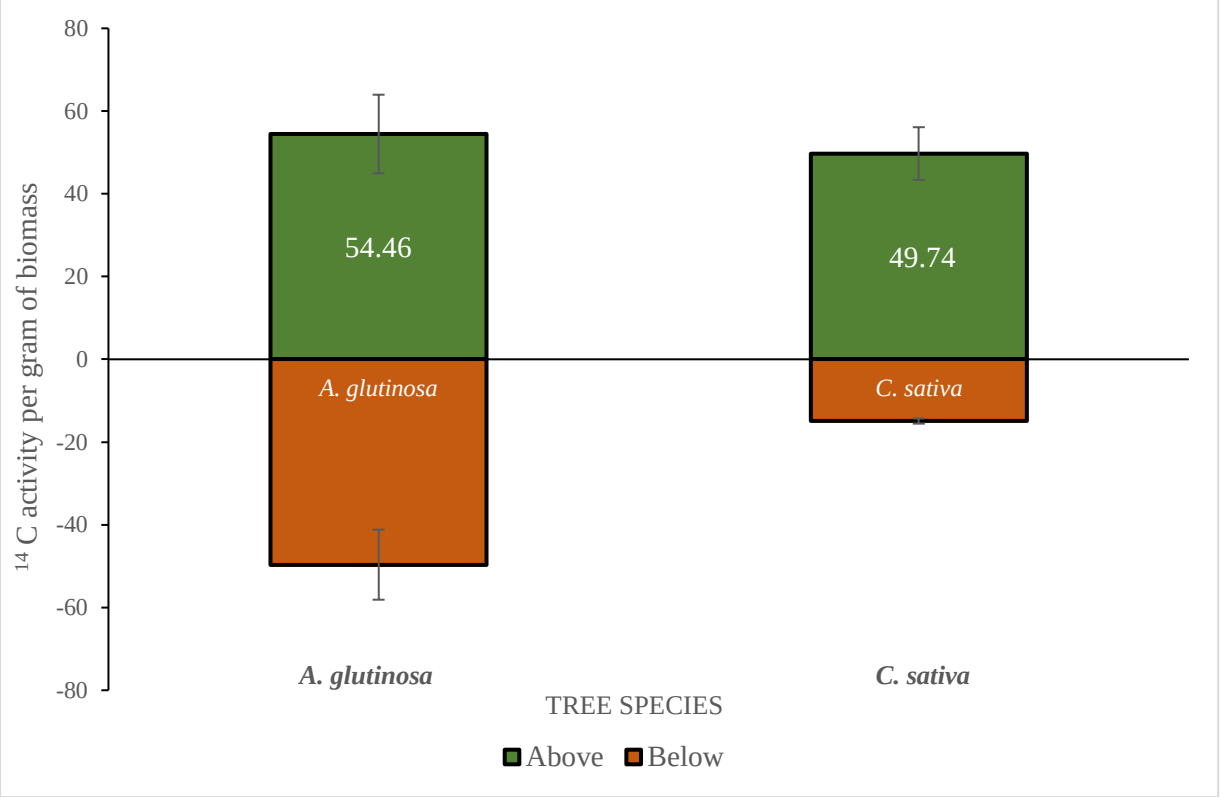

Figure 6: Comparison of the above and belowground allocation of ^{14}C activity expressed as kBq per gram of dried biomass weight (grams) in the two species studied. Data are mean (n=3) ± SE.

3.8. Ground vegetation

The ^{14}C activity which was detected in the ground vegetation was also divided into above and belowground components (Figure 7). Surprisingly, no obvious differences or trends in ^{14}C content were observed in the weed species growing around the trees (P = 0.55).

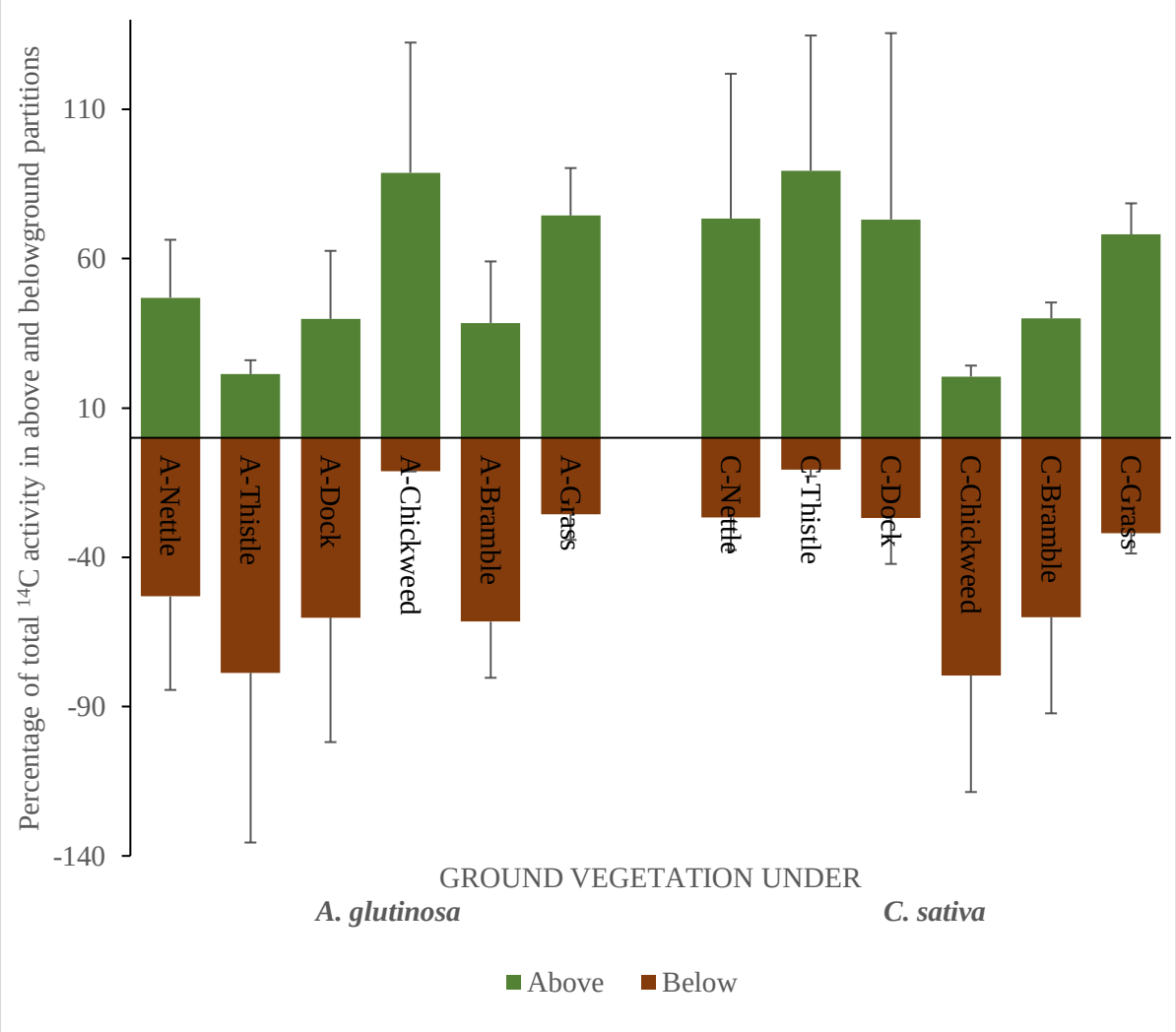

Figure 7- Percentage ^{14}C activity in the above and belowground biomass partitions of the ground vegetation located under the trees of the two species treatments. Data are mean (n = 4) ± SE.

To establish if there were differences in above and belowground ^{14}C allocation to the understorey weed species between the two species treatments, we decided to compare the species treatments as an above/ belowground activity ^{14}C % allocation ratio (Table 5). Although the species treatment appeared to have different above-belowground allocation trends (see Figure 9), the statistical analysis revealed no significant differences in ^{14}C allocation. The *P* values of the statistical analysis are also tabulated in Table 5.

Table 5- The above and belowground allocation as a percentage of total ^{14}C in the understorey plant species represented as a ratio (aboveground/ belowground). Data are mean (n= 3) ± SE.

	Aboveground-to-belowground ^{14}C activity ratio		*P-value*
Weed species	*A. glutinosa*	*C. sativa*	
Urtica dioica (Stinging nettle)	2.47 ± 0.47	2.64 ± 1.08	0.90
Cirsium vulgare (common thistle)	1.98 ± 1.05	11.25 ± 7.64	0.27
Rumex obtusifolius (broadleaved dock)	1.09 ± 0.54	1.95 ± 0.92	0.29
Cerastium fontanum (mouseear chickweed)	1.81 ± 1.04	0.74 ± 0.54	0.87
Rubus fruticosus (bramble)	15.12 ± 11.94	1.07 ± 0.46	0.41
Holcus lanatus (grass spp.)	3.54 ± 1.95	3.26 ± 1.35	0.58

4. Discussion

H_1 Transfer of C to ground vegetation will be greatest via the CMN of *A. glutinosa*, that has a symbiosis with both ecto- and arbuscular mycorrhizae, rather than *C. sativa* an obligate ectomycorrhizal species.

Although it appeared that there were differences in below- and above-ground C allocation to the weeds under the canopy of the two species treatments, statistical analysis revealed that there were no significant difference in either the total, above- or below-ground allocation to the ground vegetation. The hypothesis was therefore rejected.

The key results from this study include the results of the allocation of C (as activity per gram) for the two species. These data showed that the belowground allocation i.e. the allocation of C from the source (leaves) to the sink (roots) was higher in *C. sativa* than *A. glutinosa*. We expected that the belowground allocation in *A. glutinosa* would be greater than that of *C. sativa* as a result of the additional energetic demands of the dual mycorrhizal symbiosis (both arbuscular and ectomycorrhizal) and the bacterial symbiosis with *Frankia alni*. Many previous studies have suggested a positive relationship between the mycorrhizal/ bacterial symbiotic diversity and belowground allocation (Hobbie, 2006; Rygiewicz and Andersen, 1994;

Robinson, 2004). This suggests that the belowground C allocation in the *A. glutinosa* treatments probably further allocates the C to the fungal and bacterial partners (Schiestl-Aalto et al., 2019), whereas in *C. sativa* this C remains in the root biomass, predominantly the fine roots.

This hypothesis is supported by the allocation to hyphae in *A. glutinosa* treatments which shows that C transfer to hyphae in *A. glutinosa* is faster and more prolonged than *C. sativa*. This could therefore go some way to explaining the lack of C in the roots of *A. glutinosa* when compared to *C. sativa.* Interestingly, however, if there was increased C transfer to the hyphae in the case of *A. glutinosa* then it appears that the C must have been utilised by the fungi for hyphal growth as there were no visible differences in the ^{14}C activity recovered in soil respiration. The soil respiration data did not distinguish the heterotrophic (fungal) from the autotrophic (plant) respiration, which may have skewed the data. We would also expect that total belowground respiration would be proportionally more in the *A. glutinosa* treatments. Contrastingly, we would expect a greater proportion of the total; belowground respiration to be autotrophic (from the tree roots). Another interesting result is that you can see the huge differences in biomass allocation in the ground flora. The differences in the results will now be discussed in more detail.

4.1. ^{14}C activity in hyphal in-growth mesh bags

Biological oxidation and subsequent liquid scintillation spectrometry suggested that there was more ^{14}C activity transferred to the soil and hyphae under the *A. glutinosa* trees, however, due to large differences between replicates this trend was not found to be significant. The end of the summer/ beginning of autumn was chosen as the optimal timing period for the experiment to be undertaken as a result of the previous studies that noted as much mycorrhizal colonisation of in-growth mesh bags occurred at the end of the temperate tree growing season (July-September) as for the whole growing season from March (Wallander et al., 2001). In the same experiment Wallander et al. (2001) also noted that mesh bags placed in the ground during the temperate non-growing winter period (December-March) showed no visible signs of mycorrhizal colonisation. This is more obvious as temperate senesced trees are not allocating C belowground during periods of low photosynthetic activity (Royer et al., 2003). The autumn as the most significant period for mycorrhizal growth is supported by the presence at the time of fruiting bodies and the soils chitin: ergosterol ratio (Wallander et al., 1997). It has been

hypothesised that maximal mycorrhizal growth is concurrent with maximal fine root production (Stober et al., 2000).

Some previous studies have shed doubt on the efficacy of the in-growth mesh bag methodology (Wallander et al., 2001) in accurately estimating the biomass and ^{14}C content of the mycorrhizal fungi. Firstly, it is not known how much the mesh bag restricts the normal growth of mycorrhizal hyphae and this would be very challenging to quantify accurately (Wallander et al., 2001). Previous studies have proven that the presence of sand as a substrate in in-growth mesh bags is not a significant impediment to fungal colonisation of mesh bags as tested by the presence of similar levels of ergosterol both inside and outside the mesh bag (Ekblad et al., 1999; Ekblad, 1997). Secondly, we do not know the effect of using sand rather than soil as a substrate and if this discourages or encourages hyphal in-growth. Thirdly, the addition of phosphorus into the sand mix used in the mesh bags may have encouraged the penetration through the mesh of saprophytic fungi. If saprophytic fungi had in fact grown into the bag then ^{14}C measured and assumed to have originated for mycorrhizal fungi could have been from decomposing organic matter, bacterial microbial biomass that fed on ^{14}C enriched root exudates and then decomposed by fungi. Although the sampling technique used in this experiment was consistent and considered a standard sampling technique, it is possible that a portion of the ecto- or arbuscular-mycorrhizal community remained un-sampled and this has been previously reported as a potentially confounding factor (Taylor, 2002; Pickles and Pither, 2014).

4.2. Tree biomass data

The biomass of the tree organs showed only significant differences in the coarse root data between the two species treatments. Sampling techniques including the soil cores which 7 cm in diameter and taken within 30 cm of the soil surface and in-growth mesh bags which were located a maximum of 10 cm, concentrate on the mycorrhizal activity in the forest soil surface. The mean root weight for each tree species per soil core was calculated and then multiplied by the estimated diameter spread of the tree roots. This then gave a total tree root estimation that could be used with the other biomass calculation to investigate the percentages of ^{14}C activity in each of the tree partitions. However, it is known that many mycorrhizal species are active deeper than was sampled (Jenkins et al., 1988; Dalpe et al., 2000; Bornyasc et al., 2005). The specific soil conditions of a field trial are known to alter the root biomass, architecture, spatial heterogeneity and rooting depth of trees (Schenk, 2005; Dickie et al.,

2013). It has been found that even within individuals of the same species rooting depths can vary hugely (Stone and Kalisz, 1991; Canadell et al., 1996). The root core samples, due the experimental timing, were unfortunately taken during the autumn (the end of the growing season), which has been reported to be an inaccurate time to conduct this type of analysis (Anderson and Ingram, 1994; Böhm, 2012). The soils at Henfaes experimental farm are notoriously stony as a result of being derived from glacial till (deposited ca. 10,000 years ago) (Marston et al., 2017). Methods for fine root sampling, storing and washing, however carefully undertaken, will inevitably result in some loss of biomass. It was decided that in this field experiment this had likely happened and as a result a loss correction factor should be applied to the final belowground biomass data. The literature suggests a correction factor of between 1.25-2 depending on estimated losses due to the sampling and processing (Bohm, 1979; Anderson and Ingram, 1994). It was decided in this instance a loss correction factor of 1.5 was most appropriate, based on similar experiments and this was added to the fine root data after the soil cores calculations were completed.

Calculations of the percentage of ^{14}C which at the time of destructive harvesting reside in the various plant structures and below-ground C pools require reliable estimations of the biomass of each compartment. All the aboveground biomass was successfully removed, and by loosening the soil with a spade, some of the coarse roots were extracted from the soil matrix. However, much of the fine roots were not sampled due to the fact that the studied trees had been previously coppiced (cut at just above ground level) on several occasions it was decided that the allometric relationship between above and belowground biomass, or traditional root: shoot or above: belowground biomass relationships found in the literature would be unreliable methods to determine belowground biomass in this instance. It has been reported by some studies that soil cores can underestimate coarse root biomass (Jackson et al., 2009, Major et al., 2012), due to rarely encountering large coarse roots (Taylor et al., 2013) and as a result of limited soil volume being sampled (Levillain et al., 2011). Others, however, report no difference between this methodology and other ways of estimating such as excavated soil pits (Rau et al., 2009, Smith et al., 2013). On balance, due to its simplicity and relative ease of the calculations,the soil core methodology was chosen (Rau et al., 2009). It is hoped the methodology increased the root biomass data obtained from the root cores by reducing the distance between the tree and the sampling point using a previously described method (Sochacki et al., 2017).

4.3. ^{14}C activity above and belowground

The above and belowground allocation data (as a percentage of total ^{14}C activity) emphasises the differences in relative allocation between the two species in the field. Total aboveground ^{14}C activity was larger in the *A. glutinosa* treatments although this was not found to be significant. The total belowground data was found to be significant, with almost half the ^{14}C activity found in the *A. glutinosa* treatments than the *C. sativa* treatments. This is roughly in line with other studies. For example, Gertrudix et al. (2012) found that *C. sativa* had almost twice the C stored belowground in roots than *A. glutinosa.* This could be due to the *A. glutinosa* tripartite symbiotic partners being allocated a large percentage of the belowground C which has been experimentally demonstrated in other species (Kafle et al., 2019; Ossler et al., 2015; Wang et al., 2016).

4.4. ^{14}C activity as kBq per gram

A. glutinosa fine roots had four times as much ^{14}C activity per gram of biomass than the *C. sativa* fine roots. This could be as a result of the soil core methodology which assumes that all fine tree roots located under the canopy of trees originated from that species. As the results were mean data (n= 4) we can assume that this is unlikely to have made that much difference and therefore that either the belowground allocation is greater in *A. glutinosa* or the fact that the *A. glutinosa* treatments have less fine root biomass and therefore the relative concentration is higher. After revisiting the fine root biomass estimations, it is clear that the former is not the case, as mean fine root biomass was similar between *A. glutinosa* and *C. sativa.* Therefore, this suggests that belowground allocation of recently fixed C is more in *A. glutinosa* or that the allocation is either quicker or slower and that as we only had a single destructive harvesting event, we are unable to rule-out temporal variations between species studied.

4.5. Transfer to ground vegetation

This was perhaps the most surprising result of this experiment, we were expecting the ground vegetation to show more ^{14}C enrichment under the *A. glutinosa* than under the *C. sativa* as a result of *A. glutinosa* having both arbuscular and ecto-mycorrhizal associations and the ground vegetation being thought be predominantly arbuscular. The ^{14}C activity was found to be quite similar and with no significant differences between species treatments. Recent studies suggest that trees not only interact with distinct mycorrhizal associations, such as arbuscular and ecto-mycorrhizal symbionts, but also a profusion of diverse and disparate

fungal species located in both the rhizosphere and endosphere (Mandyam and Jumpponen, 2005; Newsham, 2011). These findings question the previous assumption that arbuscular and ectomycorrhizae form distinct fungal networks between which trading is not possible (Toju, Sato, 2018) and that the two groups are sufficiently taxonomically and functionally different to make hyphal fusion unlikely (He et al., 2006). Toju and Sato postulate that the generalist endophytic fungal from the ascomycete order Helotiales, which have known associations with both arbuscular and ecto mycorrhizal plants may have a role in mediating feedbacks between the different mycorrhizal types.

The C transfer from the pulsed trees donor roots demonstrated by these results, could be explained by the following possible processes: (1) root grafting. A root graft is when two or more trees, normally of the same or phylogenetically similar species (Graham, 1966) fuse together, allowing the transfer of water and carbohydrates (Fraser et al., 2006), this has been demonstrated experimentally with both dyes (Graham, 1960), and radio-isotopes (Fraser et al., 2006; Woods and Brock, 1964). It is possible that *A. glutinosa* and *C. sativa* form root grafts as both are in the order *Fagales*, *A. glutinosa* is in the family *Betulaceae* whereas *C. sativa* is part of the family *Fagaceae*, but they are relatively close phylogenetically. (2) The movement of C from mycorrhizal mycelia to soil (Johnson et al., 2002); (3) the rapid turnover of mycorrhizal hyphae within the soil (Staddon et al., 2003); (4) the effect of soil fauna (Klironomos and Hart, 2001; Perez-Moreno and Read, 2001; Johnson et al., 2005); (5) root exudation (Jones et al., 2004); and (6) recapture of C of rhizodeposits from either the roots of hyphae (Rangel-Castro et al., 2002; Sun et al., 1999, Toljander et al., 2007; Walker et al., 2003) processes which are mediated by both plants and mycorrhizas (Jones et al., 2004). Unfortunately, our experimental design did not allow us to determine which of these mechanisms was responsible for the transfer of C, recently fixed by the tree, to the ground vegetation.

Another possible explanation of the lack of difference in transfer to the ground vegetation from the two tree species is that the soil was recently afforested land that was previously managed as an agricultural field, which may have influenced the availability of AM fungi propagules. Arbuscular mycorrhizal propagules although fast to accumulate in vegetated soils (Gould et al., 1996), can become sparse under intensive agricultural management (Manharon et al., 2017) and as a result of fragmentation by agriculture, and lack of continuity of land-use, some temperate forest ecosystems are known to be limited by lack of fungal reproductive material (Veresogloe et al., 2017) and dispersal limitation (Zobel and Öpik, 2014).

The co-existence of multiple layers of vegetation are a unique feature of forest ecosystems and the vegetative strata are almost certainly interacting in ways not yet explicit (Sutherland et al., 2013). The interactions between vertical layers of temperate forests are postulated to be asymmetrical because although the canopy level directly influences the understorey by competition for light interception (Barbier et al., 2008). It is believed unlikely that the ground vegetation can directly alter the health of the canopy or its ability to reproduce (Grünfeld et al., 2019). It is thought that canopy trees may facilitate the formation of diverse understoreys (Veresoglou et al., 2017). The woody perennial over-storey may also act as propagules for the establishment of CMN with the AM ground vegetation species (Van der Heijden et al., 2004), although experimental data to confirm this is lacking (Grünfeld et al., 2019). Radić et al. (2012) found that in the case of a grape (*Vitis vinifera* L.) vineyard that the presence of AM weed species in the understorey significantly altered the fungal community with more diversity and different fungal species dominating. The more frequent and diverse herbaceous plants may be contributing by featuring unique and fundamentally useful functional traits and subsequently could be ecologically disproportionately important (Reiss et al., 2009) and may yet be found to interact directly with the canopy species (Gilliam, 2007). It is believed that in some cases the higher quality of understorey litter could be priming essential cycling processes in the soil, such as decomposition, N-mineralisation and nitrification (Van der Krift and Berendse, 1997; Vereoglou et al., 2011; Luo et al., 2016). The interactions between vertical layers in temperate forest ecosystems are highly complex, but it is reasonable to suggest that dual mycorrhizal trees such as *A. glutinosa* increase AMF propagules in the soil and therefore assist with the diversity, health and growth of the understorey plant species (Azcón-Aguilar and Barea, 1997; Barbier et al., 2008; Van der Heijden et al., 1998).

4.6. Spatial and temporal dynamics

The temporal and spatial dynamics appeared as measured by soil respiration traps, soil pore water, and hyphal in-growth mesh bags seemed to suggest that the plant: microbe symbiosis was pulsing carbohydrates to specific regions of soil at different times. Others studies have found similar results using a range of methodologies including high-efficiency Geiger-Müller tubes (Brownlee and Jennings, 1982), *in vivo* imaging of radioisotopes using a β-scanner (Timonen et al., 1997; Olsson and Gray, 1998), and an autoradiography system to image phosphorus transfer (Lindahl et al., 1999; Leake et al., 2001). Nutrient translocation through rhizomorphs and hyphae is known to occur at rates faster than diffusion, so some form of pumping mechanism is assumed, although not yet fully understood (Tlalka et al., 2002). It

has been suggested that the bi-directional flow of nutrients takes place across the symbiotic interfaces surrounded by the plant and fungal membranes passively whereby it is actively transported into the receiver organism (Smith and Read, 2010). The transport proteins involved in this process have not yet been isolated, however, and more research in this field is needed (Ferrol et al., 2002).

5. Conclusions

We used ^{14}C as a proxy for photo-assimilated C allocation to different parts of the trees, their symbiotic partners and the ground vegetation that was under the canopy of the two-tree species studied. The results suggest that trees possessing dual mycorrhizal symbiotic relationships (i.e., ecto- and arbuscular mycorrhizae), or tripartite symbiosis that includes N-fixing bacteria are ecologically important for the cycling and storage of C in temperate forest ecosystems. However, to further understand the C allocation dynamics of tripartite symbiosis and in particular to identify the specific mechanisms which mediate C allocation to the nitrogen-fixing bacteria arbuscular and ectomycorrhizal symbionts, further analyses would be required. Combining N-fixing species with other temperate forest species is not often considered when selecting temperate forest species in commercial and non-commercial afforestation practice. As a result, many of the potential benefits to the diversity and functionality of temperate forest ecosystems and agroforestry systems are not being exploited. Furthermore, the bilateral resource sharing between trees and the ground vegetation under their canopy has not been considered both in terms of the effect it could have of C storage and priming of leaf litter and the implications for the population and competition dynamics of temperate forest ecosystems.

6. Acknowledgements

This work was funded by NERC through Envision DTP. The authors would also like to acknowledge the role that technicians Jonathon Roberts and Sarah Chesworth had in supporting this research. The assistance and support I received in the field and laboratory from Léa Sgro and Bid Webb was invaluable, especially discussions about root sampling methodology.

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8. Appendices

8.1 ^{14}C activity as kBq per partition

Table S1- kBq per partition

	Species treatment		
	A. glutinosa	***C. sativa***	
Tree partition			***P value***
Leaves	1680.91 ± 301.02	1092.73 ± 123.08	0.15
Stem	629.2 1± 201.11	291.89 ± 105.77	0.21
Branch	5155.74 ± 1745.86	1690.85 ± 183.08	0.12
Root	3758.64 ± 1579.72	4729.91 ± 971.23	0.63
Soil respiration	0.06 ± 0.01	0.08 ± 0.02	0.47
Soil H_2O	<0.01	<0.01	0.57
Soil	52.91 ± 25.27	56.17 ± 27.42	0.93
Sand	0.02 ± 0.01	0.01 ± 0.00	0.22
Total	3811.63 ± 1571.45	4786.17 ± 944.42	0.62

Chapter 7- General discussion and future work

7.1. Overarching aims and objectives

The overarching aim of this research was to investigate the importance of plant: microbe symbioses, commonly found in temperate forest ecosystems, to the belowground cycling and storage of C in soils. Specifically, the objectives were:

1. To investigate the differences in belowground C allocation between early and late-successional temperate tree species (Chapter 3 and Chapter 6)
2. To investigate the intra and interspecific transfer of C via CMNs in temperate forest ecosystems (Chapter 4)
3. To investigate the effect of trees with tripartite mutualistic symbiotic relationships on the magnitude and allocation of C transferred via CMN (Chapter 5)
4. To investigate the differences in belowground allocation between glasshouse-based and field-based experiments (Chapter 6)

In the final chapter, I will discuss and synthesise the empirical findings presented in the thesis and relate them to overarching aims and objectives and discussing possible future research.

Objective 1: *To investigate the differences in belowground C allocation between early and late-successional temperate tree species (Chapter 3 and Chapter 6)*

We tested the hypothesis that the pioneer trees species (*A. glutinous* and *B. pendula*) allocate more C belowground because they are frequent found as primary colonisers, or colonisers of soils with poor nutritional status. Successful colonisation in these conditions requires a growth strategy that invests in belowground biomass (e.g. roots and mycorrhizal symbionts) for nutrient and water acquisition. Chapter 3 showed that in the three-tree species studied there were no differences in C allocation belowground between *C. sativa* and *A. glutinosa* or *B. pendula*. The life history character traits, and successional status of a species, are thought to be associated with specific morphological and functional traits (Leuschner and Meier, 2018). Namely, that late successional stage trees will normally develop under the shade, or partial shade, of other trees and as a result are generally found to have both a higher leaf area and lower rate of photosynthesis compared to early successional species. However, in the case of *A. glutinosa* a broader range of light tolerance enables establishment under canopy in addition to full light (Coll et al., 2008). In early successional stage trees, the maximum rate of photosynthesis is typically higher than late successional species, which would support a greater

allocation of C to belowground biomass for tree stability and access to resources. In contrast to the traditionally held view, the data presented in Chapter 3 showed that *A. glutinosa* allocated less C belowground than *B. pendula* or *C. sativa*. This unexpected finding could be explained by the differences in the mycorrhizae community structure present in the soil and those that are in symbiosis with the trees. Indeed, fruiting bodies of *Laccaria oblongospora* were observed in the experimental plots and have been reported to prevent colonisation by other species of mycorrhizae by altering the root architecture (Chen et al., 2000). Another explanation could be that this was because the experiments were conducted using seedlings that have been shown to alter their morphological traits and patterns of C allocation as a tree ages (Paz 2003; Delagrange et al. 2004; Claveau et al. 2005; Kneeshaw et al. 2006).

Smith and Read, (2010) suggest that approximately 2 to 40 % of photosynthetically fixed C is allocated below ground for either; growth, repair, or metabolic processes (e.g. respiration) by the root system. C exuded from roots may also be utilised for growth and respiration of the soil microbial community (Walker et al., 2003), or becomes incorporated into the soil organic matter pool through the process of root, hyphal and microbial turnover (Jones et al., 2009). Belowground allocation of C can have also been shown to promote root exudation and microbial activity (Tang et al., 2009). Although the rhizosphere is a microbial hotspot, comprised of a diverse microbial community, the mycorrhizal fungi are the most ecologically significant (Jones et al., 2009) that is thought, in some cases, to use up to 30 % of the photosynthetically fixed C assimilated by its symbiotic partner (Johnson et al., 2002; Leake et al., 2006). The use of C isotopes presents an opportunity to increase our knowledge of C allocation to the plant- soil system, and are revealing that C fluxes can vary temporally, according to the time of day, season or even annually. For example, during a mast year the allocation of C to the plant biomass partitions may change due to the C demand of producing seed with subsequent impacts on autotrophic and heterotrophic respiration (Brüggemann et al., 2011). The general principles of C partitioning are thought to involve molecular regulation although the mechanisms that underpin this process are not yet fully understood (Slewinski and Braum, 2010). Empirical studies have also shown that C partitioning occurs by the means of transport carbohydrates through phloem using a pressure-driven mass-flow system, whereby it can be delivered to areas of C requirement i.e. from source to sink (Van Bel, 2003). Ultimately, the allocation is not only driven by the delivery mechanism, in this case photosynthesis, but also the C demand (sink strength) of various plant tissues (Wardlaw 1969). Kayler et al., (2010) suggested that the time lag from C fixation to belowground respiration is an important

indication of the size and status of the C pools, nutritional status, biological activity and the hydration status of the plant, and not just the successional stage of the tree species.

***Objective 2**: To investigate the intra- and inter-specific transfer of C via CMNs in temperate forest ecosystems (Chapter 4).*

In chapter 4, a slightly larger transfer of C between inter-specific species combinations was observed when compared to intraspecific species combination, however there were no statistically significant differences between the two different groupings. The observed trend suggests that interspecific exchange of C and nutrients (i.e. interspecific facilitation) may be more widespread than previously thought and important to our understanding of plant community competition dynamics. DNA extracted from soil to identify mycorrhizal species identified three species, *Sphaerosporella brunnea*, *Inocybe curvipes* and *Laccaria oblongospora* as the likely candidates for the fungi responsible for mediating the transfer between the trees in the treatments. However, no statistically significant differences were found between the diversity and / or abundance of mycorrhizae present in the different tree species combinations. This suggests that C transfer between trees was unlikely to have been limited by an insufficient bank of fungal reproductive propagules present in the soil used in this study. If microbial diversity and abundance were not driving the increased transfer of C between interspecific species combinations then the transfer is most likely mediated by source: sink relationships of the multiple partners, that warrants further study to disentangle. Although it is methodologically difficult to fully quantify the costs and benefits of mycorrhizal symbiosis, particularly because of the challenges in separating root from hyphal respiration in the field (Heinemeyer et al., 2006), and the speed of transfer to belowground symbionts (Johnson et al., 2002). Individual plant species have unique responses to mycorrhizal fungal colonisation (Plett, and Martin, 2018). For this reason, many studies have chosen to compare mycorrhizal with non-mycorrhizal plants of the same species (Norman and Hooker, 2000; Stefania et al., 2018). However, this methodology is not without its own issues (Smith and Smith, 2012), such as the presence of mycorrhizal fungi stimulating root exudation (Kaiser et al., 2015). Some even suggest that it may not be possible to fully compare the responses of the non-mycorrhizal and mycorrhizal plants of the same size and species (Lendenmann et al., 2011), hence the popularity of split root experiments (Douds et al., 2000). The cost benefit analysis of mycorrhizal symbiosis can alter over time depending on climatic conditions, nutrient availability and particularly with the plant and fungal species identity (Péret et al., 2018).

The results from the two CMN experiments, Chapters 4 and 5 demonstrated that transfer between trees via CMN was not only dependant on abundance or diversity of the mycorrhizae present. Chapter 4 suggests that the energetic demands of nitrogen fixation was likely resulting in preferential allocation of donor fixed ^{14}C being allocated to the receiver nodules. Chapter 5 showed a trend whereby more transfer took place in inter- than intraspecific treatments, again suggesting that the growth demands of species at different stages led to bilateral resource sharing. We therefore suggest that the allocation of C to plant: microbe symbiotic partners by the trees appeared to be regulated by source: sink relationships, it is likely that source-sink relationships would be one of the mechanisms involved in the CMN. Another potential mechanism is mycorrhizal mediated trading, whereby nutrient exchange is mediated by the fungi at the plant: microbial interface following the biological market model (Kiers et al., 2011; Fellbaum et al, 2014), or possibly the evolutionary economic game theory (Archetti et al., 2011) whereby uncooperative partners positively selected those that were co-operative.

Objective 3: *To investigate the effect of trees with tripartite mutualistic symbiotic relationships on the magnitude and allocation of C transferred via CMN (Chapter 5)*

The results from Chapter 5 showed that if the trees receiving C via the CMN were actinorhizal, then the C sink strength mediated by the energetic demand of the process of N-fixation was large enough for C to be preferentially translocated to the root nodule via the CMN rather than to other plant organs. This C was transferred through the hyphae of the mycorrhizal hyphae, which were present in the sand tube and forming a CMN connecting the two trees through the 40 µm screen, which covered the tube ends. It is also clear from the results of Chapter 5 and Chapter 6, that plant: microbe symbiosis plays a significant role in not only the belowground transfer and storage of C in temperate ecosystems but also in the formation of plant communities and the mediation of interplant competition (Fonesca et al., 2017).

This may have been an over ambitious objective as investigating belowground root exudation and the abundance and diversity of microbes in the rhizosphere and soil, many of which are currently unculturable (Suyal et al., 2019), has been considered for some time as mission impossible due to the experimental challenges it presents (Oburger and Jones, 2018). Specifically, that analysis of the quality and quantity of rhizodeposits has been hampered by the lack of analytical technology to allow accurate estimates, and that belowground sampling of these minute chemicals into a substrate as heterogeneous as soil also presents multiple sampling challenges (Oburger and Jones, 2018). Another confounding factor is the fact that the

exuded metabolites are simultaneously being released through multiple pathways whilst simultaneously being consumed and cycled during microbial turnover as microbes excrete and respire C, or in some cases, microbial C is assimilated by others when microbes consume each other. Often studies have been conducted in hydroponic (soil less), sterile conditions, to simplify the number of potential variables, this reductionist technique can produce results that further our understanding of C cycling, although how applicable these results are to any real-life situation is still a topic of debate (Sasse et al., 2018; Klein et al., 1988). It is widely accepted that the presence of microbes at the root interface will not only stimulate increased rhizodeposition (Philips et al., 2004) but in some cases can also release compounds that increase root leakiness (Philips et al., 2004; Kudoyarova et al., 2014). To ensure constant benefit for both symbiotic partners in the relationship, these symbiotic relationships are a constant battle between both trading partners, almost akin to predator prey relationships (Oburger and Jones, 2018).

Objective 4: *To investigate the differences in belowground allocation between glasshouse-based and field-based experiments (Chapter 6)*

Our glasshouse experiments were conducted on *A. glutinosa*, *B. pendula* and *C. sativa* selected because they were the species also present in the BangorDIVERSE field experiment. Due to the size of the trees in the field experiment, only coppiced *A. glutinosa* and *C. sativa* trees were available for pulse labelling. Belowground allocation of C for *A. glutinosa* was 32 % of the total ^{14}C activity in both the glasshouse and field-based experiments, whereas for *C. sativa* the belowground C allocation was 60 % of the total ^{14}C assimilated in the glasshouse, but only 45 % of the total ^{14}C activity in the field experiment.

The allocation of C belowground was surprisingly similar for *A. glutinosa* grown in the two different experimental settings, suggesting that the climatic conditions may not influence allocation of C in this species. In contrast, for the *C. sativa* 15 % more C was allocated belowground in the glasshouse experiment. These differences could be explained by the treatment of the soil in the glasshouse experiment, which was sieved to 2 mm altering the soil structure and influencing the soil microbial communities. The mixing and alteration of soil structure is similar to the treatment of soils during tillage by industrial agriculture. Pedology and edaphology both have their origins in agricultural science and as a result, the majority of experimentation of soil biological interactions have been conducted in agricultural soils. However, agricultural soils are altered through various management practices designed to

increase the productivity of a chosen crop species (Thompson, 2017). Agricultural soils are routinely ploughed, mixed and disturbed before the inevitable need for amendment and/or fertilisation to replace the nutrition removed by the harvesting of the agricultural crops and their by-products (Gold, 2016). This processing undoubtedly alters the nutrient dynamics by destroying microsites, which are known to protect C from microbial interactions (Keiluweit et al., 2017), the sieving process replicates this to some extent.

Another possible explanation is that the black plastic plant pots used were in direct sunlight, which may have artificially increased microbial activity influencing microbial metabolic rates and efflux of CO_2. Microbial biomass C turnover rates ranges from 7 to 95 days (Kaštovská and Šantrůčková, 2007) whereas in mycorrhizal fungi it ranges from 5 to 9 days, indicating a comparative expeditious flux of C (Godbold et al., 2006). Turnover times of the soil microbial community can be hard to quantify as often microbial C is consumed by other microbes before being respired (Jones et al., 2009). However, many alternative mechanisms have been postulated in the literature to explain C allocation in different experimental systems (Řezáčová et al., 2017). For example: (i) the choice of model host plant and species of mycorrhizae partner in symbiosis (Pearson and Jakobsen, 1993; Lerat et al., 2003; Lendenmmann et al., 2011); (ii) developmental stage of the symbiosis (Wright et al., 1998); (iii) the volume of the pots (if grown in pots); length of the pulse and chase periods (Řezáčová et al., 2017); and (iv) the environmental conditions (Slavíková et al., 2017).

1.2 The importance of plant: microbe symbiosis in shaping temperate forest ecosystems

Plant microbe symbiosis is known, not only to stimulate belowground C allocation and therefore rhizodeposition and C storage in soils, but can also indirectly affect C capture by the photobionts by indirectly shaping their plant communities through many, not fully understood, mechanisms (Kandlikar et al., 2019). This section will briefly discuss the primary ways in which belowground symbiotic microorganisms have and do indirectly affect the temperate terrestrial primary production through modifying and mediating plant community dynamics.

It is widely accepted that 400 million years ago, the first plants left the sea to begin to inhabit the terrestrial ecosystems and that early forms of arbuscular mycorrhizal plant microbe symbiosis were fundamental to facilitating this process (Remy et al., 1994; Brundrett et al., 2002). The majority of terrestrial plants have retained these beneficial symbiotic relationships (Harley and Harley 1987; Wang and Qui 2006; Smith and Read 2010), with current estimates

of between 74 – 82 % of global plants remaining dependant on this relationship (Brundrett 2009). Over the proceeding millennia the plant species without AM symbiosis have developed a suite of alternative survival strategies including; ericoid, ecto-, and orchid mycorrhizal symbiosis, relationships with nitrogen-fixing bacteria, endophytic fungal and bacterial symbiosis, carnivory, cluster roots and parasitism (Brundrett 2009; Lambers et al., 2011, Lambers and Teste 2013). In some cases, the AM relationships crossed the line from beneficial to the plant to antagonistic (Brundrett 2009; Lambers et al., 2011; Lambers and Teste 2013) and in some cases even pathogenic (Hentschel et al., 2000; Lutzoni et al., 2018).

It is known that plant symbiont can alter the rhizosphere by altering; the plant immune responses (Jacott et al., 2017), hormones (Egamberdieva et al., 2017), exudate composition (Ulrich et al., 2019), and defences to herbivory (Rasmann et al., 2017). It has been suggested that the plant symbiont is also fundamental to shaping the rhizosphere and diversity and functionality of the plant-associated microbiome (Uroz et al., 2019). The topic of succession in plant communities has long been a topic considered fundamental to ecology (Walker and Del Moral, 2003; Prach and Walker, 2011). The concept that symbiotic relationships alter successional trajectory is fundamentally important, not only to understanding how plant communities have and may change over time (Knelman et al., 2018; Allen et al., 2018), but also in the restoration of contaminated and disturbed ecosystems (Pulsford et al., 2016) such as the recolonisation of spoil heaps created by past industrial mining (Frouz et al., 2008; Prach and Walker 2011). Previously the role of aboveground and belowground interactions had been largely overlooked particularly in the biogeochemical cycling and storage of fundamental elements such as C (Shri et al., 2019) and N (Moreau et al., 2019).

C flows not only from trees to fungi, but also from fungi to tree although historically as this is a less frequent study subject (Řezáčová et al., 2017). However up to ten percent of global plant species are at least partially mycoheterotrophic, which means they receive a C benefit from the fungi for the majority of their lifecycle (Leake, 2005). C radio-isotope experimentation (^{14}C) has demonstrated a C flux from fungus to plant in green orchids (*Goodyera repens*) in association with mycorrhizal fungi *Ceratobasidium cornigerum* (Cameron et al., 2008). Although probably less common and therefore ecologically significant there is also proof that fungi can supply some plants with C in the form of amino acids (Aduzinadah and Read, 1986). This form of C transfer which is atypical may yet be significant to the C trading of mycorrhizal associations (Taylor et al., 2004) and may yet be discovered in other forms of mycorrhizal association (Johnson, 2008). Recent methodological developments, such as next generating

sequencing and the expansion of online DNA datasets are allowing us to identify previously unknown microbial diversity and inevitably new forms of symbiotic relationships (Balestrini and Lumini, 2018).

The importance of symbiotic microbial relationships to maintaining diverse and healthy plant populations especially under stress conditions in both agricultural systems (Arora, 2018) and forest ecosystems (DeLuca et al., 2019) is now irrefutable (Spinosa, 2008; Pickles et al., 2020). However anthropogenic activities are now threatening the microorganisms that underpin a healthy global ecosystem (Cavicchioli et al., 2019). Threats to microorganisms are complex and varied and include; climate change (Cavicchioli et al., 2019), pollution (Xavier and Germinda, 1999), N deposition (Regus et al., 2017; Lilleskov et al., 2019) and agricultural activities (Nelkner, et al., 2019). For instance, tillage damages the richness of AM fungi and potentially results in the loss of some of the services AM fungi provide to agriculturally desirable plant hosts. The phylogenetic under dispersion in an AM fungal community, indicated by the presence of few AM fungal genera or families, could be used as an indicator of where AM fungal communities may be limited in functional diversity, if phylogeny can be linked with functional complementarity and AM fungal coexistence (Maherali and Klironomos 2007). Finally, the application of niche theory and temporal community dynamics to AM fungal communities would greatly improve the knowledge base as to how environmental heterogeneity may support AM fungal diversity and thus the overall facilitative effects of an AM fungal community. Nonetheless, although the facilitative, and to a lesser extent antagonistic, effects of AM fungi have been long studied, the mechanisms that control them in a predictable fashion for future ecological application have yet to be fully realized.

An idea known as the Hologenome concept has been postulated (Zilber-Rosenberg and Rosenberg, 2008) which argues that advances in both microbiology and discoveries revealed by novel DNA extraction techniques across all biological fields are suggesting that we can no longer consider animals and plants as individuals all are holobionts comprised of a host individual with multiple symbiotic microbes. As we have discussed microbes are not only on the external surfaces of organisms, but within the body fluids (exosymbionts), and also inside many plant and animal cells (endosymbionts). The human microbiota is thought to contain ten times more microbial cells than the number of human cells (Wenner, 2007) and these microbes are now associated with both mental and physical health (Falony et al., 2019). Plants have been found to have 10^8 bacteria per gram of leaf (Lindow and Brandl, 2003), whereas the rhizosphere can contain up to 10^9 bacteria and 10^6 fungal cells per gram of soil (Forster, 1988). In order for

these microbiotas to be involved directly in the evolution of organisms they would need to be passed down over generations and this has been demonstrated through many mechanisms (Rosenberg and Zilber-Rosenberg, 2011). These include vegetative reproduction (Rosenberg and Zilber-Rosenberg, 2016), cytoplasmic inheritance (Dawid and Blackler, 1972), eggs (Baumann et al., 1995), via faeces (Engel and Moran, 2013), contact during and after birth (Gilbert et al., 2014) as well as many other possible vectors.

2. Future research

Suggestions for future research in this field include more radioisotope work with both N and C, to get a greater understanding of how CMN transfer N fixed by N-fixation in roots nodules between trees intra- and inter-specifically to further investigate the most interesting results from Chapter 4, namely that the CMN plant symbiosis preferentially allocated C to the root nodule of the receiver tree. More field-based research is required to further investigate transfer from ectomycorrhizal only trees to arbuscular mycorrhizal ground vegetation to establish which of the potential mechanisms is responsible for the unexpected results measured in Chapter 6. To get a sense for what is happening at a greater scale these experiments could be replicated using other soils in temperate forests both in the UK and aboard. More species could be investigated and common combinations in order to assist in the creation of soil C modelling software which will be essential in working out where the most efficient gains can be made in sequestering more C belowground in temperate forest for the least financial cost.

More research needs to be conducted to quantify C storage under species mixes that occur commonly in temperate forest ecosystems. This will allow the development of more accurate terrestrial C storage models. In the same way as we are improving aboveground yield models to account for the modern drive away from monoculture clear-cut silvicultural systems and towards forms of continuous cover forestry. Continuous cover forestry increases the importance of understanding how species diversity of both primary producing and microbial components of the plan: microbe symbiosis can mitigate anthropogenic C emissions. There is also a need for more integration of research within the field of plant: microbe symbiotic partnerships. Research has historically focussed in on specialised areas as different scales, which has led to an incomplete understanding of the highly complex mechanisms mediating symbiosis (Ferlian et al., 2018) and in particular how these relationships can help us to achieve

sustainable food production and possibly help mitigate some of the detrimental effects of human driven climate change (Pickles et al., 2020).

3. Conclusions

The empirical data presented in this thesis suggests that the presence of plant microbe symbiosis alters the plant community's allocation belowground, root exudation and ultimately C cycling and storage in temperate forest ecosystems. Consistently throughout the experimental chapters, *C. sativa* has shown greater belowground allocation of C than *A. glutinosa* or *B. pendula*. We therefore suggest that of the three species studied *C. sativa* would be best to maximise C sequestration potential belowground. These results also highlight how little is known about tree belowground allocation across the temperate forest biome. If we are to improve C accountancy and global circulatory models more experimentation of this nature is required. A better understanding of how species choices can improve C draw down under anthropogenically driven climatic change is essential. We postulate from our results that the allocation of C to the tree organs is determined by a combination of supply and demand across a source: sink gradient and the interplay of the two or more symbiotic partners constantly vying to ensure continued benefit from the partnership. It is also understood that the source sink strength of relative biomass partitions will be different amongst, or between species, and at different times during the tree's lifecycle. Our studies suggested that although the presence of actinorhizal root nodules did not seem to alter the overall belowground allocation, it was obvious that the C demand of the N-fixation process was modifying the source: sink relationships in those treatment sufficiently for the plant: microbe continuum to preferentially allocate C to the root nodule of *A. glutinosa* via the CMN. Forest ecology has traditionally neglected to fully account for the importance of belowground symbiotic relationships and as we learn more about their ability to facilitate resource sharing amongst and between species we will also have to find new ways to protect them from the pressures the future may hold for them.

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8. Appendices

This section will provide some backgrounds photographs of the experimental design and construction.

1. Experimental design and construction photos of Chapter 3

Plate 1: Memorial roof glasshouse 3 with experimental Chapter 3 and 4, in progress (taken 18/05/16).

Plate 2: Chapter 3 bisected plant pot with 39 micron screen secured to exclude tree roots from half the pot volume.

Plate 3: 72 pots completed for the experimental Chapter 3.

Plate 4: An *A. glutinosa* (common alder) tree potted in the bisected pot in air-dried soil that had been sieved to 4 mm to be used in Chapter 3.

Plate 5: A *C. sativa* (sweet chestnut) tree potted in the bisected pot in air-dried soil that had been sieved to 4 mm to be used in Chapter 3.

Plate 6: *C. sativa* (sweet chestnut) potted into a bisected pot for experimental Chapter 3 (taken 18/05/16)

2. Experimental design and construction photos of an experiment which does not appear in the thesis

This Perspex slab experiment was a failed attempt to visualise ^{14}C activity being exuded, using a phosphor imaging machine to photograph the fine roots after a ^{14}C pulse-chase had been applied. Unfortunately after constructing the Perspex slabs and growing the three species for 6 months, it was discovered the phosphor imaging machine wasn't working.

Plate 7: Perspex cut up ready for slab construction

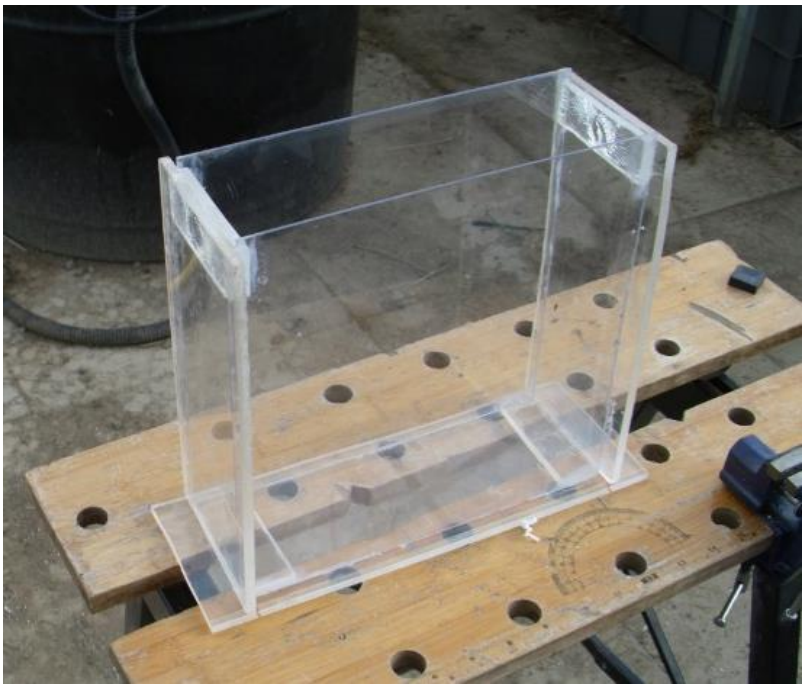

Plate 8: First Perspex slab constructed

Plate 9: reinforced Perspex slabs

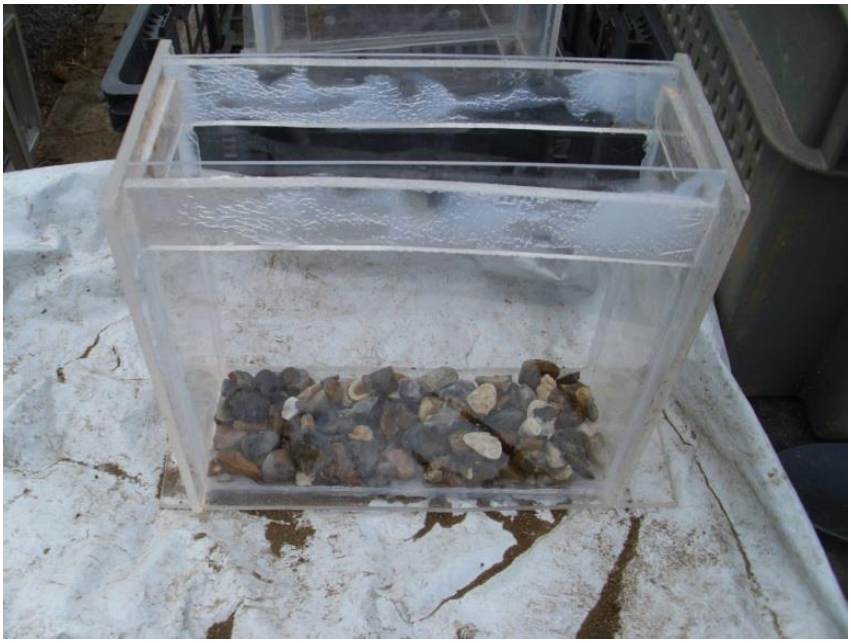

Plate 10: stones were placed in the bottom of the slabs to allow drainage and prevent anaerobic conditions rom damaging the roots

Plate 11: three species of tree potted up in the slabs

Plate 12: Growth of trees in slabs (taken 18/05/2016)

3. Experimental design and construction photos of Chapter 4 showing the Perspex tubes interconnecting pots (transfer via. common hyphal networks)

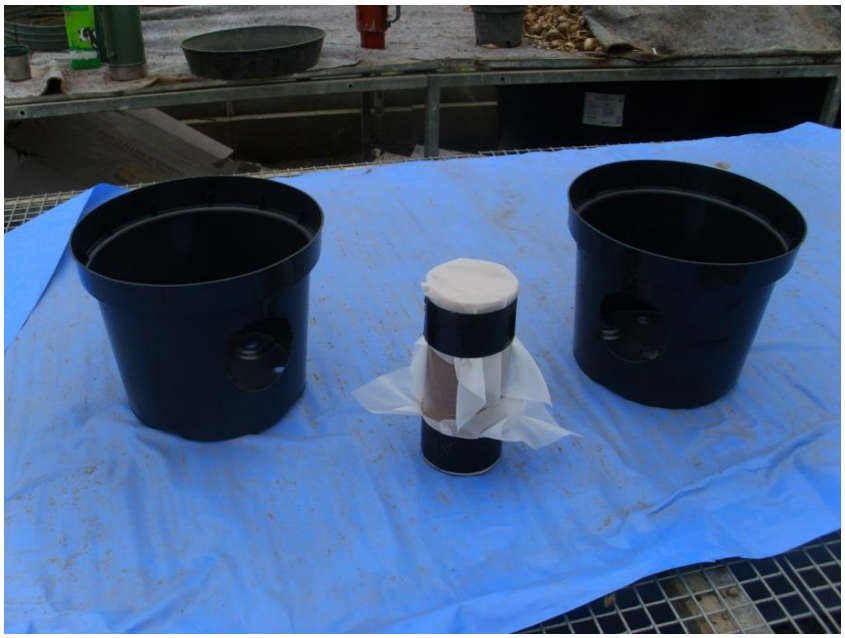

Plate 11: 48 pots had elliptical holes which matched the diameter of the interconnecting tubing

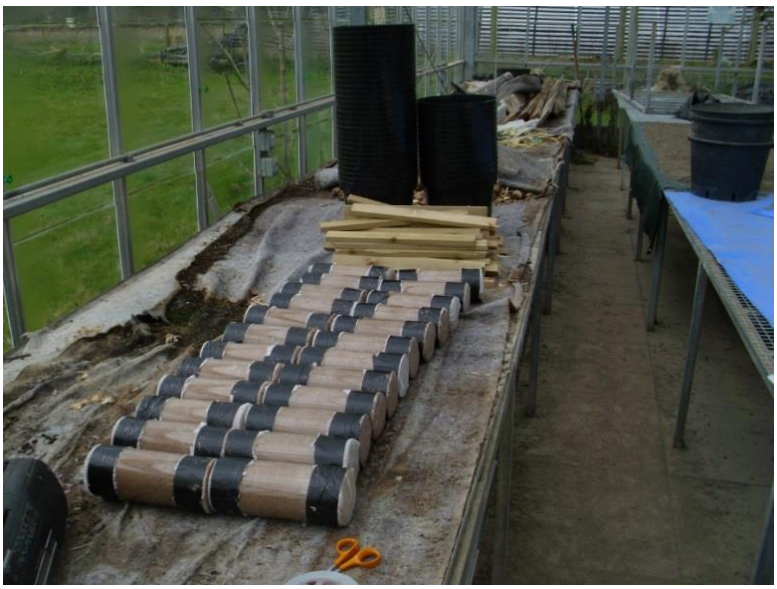

Plate 12: Interconnecting tubing was each filled with 2kg of sand and 20g of bone-meal, then the ends were covered in 40 micron screen and secured with fabric tape

Plate 13: pots were then stapled together with wooded supporting struts to prevent mycorrhizal hyphal from being damaged in transit during the experiment

Plate 14: Interconnecting tubing was each filled with 2kg of sand and 20g of bone-meal, then the ends were covered n 40 micron screen

Plate 15: Pots were then panted with the trees and left to grow and develop mycorrhizal