CHARACTERIZING ENVIRONMENTAL, TEMPORAL AND SPATIAL SCALING OF RHIZOSPHERE FUNGI IN BIOENERGY CROPS; AND THEIR ROLE IN BELOWGROUND CARBON CYCLING

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Declaration

I declare that the work presented in this thesis was conducted by me under the direct supervision of Doctor Gary D. Bending, Doctor Niall McNamara and Doctor Christopher van der Gast with the exception of those instances where the contribution of others has been specifically acknowledged. The work in this thesis has not been submitted previously for any other degree.

Christopher James Barnes

List of Abbreviations

$^{12}C^{+13}C$ - Carbon
$^{13}C/^{14}C$ - Carbon isotope with an atomic number of 13/14
$^{13}CO_2/^{14}CO_2$ - Carbon dioxide with a carbon 13/14 isotope
ABS - Acrylonitrile butadiene styrene
ANOSIM - Analysis of similarity
ANOVA - Analysis of variance
AM - Arbuscular mycorrhizal fungi
C - Carbon
CRDS - Cavity ring down system
CO_2 - Carbon dioxide
DNA - Deoxyribonucleic acid
DGGE - Denaturing gel gradient electrophoresis
ECD - Electron Capture Device
ECM - Ectomycorrhizal fungi
ErM - Ericoid mycorrhizal fungi
FACE - Free atmospheric CO_2 enrichment
FID – Flame ionisation detector
GC - Gas chromatography
GC-MS - Gas chromatography mass spectrometry
ICP - inductively coupled plasma optical emission spectrometer
IRMS - Isotope ration mass spectrometry
ITS – Internal transcribed spacer non-functional region of rRNA
K – Potassium
MID – Multiplex identifier
Mg - Magnesium
N – Nitrogen
NH_4 - Ammonium
NO_3 – Nitrate
P – Phosphorus
PAR – Photosynthetically active radiation
PDB – Vienna PeeDee Belemnite
PCR – Polymerase chain reaction
PLFA - Phospholipid-fatty acid analysis
PTFE – Polytetrafluoroethylene
OTU – Operational taxonomic unit
RDA - Redundancy analysis
Sh – Shannon's diversity index
Si – Simpson's diversity index
SIP - Stable isotope probing for isotopes from DNA, RNA and lipids
SSIM - Small Sample Inlet Module
SOC - Soil organic carbon
SRC - Short rotation coppice
TRF - Terminal restriction fragment
TRFLP - Terminal restriction fragment length polymorphism

Summary

The rhizosphere consists of plant roots and the adjoining soil, which contains a functionally and genetically rich fungal community. The obligate plant symbionts, the mycorrhizal fungi, have been shown to receive substantial quantities of plant-derived C and play an important role in belowground C dynamics. The flux and residence time of C is however likely to be highly species-specific for rhizosphere fungi, and therefore their abundance and composition will likely have important implications on C storage belowground. Rhizosphere fungal community formation is extremely complex and despite being an area of intense research, current understanding is limited.

The composition and abundance of rhizosphere fungi have been shown to vary with temporal and environmental parameters, and potentially geographical separation. However, no studies to date have analysed these parameters simultaneously to isolate the independent effects of each. Clone libraries in conjunction with TRFLP were performed before progressing to 454-pyrosequencing to profile the rhizosphere fungal community of a short rotation coppice (SRC) willow field site. In this work, distinct seasonal fungal assemblages were shown, with N availability having a large effect in summer and geographical distance effects in autumn sampling points. Additionally, a rare large transition in the composition of the rhizosphere fungi was also demonstrated, which was most likely driven by extreme rainfall earlier in the growing season of the year of transition. Finally, using ^{13}C-labelled-CO_2 the belowground movement of recently derived photo-assimilates was shown to differ between *Miscanthus* x *giganteus* and SRC willow, however no significant fluxes were associated with rhizosphere fungal pathways in either crop. Results from this work demonstrate that some of the considerable complexity of microbial communities could have been overlooked in previous community analyses, whilst the flow of C within through mycorrhizal pathways maybe less important in bioenergy cropping systems compared to other ecosystems.

CHAPTER I: INTRODUCTION AND REVIEW OF THE LITERATURE

1.1 General introduction to carbon cycling and bioenergy crops

1.1.1 Global climate change dynamics

Rising average global temperatures have been linked with increasing concentrations of greenhouse gases (Archer and Pierrehumbert 2013). CO_2 is the most abundant greenhouse gas, with an average of 3.3 Gt C yr^{-1} present in the atmosphere (IPCC, 2007). Anthropogenic greenhouse gas emissions have raised atmospheric CO_2 from 280 to nearly 400 parts per million since 1850 (Alonso et al. 2005). The detrimental effects of greenhouse gases have since been recognised in policy, with the Kyoto agreement being a landmark piece of legislation attempting to tackle the problem (UNEP, 1997). However, considerably greater C is found within terrestrial ecosystems than in the atmosphere, with 2011 Gt C estimated to be found within the soil-weathering zone alone (IPCC report, 2007). Whilst terrestrial C can be stored within the biomass of living organisms, substantial quantities are stored within the soils as organic matter (Kirschbaum 1995). Respiration from soils is a substantial C input into the atmosphere, with 1015 Gt C released from soils each year (Raich and Potter 1995), with a number of factors affecting the turnover time of carbon stored within terrestrial systems including, net primary production, climatic factors and edaphic properties (Gallardo and Schlesinger 1994). The conversion of land from natural ecosystems to agriculture nearly universally causes loss of C from the soil into the atmosphere (Murty et al. 2002). Given that agriculture occupies 40% of all usable land, this process is likely to be a considerable anthropogenic contribution to atmospheric C (Guo and Gifford 2002). Consequently, many recent studies have focused on increasing the carbon stored in managed lands, so as to increase the comparability of their C storage function with some natural ecosystems (Ramankutty and Foley 1999; Cannell 2003).

1.1.2 Sustainable energy production in the UK

Energy security is one of the biggest issues facing the world over the next century. The exponential population growth along with rising standards of living has increased demand on finite quantities of fossil fuels across the globe (Asif and Muneer 2007). Fossil fuels are predicted to run out within 1 or 2 generations, with oil, coal and gas predicted to be depleted within 35, 107 and 37 years respectively (Shafiee and Topal 2009). Declining birth rates coupled with increased efficiency measures has stabilised UK energy demand, which is predicted to remain around 6 billion GJ yr^{-1} until the year 2030 (DECC, 2007). However this supply will still need to be met, with the majority still supplied from dwindling non-renewable sources.

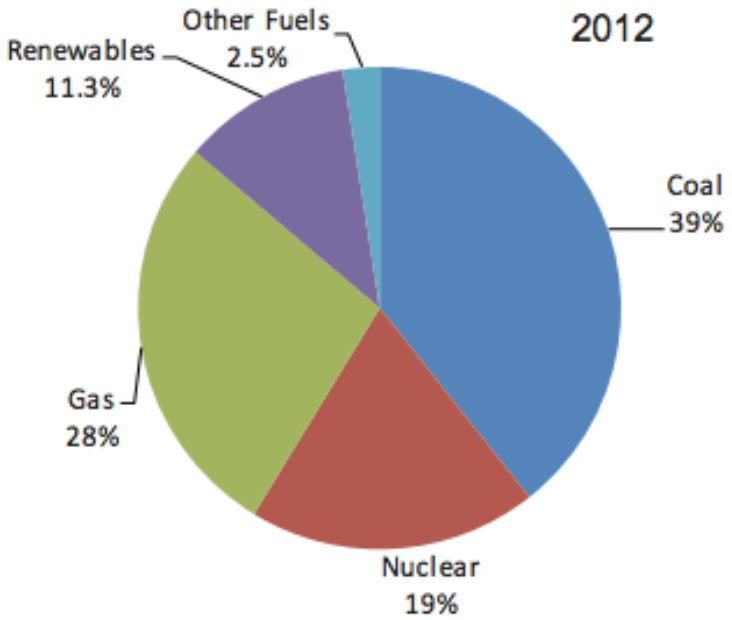

Figure 1.1 Sources of UK electricity generation (DECC Statutory Security of Supply Report, 2013).

The increasing scarcity of fossil fuels has led governments and energy providers to invest in alternative energy supplies to ensure future stability. Nuclear fission can create large quantities of power, operating in a similar mold to fossil fuelled power plants. However with safety concerns, public opinion of nuclear power is extremely negative (Corner et al. 2011). Renewable energy sources such as solar, hydroelectric and wind power offer low emission sustainable alternatives (Dincer 2000). However, a large initial investment is required before energy production, which is often lower than that for traditional energy sources (Varun, Bhat, and Prakash 2009). Governmental incentives and subsidies have been implemented in order to stimulate renewable production, and as of 2011, 11.3% of UK electricity production (4.3% of all energy production) came from renewables (Fig1.1, DECC report, 2013).

Biomass energy offers a useful supplement in the search to reduce fossil fuel dependency. Bioenergy crops can be grown and harvested rapidly for combustion, whilst potentially sequestering carbon during their lifetime. Biomass can also be fed into coal-fired power plants to supplement fossil fuels. This has the advantage of being easy to do, with very little modification to current power generation infrastructure. Biomass can also be converted into liquid fuels and utilized in much the same way as petroleum and diesel (Hammond, Kallu, and McManus 2008). Whilst the energy transfer of this is less favorable than electrical generation, other renewables such as solar, wind and hydroelectric are limited in their capacity to fulfill this role (Rowe, Street, and Taylor 2009).

1.1.3 Potential benefits of bioenergy crops

In addition to energy production, bioenergy crops offer a unique opportunity to convert low yielding agricultural land into low maintenance energy crop production as much less fertilizer is required for growth (Hill et al. 2006). Latest figures show that biomass energy accounts for 82% of total UK renewable energy, a figure that is rising (DECC report, 2013). However there are some contentious issues in the growing of biofuels, especially when they replace food crops. With increased food and housing needs, these tend to be prioritized over biofuel production for available land. Whilst affluent nations have a food surplus for many crops, reductions in food production could lead to more frequent and intense 'spikes' in food prices, disproportionately affecting the poorest (Trostle 2010). However there are many examples where biofuels are economically and ethically viable. For example there are 66,000 ha of brown field land that is currently not used in the UK alone (Hartley et al. 2009). Unlike in food production, these crops can be grown on polluted or contaminated land and could even be useful in bioremediation (Lehmann and Stahr 2007) and thus there is substantial scope for growing bioenergy crops in conjunction with food crops to optimize land utilization.

Energy production from first generation bioenergy crops was reliant on the fermentation of biomass to sugars, which is a highly inefficient process. Second-generation technology uses enzymes to separate plant lignin and cellulose, which can then be fermented as with first-generation crops. Biofuel can therefore be produced from any plant material, markedly improving efficiency whilst allowing a 90% reduction in CO_2 release to the atmosphere compared to petroleum utilization (Blanco-Canqui and Lal 2007). However, this process still only yields around 35% of potential energy from *Miscanthus* with less still for willow (de Visser, Vianden, and Schnyder 1997; Hammond, Kallu, and McManus 2008). Intensive research into improved microbiological and enzymatically degradation of lignin is currently being undertaken, and this is likely to significantly improve the efficacy of the process in the near future (Jørgensen, Kristensen, and Felby 2007).

In order to maximize the carbon sequestration potential of bioenergy crops, high belowground carbon allocation needs to be coupled with lower turnover rates of C within the soil. The increases in SOC associated with bioenergy crops over arable crops can occur via many mechanisms. Arable crops have been bred to yield particularly high aboveground biomass and the transition to bioenergy crops increases the proportion of photo-assimilates that are allocated belowground (Fornara and Tilman 2008; Anderson-Teixeira et al. 2013). Also, bioenergy crops do not undergo regular tillage, which decreases soil aeration and consequently lowers the rate of microbial breakdown of soil organic matter (Grandy and Robertson 2007). Bioenergy crops also invest in deep roots within the soil profile, where C turnover is significantly lower (Jobbágy and Jackson 2000; Matamala et al. 2008). Finally, changes in quantity and quality of litter input (C:N ratio, lignin content, phenolic compound content) can increase the rate of C sequestration through decreased turnover times (Blanco-Canqui and Lal 2007).

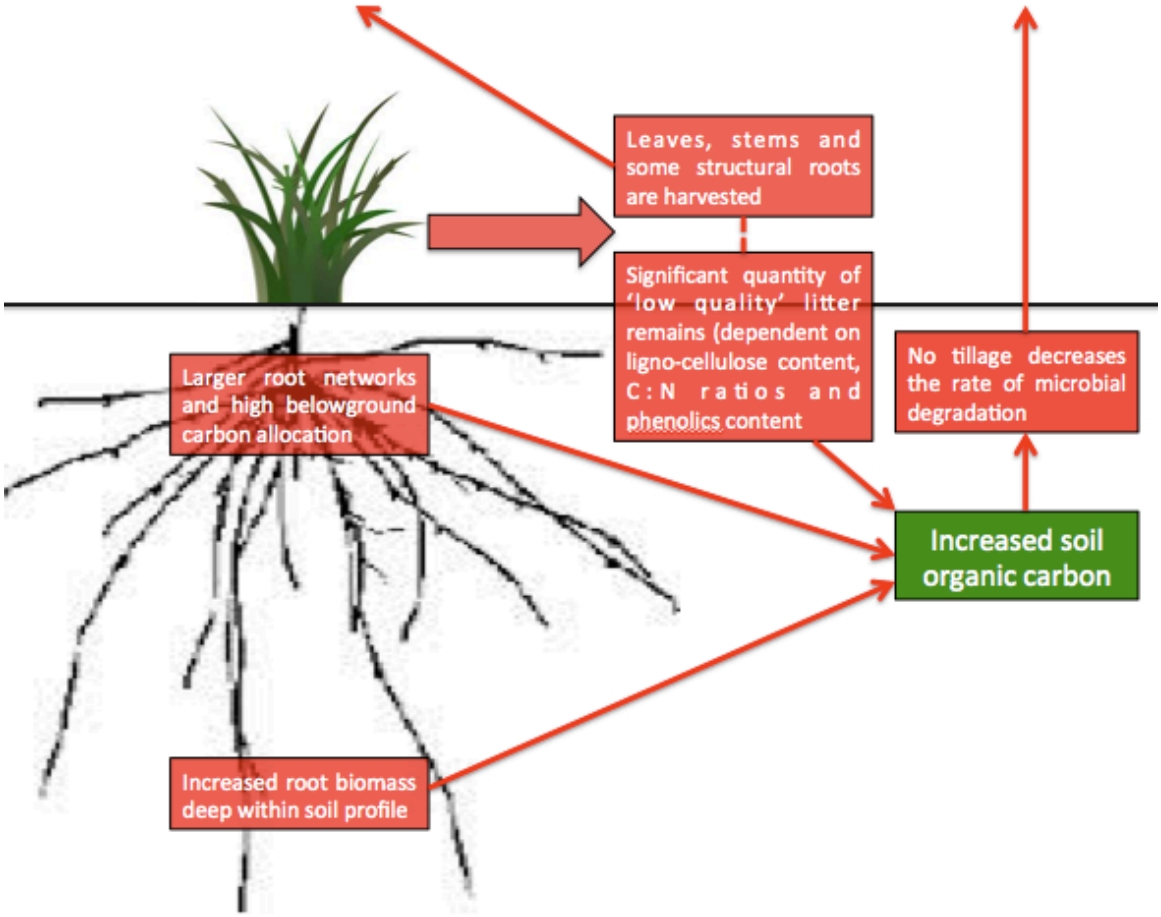

Figure 1.2 Mechanisms for enhanced carbon sequestration by bioenergy crops over arable crops.

In addition to perennial and annual crop differences, different bioenergy crops will also differ in their C sequestration potential. In the UK, grasses such as *Miscanthus* x *giganteus* and switchgrass are commonly grown on commercial scale for bioenergy production, whilst woody tree species such as short rotation coppice (SRC) willow and poplar are also utilized. SRC willow was shown to yield between 2.4-18 t ha^{-1} yr^{-1} of dry matter and sequestered around 1.6 t C ha^{-1} yr^{-1} of from the atmosphere (Taylor and Bunn, 2000; Armstrong 2003). Modelling of SRC willow across the UK predicted yields of between 2 – 42 t ha^{-1} yr^{-1} of dry matter with sequestration rates of 0.2-1.1 t C ha^{-1} yr^{-1} (Grogan and Matthews 2002). More work has been performed on the C sequestration potential of *Miscanthus,* which was shown to yield between 10 to 40 t ha^{-1} yr^{-1} of dry matter (Lewandowski et al. 2000), sequestering 1.63 t C ha^{-}

^{1} yr^{-1} (Kahle et al. 2001), which equates to around 15 GJ ha^{-1} of energy. During the 2000-2006 period, 4600 ha of *Miscanthus* was planted with a further 5400 ha of the perennial grass established in 2007 (NFFCC, 2006; Defra, 2008). It was also calculated that throughout the EU15 area (the countries in the EU prior to the 2004 accession of 10 additional countries), 12 M ha^{-1} of potential ground could be converted to *Miscanthus* to sequester a total of 76 Mt C yr^{-1}, which would reduce EU15 CO_2 emissions by 9%, and significantly contribute to carbon reductions commitments (Clifton-Brown et al. 2000). However the saturation point of European soils with SOC has yet to be determined and the availability of land is likely to be considerably less than that needed in order to meet ambitious targets (Clifton-Brown, Breuer, and Jones 2007).

1.2 Introduction to the rhizosphere

The rhizosphere consists of roots and the surrounding few millimeters of soil, which serves as a hub of interaction between above and belowground ecosystems. Whilst the rhizosphere is considered the communion between active roots and surrounding soil, the vast majority of soil in the weathering soil layer has been directly within the rhizosphere within the previous 100 years (Cardon and Whitbeck 2011). An enormous array of archea, protists, bacteria and animals reside within the rhizosphere, and these communities are connected functionally, interacting across space and time. The rhizosphere is a source and sink for a range of ecologically important compounds, such as sugars and polysaccharides, amino acids and enzymes (Belnap, Hawkes, and Firestone 2003; Pinton, Varanini, and Nannipieri 2007). Consequently the rhizosphere has an influence far beyond itself. Given the pivotal role the rhizosphere has within soil ecosystems, it has been the focus of a great deal of research from mycologists, plant scientists, microbiologists and agronomists, with particular interest in the structure and of its associated microbial communities.

1.2.1 The fungi of the rhizosphere

The fungal community composition of the rhizosphere differs substantially from the bulk soil (Smalla et al. 2001). Mycorrhizal fungi are considered one of the most important groups within soil communities and have received the majority of research on rhizosphere fungi. They are also the dominant microbial group by biomass within the rhizosphere fungi (Högberg and Högberg 2002). Mycorrhizas consist of the association between a plant and specific soil fungi which are obligate symbionts, relying solely on their hosts as a C source. Mycorrhizal associations can either be intracellular (e.g. arbuscular (AM) and ericoid (ErM) associations), or extracellular (e.g. ectomycorrhizal (ECM) associations). AM fungi are the oldest mycorrhizal associations, originating 460 million years ago (Simon et al. 1993). AM hyphae penetrate into plant cortical root cells, forming arbuscules, which are branched structures that are the site of interactions between the host and symbiont. Extraradical hyphae penetrate from the root cells forming an expanse of hyphae within the soil matrix (Bago et al. 1998). ErM form fungal coils within the epidermal cells of their Ericaceous hosts, which also form hyphal networks outside of the root cells (Smith and Read 2010). In addition to AM and ErM, there is also a diverse community of non-mycorrhizal endophytes that colonize root cells (Hyde and Soytong 2008). ECM fungi are the most recent mycorrhizal association to evolve, originating 130 million years ago (Berbee and Taylor 1993). These fungi do not penetrate root cells, instead forming sheaths around root tips (Duddridge 1986). Their hyphae form a 'Hartig net', an extensive latticework of hyphae that connects epidermal and cortical root cells throughout the root system (Blasius et al. 1986).

In return for receipt of plant-derived metabolites, mycorrhizal fungi offer their plant hosts numerous growth benefits. Increased root colonization by both ECM and AM fungi has been linked with increased water and nutrient scavenging, with N (nitrogen) and P (phosphorus) plant content shown to be significantly higher than uninoculated controls (Kucey 1987; Hodge, Campbell, and Fitter 2001). Mycorrhizal fungi have also been linked to increased disease resistance of their host, limiting

colonization of phytopathogenic endophytes (Liu et al. 2007). In addition to direct host benefits, mycorrhizal fungi have been shown to alter fundamental soil properties, with improved soil stability and moisture retention linked to binding of the soil matrix by mycorrhizal hyphal networks (Bearden and Petersen 2000). Non-mycorrhizal endophytes have been reported as minor or secondary pathogens, however the common occurrence of potentially phytopathogenic endophyte populations in both healthy and diseased individuals confounds this relationship (Peláez et al. 1998). Additionally, dark septate endophytes have been linked to increased host N uptake, and the overall role of non-mycorrhizal endophytes is much less clearly understood than for mycorrhizal fungi (Upson, Read, and Newsham 2009). Finally saprophytic fungi also perform an integral role within the ecosystem, breaking down soil organic matter, which ultimately increases the availability of nutrients to the soil biota (Bolton, Fredrickson, and Elliott 1992).

AM fungi can form associations with over 80% of land plant species (Peay et al. 2007), whilst ECM associations are limited to specific gymnosperm and angiosperms taxa, and are associated with just 2% of plant species (Schüβler, Schwarzott, and Walker 2001). The ErM fungi are even more host-limited and are only able to form associations with a few families of the Ericales plant order (Straker 1996). As such, mycorrhizal fungi are globally distributed. Ericoid fungal associations tend to be abundant at high latitudes and altitudes, in addition to nutrient poor regions such as boreal forests, heathlands and bogs (Cullings 1996). ECM associations are abundant in temperate ecosystems such as boreal woodlands, whilst grasslands tend to be dominated by arbuscular mycorrhizas (Treseder and Cross 2006; Vellinga, Wolfe, and Pringle 2009). The large quantities of land used in agriculture has also significantly impacted upon global mycorrhizal distributions, with many arable crops only able to form AM associations (Öpik et al. 2006). Whilst non-mycorrhizal endophytes can form associations with nearly all land plants, host preferences have also been shown and their global spatial distribution remains unclear (Cannon and Simmons 2002; Burgess et al. 2004).

1.2.2 Recent technologies have greatly improved the profiling of microbial communities

Previous investigations into soil microbial communities have been limited by experimental procedures that cannot identify and quantify large numbers of individuals. Characteristic groups, such as mycorrhizal fungi have morphologically distinct structures such as sporocarps and spores, thus have subsequently received the majority of research focusing on the role and distribution of rhizosphere biota. Fruiting bodies have most often been used to investigate spatial and temporal scaling of ECM fungi. However the abundance of sporocarps (fruiting bodies) may not reflect the belowground biomass, with allocation of energy to sporocarp formation differing between species, and some species forming sporocarps infrequently, which may not occur at all within the duration of study periods (Mollina and Trappe, 1986; Richard et al. 2005). Morphotyping of mycorrhizal roots and spores has also been used to investigate ECM and AM communities. However there is a degree of observer subjectivity and diversity is under represented due to poor taxonomic resolution (Smith et al, 2002). Spores are also persistent structures, and therefore their composition and abundance will respond slowly to change (Bever et al. 2001). DNA 'fingerprinting' techniques such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (TRFLP) cheaply capture much more of the diversity and abundance of taxa than traditional techniques (Allmér et al. 2006). However taxa identities are unknown, which limits the biological inferences that can be made from these datasets. Relatively recently developed high throughput sequencing techniques have the ability to improve the characterization of microbial communities, as they can assess diversity whilst providing relative abundance data for named taxa. Whilst studies have begun to pioneer these technologies in microbial ecology, the potential of these technologies has yet to be fully utilized in understanding microbial community assembly (Buée, Boer, et al. 2009; Lim et al. 2010; Tedersoo, May, and Smith 2010; Dumbrell et al. 2011).

1.2.3 Rhizosphere fungal diversity

Traditional morphotyping of spores and roots found that there was considerable diversity within the rhizosphere fungal community (Bruns 1995; Colgan III et al. 1999; Coughlan et al. 2000). However, with the advent of molecular techniques, orders of magnitude higher levels of rhizosphere fungal richness have been discovered. 454-pyrosequencing revealed 470 different fungal taxa within the rhizosphere of a single plant species (Blaalid et al. 2012). Despite host limitations, further studies revealed that 70 different AM fungal taxa were assigned in a mixed grassland alone (Dumbrell et al. 2011), whilst over half of the 600-1000 fungal OTUs found within a temperate woodland were ECM genotypes (Buée, Boer, et al. 2009). Studies of endophyte taxa richness also revealed 551 OTUs within a locally distributed single tree species (Zimmerman and Vitousek 2012). Given this exceptionally high diversity across rhizosphere fungal groups, considerable sampling numbers are needed if a significant proportion of the community is to be detected by analyses.

1.2.4 Rhizosphere fungal composition varies with a plethora of environmental, temporal and spatial parameters

In addition to the substantial diversity found within the fungi of the rhizosphere, the community composition also varies greatly. Whilst the aboveground biota significantly affects the rhizosphere community (De Deyn and Van der Putten 2005), the complex interactions between a variety of other environmental, spatial and temporal factors will also determine the rhizosphere fungal community composition (Marschner et al. 2002; Marschner, Crowley, and Yang 2004; Rosendahl 2008).

1.2.5 Environmental effects on rhizosphere community

Abiotic factors have substantial effects on the rhizosphere fungal community, with changing edaphic properties shown to ubiquitously affect soil microbial communities (Griffiths et al. 2011; Hazard et al. 2013; Anderson, Genney, and Alexander 2014). pH has been linked with rhizosphere fungal diversity and abundance, with low soil pH associated with significantly reduced diversity (An *et al*, 2008). P and N also significantly impact upon the community, with increasing concentrations linked to reductions in both diversity and biomass across rhizosphere fungal groups (Jensen and Jakobsen 1980; Kahiluoto et al. 2001; Nilsson et al. 2004; Hijri et al. 2006; Smith and Read 2010).

Land management has an extremely large effect on edaphic properties. Extremes in pH and P, which can arise through intensive farming and regular fertilizer application, have been shown to have a particularly large effect on rhizosphere fungal composition (An et al. 2008; Gosling et al. 2013). Additionally, a study comparing AM communities under organic and conventional land management found a reduction in diversity under conventional land management (van der Gast et al. 2011), and these anthropogenic effects have been suggested to be a serious threat to belowground diversity, particularly in higher colonizing mycorrhizal systems (Treseder and Cross 2006; Turrini and Giovannetti 2012). Whilst further investigations into this phenomenon are required, it is clear that some land management strategies can have negative impacts on soil communities.

1.2.6 Temporal effects on the rhizosphere community

Climatic factors intrinsically vary with season and have been shown to directly influence the rhizosphere community, with rainfall, temperature and soil moisture all found to impact upon both diversity and abundance of mycorrhizal fungi and non-mycorrhizal endophytes (Anderson, Liberta, and Dickman 1984; Wang et al.

1993; Coughlan et al. 2000; Lovelock, Andersen, and Morton 2003; Tchabi et al. 2008; Zimmerman and Vitousek 2012). Both alpha and beta diversity of AM fungi have specifically been shown to negatively correlate with rainfall and daylight hours (Allen et al, 1998; Dumbrell et al, 2011). However, response to climatic parameters has also been shown to be highly species specific, with different fungal taxa demonstrating a range of responses to changing climatic variables, demonstrating a need for studies to focus beyond community level shifts (Merryweather and Fitter 1998; Helgason, Fitter, and Young 1999; Hijri et al. 2006).

Longer-term transitions in rhizosphere communities also occur over many years. Successional patterns were demonstrated in both AM fungi and ECM fungi, in coastal dunes and retreating glacier zones respectively (Bever et al. 2001; Hart, Reader, and Klironomos 2001). However, as the aboveground biomass also underwent successional changes, it is unclear whether the rhizosphere fungi were the 'passenger' or 'driver' of these transitions. However, in a mixed population of 2 and 5-year-old *Tetragastus panamensis*, age-specific arbuscular interactions were also found, with this plant-host maturation stage is likely having a notable effect on the overall community composition (Daniell et al. 2001; Husband et al. 2002). Similarly, successional patterns in ECM fungi were associated with the age of tree stands (Last et al. 1984; Keizer and Arnolds 1994; Visser 1995). Whilst the exact mechanisms behind this succession of ECM fungi are unclear, there is some evidence that changing root densities and differences between the dissemination and colonization rates of individual species will influence ECM distribution over time (Bastide, Piché, and Kropp 1995; Högberg et al. 1999; Pickles et al. 2010; Blaalid et al. 2012).

1.2.7 Distance effects on the rhizosphere community

In addition to environmental and temporal factors, the geographic distance between AM fungal communities has previously been shown to correlate with differences in the community composition between the two (Green et al. 2004; van der Gast et al. 2011). However, other studies of AM fungi have not found significant distance effects and the importance of distance as a determinant of community composition is still of some debate (An et al. 2008; Hazard et al. 2013). A meta-analysis of AM fungi found distinct assemblages between continents, indicating that long-distance dispersal events are relatively rare (Kivlin, Hawkes, and Treseder 2011). However communities were still found with surprisingly high similarity at the global scale, particularly at environmental extremes. This suggests that the relationship between geographic distance and the community is complex, with distance effects interacting with a wealth of environmental and temporal factors to determine the rhizosphere community composition.

1.3 The movement of carbon belowground and the role of rhizosphere fungi

Given the potential C sequestration by bioenergy crops, much interest has arisen in the mechanisms behind noted increases in the SOC beneath bioenergy crops. Soil C can exist within the biota, as part of roots or microbes, or as part of the bulk soil (Richert et al. 2000). Whilst the majority of soil C is thought to be plant-derived (Crow et al. 2009), significant quantities have also been shown to originate from rhizosphere fungal pathways (Rillig et al. 2003). However, the understanding of how recently derived photo-assimilates flows through plant and rhizosphere fungal pathways and contribute to long-term carbon pools remains limited.

1.3.1 Measuring belowground carbon flux and residence times

Measuring the flow of C in belowground ecosystems is considerably more challenging then doing so aboveground, since changes in SOC are hard to detect, the C pool is large compared to annual inputs of SOC, which also has a long turnover time. Therefore changes in SOC are often too small to detect within the duration of experiments (Hungate et al. 1995). The radioactive isotope ^{14}C occurs in trace quantities within the atmosphere and can be used to measure the flow and storage of photo-assimilates. Incubation of plants in a $^{14}CO_2$ enriched atmosphere will subsequently induce a change in the isotopic composition of photo-assimilates derived during the incubation period, which can be followed through the plant and wider environment. This signal can be tracked in different compartments over time to quantify the fate and longevity of carbon within the entire soil system. However, due to the inherent danger associated with radioactive isotopes, these studies are restricted to the laboratory. ^{13}C is a naturally occurring stable isotope, accounting for just 1.1 % of all carbon in the atmosphere and $^{13}CO_2$ can be used safely in a similar manner to $^{14}CO_2$, to perturb the isotope ratio of plant photo-assimilates.

Stable isotope probing (SIP) experiments have traditionally been performed with closed top chambers, enclosing plants with the labelled gas. However these chambers suffer from 'greenhouse effects', which alter temperature, humidity, light penetration, rainfall, and CO_2 levels, which are often drawn down to below atmospheric levels before label is introduced (Talhelm et al. 2007), all of which may effect plant behavior. Whilst open topped chambers have been used to counteract this, the Web-FACE (Free-air CO_2 enrichment) system delivers $^{13}CO_2$ through a series of porous tubes running through the canopy of trees (Fig1.3; Talhelm et al. 2007; Gamnitzer, Schäufele, and Schnyder 2009). Enriched gases are however expensive, and non-enclosed systems require greater quantities of label to obtain the same level of enrichment of assimilates as closed chambers, and are therefore often prohibitively expensive.

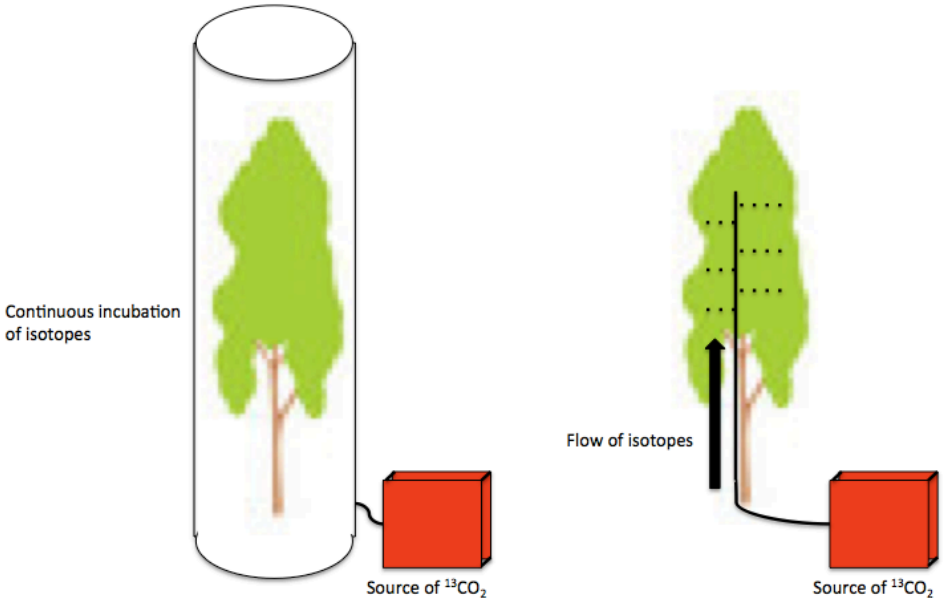

Figure 1.3 (Left) Closed-top chamber. Isotopes are enriched within a static chamber with no airflow. (Right) Web-FACE design alternative to enclosed chambers. Continuous flow of labelled gases ensures stable isotopic enrichment while plants are still exposed to natural conditions (Adapted from Talhelm et al. 2007).

Once fixed, labelled assimilates can be followed through plants into bulk soil and microbes. Bulk soil and large structures such as roots and ECM mycelium can be sampled directly via gas chromatography-mass spectrometry (GC-MS) to investigate enrichment over time. The increasing availability of isotopic analysis with mass spectrometry has facilitated increases in the frequency and sampling numbers achievable in labelling experiments (Rytter and Hansson 1996). Phospholipid fatty acid analysis in conjunction with stable isotope probing (SIP-PLFA) allows for both the estimation of total biomass associated with microbial groups and the relative enrichment of each (Chen et al. 2008). DNA and RNA techniques in conjunction with SIP allow for greater resolution between taxonomic groups whilst still providing enrichment information. However very high quantities of label are needed for this to be performed successfully (Radajewski et al. 2000; Griffiths et al. 2004; Manefield et al. 2007).

1.3.2 Carbon fixation by plant hosts

Photosynthesis is the primary source of metabolites in green plants, and the rate of photosynthesis will vary between species. The maximum photosynthetic activity of leaves, leaf density and leaf turnover rates all need to be assessed if the *in situ* carbon fixation potential of plants is to be accurately calculated (Brodribb, Feild, and Jordan 2007). Once fixed, carbon is transported from photosynthetic tissues to support growth and maintenance across the plant. Newly synthesized carbohydrates (sugars) in the phloem are detectable 4 hours after fixation, peaking after just 16 hours (de Neergaard and Gorissen 2004). Turnover of aboveground carbon is also rapid, with up to an 83 % reduction in the shoot concentration of assimilates just 3-4 days after being fixed in grasses and clover species (Johnson et al. 2002). Low levels of recently fixed C however remain stable within the shoots for over a month, being incorporated into the stem biomass (de Neergaard and Gorissen 2004). Additionally, it is the flow of recently derived photosynthates that support plant fruiting bodies production rather than stored carbohydrates (Wessels, 1993).

1.3.3 Belowground fate of recently assimilated carbon

Photo-assimilates enter the belowground system rapidly from the aboveground biomass, with photo-assimilates detectable in the roots within hours of synthesis in grass species (Ignacio Rangel-Castro et al. 2004) and within 2 days in mature trees (Högberg et al. 2007). Up to 47.5 % of all fixed carbon was detectable within the roots of *Lolium perenne* (Kuzyakov and Domanski 2002), and up to 26 % for various tree species (Epron et al. 2011), representing a considerable sink of carbon for plants. Once it enters the roots, photo-assimilates have multiple potential fates. They can be utilized in active cellular processes within the root (and thus released as CO_2) or incorporated into the root biomass. Rhizodeposition, the release of materials such as sugars from roots into the soil, represents a significant sink of plant-derived

assimilate (Richert et al. 2000). The turnover of dead biomass (Bowden et al. 1993) and the mineralization of rhizodeposits by soil microbes will subsequently release the majority assimilates back into the atmosphere as CO_2 (Rosling, Lindahl, and Finlay 2004). *In situ* analysis of mature *Pinus sylvestris* L. showed that between 7-10 % of assimilates were respired from the soil within a week of synthesis, whilst 10-15 % of assimilates were respired from the soil of mature beech trees within the same time, thus the mineralization of fresh C allocated belowground comprised a substantial proportion of overall soil respiration in both systems (Plain et al. 2009; Högberg et al. 2010). Respiration of photo-assimilates occurs rapidly after fixation by plants and soil microbes, with fresh assimilates emerging from the soil after just 4 hours after assimilation and peaking within 2 days from tree dominated ecosystems (Plain et al. 2009). The turnover was also rapid, with trace quantities of the C detectable in soil respiration just 1 week after fixation.

A significant proportion of soil C is plant-derived, with the stabilization of C associated with plant biomass and exudates allowing plant-derived C to persist within the soil for longer-time periods (Crow et al. 2009). The stabilization of C within roots and litter biomass allows for slow but substantial inputs into long-term belowground C pools (Kavvadias et al. 2001; Janssens et al. 2002). However, rhizodeposition can rapidly contribute photo-assimilates into the soil C pools. The associated microbial biomass and bulk soil of clover and grass were significantly enriched 4 days after labelling in controlled environments, remaining so for more than 30 days post synthesis (de Neergaard and Gorissen 2004), whilst the soil microbes were enriched 2 days after labelling in laboratory grown mature rice plants (Wu et al. 2009). Additionally, the microbial biomass associated with mature trees were also enriched with recently derived photo-assimilates 2 days after labelling in environmental samples (Epron et al. 2011). Given such a short time frame between fixation and label's appearance in microbial pools, rhizodeposition rather than the turnover of plant biomass is likely to be the source of these C fluxes. Whilst approximately 20 % of all fixed carbon was rhizodeposited by maize, the majority was respired by soil microbes, however between 2 and 5 % of net photo-

assimilates were retained in soils throughout the growing season (Hütsch, Augustin, and Merbach 2002), suggesting a significant contribution of fresh photo-assimilates directly into SOC via rhizodeposition.

1.3.4 Belowground flux and residence time of recently derived photo-assimilates varies with the environment

Whilst it has been shown that large quantities of C are transferred belowground, a variety of factors will influence the flux and residence time of recently derived photo-assimilates. The above and belowground allocations of photo-assimilates is highly host-specific, which was shown to vary between species as well as between plant types (de Neergaard and Gorissen 2004; Dannoura et al. 2011). Reductions in soil C input have also been shown with increasing soil pH and nutrient content, through decreased root growth and decomposition (Rosling, Lindahl, and Finlay 2004; Ignacio Rangel-Castro et al. 2004), although increasing soil nutrients may also reduce the proportion of total assimilates that are transported belowground (Stewart and Metherell 1999; Mack et al. 2004). Additionally, the proportion of total fixed C allocated belowground also varies seasonally, with a greater percentage being allocated outside the peak aboveground growing season (Saggar and Hedley 2001; Dannoura et al. 2011).

1.3.5 Mycorrhizal fluxes and effect on soil organic carbon

In addition to plant pathways, mycorrhizal fungi can redirect C directly from roots into fungal pathways. Whilst mycorrhizal fungi have been linked with many beneficial effects on plants, they require significant proportions of C from their hosts. Heinemeyer et al. (2006) estimated the respiration of the extra-radical mycelium of AM fungus to be 0.2 μg C g^{-1} d^{-1}, whilst Ettema, Lowrance, and Coleman (1999) reported ECM mycelium (which are generally thicker as they tend to form

rhizosmorphs) to have hyphal respiration of 40 μg C g^{-1} d^{-1}. Conservative estimates of ECM biomass suggest that 32 % of the soil microbial biomass was extramatrical ECM mycelium, accounting for approximately 145 kg ha^{-1} (Högberg and Högberg 2002). Additionally, Leake et al. (2001) calculated that every nmol of C found in mycorrhizal mycelium (excluding the mycorrhizal mantle on roots), reduced the amount of C found in plants by 2.6 nmol. Given that these fungi are obligate symbionts that are solely reliant on their hosts for C, these fungi are clearly significant sinks of C from their hosts.

Both AM and ECM have been experimentally found to be substantial sinks plant derived photo-assimilates, with up to 20% of plant C associated with mycorrhizal pathways (Leake et al. 2001; Högberg and Högberg 2002; Smith and Read 2010), whilst the total cost of supporting AM extraradical hyphae alone was shown to be between 0.8 % and 1 % of net photosynthates (Jakobsen and Rosendahl 1990; Heinemeyer et al. 2006). The flow of assimilates through fungal pathways is also rapid, with the external mycelium of seedling of *Pinus sylvestris* L. shown to receive photo-assimilates just 24 hours after photosynthesis, whilst this figure is between 2 and 4 days for mature trees (Leake et al. 2001; Högberg et al. 2007). Unsurprisingly given the intimate nature of mycorrhizal associations, photo-assimilates were found in roots and hyphae simultaneously. However, unlike the rapid rise and fall of recently derived photosynthates found in soil respiration, assimilates in extra-radical hyphae do not peak until 8 days after photosynthesis, suggesting a mycorrhizal role in mid to long-term C cycling (Leake et al. 2001).

Nearly all studies to date have measured the net transfer of C between root and mycorrhizal fungus. Only one study of mycorrhizal fungi has measured the flow from fungus-to-plant as well as plant-to-fungus (Cameron et al. 2008), with nearly all work to date assuming this upward flow of C is negligible (Johnson et al. 2002). Orchids have been shown to be dependent upon fungal symbionts to supply C in early development, with C rich molecules such as glutamine passing from the symbionts to the plant host (Smith and Read 2010). Assessments of C allocation

from plants into fungi may underestimate the flux of C allocated to the mycorrhizal symbionts, with a significant proportion of C provided to the fungi being returned to the plant host in other forms. This flow of C from fungus to the plant was found to be a maximum of 20% of input, however there is likely to be considerable variability in this figure with different plant-hosts and types of mycorrhizal association (Cameron et al. 2008).

The presence of mycorrhizal fungi has also been shown to affect SOC content. There was a tenfold reduction in SOC concentration and soil respiration derived from assimilates when AM hyphae were excluded from the soil of a grassland in Scotland (Johnson et al, 2001). Whilst lime treated pastures were shown to have both greater mycorrhizal abundance and higher concentration of recent assimilates in soil effluxes than untreated controls, there was a significant reduction in SOC with increased AM abundance (Staddon 2003), suggesting the relationship between mycorrhizal fungi and SOC storage may be more complex than previously thought. However, experimental methodologies greatly differed between these studies, with Staddon (2003) performed in pastureland, whilst Johnson and colleagues work was performed under controlled environmental conditions. Therefore considerably more work is needed to understand the role of mycorrhizal fungi in belowground movement of carbon and their effect on storage within the soil.

1.4 The understanding of rhizosphere fungal community assemblages has important functional implications

Given the variable functions of rhizosphere fungi, it is unsurprising that changes in the fungal assemblage will also affect rhizosphere functioning (Wardle et al. 2004; Heemsbergen et al. 2004; Mikola et al. 2005). For example, increasing symbiont diversity and colonization is associated with increasing nutrient uptake (Abuzinadah, Finlay, and Read 1986; Coleman, Bledsoe, and Lopushinsky 1989; Arnebrant 1994; Keller 1996; Agerer 2001), whilst increasing phytopathogenic species is indicative of disease (Espinoza et al. 2008). However, the composition within rhizosphere fungal groups will also affect rhizosphere functioning. Different mycorrhizal fungal species have been shown to have variable C costs associated with the same nutrient benefits, such as P provision (Kiers et al. 2011). Therefore the relative abundance of each of these different 'quality' symbionts within the rhizosphere will likely affect the overall cost of supporting the rhizosphere to the plant. However, this work was performed in a highly simplified laboratory model, with only 2 fungal taxa competing for resources from a single root culture. The substantial diversity of both the aboveground biomass and their mycorrhizal fungal partners found within environmental systems makes the understanding of how the differing rhizosphere fungal assemblages effect ecosystem functioning enormously complex.

1.5 Aims and Objectives

The overall aim of this work was to characterize the temporal and spatial diversity of rhizosphere fungal communities within bioenergy crops within the UK, and to determine their role in belowground C cycling. Chapter 2 was aimed at understanding the role of environmental heterogeneity and spatial scaling effects in shaping rhizosphere fungal community composition.

Objective: To investigate the relative roles of geographical, temporal and environmental factors on rhizosphere fungal community assembly in SRC willow.

Chapter 3 was aimed at understanding the role of long-term temporal shifts on the rhizosphere fungal community.

Objective: To investigate temporal community dynamics of fungi within the rhizosphere of SRC willow over a 3-year sampling period.

Chapter 4 aimed to quantify the role of rhizosphere fungi in cycling C within the rhizosphere of short rotation coppice willow and *Miscanthus* x *giganteus*.

Objective: To compare the flux and residence of recently derived photo-assimilates through AM and ECM pathways of *Miscanthus* and SRC willow respectively.

CHAPTER II: INVESTIGATING ENVIRONMENTAL, GEOGRAPHICAL AND SEASONAL VARIATION IN THE STRUCTURE OF RHIZOSPHERE FUNGI

2.1 Abstract

The fungal communities that inhabit the rhizosphere are formed from a functionally and genetically diverse range of species. Rhizosphere fungi have been shown to impact the productivity of aboveground biomass and the rates of soil biogeochemical cycles. Whilst these effects have been shown to vary between community assemblages, and a substantial amount of research has been undertaken to investigate the factors that affect rhizosphere community assembly. The rhizosphere fungal community composition is known to be influenced by spatial and temporal factors, however the role of geographical distance is less clearly understood. The aim of this study was to investigate the factors that regulate rhizosphere fungal community assembly, identifying the independent effects of spatial, temporal and environmental parameters within a bioenergy cropping system. Soil and root samples were taken from 160m line transects in a short rotation coppice willow field in Brattleby (Lincolnshire, UK). Sampling was performed in October 2010, July, August and October of 2011. DNA was extracted from roots and used for terminal restriction fragment length polymorphism (TRFLP) analysis using general fungal ITS1F and ITS4 primers. Partial Mantel and redundancy analyses (RDAs) were used to correlate the metadata against the communities. While the results demonstrated that environmental and temporal factors affected rhizosphere fungal community composition, a strong seasonal-dependent geographical effect was also detected. This distance effect was more pronounced in the October 2010 and October 2011 communities, whilst nitrogen availability was more important in the summer months. pH also nearly ubiquitously influenced rhizosphere composition across the year. Given that both the rhizosphere fungal assemblages and the factors that regulate assembly vary throughout the year, future investigations into rhizosphere community composition would greatly benefit from a temporal component to sampling strategies.

2.2 Introduction

Since MacArthur and Wilson's (1967) work on island biogeography, it has been scientific dictum that the geographical distance between samples will affect the composition and similarity of their ecological communities, with decreasing community similarity with increasing distances. However, Baas-Becking (1934) stated 'everything is everywhere, but, the environment selects' for microorganisms, fundamentally differing from the traditional ecology of macroorganisms. For this to be true, changing distances between sampling locations, even on a global scale, will not affect microbial communities. This idea is logical, as microorganisms are small and easily dispersed. They also have a quick generation time, with the potential to form populations rapidly from very low numbers. Consequently they are considerably less likely to suffer from dispersal limitation compared to macroorganisms (Martiny et al. 2006). However there are a growing number of publications demonstrating distance effects on microbial communities, with a recent focus on the extent of these effects (Horner-Devine et al. 2004; Soininen, McDonald, and Hillebrand 2007; Bell 2010).

Rhizosphere fungi are functionally and genetically diverse, playing an integral role in connecting the aboveground biomass to the belowground ecosystem. Mycorrhizal fungi are obligate root symbionts, which often dominate the rhizosphere microbial biomass (Kucey 1987; Smalla et al. 2001). They have been shown to be a significant sink of plant derived carbon, but are associated with increased nutrient uptake and disease resistance (Heinemeyer et al. 2006; Liu et al. 2007; Högberg et al. 2010). AM, ErM and ECM are some of the most abundant mycorrhizal types, with the majority of AM and ErM associations biomass predominately found within roots (Kinden and Brown 1975; Perotto et al. 1995), or as ECM that form sheaths around root tips (Alexander and Högberg 1986). Conversely, phytopathogenic fungal species, which also reside within the rhizosphere, are associated with disease and ultimately reductions in plant growth (Espinoza et al. 2008). Additionally, saprophytic fungi decompose organic matter, thereby increasing SOC and macro-nutrient turnover

within the rhizosphere (Niklaus et al. 2001). Given the strong and variable effect the rhizosphere fungal community can have on aboveground biomass, and the potential for shifts in composition to influence ecosystem function, the understanding of how these communities assemble is of ecological importance (Berg and Smalla 2009).

Whilst many individual environmental parameters have been shown to correlate with changes in soil microbial communities, the understanding of how soil communities assemble within the environment is still in its infancy (Van Der Heijden, Bardgett, and Van Straalen 2008; Griffiths et al. 2011). The community composition of both ECM and AM fungi have been shown to be affected by edaphic properties (Anderson, Liberta, and Dickman 1984; Escudero and Mendoza 2005; Buée, Vairelles, and Garbaye 2005; Tchabi et al. 2008; Fitzsimons, Miller, and Jastrow 2008). Soil pH in particular is almost ubiquitously important for rhizosphere communities, affecting both composition and function (Coughlan et al. 2000; Högberg et al. 2001; Dickie, Xu, and Koide 2002; Tedersoo, May, and Smith 2010). Other soil nutrients also influence rhizosphere fungal community composition, with increasing K, P and N soil concentrations shown to reduce rates of root colonization by mycorrhizal fungi (Jensen and Jakobsen 1980; Treseder and Cross 2006; Gosling et al. 2013). Additionally, plant nutritional status is also linked with mycorrhizal communities, with increasing host P status linked to increased mycorrhizal abundance (Smith and Read 2010).

Both short and long-term temporal shifts have been shown in rhizosphere fungal communities. Seasonality has been shown to affect rhizosphere fungi, as determined by measuring AM spore abundance and distribution (Allen et al. 1998; Merryweather and Fitter 1998). AM fungi were later found to have distinct summer-winter assemblages (Dumbrell et al. 2011), but still considerably less is known about seasonal effects on rhizosphere community composition compared to environmental factors and there is much uncertainty behind the mechanisms driving these changes (Last et al. 1984; Pickles et al. 2010). Rainfall, soil temperature and the likelihood of freeze-thawing events fluctuate seasonally and

may directly affect soil and rhizosphere communities, whilst changing biotic factors, such as plant growth and vegetation composition will also directly affect rhizosphere biota (Piceno and Lovell 2000; Smalla et al. 2001). Longer-term shifts in rhizosphere communities also occur, with discrete mycorrhizal associations forming with plant maturation stage (Daniell et al. 2001; Bergemann and Miller 2002; Husband, Herre, and Young 2002), whilst changes in root density (Bergemann and Miller 2002; Pickles et al. 2010) and neighbour sharing of symbionts have also been linked with long-term changes in ECM fungi (Högberg et al. 1999).

The affects of geographic separation on the rhizosphere community composition have begun to be investigated at the local (a single field site), regional (different field sites within a single land mass) or global (between continents), however there is still much uncertainty about the extent of these effects. Community similarity in both ascomycete fungi and AM fungi has been shown to decrease with increasing geographic distances apart, even when changing environmental and climatic factors have been accounted for (Green et al. 2004; van der Gast et al. 2011; Horn et al, 2014). Kivlin, Hawkes, and Treseder (2011) found strong evidence for distance effects occurring between continents, suggesting that mixing events at a global scale are relatively rare in AM fungi. In contrast, recent studies into AM fungi by An et al. (2008) and Hazard et al. (2013) found that distance had no significant effect on AM fungus community composition at the regional scale. The majority of this previous research has focused on spatial scaling at the global and regional scale, with spatial scaling effects at the local level often lost through 'pooling' of samples from the same sample site (An et al. 2008; Hazard et al. 2013). Whilst other studies have begun to compare the extent of local spatial scaling effects with larger geographical scales, comprehensive analyses have yet to be performed (Green et al. 2004, Horn et al. 2014).

Previous investigations have predominantly focused on community shifts within single microbial groups; with both AM fungi and ECM fungi the main target of research within the rhizosphere. However these groups are plant symbionts and

represent a sub-component of total rhizosphere diversity, and dynamics of the whole rhizosphere fungal community, which also includes saprotrophs and pathogens, has been very poorly characterized (Marsh and Schultze 2001; Dickie, Xu, and Koide 2002). Community fingerprinting techniques, such as TRFLP analysis allow for surveys of diversity across the rhizosphere to be performed, providing improved taxonomic resolution when compared to morphological techniques (Sanders 2004; Rosendahl 2008). Additionally, previous studies have tended to analyze metadata parameters against the rhizosphere fungal communities independently, ignoring potential interactions between them (Lingfei, Anna, and Zhiwei 2005; Jumpponen et al. 2010). However, the substantial autocorrelation between many environmental parameters may lead to incorrect parameters being associated with community shifts. Disentangling environmental parameters is a complex process, but analyses such as partial Mantel and direct ordination (through canonical correspondence analysis or redundancy analysis) have been previously used to attempt to limit the effects of autocorrelation within community studies (Tuomisto and Ruokolainen 2006; Lekberg et al. 2007; Hazard et al. 2013).

The aim of this work was to investigate the relative importance and interactions of distance, time and environmental effects for determining the structure of rhizosphere fungi at the local level. A short rotation coppice willow plantation in Brattleby, Lincolnshire was selected for sampling. Rhizosphere samples were collected over line transects within the field site, at 4 time points between October 2010 and October 2011. DNA was extracted and TRFLP analysis with ITS primers used to assess changes in fungal community composition, whilst clone libraries were also established using ITS primers to identify abundant fungal taxa (Gardes and Bruns 1993; Dickie, Xu, and Koide 2002).

Hypothesis: The geographical distance between rhizosphere fungal populations will have a significant effect on their community similarity, which will vary both temporally and with environmental conditions.

2.3 Methods

2.3.1 Study Site and Experimental Design

The field site was a short rotation coppice (SRC) willow plantation in Brattleby, Lincolnshire, UK (94225, 81108 British decimal degrees). The underlying soil type is a fine loam over clay, with approximately 25 %, 29 % and 49 % clay, sand and silt respectively. The 30-year mean air temperature was 9.9 ˚C (Bottoms, unpublished). The site varies extensively in pH, ranging from 5.5 – 7.2, and has a mean total C and N content of 1.81 % and 0.28 % respectively (Bottoms, unpublished). The willow was planted in 2000 at a planting density of 15,000 stools ha^{-1} and covered approximately 9.44 ha. Previously the land was rotated between wheat and oilseed rape for at least 20 years before conversion to *Salix viminalis*. Paired rows of trees were planted at 0.75 m apart on the 'hollow', and 1.5 m between 'humps'. Different varieties of SRC willow were planted to prevent disease spread, with Tora (60 %) being the most abundant (the others being Bjorn (10 %), Bowles Hybrid (10%), Jorr (10 %) and Jorunn (10 %)). The crop was coppiced first in 2001, then in 2004, 2007, and 2010 with yields of 20, 26 and 19 t ha^{-1} respectively. The growing season is between March and September for SRC willow in the UK. The site received concentrated PK fertilizer (Fibrophos) at 660 kg ha^{-1} at establishment, with 20 tonnes of each compost and lime applied to the field site in February 2010. No further exogenous nutrient input was applied during the course of the experiment.

A single row of the willow was used for line transects, which started from the southern edge of the field heading north. In order to avoid edge effects, transects started 25 m along the row, and 8 samples were taken every 20 m across the row, spanning 160 m (Fig2.1). Subsequent transects shifted sequentially 3 m north of the previous sampling in order to avoid repeat sampling of disturbed soil. At each location, 4 subsamples were taken in a cross shape 1 m around a central point. These consisted of 4.5 cm diameter soil cores that were 15 cm deep (van Wald

equipment, Netherlands). Transects were taken in October 2010, July, August and October 2011.

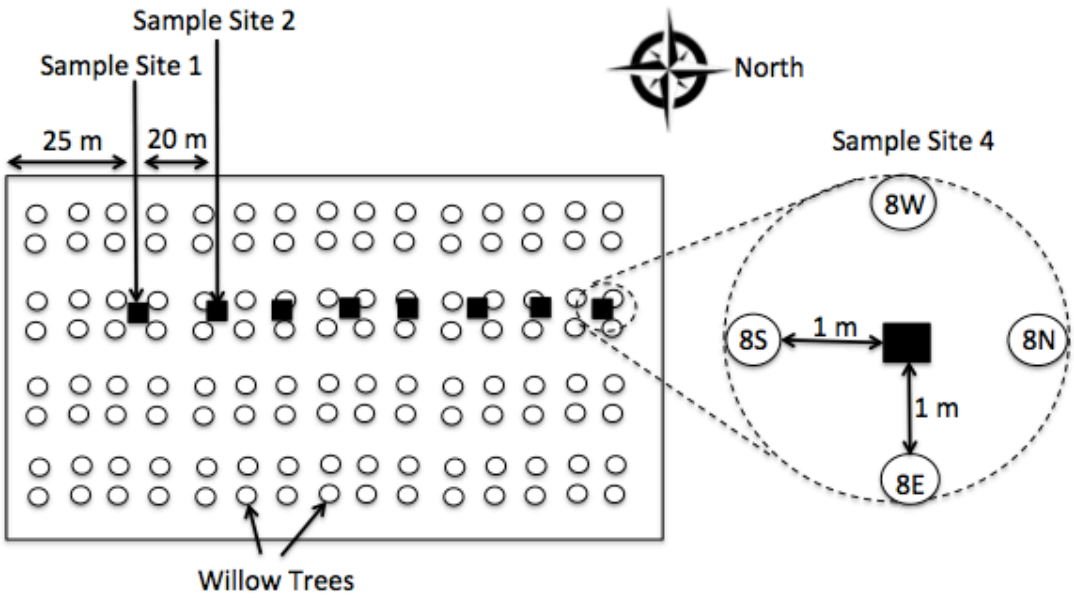

Figure 2.1 Schematic of the line transects. Transects headed north along a single paired row of the willow trees. Sampling locations were 20 m apart. Sampling locations consisted of 4x15 cm deep soil cores taken at 1 m distances around the central point.

2.3.2 Sample Preparation

Soil was softened by soaking at 19 ˚C for one hour in deionized water, before roots were hand extracted using forceps. Non-senescent fine roots were selected by morphology (lighter color and branched structure with the presence of fine root tips). Those roots of less than 2 mm diameter were washed on a 6 mm sieve in order to remove adhering soil. Roots were then cut into 1 cm lengths and mixed thoroughly, before 0.5 g was taken for DNA extraction using a soil extraction kit (MP Biomedicals, Cambridge).

2.3.3 Soil Nutrient Analysis

For mineral analysis, 100 g of soil was air dried for one week before being ground and sieved to <2 mm particle size. To measure pH, 10 mg of soil was added to 25 ml deionized water and the mixture shaken for 15 m before measurement using a pH meter (Accumet AB15, Fischer Scientific). In order to measure extractable P, 5 g of finely ground soil was incubated for 30 m in 0.5 M $NaHCO_3$, before analysis on an inductively coupled plasma optical emission spectrometer (ICP; HORIBA, Jobin-Yvon; Olsen, 1954). K and magnesium (Mg) were extracted by incubating 10 g of soil with 1 M NH_4NO_3 for 30 m, followed by centrifugation. Concentrations of K and Mg in the supernatant were measured using ICP (Bremner and Keeney 1965). NH_4, and NO_3 were extracted by incubating 20 g of soil with 10.5% K_2SO_4 for 2 hr, and following centrifugation, measured in supernatant using a FIAstar 500 flow injection analyser system (LECO, USA; Hendriksen and Olsen; 1970; Bremner and Keeney 1965). Total C and N analysis was performed using a LECO CN2000 C and N analyzer.

2.3.4 Clone Library Preparation

A clone library was established to identify common fungal taxa that reside within the rhizosphere and to ensure that the TRFLP method selectively profiled fungi. 1 µl of a 25 ng µl DNA solution from each subsample from the October 2010 line-transect was combined into a single pooled DNA sample. The fungal internal transcribed spacer region (ITS) was amplified by polymerase chain reaction (PCR) using ITS1F (5-CTTGGTCATTTAGAGGAAGTAA-3) and ITS4 (5-TCCTCCGCTTATTGATATGC-3') primers (GeneAmp PCR System 9700, Applied Biosystems; Gardes and Bruns 1993). PCR was also performed using AML1 (5'-ATCAACTTTCGATGGTAGGATAGA) and AML2 (5'-GAACCCAAACACTTTGGTTTCC-3') primers to specifically investigate the presence of arbuscular mycorrhizal fungi (Lee, Lee, and Young 2008). The programme for PCR consisted of: 5 min at 92 ˚C; followed by 25 cycles of 30 s at 92 ˚C, 90 s at 56 ˚C, followed by 30 s at 72 ˚C; a final extension of 5 min at 72 ˚C for both primer sets. PCR products were purified using a QIAquick PCR purification kit

(QIAGEN). Purifications were ligated to the pDrive Cloning Vector plasmid and transfected into QIAGEN EZ competent cells as per manufacturer's instructions (QIAGEN). Using the blue/white colour of the colonies to identify successfully transformed cells, 96 samples were taken for Sanger sequencing (QIAgen, UK; ABI 3130xl, Life Technologies, UK; Warwick Genomics Centre, Wellesbourne Campus, UK).

Ends were trimmed from the resulting sequences to avoid ambiguous bases, with approximately 35 base pairs (bp) excised at each end. Sequences that had no ambiguous bases and a minimum of 300 bp were used for taxonomic identification. BLAST searches were used for identification, using the 'Nucleotide collection' database of the National Centre for Biotechnology Information and the sequence with highest similarity was recorded (to a minimum of 97 % sequence similarity to ensure a high quality assignment (Altschul 1990), otherwise sequences were left as unassigned).

2.3.5 TRFLP protocol

Labelled ITS1F-(6FAM) and ITS4-(TET) primers were used to investigate ECM fungal communities (Gardes and Bruns 1993), whilst AML1-(HEX) and AML2–(TET) primers were used to investigate AM fungi (Gardes and Bruns 1993; Lee, Lee, and Young 2008). 25 ng of DNA from each of the subsamples from the October 2010 transect was used as a template for each PCR reaction. PCR was performed using 25 ng of DNA from each subsample in a total volume of 50 µl, which included 47 µl of Megamix (Microzone, Haywards Heath, UK), 1 µl of forward and reverse primers, and 1 µl of 25 ng μl^{-1} template DNA from samples. PCR cycling conditions for both ITS and AML primer reactions followed the same protocol as the clone library amplifications.

Clone library sequences were used to find optimal restriction enzymes for TRFLP analysis, which were selected on the basis of the number of restriction sites found within the sequenced ITS regions. Consequently ITS amplicons underwent digestion with *Hpy8I* (Fermentas, UK) during TRFLP analysis. 20 µl reactions contained 200 ng of DNA, 2 units of enzyme and 2 µl of x10 manufacturers buffer before reactions were equilibrated to 20 µl using molecular grade water (MO BIO Laboratories, CA, USA). Following digestion samples were run through sephadex columns for further purification (Sigma-Aldrich, Germany). 4 µl of digested and purified samples were then loaded on capillary sequencer (ABI 3010, Applied Biosystems, UK). Samples were run with GeneScan 1200 LIZ ladder (Applied Biosystems, UK), which acted as an internal standard for assessing fragment length.

Genemarker v1.50 (Softgenetics, PA, USA) was used to quantify the number and height of the resulting TRFLP peaks, which were exported to MS Excel (Microsoft, USA) for further analysis. Baseline noise was considered to be 50 fluorescence units, and consequently peaks lower than this were removed from the analysis. Samples were then normalized by the conversion of peaks to percentage of relative abundance of the total fluorescence area.

2.3.6 Statistical Analyses

Relative abundance data produced via TRFLP underwent arcsine transformations in order to homogenize variation before all statistical analyses were performed. Differences between the numbers of TRF were calculated using one-way analysis of variance (ANOVA), whilst Simpson's and Shannon's diversity indices were used to assess alpha diversity (XLSTAT 2002, Adinsoft, USA). Simpson's index is a dominance measure for which presence of rare species has a limited effect on the measure, whilst Shannon's index assumes all species are present and randomly sampled, thus emphasizes the effect of rare taxa (Magurran 2004). Simpson's and

Shannon's indices were calculated using the following equations (Simpson 1949; Pielou 1966):

Simpson's Index:

$$D_s = \frac{N(N-1)}{\sum n_i(n_i-1)}$$

Where N = the total number of individuals of all taxa

n_i = the total number of individual taxa i

Shannon's Index:

$$H' = -\sum p_i \ln p_i$$

Where p_i = the proportion of individuals of taxa *i*

Bray-Curtis similarity matrices (Bray and Curtis 1957) were created from TRFLP data using XLSTAT 2002. Similarity matrices were also created for soil nutrient concentrations, whilst a third Euclidean matrix was generated from the paired geographical distance between samples. The paired distance matrix and the Bray-Curtis similarity matrix for the TRFLP data were used to generate distance-decay measures as previously described by Green et al. (2004). In this method, an exponential gradient was calculated by plotting the similarity values of the community against geographical distance separation, giving the distance-decay rate. Differences between the rates of decay were tested using the *t*-distribution method (Fowler, Cohen, and Jarvis 1998). Correlations between nutrients and individual TRF were investigated using the Pearson product moment correlation coefficient in Excel 2011 (Microsoft, USA). Partial Mantel statistics were performed after Bray-Curtis matrices were formed from the community and soil data, whilst a third matrix was created from the geographical distances between samples (XLSTAT 2002). Communities were compared to both soil nutrients and geographical

distance independently, whilst the variation of the other matrix (conditional variance) was accounted for.

For direct ordination, community data underwent arcsine transformations before undergoing redundancy analysis with integrated forward selection analysis (Canoco v5.0, Wageningen, Netherlands; Braak and Šmilauer 2002). Forward interactive selection was performed to obtain significantly correlating explanatory variables with the ordination analysis of the community (Wollenberg 1977). The total variation explained in the ordination analysis as well as the variation explained by each explanatory variable was calculated during the analysis.

2.4 Results

2.4.1 Edaphic Properties

Biogeochemical data was taken for each of the 4 subsamples from within each sampling site. There were strong gradients in pH, P, K and % N across the transect which were conserved across all seasons (Fig2.2a-d). P, K and % N inversely correlated with pH. pH ranged from 5.3 to 7.0 across the 160 m transect in all seasons. Bioavailable P halved from 80 mg kg^{-1} to 35 mg kg^{-1} in October 2010, and from 40 to 20 mg kg^{-1} in the following seasons. Similarly, % N was much higher in October 2010, halving from an average of 0.32 % N in 2010 to 0.17 %. A gradient in % N was maintained across transects in 2011, falling from an average of 0.15 % to 0.5 % N. Available K was present at higher concentrations in sites 1-4 across transects, from mg kg^{-1} to 200 mg kg^{-1} in sites 5-8. % C and Mg concentrations remained stable throughout time and space (Fig2.2e and 2.2f).

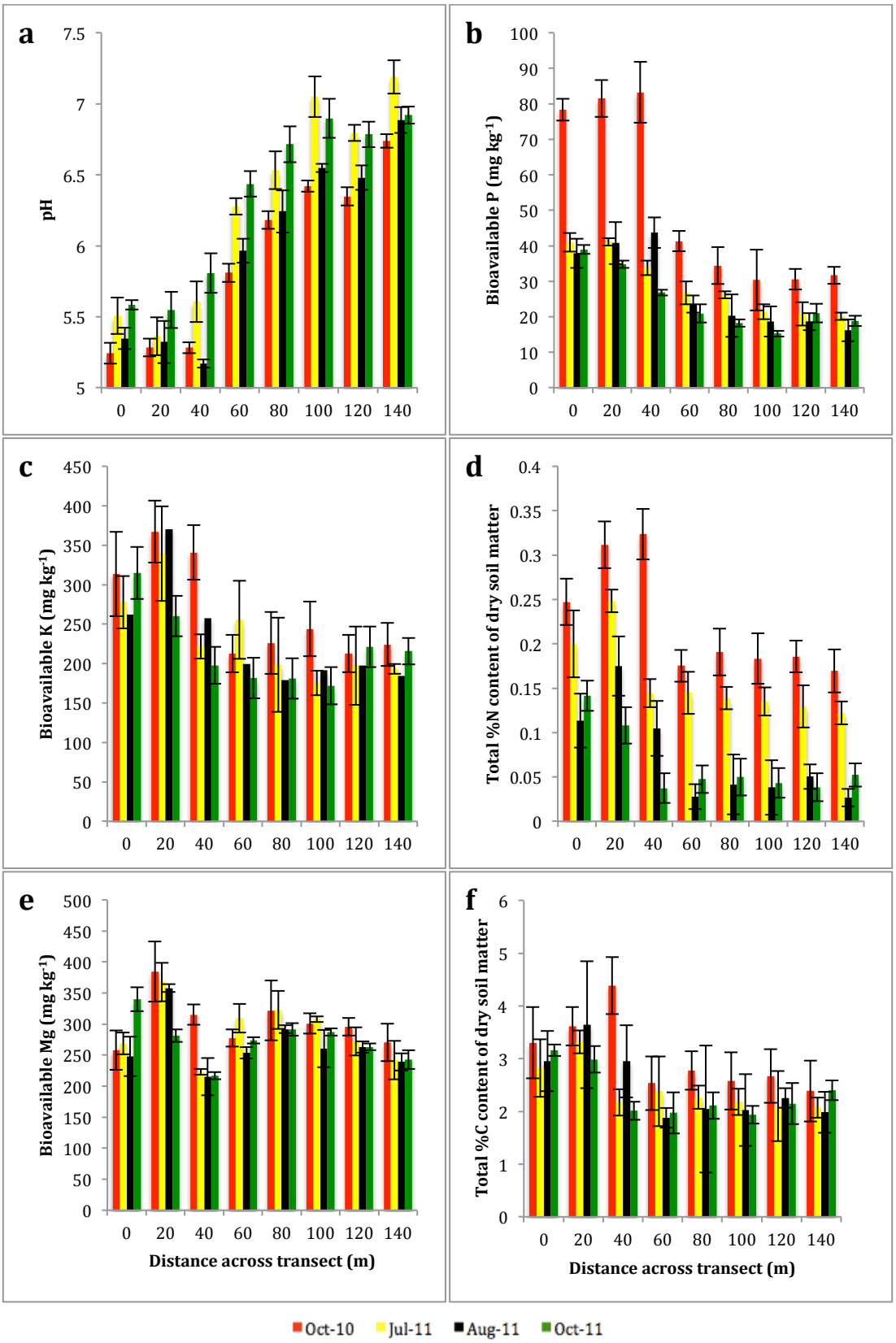

Figure 2.2 (a) pH, (b) Bioavailable P, (c) Bioavailable K, (d) %N, (e) Mg and (f) %C across the 8 transect sampling locations. Error bars are ± 1 S.E.M.

2.4.2 Identifying dominant fungal species in the rhizosphere in SRC willow

A clone library established from pooled October 2010 samples yielded 67 sequences that passed quality filtering from a possible 96. Over 80 % of sequences by abundance belonged to ECM taxa. The sequences aligned to 33 different genotypes spanning 12 different genera. At species level, the library was dominated by the ECM taxa *Pulvinula constellatio* and *Sebacina* genotypes, which represented 17.9 % and 16.4 % of sequence abundance respectively (Table 2.1). PCR for arbuscular fungi using AML1 and AML2 primers was not successful and consequently a clone library could not be established.

Table 2.1 Assigned identity and abundance of rhizosphere fungi established from the October 2010 clone library.

Accession number	Taxonomy*	Ecological Function	Relative abundance (%)
AF289074.1	*Pulvinula constellatio*	ECM	17.9
HQ212339.1	*Sebacina sp.1*	ECM	16.4
HQ604731.1	*Cortinarius fulvescens*	ECM	6.0
DQ102683.1	*Cortinarius saniosus HL90-235*	ECM	4.5
GU180308.1	*Trechisporales sp.1*	ECM	4.5
JF519477.1	*Trechisporales sp.2*	ECM	4.5
JF519135.1	*Trechisporales sp.3*	ECM	3.0
AY204588.1	*Alatospora acuminata*	Saprophyte	3.0
FN428924.1	*Cryptococcus podzolicus*	Saprophyte	3.0
GU934581.1	*Neonectria radicicola*	Phytopathogenic	3.0
HQ604845.1	*Russula bicolor*	ECM	3.0
JF519274.1	*Sebacinales sp.*	ECM	3.0
AB488490.1	*Exophiala sp. NH1238*	Animal Pathogen	1.5
AF062813.1	*Myxotrichum chartarum*	ECM	1.5
AF178540.1	*Dictyochaeta fertilis*	ECM	1.5
AF486119.1	*Phialophora finlandia*	Saprophyte	1.5
AJ534912.1	*Tomentella sp. O41*	ECM	1.5
DQ102683.1	*Cortinarius saniosus*	ECM	1.5
DQ195592.1	*Thelephorales sp. A.Becerra 10*	ECM	1.5
EU883430.1	*Tetracladium maxilliforme*	ECM	1.5
FJ210748.1	*Trechisporales sp.*	ECM	1.5
FJ439591.2	*Hypocreales sp. GMG_PPb3*	ECM	1.5
FJ481036.1	*Neonectria radicicola*	ECM	1.5
FN428924.1	*Cryptococcus podzolicus*	ECM	1.5
GU233374.1	*Dermocybe sp. PDD 94202*	ECM	1.5
GU479339.1	*Hymenogaster populetorum*	ECM	1.5
GU550111.1	*Cortinarius flexipes*	ECM	1.5
GU566260.1	*Truncatella angustata*	Phytopathogen	1.5
HQ212355.1	*Sebacina clone 8_p14*	ECM	1.5
HQ630994.1	*Chaetosphaeria sp. TMS-2011*	ECM	1.5
U83475.1	*Thelephoraceae sp. 'Taylor #11'*	ECM	1.5

***Taxonomic identity assigned by BLAST search (minimum 97% similarity) using the reference nucleotide database from the NCBI bioinformatics resources and ecological function was noted where possible. Frequency is the proportion of the 67 sequences associated with each OTU. Abbreviations: ECM = ectomycorrhizal fungi**.

2.4.3 Assessing alpha and beta diversity

From the TRFLP analysis performed on the roots, 273 different TRF were identified over the 4 seasons (Table 2.2). TRF averages between transects did not differ (P=0.537), ranging between 50-58 per sample site. Mean TRF diversity was significantly different between sites (P=0.023), with site 4 consistently having fewer TRF than other sites within the transects.

Table 2.2 Average number of TRF across the line transects using ITS1F and ITS4 primers.

Sample Site	Combined	Oct-10	Jul-11	Aug-11	Oct-11
1	49.8	44.0	43.0	53.0	59.0
2	34.5	22.0	41.3	48.5	26.3
3	51.9	68.3	34.5	53.0	51.7
4	31.8	30.8	25.0	-*	39.8
5	61.6	83.3	78.8	37.0	47.5
6	42.9	34.8	50.8	48.0	38.0
7	63.1	46.8	83.8	68.8	53.0
8	53.5	62.0	49.5	56.7	45.8
Average	49.3	49.0	50.8	52.1	45.1
Standard error of the mean	2.9	5.2	5.1	1.5	2.6

***PCR unsuccessful.**

Alpha diversity indices allow for comparisons of diversity between sample sites, therefore Simpson's and Shannon's indices were calculated. From the Simpson's indices of the TRFLP data (Table 2.3), diversity remained very stable across seasons, with no significant differences between transects (P=0.602). However, there was significant variation between the sampling locations within transects (P=0.010). Site 4 had the least alpha diversity in all transects, whilst sites 5 and 7 had higher than average diversity. The Shannon's index did not vary between seasons or between sites (P=0.234, P=0.053).

Table 2.3 Shannon's and Simpson's alpha indices of diversity at each sampling location across each transect.

	Combined		Oct-10		Jul-11		Aug-11		Oct-11	
Sample Location	**Sh**	**Si**	**Sh**	**Si**	**Sh**	**Si**	**Sh**	**Si**	**Sh**	**Si**
1	1.08	578.5	1.13	279.6	1.19	173.9	0.83	1689.3	1.18	171.2
2	1.10	367.8	0.76	369.2	1.22	145.0	1.12	856.6	1.3	100.4
3	1.28	107.4	1.25	80.6	1.15	232.0	1.38	62.1	1.33	54.8
4	1.09	443.1	1.01	875.2	1.02	273.8	-*	-*	1.24	180.5
5	1.13	458.4	1.02	97.7	1.19	83.2	1.05	1607.3	1.26	45.3
6	1.24	201.0	1.14	401.2	1.31	103.1	1.17	220.0	1.34	79.8
7	1.24	106.0	1.29	131.4	1.36	21.0	1.07	134.6	1.24	137.0
8	1.28	152.2	1.45	40.6	1.37	74.9	1.01	380.1	1.29	113.3
Average	1.18	301.8	1.13	284.4	1.23	138.4	1.09	707.1	1.27	110.2
Standard Deviation	0.09	182.9	0.21	274.0	0.12	85.0	0.17	693.5	0.05	50.2

***PCR unsuccessful. Abbreviations: Sh = Shannon's index value, Si = Simpson's index value.**

Beta diversity assesses the rate of change in diversity across a landscape. Distance-decay rates were performed to quantify the breakdown in community similarity across each transect. August 2011 had a lower rate of decay in community similarity when compared to the July 2011 (P<0.001) and October 2010 (P<0.001) and 2011 (P<0.001) samples (Fig2.3). Whilst August 2011 differed significantly from all other seasons, there were no significant differences between any of October 2010, July 2011 and October 2011 (Table 2.4).

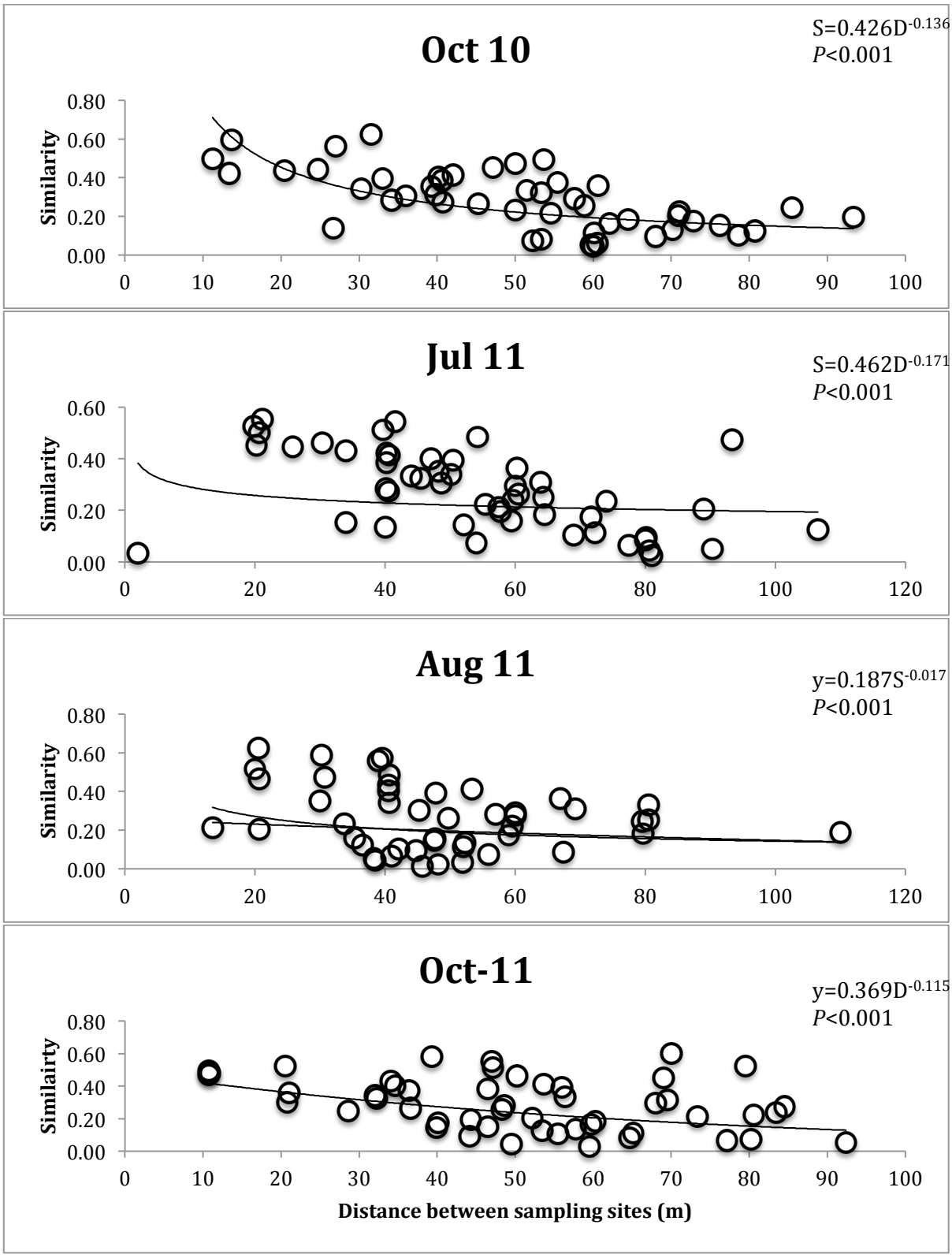

Figure 2.3 The distance-decay of fungal community similarity through over time. Given are Bray-Curtis similarity (*S*) values. Averages of distance (*D*) were taken across similarity values within intervals of 0.01. In each instance, the distance-decay power law equation is given $S = cD^d$ (*d* is rate of decay) calculated from all values.

Table 2.4 Table of *P*-values testing for significant differences between distance-decay rates (Figs2.3a–d) using the *t*-distribution method (Fowler *et al*, 1998).

	Oct-10	Jul-11	Aug-11	Oct-11
Oct-10	-	0.9206	**<0.001**	0.1585
Jul-11		-	**<0.001**	0.1595
Aug-11			-	**<0.001**
Oct-11				-

Bold indicates significance (*P*<0.05).

2.4.4 Investigating distance and edaphic effects on the rhizosphere fungal community

Individual TRF abundance was correlated against nutrients to determine individual taxa that were associated with shifts in community composition (Table 2.5). pH was the greatest influence across the dataset, correlating significantly with 25 individual taxa ($P<0.001$), with distance across transects also significantly correlating with 14 individual taxa ($P<0.001$). pH was consistently important across each transect, correlating with the most taxa in both October samples, and was also correlated with 19 and 9 taxa in July 2011 and August 2011 respectively. Distance played a variable role throughout the year, significantly correlating with 20 individual TRF in both October and July 2011 transects, whilst not correlating with any TRF in August 2011. Whilst NO_3, NH_4, % N and K all correlated with many taxa across in individual transects, no consistent trend was found throughout sampling. Mg and % C correlated with very few TRF in the combined or individual transects.

Table 2.5 Number of significantly correlating TRF with each metadata parameter (*P*<0.01).

Parameter	Combined	Oct-10	Jul-11	Aug-11	Oct-11
pH	25	8	19	9	22
Bioavailable P (mg/kg)	7	2	16	19	10
Bioavailable K (mg/kg)	7	3	12	3	4
Bioavailable Mg (mg/kg)	2	1	2	7	4
%N	4	0	10	10	6
%C	6	2	4	6	5
NO_3 (mg/kg)	5	6	11	4	6
NH_4 (mg/kg)	5	8	12	12	9
Distance	14	6	20	0	20

A total of 273 different TRF were used for correlation analysis.

Metadata parameters were correlated against taxa richness (Table 2.6). Taxa richness positively correlated with pH, as it increased from acidic (5.2) to neutral (7.2) across transects (*P*<0.001). K also negatively correlated with number of TRF (*P*=0.013), demonstrating decreasing fungal diversity with increasing K concentration. Distance also correlated positively with number of TRF at each sampling location, with increasing taxa richness across transects (*P*=0.002). P, K, Mg, %N, %C, NO_3 and NH_4 did not have an effect on taxa richness.

Table 2.6 Correlations of environmental parameters with total number of TRF.

Parameter	pH	P (mg/kg)	K (mg/kg)	Mg (mg/kg)	%N	%C	NO^3 (mg/kg)	NH^4 (mg/kg)	Distance
R^2	**0.209**	-0.146	**-0.198**	-0.076	-0.066	-0.086	-0.028	-0.077	**0.261**
P	**0.009**	0.051	**0.013**	0.2	0.231	0.17	0.379	0.195	**0.002**

Bold indicates significant correlation (*P*<0.05).

Bray-Curtis similarity matrices formed from the TRFLP data were correlated against the Bray-Curtis matrices of soil parameters and Euclidean distance matrices. The community data from October 2010, July 2011 and October 2011 did not correlate with soil properties (Table 2.7), in contrast to August 2011. Community data from the TRFLP was also correlated against distance, whilst variation in soil nutrients was accounted for, which significantly correlated with changes in rhizosphere community across all transects.

Table 2.7 Partial Mantel statistics correlating distance and soil parameters to the community data, for each transect.

Variable	Conditional variable	Oct-10		Jul-11		Aug-11		Oct-11	
		P-value	R	*P*-value	R	*P*-value	R	*P*-value	R
Distance	Soil Parameter	0.125	-0.051	0.065	0.044	**< 0.001**	**-0.046**	0.871	-0.049
Soil Parameters	Time	**<0.001**	**0.059**	**<0.001**	**0.046**	**<0.001**	**-0.049**	**<0.001**	**0.039**

Each variable was correlated against the rhizosphere fungal community data, whilst accounting for variance in the conditional variable. Bold indicates significant correlation (*P*<0.05).

RDAs were performed on the TRFLP data with integrated forward selection of significant explanatory variables (Table 2.8). Soil pH was consistently important in determining the community structure, with the community composition in every season but October 2011 being affected. Distance correlated with community variation in both October 2010 and October 2011, but not in August 2011 and July 2011. Instead nitrogen availability, in the form of NO_3 and NH_4 significantly correlated with the community variation in July 2011 and August 2011 respectively.

Table 2.8 Redundancy analysis determining the variation of the rhizosphere fungal communities from TRFLP of each transect explained by metadata parameters.

	Oct-10		Jul-11		Aug-11		Oct-11	
	Variation explained (%)	*P*	Variation explained (%)	*P*	Variation explained (%)	*P*	Variation explained (%)	*P*
pH	12.7	0.002	16.1	0.002	9.4	0.002	-	-
Distance	6.2	0.004	-	-	-		4.5	0.002
NO_3	-	-	5.7	0.014	-	-	-	-
NH_4	-	-	-	-	7.7	0.011	-	-
P	-	-	-	-	-	-	12.8	0.047
Undeter-mined	81.1		78.2		82.9		82.7	

2.4.5 Investigating temporal effects on the rhizosphere fungal community

Partial Mantel statistics for the combined dataset significantly correlated distance, but not nutrients, to variation in the community (Fig2.9). Redundancy analysis was also performed on the combined community data across each of the 2010 and 2011 transects (Fig2.10). pH explained the greatest amount of variation within the system, accounting for 7.4 % of all fungal community variation. Each season independently correlated with the community structure, accounting for a further 6.5 % cumulative variation within the rhizosphere fungal community. Time also independently correlated with changes in community composition, which demonstrates significant differences between the two October samples. NO_4 concentration was also linked to changes in the community, explaining a further 1.4 % of variation.

Table 2.9 Partial Mantel statistics correlating distance and soil parameters to the combined community data from the TRFLP.

Variable	Conditional variable	*P-value*	R
Soil Parameters	Distance	0.116	-0.044
Distance	Soil Parameters	**<0.001**	**-0.047**

Each variable was correlated against the rhizosphere fungal community data, whilst accounting for variance in the conditional variable. Bold indicates significant correlation (*P*<0.05)

Table 2.10 Redundancy analysis determining the variation of the combined rhizosphere fungal communities from TRFLP explained by metadata parameters.

Parameter	Variation Explained (%)	*P*
pH	7.4	0.002
Aug	4.3	0.002
July	1.6	0.002
October	1.6	0.002
Time	1.5	0.004
NO_3	1.4	0.010
Undetermined	83.8	

2.5 Discussion

The results demonstrate for the first time that microbial communities vary with edaphic parameters and time simultaneously, but also show evidence of dispersal limitation at the local geographic scale. The rhizosphere fungal community of the willow was dominated by ECM genotypes (by relative abundance), whose composition and biomass is known to vary seasonally (Escudero and Mendoza 2005; Jumpponen et al. 2010) and with edaphic properties such as pH, P, K and N (Buée, Vairelles, and Garbaye 2005; Tchabi et al. 2008; Fitzsimons, Miller, and Jastrow 2008). In this work, distinct rhizosphere fungal assemblages were shown to form throughout the year, with the factors which regulated their assembly also varying throughout the year. Sampling time significantly affected the rhizosphere fungal community composition, with communities sampled in July and August having separate community compositions, whilst the two October samples together had a significant combined composition. When seasonal communities were investigated separately, the rhizosphere fungal compositions were found to display prevalent distance effects in the October assemblages, whilst nitrogen sources in the forms of NH_4 and NO_3 were most important in determining composition in the summer assemblages. These factors remained relatively stable throughout the sampling period, indicating that seasonal variation may not be entirely driven by climatic parameters.

The geographic separation between sampling locations correlate with community variation within the rhizosphere fungal community, but this was of varying importance throughout the year. Distance between samples significantly correlated with the community variation in both October samples using redundancy analyses, explaining 6.2 % and 4.5 % of community variation respectively, whilst distance also correlated with the community variation in both October transects using partial mantel statistics. Distance also correlated with the abundance of around 10 % of taxa abundance and with overall taxa richness in both the October samples, which is in agreement with a growing number of studies that suggest that the Baas-Becking

statement does not hold true, even at the local scale for rhizosphere fungi (Green et al. 2004; Lekberg et al. 2007; van der Gast et al. 2011; Kivlin, Hawkes, and Treseder 2011). Studies which have not found a distance effect on community composition have investigated this relationship with single time points, and consequently may have missed the 'window' in time when distance effects are significant (An et al. 2008, Hazard et al. 2013).

Increasing P, K and N have been shown to decrease mycorrhizal colonization and diversity, whilst changes in pH have been shown to have a near ubiquitous effect on soil microbial community composition (Treseder and Cross 2006; Griffiths et al. 2011; Gosling et al. 2013). The strong gradients in P, pH and K found across transects also allowed for insightful investigations of the effects of edaphic factors on the community, and pH was subsequently shown to correlate with the abundance of nearly 10 % of all taxa. pH was also shown to consistently correlate with community level shifts, and is concurrent with a wealth of literature suggesting pH is a major factor determining rhizosphere fungal community assembly (Griffiths et al. 2011; Dumbrell et al. 2011; Hazard et al. 2013). Additionally, the gradient in K also inversely correlated with species richness, suggesting a negative effect on rhizosphere fungi with increasing nutrient content. Whilst the gradient in P caused no change in taxa richness, differences in P were shown to cause specific compositional shifts within the rhizosphere fungal community. The large gradients in pH, P and K across transects were likely caused by the poor distribution of fertilizer in spring 2010, and demonstrates the impact that land management practices have on soil microbes.

Willow trees have the potential to form associations with both AM and ECM fungi, in addition to interacting with a plethora of saprophytic and phytopathogenic fungal species that reside in the rhizosphere (Jones, Durall, and Tinker 1990; Trowbridge and Jumpponen 2004; Van Der Heijden, Bardgett, and Van Straalen 2008). ECM fungi were by far the most abundant rhizosphere fungus group, with 25 ECM phylotypes identified, comprising over 80 % of the fungal ITS clone library, whilst

no AM strains were identified. Both of the dominant ECM taxa, *Cortinarius* spp. and *Sebacina* spp. are commonly associated with willow trees in environmental samples (Keizer and Arnolds 1994; van der Heijden, Vries, and Kuyper 2000; Ryberg, Larsson, and Molau 2009; Fujiyoshi et al. 2011). A Chao1 estimate of 32 was calculated for just the ECM fungi within the system (Chao 1987), which is similar to morphological based estimates of ECM in a range of natural forest stands (Bruns 1995). However, given that a substantial proportion of the community remains unidentified, greater sequencing depth and sample numbers are needed before taxonomically identified fungal assessments of diversity and community assembly can be accurately produced.

Previous investigations into ECM fungal diversity has largely used sporocarps collected within a narrow timeframe, and these approaches significantly underrepresent diversity (Colgan III et al. 1999). Morphotyping approaches are also limited in their taxonomic resolution and are highly subjective (Gardes and Bruns 1996; Horton and Bruns 2001; Shi et al. 2012). Community fingerprinting techniques such as TRFLP allow for large proportions of microbial diversity to be assessed simultaneously in large numbers of samples (Sanders 2004). However taxa identities remain unknown, unless time intensive strategies such as the sequencing of sporocarps and mycelial isolates are used in conjunction with fingerprinting (Allmér et al. 2006). In this work we have shown substantial shifts in the composition of rhizosphere community but limited changes in alpha diversity. Therefore biological interpretations from this data are limited. The implementation of high throughput sequencing techniques has the potential to solve both the inadequate sequence number of clone libraries and the lack of taxonomic information given in TRFLP (Buée et al. 2009). Whilst some studies have pioneered use of next generation sequencing to investigate the diversity of soil fungi, the potential of these technologies for enhancing our understanding of microbial communities has yet to be fully exploited (Dumbrell et al. 2009; Tedersoo, May, and Smith 2010; Lim et al. 2010; Dumbrell et al. 2011).

RDAs perform direct ordinations, simplifying the vast array of individual taxa data into a smaller number of components based on shared variation. However in doing so, much of the variation associated with individual taxa is lost. Even in the simplified community datasets from the redundancy analyses, only around 20% of the variation was explained by the metadata. Whilst the amount of variation from these statistics that can be explained is often low, there are a number of reasons for this. Generalist species that can survive across wide ranges in environmental parameters, such as pH and P, will often be unaffected by the level of variation found between sampling sites (Koch, Croll, and Sanders 2006; An et al. 2008; Gosling et al. 2013). There will also be local differences in sunlight, soil temperature and moisture, and whilst many of these parameters can be measured broadly, comprehensive fine-scale measurements are often simply beyond the capabilities of an experiment's resources. Finally, environmental systems always have large amounts of 'stochasticity' within them, which cannot be easily explained by measuring any parameter (Dumbrell et al. 2009).

Differing rhizosphere fungal assemblages will likely have differing effects on plant growth and in nutrient cycles. Therefore the understanding of how rhizosphere fungal assemblages form is of importance in understanding ecosystem functioning. However, to date no studies have attempted to simultaneously address spatial, temporal and environmental variation of microbial communities. Through this work, we have shown that selection pressures on the rhizosphere fungi change throughout the year, so that each time point has its own unique fungal assemblage. Also, community composition was affected by distance, even at the local level, with the effects particularly prevalent in the autumn. Our results show that temporal and local geographical variations, which are often neglected in studies of microbial diversity, have a significant impact on rhizosphere fungal community composition. Future studies of rhizosphere fungal community formation should not underestimate potential distance effects at the local geographic sale. In addition, sampling strategies would also benefit from multiple time points to fully characterize variation in the rhizosphere communities within ecosystems.

CHAPTER III: DETERMINING TEMPORAL VARIABILITY IN THE STRUCTURE OF RHIZOSPHERE FUNGAL COMMUNITIES IN SHORT ROTATION COPPICE WILLOW

3.1 Abstract

Rhizosphere fungi represent a key link between aboveground biomass and the bulk soil. These fungi perform a variety of functions and changes to community composition can affect overall rhizosphere functioning. Previous attempts to explain the factors that regulate rhizosphere fungal assembly have generally been performed over a single growing season, and only explain a low proportion of community variability. In this work, rhizosphere fungal spatio-temporal dynamics were investigated over a 3-year period in short rotation coppice (SRC) willow. Line transects consisting of soil cores taken every 20 m for 160 m were collected in October 2010, July 2011, October 2011, July 2012 and October 2012. DNA was extracted from live roots and their associated rhizosphere fungal communities. Pyrosequencing of the internal transcribed spacer region was performed to investigate fungal community composition over the sampling period. Investigations into community level shifts were performed via partial Mantel statistics, multidimensional scaling and redundancy analysis (RDA), whilst changes in individual operational taxonomic units (OTUs) were also investigated. While pH had an almost ubiquitous effect on the composition of rhizosphere communities, the structure of communities sampled in 2012 markedly differed to those of previous sampling events. This large transition in community structure was shown to dwarf the other factors that regulated community assembly, and persisted throughout the growing season of 2012. While reads assigned to ectomycorrhizal (ECM) fungi dominated the rhizosphere, their overall abundance and diversity fell substantially in 2012 compared to previous years. The exception to this trend was the number of reads assigned to *Cortinarius disemospermus*, which rose from low abundance in the 2010 and 2011 communities to comprising of a mean abundance of nearly 20 % of the 2012 communities. Reads assigned to the dark septate endophyte *Phialocephala*

fortinii and the phytopathogenic *Truncatella angustata* endophyte genotypes also increased in abundance in 2012. Whilst many environmental parameters such as edaphic and climatic factors remained stable throughout the duration of the experiment, a series of heavy rainfall events occurring early in the growing season of 2012 may have been the main driver behind the dramatic compositional change in the rhizosphere community. Given the decline of ECM fungi and rise of potentially pathogenic fungal genotypes within the rhizosphere, the compositional shift that occurred in 2012 may have had a negative impact on the aboveground biomass.

3.2 Introduction

Since early investigations into the plant communities of sand dunes revealed succession patterns in the ecology of plants (Cowles 1899), the transition between early pioneer species to a climax community has been shown in a variety of ecosystems (Shelford 1911; Gleason 1926; Clements 1936). However the understanding of how microbial communities develop over time poses a considerably greater challenge than for macro-organisms, since they are exceptionally complex, are not readily accessible and are difficult to resolve and identify (Martiny et al. 2006; Blaalid et al. 2012). Previous experiments to investigate temporal variation in microbial communities have often been over a single growing season or have had few temporal replicates (Peay, Kennedy, and Bruns 2011). Even those that have continued for a number of years have generally been limited to investigating specific groups within the soil microbial community or have used molecular techniques that are limited in the taxonomic information they provide (Daniell et al. 2001; Husband, Herre, and Young 2002). Consequently, the understanding of long-term changes in rhizosphere communities over multiple years is severely limited, and the understanding of interactions between specific individuals or groups of taxa within communities is particularly poorly characterized.

The rhizosphere represents the roots and the narrow adjoining region of soil, with its associated microbial community. It is the primary location of aboveground-belowground interactions and is rich in bacterial and fungal diversity (Buée et al. 2009). Whilst the spatial and temporal dynamics of rhizosphere fungi have been the focus of many studies, these factors have rarely been investigated simultaneously (Koide et al. 2007; Dumbrell et al. 2009; Pickles et al. 2010). Fungi within the rhizosphere interact directly and indirectly with plant root cells, performing a variety of functions. Arbuscular mycorrhizal (AM) and ECM fungi are some of the best characterized rhizosphere fungal groups, and form symbiotic mycorrhizal relationships with their hosts. AM fungi can form associations with over 80 % of land plant species, whilst ECM fungi are limited to woody plant species (Torti, Coley, and Kursar 2001; McGuire et al. 2008). Mycorrhizal fungi have been shown to confer plant growth benefits, such as increasing host uptake of nutrients and providing disease resistance, but at a cost of C metabolites from the plant (Kucey 1987; Cordier et al. 1998; Liu et al. 2007; Sieber 2007). Additionally non-mycorrhizal root endophytes can have strong growth benefits and costs to plants, with symbiotic, saprophytic and phytopathogenic interactions recorded (Kucey 1987; Cairney and Meharg 2002; Bolwerk et al. 2003). Therefore changing the composition and abundance of fungal communities within the rhizosphere will have important implications for the rhizosphere at the functional level.

The rhizosphere fungi interact, competing for space and resources (Visser 1995). Whilst rhizosphere fungal composition can be affected by host limitation and preferences, it can also vary with a plethora of environmental parameters. Temporal changes in communities can be considered as seasonal variation or as longer-term transitions over several years. There is a wealth of literature showing AM fungal seasonal variations in spore composition, hyphal abundance and root colonization rates (Bever et al. 2001; Escudero and Mendoza 2005; Bohrer, Friese, and Amon 2004), whilst distinct seasonal assemblages have also been found within AM communities using pyrosequencing (Dumbrell et al. 2011). Morphotyping and pyrosequencing studies of ECM have also shown compositional changes within the

course of a year (Koide et al. 2007; Courty et al. 2008; Pickles et al. 2010; Peay, Kennedy, and Bruns 2011). For non-mycorrhizal endophytes, annual community shifts have been found inconsistently, suggesting factors other than time may be more important in determining the community composition (Arnold et al. 2003). These seasonal variations in the rhizosphere community have often been linked to climatic factors, with rainfall, soil moisture, temperature and hours of sunlight shown to correlate strongly with shifts in AM and ECM communities (Hacskaylo, Palmer, and Vozzo 1965; Hutchison 1990; Gehring et al. 1998; Domisch, Finér, and Lehto 2002; Jany, Martin, and Garbaye 2003).

In addition to seasonal variation, long-term temporal shifts in the structure of AM and ECM fungal communities have also been found, spanning across many years. For example, in mixed-aged *Tetragastris panamensis* grasslands, age-exclusive AM associations were found, suggesting a host maturation stage effect on the community (Daniell et al. 2001; Husband et al. 2002). Similarly, host age effects were also found in the ECM fungi of monodominant tree stands (Last et al. 1984; Visser 1995), whilst a succession pattern in the aboveground biota of land freed by retreating glaciers was matched by a succession pattern in the ECM community (Blaalid et al. 2012). Common early-stage and late-stage ECM genotypes have subsequently been identified, based on the time they generally occur within ECM community series (Deacon and Fleming, 1972; Bergemann and Miller 2002). Whilst differing colonization and dispersal rates have been proposed as a mechanism for temporal shifts in the composition of ECM fungus communities, changes in root density may also impart a temporal effect (Pickles et al. 2010).

Edaphic factors such as pH (Anderson, Liberta, and Dickman 1984; Coughlan et al. 2000; Fitzsimons, Miller, and Jastrow 2008), P (Mandyam and Jumpponen 2005) and NO_3 concentrations (Escudero and Mendoza 2005; Treseder and Cross 2006; Jensen and Jakobsen 1980) have also been shown to have significant effects on the composition and abundance of rhizosphere fungal communities. However soil parameters will also change over time. For example extremes in P and K can be

caused by fertilization, whilst loss of nutrients by runoff and utilization by biota can cause rapid declines (Moore, McCann, and de Ruiter 2005). Intensive fertilizer application can also lead to acidification of soils (Peters et al. 2011). These anthropogenically induced changes in pH and soil nutrients have been shown to have particularly large effects on both the composition of rhizosphere fungi and root colonization rates (Danielson, 1991; An et al, 2008; Turrini and Giovannetti, 2012; Gosling et al, 2013). Other mechanisms such as the turnover of organic matter (Dungait et al. 2012), deposition of nutrients from the atmosphere and the weathering of inorganic rock minerals (Newman 1995) also drive changes in edaphic properties over time, making the soil a dynamic environment which subsequently impacts upon the rhizosphere community. However bioenergy crops receive limited quantities of fertilizer during their lifetime (Cannell 2003), potentially allowing greater rhizosphere fungal colonization and diversity compared to other more intensively managed agro-ecosystems.

Experimental techniques have previously limited the power of investigations into the organization and functioning of soil microbial communities. Molecular techniques such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (TRFLP) are cheap and capture much more of the rhizosphere fungal diversity and abundance than methods that rely on taxonomic identification of biological structures (Allmér et al. 2006). However taxa identities are not resolved using these approaches, although this can be achieved by coupling molecular fingerprinting techniques with relatively time consuming and expensive Sanger sequencing of clone libraries (see Chapter 2). This severely limits the biological inferences that can be made from these datasets, potentially overlooking important interactions between community members. It is only with the recent advent of high throughput sequencing techniques that OTUs within the community can be taxonomically defined to a high resolution, whilst sampling large enough numbers to simultaneously assess diversity. Whilst a small number of studies have pioneered the use of high-throughput sequencing for profiling rhizosphere fungal communities, the potential of these techniques for enhancing the

understanding microbial community assembly have yet to be fully utilized (Buée et al. 2009; Dumbrell et al. 2011).

The work performed in Chapter 2 demonstrated that the willow field site had a diverse rhizosphere fungal community that was dominated by ECM (Fig 2.1), however there were also other functional groups present such as saprophytes and plant pathogens. Additionally, whilst seasonal variation was suggested over the 2-year sampling period, further temporal samples are needed to assess the long-term community development. Using 454-pyrosequencing, Chapter 3 expands upon Chapter 2 through utilizing the taxonomic information provided by high-throughput sequencing to understand the temporal development across the rhizosphere fungal groups, including symbionts, pathogens and saprophytes. Sampling was also extended to a 3-year period in order to assess the persistence of the seasonal assemblages demonstrated in Chapter 2. Soil cores were taken across line transects at 5 time points over the duration of the experiment and DNA was extracted from roots was used to pyrosequence the rhizosphere community. Both community level shifts and changes in specific out abundances were analysed over time, in addition to edaphic and geographical parameters, in order to investigate the factors that regulate rhizosphere fungal community assemblage over the 3-year sampling period.

Hypothesis: Temporal variation, in addition to changing environmental and geographical distance effects, significantly influence the rhizosphere fungal community composition within and between fungal functional groups such as mycorrhizal fungi, pathogens and saprophytes.

3.3 Materials and Methods

3.3.1 Study site and experimental design

Sampling was performed in the SRC willow plantation in Brattleby, Lincolnshire, UK, as described in Chapter 2 (2.3.1). The October 2010, July 2011, and October 2011 DNA samples analysed in Chapter 2 were used to perform pyrosequencing, with the addition of new transects taken in July 2012 and October 2012. These new line transects were conducted as previously described, consisting of 8 sampling locations, each of the 4 subsamples taken at 1 m distances from a central point, whilst each sampling location was 20 m apart. Sub samples were soil cores (2 cm diameter) collected to a depth of 15 cm. Unlike the Chapter 2 TRFLP work, subsamples were pooled at each sampling location giving 8 samples per time point in the community analysis. In total 40 samples spanning the 3-year period underwent pyrosequencing.

3.3.2 Soil Nutrient Analysis

Soil nutrient analyses were carried out as outlined in chapter 2 (2.3.3). pH and bioavailable, Mg (mg kg^{-1}), P (mg kg^{-1}) and K (mg kg^{-1}) were determined as the mean at each location, calculated from the 4 subsamples.

3.3.3 Weather data collection

Meteorological data was provided by weather stations located centrally within each of the willow and *Miscanthus* field sites. These consisted of a cup anemometer, wind vane, air and wet bulb temperatures (Didcot Instruments AWS, Didcot, UK) and a rain gauge (Rimco, Malton, UK). Data was logged as 30 min averages with the

exception of rainfall, which was logged as the 30 min total, all of which used CR10 data loggers (Campbell Scientific, UT, USA).

3.3.4 DNA Preparation

Root extraction, selection, washing and DNA extraction were performed as described in Chapter 2. After DNA extraction, the 4 subsamples at each sampling location were equilibrated to 25 ng μl^{-1} using a nanodrop ND1000 (Fischer Scientific, MT, USA) and 10 µl of each was pooled to make the DNA template used for pyrosequencing.

3.3.5 Bar-coded pyrosequencing

Pooled DNA extractions underwent further equilibration to ensure samples were 25 ng μl^{-1} before 1 µl was used for nested PCR. For the fungal specific reaction, the 550-750 base pair internal transcribed spacer fungal hypervariable region underwent amplification using ITS1F (5-CTTGGTCATTTATTTAGAGGAAGTAA-3) and ITS4 (5-TCCTCCGCTTATTGATATGC-3') primers in an initial polymerase chain reaction using MyTaq HS DNA polymerase (Bioline, USA; Gardes and Bruns 1993). The ITS region has previously been used successfully in pyrosequencing studies of fungi (Buée et al. 2009). PCR reactions consisted of: 1 µl of DNA template, 2 mM dNTPs, 10 pmol of each primer, whilst thermocycler conditions were 95 ˚C for 5 min; 40 cycles of 95 ˚C for 30 s, 55 ˚C for 30 s, 72 ˚C for 0.5 min; and 72 ˚C for 5 min, using a Biometra TJ3000 thermocycler (Biometra, Germany). A secondary semi-nested PCR reaction was performed in order to add the fusion primer necessary for pyrosequencing. Fusion primers consisted of: GS FLX LR70-specific adapter A, a multiplex identifier (MID), and a new forward primer, a modified version of the universal M13 primer. The fusion primers used in the secondary reaction were: forward 5'-*GTGTGAAATTGTTACGCT* (10-bp MID) CTTGGTCATTTAGAGGAAGTAA-3 and reverse

5’ TCCTCCGCTTATTGATATGC-3’. The forward primer comprised of the A adapter (in italic type) for the pyrosequencing reaction, the 10-bp MID is part of Roche’s extended MID set (www.454.com) and the final part is the modified M13 primer. The reverse primer consists of the fusion adapter B only. The secondary PCR was performed in a volume of 25 µl, and consisted of: 1 µl of DNA template, 2 mM dNTPs, 10 pmol of each primer, whilst thermocycler conditions were: 94 ˚C for 1 min 40 s; 40 cycles of 95 ˚C for 20 s, 55˚C for 20 s and 72 ˚C for 20 s; and 95˚C for 10 min. Sample concentrations were then calculated by SYBR gold based quantitation (Shmidazu, Japan), before 2 plates of 20 equimolar concentrations of MID tagged samples were loaded onto a Roche 454 GS Junior pyrosequencer (454 Life Sciences/Roche Applied Biosystems, Nurley, NJ, USA) and sequenced at Micropathology Ltd (Coventry, UK).

3.3.6 Processing of pyrosequencing data

Sequencing data underwent denoising with Acacia-1.52 software (Bragg et al. 2012), reducing the number of sequences from 127,457 to 100,581. The software package ‘Quantitative insights into microbial ecology’ (QIIME v1.7.0, USA) was used to perform the remaining sample processing (Caporaso et al. 2010). Briefly, samples of the 2 runs underwent demultiplexing and were combined into a single sequencing file, before the orientation was adjusted for those in the reverse complementary form (Courtemanch 1996). OTUs were picked and from these reads using UCLUST (Edgar, 2010) and a consensus representative sequence was created from each OTU. A muscle alignment was performed on the data from the representative set picked earlier in the pipeline. Taxonomy was assigned at the 97% level using the 27.08.2013 release of the ITS fungal database for QIIME, from the UNITE project (Wang et al. 2007). The aligned file and the taxonomy file were combined, creating the final OTU table (in .biom format). The table was exported to MS excel, giving the taxonomic assignment and relative abundance for each sample. An initial 100,581 sequences formed 5,900 OTUs. Due to the lack of an aligned sequence dataset with

the UNITE database, no chimera checking was able to be performed, however OTUs of 3 or less were removed to limit artificially inflating diversity estimates with chimeras (Huse et al. 2007), leaving a total of 94,083 sequences aligning to 994 different OTUs.

3.3.7 Statistical Analyses

Relative abundance data underwent arcsine transformations in order to homogenize variation before all statistical analyses were performed. Sequences were subsampled for each location to the lowest number of reads at an individual sampling point (1157) using RStudio (R Foundation, Austria), before OTU richness, Simpsons diversity index, Shannon's diversity index and Chao1 estimates were produced (Simpson, 1949; Pielou 1966; Chao 1987). One-way analyses of variance (ANOVA) were performed on the OTU data at the phylum, family, genus and species levels, analyzing variation between sampling times (XLStat 2002, Adinsoft, USA). One-way ANOVAs were also used to test for differences in climatic and nutrient parameters. Additionally, due to the distinct communities in samples taken in 2012 compared to the 2010 and 2011 samples, the greatest 2.5% of OTUs decreasing and increasing in mean abundance in 2012 were identified before one-way ANOVAs were performed to test for significant variation of each between sampling times (OTUs clustered at the species level; XLStat, 2002, Adinsoft, France). Post-hoc analyses (Tukey tests) were performed when significant variation was found in these ANOVA analyses.

The community data produced by pyrosequencing was used to create Bray-Curtis similarity matrices (Bray and Curtis 1957), before multidimensional scaling analysis was performed. One-way analyses of similarity (ANOSIMs) were performed to correlate metadata parameters to the MDS output (Primer6 v.6.1.9, Primer-E-Ltd, UK). Additionally, partial Mantel statistics were performed to correlate the Bray-Curtis matrix of the community data against the Bray-Curtis matrix of the soil

nutrients, the Euclidean distance matrix and the time matrix, which was the difference in weeks between the collection of samples. Each metadata matrix was correlated against the community matrix using partial mantel statistics, with the variation in the other metadata matrices each separately accounted for (conditional variation) (XLSTAT 2002; Tuomisto and Ruokolainen 2006). RDA with forward selection was also performed to correlate metadata to changes in the rhizosphere fungal communities, whilst limiting the autocorrelation between metadata parameters (CANOCO 5.0; Borcard et al. 2004; Braak and Šmilauer 2002). However this was direct ordination from OTU data, rather than from Bray-Curtis matrices.

3.4 Results

3.4.1 Edaphic properties

Biogeochemical data were calculated as the mean of the 4 subsamples at each sampling location. There were strong gradients in pH, P (mg kg^{-1}) and K (mg kg^{-1}; Fig3.1a-d). pH ranged from 5.2 to 7.2, with sites 1 to 3 having significantly (P<0.001) lower pH on average than sites 5-8. Bioavailable P significantly varied between sampling locations across transects (P=0.022), halving from 40 mg kg^{-1} in sites 1-3 to 20 mg kg^{-1} in sites 5-8, but it also varied significantly (P=0.044) between sampling times, with considerably higher quantities in the October 2010 transect, in which it ranged from approximately 80 mg kg^{-1} in sites 1-3 to 40 mg kg^{-1} in sites 4-8. Similarly, K significantly varied (P=0.033) between sampling locations across transects, from approximately 300 mg kg^{-1} in sites 1-3 to 200 mg kg^{-1} in sites 5-8, with the gradient not significantly varying between sampling times (P=0.728). No clear trends were found in bioavailable Mg distribution, although there was some variability between sites, with concentrations ranging between 225 mg kg^{-1} and 400 mg kg^{-1}.

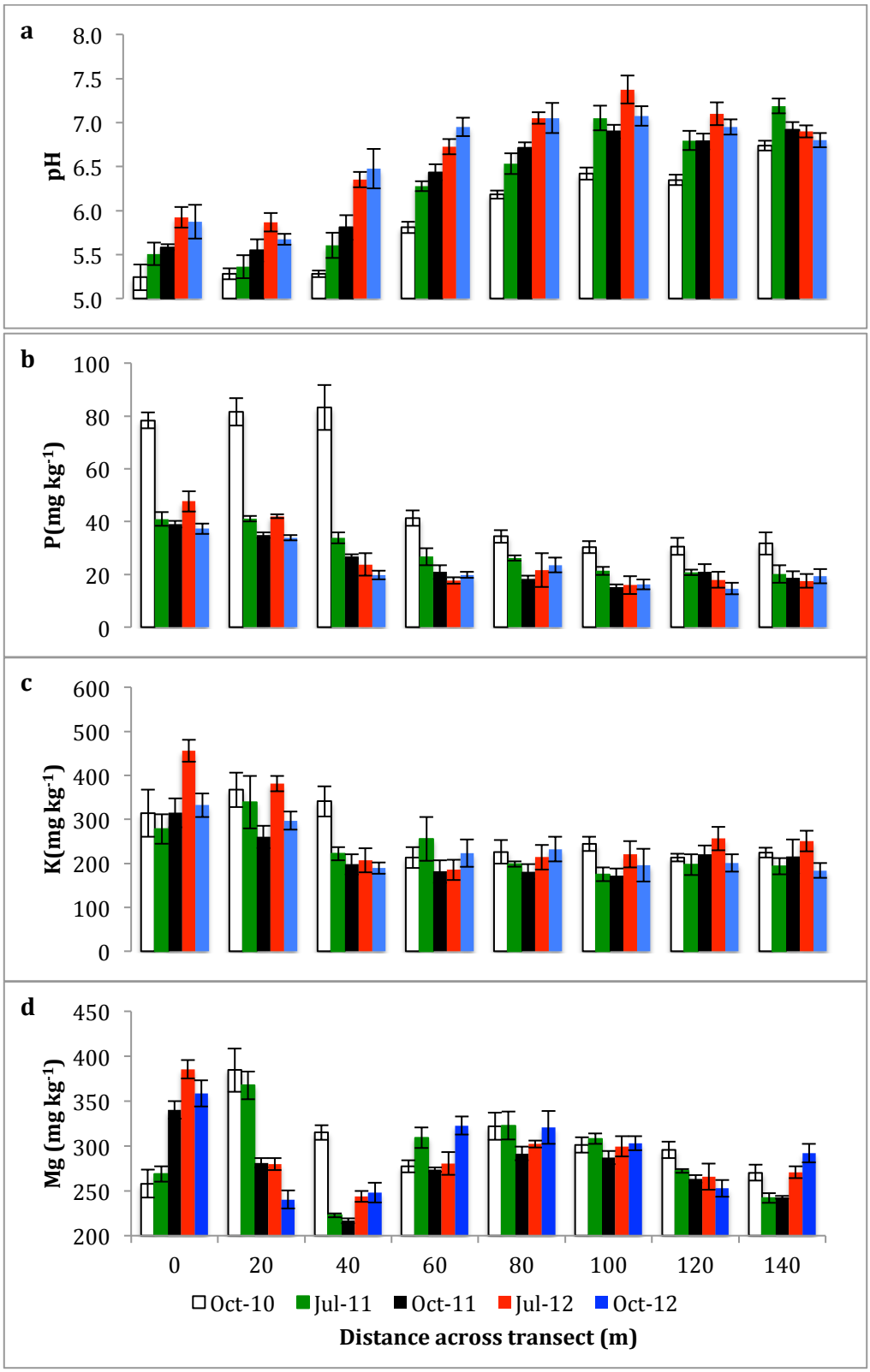

Figure 3.1 Nutrient data from the October 2010, July 2011, October 2011, July 2012 and October 2012 transects a.) pH b.) P (mg kg^{-1}) c.) K (mg kg^{-1}) d.) Mg (mg kg^{-1}). Error bars are ±1 S.E.M.

3.4.2 Climatic data

Monthly average temperature was recorded at the Brattleby site, commencing in January 2010 and finishing in December 2013 (Fig3.2a). Unsurprisingly, the average daily temperature varied throughout the year (P<0.001), rising from around 1.0 ˚C in January to 15.0 ˚C in August. Temperature between years also varied significantly (P=0.048), with 2011 having a warmer spring and autumn compared to the other years. Rainfall did not differ throughout the year (P=0.600), with no consistent wet and dry parts (Fig3.2b). However monthly precipitation strongly differed between years (P=0.025), with 2012 having substantially higher rainfall in May (140 mm/month) and July (130 mm/month), over double that of the previous year's averages.

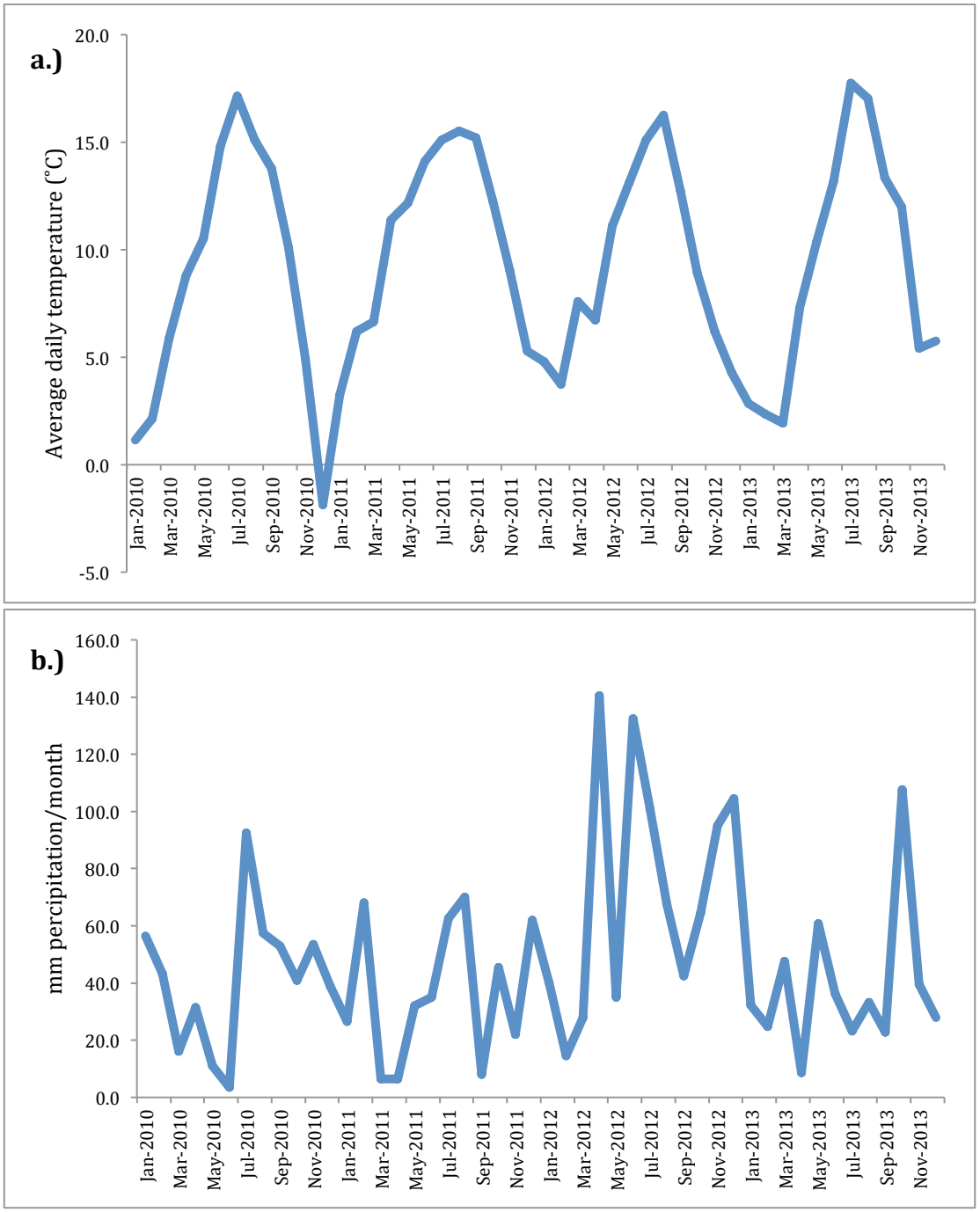

Figure 3.2 Weather data from the willow site over the 3-year sampling period. a.) Monthly average temperature (˚C) and b.) Monthly average rainfall (mm of precipitation/month).

3.4.3 Investigating fungal diversity over time using 454-pyrosequencing

Differences in the number of reads were assessed between both sampling locations and sampling times (Table 3.1). Whilst the number of reads at each sampling location were not significantly different (*P*=0.458*)*, there were significant differences between the sampling times (*P*=0.010). Tukey tests showed that October 2010 had a significantly higher number of reads than all other sampling times with the exception of July 2012. Before diversity measures were produced, sequences were subsampled to 1157 reads, the lowest number of reads for a site within the comparisons. Chao1 estimates (an estimate of total number of different OTUs within a sample) were however calculated for each sample, and did not differ between seasons (*P*=0.426*)* or sampling sites (*P*=0.729). There were also no significant differences in the number of different reads assigned (OTU richness) between sampling locations (*P*=0.458) or sampling times (*P*=0.418). There were also no significant differences in Shannon's or Simpson's indices between sampling locations or sampling times (Shannon's *P*=0.699 and *P*=0.087, Simpson's *P*=0.623 and *P*=0.053 respectively).

Table 3.1 Average OTU Richness, Simpson's diversity index, Shannon's diversity index and Chao1 estimates from pyrosequencing data for each sampling time.

Season	OTU Richness	Simpson's	Shannon's	Chao1 Estimate
Oct-10	98.29	8.18	2.79	1073.06
Jul-11	93.93	6.7	2.75	604.64
Oct-11	102.7	9.16	2.97	747.00
Jul-12	101.81	4.82	2.36	1005.58
Oct-12	114.43	9.7	3.03	854.87
Average	102.23	7.71	2.78	857.03
Standard Deviation	7.64	1.98	0.26	190.10

3.4.4 Investigating spatial, temporal and environmental factors controlling rhizosphere fungal assemblage

Partial Mantel statistics were performed to correlate community variation with time, soil parameters and distance. Each factor was correlated against the community whilst the variations of the other metadata matrices were each separately accounted for (i.e. serving as the conditional variable). The sampling time (in weeks after October 2010 that sampling took place) correlated significantly with community structure, even when both distance (P<0.001) and soil parameters (P<0.001) were used as the conditional variable (Table 3.2). Distance also correlated with variation in community structure, independently of both time (P=0.002) and soil parameters (P=0.006), whilst soil parameters did not correlate with community structure when the variation of distance (P=0.523) or time (P=0.417) was accounted for.

Table 3.2 Partial Mantel statistics correlating the Bray-Curtis matrices generated from the rhizosphere fungal community data to the Bray-Curtis matrix for nutrients, a Euclidean distance matrix and a sampling time matrix.

Variable	Conditional variable	*P*-value	Global R-values
Soil Parameters	Distance	*0.523*	-0.037
Soil Parameters	Sampling time	*0.417*	-0.045
Distance	Soil Parameters	***0.006***	**0.045**
Distance	Sampling time	***0.002***	**-0.043**
Sampling time	Soil Parameters	***<0.001***	**-0.042**
Sampling time	Distance	***<0.001***	**0.049**

The variable was correlated against the Bray-Curtis similarity matrix from the community data whilst variation in the conditional variable column was accounted for.

3.4.5 Non-metric multidimensional scaling analysis of rhizosphere fungal communities

A Bray-Curtis similarity matrix was created from the pyrosequencing data of the rhizosphere community, before multidimensional scaling was performed. One-way ANOSIMs were subsequently used to correlate metadata with the community similarity (Table 3.3), which revealed significant relationships between rhizosphere fungal community and time (number of weeks since sampling started; P=0.001), season (Fig3.3; P=0.018), pH (P=0.002) and P (P=0.003), whilst there were no relationships with distance (P=0.180), K (P=0.123) and Mg (P=0.190).

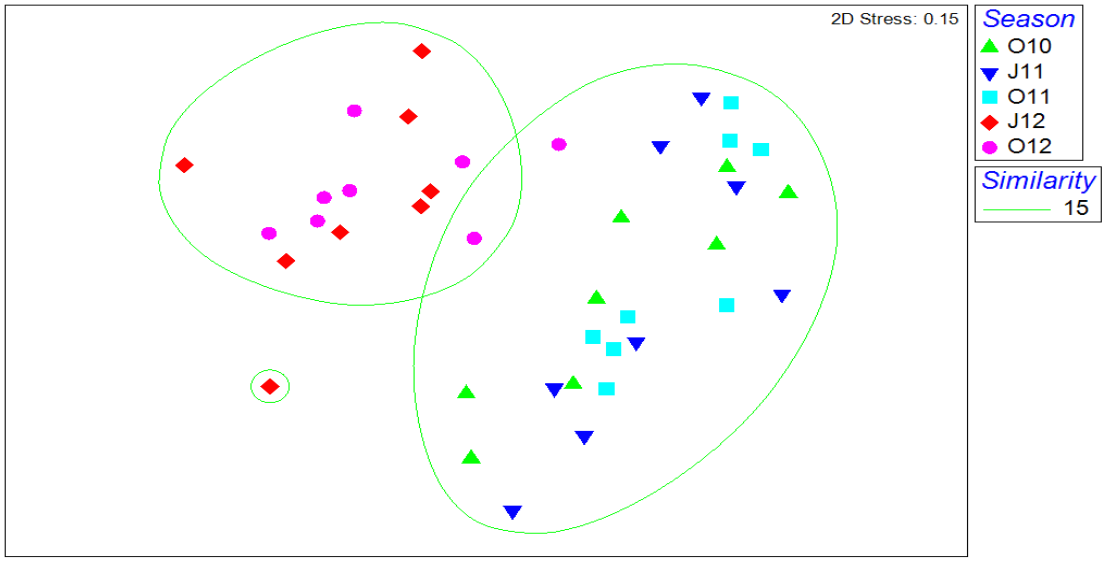

Figure 3.3 Non-metric dimensional scaling showing clustering based on similarity (given as a percentage) of the rhizosphere fungal communities between transects. Abbreviations: O10 = October 2010, J11 = July 2011, O11 = October 2011, J12=July 2012, O12=October 2012.

Table 3.3 ANOSIMs analyzing the MDS from the rhizosphere fungal community to metadata parameters.

Variable	Global – *R* value	*P-value*
Time	0.429	**0.001**
Distance	0.055	0.180
Season	0.052	**0.018**
pH	0.187	**0.002**
P	0.207	**0.003**
K	0.076	0.123
Mg	0.046	0.190

Bold *P*-values indicates significance (*P*<0.05).

RDA was performed with forward selection to correlate metadata parameters to changes in the rhizosphere fungal community, whilst limiting the effects of autocorrelation within the metadata parameters. From this analysis, time between sampling (*P*=0.002), and not season, was selected as a significantly correlating temporal component, whilst pH (*P*=0.002) was the only environmental parameter linked to community structure (Table 3.4).

Table 3.4 Redundancy analysis determining the variation of the rhizosphere fungal community against metadata parameters.

Parameter	*P*	Variance explained (%)
Time	0.002	14.1
pH	0.002	9.9
Total		24.0
Undetermined		76.0

3.4.6 Investigating phylogenetic changes in community composition over time

Reads assigned to the Ascomycota and Basidiomycota formed the largest phyla by mean relative abundance of combined data from all sub samples, comprising 47.9 % and 30.1 % of sequences taken from the rhizosphere (Fig3.4), whilst reads assigned to the Zygomycota formed 0.2 % of sequences. There were no reads assigned to the remaining fungal phyla, including the Glomeromycota. In addition to the identified taxa, 15.2 % of sequences could only be assigned to the fungal kingdom and a further 6.5 % were unassigned to any taxonomic group within the fungal kingdom.

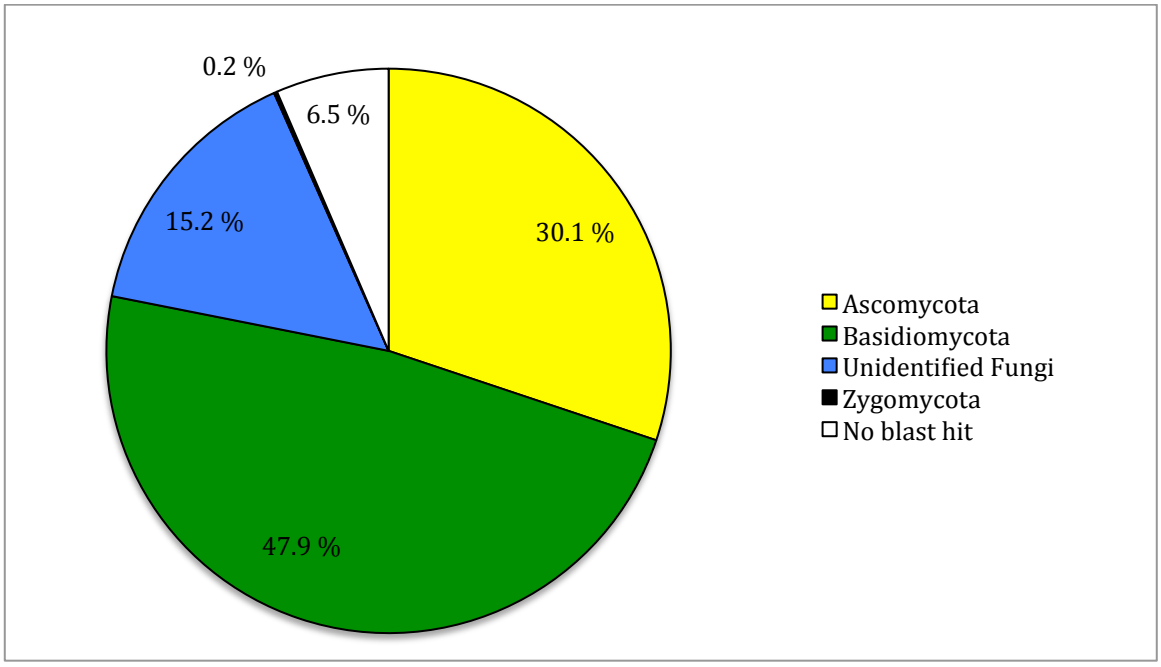

Figure 3.4 Mean relative abundances of reads assigned to fungal phyla across all rhizosphere samples collected between 2010 and 2012. Sequence taxonomy was assigned by RDP classifier at 97 % similarity, using the UNITE 27.08.2013 database.

Reads were assigned at the phylum level and changes in composition were investigated over time (Fig3.5). Reads assigned to the Basidiomycota formed the largest phylum by abundance in October 2010, July 2011 and October 2011, whilst reads assigned to the Ascomycota comprised the largest proportion of reads in July 2012 and October 2012 (Fig3.5a). The proportion of reads assigned to the

Basidiomycota significantly declined between 2010/11 and 2012 (P=0.011), with means of 57.4 % to 34.0 % respectively, whilst the abundance of Zygomycota sequences significantly increased over this period (P<0.001; Fig3.5b), from under 0.01 % in 2010/11 to a mean of 0.45 % in 2012. Reads assigned to the Ascomycota, unidentified fungi and the no blast hit groups did not vary significantly over time.

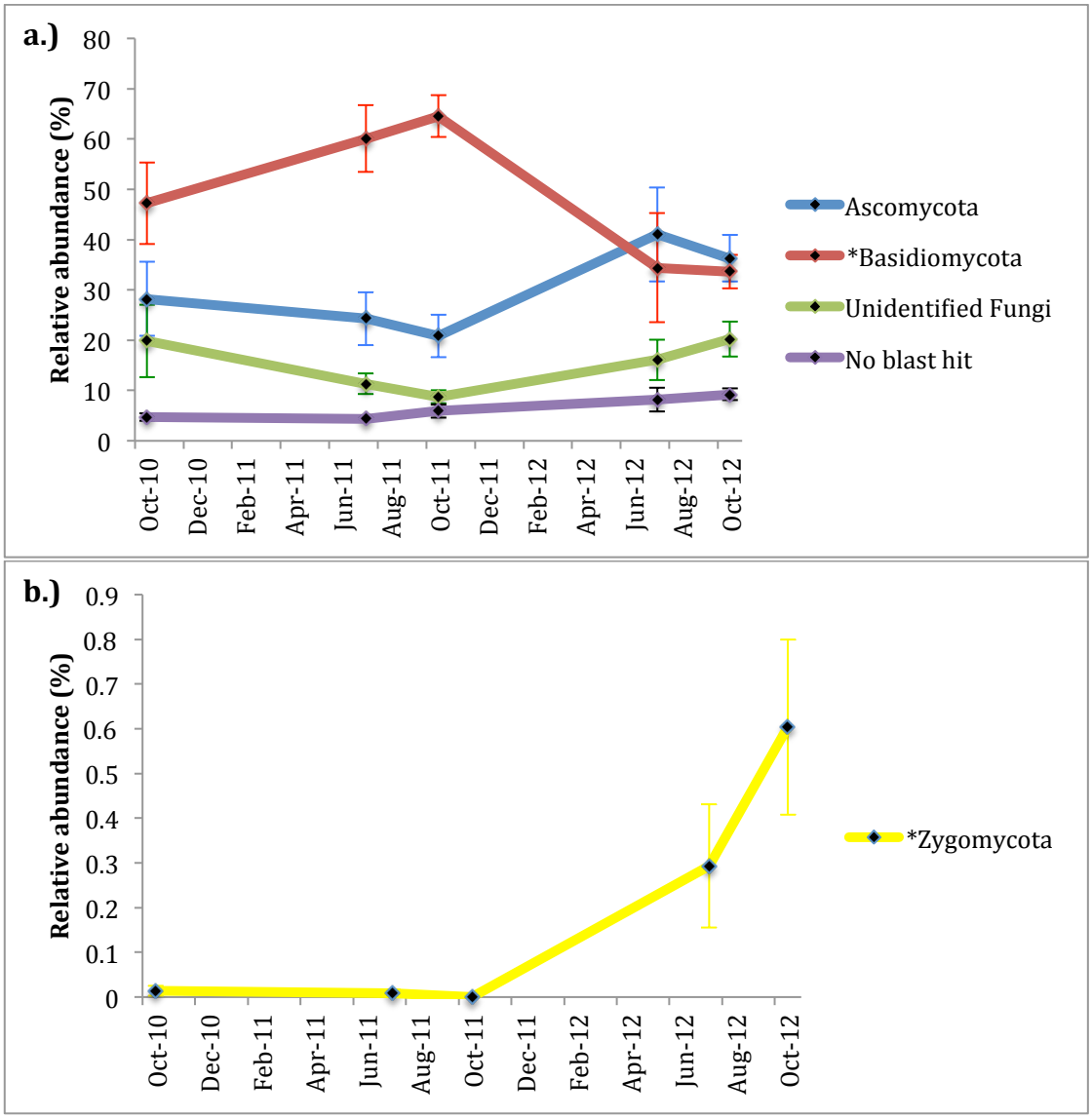

Figure 3.5 Mean relative abundance of reads assigned to phyla at each sampling time a.) Ascomycota, Basidiomycota, Unidentified fungi and No blast hit b.) Zygomycota are included separately due to low relative abundance. Sequence taxonomy was assigned by RDP classifier at 97 % similarity, using the UNITE 27.08.2013 database. Error bars are ±1.S.E.M.

Reads were assigned at the family level and the variation in relative abundances investigated over time (Fig3.6). Within the reads assigned to the Ascomycota, there were significant increases over time for the Vibrisseaceae (P=0.046), Amphisphaeriaceae (P=0.012) and Tuberaceae (P=0.037) increasing by approximately 19.0 %, 3.2 % and 1.6 % from October 2010 to October 2012 (Fig3.6a), while the Pyronemataceae sigificantly decreased (P<0.001) by 6.5 % from peak abundance in July 2011 to October 2012. Within reads assigned to the Basidiomycota, only the ECM families significantly decreased with time, with the Bolbitiaceae (P<0.001) decreasing by 16.9 %, from a peak of 17.9 % in July 2011 to 1.0 % in October 2012, whilst the Tricholomataceae (P=0.019) decreased from 2.3 % to 0.1 % over the same period (Fig3.6b).

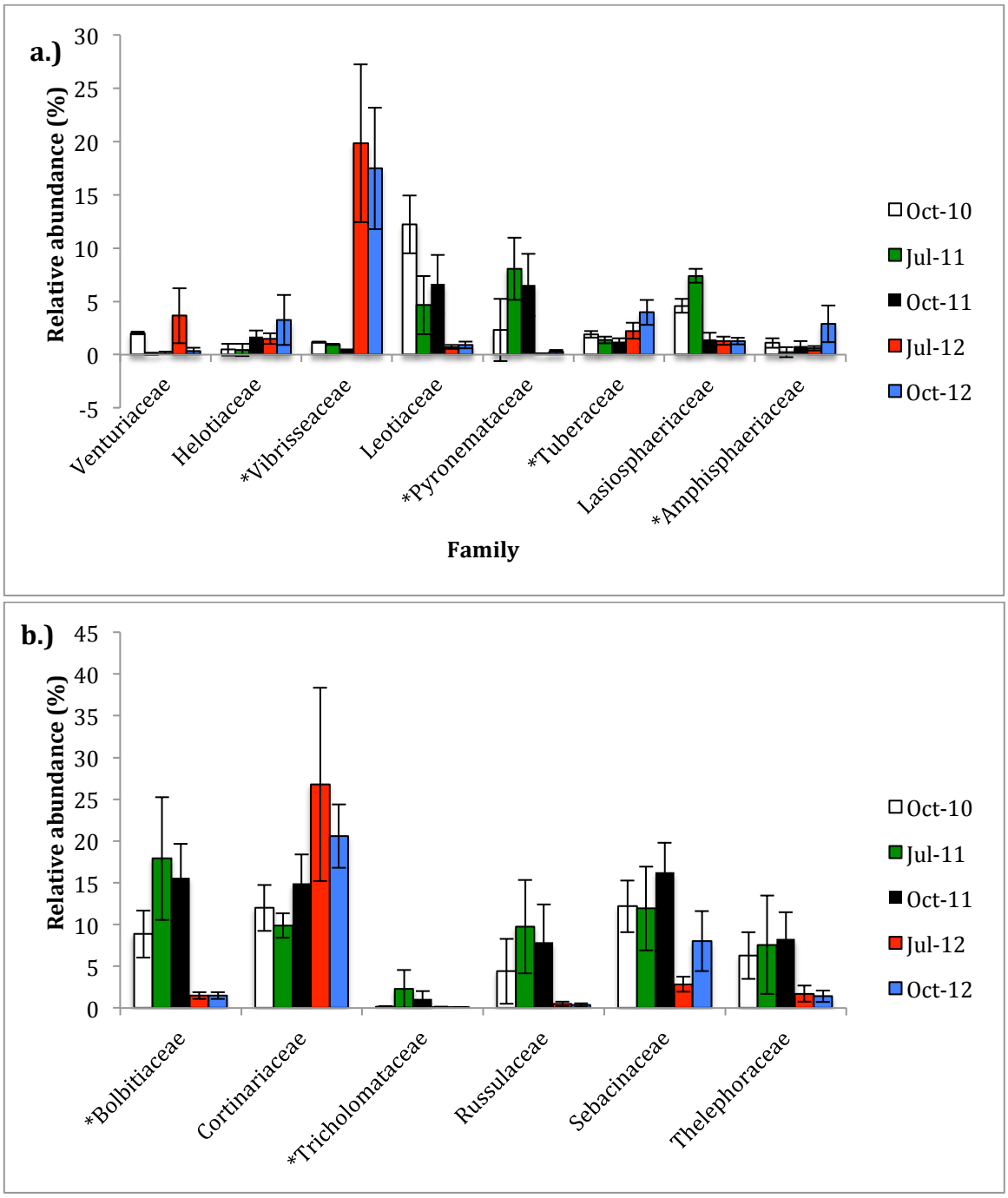

Figure 3.6 Relative abundance of reads assigned to each family over for each sampling time. For clarity, only those with an average relative abundance above 0.5% are shown a.) Ascomycota b.) Basidiomycota. Sequence taxonomy was assigned by RDP classifier at 97% similarity, using the UNITE 27.08.2013 database. Error bars are ±1.S.E.M. *Denotes family significant variation between transects.

3.4.7 Investigating key OTU changes between 2010/11 and 2012

In order to investigate the key OTUs behind the transition of the community in 2012, the mean difference in abundance between 2010/11 and 2012 was found and the OTUs (identified to the species level) with the greatest increase and decrease underwent one-way ANOVA to investigate the significance of variation between sampling times (Table 3.5). Of the 50 OTUs that underwent analysis, 11 significantly varied between sampling points, with 4 OTUs decreasing and 7 increasing in abundance in 2012 compared to 2010/11. Reads assigned to Ascomycete fungi *Lichenella iodopulchra* had the largest mean decline in 2012 compared to 2010/11, falling by a mean abundance of 11.4 %. Reads assigned to the ECM species *Hebeloma sp.* (P=0.024), *Naucoria salicis* (P=0.049) and *Otidea apophysata* (P=0.037) all decreased in abundance in the 2012 communities compared to 2010/11, decreasing by a mean abundance of 3.3 %, 0.7 % and 0.3 % respectively, whilst reads assigned to the *Pezizales sp.* (P=0.019) significantly increased in abundance, with a mean increase of 2.2 % in 2012. Additionally, reads assigned to ECM genotype *C. disemospermus* dramatically increased in mean abundance to 20.0% (P<0.001) in 2012, from a mean of just 3.2% in 2010/2011. Reads assigned to the dark septate endophyte *Phialocephala fortinii (P*<0.001) and the phytopathogenic endophyte *Truncatella angustata* (P=0.003) also both significantly increased in relative abundance in 2012 by a mean of 2.4 % and 16.3 % respectively compared to previous years. There were significant increases in relative abundance of a further 2 OTUs that could only be taxonomically assigned to the fungal kingdom (P=0.003 and P<0.001) and another OTU (P<0.001), which was completely unassigned even at the kingdom level, with increases in mean abundance in 2012 compared to 2010/11 transects by 0.7 %, 5.7 % and 3.6 % respectively.

Table 3.5 OTUs (analysed at the species level) that significantly varied between sampling times listed with taxonomic information, average change in relative abundance in 2012 compared to 2010/2011, *P*-value and putative biological function.

Taxonomy	Phylum	Average Change 2012*	*P*-value	Biological Function
Lichinella iodopulchra	Asc.	-11.39	*0.024*	Lichen-like
Hebeloma *sp.*	Bas.	-3.33	*0.049*	ECM
Naucoria salicis	Bas.	-0.69	*0.037*	ECM
Otidea apophysata	Asc.	-0.33	*0.003*	ECM
Unidentified fungal sp.3	-	0.72	*0.030*	-
Pezizales *sp.*	Asc.	2.19	*0.019*	ECM
Truncatella angustata	Asc.	2.39	*0.003*	Phytopathogenic
Unidentified fungal sp.5	-	5.68	*< 0.001*	-
No blast hit	-	3.57	*< 0.001*	-
Phialocephala fortinii	Asc.	16.31	*< 0.001*	Endophyte
Cortinarius disemospermus	Bas.	17.62	*< 0.001*	ECM

The mean difference between 2010/11 and 2012 was investigated and the OTUs (identified to the species level) with the greatest 2.5% increase and decrease underwent one-way ANOVA and the subsequent *P*-values produced are listed. Sequence taxonomy was assigned by RDP classifier at 97% similarity using the UNITE 27.08.2013 database. Abbreviations: Asc. = Ascomycota Bas. = Basidiomycota, ECM = Ectomycorrhizal fungi.

3.4.8 Investigating Mycorrhizal fungal community dynamics

Members of the Ascomycota, Basidiomycota and Zygomycota can form ECM associations, whilst other fungi perform a variety of other functions. Reads assigned to ECM families were selected across phyla to investigate changes in their OTU richness over time (Fig3.7). The OTU richness for ECM families revealed that 5 of the 9 differed significantly between sampling times, with the Bolbitiaceae (P<0.001), Cortinariaceae (P<0.001), Russulaceae (P=0.047), Pyronemataceae (P=0.003) and Tuberaceae (P=0.038) all significantly lower in OTU richness in 2012 than 2010/11, decreasing by approximately 4, 27, 2, 2 and 2 OTUs per family between 2010/11 and 2012. There were significant increases in reads assigned to the Thelephoraceae (P=0.007) and the Trichosporonaceae (P=0.043) families, which increased by approximately 3 and 2 OTUs per family in the 2012 communities compared to 2010/11. Only the OTU richness of reads assigned to the Sebacinaceae (P=0.369) and Helvellaceae (P=0.558) families did not significantly differ between sampling times. Additionally, the cumulative abundance of reads assigned to ECM OTUs was also assessed for changes over time (Fig3.8b), which demonstrated that while ectomycorrhizal fungi increased in relative abundance in 2011 compared to 2010, they declined substantially below 2010 levels in both 2012 sampling times (P<0.001).

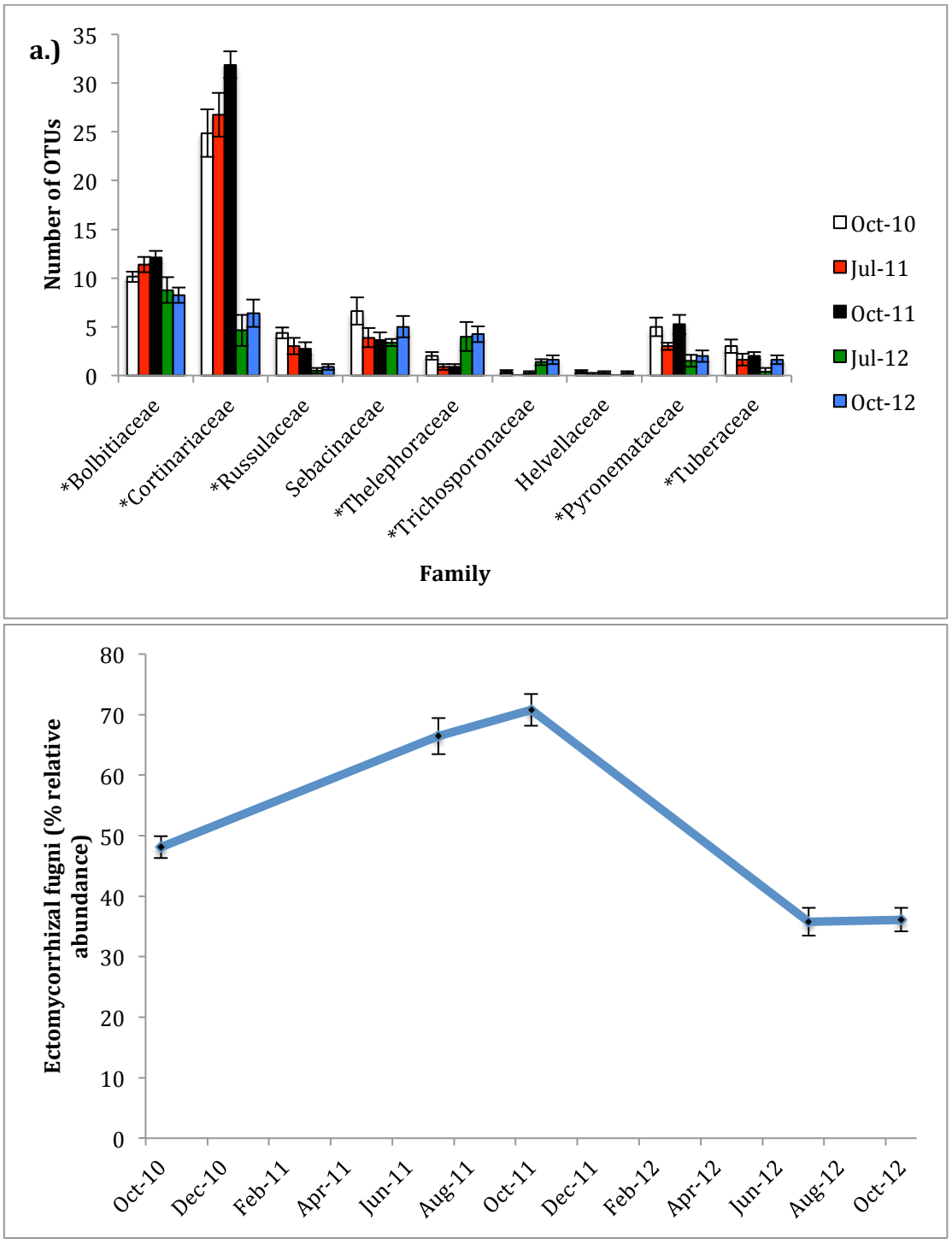

Figure 3.7a.) OTU richness of reads assigned to the ectomycorrhizal families in the rhizosphere of SRC willow between 2010 and 2012. *Denotes significant differences between transects b.) Cumulative relative abundance of mycorrhizal fungi within the willow rhizosphere between 2010 and 2012. Sequence taxonomy was assigned by RDP classifier at 97 % similarity using the UNITE 27.08.2013 database.

3.5 Discussion

This work demonstrates that although the rhizosphere fungal community varies with environmental and geographical parameters, it is infrequent but large transitions that have the largest effect on the community over time, potentially driven by environmental extremes. Whilst the only study to date that has compared spatio-temporal variation of rhizosphere communities using high-throughput sequencing found distinct seasonal assemblages, sampling was performed over a single year and thus lacked year-on-year comparisons (Dumbrell et al. 2011). In Chapter 2, distinct seasonal fungal assemblages were found in the SRC willow rhizosphere community. However the 3-year community analysis revealed that less frequent but large transitions occurred in time that caused far greater variation in rhizosphere fungal composition than those associated with seasonality. This transitional effect persisted throughout the growing season of 2012, with the July 2012 and October 2012 assemblages sharing considerably more community similarity to each other than to all other sampling points. Edaphic properties were also shown to impact upon the rhizosphere fungal community composition, with pH of particular importance, supporting a wealth of literature on the topic (McAfee and Fortin 1987; Jonsson et al. 1999; Buée, Vairelles, and Garbaye 2005; Buée, Reich, et al. 2009; Gosling et al. 2013; Hazard et al. 2013). However, whilst pH was hypervariable (ranging from 5.2 to 7.0) between sampling locations, pH and other edaphic properties generally remained stable throughout the duration of the experiment, thus were unlikely to play a role in the rhizosphere fungal community transition between 2010/11 and 2012.

The rhizosphere fungal community of the SRC willow was dominated by reads assigned to the Ascomycota and Basidiomycota, with the Zygomycota also consistently forming a small part of the community. Whilst willow can form AM associations, no reads were assigned to the Glomeromycota within the community, suggesting very low occurrence of AM associations within the willow roots. Many of the reads assigned to the ascomycete, and most of the basidiomycete fungi, were

from ECM fungal families, accounting for a combined average of over 50 % of the total fungal abundance across all transects.

There are a number of mechanisms which may drive long-term transitions in ECM communities. Sharing of hyphae between hosts has been shown to effect ECM fungal community development over time (Bastide, Piché, and Kropp 1995). However the aboveground biomass consisted of 6 closely related genotypes of the same age, and therefore hyphal sharing is unlikely to drive these transitions. Similarly, increasing root density as stands mature has been suggested as another mechanism behind changing ECM composition (Pickles et al. 2010; Peay, Kennedy, and Bruns 2011). However the field site was well established at the start of the experiment in 2010, therefore whilst the effects of changing root density cannot be dismissed, the large transition in the rhizosphere fungal community in 2012 compared to previous years is unlikely to reflect a changes in root density (Bergemann and Miller 2002). Interestingly, heavy rainfall has been linked to increasing AM root colonization in vineyards (Schreiner 2005) and tropical forests (Allen et al. 1998), and increasing soil moisture was also shown to increase AM infection in the laboratory (Lodge 1989), whilst ECM fungi were shown to be inversely correlated with AM infection, and thus reduced in abundance with increasing soil moisture. ECM have also been loosely characterised as early or late-stage genotypes based on their order of appearance on root systems (Mason, Last, and Wilson 1987). The late-stage families Russulaceae and Bolbitiaceae decreased in abundance and diversity over time whilst the early-stage Thelephoraceae family increased in OTU richness (Deacon 1992; Bergemann and Miller 2002). Therefore an alternate hypothesis is that a rain induced perturbation event occurred early in the growing season of 2012, which partially 'reset' the successional pattern of the ECM community. This would explain both the decline in reads assigned to the ECM fungi and the potential increase in early-stage ECM fungal diversity in later sampling points. Additionally, the reads assigned to the ECM genotype *C. disemospermus* was the largest beneficiary of the transition in 2012, increasing by a mean abundance of 17.6 % compared to the previous 2 years. This could be indicative of a large change in competition dynamics

within the ectomycorrhizal fungus community, with *C.disemospermus* a particularly resilient genotype to very wet soils.

Whilst over half of the reads assigned to the fungi of the rhizosphere were ECM genotypes by abundance, substantial saprophyte, non-mycorrhizal endophytes and phytopathogenic fungal populations were also found, which have been shown to form a complex web of interactions amongst each other *in situ* (Smith and Read 2010). *P. fortinii* has been found in high abundance in the abiotically stressful environments of the artic and subarctic (Queloz et al. 2005; Roberts et al. 2009), and has also been linked to increased NO_3 uptake by their hosts (Newsham 2011). Therefore active host selection for optimal symbionts may drive the marked increase in the reads assigned to *P. fortinii* in 2012. There was also a general increase in reads assigned to endophytes in 2012 compared to previous years, including the pathogenic *Truncatella angustata*. ECM fungi form sheaths around root tips which have been hypothesised to form physical barriers to endophyte colonization (Reininger and Sieber 2012). Therefore the decline in reads assigned to ECM could allow endophytes such as *P. fortinii* and *T. angustata* that were present in low abundance to opportunistically colonize the roots in their absence (Kucey 1987; Cordier et al. 1998; Erbilgin and Raffa 2002; Liu et al. 2007).

In this work, 454-pyrosequencing was used to analyse the across fungal community of the rhizosphere, rather than focusing on single fungal groups such as the mycorrhizal fungi or non-mycorrhizal endophytes. However since pyrosequencing only provides relative abundance data, fungal biomass remains unknown. Reads assigned to the non-mycorrhizal endophytes and phytopathogenic taxa were present across all time points in low abundance, but increased in the 2012 communities. However it remains possible that biomass was stable amongst these groups, but due to the reduction in ECM fungi, they increase in abundance in relative terms. This could have a profound effect for predicting the health and functioning of the rhizosphere. Increasing phytopathogenic fungi could be indicative of disease, whilst increasing colonization by dark septate endophytes could be indicative that

the host is under abiotic stress (Malinowski and Belesky 2000; Waller et al. 2005). Future experiments could employ measures of biomass such as qPCR with pyrosequencing to quantify fungal biomass, whilst shotgun metagenomics could also be performed instead of amplicon-based sequencing to limit PCR bias between taxonomic groups (Anderson, Campbell, and Prosser 2003). The power of both amplicon-based and metagenomic studies will however be limited by the reference databases, which for fungi are particularly poorly annotated compared to bacteria and other eukaryotes.

The fungi within the rhizosphere perform a range of functions, and compositional changes within the rhizosphere fungi will be reflected in the overall functioning of the rhizosphere. In this experiment, edaphic parameters such as pH were shown to influence rhizosphere fungal community composition. However the evidence also points to large and infrequent transitions driven by short-lived extreme environmental stresses such as heavy rainfall, which allow rare taxa to proliferate and dominate the post-stress community for at least the duration of the growing season. Therefore single sampling time points will always be limited for providing understanding of rhizosphere fungal community assembly. The greater taxonomic resolution given through next generation sequencing techniques was used to highlight subtle but potentially important changes in community composition which were hidden within larger taxonomic units, such as those which took place within the reads assigned to the Cortinariaceae family. Finally, whilst the reads assigned to the ECM fungal community changed in composition and structure, this was associated with changes to the diversity and relative abundance of other fungal groups. These interactions are poorly characterised but here changes in the abundance of ECM potentially permitted increased endophyte (both symbiotic and phytopathogenic) colonization. To date, few studies have investigated the long-term dynamics of the rhizosphere, and none have implemented next generation sequencing. Additional studies using these techniques to investigate rhizosphere community development over several years would greatly assist in both elucidating

the mechanisms behind large transitions and also determining the persistence within the community of key taxa over time.

CHAPTER IV: COMPARING THE FLUX AND RESIDENCE TIME OF RECENTLY DERIVED PHOTO-ASSIMILATES THROUGH ECTOMYCORRHIZAL AND ARBUSCULAR MYCORRHIZAL PATHWAYS

4.1 Abstract

Bioenergy crops are a growing source of land use within the UK, and it is a long-term research target to utilize them in sequestering carbon (C) from the atmosphere into soil. Whilst overall soil organic carbon (SOC) increases have been demonstrated in these crops, the mechanisms behind these increases remain relatively unknown. Mycorrhizal fungi are obligate plant symbionts that are auxotrophic for C, which they derive from their hosts, and are substantial sinks of recently derived photo-assimilates in a variety of plants. Although, a significant quantity of SOC is thought to be fungal-derived, the proportion derived from mycorrhizal fungi remains unclear. In this work, the flux and residence time of recently derived photo-assimilates associated with the ectomycorrhizal (ECM) and arbuscular mycorrhizal (AM) fungal pathways were quantified in short rotation coppice (SRC) willow and *Miscanthus* respectively. This involved performing the world's largest (by volume) pulse labelling experiment, in which 4 x 45 m^3 blocks of SRC willow and *Miscanthus* x *giganteus* were labelled with 99%-labelled-CO_2 simultaneously, in August 2012 in Brattleby (Lincolnshire, UK). Cores inserted into the field prior to the growing season were used to determine ^{13}C enrichment of soil effluxes and bulk soil in rhizosphere (roots and hyphae), hyphal-only, and root and hyphal free soil. Results from this work demonstrated that *Miscanthus* roots were more enriched with ^{13}C than willow roots, but that soil effluxes and SOC were more enriched in ^{13}C in the willow than the *Miscanthus*. However the hyphal inclusion cores did not show a significant difference in ^{13}C enrichment of soil effluxes or bulk soil relative to the root and hyphal exclusion cores in either crop. These results suggest that willow roots are more active than the *Miscanthus* roots, and that the rapid flow of recently derived photo-assimilates significantly contributes to medium-term SOC pools in

the willow crop but not *Miscanthus*. Finally, whilst mycorrhizal fungi have been shown to be a major component of belowground C cycling in other ecosystems, mycorrhizal fungal pathways may potentially be less important in the distribution and storage of photo-assimilates in bioenergy crops, although some experimental uncertainties remain.

4.2 Introduction

The accumulation of C in the soils beneath bioenergy crops has been extensively investigated as a method of mitigating atmospheric CO_2 (Campbell et al. 2008; Zimmermann, Dauber, and Jones 2012; Walter, Don, and Flessa 2014). Despite contentious land use issues, increasing quantities of bioenergy crops are being grown within the UK, with *Miscanthus x giganteus* and SRC willow being two of the most widely grown (Rowe, Street, and Taylor 2009). Each has been shown to sequester around 1.6 t C ha^{-1} yr^{-1} (Taylor and Bunn 2000; R. D. Armstrong et al. 2003), however the processes behind this SOC accumulation have yet to be defined and quantified. The majority of SOC in other ecosystems has been shown to be plant-derived (Crow et al. 2009), and multiple mechanisms are associated with its formation. As plant biomass decomposes, a proportion of C will remain within long-term soil C pools in recalcitrant forms. Additionally, the rhizodeposits, which are organic compounds released from plant roots, have also been shown to significantly contribute to SOC pools beneath tree and grass species (Cardon 1995; Kuzyakov and Domanski 2002; Neergaard, Porter, and Gorissen 2002; Hütsch, Augustin, and Merbach 2002). However as organic materials are mineralised, most C is returned to the atmosphere, with only small amounts stabilised as SOC (Rosling, Lindahl, and Finlay 2004). Increases in SOC can therefore be achieved through increased C input into the soil or through decreasing the rate of turnover of C within it.

Up to half of all fixed C has been shown to be redirected belowground in a range of plants (Kuzyakov and Domanski 2002; Epron et al. 2011), which drives root growth

and maintenance (Ignacio Rangel-Castro et al. 2004). This flux of recently derived photo-assimilates has been shown to be a major driver of belowground processes, accounting for up to half of all C present in soil respiration (Chambers et al. 2004). Mycorrhizal fungi are the most abundant microbes in the rhizosphere (Högberg and Högberg 2002), and are auxotrophic, requiring plant-derived C in the form of sugars and other labile C compounds from their host (Derrien, Marol, and Balesdent 2004; Heinemeyer et al. 2006). Consequently, mycorrhizal fungi have been shown to receive substantial quantities of C fixed by plants, with up to 5-20 % of all fixed carbon translocated to arbuscular mycorrhizal (AM) fungi (Leake et al. 2001; Smith and Read 2010), whilst tree girdling experiments to limit ectomycorrhizal fungi (ECM) growth reduced overall soil respiration by 56 % in *Pinus sylvestris* L. (Högberg and Högberg 2002).

The growth and subsequent turnover of mycorrhizal fungi contributes to SOC pools, with the overall residence time of C associated with ECM fungi shown to be 4 to 5 years (Treseder et al. 2004). However the residence time of C associated with different mycorrhizal structures varies, being just 5 to 6 days for extraradical hyphae, but considerably longer for spores and thicker hyphae (Godbold et al. 2006; Rooney et al. 2009). Additionally, glomalin, which is thought to be a glycoprotein produced by AM fungi, has been shown to have a residence time of many years, thereby contributing to long-term C pools (Rillig et al. 2003). The composition of mycorrhizal structures and exudates will vary between AM fungi and ECM fungi, as well as being highly species specific and thus, the residence time of fungal associated C will likely vary between and within ecosystems (Treseder et al. 2004). Whilst large quantities of soil C have been demonstrated to be fungal derived (Rillig et al. 2001), the origin and turnover of C from mycorrhizal fungi is still relatively poorly understood (Johnson et al. 2002; Staddon 2003; Rilig et al. 2003; Godbold et al. 2006; Högberg et al. 2010; Orwin et al. 2011).

The intimate nature of the association formed between plant roots and mycorrhizal fungi makes separating their roles in belowground C cycling a challenging process,

and in natural conditions, the two are almost inseparable. However fine meshes can be utilized to selectively allow colonization of soil by roots or extraradical hyphae alone (Godbold et al. 2006; Heinemeyer et al. 2006; Moyano, Kutsch, and Schulze 2007; Nottingham et al. 2010). In particular, meshes with a pore of between 22 – 35 μm have been successfully used to allow mycorrhizal hyphal penetration whilst excluding fine roots. The flow of plant-derived C into root exclusion zones is therefore limited to that from fungal pathways and thus can be used to quantify the C flux associated with extraradical hyphae. However many mycorrhizal structures exist either intracellularly within root cells or as sheaths over root tips and consequently a significant proportion of mycorrhizal fungal biomass will be excluded from hyphal-only cores (Duddridge 1986; Bago et al. 1998).

Investigating belowground C cycling is technically demanding due to soil inaccessibility, therefore sampling is often destructive. Additionally, soil C pools are also extremely large and generally change slowly in response to environmental factors (Hungate et al. 1995). Therefore changes in SOC are very small in experimental time frames, whilst the inherent 'patchiness' of SOC across landscapes further confounds investigations (Franzluebbers, Stuedemann, and Schomberg 2000). However, the ever-increasing availability of isotopic analyses has allowed for the greatly improved detection of changes in SOC. Differences in fixation rates of naturally occurring ^{12}C and ^{13}C isotopes between C3 and C4 plants has allowed for long-term changes in SOC to be accurately measured under changing land uses (Hobbie 2006). However, the exogenous addition of $^{13}CO_2$ can also be used to perturb the isotopic signature of plant photo-assimilates produced during the period of incubation (Del Galdo et al. 2003). This spike in ^{13}C isotopic concentration can be followed through the ecosystem via stable isotope probing (SIP) techniques. Label can be quantified directly from soil respiration, bulk soil and roots with isotope-ratio mass spectrometry (IRMS), whilst stable isotope probing of phospholipid fatty-acids (SIP-PLFA) can also be performed from bulk soil to follow the transition of assimilates through microbial communities. These lipid biomarkers

are arguably linked to specific groups of soil biota such as fungi, Gram-positive bacteria and Gram-negative bacteria (Frostegård, Tunlid, and Bååth 2011).

In this experiment, the flux and residence time of recently derived photo-assimilates associated with the AM and ECM fungal pathways were compared in *Miscanthus* x *giganteus* and SRC willow bioenergy plantations respectively (Burns, unpublished; Chapters 2 and 3). Soil cores were established to partition plant-soil C fluxes within each crop, using fine root and hyphal inclusion cores, hyphal inclusion-root exclusion cores and root and hyphal exclusion cores. Pulse labelling was applied to 4 x 45 m^3 chambers within each crop, using 99.9 % ^{13}C-labelled-CO_2. The ^{13}C present in soil effluxes was assessed over time in the different treatment cores, whilst incorporation into the bulk soil and roots was also assessed in order to quantify the movement and storage of recently derived photo-assimilates through mycorrhizal pathways.

Hypothesis: The rhizosphere fungi of both *Miscanthus* x *giganteus* and SRC willow are significant sinks of recently derived photosynthates, whilst the flux and residence of C associated with each will differ.

4.3 Materials and Methods

4.3.1 Field site

The bioenergy plantation in Brattleby, Lincolnshire, UK (94225, 81108 British decimal degrees) supports an adjacent *Miscanthus* x *giganteus* to the SRC willow crops, with the willow site described in Chapter 2 (2.1.1) used for this work. The *Miscanthus* site consists of a field of approximately 11.56 ha, with an underlying soil type of clay-loam with approximately 27 %, 29 % and 49 % clay, sand and silt respectively. The soil contained 1.5 % and 0.3 % of C and N respectively, and had a pH that ranged from 6.8 to 7.3 (Bottoms, unpublished). Prior to planting, the site

had 3-year rotations between wheat and oilseed rape. The site had a 20-year mean annual precipitation of 605 mm and temperature of 9.9˚C. In 2006 the *Miscanthus* was planted at intervals of about 1 m between rhizomes. During planting, the field was ploughed, before power harrowing and herbicide application (Roundup, Monsanto). Unlike the willow, *Miscanthus* was harvested annually yielding 5.7, 10.7, 9.7 and 9.1 t ha^{-1} between 2009 and 2012. 660 kg ha^{-1} of PK fertilization (Fibrophos, Hatcher fertilisers, UK) was added in the spring of 2010 with no further application throughout the duration of this experiment.

4.3.2 Partitioning soil communities

Cores were constructed from acrylonitrile butadiene styrene (ABS) soil pipe that was 8 cm in diameter and 20 cm in depth (Fig4.1). 4 circular 'windows' of 4 cm in diameter were evenly distributed around the circumference of cores. This pattern was repeated 3 times over the length of the core, leaving 12 'windows' in total. Nylon meshes of differing pore sizes (Plastok Services, The Wirral) were used to selectively exclude sections of the belowground community. Using 2 mm meshes allowed for the penetration of all but coarse roots to the inner soil. Reducing pore size to 35 µm was performed to retain ECM and AM extraradical hyphal penetration but limit fine root penetration. A 1 µm mesh was used to exclude both roots and hyphae (Johnson, Leake, and Read 2001; Moyano, Kutsch, and Schulze 2007; Nottingham et al. 2010). For brevity, from this point onwards cores surrounded by the 2 mm mesh will be referred to as 'R^+/H^+', the 35 µm mesh as 'R^-/H^+ and the 1 µm mesh will be referred to as 'R^-/H^-'. Cores were filled with soil from each crop, which was removed in January 2012 from the upper 20 cm of each their respective field sites, before sieving through a 10 mm sieve and large roots removed. 1.8 kg of soil was added to the willow cores and 2.1 kg to the *Miscanthus*. Before cores were inserted 50 ml of deionized water was added to cores.

During the insertion of cores, a 15 cm deep hole was created using an 8 cm in diameter Dutch soil auger to which cores slid into the empty space. To ensure a good contact between the core and surrounding soil, a mixture of 3 parts deionized water and 1 part extracted soil was poured around the circumference of the inserted cores. Additionally within each block, solid soil collars consisting of pipe 4 cm in length by 8 cm in diameter were inserted into the ground at the same time as core insertion, with these serving as an adapter for measuring respiration rates of undisturbed soil (Fig4.1).

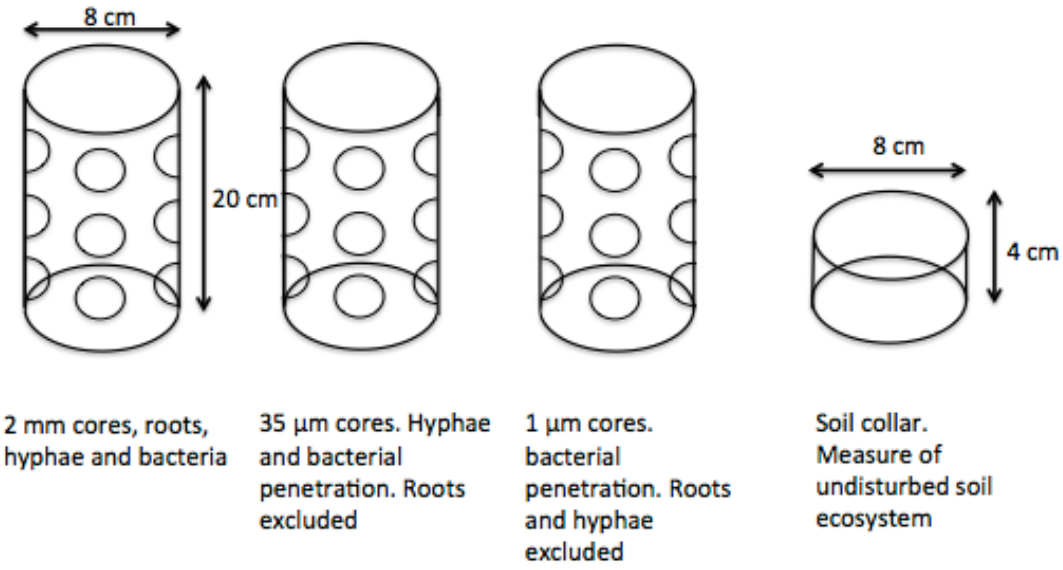

Figure 4.1 Meshes of varying pore size surrounded cores, which allowed for the selective colonization of roots and hyphae. Soil collars were also employed to measure undisturbed soil.

4.3.3 Sampling blocks structure

The willow was distributed in paired-rows whilst the *Miscanthus* formed more clustered biomass. Consequently, an area of 2.5 m (wide) x 6 m (length) was chosen for blocks, covering whole paired-rows of trees in the willow or several clusters of the *Miscanthus,* whilst also allowing large numbers of cores to be inserted within blocks. The willow consisted of a mixture of highly related genotypes, therefore

blocks containing only the dominant genotype, *Tora* (30% by abundance), were chosen to limit variability between sites. Additionally, since soil pH was hypervariable across the willow field, blocks between 5.5 and 6.5 were selected for use in experiments. 4 blocks in willow and *Miscanthus* crops were eventually established. Each block had 23 sampling locations allocated, and were filled in a random order with 7 of each core type (R^+/H^+, R^-/H^+ and R^-/H^-) and 2 soil collars. Core locations were a maximum of 0.2 m from the nearest plant and a maximum of 0.4 m from the next nearest mature plant, allowing multiple potential entry sources of roots to cores. Cores were also a minimum of 0.2 m away from their nearest neighbouring core (Fig4.2). Willow cores were inserted on the 30.3.12 whilst the *Miscanthus* core had to be inserted later due to a late harvest, on the 23.4.12.

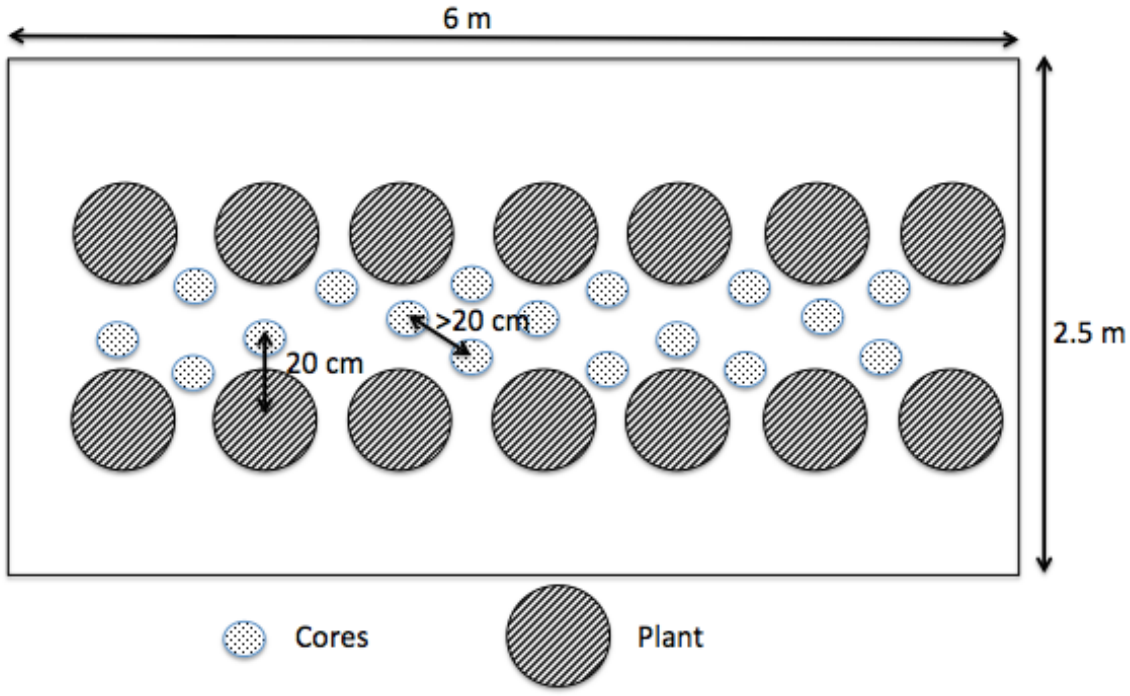

Figure 4.2 4 blocks of cores were established within each of the *Miscanthus* and willow plantations. 23 core locations were identified within each block before cores and control collars were randomly allocated. Each core was within 20 cm of the nearest plant and within 40 cm of the next nearest, whilst being a minimum of 20 cm away from any other core.

4.3.4 Core monitoring before labelling

Total respiration was measured after cores were established, using an EGM-4 infrared gas analyser with an SRC-1 attachment (PP Systems, Amesbury, USA). Adapters measuring 17 cm (height) x 8 cm (diameter) were built to house SRC-1 units (Fig4.3). The base of the adapters had a flat 20 cm x 20 cm panel with an 8 cm diameter circular hole cut in the bottom, which was aligned over cores. Additionally a 20 cm (length) x 20 cm (width) x 2 cm (depth) foam disc, which also had an 8 cm diameter hole was slid tightly over the core's protruding edge, forming a flat surface over which the adapter and SRC-1 was placed.

IRGA measurements were taken in the *Miscanthus* and willow simultaneously by 2 researchers working independently. Sampling took place between 11:00 and 13:00 hours, with both block order and core order randomized to prevent a systematic temporal bias. Total respiration measurements were initially taken 2 weeks after insertion, with increasing frequency throughout the sampling period, initially once a month in April, before increasing to a biweekly rate in May and June. Whilst the total respiration of only 2 cores of each type were normally analysed at each sampling point, every core within each block had respiration measured on the last sampling point prior to pulse-labelling (23.07.12). Subsequently, cores nearest to the average respiration rate of each treatment type, within each block, were selected for gas sampling throughout the pulse labelling sampling.

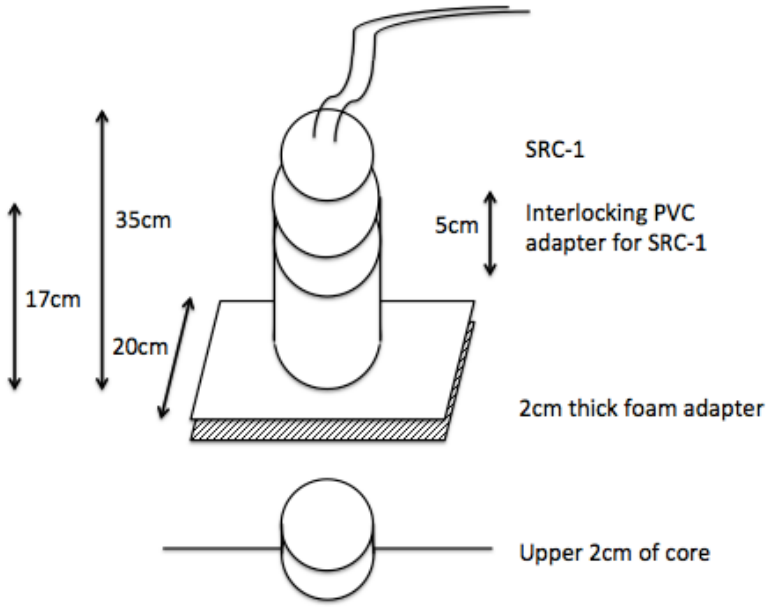

Figure 4.3 Total core respiration was sampled using an infrared gas analyser. An adapter was created to house an SRC-1, which rested on foam discs and allowed for an airtight seal to be formed over cores.

4.3.5 Pulse labelling

Chamber design was similar to that of previous *in situ* ^{13}C pulse labelling experiments (Subke et al. 2009; Biasi et al. 2012). Tents 6 m (length) x 2.5 m (width) x 3 m (height) were constructed at each sampling block from transparent plastic (<90% PAR)and aluminium poles, forming labelling chambers (Fig4.4). Tents were tall enough to cover the willow trees with minimal compaction of the upper branches. A water-cooled 7.3kW air conditioner that can move air at a rate of 1,450 m^3 hr^{-1} was employed in each tent, with 2 further tripod fans inside to further enhance air movement. Consequently each chamber had a generator to provide power to the fans and air conditioners.

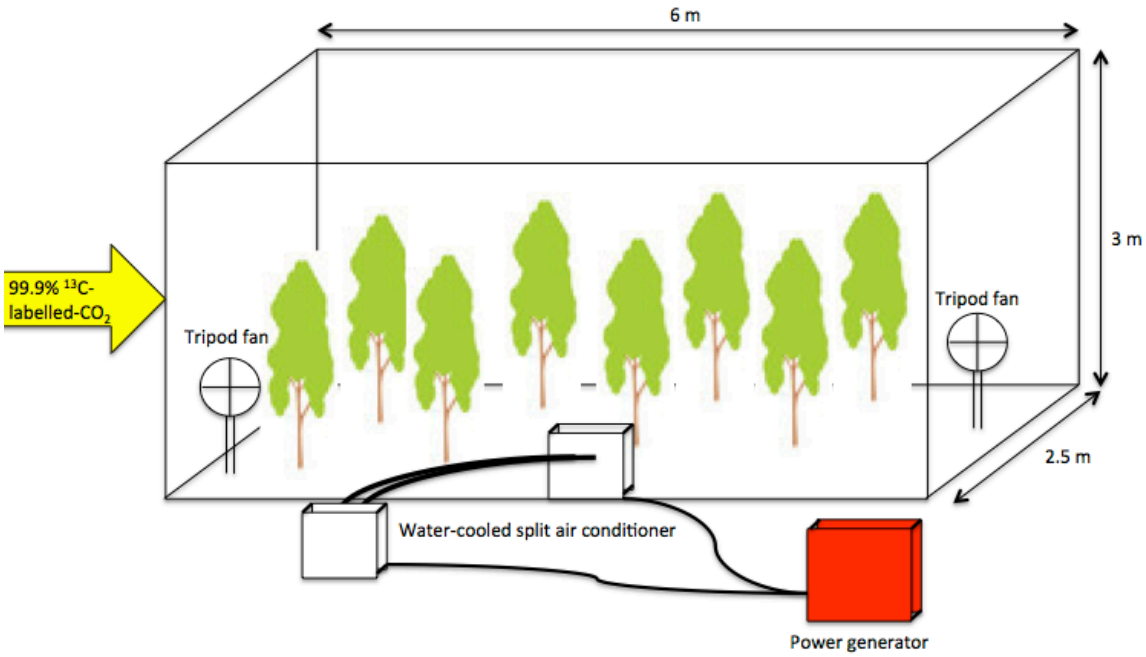

Figure 4.4 Aluminium poles were covered in transparent plastic (<90% PAR) and sealed at the bottom with sandbags. Air conditioners were employed to keep tents at ambient temperatures whilst additional fans ensured homogenous cooling. Labelling was performed on the 28.08.12 for 4.5 hours, starting at 08:00 hrs.

Labelling commenced 21 weeks after core insertion in the willow and after 18 weeks in the *Miscanthus*. Labelling was performed on 23.08.12. Enrichment with 99% ^{13}C-labelled-CO_2 was performed for 4.5 hours, commencing at 08:00. Through the addition of sandbags at each block, an airtight seal was created around the bottom of the chambers (Fig4.5). Real time carbon ppm values and $^{13}C/^{12}C$ ratios for all tents were provided during the labelling using a Picarro $^{13}CO_2$ analyser (Picarro G-2131-*I* Analyzer Series Isotopic CRDS (Cavity Ring Down) system, Picarro Inc, CA, USA). Samples were delivered to the Picarro in a flow-through system, which comprised of polytetrafluoroethylene (PTFE) sampling lines, flow controllers and flow monitors. The Picarro was run from a mobile laboratory (McNamara et al. 2002), which in turn had its own generator. CO_2 was initially drawn down to below 200 ppm before 17L of pure $^{13}CO_2$ was added in sequential batches. Immediately after labelling, the transparent plastic was removed to allow equilibration of chambers with the environment.

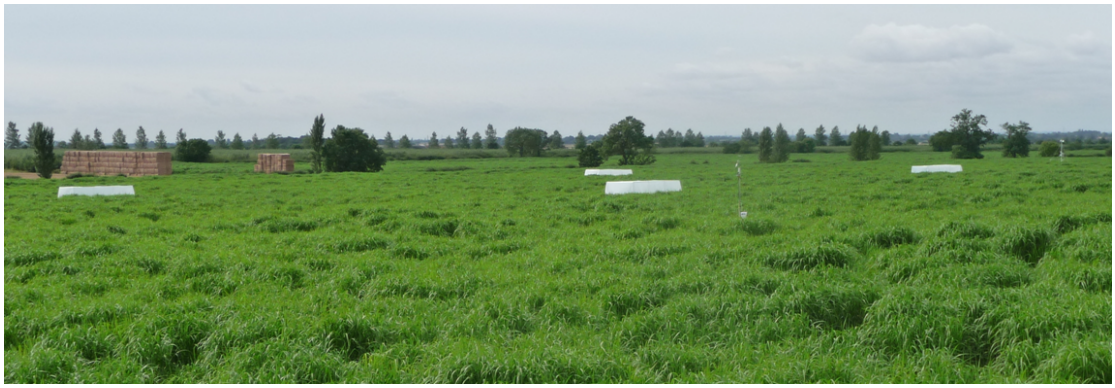

Figure 4.5 The tops of the 3 m labelling chambers sticking above the 2.5 m tall *Miscanthus* crop.

4.3.6 Post-pulse gas sampling

Gas sampling performed during the pulse labelling period used open-bottomed chambers that were attached to cores immediately prior to sampling, and soil effluxes were sampled as they accumulated. Rubber seals around cores served as a connector between cores and open-bottomed chambers, allowing an airtight connection (Fig4.6). Lids had rubber septa through which a 0.5 µm gauge syringe was used to take 20 ml gas samples (6.85% of total headspace volume) at 0, 15, 30 and 45 minutes post enclosure (Suba-Seal, Sigma-Aldrich, UK). These were transferred to a 12 ml gas-tight borosilicate glass vial (Labco, Lampeter, UK) for subsequent analysis. Gas samples were analysed separately for CO_2 concentration (ppm) and ^{13}C isotopic enrichment. 10 ml of gas was removed from each sample into a Small Sample Inlet Module (SSIM), and IRMS was performed to measure the $^{12/13}C$ ratio via the Picarro G2131-*I* CRDS. Between every sample a calibration gas standard (414 C ppm and -9.98‰) was analysed. Gas chromatography (GC) was performed on a further 7 ml of each gas sample, to give a $^{12+13}C$ ppm value using a PerkinElmer Austosystem XL Gas Chromatograph (PerkinElmer, USA) fitted with a Flame Ionisation Detector (FID) operating at 130 ˚C and Electron Capture Device (ECD) operating at 360 ˚C. A stainless steel Porapak Q 50-80 mesh column (length 2 m, outer diameter 3.17 mm) that was maintained at 60 ˚C was fitted to the GC. Each

run on the autosampler consisted of 32 samples with an additional 8 samples of calibration gases of known C concentrations of which samples were subsequently correlated against (Case et al. 2012).

At each sampling point, one core of each treatment type underwent gas sampling and isotopic analysis within each block. Sampling of ^{13}C in respiration was initially performed the day prior to labelling, then at 6 and 24 hours after labelling, followed by gas sampling at 2,4, 7, 14, 28, 42 and 104 days post-pulse.

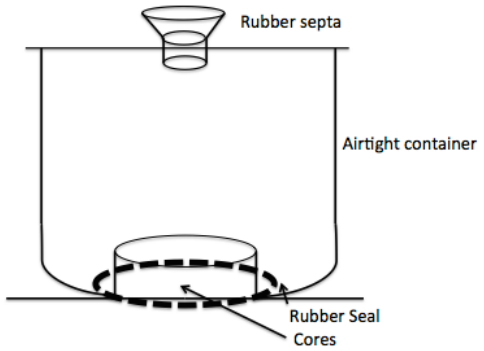

Figure 4.6 Cores were enclosed in an airtight chamber, with a rubber seal interlocking the bottom of the chamber to cores. Gas samples were taken via syringes through rubber septa within the lids.

4.3.7 Post-pulse bulk root and soil analysis

Core extractions were performed before labelling, followed by extractions at 6 and 24 hours, 7, 28, 104 and 208 days after labelling. Extraction of cores consisted of cutting around the length of the outer circumference with a flat blade, before pliers were used to remove the severed core. One core of each treatment type was randomly removed from each block, whilst collars were only used in gas sampling and were not extracted. Once removed, soil from within the cores was mixed and split into 4 even samples before freezing. Prior to analysis, two of the bags were defrosted overnight at 4˚C. A 50 ml tube was filled with soil before returning to -20 ˚C then freeze-dried for 3 days and cryo-milled before ^{13}C analysis. The remaining

soil was weighed and soaked for one hour in 19 ˚C deionised water, before roots were manually removed using forceps. Live and dead roots were separated by morphology (a lighter colour and the presence of fine root tips were considered live), and fresh weight determined to give an estimate of live root biomass, before being frozen at -20 ˚C again. A 0.10 g aliquot of the live roots was freeze-dried overnight, and then cryo-milled before ^{13}C analysis.

Freeze dried and cryo-milled soil and root samples underwent combustion in a Costech ECS4010 Elemental Analyser (Costech Analytical Technologies Inc, CA, USA) before analysis in the Picarro CRDS system, using a split-flow interface method (Balslev-Clausen et al, 2013). Between 2-3 mg of root samples or between 20-50 mg of bulk soil were weighed into ultra clean pressed tin cups (Elemental MicroAnalysis, UK) before being loaded into a Zero N-Blank, 50-position carousel austosampler and combusted. Evolved CO_2 and nitrogen oxides are passed through a reduction column (HayeSep Q Porous Polymer, 3 m) to separate gases (CO_2, CO, Ar, N_2, NO) before a caddy split flow interface passed gases into the Picarro CRDS analyser for ^{13}C analysis. Standards of a known bulk soil, beet sugar and cane sugar that had a range of $\partial^{13}C$ values were used as controls on each run, with samples calibrated against these.

4.3.8 Calculating pulse derived ^{13}C in soil respiration

Isotope-ratio mass spectrometry was performed in order to give $\partial^{13}C$ values, an isotopic signature of the ratio of ^{13}C and ^{12}C parts per thousand (‰) relative to a standard value for Vienna PeeDee Belemnite (PDB), which has an $R_{standard}$ value of 0.0112372 heavy to light ratio (using Equation (1) Boutton, 1991; Lu et al, 2004).

Equation 1:

$$\delta^{13}C_{sample} = (((^{13}C/^{12}C_{sample})/(^{13}C/^{12}C_{PDB}) - 1) \times 1000)$$

Atom % is the number of atoms of a given isotope in 100 atoms of an element (Ward et al, 2009), and $\partial^{13}C$ values were converted to atom % using equation 2.

Equation 2:

$$\text{Atom \%} = (100 \times AR \times (\delta^{13}C/1000 + 1)) / (1 + AR \times (\delta^{13}C/1000 + 1))$$

Where AR = 0.0112337, the absolute $^{12}C/^{13}C$ ratio of PDB and $\partial^{13}C$ = standard delta value of sample.

Using equation 2, ^{12}C and ^{13}C components to respiratory fluxes over the 45 minute incubation period were calculated before the ^{12}C and ^{13}C flux rates could be calculated (using equations 3 and 4).

Equation 3:

$$C_m = (C_v \times M \times P) / (R \times T)$$

Where C_m = Mass per volume concentration (μg CO_2-C / L), C_v = CO_2 concentration by volume (mixing ratio, ppmv CO_2 - C), M = Molecular weight of CO_2, P = Barometric pressure (atm), R = Ideal gas constant defined as 0.08205746 L atm K^{-1} mol^{-1} and T = Air temperature at the time of sampling (K).

Equation 4:

$$F = (V \times C_{rate})/A$$

Where F = Gas flux (mg CO_2 – C m^{-2} $h^{-1)}$, V = Internal volume of the enclosure (m^3), C_{rate} = Change in gas concentration over enclosure period (mg CO_2 m^3 h^{-1}) and A = area of collar enclosed soil surface (m^2).

Due to endogenous background ^{13}C found in natural ecosystems, the excess ^{13}C fluxes were calculated as the accumulation of ^{13}C beyond that of changes in natural abundances. The accumulation of ^{13}C in each crop was modelled from the pre-pulse data as the exponential function from each crop, and the change in natural

abundance of isotopes accounted for in an equivalent flux rate for each sample (shown in Equation 5).

Equation 5:

$$^{13}C_{Excess}\ Flux\ (\mu g\ m^{-2}\ h^{-1}) = {}^{13}C_{Pulse\ labelling}\ flux - {}^{13}C_{Natural\ Abundance}\ flux$$

4.3.9 PLFA Extractions

Microbial community structure was assessed by analysis of ester-linked PLFAs. 1.0 g of freeze-dried soil was sieved to 2 mm and roots removed before extraction, whilst 0.3 g of fresh roots from corresponding cores also underwent extraction. Soil from all pre-pulse cores was used to investigate the fungal colonization in R^-/H^+ cores, which were compared to R^-/H^- cores. Bulk soil from all R^+/H^+ cores and roots were analysed separately for samples up to 3 months after labelling. Lipid extraction of a total of 96 samples was performed, with 60 from bulk soils and a further 36 from the extracted roots.

Total lipid was extracted using a modified Bligh and Dyer technique (Frostegard *et al*, 1991). Lipids were collected on a silica column. Neutral and glycolipids were eluted from the column with chloroform and acetone and discarded. Polar lipids were eluted with methanol, collected and transmethylated to their fatty acid methyl esters, which were quantified and identified by chromatographic retention time and mass spectral comparison using 13LO (methyl tridecanoate) and 19L0 (methyl nonadecanoate fatty acid as an internal standard using a gas chromatograph (6890 series, Agilent Technologies, UK). Oven temperature ramped from 100˚C to 160˚C at 20˚C min $^{-1}$ and was held for 32 minutes.

4.3.10 Statistical analysis

One-way analysis of variance (ANOVA) was performed to test the significance of differences between treatments types and changes over time in root densities, total soil effluxes (measured by IRGA and GC), $\partial^{13}C$ soil effluxes and excess ^{13}C effluxes, and when significance was found, a Tukey post-hoc test was performed (XLSTAT, 2006, Adinsoft, France). Additionally, paired t-tests were used to test for differences in these parameters between willow and *Miscanthus* treatments (XLSTAT).

4.4 Results

4.4.1 Measuring respiration pre-pulse

Total respiration ranged from between 0.08 and 1.03 g CO_2 m^{-2} h^{-1} for willow and 0.05 and 0.80 g CO_2 m^{-2} h^{-1} for *Miscanthus*, both of which significantly increased throughout the sampling period (P<0.001 and P<0.001 respectively, Fig4.7). Despite increasing respiration rates over time, there were no significant differences between any treatment types in either crop (P=0.787 and P=0.770 for willow and *Miscanthus* respectively), whilst there was also no significant difference in total respiration rates between the *Miscanthus* and willow (P=0.373).

4.4.2 Miscanthus and willow root biomass

Roots were extracted from each core after destructive sampling and live root densities were calculated under each treatment type. Willow R^+/H^+ cores had significantly more roots than the R^-/H^+ and R^-/H^- cores (P<0.001), with over 12 g roots kg^{-1} of soil in the R^+/H^+ treatment compared to under 1 g roots kg^{-1} of soil in each of the other treatments (Fig4.8a). Root biomass did not change significantly over time in any of the willow treatments (P=0.357). *Miscanthus* live root densities

also significantly differed between treatments (P<0.001), with the R^+/H^+ treatment having an average of over 5 g roots kg^{-1} soil, considerably more than the R^-/H^+ and R^-/H^- cores, which both had an average of less than 0.1 g roots kg^{-1} soil (Fig4.8b). As with the willow, there was also no change in live root density in *Miscanthus* treatments over time (P=0.580). The willow R^+/H^+ treatment also contained on average a greater live root density (P<0.001) than *Miscanthus* R^+/H^+ treatments.

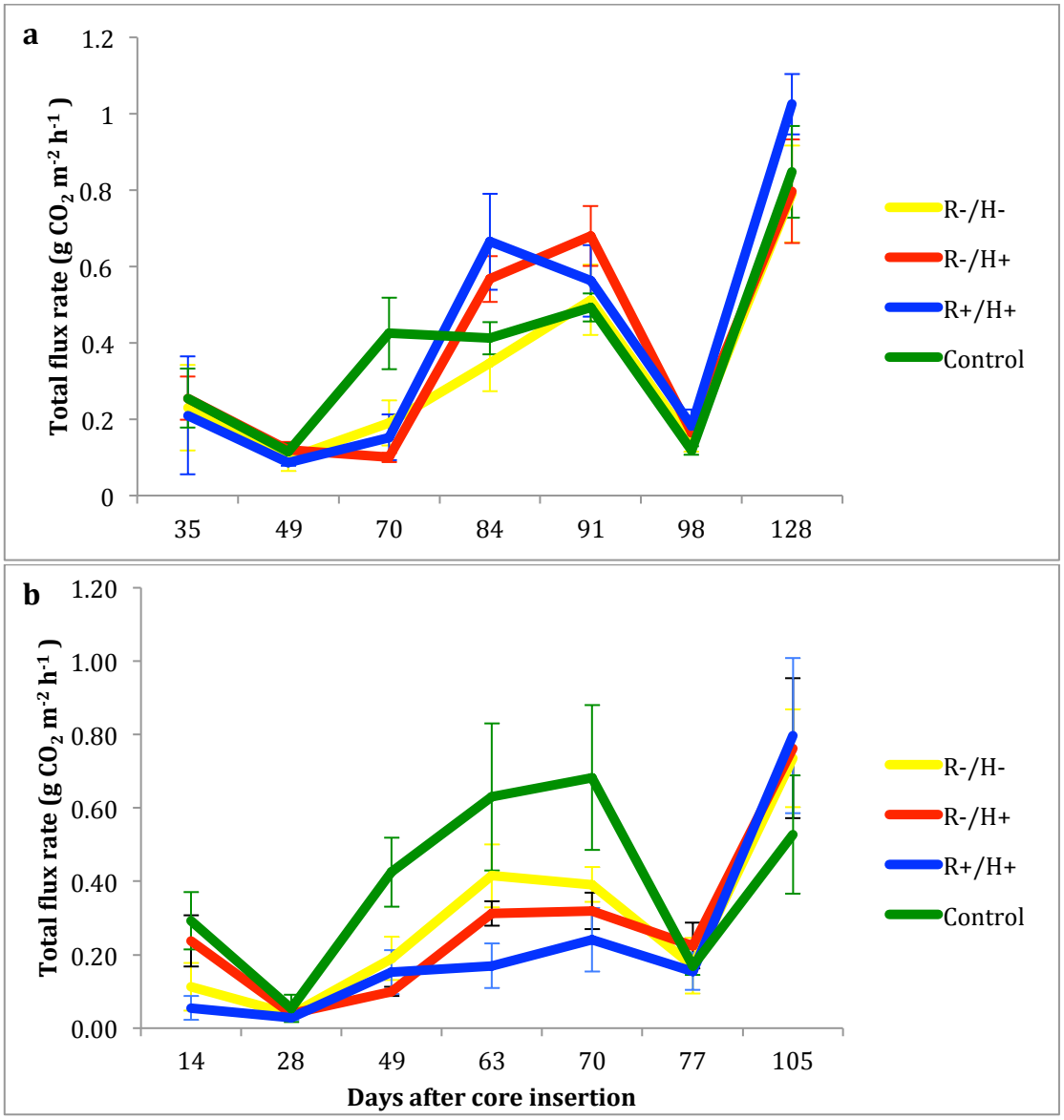

Figure 4.7 Prior to pulse labelling total respiration rates monitored with IRGAs in a) Willow b) *Miscanthus*. R^+/H^+ = roots and hyphal inclusion, R^+/H^+ = hyphal inclusion only, and R^+/H^+ = root and hyphal exclusion. Error bars are ± 1 S.E.M.

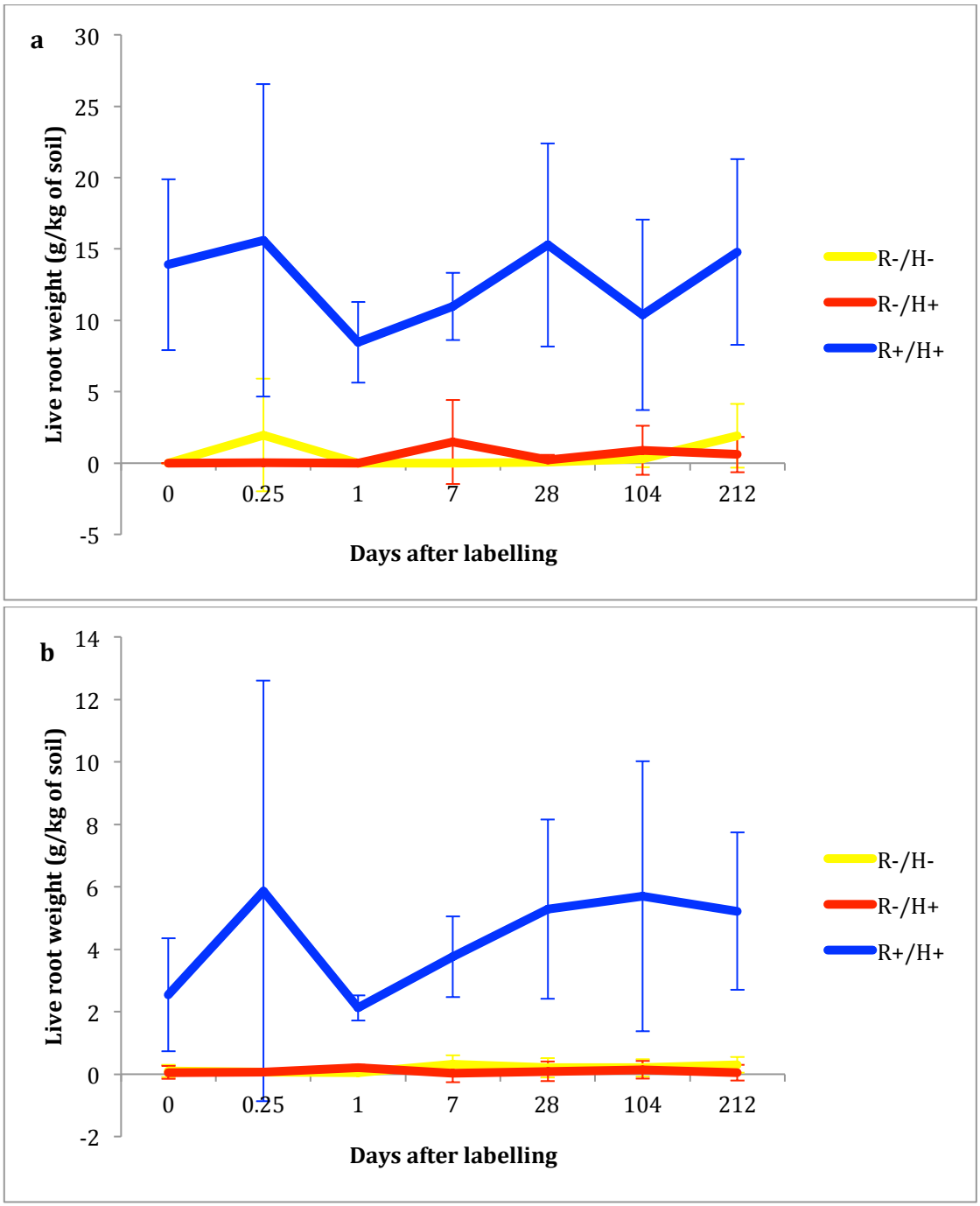

Figure 4.8 Root density under each treatment type during the labelling period in a.) Willow b.) *Miscanthus*. R^+/H^+ = roots and hyphal inclusion, R^+/H^+ = hyphal inclusion only, and R^+/H^+ = root and hyphal exclusion. Error bars are ± 1 S.E.M.

4.4.3 Gas samples from pulse labelling

$\partial^{13}C$ values of soil respiration significantly increased under all treatments in the willow (P<0.001), reaching a maximum of 120 ‰ 48 hours after labelling, before declining to pre-pulse levels between 7 and 14 days after pulsing in all treatments (Fig4.10a). However no significant differences in the $\partial^{13}C$ values of soil effluxes were detected between any treatment types in the willow (P=0.109). The ^{13}C flux elevated above natural abundances, referred to as the excess ^{13}C flux, also significantly increased after labelling in the willow (P<0.001), peaking 48 hours after labelling (Fig4.11a) to a maximum of 11.7 μg CO_2-C m^{-2} h^{-1} in the H^+/R^+ treatment, which was significantly higher (P<0.041) than all other treatment types (maxima of 5.8 μg CO_2-C m^{-2} h^{-1} μg and 7.4 CO_2-C m^{-2} hr^{-1} in the R^-/H^+ and R^-/H^- respectively). However, excess ^{13}C enrichment of the soil flux also returned to basal levels in all treatments between 7 and 14 days after labelling in the willow.

$\partial^{13}C$ values of *Miscanthus* soil respiration significantly increased after labelling in all treatments (P<0.001), peaking between 24 and 36 hours post-pulse (Fig4.10b), however no significant differences between treatments were detected (P=0.153). Excess ^{13}C soil fluxes were also calculated for the *Miscanthus*, which also demonstrated significant enrichment after labelling (P<0.001), peaking at around 2 μg CO_2-C m^{-2} h^{-1} in all treatments between 24 and 36 hours after labelling (Fig4.11b), with no significant differences between treatments (P=0.669). Both the excess ^{13}C soil flux and overall ^{13}C enrichment of soil effluxes returned to pre-pulse levels within 7 days after labelling in all cores in the *Miscanthus*.

Both crops demonstrated rapid movement of label belowground, with excess ^{13}C detectable in soil effluxes just 6 hours after labelling. However, peak enrichment of soil effluxes occurred between 24 and 36 hours after labelling in the *Miscanthus*, and at 48 hours in the willow. Additionally, no excess ^{13}C was detectable from the *Miscanthus* soil effluxes 7 days after labelling, whilst label remained detectable in the willow for between 7 and 14 days post-pulse. Both the $\partial^{13}C$ values and excess

^{13}C of soil respiration were however significantly larger in the willow compared to the *Miscanthus* ($P<0.001$ and $P<0.001$ respectively).

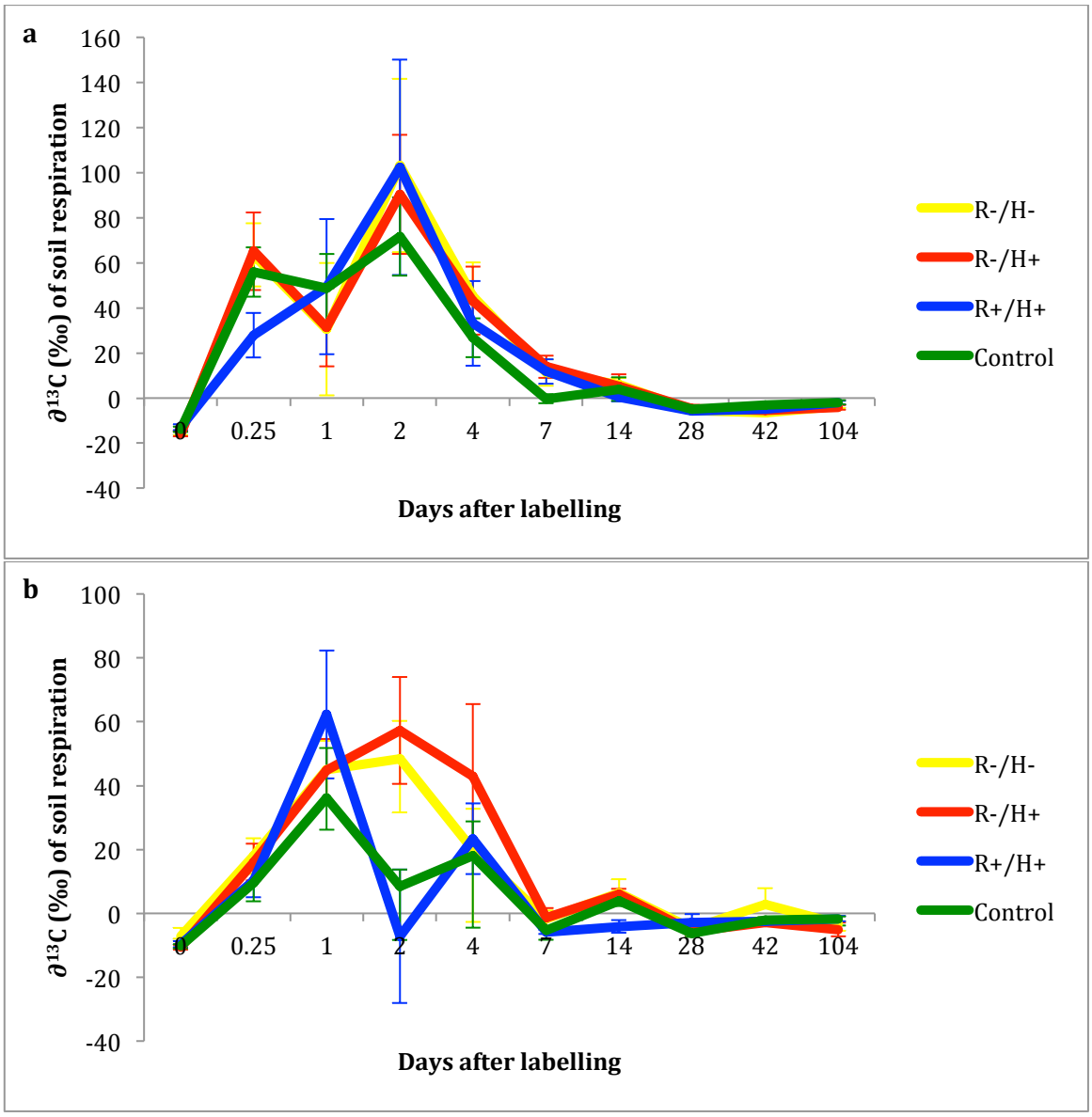

Figure 4.9 $\partial^{13}C$ Enrichment of soil effluxes (in ‰) during the labelling period a) willow and b) *Miscanthus*. R^+/H^+ = roots and hyphal inclusion, R^+/H^+ = hyphal inclusion only, and R^+/H^+ = root, hyphal exclusion and PP = pre pulse. Error bars are ± 1 S.E.M.

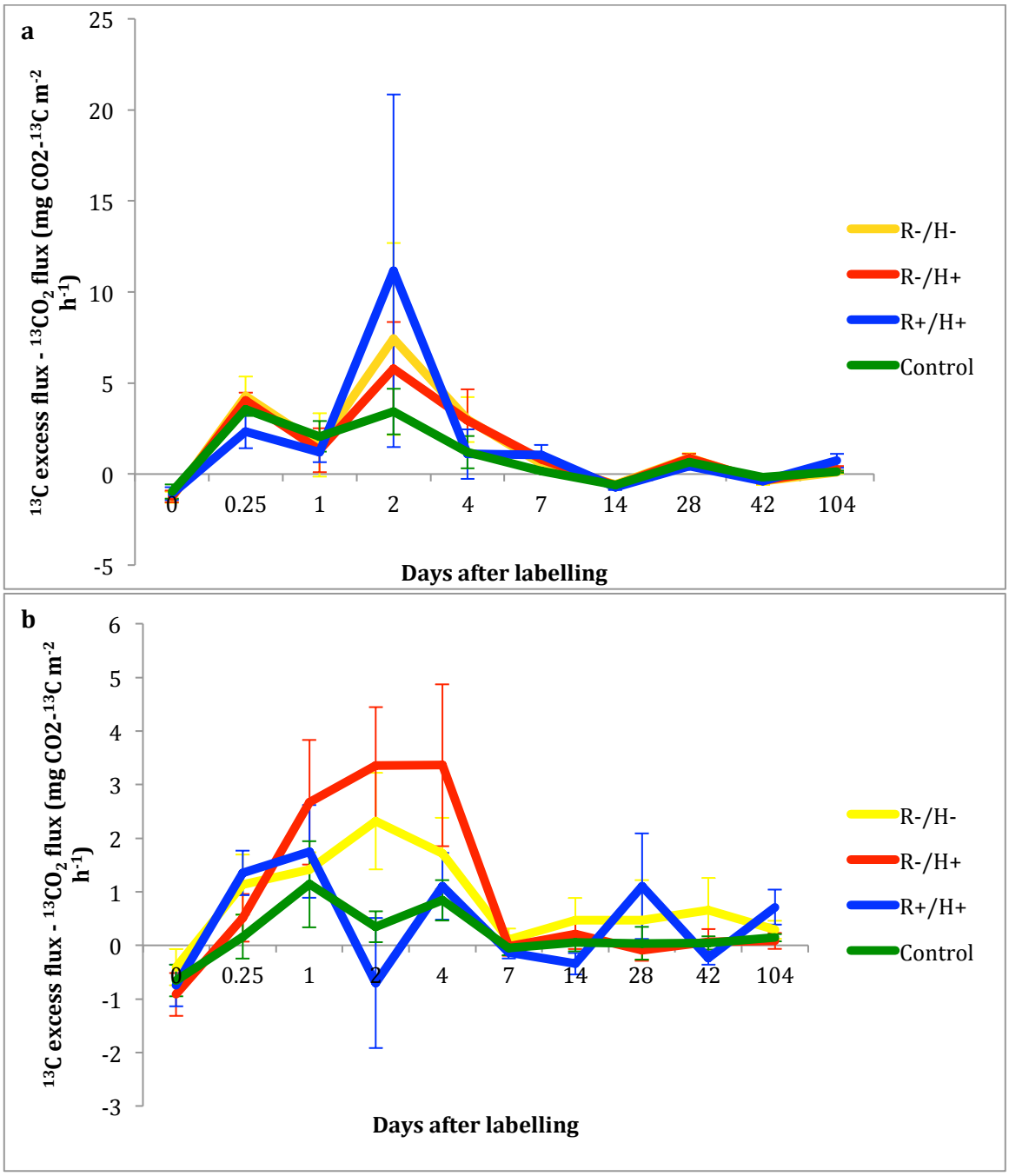

Figure 4.10 Excess ^{13}C soil fluxes for each treatment time during the labelling period a) willow and b.) *Miscanthus*. R^+/H^+ = roots and hyphal inclusion, R^+/H^+ = hyphal inclusion only, R^+/H^+ = root and hyphal exclusion and PP = pre pulse. Error bars are ± 1 S.E.M.

4.4.4 Bulk soil and root samples

The $\partial^{13}C$ values were calculated for the bulk soil of each treatment type in both crops. The willow bulk was significantly more enriched in the R^+/H^+ treatment compared to the other treatment types (Fig4.11a; P=0.037), rising from 26.8 ‰ to -23.5 ‰ in the R^+/H^+ treatment, which peaked 1 month after labelling (P<0.001). The *Miscanthus* bulk soil ^{13}C enrichment however did not differ between treatment types or vary over time (Fig4.11b; P=0.431 and P=0.678 respectively).

Willow roots taken from the R^+/H^+ cores were substantially ^{13}C enriched after labelling (Fig4.11c), with $\partial^{13}C$ values significantly increasing by 29.4 ‰ at its peak 1 month after labelling (P=0.002). Roots remained 10 ‰ more enriched than pre-pulse 7 months after labelling. *Miscanthus* root $\partial^{13}C$ values significantly increased by 34.0 ‰ at its peak 1 week after labelling (P=0.022), and remained 17.3 ‰ more enriched than pre-pulse 7 months after labelling. *Miscanthus* roots were also significantly more enriched than the corresponding willow roots (P<0.001).

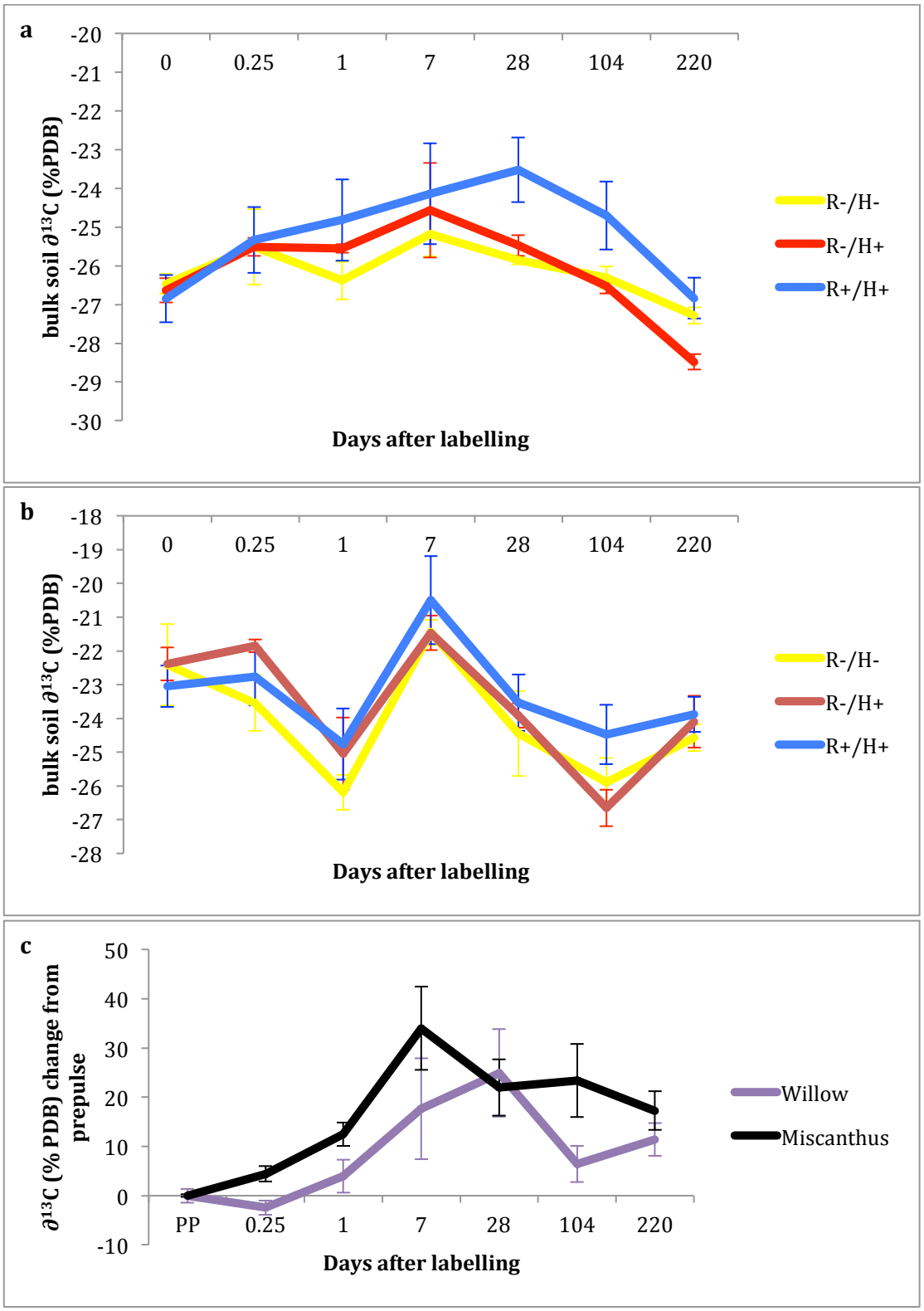

Figure 4.11 Enrichment (in $\partial^{13}C$) for each treatment during the labelling period in a) willow bulk soil, b) *Miscanthus* bulk soil c) Roots from both willow and *Miscanthus*. PP = pre pulse. Error bars are ± 1 S.E.M.

4.4.5 Phospholipid Fatty-Acid analysis of bulk soil and roots

Phospholipid Fatty-Acid analysis was performed on roots and bulk soil from the R^+/H^+ cores to characterize the transfer of label through the microbial community. Additionally, the R^-/H^+ and R^-/H^- were extracted from the pre-pulse sampling to investigate fungal colonization rates. Unfortunately, the quality of data produced from gas chromatography was poor and no samples underwent I-RMS. Of the 96 samples that were extracted, only 48 samples were used for gas chromatography, of which 44 were from soil samples and 4 were roots. Of the samples analysed, only 19 were of high enough quality to be used in community analysis (Fig4.11a), which were all soil extracts. 7 soil extracts had very large contaminant peaks at very low retention times (Fig4.11b) which increased baseline noise to an extent that peaks could no longer be reliably interpreted. A further 18 soil samples and all root samples had low diversity and low peak intensity making further analysis impossible (Fig4.11c, Fig 4.11d).

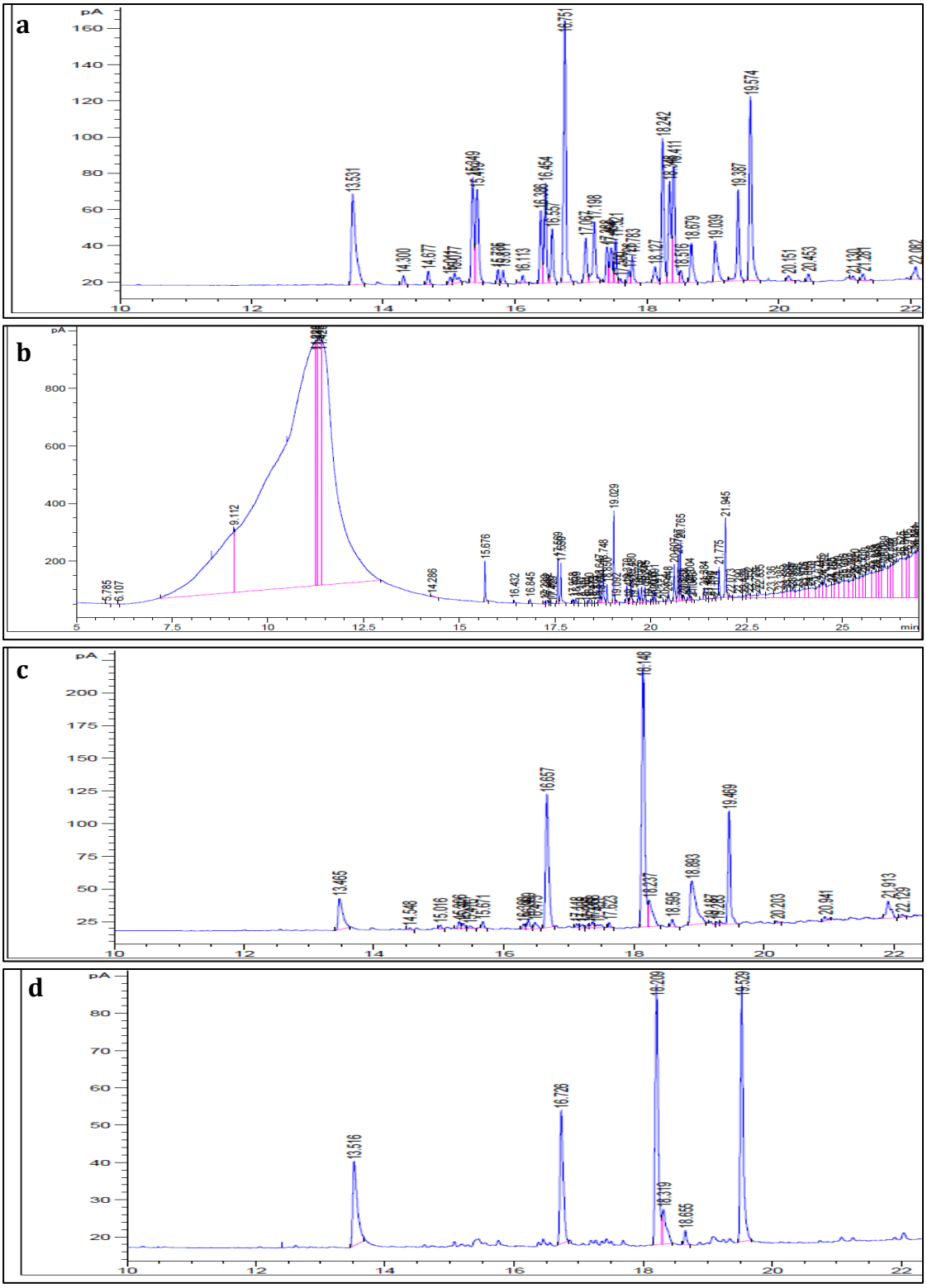

Figure 4.12 PLFA analysis from roots and soil. a.) A successful extraction from willow bulk soil b.) Sample with large contaminant in bulk *Miscanthus* soil c.) Low peak concentrations from willow bulk soil d.) Low peak numbers in samples extracted from roots.

4.5 Discussion

In this work, the flow of recently derived photo-assimilates was shown to significantly contribute to C pools within the bulk soil of SRC willow, but not in *Miscanthus*. Previous work in Maize revealed that around 20 % of all fixed carbon underwent rhizodeposition into the soil and between 2-5 % was retained in soil for at least a single growing season (Hütsch, Augustin, and Merbach 2002). Whilst significant quantities of fresh assimilates were found belowground in both the willow and *Miscanthus*, its distribution differed between crops. *Miscanthus* roots were more enriched than willow per unit mass, whilst also retaining a greater proportion of the label throughout the 7-month post-pulse monitoring period. Soil effluxes from willow treatments were considerably more enriched with ^{13}C than *Miscanthus*, suggesting greater use of recently derived photo-assimilates by willow roots in driving cellular processes. The long-term enrichment of *Miscanthus* roots coupled with the lack of enrichment of bulk soil is in agreement with previous studies of rhizomatous plants, that fresh-assimilates are stored long-term as carbohydrates in the belowground biomass (Chaudhary et al. 2012) and have low rates of rhizodeposition (Wiesenberg et al. 2012).

Despite the significant movement of recently derived photo-assimilates belowground in both crops, there was no evidence of ecologically significant fluxes through fungal pathways. However both AM and ECM have been shown to receive significant amounts of C from their host plants and contribute to soil effluxes (Jakobsen and Rosendahl 1990; Leake et al. 2001; Högberg and Högberg 2002; Moyano, Kutsch, and Schulze 2007; Nottingham et al. 2010). AM and ECM hyphae have been shown to utilize 0.003μg C m^{-1} h^{-1} and 40 μg m^{-1} h^{-1} respectively (Ettema, Lowrance, and Coleman 1999; Heinemeyer et al. 2006), therefore given the abundance of mycorrhizal fungi within the rhizospheres of both *Miscanthus* (Burns et al, unpublished) and willow (Chapter 2 and 3), it would be expected that mycorrhizal fungi are substantial sinks of recently derived photo-assimilates within these crops (Högberg and Högberg 2002; Heinemeyer et al. 2006). Additionally, the

bulk soil enrichment also did not differ between R^-/H^+ and R^-/H^- treatments in either crop, despite significant proportions of soil C been shown to be fungal derived in other systems (Rillig et al. 2003) and the overall effects of mycorrhizal fungal colonization on total SOC remains less clear (Durall, Jones, and Tinker 1994; Neergaard, Porter, and Gorissen 2002; Johnson et al. 2002; Staddon 2003; Godbold et al. 2006; Högberg et al. 2010).

There are a number of potential reasons for there being no difference in ^{13}C enrichment of soil effluxes and bulk soil between R^-/H^+ and R^-/H^- cores. The failure of mycorrhizal hyphae to colonize R^-/H^+ cores is the most obvious explanation. However selectively permeable membranes have been used in a range of laboratory and field studies studying grasses and trees, although none in commercial bioenergy plantations, and particularly on this experimental scale (Godbold et al. 2006; Heinemeyer et al. 2006; Moyano, Kutsch, and Schulze 2007; Nottingham et al. 2010). Previous experiments investigating hyphal fluxes using root exclusion cores have generally been between 16-18 mm in diameter, notably smaller than the 80 mm diameter cores used within this work (Johnson, Leake, and Read 2001; Lukac, Calfapietra, and Godbold 2003; Godbold et al. 2006). However increased fluxes associated with AM hyphal colonization have also been demonstrated in cores of up to 160 mm in diameter (Nottingham et al. 2010). ECM have also been characterized by their colonization/dissemination rates of plants over time, with long-distance exploration types hypothesized to be the most likely ECM species to colonize available space, and these were common within SRC willow (Bergemann and Miller, 2002). Given the core size and composition of mycorrhizal fungi present within the willow, hyphal colonization of cores would be expected. PLFA was performed to quantify fungal biomass with the R^-/H^+ and R^-/H^- treatments, which was to be used as an indicator of successful hyphal colonization. However, as PLFA was unsuccessful, there was no quantification of fungal biomass within cores, thus it remains unclear whether hyphae colonized the R^-/H^+ treatments or whether the movement of fresh photo-assimilates through fungal pathways is very low. Repeating experiments with smaller diameter cores would however reduce the

average distance between the core interior and the external environment, increasing the probability of successful hyphal colonization.

The SOC increases under the willow R^+/H^+ treatment demonstrate root effects on C movement and storage. However there were no differences in total respiration between treatment types and limited differences in the ^{13}C enrichment of soil effluxes, despite root respiration shown to substantially contribute to overall soil respiration in a range of ecosystems (Ewel, Cropper.Jr., and Gholz 1987; Bloom, Sukrapanna, and Warner 1992; Rochette, Flanagan, and Gregorich 1999). Root densities and total soil respiration measurements from the R^+/H^+ cores were however comparable to previous *in situ* studies for willow and *Miscanthus* (Rytter 2001; Hendrick and Pregitzer 1996; Pacaldo, Volk, and Briggs 2011). Investigations into the root dynamics of these bioenergy crops revealed that only 64 % of willow roots reside in the upper 20 cm of the soil profile, while in *Miscanthus* only 28 % of roots are found in the upper 30 cm of soil (Rytter and Hansson 1996; Neukirchen et al. 1999). Therefore a significant proportion of total soil respiration and ^{13}C found in effluxes from cores may actually originate from roots deep within the soil profile, which passes through the soil and enters into sampling chambers. This would confound respiration values from the cores, explaining the lack of treatment effects on both isotopic and non-isotopic respiration measurements associated with root and hyphal colonization. However the upward movement of transient gases would not affect SOC content, thus would not explain the limited differences in bulk soil ^{13}C enrichment between treatment cores.

Understanding SOC accumulation beneath bioenergy crops is essential for assessing their long-term potential for mitigating atmospheric CO_2. In this work, the flow of recently derived photo-assimilates from willow roots was shown to significantly contribute to SOC pools, but this was not the case in *Miscanthus*. Relative to willow, *Miscanthus* roots were shown to have a greater proportion of fresh assimilates per unit mass of root, and these assimilates also had a greater residence time within the *Miscanthus* roots. Willow roots demonstrated higher rates of release of photo-assimilates into the soil or respiration associated with root cells, than *Miscanthus.*

Additionally, despite substantial quantities of photo-assimilates being redirected to the roots, fungal symbionts did not appear to receive ecologically significantly quantities of C in either crop. However, due to the pioneering scale of this work, some uncertainty remains regarding these results. Finally, whilst the use of isotopic analyses allowed for subtle changes in enrichment of SOC to be detected, further pulse labelling experiments with stable isotope probing of lipids, DNA and RNA will allow for powerful investigation into the biological fate of photo-assimilates in soils, including the role of mycorrhizal fungi.

CHAPTER V: GENERAL DISCUSSION

5.1 General Overview of Findings

This work shows that fungi inhabiting the willow rhizosphere represent a diverse community, with ectomycorrhizal fungi (ECM) comprising the dominant component (by relative abundance). Whilst the community was affected by environmental and temporal parameters, for the first time this study shows a simultaneous seasonal-dependent geographical distance effect. However, in spite of mycorrhizal fungi forming substantial components of the rhizosphere of the willow, and potentially also the *Miscanthus,* bioenergy cropping systems, no significant fluxes of recently derived photo-assimilates were associated with these fungal pathways.

When shifts in the composition of the rhizosphere fungi were investigated at the community level using both pyrosequencing and TRFLP analyses, edaphic properties were linked to variance in rhizosphere fungal composition, with pH a nearly constitutively important factor. This is consistent with an abundance of studies across soil microbial groups (Buée et al. 2009; Griffiths et al. 2011; Hazard et al. 2013). However performing multiple sampling time points also increased the number of environmental parameters that were linked to variation in the rhizosphere fungal community (Table 2.8). Whilst nitrogen (in the form of NH_4 and NO_3) was found to be one of the most important parameters in the summer samples, K and P availability were also shown to have limited effects on community formation throughout the year. However a pronounced distance effect was also found, but only within the October transects, which further adds to the growing number of studies that suggest the Baas-Becking statement does not hold true for soil fungi (Green et al. 2004; van der Gast et al. 2011; Kivlin, Hawkes, and Treseder 2011; Horn et al. 2014).

Through the work performed in Chapters 2 and 3, seasonal and longer-term variation was found within the rhizosphere fungal community, and these studies can

be linked to provide a model of how the community changed over an extended period of time (Fig5.1). In Chapter 2, seasonal assemblages were shown to cycle throughout the year. Seasonal assemblages were compositionally similar between years, but varied throughout the year, as did the factors that regulated their assembly. However, Chapter 3 demonstrated that large transitions also occurred in time, potentially driven by extreme weather events such as heavy rainfall. This transition occurred across seasonal assemblages, consequently producing a new, compositionally distinct, set of seasonal assemblages. A key question that remains unanswered is the persistence of these large transitions. They were shown to persist throughout one year, however it is unknown if they eventually return to their previous assemblages. Most research into the effects of disturbance events on soil microbial communities *in situ* has been performed when the community is subjected to a constant change in selection pressure such as fertilization or the addition of heavy metals (Drijber et al. 2000; Müller et al. 2002; Marschner, Crowley, and Yang 2004; van der Gast et al. 2011). However events such as the heavy rainfall suspected of causing the compositional shifts in Chapter 3 confer only temporary effects on the community and to date, no study has investigated the role of environmental extremes in determining community assembly of soil microbes over multiple years.

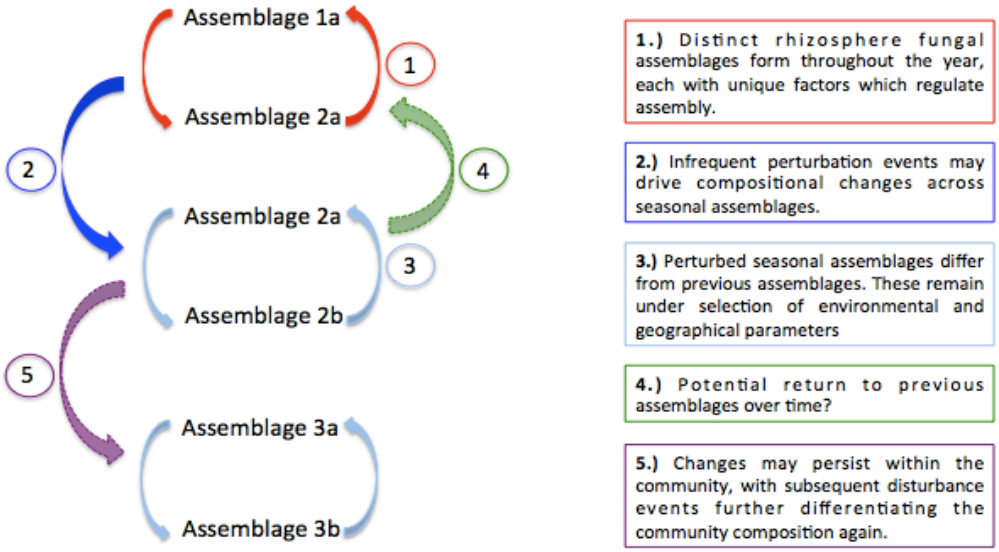

Figure 5.1 The transition between rhizosphere fungal assemblages with time.

In this work, the community was characterized with a clone library in Chapter 2 (Fig2.1) and pyrosequencing in Chapter 3 (Fig3.1). Both techniques demonstrated that the rhizosphere fungal community of SRC willow was dominated by ECM phylotypes by relative abundance (Table 3.1). Specifically, *Sebacina* and *Cortinarius* phylotypes were abundantly assigned in both databases. However, the *Pulvinula constellatio* phylotype, which was most abundant in the clone library, was extremely rarely assigned in the pyrosequencing datasets (<0.1% average relative abundance). The discrepancies between the two sequencing techniques most likely arose through the use of separate reference databases. Despite the limited number of sequences produced, the significant overlap in the taxa identified from the clone library and pyrosequencing suggests that clone libraries provide a useful 'snapshot' into abundant taxa within the community. Previous investigations into the ECM fungal communities of monodominant temperate woodlands using sporocarps have also found that *Trichisporales*, *Cortinarius* and *Russula* species were also common symbionts within temperate woodlands; with the agreement of molecular and morphological techniques providing further confidence in the results (Bruns 1995; van der Heijden, Vries, and Kuyper 2000; Trowbridge and Jumpponen 2004).

Rhizosphere fungal diversity was assessed using TRFLP and pyrosequencing. Pyrosequencing produced significantly higher estimates of taxa richness compared to TRFLP (paired t-test, $P<0.001$; Table 5.1). This is unsurprising, as TRFLP is based on the electrophoresis of amplicons that have undergone restriction digests. A significant proportion of these amplicons may not have the restriction sites for the restriction enzymes used in analysis, and furthermore amplicons of the same length will not be differentiated, and both these drawbacks of TRFLP will reduce the diversity estimates produced. However taxa richness did not differ between transects in either the TRFLP or the pyrosequencing, and no obvious skews were found in taxa richness between the two techniques, suggesting that TRFLP remains a good estimate of microbial diversity despite these limitations.

Table 5.1 Taxa richness generated from TRFLP and OTU richness generated from pyrosequencing, averaged across each transect.

Season	TRFLP	Pyrosequencing
Oct-10	48.97 ± 3.67	98.29 ± 6.83
Jul-11	50.82 ± 3.64	93.93 ± 12.75
Aug-11	52.13 ± 5.70	-*
Oct-11	45.11 ± 1.82	102.7 ± 7.92
Jul-12	-*	101.81 ± 4.38
Oct-12	-*	114.43 ± 10.38
Average	49.26 ± 2.70	102.23 ± 8.45

Values are ± 1 S.E.M. *No measurement taken

This work has also shown that ECM fungi dominate the SRC willow, whilst arbuscular mycorrhizal (AM) fungi have been shown to be present in the rhizosphere of *Miscanthus* (Fig2.1 and Fig3.8; and Burns et al, unpublished). The belowground utilization of recently derived photo-assimilates was also shown to markedly differ between the willow and *Miscanthus* crops, however the flow through both fungal pathways was negligible (Fig4.7, Fig4.9-11, Fig5.2). There are a number of experimental reasons that may explain the limited differences associated with roots and hyphal exclusion in the isotopic enrichment of soil effluxes and bulk soils (Högberg et al. 2001; Johnson et al. 2002; Heinemeyer et al. 2006). Mycorrhizal fungi have been shown to receive substantial quantities of C in a range of different systems and given the uncertainties regarding the results obtained in Chapter 4, the importance of mycorrhizal fungi within the belowground C cycles of the *Miscanthus* and willow crops is likely to be greater than the results within this work suggest. Additionally, the composition of mycorrhizal fungi will impact upon the total quantity of C received from the host, which varies between both mycorrhizal type and species (Högberg et al. 2001; Johnson et al. 2002; Kiers et al. 2011). Whilst seasonal differences have been shown in the turnover of soil C beneath various tree species (Epron et al. 2011), the marked seasonal variation within the rhizosphere fungal community shown within Chapter 2 may also significantly contribute to these differences in total soil flux and turnover time of fresh photo-assimilates.

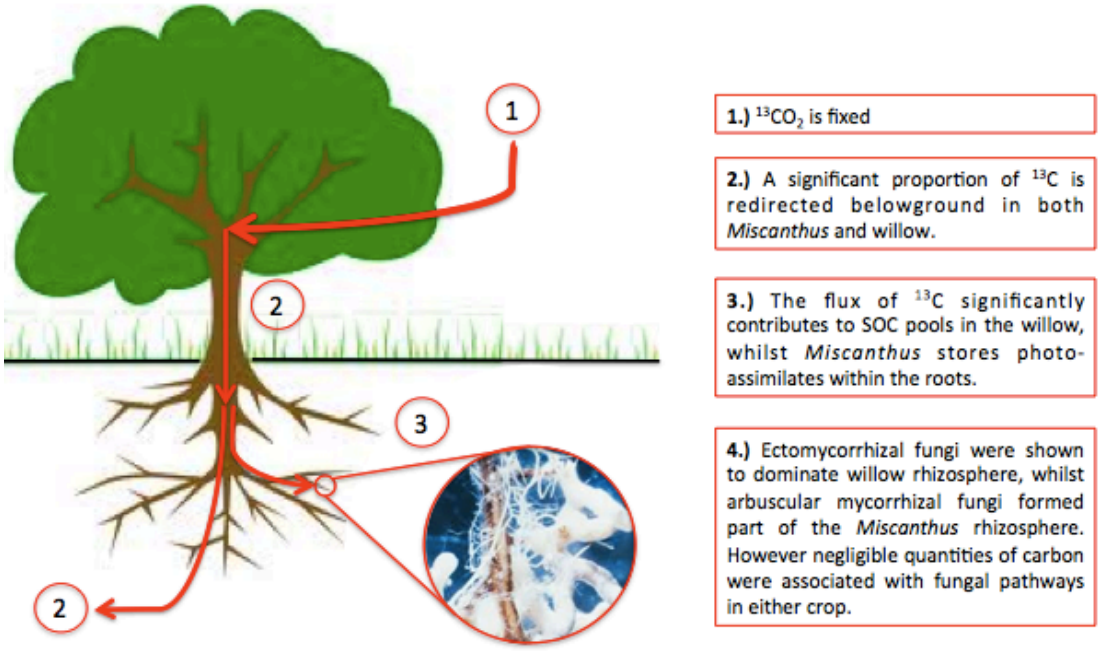

Figure 5.2 Transfer of $^{13}CO_2$ label through the *Miscanthus* and willow belowground.

5.2 Broad Implications of work

5.2.1 Improving abilities to characterize microbial communities

Technological advances have assisted in the understanding of microbial communities. Pyrosequencing provides useful taxonomic information that TRFLP does not, negating the need to perform expensive and time consuming additional analyses such as clone libraries in parallel with community fingerprinting techniques. This work has shown that alpha diversity remained unchanged across all time points. However important compositional changes occurred between time points in datasets established from both TRFLP and 454-pyrosequencing analyses. The lack of taxonomic information provided by TRFLP clearly limits the potential of these results. Sequencing techniques greatly enhance the biological interpretation of community profiling, which in this work allowed for important fluctuations specifically within the mycorrhizal communities to be followed. Whilst methods

such as DGGE and TRFLP provide useful insights into community level change within microbial populations, future studies would significantly benefit from the transition from these techniques to next generation sequencing.

5.2.2 Furthering the understanding of rhizosphere fungal community assembly

Both the rhizosphere fungal community composition and the factors that regulate assembly varied seasonally. There was considerable variation in the rhizosphere community linked to seasonality, in addition to geographical and edaphic parameters. Thus the environment needs to be comprehensively characterised over time in order to capture all the variation in both community composition and factors that regulate assembly. Additionally, many metadata parameters are intimately linked and will subsequently autocorrelate; therefore an in-depth understanding of the interactions within environmental parameters is also needed to fully understand community assembly. Finally, given the relatively rare but substantial transitions in the rhizosphere fungal communities that occur, infrequent but long-term monitoring of rhizosphere communities would assist in identifying the mechanisms behind large community changes.

5.2.3 Linking rhizosphere fungal assemblages to functioning at an ecosystem level

The increasing ability to identify and quantify large numbers of fungi that resides within the rhizosphere provides an excellent opportunity to start to link rhizosphere functioning to composition, and potentially understanding how changes compositionally changes may effect biogeochemical cycles and aboveground biomass. However, this is exceptionally complex, as many organisms are poorly characterised taxonomically and functionally. Whilst a complete understanding of structure-function relationship of rhizosphere fungi maybe some way off, future experiments investigating changing abundances between fungal groups such as

mycorrhizal, phytopathogenic and saprophytic fungi and the effects of these changes on nutrient cycles or aboveground biomass may yield insightful observations about the importance of the rhizosphere fungi within ecosystems.

5.3 Future direction of study

This work has emphasized a range of further questions for future research to answer regarding rhizosphere fungal community formation and its subsequent function in belowground carbon cycling within bioenergy cropping systems. Future studies need to go beyond simply describing communities within a location at single time points, but analyze changes in communities against the variety of external parameters such as edaphic, temporal, climatic and biotic factors that may regulate community assemblage. Additionally, further studies are required to assess the structure-function relationship of rhizosphere fungal communities within belowground C cycling of bioenergy cropping systems. There are many important unanswered questions within these areas, including:

5.3.1 Are geographical distance effects on the rhizosphere fungal communities seasonal in other ecosystems?

In our study, we provide further support that distance effects do occur in microbial communities. However, geographical effects were seen predominantly after the peak growing season. Given that a number of studies have shown that both the composition of rhizosphere fungi and the factors that regulate assembly vary throughout the year, the relative importance of geographical distance effects may also vary throughout the year in other ecosystems. Whilst distance effects have been found in many studies, others such as An et al. (2008) and Hazard et al. (2013) found limited effects in AM fungi. However, both investigations used single sampling time points, early in the growing season. It is therefore possible that distance effects do occur within these communities and that the lack of a temporal aspect to

sampling limits the discovery of these. It is only by investigating the effect of geographical separation on the compositions of the rhizosphere fungal communities over multiple time points in a range of ecosystems, and potentially revisiting site of previous these previous studies, can this question be answered definitively.

5.3.2 What is the extent of local geographic effects on rhizosphere fungal communities compared to those at the regional and global scale?

Whilst a limited number of studies of spatial scaling in microbial communities have analysed across local, regional and continental geographic scales in ascomycete and AM fungi, these have also been subject to considerable variation in geological, topological and aboveground biomass characteristics, adding considerable complexity to analyses (Green et al. 2004; Kivlin, Hawkes, and Treseder 2011). Given the extensive community variation and geographical distance effects found at the small scale, comprehensive characterization of these communities needs to be performed before distance effects at larger scales can be fully understood. Monoculture bioenergy crops provide useful insights into rhizosphere fungi, as they remain uniform in aboveground biomass, whilst limited fertilizer application limits the extremes in P and K present in other managed ecosystems.

5.3.3 Do large environmental extremes dramatically shift rhizosphere fungal community composition? Do they have a long-term effect on fungal assemblages?

In this work, there was a dramatic shift in rhizosphere composition in 2012, which was most likely due to extremely heavy rainfall during the early growing season, however this link could not be definitively confirmed. Whilst extremes in edaphic properties such as pH and P have been shown to have disproportionately large effects on the community composition, these apply constant selection pressure on the rhizosphere. Environmental events such as ground frosts, droughts and heavy

rainfall can exhibit strong but relatively short-term stresses on ecosystems. A limited number of studies have shown weather events such as heavy rainfall affect mycorrhizal hyphal colonization and spore counts (Merryweather and Fitter 1998; Zhao et al. 2001). However little is known about what may drive large transitions in the rhizosphere fungal communities, or whether they permanently change, or return (fully or partially) to their previous assemblages. Further studies are needed in order to assess the frequency, mechanisms and persistence of these large community changes, with observations within environmental surveys tested within controlled environments to further isolate and confirm the 'true' determinants and effects of these community transitions.

5.3.4 Investigating changing taxonomic compositions of rhizosphere fungal assemblages

Much research has focused on community level shifts or has focused on the interactions between small numbers of rhizosphere fungal groups. However understanding the taxonomic shifts behind community level transitions in the rhizosphere is also of importance. Studies of entire rhizosphere fungal communities remain in their infancy, with relatively recent next generation sequencing techniques able to elucidate the key taxal changes behind overall changes in rhizosphere communities. These could provide useful biological inferences about the health and function of the rhizosphere, whilst used to highlight potentially fruitful interactions to be investigated with laboratory experiments.

5.3.5 Does the flow of recently derived photosynthates through root fungal pathways significantly contribute to SOC pools in bioenergy crops?

Arguably a key function of rhizosphere fungi is in belowground C cycling. Whilst the emphasis of many studies has been on plant and root inputs into soil organic carbon

(SOC), or simply overall SOC changes over time, considerably less is known about rhizosphere fungal input. Glomalin and other fungal exudates have very long residence time in soil, with Rillig et al. (2003) predicting that these contribute to long-term soil carbon pools. Whilst studies have found significant quantities of recently derived photo-assimilates do pass through mycorrhizal pathways, few have investigated their role in SOC accumulation, with those that have producing conflicting results (Johnson et al. 2002; Neergaard, Porter, and Gorissen 2002; Staddon 2003). Further experiments in both managed and natural lands are required to clarify and quantify the role of rhizosphere pathways in belowground carbon storage, whilst their role in bioenergy crops is of particular current interest.

VI: REFERENCES

Abuzinadah RA, Finlay RD, Read DJ. (1986). The role of proteins in the nitrogen nutrition of ectomycorrhizal plants. *New Phytol* **103**:495–506.

Agerer R. (2001). Exploration types of ectomycorrhizae. *Mycorrhiza* **11**:107–114.

Alexander IJ, Högberg P. (1986). Ectomycorrhizas of tropical angiospermous trees. *New Phytol* **102**:541–549.

Allen EB, Rincon E, Allen MF, Pérez-Jimenez A, Huante P. (1998). Disturbance and seasonal dynamics of mycorrhizae in a tropical deciduous forest in Mexico. *Biotropica* **30**:261–274.

Allmér J, Vasiliauskas R, Ihrmark K, Stenlid J, Dahlberg A. (2006). Wood-inhabiting fungal communities in woody debris of Norway spruce (*Picea abies* (L.) *Karst.*), as reflected by sporocarps, mycelial isolations and T-RFLP identification. *FEMS Microbiol Ecol* **55**:57–67.

Alonso AP, Vigeolas H, Raymond P, Rolin D, Dieuaide-Noubhani M. (2005). A new substrate cycle in plants. evidence for a high glucose-phosphate-to-glucose turnover from *in vivo* steady-state and pulse-labeling experiments with [^{13}C]glucose and [^{14}C]glucose. *Plant Physiol* **138**:2220–2232.

Altschul S. (1990). Basic local alignment search tool. *J Mol Biol* **215**:403–410.

Anderson IC, Campbell CD, Prosser JI. (2003). Potential bias of fungal 18S rDNA and internal transcribed spacer polymerase chain reaction primers for estimating fungal biodiversity in soil. *Environ Microbiol* **5**:36–47.

Anderson IC, Genney DR, Alexander IJ. (2014). Fine-scale diversity and distribution of ectomycorrhizal fungal mycelium in a Scots pine forest. *New Phytol* **201**:1423–1430.

Anderson RC, Liberta AE, Dickman LA. (1984). Interaction of vascular plants and vesicular-arbuscular mycorrhizal fungi across a soil moisture-nutrient gradient. *Oecologia* **64**:111–117.

Anderson-Teixeira KJ, Masters MD, Black CK, Zeri M, Hussain MZ, Bernacchi CJ, *et al.* (2013). Altered belowground carbon cycling following land-use change to perennial bioenergy crops. *Ecosystems* **16**:508–520.

An G-H, Miyakawa S, Kawahara A, Osaki M, Ezawa T. (2008). Community structure of arbuscular mycorrhizal fungi associated with pioneer grass species *Miscanthus sinensis* in acid sulfate soils: Habitat segregation along pH gradients. *Soil Sci Plant Nutr* **54**:517–528.

Archer D, Pierrehumbert R. (2013). The warming papers: The Scientific Foundation for the climate change forecast. John Wiley & Sons.

Armstrong RD, Millar G, Halpin NV, Reid DJ, Standley J. (2003). Using zero tillage, fertilisers and legume rotations to maintain productivity and soil fertility in opportunity cropping systems on a shallow vertosol. *Aust J Exp Agric* **43**:141.

Arnebrant K. (1994). Nitrogen amendments reduce the growth of extramatrical ectomycorrhizal mycelium. *Mycorrhiza* **5**:7–15.

Arnold AE, Mejía LC, Kyllo D, Rojas EI, Maynard Z, Robbins N, *et al.* (2003). Fungal endophytes limit pathogen damage in a tropical tree. *Proc Natl Acad Sci* **100**:15649–15654.

Asif M, Muneer T. (2007). Energy supply, its demand and security issues for developed and emerging economies. *Renew Sustain Energy Rev* **11**:1388–1413.

Baas-Becking LGM. (1934). Geobiologie, of inleiding tot de milieukunde: Met literatuurlijst en ind. Van Stockum.

Bago B, Azcón-Aguilar C, Goulet A, Piché Y. (1998). Branched absorbing structures (BAS): a feature of the extraradical mycelium of symbiotic arbuscular mycorrhizal fungi. *New Phytol* **139**:375–388.

Bastide PY de la, Piché Y, Kropp BR. (1995). Vegetative interactions among mycelia of *Laccaria bicolor* in pure culture and in symbiosis with *Pinus banksiana*. *Can J Bot* **73**:1768–1779.

Bearden BN, Petersen L. (2000). Influence of arbuscular mycorrhizal fungi on soil structure and aggregate stability of a vertisol. *Plant Soil* **218**:173–183.

Bell T. (2010). Experimental tests of the bacterial distance–decay relationship. *ISME J* **4**:1357–1365.

Belnap J, Hawkes CV, Firestone MK. (2003). Boundaries in miniature: two examples from soil. *BioScience* **53**:739–749.

Berbee ML, Taylor JW. (1993). Dating the evolutionary radiations of the true fungi. *Can J Bot* **71**:1114–1127.

Bergemann SE, Miller SL. (2002). Size, distribution, and persistence of genets in local populations of the late-stage ectomycorrhizal basidiomycete, *Russula brevipes*. *New Phytol* **156**:313–320.

Berg G, Smalla K. (2009). Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. *FEMS Microbiol Ecol* **68**:1–13.

Bever JD, Schultz PA, Pringle A, Morton JB. (2001). Arbuscular mycorrhizal fungi: More diverse than meets the eye, and the ecological tale of why the high diversity of ecologically distinct species of arbuscular mycorrhizal fungi within a single community has broad implications for plant ecology. *Bioscience* **51**:923–931.

Biasi C, Pitkämäki AS, Tavi NM, Koponen HT, Martikainen PJ. (2012). An isotope approach based on ^{13}C pulse-chase labelling vs. the root trenching method to separate heterotrophic and autotrophic respiration in cultivated peatlands. *Boreal Environ Res* **17**:184–192.

Blaalid R, Carlsen T, Kumar S, Halvorsen R, Ugland KI, Fontana G, *et al.* (2012). Changes in the root-associated fungal communities along a primary succession gradient analysed by 454 pyrosequencing: Primary succession of root-associated fungi. *Mol Ecol* **21**:1897–1908.

Blanco-Canqui H, Lal R. (2007). Soil and crop response to harvesting corn residues for biofuel production. *Geoderma* **141**:355–362.

Blasius D, Feil W, Kottke I, Oberwinkler F. (1986). Hartig net structure and formation in fully ensheathed ectomycorrhizas. *Nord J Bot* **6**:837–842.

Bloom AJ, Sukrapanna SS, Warner RL. (1992). Root respiration associated with ammonium and nitrate absorption and assimilation by Barley. *Plant Physiol* **99**:1294–1301.

Bohrer KE, Friese CF, Amon JP. (2004). Seasonal dynamics of arbuscular mycorrhizal fungi in differing wetland habitats. *Mycorrhiza* **14**:329–337.

Bolton H Jr, Fredrickson JK, Elliott L. (1992). Microbial ecology of the rhizosphere. pp27–63.

Bolwerk A, Lagopodi AL, Wijfjes AH, Lamers GE, Chin-A-Woeng TF, Lugtenberg BJ, *et al.* (2003). Interactions in the tomato rhizosphere of two *Pseudomonas* biocontrol strains with the phytopathogenic fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *Mol Plant Microbe Interact* **16**:983–993.

Borcard D, Legendre P, Avois-Jacquet C, Tuomisto H. (2004). Dissecting the spatial structure of ecological data at multiple scales. *Ecology* **85**:1826–1832.

Bowden RD, Nadelhoffer KJ, Boone RD, Melillo JM, Garrison JB. (1993). Contributions of aboveground litter, belowground litter, and root respiration to total soil respiration in a temperate mixed hardwood forest. *Can J For Res* **23**:1402–1407.

Braak C ter, Šmilauer P. (2002). CANOCO reference manual and CanoDraw for Windows user's guide: software for canonical community ordination (version 4.5). *Sect Permut Methods Microcomput Power Ithaca N Y*.

Bragg L, Stone G, Imelfort M, Hugenholtz P, Tyson GW. (2012). Fast, accurate error-correction of amplicon pyrosequences using Acacia. *Nat Methods* **9**:425–426.

Bray JR, Curtis JT. (1957). An ordination of the upland forest communities of Southern Wisconsin. *Ecol Monogr* **27**:325–349.

Bremner JM, Keeney DR. (1965). Steam distillation methods for determination of ammonium, nitrate and nitrite. *Anal Chim Acta* **32**:485–495.

Brodribb TJ, Feild TS, Jordan GJ. (2007). Leaf maximum photosynthetic rate and venation are linked by hydraulics. *Plant Physiol* **144**:1890–1898.

Bruns TD. (1995). Thoughts on the processes that maintain local species diversity of ectomycorrhizal fungi. *The Significance and Regulation of Soil Biodiversity*, Collins, HP, Robertson, GP, & Klug, MJ (eds) Developments in Plant and Soil Sciences, Springer Netherlands, pp. 63–73.

Buée M, Boer WD, Martin F, Overbeek L van, Jurkevitch E. (2009). The rhizosphere zoo: An overview of plant-associated communities of microorganisms, including phages, bacteria, archaea, and fungi, and of some of their structuring factors. *Plant Soil* **321**:189–212.

Buée M, Reich M, Murat C, Morin E, Nilsson RH, Uroz S, *et al.* (2009). 454 Pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. *New Phytol* **184**:449–456.

Buée M, Vairelles D, Garbaye J. (2005). Year-round monitoring of diversity and potential metabolic activity of the ectomycorrhizal community in a beech (*Fagus silvatica*) forest subjected to two thinning regimes. *Mycorrhiza* **15**:235–245.

Burgess TI, Wingfield MJ, Wingfield BD. (2004). Global distribution of *Diplodia pinea* genotypes revealed using simple sequence repeat (SSR) markers. *Australas Plant Pathol* **33**:513–519.

Cairney JW, Meharg AA. (2002). Interactions between ectomycorrhizal fungi and soil saprotrophs: implications for decomposition of organic matter in soils and degradation of organic pollutants in the rhizosphere. *Can J Bot* **80**:803–809.

Cameron DD, Johnson I, Read DJ, Leake JR. (2008). Giving and receiving: measuring the carbon cost of mycorrhizas in the green orchid, *Goodyera repens*. *New Phytol* **180**:176–184.

Campbell JE, Lobell DB, Genova RC, Field CB. (2008). The global potential of bioenergy on abandoned agriculture lands. *Environ Sci Technol* **42**:5791–5794.

Cannell MGR. (2003). Carbon sequestration and biomass energy offset: theoretical, potential and achievable capacities globally, in Europe and the UK. *Biomass Bioenergy* **24**:97–116.

Cannon PF, Simmons CM. (2002). Diversity and host preference of leaf endophytic fungi in the Iwokrama Forest Reserve, Guyana. *Mycologia* **94**:210–220.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, *et al.* (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* **7**:335–336.

Cardon ZG. (1995). Influence of rhizodeposition under elevated CO_2 on plant nutrition and soil organic matter. *Plant Soil* **187**:277–288.

Cardon ZG, Whitbeck JL. (2011). The rhizosphere: An ecological perspective. Academic Press, UK. pp57-71.

Case SDC, McNamara NP, Reay DS, Whitaker J. (2012). The effect of biochar addition on N_2O and CO_2 emissions from a sandy loam soil – The role of soil aeration. *Soil Biol Biochem* **51**:125–134.

Chambers JQ, Tribuzy ES, Toledo LC, Crispim BF, Higuchi N, dos Santos J, *et al.* (2004). Respiration from a tropical forest ecosystem: partitioning of sources and low carbon use efficiency. *Ecol Appl* **14**:72–88.

Chao A. (1987). Estimating the population size for capture-recapture data with unequal catchability. *Biometrics* **43**:783–791.

Chaudhary DR, Saxena J, Lorenz N, Dick RP. (2012). Distribution of recently fixed photosynthate in a switchgrass plant-soil system. *Plant Soil Environ-UZEI Czech Repub*.

Chen Y, Dumont MG, McNamara NP, Chamberlain PM, Bodrossy L, Stralis-Pavese N, *et al.* (2008). Diversity of the active methanotrophic community in acidic peatlands as assessed by mRNA and SIP-PLFA analyses. *Environ Microbiol* **10**:446–459.

Clements FE. (1936). Nature and structure of the climax. *J Ecol* **24**:252.

Clifton-Brown JC, Breuer J, Jones MB. (2007). Carbon mitigation by the energy crop, *Miscanthus*. *Glob Change Biol* **13**:2296–2307.

Clifton-Brown JC, Neilson B, Lewandowski I, Jones MB. (2000). The modelled productivity of *Miscanthus* × *giganteus* in Ireland. *Ind Crops Prod* **12**:97–109.

Clifton-brown JC, Stampfl PF, Jones MB. (2004). *Miscanthus* biomass production for energy in Europe and its potential contribution to decreasing fossil fuel carbon emissions. *Glob Change Biol* **10**:509–518.

Coleman MD, Bledsoe CS, Lopushinsky W. (1989). Pure culture response of ectomycorrhizal fungi to imposed water stress. *Can J Bot* **67**:29–39.

Colgan III W, Carey AB, Trappe JM, Molina R, Thysell D. (1999). Diversity and productivity of hypogeous fungal sporocarps in a variably thinned Douglas-fir forest. *Can J For Res* **29**:1259–1268.

Cordier C, Pozo MJ, Barea JM, Gianinazzi S, Gianinazzi-Pearson V. (1998). Cell defense responses associated with localized and systemic resistance to *Phytophthora parasitica* induced in tomato by an arbuscular mycorrhizal fungus. *Mol Plant Microbe Interact* **11**:1017–1028.

Corner A, Venables D, Spence A, Poortinga W, Demski C, Pidgeon N. (2011). Nuclear power, climate change and energy security: Exploring British public attitudes. *Energy Policy* **39**:4823–4833.

Coughlan AP, Dalpé Y, Lapointe L, Piché Y. (2000). Soil pH-induced changes in root colonization, diversity, and reproduction of symbiotic arbuscular mycorrhizal fungi from healthy and declining maple forests. *Can J For Res* **30**:1543–1554.

Courtemanch DL. (1996). Commentary on the subsampling procedures used for rapid bioassessments. *J North Am Benthol Soc* **15**:381.

Courty P-E, Franc A, Pierrat J-C, Garbaye J. (2008). Temporal changes in the ectomycorrhizal community in two soil horizons of a temperate oak forest. *Appl Environ Microbiol* **74**:5792–5801.

Cowles HC. (1899). The ecological relations of the vegetation on the sand dunes of Lake Michigan. University of Chicago.

Crow SE, Lajtha K, Filley TR, Swanston CW, Bowden RD, Caldwell BA. (2009). Sources of plant-derived carbon and stability of organic matter in soil: implications for global change. *Glob Change Biol* **15**:2003–2019.

Cullings KW. (1996). Single phylogenetic origin of ericoid mycorrhizae within the Ericaceae. *Can J Bot* **74**:1896–1909.

Daniell TJ, Husband R, Fitter AH, Young JPW. (2001). Molecular diversity of arbuscular mycorrhizal fungi colonising arable crops. *FEMS Microbiol Ecol* **36**:203–209.

Dannoura M, Maillard P, Fresneau C, Plain C, Berveiller D, Gerant D, *et al.* (2011). *In situ* assessment of the velocity of carbon transfer by tracing ^{13}C in trunk CO_2 efflux after pulse labelling: variations among tree species and seasons. *New Phytol* **190**:181–192.

Deacon J. (1992). Interactions of ectomycorrhizal fungi. *Mycorrhizal Funct Integr Plant-Fungal Process*, Chapman and Hall, NY, USA. pp 1249.

Heijden MGA van der, Bardgett RD, Straalen NM van. (2008). The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecol Lett* **11**:296–310.

Derrien D, Marol C, Balesdent J. (2004). The dynamics of neutral sugars in the rhizosphere of wheat. An approach by ^{13}C pulse-labelling and GC/C/IRMS. *Plant Soil* **267**:243–253.

De Deyn GB, Putten WH. van der (2005). Linking aboveground and belowground diversity. *Trends Ecol Evol* **20**:625–633.

Dickie IA, Xu B, Koide RT. (2002). Vertical niche differentiation of ectomycorrhizal hyphae in soil as shown by T-RFLP analysis. *New Phytol* **156**:527–535.

Dincer I. (2000). Renewable energy and sustainable development: a crucial review. *Renew Sustain Energy Rev* **4**:157–175.

Domisch T, Finér L, Lehto T. (2002). Growth, carbohydrate and nutrient allocation of Scots pine seedlings after exposure to simulated low soil temperature in spring. *Plant Soil* **246**:75–86.

Drijber RA, Doran JW, Parkhurst AM, Lyon DJ. (2000). Changes in soil microbial community structure with tillage under long-term wheat-fallow management. *Soil Biol Biochem* **32**:1419–1430.

Duddridge JA. (1986). The development and ultrastructure of ectomycorrhizas. *New Phytol* **103**:457–464.

Dumbrell AJ, Ashton PD, Aziz N, Feng G, Nelson M, Dytham C, *et al.* (2011). Distinct seasonal assemblages of arbuscular mycorrhizal fungi revealed by massively parallel pyrosequencing. *New Phytol* **190**:794–804.

Dumbrell AJ, Nelson M, Helgason T, Dytham C, Fitter AH. (2009). Relative roles of niche and neutral processes in structuring a soil microbial community. *ISME J* **4**:337–345.

Dungait JAJ, Hopkins DW, Gregory AS, Whitmore AP. (2012). Soil organic matter turnover is governed by accessibility not recalcitrance. *Glob Change Biol* **18**:1781–1796.

Durall DM, Jones MD, Tinker PB. (1994). Allocation of ^{14}C-carbon in ectomycorrhizal willow. *New Phytol* **128**:109–114.

Edgar RC. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**:2460–2461.

Epron D, Ngao J, Dannoura M, Bakker MR, Zeller B, Bazot S, *et al.* (2011). Seasonal variations of belowground carbon transfer assessed by *in situ* pulse labelling of trees. *Biogeosciences* **8**:1153–1168.

Erbilgin N, Raffa KF. (2002). Association of declining red pine stands with reduced populations of bark beetle predators, seasonal increases in root colonizing insects, and incidence of root pathogens. *For Ecol Manag* **164**:221–236.

Escudero V, Mendoza R. (2005). Seasonal variation of arbuscular mycorrhizal fungi in temperate grasslands along a wide hydrologic gradient. *Mycorrhiza* **15**:291–299.

Espinoza JG, Briceño EX, Keith LM, Latorre BA. (2008). Canker and twig dieback of blueberry caused by *Pestalotiopsis* spp. and a *Truncatella* sp. in Chile. *Plant Dis* **92**:1407–1414.

Ettema CH, Lowrance R, Coleman DC. (1999). Riparian soil response to surface nitrogen input: temporal changes in denitrification, labile and microbial C and N pools, and bacterial and fungal respiration. *Soil Biol Biochem* **31**:1609–1624.

Ewel KC, Cropper. WP, Gholz HL. (1987). Soil CO_2 evolution in Florida slash pine plantations: Importance of root respiration. *Can J For Res* **17**:330–333.

Fitzsimons MS, Miller RM, Jastrow JD. (2008). Scale-dependent niche axes of arbuscular mycorrhizal fungi. *Oecologia* **158**:117–127.

Fornara DA, Tilman D. (2008). Plant functional composition influences rates of soil carbon and nitrogen accumulation. *J Ecol* **96**:314–322.

Fowler J, Cohen L, Jarvis P. (1998). Practical statistics for field biology, John Wiley & Sons Ltd: Chichester, UK.

Franzluebbers AJ, Stuedemann JA, Schomberg HH. (2000). Spatial distribution of soil carbon and nitrogen pools under grazed tall fescue. *Soil Sci Soc Am J* **64**:635.

Frostegård Å, Tunlid A, Bååth E. (2011). Use and misuse of PLFA measurements in soils. *Soil Biol Biochem* **43**:1621–1625.

Fujiyoshi M, Yoshitake S, Watanabe K, Murota K, Tsuchiya Y, Uchida M, *et al.* (2011). Successional changes in ectomycorrhizal fungi associated with the polar willow *Salix polaris* in a deglaciated area in the High Arctic, Svalbard. *Polar Biol* **34**:667–673.

Del Galdo I, Six J, Peressotti A, Francesca Cotrufo M. (2003). Assessing the impact of land-use change on soil C sequestration in agricultural soils by means of organic matter fractionation and stable C isotopes. *Glob Change Biol* **9**:1204–1213.

Gallardo A, Schlesinger WH. (1994). Factors limiting microbial biomass in the mineral soil and forest floor of a warm-temperate forest. *Soil Biol Biochem* **26**:1409–1415.

Gamnitzer U, Schäufele R, Schnyder H. (2009). Observing ^{13}C labelling kinetics in CO_2 respired by a temperate grassland ecosystem. *New Phytol* **184**:376–386.

Gardes M, Bruns TD. (1996). Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: above- and below-ground views. *Can J Bot* **74**:1572–1583.

Gardes M, Bruns TD. (1993). ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. *Mol Ecol* **2**:113–118.

Gast CJ van der, Gosling P, Tiwari B, Bending GD. (2011). Spatial scaling of arbuscular mycorrhizal fungal diversity is affected by farming practice. *Environ Microbiol* **13**:241–249.

Gehring CA, Theimer TC, Whitham TG, Keim P. (1998). Ectomycorrhizal fungal community structure of pinyon pines growing in two environmental extremes. *Ecology* **79**:1562–1572.

Gleason HA. (1926). The individualistic concept of the plant association. *Bull Torrey Bot Club* **53**:7–26.

Godbold DL, Hoosbeek MR, Lukac M, Cotrufo MF, Janssens IA, Ceulemans R, *et al.* (2006). Mycorrhizal hyphal turnover as a dominant process for carbon input into soil organic matter. *Plant Soil* **281**:15–24.

Gosling P, Mead A, Proctor M, Hammond JP, Bending GD. (2013). Contrasting arbuscular mycorrhizal communities colonizing different host plants show a similar response to a soil phosphorus concentration gradient. *New Phytol* **198**:546–556.

Grandy AS, Robertson GP. (2007). Land-use intensity effects on soil organic carbon accumulation rates and mechanisms. *Ecosystems* **10**:59–74.

Green JL, Holmes AJ, Westoby M, Oliver I, Briscoe D, Dangerfield M, *et al.* (2004). Spatial scaling of microbial eukaryote diversity. *Nature* **432**:747–750.

Griffiths RI, Manefield M, Ostle N, McNamara N, O'Donnell AG, Bailey MJ, *et al.* (2004). $^{13}CO_2$ pulse labelling of plants in tandem with stable isotope probing: methodological considerations for examining microbial function in the rhizosphere. *J Microbiol Methods* **58**:119–129.

Griffiths RI, Thomson BC, James P, Bell T, Bailey M, Whiteley AS. (2011). The bacterial biogeography of British soils. *Environ Microbiol* **13**:1642–1654.
Grogan P, Matthews R. (2002). A modelling analysis of the potential for soil carbon sequestration under short rotation coppice willow bioenergy plantations. *Soil Use Manag* **18**:175–183.
Guo LB, Gifford RM. (2002). Soil carbon stocks and land use change: a meta analysis. *Glob Change Biol* **8**:345–360.
Hacskaylo E, Palmer JG, Vozzo JA. (1965). Effect of temperature on growth and respiration of ectotrophic mycorrhizal fungi. *Mycologia* **57**:748.
Hammond GP, Kallu S, McManus MC. (2008). Development of biofuels for the UK automotive market. *Appl Energy* **85**:506–515.
Hartley W, Dickinson NM, Riby P, Lepp NW. (2009). Arsenic mobility in brownfield soils amended with green waste compost or biochar and planted with *Miscanthus*. *Environ Pollut* **157**:2654–2662.
Hart MM, Reader RJ, Klironomos JN. (2001). Life-history strategies of arbuscular mycorrhizal fungi in relation to their successional dynamics. *Mycologia* **93**:1186.
Hazard C, Gosling P, van der Gast CJ, Mitchell DT, Doohan FM, Bending GD. (2013). The role of local environment and geographical distance in determining community composition of arbuscular mycorrhizal fungi at the landscape scale. *ISME J* **7**:498–508.
Heemsbergen DA, Berg MP, Loreau M, Hal JR van, Faber JH, Verhoef HA. (2004). Biodiversity effects on soil processes explained by interspecific functional dissimilarity. *Science* **306**:1019–1020.
Heijden E van der, Vries F de, Kuyper TW. (2000). Mycorrhizal associations of *Salix repens* L. communities in succession of dune ecosystems. I. Above-ground and below-ground views of ectomycorrhizal fungi in relation to soil chemistry. *Can J Bot* **77**:1821–1832.
Heinemeyer A, Ineson P, Ostle N, Fitter AH. (2006). Respiration of the external mycelium in the arbuscular mycorrhizal symbiosis shows strong dependence on recent photosynthates and acclimation to temperature. *New Phytol* **171**:159–170.
Helgason T, Fitter AH, Young JPW. (1999). Molecular diversity of arbuscular mycorrhizal fungi colonising *Hyacinthoides non-scripta* (bluebell) in a seminatural woodland. *Mol Ecol* **8**:659–666.
Hendrick RL, Pregitzer KS. (1996). Temporal and depth-related patterns of fine root dynamics in northern hardwood forests. *J Ecol* **84**:167–176.
Hendriksen A, and Selmer-Olsen AR. (1970) Automatic methods for determining nitrate and nitrite in water and soil extracts - Analyst, RSC Publishing, Ca, USA pp. 514-518
Hijri I, Sýkorová Z, Oehl F, Ineichen K, Mäder P, Wiemken A, *et al.* (2006). Communities of arbuscular mycorrhizal fungi in arable soils are not necessarily low in diversity: Mycorrhizal communities in arable soils. *Mol Ecol* **15**:2277–2289.

Hill J, Nelson E, Tilman D, Polasky S, Tiffany D. (2006). Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. *Proc Natl Acad Sci* **103**:11206–11210.

Hobbie EA. (2006). Carbon allocation to ectomycorrhizal fungi correlates with belowgroound allocation in culture studies. *Ecology* **87**:563–569.

Hodge A, Campbell CD, Fitter AH. (2001). An arbuscular mycorrhizal fungus accelerates decomposition and acquires nitrogen directly from organic material. *Nature* **413**:297–299.

Högberg MN, Briones MJI, Keel SG, Metcalfe DB, Campbell C, Midwood AJ, *et al.* (2010). Quantification of effects of season and nitrogen supply on tree below-ground carbon transfer to ectomycorrhizal fungi and other soil organisms in a boreal pine forest. *New Phytol* **187**:485–493.

Högberg MN, Högberg P. (2002). Extramatrical ectomycorrhizal mycelium contributes one-third of microbial biomass and produces, together with associated roots, half the dissolved organic carbon in a forest soil. *New Phytol* **154**:791–795.

Högberg P, Högberg MN, Göttlicher SG, Betson NR, Keel SG, Metcalfe DB, *et al.* (2008). High temporal resolution tracing of photosynthate carbon from the tree canopy to forest soil microorganisms. *New Phytol* **177**:220-228

Högberg P, Högberg MN, Quist ME, Ekblad A, Näsholm T. (1999). Nitrogen isotope fractionation during nitrogen uptake by ectomycorrhizal and non-mycorrhizal *Pinus sylvestris*. *New Phytol* **142**:569–576.

Högberg P, Nordgren A, Buchmann N, Taylor AFS, Ekblad A, Högberg MN, *et al.* (2001). Large-scale forest girdling shows that current photosynthesis drives soil respiration. *Nature* **411**:789–792.

Horner-Devine MC, Lage M, Hughes JB, Bohannan BJM. (2004). A taxa–area relationship for bacteria. *Nature* **432**:750–753.

Horn S, Caruso T, Verbruggen E, Rillig MC, Hempel S. (2014). Arbuscular mycorrhizal fungal communities are phylogenetically clustered at small scales. *ISME J*. **8**:2231-2242

Horton TR, Bruns TD. (2001). The molecular revolution in ectomycorrhizal ecology: peeking into the black-box. *Mol Ecol* **10**:1855–1871.

Hungate BA, Jackson RB, Field CB. (1995). Detecting changes in soil carbon in CO_2 enrichment experiments. *Plant Soil* **187**:135–145.

Husband R, Herre EA, Turner SL, Gallery R, Young JPW. (2002). Molecular diversity of arbuscular mycorrhizal fungi and patterns of host association over time and space in a tropical forest. *Mol Ecol* **11**:2669–2678.

Husband R, Herre EA, Young JPW. (2002). Temporal variation in the arbuscular mycorrhizal communities colonising seedlings in a tropical forest. *FEMS Microbiol Ecol* **42**:131–136.

Huse SM, Huber JA, Morrison HG, Sogin ML, Welch DM. (2007). Accuracy and quality of massively parallel DNA pyrosequencing. *Genome Biol* **8**:R143.

Hutchison LJ. (1990). Studies on the systematics of ectomycorrhizal fungi in axenic culture: The enzymatic degradation of selected carbon and nitrogen compounds. *Can J Bot* **68**:1522–1530.

Hütsch BW, Augustin J, Merbach W. (2002). Plant rhizodeposition — an important source for carbon turnover in soils. *J Plant Nutr Soil Sci* **165**:397–407.

Hyde K, Soytong K. (2008). The fungal endophyte dilemma. *Fungal Divers* **33**:163–173.

Ignacio Rangel-Castro J, Prosser JI, Scrimgeour CM, Smith P, Ostle N, Ineson P, *et al.* (2004). Carbon flow in an upland grassland: effect of liming on the flux of recently photosynthesized carbon to rhizosphere soil. *Glob Change Biol* **10**:2100–2108.

Jakobsen I, Rosendahl L. (1990). Carbon flow into soil and external hyphae from roots of mycorrhizal cucumber plants. *New Phytol* **115**:77–83.

Janssens IA, Sampson DA, Curiel-Yuste J, Carrara A, Ceulemans R. (2002). The carbon cost of fine root turnover in a Scots pine forest. *For Ecol Manag* **168**:231–240.

Jany J-L, Martin F, Garbaye J. (2003). Respiration activity of ectomycorrhizas from *Cenococcum geophilum* and *Lactarius* sp. in relation to soil water potential in five beech forests. *Plant Soil* **255**:487–494.

Jensen A, Jakobsen I. (1980). The occrrence of vesicular-arbuscular mycorrhiza in barley and wheat grown in some Danish soils with different fertilizer treatments. *Plant Soil* **55**:403–414.

Jobbágy EG, Jackson RB. (2000). The vertical distribution of soil organic carbon and its relation to climate and vegetation. *Ecol Appl* **10**:423–436.

Johnson D, Leake JR, Ostle N, Ineson P, Read DJ. (2002). *In situ* $^{13}CO_2$ pulse-labelling of upland grassland demonstrates a rapid pathway of carbon flux from arbuscular mycorrhizal mycelia to the soil. *New Phytol* **153**:327–334.

Johnson D, Leake JR, Read DJ. (2001). Novel in-growth core system enables functional studies of grassland mycorrhizal mycelial networks. *New Phytol* **152**:555–562.

Jones MD, Durall DM, Tinker PB. (1990). Phosphorus relationships and production of extrametrical hyphae by two types of willow ectomycorrhizas at different soil phosphorus levels. *New Phytol* **115**:259–267.

Jonsson T, Kokalj S, Finlay R, Erland S. (1999). Ectomycorrhizal community structure in a limed spruce forest. *Mycol Res* **103**:501–508.

Jørgensen H, Kristensen JB, Felby C. (2007). Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. *Biofuels Bioprod Biorefining* **1**:119–134.

Jumpponen A, Jones KL, David Mattox J, Yaege C. (2010). Massively parallel 454-sequencing of fungal communities in *Quercus* spp. ectomycorrhizas indicates seasonal dynamics in urban and rural sites. *Mol Ecol* **19**:41–53.

Kahiluoto H, Ketoja E, Vestberg M, Saarela I. (2001). Promotion of AM utilization through reduced P fertilization. *Plant Soil* **231**:65–79.

Kahle P, Beuch S, Boelcke B, Leinweber P, Schulten H-R. (2001). Cropping of *Miscanthus* in Central Europe: biomass production and influence on nutrients and soil organic matter. *Eur J Agron* **15**:171–184.

Kavvadias VA, Alifragis D, Tsiontsis A, Brofas G, Stamatelos G. (2001). Litterfall, litter accumulation and litter decomposition rates in four forest ecosystems in northern Greece. *For Ecol Manag* **144**:113–127.

Keizer PJ, Arnolds E. (1994). Succession of ectomycorrhizal fungi in roadside verges planted with common oak (*Quercus robur* L.) in Drenthe, The Netherlands. *Mycorrhiza* **4**:147–159.

Keller G. (1996). Utilization of inorganic and organic nitrogen sources by high-subalpine ectomycorrhizal fungi of *Pinus cembra* in pure culture. *Mycol Res* **100**:989–998.

Kiers ET, Duhamel M, Beesetty Y, Mensah JA, Franken O, Verbruggen E, *et al.* (2011). Reciprocal rewards stabilize cooperation in the mycorrhizal symbiosis. *Science* **333**:880–882.

Kinden DA, Brown MF. (1975). Electron microscopy of vesicular-arbuscular mycorrhizae of yellow poplar: Intracellular hyphae and vesicles. *Can J Microbiol* **21**:1768–1780.

Kirschbaum MUF. (1995). The temperature dependence of soil organic matter decomposition, and the effect of global warming on soil organic C storage. *Soil Biol Biochem* **27**:753–760.

Kivlin SN, Hawkes CV, Treseder KK. (2011). Global diversity and distribution of arbuscular mycorrhizal fungi. *Soil Biol Biochem* **43**:2294–2303.

Koch AM, Croll D, Sanders IR. (2006). Genetic variability in a population of arbuscular mycorrhizal fungi causes variation in plant growth. *Ecol Lett* **9**:103–110.

Koide RT, Shumway DL, Xu B, Sharda JN. (2007). On temporal partitioning of a community of ectomycorrhizal fungi. *New Phytol* **174**:420–429.

Kucey RMN. (1987). Increased phosphorus uptake by wheat and field beans inoculated with a phosphorus solubilizing *Penicillium bilaji* strain and with vesicular-arbuscular mycorrhizal fungi. *Appl Environ Microbiol* **53**:2699–2703.

Kuzyakov Y, Domanski G. (2002). Model for rhizodeposition and CO_2 efflux from planted soil and its validation by ^{14}C pulse labelling of ryegrass. *Plant Soil* **239**:87–102.

Last FT, Mason PA, Ingleby K, Fleming LV. (1984). Succession of fruitbodies of sheathing mycorrhizal fungi associated with *Betula pendula*. *For Ecol Manag* **9**:229–234.

Leake JR, Donnelly DP, Saunders EM, Boddy L, Read DJ. (2001). Rates and quantities of carbon flux to ectomycorrhizal mycelium following ^{14}C pulse labeling of *Pinus sylvestris* seedlings: effects of litter patches and interaction with a wood-decomposer fungus. *Tree Physiol* **21**:71–82.

Lee J, Lee S, Young JPW. (2008). Improved PCR primers for the detection and identification of arbuscular mycorrhizal fungi. *FEMS Microbiol Ecol* **65**:339–349.

Lehmann A, Stahr K. (2007). Nature and significance of anthropogenic urban soils. *J Soils Sediments* **7**:247–260.

Lekberg Y, Koide RT, Rohr JR, Aldrich-Wolfe L, Morton JB. (2007). Role of niche restrictions and dispersal in the composition of arbuscular mycorrhizal fungal communities. *J Ecol* **95**:95–105.

Lewandowski I, Clifton-Brown JC, Scurlock JMO, Huisman W. (2000). *Miscanthus*: European experience with a novel energy crop. *Biomass Bioenergy* **19**:209–227.

Lim YW, Kim BK, Kim C, Jung HS, Kim B-S, Lee J-H, *et al.* (2010). Assessment of soil fungal communities using pyrosequencing. *J Microbiol* **48**:284–289.

Lingfei L, Anna Y, Zhiwei Z. (2005). Seasonality of arbuscular mycorrhizal symbiosis and dark septate endophytes in a grassland site in southwest China. *FEMS Microbiol Ecol* **54**:367–373.

Liu J, Maldonado-Mendoza I, Lopez-Meyer M, Cheung F, Town CD, Harrison MJ. (2007). Arbuscular mycorrhizal symbiosis is accompanied by local and systemic alterations in gene expression and an increase in disease resistance in the shoots. *Plant J* **50**:529–544.

Lodge DJ. (1989). The influence of soil moisture and flooding on formation of VA-endo-and ectomycorrhizae in *Populus* and *Salix*. *Plant Soil* **117**:243–253.

Lovelock CE, Andersen K, Morton JB. (2003). Arbuscular mycorrhizal communities in tropical forests are affected by host tree species and environment. *Oecologia* **135**:268–279.

Lukac M, Calfapietra C, Godbold DL. (2003). Production, turnover and mycorrhizal colonization of root systems of three *Populus* species grown under elevated CO_2 (POPFACE). *Glob Change Biol* **9**:838–848.

MacArthur RH. (8). Wilson, EO (1967): The theory of island biogeography. *Monogr Popul Biol* **1**.

Mack MC, Schuur EAG, Bret-Harte MS, Shaver GR, Chapin FS. (2004). Ecosystem carbon storage in arctic tundra reduced by long-term nutrient fertilization. *Nature* **431**:440–443.

Magurran AE. (2004). Measuring biological diversity. *Afr J Aquat Sci* **29**:285–286.

Malinowski DP, Belesky DP. (2000). Adaptations of endophyte-infected cool-season grasses to environmental stresses. *Crop Sci* **40**:923.

Mandyam K, Jumpponen A. (2005). Seeking the elusive function of the root-colonising dark septate endophytic fungi. *Stud Mycol* **53**:173–189.

Manefield M, Griffiths R, McNamara NP, Sleep D, Ostle N, Whiteley A. (2007). Insights into the fate of a ^{13}C labelled phenol pulse for stable isotope probing (SIP) experiments. *J Microbiol Methods* **69**:340–344.

Marschner P, Crowley D, Yang CH. (2004). Development of specific rhizosphere bacterial communities in relation to plant species, nutrition and soil type. *Plant Soil* **261**:199–208.

Marschner P, Neumann G, Kania A, Weiskopf L, Lieberei R. (2002). Spatial and temporal dynamics of the microbial community structure in the rhizosphere of cluster roots of white lupin (*Lupinus albus* L.). *Plant Soil* **246**:167–174.

Marsh JF, Schultze M. (2001). Analysis of arbuscular mycorrhizas using symbiosis-defective plant mutants. *New Phytol* **150**:525–532.

Martiny JBH, Bohannan BJM, Brown JH, Colwell RK, Fuhrman JA, Green JL, *et al.* (2006). Microbial biogeography: putting microorganisms on the map. *Nat Rev Microbiol* **4**:102–112.

Mason P, Last F, Wilson J. (1987). Ectomycorrhizal fungi. In: Cambridge University Press, p. 253.

Matamala R, Jastrow JD, Miller RM, Garten CT. (2008). Temporal changes in C and N stocks of restored prarie: Implications for C sequestration strategies. *Ecol Appl* **18**:1470–1488.

McAfee BJ, Fortin JA. (1987). The influence of pH on the competitive interactions of ectomycorrhizal mycobionts under field conditions. *Can J For Res* **17**:859–864.

McGuire KL, Henkel TW, Cerda IG de la, Villa G, Edmund F, Andrew C. (2008). Dual mycorrhizal colonization of forest-dominating tropical trees and the mycorrhizal status of non-dominant tree and liana species. *Mycorrhiza* **18**:217–222.

McNamara N, Benham D, Sleep D, Grant H, Stott A. (2002). Development of a trace gas stable isotope capture system in a mobile laboratory for temporal and spatial sampling of field and laboratory experiments. *Rapid Commun Mass Spectrom* **16**:2165–2171.

Merryweather J, Fitter A. (1998). The arbuscular mycorrhizal fungi of *Hyacinthoides non-scripta*: Diversity of fungal taxa. *New Phytol* **138**:117–129.

Mikola J, Nieminen M, Ilmarinen K, Vestberg M. (2005). Belowground responses by AM fungi and animal trophic groups to repeated defoliation in an experimental grassland community. *Soil Biol Biochem* **37**:1630–1639.

Moore JC, McCann K, de Ruiter PC. (2005). Modeling trophic pathways, nutrient cycling, and dynamic stability in soils. *Pedobiologia* **49**:499–510.

Moyano FE, Kutsch WL, Schulze E-D. (2007). Response of mycorrhizal, rhizosphere and soil basal respiration to temperature and photosynthesis in a barley field. *Soil Biol Biochem* **39**:843–853.

Müller AK, Westergaard K, Christensen S, Sørensen SJ. (2002). The diversity and function of soil microbial communities exposed to different disturbances. *Microb Ecol* **44**:49–58.

Murty D, Kirschbaum MUF, Mcmurtrie RE, Mcgilvray H. (2002). Does conversion of forest to agricultural land change soil carbon and nitrogen? A review of the literature. *Glob Change Biol* **8**:105–123.

Neergaard A de, Gorissen A. (2004). Carbon allocation to roots, rhizodeposits and soil after pulse labelling: a comparison of white clover (*Trifolium repens* L.) and perennial ryegrass (*Lolium perenne* L.). *Biol Fertil Soils* **39**:228–234.

Neergaard A de, Porter JR, Gorissen A. (2002). Distribution of assimilated carbon in plants and rhizosphere soil of basket willow (*Salix viminalis* L.). *Plant Soil* **245**:307–314.

Neukirchen D, Himken M, Lammel J, Czypionka-Krause U, Olfs H-W. (1999). Spatial and temporal distribution of the root system and root nutrient content of an established *Miscanthus* crop. *Eur J Agron* **11**:301–309.

Newman EI. (1995). Phosphorus inputs to terrestrial ecosystems. *J Ecol* **83**:713.

Newsham KK. (2011). A meta-analysis of plant responses to dark septate root endophytes. *New Phytol* **190**:783–793.

Niklaus PA, Glöckler G, Siegwolf R, Körner C. (2001). Carbon allocation in calcareous grassland under elevated CO2: a combined ^{13}C pulse-labelling/soil physical fractionation study. *Funct Ecol* **15**:43–50.

Nilsson LO, Giesler R, Bååth E, Wallander H. (2004). Growth and biomass of mycorrhizal mycelia in coniferous forests along short natural nutrient gradients. *New Phytol* **165**:613–622.

Nottingham AT, Turner BL, Winter K, van der Heijden MGA, Tanner EVJ. (2010). Arbuscular mycorrhizal mycelial respiration in a moist tropical forest. *New Phytol* **186**:957–967.

Ohtonen R, Fritze H, Pennanen T, Jumpponen A, Trappe J. (1999). Ecosystem properties and microbial community changes in primary succession on a glacier forefront. *Oecologia* **119**:239–246.

Olsen SR. (1954). Estimation of available phosphorus in soils by extraction with sodium bicarbonate. Washington D.C.: US Dept. of Agriculture, USA

Öpik M, Moora M, Liira J, Zobel M. (2006). Composition of root-colonizing arbuscular mycorrhizal fungal communities in different ecosystems around the globe. *J Ecol* **94**:778–790.

Orwin KH, Kirschbaum MUF, St John MG, Dickie IA. (2011). Organic nutrient uptake by mycorrhizal fungi enhances ecosystem carbon storage: a model-based assessment. *Ecol Lett* **14**:493–502.

Pacaldo RS, Volk TA, Briggs RD. (2011). Carbon balance in short rotation willow (*Salix dasyclados*) biomass crop across a 20-year chronosequence as affected by continuous production and tear-out treatments. *Biomass Bioenergy Crops IV Asp Appl Biol* **112**:131–138.

Peay KG, Bruns TD, Kennedy PG, Bergemann SE, Garbelotto M. (2007). A strong species-area relationship for eukaryotic soil microbes: island size matters for ectomycorrhizal fungi. *Ecol Lett* **10**:470–480.

Peay KG, Kennedy PG, Bruns TD. (2011). Rethinking ectomycorrhizal succession: are root density and hyphal exploration types drivers of spatial and temporal zonation? *Fungal Ecol* **4**:233–240.

Peláez F, Collado J, Arenal F, Basilio A, Cabello A, Díez Matas MT, *et al.* (1998). Endophytic fungi from plants living on gypsum soils as a source of secondary metabolites with antimicrobial activity. *Mycol Res* **102**:755–761.

Perotto S, Peretto R, Faccio A, Schubert A, Bonfante P, Varma A. (1995). Ericoid mycorrhizal fungi: cellular and molecular bases of their interactions with the host plant. *Can J Bot* **73**:557–568.

Peters GM, Wiedemann S, Rowley HV, Tucker R, Feitz AJ, Schulz M. (2011). Assessing agricultural soil acidification and nutrient management in life cycle assessment. *Int J Life Cycle Assess* **16**:431–441.

Piceno YM, Lovell CR. (2000). Stability in natural bacterial communities: Plant resource allocation effects on rhizosphere diazotroph assemblage composition. *Microb Ecol* **39**:41–48.

Pickles BJ, Genney DR, Potts JM, Lennon JJ, Anderson IC, Alexander IJ. (2010). Spatial and temporal ecology of Scots pine ectomycorrhizas. *New Phytol* **186**:755–768.

Pielou EC. (1966). The measurement of diversity in different types of biological collections. *J Theor Biol* **13**:131–144.

Pinton R, Varanini Z, Nannipieri P. (2007). The rhizosphere: biochemistry and organic substances at the soil-plant interface, Second Edition. CRC Press.

Plain C, Gerant D, Maillard P, Dannoura M, Dong Y, Zeller B, *et al.* (2009). Tracing of recently assimilated carbon in respiration at high temporal resolution in the field with a tuneable diode laser absorption spectrometer after *in situ* $^{13}CO_2$ pulse labelling of 20-year-old beech trees. *Tree Physiol* **29**:1433–1445.

Queloz V, Grunig CR, Sieber TN, Holdenrieder O. (2005). Monitoring the spatial and temporal dynamics of a community of the tree-root endophyte *Phialocephala fortinii. New Phytol* **168**:651–660.

Radajewski S, Ineson P, Parekh NR, Murrell JC. (2000). Stable-isotope probing as a tool in microbial ecology. *Nature* **403**:646–649.

Raich JW, Potter CS. (1995). Global patterns of carbon dioxide emissions from soils. *Glob Biogeochem Cycles* **9**:23–36.

Ramankutty N, Foley JA. (1999). Estimating historical changes in global land cover: Croplands from 1700 to 1992. *Glob Biogeochem Cycles* **13**:997–1027.

Reininger V, Sieber TN. (2012). Mycorrhiza reduces adverse effects of dark septate endophytes (DSE) on growth of conifers. *PLoS ONE* **7**:e42865.

Richard F, Millot S, Gardes M, Selosse M-A. (2005). Diversity and specificity of ectomycorrhizal fungi retrieved from an old-growth Mediterranean forest dominated by *Quercus ilex*. *New Phytol* **166**:1011–1023.

Richert M, Saarnio S, Juutinen S, Silvola J, Augustin J, Merbach W. (2000). Distribution of assimilated carbon in the system *Phragmites* australis-waterlogged peat soil after carbon-14 pulse labelling. *Biol Fertil Soils* **32**:1–7.

Rillig MC, Ramsey PW, Morris S, Paul EA. (2003). Glomalin, an arbuscular-mycorrhizal fungal soil protein, responds to land-use change. *Plant Soil* **253**:293–299.

Rillig MC, Wright SF, Nichols KA, Schmidt WF, Torn MS. (2001). Large contribution of arbuscular mycorrhizal fungi to soil carbon pools in tropical forest soils. *Plant Soil* **233**:167–177.

Roberts P, Newsham KK, Bardgett RD, Farrar JF, Jones DL. (2009). Vegetation cover regulates the quantity, quality and temporal dynamics of dissolved organic carbon and nitrogen in Antarctic soils. *Polar Biol* **32**:999–1008.

Rochette P, Flanagan LB, Gregorich EG. (1999). Separating soil respiration into plant and soil components using analyses of the natural abundance of carbon-13. *Soil Sci Soc Am J* **63**:1207.

Rooney DC, Killham K, Bending GD, Baggs E, Weih M, Hodge A. (2009). Mycorrhizas and biomass crops: opportunities for future sustainable development. *Trends Plant Sci* **14**:542–549.

Rosendahl S. (2008). Communities, populations and individuals of arbuscular mycorrhizal fungi. *New Phytol* **178**:253–266.

Rosling A, Lindahl BD, Finlay RD. (2004). Carbon allocation to ectomycorrhizal roots and mycelium colonising different mineral substrates. *New Phytol* **162**:795–802.

Rowe RL, Street NR, Taylor G. (2009). Identifying potential environmental impacts of large-scale deployment of dedicated bioenergy crops in the UK. *Renew Sustain Energy Rev* **13**:271–290.

Ryberg M, Larsson E, Molau U. (2009). Ectomycorrhizal diversity on *Dryas octopetala* and *Salix reticulata* in an alpine cliff ecosystem. *Arct Antarct Alp Res* **41**:506–514.

Rytter R-M. (2001). Biomass production and allocation, including fine-root turnover, and annual N uptake in lysimeter-grown basket willows. *For Ecol Manag* **140**:177–192.

Rytter R-M, Hansson A-C. (1996). Seasonal amount, growth and depth distribution of fine roots in an irrigated and fertilized *Salix viminalis* L. plantation. *Biomass Bioenergy* **11**:129–137.

Saggar S, Hedley CB. (2001). Estimating seasonal and annual carbon inputs, and root decomposition rates in a temperate pasture following field ^{14}C pulse-labelling. *Plant Soil* **236**:91–103.

Sanders IR. (2004). Plant and arbuscular mycorrhizal fungal diversity – are we looking at the relevant levels of diversity and are we using the right techniques? *New Phytol* **164**:415–418.

Schreiner RP. (2005). Spatial and temporal variation of roots, arbuscular mycorrhizal fungi, and plant and soil nutrients in a mature Pinot Noir (*Vitis vinifera* L.) Vineyard in Oregon, USA. *Plant Soil* **276**:219–234.

Schüßler A, Schwarzott D, Walker C. (2001). A new fungal phylum, the Glomeromycota: phylogeny and evolution. *Mycol Res* **105**:1413–1421.

Shafiee S, Topal E. (2009). When will fossil fuel reserves be diminished? *Energy Policy* **37**:181–189.

Shelford VE. (1911). Ecological Succession: Stream fishes and the method of physiographic analysis. *Biol Bull* **21**:9–35.

Shi P, Abbott LK, Banning NC, Zhao B. (2012). Comparison of morphological and molecular genetic quantification of relative abundance of arbuscular mycorrhizal fungi within roots. *Mycorrhiza* **22**:501–513.

Sieber TN. (2007). Endophytic fungi in forest trees: are they mutualists? *Fungal Biol Rev* **21**:75–89.

Simpson HE. (1949). Measurement of diversity. *Nature* **163**:688.

Simon L, Bousquet J, Lévesque RC, Lalonde M. (1993). Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants. *Nature* **363**:67–69.

Smalla K, Wieland G, Buchner A, Zock A, Parzy J, Kaiser S, *et al.* (2001). Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: Plant-dependent enrichment and seasonal shifts revealed. *Appl Environ Microbiol* **67**:4742–4751.

Smith SE, Read DJ. (2010). Mycorrhizal symbiosis. Academic Press.

Soininen J, McDonald R, Hillebrand H. (2007). The distance-decay of similarity in ecological communities. *Ecography* **30**:3–12.

Staddon PL. (2003). The speed of soil carbon throughput in an upland grassland is increased by liming. *J Exp Bot* **54**:1461–1469.

Stewart DPC, Metherell AK. (1999). Carbon (^{13}C) uptake and allocation in pasture plants following field pulse-labelling. *Plant Soil* **210**:61–73.

Straker CJ. (1996). Ericoid mycorrhiza: ecological and host specificity. *Mycorrhiza* **6**:215–225.

Subke J-A, Vallack HW, Magnusson T, Keel SG, Metcalfe DB, Högberg P, *et al.* (2009). Short-term dynamics of abiotic and biotic soil $^{13}CO_2$ effluxes after *in situ* $^{13}CO_2$ pulse labelling of a boreal pine forest. *New Phytol* **183**:349–357.

Talhelm AF, Qadir SA, Powers MD, Bradley KL, Friend AL, Pregitzer KS. (2007). ^{13}C labeling of plant assimilates using a simple canopy-scale open air system. *Plant Soil* **296**:227–234.

Taylor G, Bunn SM. (2000). Identifying biotechnological and biological research priorities for woodfuel. In *Short Rotation Coppice and Wood Fuel Symposium: from Research to Renewable Energy*, Armstrong, AT & Claridge, J (eds), Forestry Commission.

Tchabi A, Coyne D, Hountondji F, Lawouin L, Wiemken A, Oehl F. (2008). Arbuscular mycorrhizal fungal communities in sub-Saharan Savannas of Benin, West Africa, as affected by agricultural land use intensity and ecological zone. *Mycorrhiza* **18**:181–195.

Tedersoo L, May TW, Smith ME. (2010). Ectomycorrhizal lifestyle in fungi: global diversity, distribution, and evolution of phylogenetic lineages. *Mycorrhiza* **20**:217–263.

Torti SD, Coley PD, Kursar TA. (2001). Causes and consequences of monodominance in tropical lowland forests. *Am Nat* **157**:141–153.

Treseder KK, Cross A. (2006). Global distributions of arbuscular mycorrhizal fungi. *Ecosystems* **9**:305–316.

Treseder KK, Masiello CA, Lansing JL, Allen MF. (2004). Species-specific measurements of ectomycorrhizal turnover under N-fertilization: combining isotopic and genetic approaches. *Oecologia* **138**:419–425.

Trostle R. (2010). Global agricultural supply and demand: Factors contributing to the recent increase in food commodity prices (rev. Ed.). DIANE Publishing.

Trowbridge J, Jumpponen A. (2004). Fungal colonization of shrub willow roots at the forefront of a receding glacier. *Mycorrhiza* **14**:283–293.

Tuomisto H, Ruokolainen K. (2006). Analyzing or explaining beta diversity? Understanding the targets of different methods of analysis. *Ecology* **87**:2697–2708.

Turrini A, Giovannetti M. (2012). Arbuscular mycorrhizal fungi in national parks, nature reserves and protected areas worldwide: a strategic perspective for their *in situ* conservation. *Mycorrhiza* **22**:81–97.

Upson R, Read DJ, Newsham KK. (2009). Nitrogen form influences the response of *Deschampsia antarctica* to dark septate root endophytes. *Mycorrhiza* **20**:1–11.

Varun, Bhat IK, Prakash R. (2009). LCA of renewable energy for electricity generation systems—A review. *Renew Sustain Energy Rev* **13**:1067–1073.

Vellinga EC, Wolfe BE, Pringle A. (2009). Global patterns of ectomycorrhizal introductions. *New Phytol* **181**:960–973.

Visser R de, Vianden H, Schnyder H. (1997). Kinetics and relative significance of remobilized and current C and N incorporation in leaf and root growth zones of *Lolium perenne* after defoliation: assessment by ^{13}C and ^{15}N steady-state labelling. *Plant Cell Environ* **20**:37–46.

Visser S. (1995). Ectomycorrhizal fungal succession in jack pine stands following wildfire. *New Phytol* **129**:389–401.

Waller F, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M, *et al.* (2005). The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. *Proc Natl Acad Sci U S A* **102**:13386–13391.

Walter K, Don A, Flessa H. (2014). No general soil carbon sequestration under Central European short rotation coppices. *GCB Bioenergy*.

Wang GM, Stribley DP, Tinker PB, Walker C. (1993). Effects of pH on arbuscular mycorrhiza: Field observations on the long-term liming experiments at Rothamsted and Woburn. *New Phytol* **124**:465–472.

Wang Q, Garrity GM, Tiedje JM, Cole JR. (2007). Naïve bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* **73**:5261–5267.

Wardle DA, Bardgett RD, Klironomos JN, Setälä H, Putten WH van der, Wall DH. (2004). Ecological linkages between aboveground and belowground biota. *Science* **304**:1629–1633.

Wiesenberg GLB, Schneckenberger K, Schwark L, Kuzyakov Y. (2012). Use of molecular ratios to identify changes in fatty acid composition of *Miscanthus* × *giganteus* (Greef et Deu.) plant tissue, rhizosphere and root-free soil during a laboratory experiment. *Org Geochem* **46**:1–11.

Wollenberg AL van den. (1977). Redundancy analysis an alternative for canonical correlation analysis. *Psychometrika* **42**:207–219.

Wu WX, Liu W, Lu HH, Chen YX, Devare M, Thies J. (2009). Use of ^{13}C labeling to assess carbon partitioning in transgenic and nontransgenic (parental) rice and their rhizosphere soil microbial communities. *FEMS Microbiol Ecol* **67**:93–102.

Zhao Z-W, Xia Y-M, Qin X-Z, Li X-W, Cheng L-Z, Sha T, *et al.* (2001). Arbuscular mycorrhizal status of plants and the spore density of arbuscular mycorrhizal fungi in the tropical rain forest of Xishuangbanna, southwest China. *Mycorrhiza* **11**:159–162.

Zimmerman NB, Vitousek PM. (2012). Fungal endophyte communities reflect environmental structuring across a Hawaiian landscape. *Proc Natl Acad Sci* **109**:13022–13027.

Zimmermann J, Dauber J, Jones MB. (2012). Soil carbon sequestration during the establishment phase of *Miscanthus* × *giganteus*: a regional-scale study on commercial farms using 13C natural abundance. *GCB Bioenergy* **4**:453–461.