PLANT FOLIAR PHOSPHORUS AND SILICON CONTENT CHANGES IN RESPONSE TO ROOT FUNGAL COMMUNITIES AND SILICON ENRICHMENT

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Abstract

Arbuscular mycorrhizal fungi (AMF) are plant root symbionts that supply limiting nutrients, largely phosphorus (P), to plants in exchange for carbon. Silicon (Si) is an important defence element for plants and several reports have observed a relationship between AMF colonisation and Si uptake. It is unknown how diverse soil microbial communities affect foliar Si and P concentrations, and whether improvements in foliar Si concentrations due to AMF colonisation are observed in field conditions. This is the first study to document the dual effects of AMF and Si application in a non-crop species (*Brachypodium sylvaticum*) and the effect of different microbial communities, on plant uptake and deposition of Si and P.

An initial glasshouse experiment used a single species AMF inoculum in combination with a Si enrichment treatment to investigate the effect on foliar Si and P concentration. The results showed that AMF improved the uptake of Si and P compared to non-colonised plants, but that different mechanisms for uptake are likely. Introducing microbial communities isolated from agricultural and woodland environments as inocula in a controlled environment showed that microbial diversity alters the efficacy of Si and P supply, and that improvements in the supply of these were not directly related to AMF colonisation. Finally, *B. sylvaticum* plants from woodland were sampled across two years. The results of this sampling did not show any benefit of AMF on Si and P uptake, but did reveal significant differences in P concentration over time irrespective of fungal colonisation.

Comparisons between studies using high throughput sequencing demonstrates that the methods commonly used in mycorrhizal studies may be overlooking important interactions with un-recorded organisms in the soil and roots of experimental plants. Ecologically relevant studies incorporating long-term repeated sampling are required to fully understand how microbial communities can improve Si and P nutrition in plants.

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Author's Declaration

I, Rosemary Erin Haskell, declare that all the material detailed in this thesis is my own work, and written by myself. This work has not previously been presented for an award at this, nor any other University. All sources are acknowledged as References.

1 General Introduction

1.1 Rationale

Plant yield improvements over the previous century have been the result of technical and chemical advances in agricultural practice, alongside highly selective plant breeding. Despite continued investment into agricultural research and development, yields of global staple crops have plateaued in recent decades (Ray *et al.*, 2012; Grassini *et al.*, 2013). The global application of chemical fertilisers, pesticides and herbicides in intensive agriculture has negatively affected natural floral (Schmitz *et al.*, 2014), faunal (Beketov *et al.*, 2013) and microbial (Jacobsen & Hjelmsø, 2014; Nettles *et al.*, 2016) communities, as well as the abiotic soil environment (Pagliai *et al.*, 2004; Abdollahi *et al.*, 2014). Balancing demands to increase food supply for a growing population, safeguarding agricultural productivity under a warming climate and limiting biodiversity loss has led to the movement of 'ecological intensification' of agriculture (Cassman, 1999; Bommarco *et al.*, 2013). Chemical interventions to improve plant productivity have typically attempted to replicate and enhance existing processes previously achieved through natural ecosystem functions, particularly symbiotic relationships (Bommarco *et al.*, 2013). The ecological intensification movement seeks to incorporate and enhance natural ecosystem functions to improve plant productivity to achieve multiple benefits for plant productivity, rather than trying to artificially replicate singular functions (Bommarco *et al.*, 2013; Bowles *et al.*, 2016).

Soil microbial communities are crucial in the functioning of healthy ecosystems, with rhizosphere organisms playing an important role in defence and nutrient acquisition. Understanding the processes, turnover and diversity of the biotic component of the soil is also crucial to inform reliable climate models. Soils represent the greatest terrestrial sink of organic carbon (C), but current predictions indicate that under a warming climate CO_2 release from soils will increase, with microbes utilising formerly recalcitrant stable organic matter compounds (Frey *et al.*, 2013; Scharlemann *et al.*, 2014). Any loss of diversity as a result of climate change is also predicted to have significant negative effects on the provision of ecosystem services that are crucial in sustaining diverse plant communities and agricultural food crops (Delgado-Baquerizo *et al.*, 2016).

Understanding the functions that soil microbes can provide will potentially lead to improvements in agricultural productivity and sustainability. Improving the microbial diversity in these soils will have additional benefits for soil structure, carbon sequestration and providing pools of microbial genetic diversity, which has implications for healthy ecosystem functioning and bioprospecting at present and in the future.

1.2 Arbuscular mycorrhizal fungi

1.2.1 *Biology and structure of the arbuscular mycorrhizal symbiosis*

Plants have evolved associations with a variety of microbes that have enabled them to alleviate or overcome numerous environmental stresses. One of the most ancient (*c.*450 million years) relationships forms between plant roots and a specific group of soil fungi from the phylum Mucoromycota, sub-phylum Glomeromycotina called arbuscular mycorrhizal fungi (AMF) (Parniske, 2008; Spatafora *et al.*, 2016). The signalling pathway leading to arbuscular mycorrhizal (AM) symbiosis is shared in part with the pathway leading to the formation of the bacterial Rhizobium-legmue symbiosis, which evolved more recently, suggesting its origin in the AMF pathway (Genre & Russo, 2016). Over 80% of plants are capable of forming relationships with AMF, and the persistence of this relationship throughout evolutionary history is a testament to its benefit to plants (Smith & Read, 2010).

AMF form a key bridge between the soil environment and plants, creating an extended chemical and microbial environment around the roots; termed the 'mycorrhizosphere'(Rambelli, 1973; Linderman, 1988). The primary benefit of AMF to plants is to provide limiting nutrients from the soil, predominately phosphorus (P), but also nitrogen (N), and in return the fungus receives plant sugars on which is it dependent for survival (Smith & Smith, 2011; Hodge & Storer, 2014). P is one of the most limiting nutrients for plants, second only to N. Roots are only able to take up and assimilate P as orthophosphate (inorganic P (P_i)), which is highly immobile in the soil profile and therefore leads to rapid formation of depletion zones surrounding plant roots (Smith *et al.*, 2011). While the majority of plants are capable of forming the AMF symbiosis, the environmental conditions are not always conducive to their formation. In high intensity agricultural systems, plants are typically supplied with a volume of nutrients sufficient for their growth throughout the growing season, therefore reducing the reliance on AMF associations (Jensen & Jakobsen, 1980; Koide, 1985; Santos *et al.*, 2006). Further to this, agricultural practices such as monoculture production, applications of pesticides and repeated tilling of soil all lead to a depauparate AMF community (Helgason *et al.*, 1998; Daniell *et al.*, 2001). Agricultural systems apply fertilisers to maintain growth and yield of crops, but the rock phosphate source for fertiliser production is finite and predicted to be exhausted in as little as 30 years' time (Branscheid *et al.*, 2010; Cordell & White, 2011). Restoring a functional rhizosphere community, including AMF, in agricultural systems will offer an alternative to costly and unsustainable fertilisation practices.

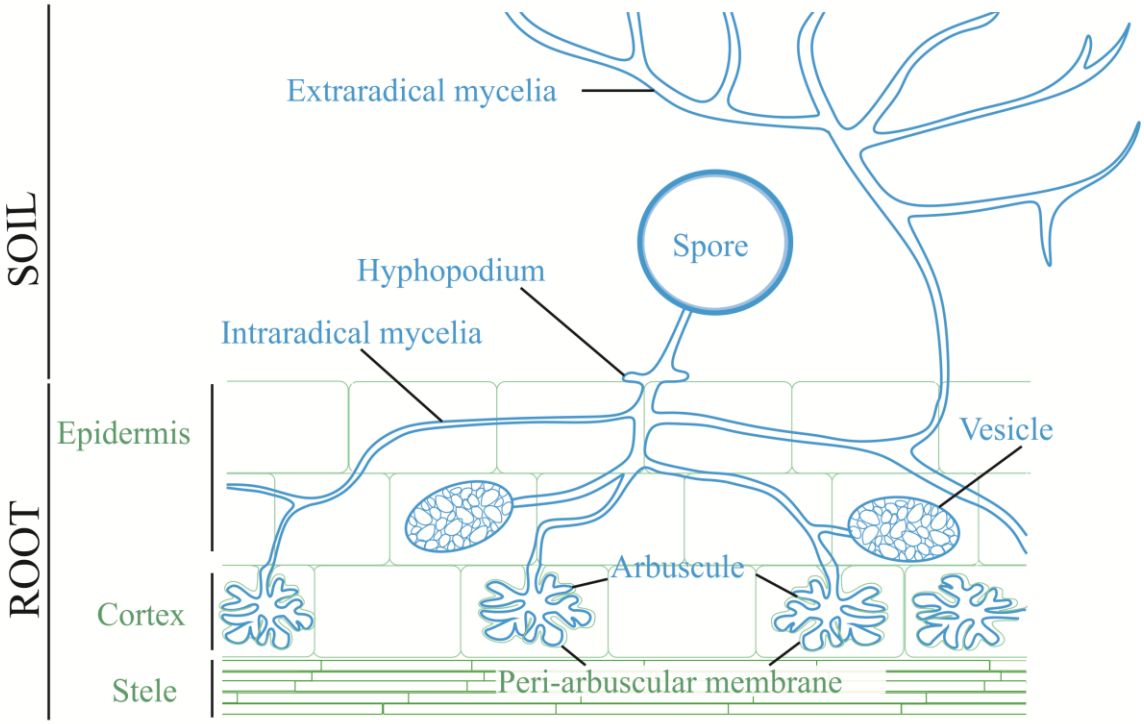

Figure 1: Diagrammatical representation of the arbuscular mycorrhizal symbiosis in a latitudinal cross section of root. Labels and structures in blue represent fungal structures while labels and structures in green represent plant structures.

The key features of an AM symbiosis is shown in Figure 1. The system is composed broadly of four to five parts, an arbuscule and peri-arbuscular membrane (PAM) which form within the root cortical cells, storage vesicles, and the intra- and extra-radical mycelia (IRM/ERM) which form within and outside the root respectively. The arbuscule is a highly differentiated and branched hyphal (fungal cell) extension that expands to fill a plant root cortical cell (Smith & Read, 2010). This structure is surrounded by the PAM which is generated by the plant host tissue. The exchange of compounds required for a successful symbiosis occurs at the PAM and therefore this structure has a large surface area, closely following the extensions of the arbuscule and contains a high density of transporters (Pumplin & Harrison, 2009). Vesicles occur in certain species of AMF and are the storage structures of the symbiosis, once sugars are acquired by the fungus they are converted to glycogen and lipids for transport and storage in vesicles (Roth & Paszkowski, 2017). The IRM extends from the point where a fungal structure called the hyphopodium penetrates the plant root, towards to the arbuscule. Finally, the ERM is the fourth essential part of the AM symbiosis. This is a structure comprised of branching fungal hyphae that spreads into the soil around the root acting as an extension of the plant root system

capable of reaching beyond the depletion zone that forms around plant roots. The smaller diameter (~4μm) of AMF hyphae (collectively mycelia) compared to fine plant roots (>2mm) enables access to smaller soil pores and otherwise inaccessible P_i from the soil (Miller *et al.*, 1995; Smith & Smith, 2011).

1.2.2 *Diversity and distribution of AMF species*

To date approximately 300 species of AMF have been defined using conventional identification techniques, predominantly based on spore morphology (Robinson-Boyer *et al.*, 2009; Schüßler & Walker, 2010). There are a number of difficulties in identifying AMF, particularly within plant roots. Hyphal morphology is more difficult to distinguish and identification beyond the genus level is not possible, therefore a difference in spore production between species can lead to inaccurate estimates of abundance and diversity (Merryweather & Fitter, 1998; Sanders, 2004). There has been a transition over the last twenty years to apply comprehensive sequencing techniques to analyse the diversity of AMF within plant roots to obtain more accurate estimates of fungal diversity (Taylor *et al.*, 2017). The application of DNA sequencing technology to diversity studies have revealed the presence of AMF species in plant roots that have never been successfully documented in spore surveys in the immediate area (Clapp *et al.*, 1995). With the ever decreasing costs of sequencing technologies, DNA-based identification has rapidly become the preferred method for analysing AMF diversity. This has been promoted by the provision of a curated database exclusively for Glomeromycotan DNA sequences, data from which has enabled detailed analyses on the global distribution of AMF (Öpik *et al.*, 2010; Davison *et al.*, 2015).

AMF are termed 'host generalists' due to their ability to colonise the majority of terrestrial plants and simultaneously colonising multiple hosts (Smith & Read, 2010). However, recent evidence suggests that there is some discrimination at an ecosystem level (Davison *et al.*, 2011) and arising from dispersal limitation (Davison *et al.*, 2016) and host relatedness (Reinhart & Anacker, 2014). This evidence would suggest that AMF diversity may show different patterns of diversity similar to plants, but at a broader scale.

1.2.3 *AMF and plant stress tolerance*

Colonisation by AMF can also induce changes in plant immune responses and chemistry, an effect that is broadly referred to as mycorrhizal induced resistance (MIR), but effects are variable depending on species and combinations and the mechanisms are not mutually exclusive (Figure 3, Pozo *et al.*, 2009; Cameron *et al.*, 2013). Colonisation by AMF induces a complex chemical interchange where microbe-associated molecular patterns (MAMPs) stimulate the plants immune system (Zhang & Zhou, 2010). This results in the release of plant phytohormones, which are subsequently suppressed, but this effectively primes the plant against

unwanted intruders (Kapulnik *et al.*, 1996; Jung *et al.*, 2012). In particular, Jasmonic Acid (JA) and derivatives have been correlated with MIR, and proposed as systemic signalling molecules (Jung *et al.*, 2012; Hilou *et al.*, 2014). The JA pathway typically targets necrotrophic fungi and chewing herbivores and so AMF primed defences are particularly effective against these groups (Pozo *et al.*, 2009; Nabity *et al.*, 2013). Transcription analyses of AM plants show that the majority of up-regulated genes are predicted to have a role as response/signalling genes for defence or stress, irrespective of AM identity (Liu *et al.*, 2007b; Gerlach *et al.*, 2015). However, numerous studies have shown that in stressed or challenged plants, different plant-AM fungal combinations appear to elicit different responses in the plants chemistry, even at a strain level (Newsham *et al.*, 1995).

AMF also improve soil structure physically, by increasing soil aggregate size, biologically by altering soil microbial and invertebrate communities and chemically through altered root and hyphal exudates (Rillig & Mummey, 2006; Leifheit *et al.*, 2014). In drying soils shrinkage can lead to smaller soil pore spaces that are inaccessible to plant roots, but not the ERM which have a smaller diameter (Whitmore & Whalley, 2009). Soil structure, relating to soil pore and aggregate size, is crucial in plant water and nutrient acquisition and also on root anchorage (Rillig & Mummey, 2006).

AM host plants also exhibit increased tolerance to osmotic (Augé *et al.*, 2015) and salinity (Porcel *et al.*, 2012) stresses, and heavy metal toxicity (Hildebrandt *et al.*, 2007; Miransari, 2017). AM plants also exhibit improved stomatal conductance, sustained photosynthetic activity, higher levels of proline and soluble sugars which all contribute to an improved water status (Augé, 2001; Bárzana *et al.*, 2015). Direct transport of water from the soil to the plant via mycorrhizal hyphae has been recognised since the 1980's, but it is only since 2009, with the discovery of water transport channels, aquaporins (AQP) in AMF that the mechanism of this has been determined (Duddridge *et al.*, 1980; Ruiz-Lozano & Azcón, 1995; Aroca *et al.*, 2009). Fungal AQP gene expression within the ERM has also been shown to respond to drought stress. AMF hyphal (ERM) growth increased the expression of two AMF AQP genes (*RiAQPF1, RiAQPF2*) were upregulated in response to drought conditions in maize and axenically grown carrot roots, resulting in improvements in plant water content (Li *et al.*, 2013b,a). Use of split pot experiments demonstrate that AMF colonisation in roots within water stressed compartments led to alterations in plant AQP gene expression, resulting in improved water uptake, but not in non-colonised controls or in colonised compartments not exposed to stress (Bárzana *et al.*, 2015). The same two AQP genes were measured in the AMF *Rhizophagus intraradices* colonising tomato plants, but only *RiAQPF2* showed upregulation in response to the drought treatment and this was accompanied by an upregulation in a plant AQP gene (*LeNIP3;1*) which was not observed in the control treatments (Chitarra *et al.*, 2016). This

demonstrates that AM can improve root functional responses in response to environmental stimuli at a small spatial scale, and that there may be some synchrony between AMF and plant AQP regulation.

AMF AQP may also be crucial in filling in the gaps in the P and N uptake pathway. The uptake of phosphate by AMF occurs though uptake and accumulation of polyphosphate (PolyP), although P nutrition is commonly cited as the main benefit of AMF to plant hosts, the exact mechanisms for transport are still under contention (Smith & Smith, 2011). Kikuchi *et al*. (2016) identified three novel AQP in AMF *Rhizophagus clarus,* one of which, RcAQP3, was implicated in ammonium uptake. This study showed a strong correlation between rates of PolyP translocation and *RcAQP3* gene expression. Plant transpiration rates were shown to stimulate the expression and activity of *RcAQP3* and control the water flow through the hyphae, particularly through the tubular vacuoles, where PolyP accumulates. PolyP is then translocated through the hyphae towards the roots. Through the knock-out of *RcAQP3* and measurement of plant response, Kikuchi *et al.* (2016) have shown the importance of fungal AQP within the function of the AM symbiosis.

The benefits of the AM symbiosis for plant growth are numerous, and the mechanisms for these improvements are progressively being revealed. The effects of AM on AQP gene regulation and AQP channel gating which enable plants to combat and tolerate abiotic stresses have opened the avenue for investigating AM impact on other processes regulated by AQP. AQP facilitate passive transport of water, but also a number of small uncharged solutes and gases such as carbon dioxide (Uehlein *et al.*, 2003), urea (Beitz *et al.*, 2006), ammonia (Jahn *et al.*, 2004), hydrogen peroxide (Bienert *et al.*, 2007), boric acid (Takano *et al.*, 2006), silicic acid (Ma *et al.*, 2006), and arsenic (Zhao *et al.*, 2009) across a natural water or electrochemical gradient. The diverse role of these compounds in the plant range from osmotic regulation, nutrition, stress signalling and plant defence.

1.3 Silicon

1.3.1 *Silicon availability, uptake and deposition in plants*

Elemental silicon (Si) is abundant in soils, but plants can only take up Si in the soluble form silicic acid ($Si(OH)_4$) which is present in soils at concentrations of 0.1mM-0.6mM, However, soil concentrations of $Si(OH)_4$ are lower than this in areas of high rainfall and alkalinity and at extreme temperatures, where plant available Si is deficient (McKeague & Cline, 1963; Haynes, 2014). Silicic acid availability can be affected by numerous environmental factors, such as soil microbial (Alfredsson *et al.*, 2016) and faunal (Bityutskii *et al.*, 2016) communities, pH (Gocke *et al.*, 2013), and litter degradation (Carey & Fulweiler, 2016). Litter degradation is significant, particularly in an agricultural context where plant residues are often removed post-harvest. The

dissolution of biogenic Si ($SiO_2.nH_2O$) fixed in phytoliths replenishes the bioavailable Si component in the soil more rapidly than occurs through abiotic weathering processes (Guntzer *et al.*, 2012b; Carey & Fulweiler, 2016). Some agricultural soils are thus deficient in Si and there is increasing interest in the use of Si application to crop systems as a way to increase Si uptake by crops and hence their resistance to both biotic and abiotic stresses (Haynes, 2014; Meharg & Meharg, 2015; Cooke *et al.*, 2016).

Si uptake by plants has historically been thought to have no associated yield penalties, even when accumulated in excess, as shown in multiple cases under experimental conditions (Fauteux *et al.*, 2005; Ma & Yamaji, 2006; Currie & Perry, 2007). However, a recent study comparing the Si concentration, growth rate and plant size of modern crop cultivars and ancestral landraces suggests that there is a penalty to the uptake and accumulation of Si, where high Si accumulating plants were on average 15% smaller than those with low Si accumulation (Simpson *et al.*, 2017). This yield penalty is predicted to be associated with the active transport and deposition of Si once inside the plant tissues.

The uptake and transport of Si in grass plants is summarised in Figure 2. Si transport from the soil is achieved through the uptake of $Si(OH)_4$ through a constitutively expressed AQP channel (Lsi1) located in the mature roots, more than 10mm from root tips (Ma *et al.*, 2006; Yamaji & Ma, 2007). In plants there are five sub-groups within the AQP MIP family and the Si channel (Lsi1) is classified within the Nodulin 26-like intrinsic protein (NIP) group (Ma *et al.*, 2006; Xu *et al.*, 2013). Originally identified in rice, homologs of the *Lsi1* gene have been identified in maize (Mitani *et al.*, 2009), pumpkin (Mitani *et al.*, 2011), wheat (Montpetit *et al.*, 2012) and other important crop species (Maurel *et al.*, 2015). After passive assimilation into the root through Lsi1, $Si(OH)_4$ is then actively transported through the endodermal cells across the casparian strip by Lsi2, a Si efflux transporter. The localisation of Lsi1 and Lsi2 is different in rice compared to other grass species, due to the difference in root structure. In maize and wheat and in other grass species lacking arenchyma, Si can be taken up by the epidermal, cortical and endodermal cells, while only exodermal cells contain the Lsi1 AQP channel in rice (Yamaji & Ma, 2007; Mitani *et al.*, 2009; Montpetit *et al.*, 2012). Transport of $Si(OH)_4$ through the root tissues to the stele occurs through the symplast until being transported into the stele by Lsi2 in wheat and maize, although the xylem loading transporter or channel has yet to be identified in any plant species (Ma *et al.*, 2011). Transport to aerial plant parts occurs through the xylem at a rate determined by transpiration (Ma *et al.*, 2007; Kumar *et al.*, 2017a), although uptake and deposition is inducible by damage to plant tissues and likely related to induction of active Si transporter Lsi2 (McLarnon *et al.*, 2017). Silicic acid is released from the xylem into foliar xylem parenchyma by an active process via the transporter Lsi6 (Yamaji *et al.*, 2008). The $Si(OH)_4$ then polymerises, with highest concentrations occurring in ‘silica cells’ where the cells

become fully mineralised, a process that only occurs during cell development of young leaves (Motomura *et al.*, 2006; Kumar *et al.*, 2017a). While transport to the leaves is dependent on transpiration, the silicification and subsequent death of these cells is determined by a currently unknown biological process, possibly involving peptides, proteins or sugars that condense silicic acid to a solid form in foliar cells and phytoliths (Kumar *et al.*, 2017a). There is also variety in the mechanism of Si deposition across cell types, which is passive at the cell wall of transpiring plant cells, and active in non-transpiring tissues and in specialised silica cells independent of transpiration (Kumar *et al.*, 2017b).

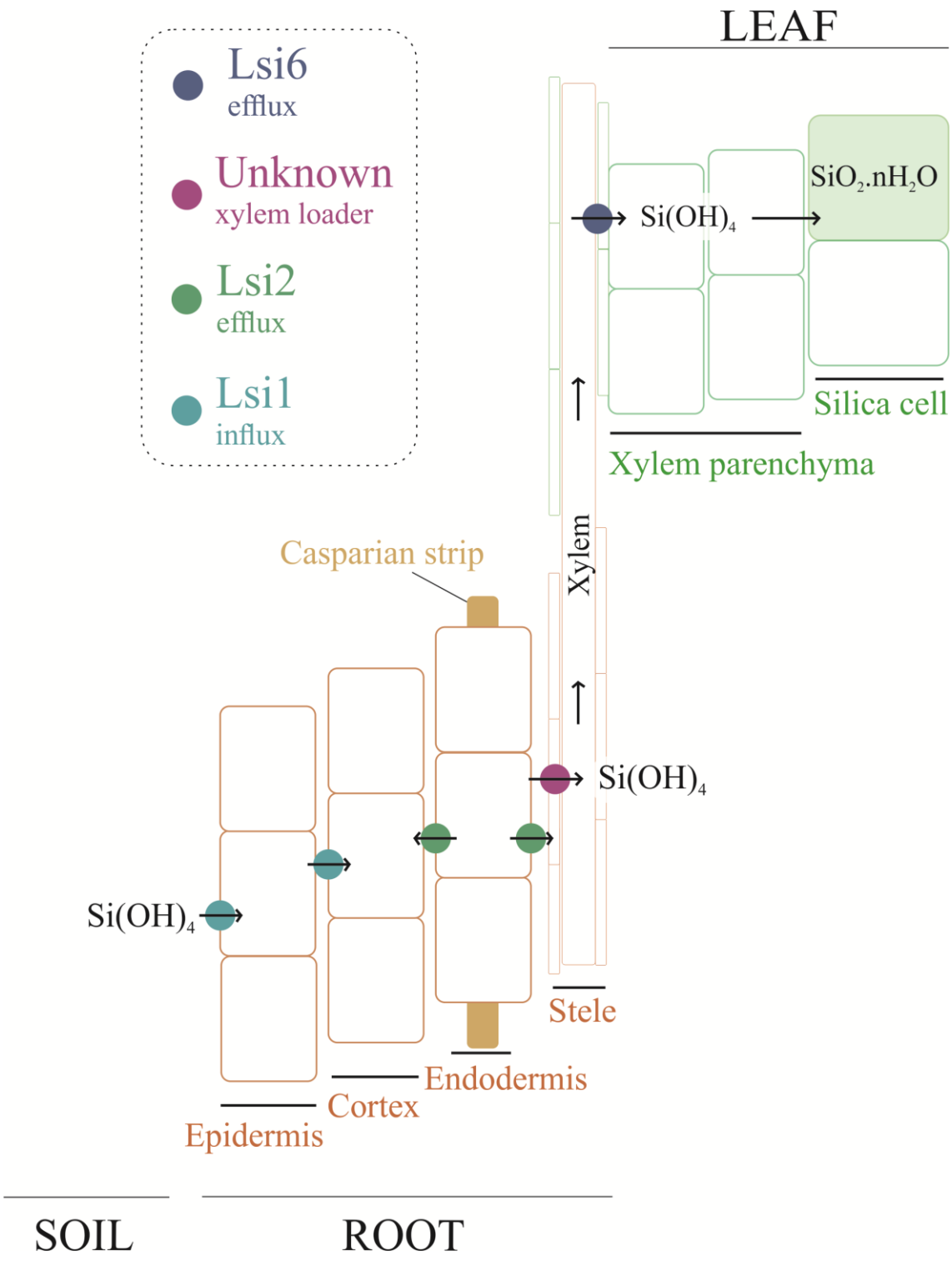

Figure 2: Transport of silicic acid $Si(OH)_4$ from the soil to leaf silica bodies. Schematic represents the transport pathway of wheat and maize plants. Adapted from Ma *et al.* (2011)

1.3.2 *Si and plant defence*

Despite being classed as "non-essential" in most plants, Si has important prophylactic effects against many pests and pathogens (Massey *et al.*, 2006; van Bockhaven *et al.*, 2013; Hartley & DeGabriel, 2016). Traditionally, Si defences have been associated with structural defence. Phytoliths are foliar insoluble silica deposits that strengthen the leaf surface, reducing plant palatability and digestibility by herbivores (Massey & Hartley, 2006), and epidermal penetration by pathogens (Kim *et al.*, 2002). Si uptake is variable across species, with grasses (Poaceae family) typically having the highest Si concentrations and investing most heavily in Si based physical defences such as silicification of leaf hairs (trichomes) and phytoliths (Epstein, 1999). The uptake of Si is constitutive but can be increased upon stimulation by herbivory, with plants that have been previously damaged typically having higher foliar Si concentrations than undamaged plants (Massey *et al.*, 2007b). Deposition of Si can also vary between and within species. Phytoliths differ in structure between species and the patterns of Si deposition on the leaf surface is also very variable. Intraspecific variations in Si deposition also occurs, where less palatable varieties have been shown to invest more in trichome silicification than softer, more palatable varieties (Hartley *et al.*, 2015).

The mechanisms for Si related defences can be physical, biochemical, molecular or any combination of these. Soluble Si in the plant also plays a role in plant protection, priming plant defence pathways, leading to enhanced levels of defence hormones when plants are attacked (Ghareeb *et al.*, 2011; Ye *et al.*, 2013; van Bockhaven *et al.*, 2015; Reynolds *et al.*, 2016). The priming of plant defences by Si is thought to be similar in ways to AMF, with Si based defences also stimulating the hormone pathways involving salicylic acid (SA) and JA (van Bockhaven *et al.*, 2013). In the absence of stress, Si-supplemented plants are generally indistinguishable from controls but when threatened or stressed Si-treated plants have more rapid and enhanced induction of chemical defences (Fauteux *et al.*, 2005; Ye *et al.*, 2013). Other chemical and molecular defence theories have been suggested, including Si interacting subtly with iron (Fe) homeostasis and links to Fe related redox-defences (Islam & Saha, 1969; Liu *et al.*, 2007a). Also, Si has been shown to influence accumulation of photorespiratory enzymes associated with defence, and interaction with elemental co-factors of enzymes involved in immune signalling (Fauteux *et al.*, 2005; Nwugo & Huerta, 2011; Kangasjärvi *et al.*, 2012). This response has also been implicated in the improved tolerance of Si supplemented plants on heavy metal tolerance. Si enriched plants have demonstrated enhanced tolerance to a range of abiotic stresses including low temperatures (Liang *et al.*, 2008), drought (Eneji *et al.*, 2008), salinity (Li *et al.*, 2015) and various heavy metal soil contaminants (Adrees *et al.*, 2015).

1.4 AMF and Si

Alternative methods to Si addition that improve Si availability for crop uptake are attracting interest, and given their well-recognised benefits in P, N and water uptake, attention has turned to the role of AMF in Si uptake, particularly because Si does not decrease the ability of AMF to colonise plants as previously thought (Garg & Bhandari, 2015; Maurel *et al.*, 2015). AMF have been shown to induce the expression of plant AQPs, including AQP from the NIP sub-family to which the Si transporter belongs. If AM plants have increased Si compared to un-colonised plants, then they should have increased protection against a range of environmental stresses. Both AMF and Si have unique benefits improving plant tolerance to multiple stresses, involving different plant hormonal responses and physical structures. Application of AMF and Si in tandem has the potential to provide a holistic and broad coverage of defences against plant attackers and abiotic stresses (Figure 3).

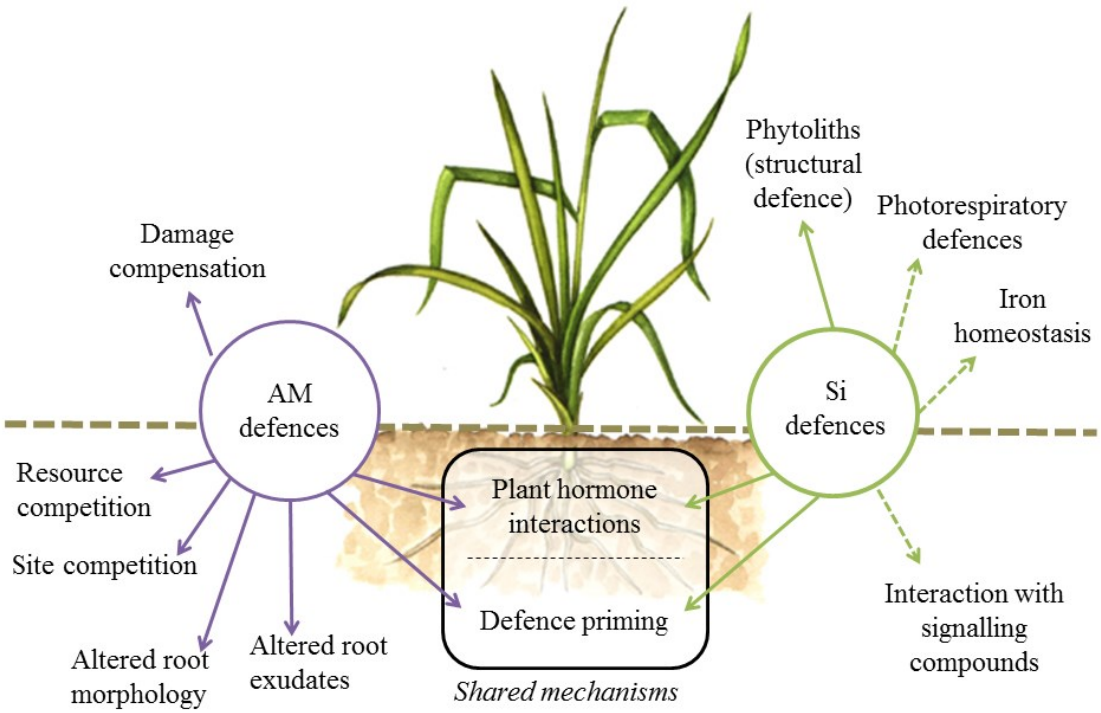

Figure 3: Summary of defence mechanisms in plants as a result of AM fungal interactions and Si application, mechanisms spatially organised to their main areas of impact.

AMF have been shown to have elevated Si concentrations in spores and hyphae under salinity stress, which demonstrates that there is a mechanism present to take up Si either passively or

actively in both these structures (Hammer *et al.*, 2011). The mechanism of this uptake is unknown and transport of any assimilated Si to plant hosts is also unknown (Hammer *et al.*, 2011). There is evidence that ectomycorrhizal fungi (ECM) can mobilise Si in the soil profile, resulting in increased plant root concentrations under certain conditions, a mechanism that may also be true for AMF, which have demonstrated a considerable effect on soil chemistry in the mycorrhizosphere (Rillig & Mummey, 2006; van Hees *et al.*, 2006). Improvements in Si in AM plants have been observed under heavy metal stress (Turnau *et al.*, 2007) and manganese stress (Nogueira *et al.*, 2002) also, compared to non-colonised counterparts.

Experiments that have directly tested the effect of Si and AMF application on Si uptake are few, but show promising interactive effects. The earliest study looked at Si uptake in mycorrhizal soybean (*Glycine max*), and demonstrated higher foliar Si concentrations in AMF colonised plants and, unlike other elements measured, Si uptake rates were not directly related to P application and uptake suggesting an alternative method of uptake (Yost & Fox, 1982). By contrast, Kothari and associates (1990) found that foliar Si concentration was lowest in maize (*Zea mays*) colonised by AMF *Glomus mosseae* (*Funneliformis mosseae*), but accounting for differences in root characteristics, mycorrhizal plants demonstrated improved Si uptake efficiency compared to non-colonised controls (Kothari *et al.*, 1990). Using *Rhizophagus irregularis*, Oye Anda *et al* (2016) demonstrated improved plant growth and Si uptake in colonised banana (*Musa acuminata*) plants, and that uptake was responsive to addition of a soluble Si solution.

The positive effect of AMF on plant Si uptake is also seen under stress conditions. Si application and mycorrhizal colonisation in chickpea (*Cicer arietinum*) subjected to salt stress decreased Na^+ uptake, while also improving yield and nutrient uptake relative to controls (Garg & Bhandari, 2015). In isolation, Si addition was found to be less effective than AMF for improving plant productivity, but was beneficial in improving plant K+/Na+ ratio, while AMF addition improved the uptake of Si yielding a joint benefit (Garg & Bhandari, 2015). Similarly, (Garg & Singh, 2017) also showed that the colonisation of AMF *R. irregularis* further improved Si uptake under cadmium and zinc stress in two genotypes of pigeonpea (*Cajanus cajan*) The results of these two latter studies demonstrate that positive effects of Si uptake and AMF colonisation are achievable in non-Si accumulating plant species, and across a number of plant growth limiting conditions (Garg & Bhandari, 2015; Garg & Singh, 2017).

Two recent studies have demonstrated that sugarcane plants colonised by AMF exhibit significantly higher Si concentrations in roots, particularly in low-Si treatments (Frew *et al.*, 2017a,b). This effect is consistently found in both commercial and native AMF communities (Frew *et al.*, 2017a). Root feeding insect performance was also measured in these studies, but

AMF colonisation appeared to most significantly affect insects in the low-Si soil treatments (Frew *et al.*, 2017a,b). Where two varieties of sugar cane were used, AMF only decreased the canegrub performance when Si availability was low and only in one variety of sugarcane tested (Frew *et al.*, 2017b). It would be expected that improved Si concentration caused by AMF Si acquisition would have the same effect as addition of a Si treatment, however there may be more complex regulation in the process, and be dependent on host and fungal combinations. The addition of the Si treatment may be bringing about other benefits; this study did not amend the pH of the Si solution addition, so change in soil chemical conditions may have an effect on the soil grub performance. If AMF are able to enhance plant Si concentrations, either through improving plant uptake efficiency or through direct transport through the AMF hypha, this could benefit plant health and productivity (Figure 3, Guntzer *et al.*, 2012).

1.5 Illumina MiSeq Amplicon Sequencing

Next-generation sequencing (NGS) as a tool for capturing microbial diversity is being applied in a variety of biological disciplines (Caporaso *et al.*, 2012; Faith *et al.*, 2013; Adams *et al.*, 2015). While platforms such as Illumina MiSeq are often used on randomly fragmented DNA, adaptations to the method and sample preparation are enabling analyses to be carried out on specific parts of the genome used for species barcoding with novel methods to overcome the limitations of the technology to sequence short fragments (Caporaso *et al.*, 2012; Taylor *et al.*, 2017). The Illumina Mi-Seq is a powerful benchtop sequencer, capable of generating 17 million pairs of reads using paired 250 nucleotide sequences (Kozich *et al.*, 2013). It works by binding amplicons to a sequencing flow cell through the use of primer adaptations. Complementary base-pairs are called and emit a fluorescent label on incorporation into the DNA strand which is recorded by the analyser. Similar sequences cluster together and will correspond to operational taxonomic units (OTUs) generated in downstream processing (Quail *et al.*, 2012). The number of reads assigned to the cluster is quantifiable so relative abundance can be assessed. There are two main approaches used to analyse rRNA gene sequences, either using a single or double PCR approach. This section will describe in detail the double-PCR method.

Preparing samples for amplicon NGS involves several additions to the standard primers used to amplify specific regions. These adaptations are applied in a two-step process (Kozich *et al.*, 2013,Figure 4). Through an initial PCR to amplify the region of interest, the standard primers are attached to specialised sequencing primers and twelve random bases. The sequencing process clusters fragments together based on sequence similarity of the initial bases in the fragment, but because the primers remain adhered to the DNA fragment, and conserved regions make up the early parts of the DNA fragment, differentiation between sequences does not occur until further into the fragment. The twelve random bases create variable sequence beyond the

primers that ensures the sequences are read further along the strand to distinguish sequences and form clusters based on the variable regions of the DNA fragment. This enables separate colonies to be distinguishable by the imaging on the MiSeq chip by reading the sequence of a single read within a colony at each location on the chip. In a second PCR, barcodes (6bp) are added that bind to the sequencing primers and allow the identification of the sequences in downstream processing. Adaptors are also added that are complementary to stubs on the sequencing flow cell so that the fragments adhere to the surface and can be read.

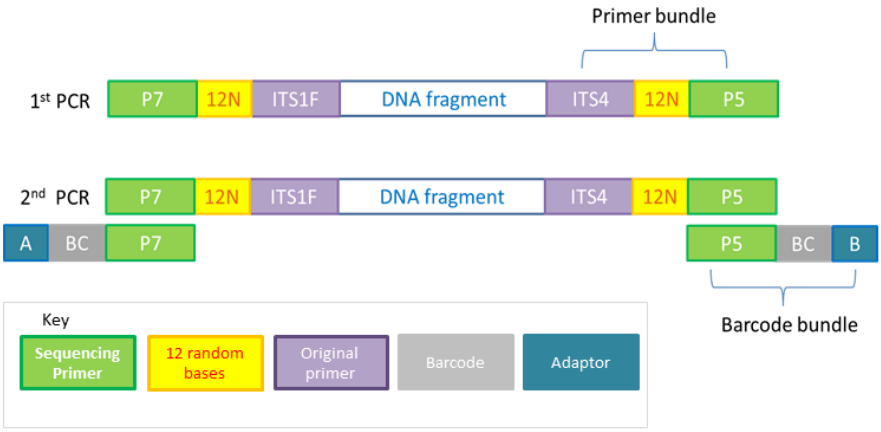

Figure 4: Adaptations of a DNA fragment for amplicon-based processing using the Illumina Miseq platform

1.6 Thesis aims and hypotheses

AMF have a significant impact on the growth and nutrition of plants. The colonisation of plants by AMF leads to significant changes in plant growth and performance, alongside a complex alteration of plant gene expression relating to the defence and nutrient uptake pathways. There has been significant evidence for a link between Si uptake and colonisation by AMF published in the last two to three years (Garg & Bhandari, 2015; Oye Anda *et al.*, 2016; Garg & Singh, 2017; Frew *et al.*, 2017b). These studies have demonstrated the ability of AMF to improve Si uptake of a variety of crop species, and under stress and non-stress conditions. However, the mechanisms of the uptake are still unknown. Although AMF spores and hyphae have been shown to accumulate Si (Hammer *et al.*, 2011), AMF also are capable of regulating plant AQP gene expression and post-translational gating of AQP which may also lead to improvements in Si uptake (Chitarra *et al.*, 2016; He *et al.*, 2016). This study aimed to develop understanding of

the mechanism by which Si uptake and deposition is improved by AMF by comparing Si and P uptake patterns in plants.

Studies using single-species and single-isolate AMF inocula have shown improvements in Si uptake in crop species but to date no studies have examined the effect of different microbial communities on the uptake of Si. In this study, the application of high-throughput sequencing has enabled an accurate assessment of the diversity of root colonising fungi in controlled and field samples, which has highlighted some potential novel interactions between plant growth and nutrition and particular microbial species. Previous studies have also focussed purely on crop species, but understanding microbial and Si interactions in the natural environment is important to improve understanding of a key pathway in plant defence and community dynamics. The inclusion of diverse microbial communities and field sampling has enabled comparisons to be made between glasshouse and natural environments to reveal how important AMF are within natural communities. This study therefore aimed to understand the tripartite link between growth of a non-crop plant, silica uptake and microbial communities, with a special focus on AMF.

Through the use of a variety of experiments at different scales and levels of complexity, progressing to a study of plants in their natural environment, this thesis aims to investigate the following hypotheses:

1. Colonisation of plant roots by AMF will improve Si and P uptake and foliar concentration.

2. The application of high-throughput sequencing methods to plant root DNA extracts from glasshouse experiments and field studies will accurately document the root colonising fungal communities and enable detailed analysis of species specific contributions to plant growth and nutrition. It is expected that despite differences in diversity, AMF abundance will remain a significant factor in Si and P uptake and concentration.

3. Comparative analysis of the experiments in this thesis will reveal whether commonly applied AMF treatments in glasshouse conditions are valuable in replicating plant response in the natural environment. It is predicted that using natural inocula in controlled conditions will generate more ecologically relevant results.

2 Methods development

This chapter has been included to provide context to the three main data chapters and to document preliminary experiments that had a significant impact on the direction of the research. It also includes a detailed overview of the sample preparation and bioinformatics for the next-generation sequencing data that is used in Chapters 4 and 5.

2.1 Substrate choice experiment

2.1.1 *Introduction and background*

Substrates suitable for plant growth naturally differ in their chemical composition depending on the parent material, and how readily available silicon (Si) compounds are available for plant uptake. For example, aluminosilicate clay material is more susceptible to weathering than quartz so sandy soils will have higher availability of soluble Si compared to clay-soils (Hodson & Evans, 1995). Substrates will also vary in pH which will impact the bioavailability of important compounds such as inorganic P (P_i) and silicic acid ($Si(OH)_4$) for plant growth, and affect microbial activity also (Ehrlich *et al.*, 2010). Availability of Si in substrates can vary widely between and within different substrates, for example soil concentrations are 1-0.6mM (Epstein, 1999) and the dissolution of Si also varies with pH and is highest at low pH (~4-7) in soil and clay (Golubev *et al.*, 2006; Haynes, 2014).

This experiment analysed four common plant growth substrates to determine their effect on plant growth, Si content, and how they interacted with a Si enrichment treatment. The results of this experiment informed the choice of substrate for future experiments involving Si uptake and its interaction with mycorrhizal fungi. Two hypotheses were proposed:

1. Plants grown in substrates with a pH range of 4-7 will have higher foliar Si concentrations than plants grown in substrates with lower or higher pH ranges.
2. Plants treated with additional Si will have a higher Si content than plants without additional Si supply.

2.1.2 *Materials and methods*

Preparation of biological materials

Winter wheat (*Triticum aestivum* var. croft (KWS-UK, Hertfordshire)) plants were grown with four different substrates: field soil from Yorkshire (Elvington), peat (J. Arthur Bowers, Lincoln, U.K) and perlite were used on their own and in combination (50:50) with terra-green (TG, calcinated attapulgite clay soil conditioner; Oil-Dri, Cambridgeshire, UK) resulting in six substrate treatments. All substrates were autoclaved (121°C, 72mins), soil and peat were

autoclaved twice with a 3 day resting period between autoclave events and prior to seed sowing while perlite and TG were autoclaved once. An additional Si treatment (Si+/Si-) was also applied to the plants, two weeks after germination. Half of the plants received 50ml of Thornton's nutrient solution with added Si ($Na_2SiO_3.9H_2O$) at 1.5mM (Si+) while the other half received a non-amended Thornton's solution. These treatments were applied twice weekly for the duration of the experiment and all pots received dH_2O throughout the experiment duration (Thornton & Bausenwein, 2000). Each treatment (substrate, Si +/-) was replicated 8 times, with a total number of 96 plants. Plants were grown in controlled glasshouse conditions (15-20°C, 16:8hrs light:dark), in a randomised block design.

Table 1: Names, formulas and final concentrations of chemicals used in the Thornton-Bausenwein nutrient solution. The final Si+ row indicates the chemical addition made for the Si+ treatments.

Stock Solution	Chemical	Formula	Final solution concentration (mg/L)
1	Ammonium nitrate	NH_4NO_3	120.00
2	Calcium chloride	$CaCl_2$	233.10
3	Magnesium sulphate heptahydrate	$MgSO_4.7H_2O$	184.85
4	Potassium sulphate	K_2SO_4	87.13
5	Sodium phosphate dihydrate	$NaH_2PO_4.2H_2O$	47.89
6	Iron(III) citrate	$FeC_6H_5O_7$	2.45
7	**Micronutrients**		
	Boric acid	H_3BO_3	3.09
	Manganese(II) sulphate tetrahydrate	$MnSO_4.4H_2O$	1.92
	Zinc sulphate heptahydrate	$ZnSO_4.7H_2O$	0.58
	Copper(II) sulphate pentahydrate	$CuSO_4.5H_2O$	0.25
	Ammonium molybdate tetrahydrate	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.09
Si+	Sodium metasilicate nonahydrate	$Na_2SiO_3.9H_2O$	426.3

Measurements for pH were taken from three randomly selected pots per treatment (n=36), at two weeks post-germination, prior to the Si treatment application, and at harvest to monitor any changes related to the addition of the Si solution. pH of the Thornton's solution with and without Si was also measured. pH measurements were taken from fresh substrate, and mixed in a 1:1 (v:v) with deionised water (dH_2O).

At harvest (49-52d after planting) leaf, shoot, and root fractions were separated and the latter rinsed thoroughly to remove residual substrate. Root length was calculated by the gridline-intersect method (Tennant, 1975; McGonigle *et al.*, 1990). Measurements were taken per field of view under a compound microscope at x200 magnification with a cross hair grid attachment to the lens. One hundred fields of view across two slides were analysed per sample, with samples randomised and allocated anonymous codes to avoid sampling bias based on the experimental condition (i.e. samples were analysed 'blind'). Mycorrhizal structures (arbuscules

(A), vesicles (V), hyphae (H), and spores (S)) were scored independently, with hyphae being scored as mycorrhizal only if seen connected to other mycorrhizal structures listed above. If hyphae were viewed that possessed a similar structure to the mycorrhizal type (i.e. free of septa/coenocytic, similar diameter) these were labelled as 'possible mycorrhizal hyphae' (PH). Non-mycorrhizal structures were also noted ('non mycorrhizal' (NM)). Scores across the two slides (to add to a sum of 100 fields of view) were then analysed for percentage mycorrhizal colonisation (MC), minimum and maximum as follows:

$$\text{Min MC (\%)} = 100/(A+V+H+S)$$

$$\text{Max MC (\%)} = 100/(A+V+H+S+PH)$$

Minimum values of colonisation were used throughout this thesis to ensure the values were most correct, and these scores were overall most consistent.

Dried green leaf material was ground (Retsch MM400 Mixer mill, Haan, Germany) and pelleted (Specac Atlas™ manual 15ton hydraulic press, Kent, UK) for Si and P analysis using an X-ray Fluorescence (XRF) gun (Niton XL3t900 GOLDD Analyser, Thermo Scientific, UK) (Reidinger et al., 2012). The XRF gun was calibrated for measurements using Si-enriched methyl cellulose and validated using certified reference material from the China National Analysis Center for Iron and Steel; 'Bush branches and leaves' (NCS DC73349) (Reidinger *et al.*, 2012).

Statistical analysis

Statistical analysis was carried out using Rstudio running R version 3.1.3 (RStudio Team, 2016). Data was checked for normal distribution using the stats::shapiro.test and for homogeneity of variance using the stats::bartlett.test. Post-hoc tests were used where appropriate; the stats::TukeyHSD was used following ANOVA tests, while the FSA::dunnTest was used with the Bonferroni method following Kruskal-Wallis tests. One-way ANOVAs were carried out to examine the effects of Si treatment on foliar Si concentration and biomass within each substrate type. A non-parametric Kruskal-Wallis test was used to determine significant differences between substrate types. Linear models incorporating block as a random factor were compared to simpler models with the dependent factors (substrate and Si treatment) using the Akaike information criterion (AIC) to determine the model that best fit the data. Changes in pH were determined with a paired t-test within substrate types and a Mann-Whitney U test across substrates. Si concentrations were arcsine transformed prior to statistical analyses, graphs are produced with the raw concentration values, but statistical tests used arcsine values where appropriate.

2.1.3 *Results*

All plants grew successfully to completion of the experiment. Plants grown in the peat substrate were much smaller in size compared to other treatments (Figure 6) and only three replicates had sufficient biomass to create a pellet for the Si analysis. The block in which plants were grown in did not affect the outcome of any statistical analyses, and this factor was removed from analyses based on the comparison of model AIC values.

The pH of substrates across the experiment was measured to monitor changes caused by the Si treatments and how this may affect plant growth and Si concentration. The pH of the Si enriched nutrient solution was not adjusted for this experiment. Silicon enriched nutrient solution (Si+) had a pH of ~10.25, significantly higher ($t_{(2)}$=-50.0, p<0.001) than the non-Si enriched nutrient solution (Si-) with a ~ pH 5.48. The addition of Si solution only had a significant impact on soil-TG substrate pH ($t_{(2)}$=-5.167, p=0.035) increasing over the duration of the experiment (Figure 5). The addition of the lower pH Si- treatment led to a significant decrease in pH ($t_{(2)}$=6.51, p=0.023) of the perlite substrate solution from an mean of 8.64 to 7.48 (Figure 5). Overall the pH of the substrates was very diverse ranging from ~3.4 in peat to ~8.3 in perlite. The addition of TG neutralised the pH, increasing or decreasing the pH of substrate solutions with the exception of the soil substrate.

The substrate had a significant impact on the total biomass of plants ($F_{(5,90)}$=59.78, p<0.01). TG addition significantly increased plant size in peat and perlite substrates compared to the single substrate treatments (Tukey adjusted P, Peat: p<0.001, Perlite: p<0.001), but decreased plant size when mixed with soil (Tukey adjusted P, p<0.001; Figure 6). This is possibly related to the

pH changes seen within these mixed substrates compared to the unmixed substrates (Figure 5).

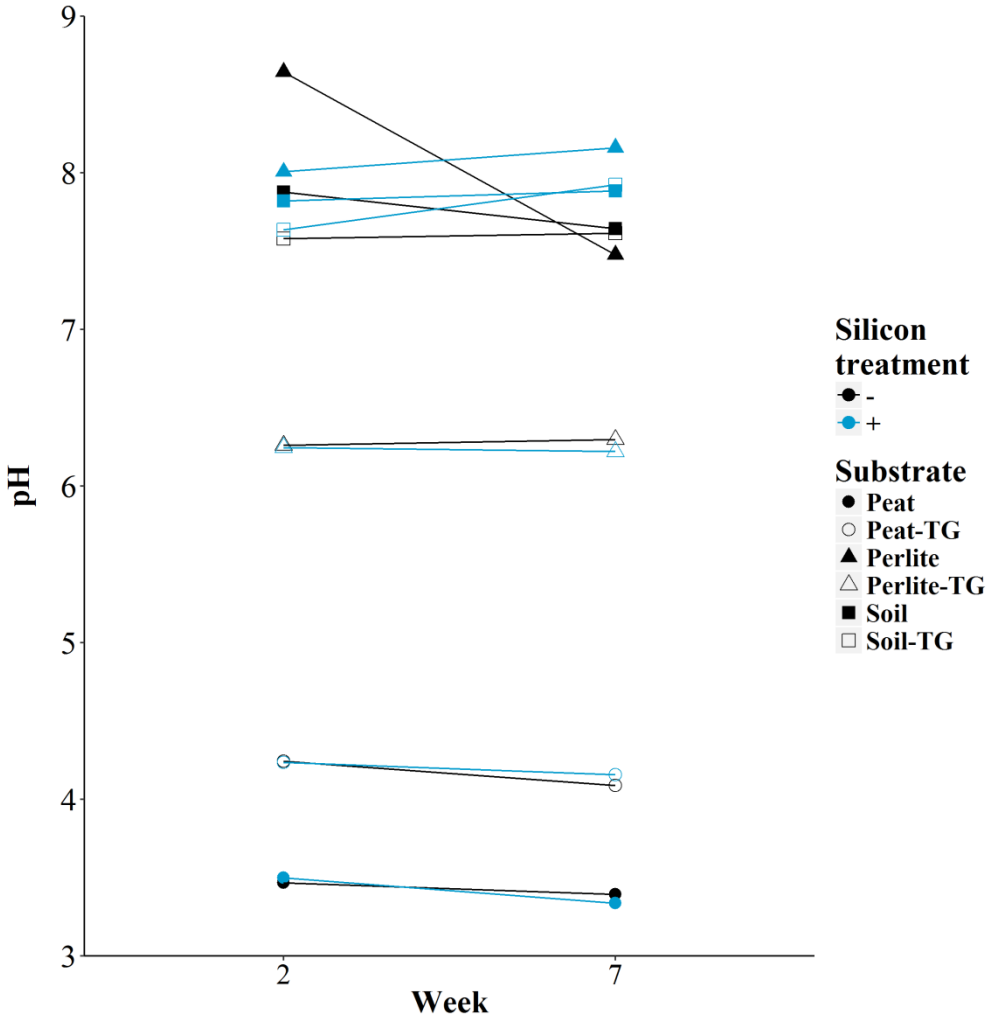

Figure 5: Change in substrate pH across the duration of the experiment at two weeks, prior to treatment with Si solution and at harvest, approximately 7 weeks after germination. Silicon (Si) treatment refers to the supply of Thornton's nutrient solution alone (Si-, black) or with added soluble Si (Si+, blue). Substrates are Peat (shaded circle), peat mixed with Terra-Green (TG, non-shaded circle), perlite (shaded triangle), perlite mixed with TG (non-shaded triangle), soil (shaded square), and soil mixed with TG (non-shaded square), n=3 for each treatment.

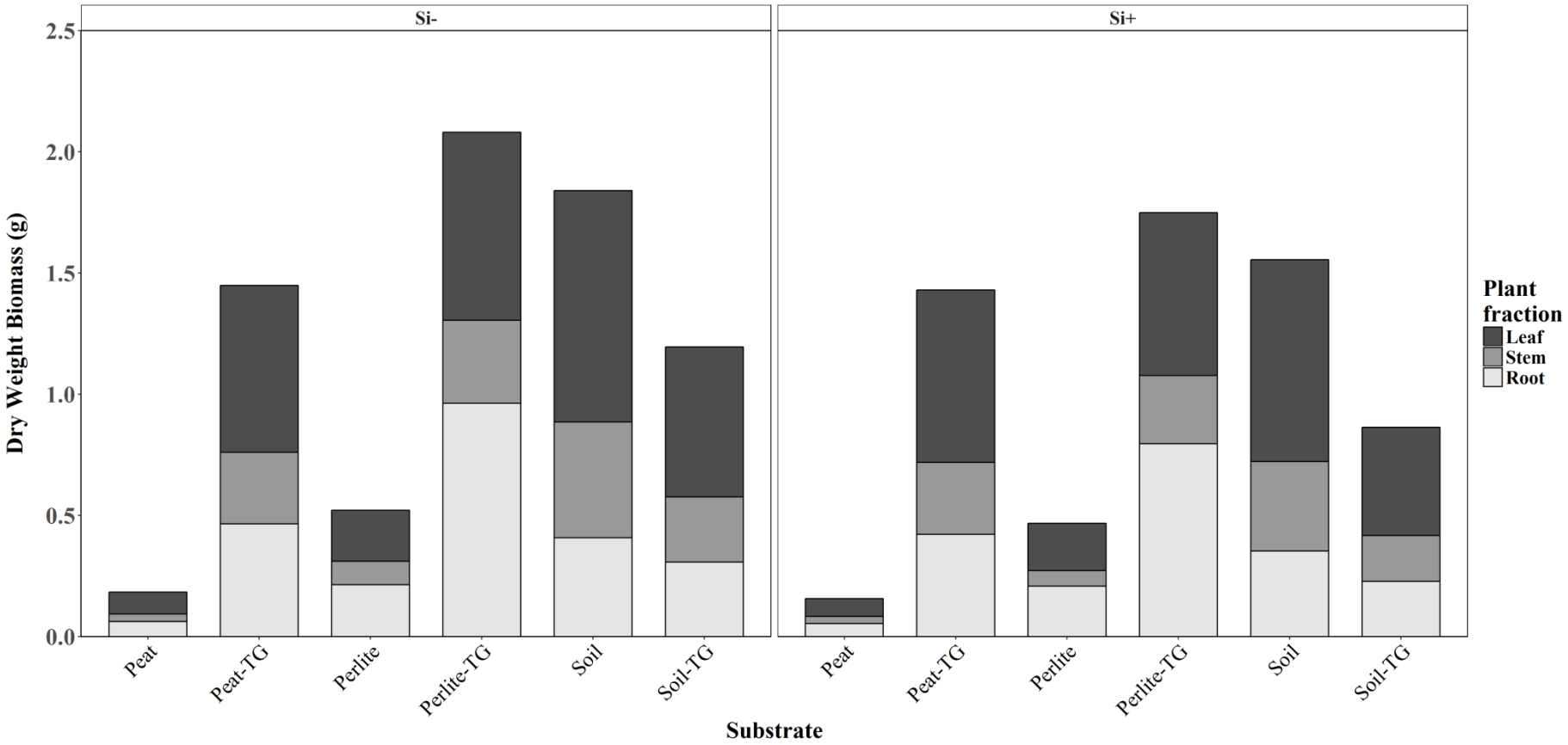

Figure 6: Dry weight biomass (g) by plant fractions (Leaf, Stem and Root) of winter wheat (*Triticum aestivum* var. Croft) grown in different substrates. The left panel shows results from plants grown without supplementary Si solution (Si-), and the right panel shows results from plants grown with weekly applications of a 1.5mM soluble Si solution (Si+), n=8 for all treatments

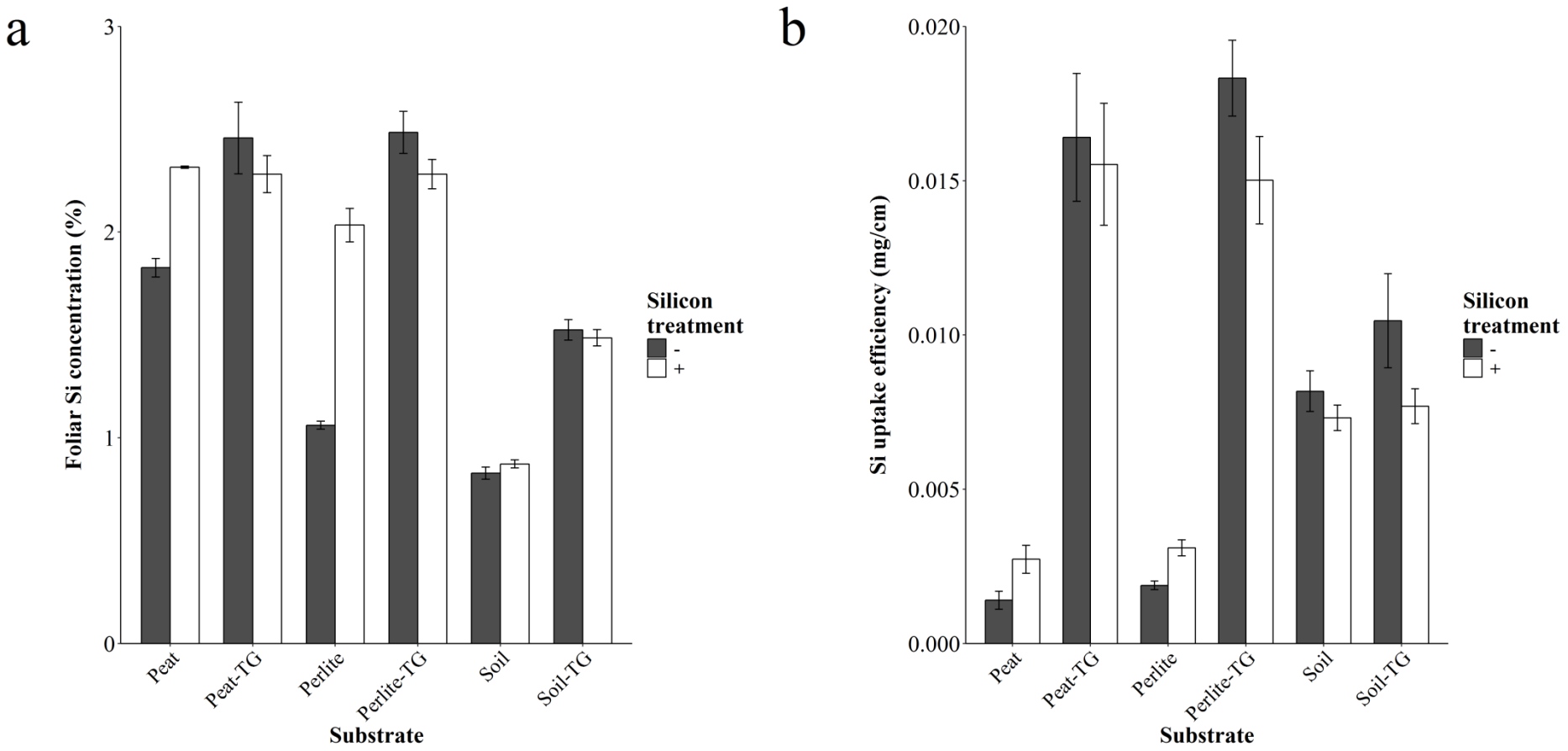

Figure 7: Foliar silicon (Si) measurements in winter wheat (*Triticum aestivum* var. Croft) grown in different substrate types; a) Foliar silicon (Si) concentration and b) Si uptake efficiency (elemental uptake per unit root length, as calculated by total foliar elemental content/root length (cm). Silicon (Si) treatment refers to the supply of Thornton's nutrient solution alone (Si-, grey bars) or with added soluble Si (Si+, white bars). Error bars represent standard error and n=8 for all treatments except for 'Peat' where n=3 for Si+ and Si

The effect of Si+ treatment was variable depending on the substrate type (Figure 7a). Plants grown in Peat and Perlite showed the most significant increases in foliar Si concentration as a result of the Si+ treatment (Peat: $F_{(1,4)}$=103.7, p<0.001; Perlite: $F_{(1,14)}$=172.42, p<0.001). Si concentration in plants grown in perlite roughly doubled with the application of the soluble Si solution (Si- 1.06%, Si+ 2.03%). When substrates were mixed with TG, there was no increase in Si concentration associated with the Si+ treatment, yielding similar concentrations to the Si- treatments (Figure 7). Plants grown in the soil substrate demonstrated the lowest Si concentrations.

To account for differences in biomass (Figure 6) Si uptake efficiency was calculated (Figure 7b). The only plants that demonstrated a significant improvement in uptake of Si due to the Si+ treatment were those grown in perlite ($F_{(1,14)}$= 16.85, p=0.001).The addition of terra-green improved the growth and Si uptake in both peat and perlite substrates, but this was not the case in the soil treatments.

2.1.4 *Discussion*

The addition of the Si+ nutrient solution had significant impacts on the pH of some substrates, particularly perlite and the soil-TG substrate mixture (Figure 5). pH appeared to be related to plant growth and the addition of TG, a clay substrate, buffered the pH of substrate. This concurrently led to improvements in plant growth and plant Si uptake efficiency in plants grown in peat and perlite substrates (Figure 6, Figure 7). This was not the case in plants grown in soil, where biomass decreased and Si uptake did not change with the addition of TG. However, pH cannot be the sole factor leading to the improvement in growth of plants grown in peat and perlite as growth and Si content and uptake were similar between the two despite their varying pH values (Figure 5, Figure 7 b). This may be due to TG improving the substrate structure and moisture content, although it should be noted that all plants received sufficient watering throughout.

There is also the potential of TG providing a source of plant-available Si, as TG is a clay mineral composed of magnesium-aluminium silicate and potentially not an inert substrate but capable of influencing plant growth and Si uptake. Interestingly, the addition of Si solution did not appear to improve the Si concentration of plants grown in substrates mixed with TG. Again, because TG is a clay-based substrate this may indicate that the Si was binding to the clay particles. The dissolution, or release of ions and Si into solution varies with pH, and for Si is highest at pH 4-7 which would explain the high Si concentrations in Peat, Peat-TG and Perlite, and also the low concentration of Soil and Soil-TG (Golubev *et al.*, 2006; Chen *et al.*, 2011). Plants grown in soil had the lowest Si concentration, but due to a lower root:shoot ratio compared to plants grown in other substrates actually outperformed peat and perlite substrates

in regards to Si uptake efficiency (Figure 7b). Excluding the treatments involving substrate mixture with terra-green, soil in isolation performed the best in terms of plant growth and Si uptake efficiency.

Aside from the quantitative results provided, there were some considerations regarding the harvesting process that influences the decision of substrates to take forward for mycorrhizal experiments. Harvesting plants from the peat substrate was complex due to the fine flecks of substrate becoming entangled in the roots and very difficult to remove compared to soil particles which easily washed away. Similarly, it was difficult to remove plant roots from the perlite substrate due to the roots growing through the perlite granules. This led to very time-consuming removal of roots in order to avoid damage so that accurate root length recordings could be obtained. This will be of particular concern in future experiments looking at the growth of mycorrhizas and their effects on plant root growth parameters.

Overall, taking into consideration the Si fertilisation by TG, yield penalties by single substrate treatments of peat and perlite and the complications arising from harvesting plants from these two substrates, soil appeared to the best substrate for use in future experiments. Sand and TG mixtures are traditionally used in mycorrhizal studies predominately to create low-nutrient conditions to encourage mycorrhizal colonisation and also to improve root removal at harvest. However, this experiment has demonstrated that the use of TG is not appropriate for Si manipulation experiments. Sand was not considered as an option due to the chemical structure containing Si. The use of soil also has the benefit of recreating more ecologically relevant conditions within glasshouse studies and so this will be the substrate taken forward for future experiments involving mycorrhizas and Si manipulation.

2.2 Plant host selection for mycorrhizal and silicon manipulation experiments.

2.2.1 *Winter wheat and mycorrhizal colonisation*

Rationale

Following on from the substrate experiment, an experiment to test the interaction between Si and arbuscular mycorrhizal fungal (AMF) colonisation on growth in winter wheat was set up. This experiment was devised to understand the change in Si accumulation in plants over time and whether mycorrhizal fungal colonisation affected Si uptake and accumulation.

Materials and methods

This experiment used a two way factorial design using application of a soluble Si application of $Na_2SiO_3.9H_2O$ at 1.5mM (Si+) or a dH_2O control (Si-) where pH of both Si- and Si+ treatments was altered to pH 7, and a commercial single species AMF inoculum, *Glomus intraradices* (syn. *Rhizophagus intraradices*, BEG72) accounting for 10% of total substrate volume (PlantWorks, UK). In addition there were three harvest time-points (4, 7, and 10 weeks after germination). The four treatments (AMF-/Si-, AMF+/Si-, AMF-/Si+, AMF+/Si+) were replicated seven times per harvest resulting in a total of 84 replicates over the course of the experiment plus eight sacrificial pots to check the progress of mycorrhizal colonisation.

At harvest, plant fractions were weighed and dried (70°C, 3 days). Fresh roots were subsampled for staining. These were cleared in 10% KOH for 3 days at 25°C, then rinsed with dH_2O and acidified for 1 hour in 1%HCl. Roots were stained in a 0.01% acid fuschin-lactic acid solution for 3days at 25°C before being rinsed once in dH_2O and stored in destain solution (1:1:14 dH2O:glycerol:lactic acid) until analysis. Samples were randomised and analysed blind for AMF intra-radical structures (arbuscules, hyphae, vesicles), at 100 fields of view per sample at x400 magnification to give a percentage of root length colonised (Nikon Eclipse 50i, Melville, NY) (McGonigle et al., 1990). Non-AM fungal root colonisation was also recorded.

Results

During the experiment sacrificial pots were sampled from to test for colonisation to inform future experimental harvest time points but no colonisation was observed in these at any point. Harvests were carried out as planned but very low mycorrhizal colonisation (<1% total root length) was recorded across the three harvest time points and across the treatments. Due to the lack of colonisation, Si analysis was performed on the control (AMF-/Si-) and Si supplemented (AMF-/Si+) alone to reduce experimental costs. Addition of Si solution did not lead to any significant differences in foliar Si concentration ($F_{(1,35)}$=3.60, p=0.066, Figure 8). Foliar Si

concentration did change over the time course of the experiment ($F_{(2,35)}$=11.53, p<0.001), increasing significantly between four week old plants to older plants (Tukey adjusted p: Week 7: p=0.028; Week 10: p<0.001), but there was no significant difference between seven and ten weeks (Tukey adjusted p: p=0.147, Figure 8). Similarly, there was a significant difference in biomass across the harvests ($F_{(2,37)}$=232.55, p<0.001), but no significant effect of Si treatment overall ($F_{(1,37)}$=2.89, p=0.098).

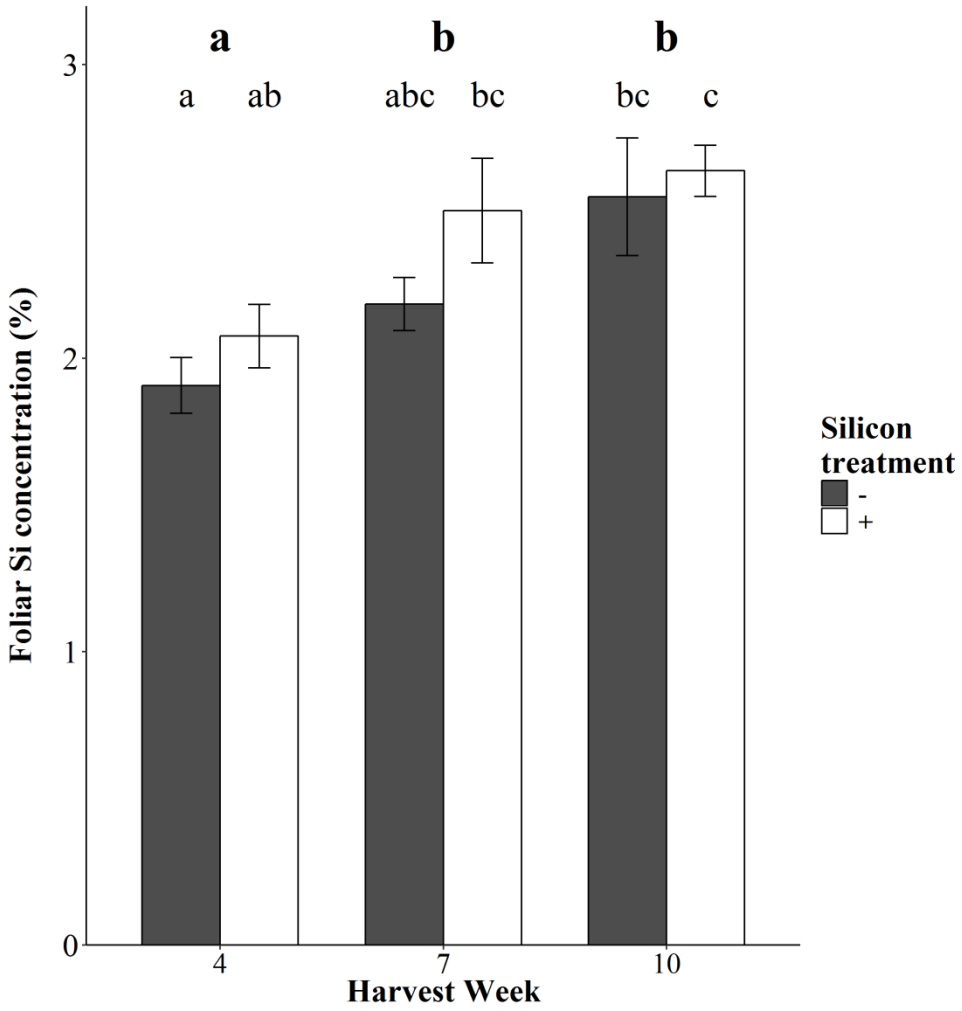

Figure 8: Foliar silicon (Si) concentration (%) of winter wheat (*Triticum aestivum* var. Croft) plants harvested at four, seven and ten weeks post-germination. The Si treatment was either a weekly application of 1.5mM Si solution (+, white bars) or an equivalent volume of deionised water (-, grey bars). Error bars represent standard error. The large, bold letters represent significant differences in Si concentration between harvests, the smaller letters represent significant differences between Si treatments across harvests.

Discussion

Overall this experiment demonstrated that Si addition alone was not necessarily beneficial to plant growth or foliar Si concentration in winter wheat plants. It also demonstrated that either this variety of winter wheat (var. Croft) was not mycorrhizal dependent, as different crop varieties can express different levels of mycorrhizal responsiveness (Lehmann *et al.*, 2012), or that the experimental materials (including the inoculum), setup and growth were not optimal for AMF colonisation. The quality of the mycorrhizal inoculum may be an issue also whereby colonisation by AMF may have been suppressed due to the presence of competing fungi in the plant roots, as non-mycorrhizal structures were noted in the mycorrhizal counts of experimental plants. Winter wheat was deemed an unsuitable plant to take forward for further experiments.

2.2.2 *Plant host screening experiment*

Rationale

Si enrichment in agricultural settings is increasing due to the substantial evidence of its benefit to plant growth across a number of different crops, and across a range of different stress conditions (Reynolds *et al.*, 2016). Enrichment of soil microbial communities in agricultural systems is also being investigated to provide plants with similar benefits in growth and defence since agricultural soils were show to be microbially depauperate (Helgason *et al.*, 1998; Thirkell *et al.*, 2017). Co-inoculation experiments investigating the dual benefits of Si and AMF in crop species have been published in recent years, demonstrating the novelty of this research area (Garg & Bhandari, 2015; Oye Anda *et al.*, 2016; Garg & Singh, 2017; Frew *et al.*, 2017b). This led to the selection of a widely used crop species as the focal species in this project; however the outcome of the previous experiment involving winter wheat was not successful in regards to AMF colonisation, which may be due to low mycorrhizal responsiveness. A transition to a non-crop species would generate more ecologically relevant results, and be important in understanding the interactions between AMF and Si uptake. This has relevance in natural systems, particularly with regards to herbivory defence and community structure which has important considerations for grassland conservation for example.

The aim of this experiment is to see how the growth and foliar Si concentration of four different native grasses is affected by colonisation by an AMF *Glomus intraradices* (BEG72, syn. *Rhizophagus intraradices*) and Si fertilisation. The results of this experiment will inform the study species of future experiments.

Materials and methods

Four different species within the Poaceae grass family were selected. *Brachypodium sylvaticum* Huds. Beauv., *Bromus erectus* Huds. (Syn. *Bromopsis erecta*), *Deschampsia cespitosa* L. P.Beauv., and *Lolium perenne* L.. These plants were chosen based on their life history traits, their amenability to colonisation by AMF and their varying Si content (Grime et al., 1988, Harley and Harley, 1987, Massey et al., 2007).

I. Preparation of biological materials

Plants were grown with addition of live (AMF+) or twice sterilised single species AMF inoculum (AMF-) and addition of Si solution (Si+) or an equivalent volume of deionised water (dH_2O) (Si-), resulting in four treatments (AMF-/Si-, AMF-/Si+, AMF+/Si-, AMF+/Si+). The AMF inoculum, *Rhizophagus intraradices* Walker & Schüßler (*Glomus intraradices* Schenck & Smith, (Schüßler and Walker, 2010)) isolate BEG72 was obtained from PlantWorks, UK in a zeolite-sand substrate containing spores and root fragments. The growth medium was field soil

from Yorkshire sterilised by two autoclave events (121°C, 72mins), with three days resting between and after autoclaving. The AMF inoculum for the AMF- treatment was also sterilised by the same method. To control for the effect of associated, non-AMF communities in the rhizosphere, the AMF inoculum was filtered (20µm mesh, followed by Whatman no.1) to obtain a bacterial filtrate, and 20ml was added to all pots to ensure uniform bacterial communities across all treatments. Seeds were sown directly in 0.5L pots, with 10% volume of AM inoculum, sterilised or live. The AMF inoculum (10% total pot volume) was added as a block, just under the soil surface based on advice received, rather than thoroughly mixing with the soil as was done previously in Section 2.2.1. This results in a dense patch of inoculum close to the location of the seed, increasing contact with the AMF propagules in the inoculum, and therefore increasing the chances of establishing a successful symbiosis. There were seven replicates per treatment (determined by an a priori power analysis test with an effect size of 0.25 (Thomas, 1997)), grown in controlled glasshouse conditions (15-20°C, 16:8 hrs light:dark), in a randomised block design. Treatments (AMF-/Si-, AMF+/Si-, AMF-/Si+, AMF+/Si+) were replicated for each grass species, therefore resulting in a total of 12 treatments, each replicated 7 times (n=84).

Si solution consisted of 1.5mM sodium silicate nonahydrate ($Na_2SiO_3.9H_2O$), with a pH amended using 0.5M HCl to match the pH of the dH_2O Si- treatment. Control treatments received equivalent volumes of dH_2O. Plants in the Si+ treatment received approximately 45mg of Si per week (exceeding natural soil concentrations and in line with similar studies (McKeague & Cline, 1963; Massey et al., 2006)), plus supplementary dH_2O when required.

At harvest leaf, shoot, and root fractions were separated and the latter rinsed thoroughly to remove residual soil. Root length was calculated by the gridline-intersect method (Tennant, 1975; McGonigle *et al.*, 1990). Root sub-samples were taken for mycorrhizal analysis, and their dry weights (DW) estimated for total root DW biomass (((remaining root fresh weight (FW) + root FW of AMF subsample) / remaining root FW) x remaining root DW).

II. Si and P measurement

Dried green leaf material was ground (Retsch MM400 Mixer mill, Haan, Germany) and pelleted (Specac Atlas™ manual 15ton hydraulic press, Kent, UK) for Si and P analysis using an X-ray Fluorescence (XRF) gun (Niton XL3t900 GOLDD Analyser, Thermo Scientific, UK) (Reidinger et al., 2012). The XRF gun was calibrated for measurements using Si-enriched methyl cellulose and validated using certified reference material from the China National Analysis Center for Iron and Steel; 'Bush branches and leaves' (NCS DC73349), and 'Spinach' (NCS ZC73013) for the Si and P concentrations, respectively (Reidinger *et al.*, 2012).

III. Symbiosis development

Roots were cleared in 10% KOH at 70°C for 30 minutes then rinsed thoroughly with dH_2O followed by staining in a 5% ink (Pelikan 4001 Brilliant Black)-5% acetic acid solution for 20 minutes also at 70°C. Root were then rinsed in 1% acetic acid solution and stored in destain solution (1:1:14 dH_2O:glycerol:lactic acid) until analysis. Prior to analysis, samples were randomised and assigned new IDs to avoid bias. Roots were mounted on glass slides in destain solution and analysed for intra-radical structures (arbuscules, hyphae, vesicles), at 150 fields of view per sample at x400 magnification to give a percentage of root length colonised (Nikon Eclipse 50i, Melville, NY,(McGonigle *et al.*, 1990)).

IV. Statistical analysis

Percentage data was arcsine transformed prior to statistical analysis. Analysis was conducted in R studio Version 3.1.3, (R Development Core Team, 2011; RStudio Team, 2016). Two Linear models were created using the lme4:lmer function (including the random term 'Block' to test for an effect of the block plants were grown in) or stats:lm function in R. The two models were compared using the Akaike Information Criterion (Akaike, 1973) to determine the model which best fits the data, and the model with the lowest score was selected for further analysis. The graphics:plot function was used to plot the data from the simple linear model to view the distribution of replicates and residuals. Data was checked for normality and homogeneity of variance using the Shapiro-Wilk normality test (stats:shapiro.test) on the residual data points of the simpler linear model and Levene's test for homogeneity of variance across groups (car:leveneTest), respectively. Data was normally distributed and the simpler model excluding 'Block' as the random term best described the data. Two-way analysis of variance (ANOVA) was carried out on mixed-effect linear models where the fixed effects were the AMF and Si treatments. Correlations were performed on transformed data using the Pearson product-moment correlation, calculated using the stats:cor.test function in R.

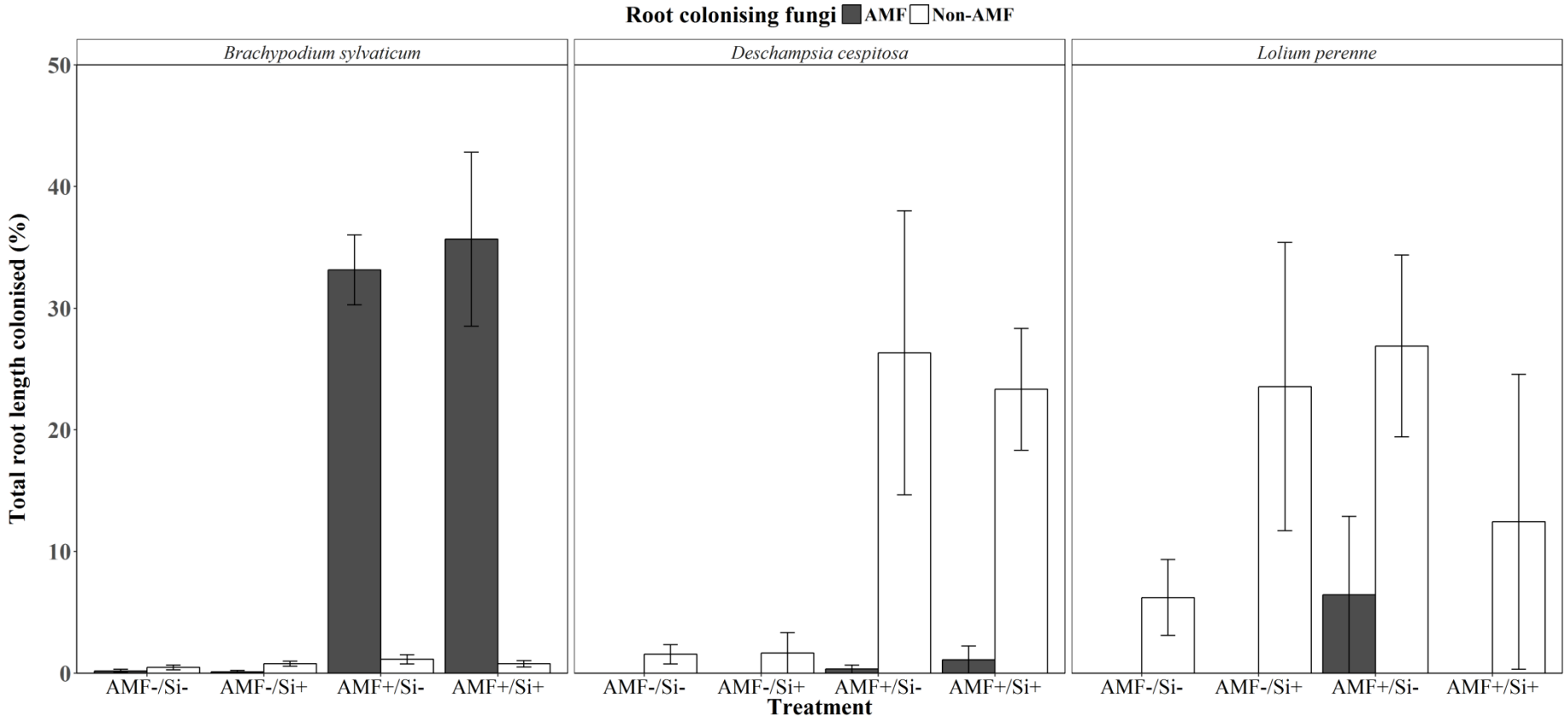

Figure 9: Total root length colonisation (%) of three grass species, *Brachypodium sylvaticm* (left panel), *Deschampsia cespitosa* (central panel), *Lolium perenne* (right panel) when grown with a live (AMF+) or sterilised (AMF-) arbuscular mycorrhizal inoculum and a silicon supplement (Si+) or water control (Si-). Grey bars represent colonisation by AMF, and white bars represent colonisation by non-AMF, error bars represent standard error.

Results

B. erectus seeds did not germinate under the conditions of this experiment after three weeks in the glasshouse. The viability of the seeds was checked on growth on moistened filter paper in Petri dishes and no growth was observed in these circumstances either. Each plant species had a different growth and emergence rate and was therefore harvested at different times to balance the risk of plants becoming pot bound with plants having insufficient biomass to be able to produce pellets for Si analyses. *L. perenne* was the most rapidly growing species and was harvested 10 weeks post-germination. *B. sylvaticum* was harvested 11-12 weeks post-germination and *D. cespitosa* 18 weeks post-germination. Roots were analysed for mycorrhizal structures, but only *B.sylvaticum* plants showed significant colonisation by AMF. Both *L. perenne* and *D. cespitosa* had within the region of 20-30% of total root length was colonised by non-AMF fungal structures, and on average 1-2% AMF colonisation (Figure 9).

Discussion

The failure of *B. erectus* to germinate and the lack of mycorrhizal colonisation in *L. perenne* and *D. cespitosa* only left *B. sylvaticum* as a viable option as a host plant to be used in future experiments. The three data chapters 3, 4, and 5 all focus on the relationship between Si and fungal colonisation in *B. sylvaticum*.

This experiment also had an impact on the methods used in subsequent experiments to introduce fungi to experimental pots. The low rates of colonisation by AMF coupled with the presence of relatively high root colonisation by other non-mycorrhizal fungi across a range of different plants led to questions about the quality of mycorrhizal inocula used in the experiment. The mycorrhizal inoculum is clearly the largest source of contaminants or non-target fungi given the lower counts in AMF- treatments. Analysis of root samples was conducted blind after samples were randomised and given arbitrary sample names to avoid bias in the counting stage. The following section discusses the implications of using commercial and stock pot inocula.

The full analysis of this experiment, focussing on *B. sylvaticum* is presented in Chapter 3.

2.3 Verifying mycorrhizal cultures

2.3.1 *Rationale*

The abundance of non-target fungal structures in experimental plant roots that was seen in early experiments was of concern. Mycorrhizal trap pots or stock pots are used to bulk up mycorrhizal inoculum using plants with high mycorrhizal susceptibility. This section details the molecular analysis of fungi in plant roots of existing stock pots and of a new stock pots set up containing the PlantWorks inoculum used in the previous two experiments (Sections 2.2.1 and 2.2.2).

2.3.2 *Materials and methods*

Mycorrhizal stock setup

A 50:50 (v:v) mixture of sand and terragreen (TG, attapulgite clay) was dampened with distilled water and sterilised by autoclaving (121°C, 72mins). Plant pots were sterilised by immersion in 8% Haychlor bleach overnight (~16hours) and rinsed thoroughly prior to potting. *Plantago lanceolata* L. seeds were sterilised by immersion and shaking in a 4% Haychlor solution for 2 minutes, and thoroughly rinsed with deionised water (dH_2O) to remove residual bleach. For each 1L pot, a 20µm mesh was cut to size and fitted in the base of the pot, 0.25g of bone meal and ~50g of original AMF inoculum (roots, spores and substrate) was mixed thoroughly with 1L of autoclaved sand:terragreen mixture and added to the pots. Ten seeds of *P. lanceolata* were added to the pots and pots were inserted into sunbags (0.2µm pore; Sigma-Aldrich (Merck), MO, USA) to prevent cross contamination, labelled and transferred to the mycorrhizal stock glasshouse (15-20°C, 16:8 light:dark). A further three stock pots were sourced from existing stocks at the University of York including a *Glomus mosseae*, *Gigaspora rosea* and an additional *G. intraradices*. The other stock pots were set up by other PhD students and researchers (~1-2 years previously) using the same method detailed above.

Mycorrhizal inocula origin and sampling

The single species mycorrhizal inoculum from Plant Works Ltd. (Kent, UK) was used directly from the source material as an inoculum source in the previous two experiments (Sections 2.2.1, 2.2.2). A sample of the original inocula in a terragreen-vermiculite (1:1, v:v) substrate was used to set up stock pots to produce further inocula for follow up experiments. The *G. mosseae* stock pot was also originally sourced from PlantWorks while the *Gig. rosea* and *G. intraradices* were from stocks originating from BioRhize Ltd. (Dijon, France), which has ceased to operate. Although the taxonomic names have been overviewed and updated recently (Schüßler & Walker, 2010), this chapter will refer to the stocks as they were identified by the companies that supplied them.

Sampling

Root samples were taken using a 2cm diameter core inserted half way down the pot, generating approximately 6cm depth of substrate and root mixture. The corer was washed thoroughly before and between samples with water followed by 70% ethanol. Roots were extracted from the substrate and washed before drying in a 70°C oven for 3 days. Samples were transferred to Eppendorf tubes with 2mm stainless steel ball bearings and milled for 1 minute (24 Hertz; Retsch MM400, Haan, Germany).

DNA extraction and Polymerase Chain Reaction (PCR)

DNA was extracted from milled root material using the PowerPlant™ DNA isolation kit (MO BIO Laboratories, Inc., CA, USA) following the manufacturers protocol with an additional step for phenolic removal. DNA was eluted in 50µl of kit buffer and concentrations were determined by NanoDrop ND-8000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). DNA was stored at -20°C until PCR amplification using a Techne TC-512 thermocycler (Bibby Scientific, Staffordshire, UK). The ribosomal RNA (rRNA) Internal Transcribed Spacer (ITS) region was amplified using the ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATG-3′) primer pair (Gardes & Bruns, 1993). Each PCR reaction mixture contained 5µl 5xGo-Taq Flexi buffer, 2µl 25mM $MgCl_2$, 0.5µl 10mM dNTP mix, 0.5µl of each primer, 0.125µl GoTaq Flexi DNA polymerase, 15.875µl dH_2O and 0.5µl template DNA. Samples were initially denatured for 4 minutes at 94 ºC, followed by 32 cycles of 94 ºC for 40 seconds, 52 ºC for 40 seconds, and 72 ºC for 60 seconds, concluded by a final extension at 72 ºC for 10 minutes. Products were checked by separation on 2% agarose gel by electrophoresis. PCR products were purified with the QIAquick PCR purification kit **(**Qiagen, CA, USA).

Bacterial cloning

DNA concentrations of the purified PCR products were quantified using the NanoDrop ND-8000 Spectrophotometer to calculate the amount of PCR product required for the ligation reaction. PCR products were mixed in a 3:1 ratio with a vector (pGEM-T Easy Vector, Promega) calculated independently for each sample to control for different DNA concentrations. Each ligation reaction contained 5µl of 2x Rapid ligation buffer, 1µl of pGEM-T Easy Vector, 1µl of T4 DNA ligase and varying volumes of the PCR product dependent on sample concentration, and dH_2O to make up to the final 10µl volume. These reactions were incubated at 4°C overnight. 50µl (100µl control) of Bacterial JM109 high-efficiency bacterial cells were added to Eppendorf tubes containing 2µl of the incubated ligation reaction, these reactions were heat shocked at 42°C for 45-50 seconds and returned to ice for two minutes. 950µl (900µl control) of Super Optimal Broth with added glucose (SOC) was added and reactions were

shaken for 1.5hours at 37°C. 100µl of reaction mixture was plated onto agar enriched with antibiotic ampicillin and X-gal for blue/white screening. Plates were incubated overnight at 37°C and white colonies demonstrating successful transformation were selected for secondary PCR under the same conditions described above. This was performed for six samples, two plates per sample and five colonies selected from each plate. PCR products were cleaned as above, DNA concentrations verified and samples diluted to 1ng/µl per 100bp for Sanger sequencing carried out by Source Bioscience (Cambridge, UK).

Sequence processing

Sequence identity was checked using the NCBI Basic Local Alignment Search Tool (BLAST) for nucleotide sequences. Sequences were trimmed using BioEdit v7.2.5 (Hall, 1999) and aligned using MEGA v6.06 (Tamura *et al.*, 2013). A Neighbour-Joining tree with five hundred bootstrap replications was generated using the MEGA package.

2.3.3 *Results*

Of the six pots that were analysed (three of *Glomus intraradices*, one of *Gigaspora rosea* and two of *Glomus mosseae*) only two produced sequences corresponding to arbuscular mycorrhizal fungi. One of the *G. intraradices* (2_BioRhize_*Glomus intraradices*, Figure 10) pots was dominated by sequences of *F. mosseae,* not the intended species. Another pot set up from the same initial starting inoculum (1_BioRhize_Glomus intraradices) however did not generate any mycorrhizal sequences. Similarly *G. rosea* (4_BioRhize_*Gigaspora rosea*) produced a sequence that corresponded to *Rhizophagus irregularis,* not the intended species (Figure 10).

Sample three (3_PlantWorks_*Glomus intraradices*), was the most recently sourced stock that was analysed, and was derived from the same material used as a mycorrhizal inoculum in Sections 2.2.1 and 2.2.2. No mycorrhizal sequences were identified in this analysis coming from this stock, but rather the sequences generated were dominated by isolates from the *Fusarium* genus, and including one sequence identified as *Serendipita* sp. (syn. *Piriformospora*). *Fusarium* is a large genus containing saprophytic and pathogenic fungi while *Serendipita* is typified by *S. indica* a plant growth promoting endophytic fungus. Other frequently isolated species across other samples included *Gibellulopsis nigrescens* (syn. *Verticillum nigrescens*), a common plant pathogen, and isolates from the *Myrothecium* genus another potential plant pathogen.

2.3.4 *Discussion*

Overall, this analysis was dominated by a few species, mostly non-mycorrhizal. It could be argued that glasshouse contamination could be causing non-mycorrhizal fungi to become dominant in these pots. However, if this was the sole reason for the contamination of the pots,

then it would be expected that the sequencing results would be uniform across all samples. However this is not strictly the case and replicates of the same pots appear to cluster together, which indicates that the source of contamination may have arisen from the production stage in the suppliers systems. For example, the sequences generated from the *Gig. rosea* pot were unique in producing sequences of an *Alternaria* sp. and *Mortierella alpina* that were not found in other samples. However, some pots that have been set up with the same starting inocula have produced different results (i.e. comparing 1 and 2_BioRhize_*Glomus intraradices*), and others have produced similar sequencing outputs (i.e. 5 and 6_PlantWorks_*Glomus mosseae*; Figure 10). This may reflect the ages of the cultures, or the difference in techniques used in the setup, as these were set up by different people.

Overall, it appears that mycorrhizal stocks, independent of age and origin, contain a variety of fungi potentially at higher abundance in the roots than the target mycorrhizal species. While some stocks did contain mycorrhizal sequences, these were not the species that were meant to be in the cultures. While some stock pot contents are checked through staining and visualisation with microscopy prior to their use as a source of mycorrhizal inoculum, distinguishing between species of AMF in the roots is difficult to do visually (Merryweather & Fitter, 1998). This suggests that without the use of molecular quantification it may be difficult to be certain of the contents of stock pots. The presence of pathogenic fungi is of concern, but potentially less significant than the presence of a plant-growth promoting fungus from the *Serendipita* genus. The inclusion of this in mycorrhizal experiments may result in it contributing to beneficial plant response in a way that makes it difficult to disentangle from the effect of mycorrhizal fungi. The colonisation of roots by AMF is said to be opportunistic and different plant species and varieties have different mycorrhizal responsiveness and susceptibility to disease. A combination of these two factors and the overall absence of sequences corresponding to AMF in the PlantWorks *G. intraradices* inoculum that was used as the AMF source in previous experiments may reveal why colonisation was so low. This review of single species mycorrhizal stocks highlights the importance of verifying the contents of mycorrhizal inoculum sources, including those supplied direct from the producers, prior to experimentation.

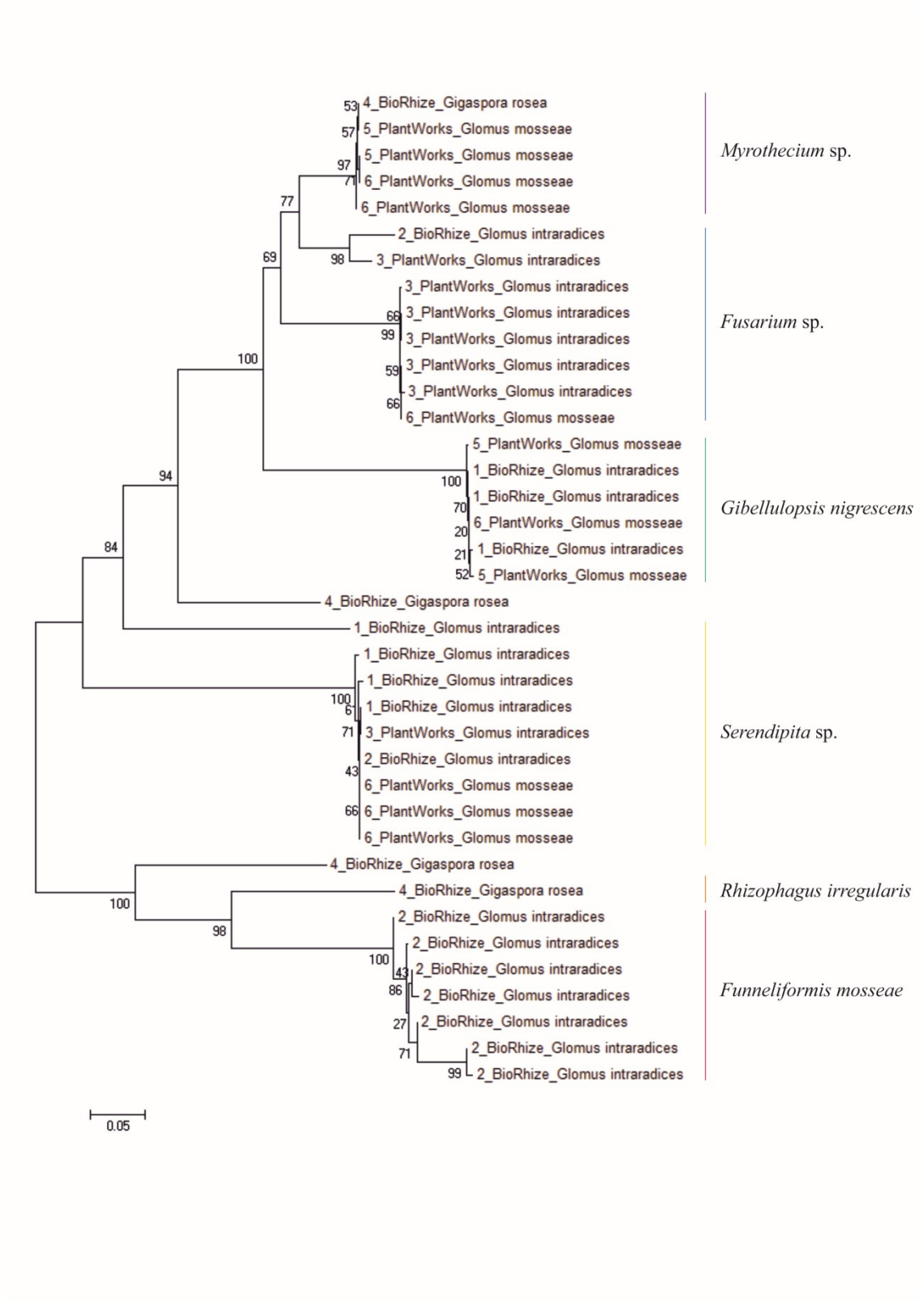

Figure 10 Neighbour-joining tree displaying relationships between sequences from different mycorrhizal stocks. 'BioRhize' and 'Plant Works' refer to the source company and the intended cultured species, *Glomus intraradices, Glomus mosseae* and *Gigaspora rosea*. Clusters are annotated on the right hand side with Blastn assigned taxonomy. Bootstrap values indicate support for nodes.

2.4 Overview of High-Throughput Sequencing methods

This section is included to present an overview of the methodology involved in the preparation, sequencing and bioinformatics of samples and data for the Illumina MiSeq sequencing platform that was used in Chapters 4 and 5.

2.4.1 *Sample preparation*

30-40mg of dried (70°C 3 days) root material was milled (Retsch MM 400, Haan, Germany) with two steel grinding balls (1cm diameter) in a 25ml steel capsule (2 minutes, vibrational frequency 24 Hertz). DNA was extracted using PowerPlant™ DNA isolation kit (MO BIO Laboratories, Inc., CA, USA). DNA was eluted in 50µl of kit buffer and concentrations were determined by NanoDrop ND-8000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). DNA was stored at -20°C until PCR amplification using a Techne TC-512 thermocycler (Bibby Scientific, Staffordshire, UK). DNA amplification was carried out in a nested PCR approach to produce enough product for sequencing. The DNA regions of interest were initially amplified using the general eukaryotic NS31 (5'-TGGAGGGCAAGTCTGGTGCC-3', Simon *et al.*, 1992)) and ITS4 (3'-TCCTCCGCTTATTGATATGC-5', Gardes & Bruns, 1993)) primer pair, which covers the 18s small subunit (SSU), ITS1, 5.8s and ITS2 regions of the rRNA operon therefore creating a high volume of template product for the nested PCR. Products of this reaction were diluted 1:100 and used for PCR amplification using general fungal primers adapted with forward and reverse Illumina Nextera adapter and linker sequence (ITS1F-ITS4) and the V4 region of the 18s small subunit (SSU) AMF specific region (NS31 (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGANNWNNNHTTGGAGGGCA-3')-AM1 (Helgason *et al.*, 1998).

Problematic PCR reactions were used where the basic PCR recipe did not produce product, or weak bands. PCR products were purified with the GenElute™ PCR Clean-Up kit (Sigma-Aldrich, St Louis, MO, USA) and DNA concentration determined by Invitrogen™ Quant-iT™ DNA high sensitivity assay Kit (Invitrogen, Life Technologies, Carlsbad, CA, USA). Sample concentrations were adjusted so all template was at the same concentration (2ng μl^{-1}) in a 10µl volume. These purified products served as the DNA template for barcoded high-throughput sequencing on the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) at the University of York, UK.

Table 2: Details of the reaction mixture and PCR cycles used on fungal DNA extracts for the initial long-read PCR (NS31-ITS4) and the PCR amplification using Illumina MiSeq specific primers (ITS

region: I11-I14, AM region: I13-I15). Kits used were Q5 ® High-Fidelity DNA Polymerase (New England Biolabs, MA, USA) and GoTaq® G2 Flexi DNA Polymerase kit (Promega, WI, USA).

Primer pairs	**Primary long-read PCR NS31-ITS4**	**Illumina MiSeq ITS1F(I11)-ITS4(I14)**	**NS31(I13)-AM1(I15)**
Reaction Mixture (25µl)	5µl 5x GoTaq® Flexi buffer	5µl 5x Q5 reaction buffer	
	2µl 25mM $MgCl_2$		
	0.5µl 10mM dNTP mix	0.5µl 10mM dNTP mix	
	0.5 µl each primer	0.5 µl each primer	
	0.125µl GoTaq® Flexi DNA polymerase	0.125µl High-Fidelity DNA polymerase	
	15.375µl dH_2O	17.375µl dH_2O *(*problematic samples: 14.875µl)*	
	1µl template DNA	1µl template DNA (diluted 1° PCR) **problematic samples: + 2.5µl BSA*	
PCR Cycle			
Initial denaturation	5min at 94°C	5min at 95°C	5min at 95°C
Cycles	30	20	20
Denaturation	30sec at 95°C	30sec at 94°C	30sec at 94°C
Annealing	30sec at 55°C	40sec at 55°C	40sec at 57°C
Extension	2min at 72°C	1m10sec at 72°C	1m10sec at 72°C
Final extension	5min at 72°C	10mins at 72°C	10min at 72°C

2.4.2 *Illumina Miseq sequencing*

Amplicons were tagged with the Illumina adapter sequence were then subject to a final round of amplification to add the unique barcode sequences. Eight cycles of PCR amplification were performed using NEBNext® High Fidelity Q5 Polymerase 2X mastermix (New England Biolabs, Ipswitch, MA) and Illumina Nextera® XT index primers (Illumina, USA). Amplicons were purified using 0.9 x by volume AMPure XP beads (Beckman Coulter, High Wycombe, UK) and eluted into low TE buffer before quantitation and pooling at approximately equimolar ratios. Samples were diluted to 4nM before denaturing with 0.2N NaOH ready for loading at ~10 pM, with a 20 % PhiX library spike (Illumina; for added sequence variety). Amplicon pools were sequenced on an Illumina MiSeq with a 600 cycle kit. The fastq (fasta files with integrated quality information) were created via the generation workflow in Illumina's BaseSpace Sequence Hub.

2.4.3 *Sequence processing*

Downstream sequence processing was carried out in PuTTY/Unix using the QIIME v.1.9.1 analysis pipeline (Caporaso *et al.*, 2010). The full pipeline for processing sequences ready for diversity analyses is detailed in Table 3. The databases used for the amplicons differed, ITS sequences were referenced against the general fungal UNITE database (v7.1, Kõljalg *et al.*, 2005; Abarenkov *et al.*, 2010) while AMF sequences were referenced against the Glomeromycota specific MaarjAM (v.0.8.1, Öpik *et al.*, 2010) database. For the sorting stage

(Step 18-19, Table 3) OTU sequences were aligned using the EMBL-EBI online Clustal Omega tool (Goujon *et al.*, 2010), which generates a simple distance phylogeny and a percentage identity matrix. OTUs that had high similarity (>90% for ITS, >95% for AM amplicons) were highlighted and taxonomy checked with BLAST (search parameters excluded environmental samples). Multiple OTUs were collapsed into a single record based on ID, and read counts aggregated if the BLAST outputs led to the same taxonomic outcome at high confidence (identical accessions or identity at >97% QC and ID in BLAST). Taxonomic attributes of OTUs previously assigned as 'unidentified' or 'unassigned' were edited to reflect the findings of the BLAST search and non-fungal OTUs were removed. Trophic guilds were assigned to group OTUs into ecologically meaningful categories using FunGuild (Nguyen *et al.*, 2016).

Table 3: QIIME analysis pipeline

	Action	Function	Description

1	Strip off 1st 13 bases	`usearch8`
		`-fastq_filter "input file name.fastq"`
		`-fastq_stripleft 13`
		`-fastqout "output file name.fastq"`
		(repeat for all sequence files)
			Remove the randomised bases added to sequences used in amplicon sequencing, so that sequences can be distinguished.

2	De-multiplexing sample files	`convert_fastaqual_fastq.py`
		`-f "input file name.fastq"`
		`-c fastq_to_fastaqual`
		(repeat for all sequence files)
			Raw fastq file (-f) contains sequence and quality information. These need to be "de-multiplexed" and split into two files, a .fastqual (quality data) file and a sequence file (.fna).

3	Create mapping files	`#SampleID  BarcodeSequence LinkerPrimerSequence`
		`E4AM  TTGGAGG  GCAAGTCTGGTGCC`
		`E4ITS TCCGTAG  GTGAACCTGCGG`
		
		(Create a separate file for each sample)
			Create a mapping file so that QIIME can distinguish between the different amplicons you've used. There is no barcode, but this assigns the first 7 bases of the primer sequence as the barcode sequence and then the rest as a linker sequence.

4	Filters out poorer sequences, and changes sequence headers	`split_libraries.py`
		`-b 7`
		`-m Step3_maps/eh1map.txt`
		`-f Step2/EH1_S66_L001_R1_001.fna`
		`-q Step2/EH1_S66_L001_R1_001.qual`
		`-o "Step4/EH1"`
		(repeat for all sequence files)
			b is barcode, defined in Step 3 (7 bases long)
			-m is the mapping file location from Step 3 (.txt)
			-f is the sequence file from Step 2(.fna)
			-q is the file with quality information from Step 2 (.fastaqual)
			-o is the file path where to create a new directory for the output (a .fna file and quality records)

5	Creates separate .fasta	`split_sequence_file_on_sample_ids.py` `-i "Step4/EH1/seqs.fna"`	To pull the two amplicon AM and ITS sequences into separate files.

	files for each amplicon within a sample	`-o "folder name"` (repeat for all sequence files)	

6	Merges all samples by amplicon to generate one file per amplicon	`cat EH1_AMF.fasta EH2_AMF etc.`
		`> concatenatedAMF.fna`
		(repeat for other amplicon)
			File names to be separated with a space only, no returns or lists.

The next five steps are to rename the within file sequence headers to make them compatible with later analyses.			
7a	`sed 's/>/>barcodelabel=/g' concatenatedAMF.fna > step7a_AMF.fna`		
7b	`sed 's/_/;/g' step7a_AMF.fna > step7b_AMF.fna`		
7c	`sed 's/ M/z/g' step7b_AMF.fna > step7c_AMF.fna`		
7d	`sed 's/z.*//' step7c_AMF.fna > step7d_AMF.fna`		
7e	`sed 's/;201/1/g' step7d_AMF.fna > step7e_AMF.fna`		
8	Groups identical seqeunces	`usearch8 -derep_fulllength step7e_AMF.fna -fastaout step8_AMF.fa -sizeout`	Input concatenated file with new headers, -fastaout to define new file name.
9	Sort clusters by size	`usearch8 -sortbysize step8_AMF.fa -fastaout step9_AMF.fa -minsize 2`	Groups sequences together based on size, excludes singletons or samples with fewer than two records (minsize 2).
10	Removes	AMF:	For AMF sequences, the MaarjAM database of Glomeromycotan sequences, and

	chimeric sequences	`usearch8 -uchime_ref step9_AMF.fa -db qiime_MaarjAM-master/maarjAM.5.fasta -strand plus -nonchimeras step10_AMF.fa`	ITS uses the UNITE database of general fungal sequences.
11	Alter OTU labels	`python fasta_number.py step10_AMF.fa OTU_ > step11_AMF.fa`	Re-numbers OTUs after the previous re-ordering and sorting steps
12	Maps new OTU file onto older file version for sample information	`usearch8 -usearch_global step7e_AMF.fna -db step11_AMF.fa -strand plus -id 0.97 -uc step12_AMF.uc`	Input file is the output of Step7e, the complete concatenated table prior to filtering. The ID parameter dictates 97% similarity between sequences. –uc is the output in the form of a mapping file for the next step.
13	Creates an OTU table	`python uc2otutab.py step12_AMF.uc > step13_AMF.txt`	The mapping file from step 12 is used to generate an OTU table.
14	Convert from .txt to .biom file	`biom convert -i step13_AMF.txt -o step14_AMF.biom --table-type "OTU table" --to-json`	The following steps require a specific .biom file format so this converts the step13 product from a text to biom file.
15	Assign taxonomy to sequences	`assign_taxonomy.py -i step11_AMF.fa -o step15_AMF/ -r maarjAM.5.fasta -t maarjAM.id_to_taxonomy.5.txt`	This stage compares the sequence file from Step 11 to the reference databases fasta files (AMF: MaarjAM, ITS: Unite) to assign taxonomy to the sequences.-o is the file path for a new directory with the taxonomy assignments and a log file.
16	Add meta data to file	`biom add-metadata -i step14_AMF.biom -o S16_AMF.biom`	This adds the sample information from the mapped sequence file from Step 14. Will create a table of OTUs counts for each sample.

		`--observation-metadata -fp step15_AMF/step11_AMF_tax_assignments.txt --observation-header OTUID,taxonomy,confidence --sc-separated taxonomy --float-fields confidence`	
17	Remove non-fungal samples	`filter_taxa_from_otu_table.py -i step16_AMF.biom -o step17_AMF.biom -n k__Plantae,k__Protista,k__Protozoa`	Filters the OTU table, removing any samples that have been assigned as plant, protist or protozoan by the reference databases. The unite database contains a minimal amount of sequences on non-fungal samples, but this will remove them if present.
18	Convert from .biom to .txt	`biom convert -i step17_AMF.biom -o step18_AMF.txt --table-type "OTU table" --to-tsv`	This needs to be converted to a text file so that the information can be visualised and extracted for the next step.
At this stage many sequences will be "unassigned" because the databases are limited and there may be unassigned non-fungal sequences that need to be removed. Sequences are checked using NCBI BlastN database to assign taxonomy manually, non-fungal samples removed, and similar samples collapsed. The Step 15 taxonomy file is also updated and re-imported for diversity analyses.			
19	Convert from .txt to .biom	`biom convert -i s18_AMF_curated.txt -o s19_AMF.biom --table-type "OTU table" --to-json`	Import the reduced and curated OTU table and convert from .txt to .biom file
20	Add sample metadata	`biom add-metadata -i step19_AMF.biom -o step20_AMF.biom`	This is the same as Step 16, but is necessary to re-add the metadata to the OTU table since both files have been altered.

		`--observation-metadata -fp step15_AMF/step11_AMF_tax_assignments.txt --observation-header OTUID,taxonomy,confidence --sc-separated taxonomy --float-fields confidence`	
21	Remove rare samples	`filter_otus_from_otu_table.py -i step20_AMF.biom -o step21_AMF.biom --min_count_fraction 0.00005`	This removes very rare samples which become problematic for normalisation techniques and can skew the data. This filters by total abundance of each OTU and removes if present at frequencies of less than 0.005% as recommended in documentation for Step 22.
22	Normalise table	`normalize_table.py -i step21_AMF.biom -a CSS -o step22_AMF.biom`	This script normalises the abundance data and is used as an alternative to rarefying samples which can remove an excessive amount of data (McMurdie *et al.*, 2014). The method CSS (cumulative sum scaling) is from the metagenomeSeq package(Paulson *et al.*, 2013)
23	Assign trophic guilds	`python Guilds_v1.0.py -otu step21_plustax.txt -db fungi -m -u`	This assigned fungal trophic guilds to the OTUs. It requires an OTU table with the taxonomic assignments included in the same file, so a new column with these is added with the header ‘taxonomy’.

3 Colonisation by *Rhizophagus irregularis* increases root uptake efficiency of silicon and phosphorus in *Brachypodium sylvaticum*

3.1 Introduction

Arbuscular mycorrhizal fungi (AMF) are obligate plant symbionts that colonise the roots of over 80% of land plants (Smith & Read, 2010). AMF exchange plant growth limiting nutrients, predominately P collected from the soil for photosynthetic carbon (Parniske, 2008). AMF mobilise P in the soil by forming networks of fine hyphae or extra-radical mycelia (ERM), which extend beyond the rhizosphere and penetrate small soil pores, thereby evading the P depletion zone that forms around plant roots, and indirectly improving soil nutrient retention (Rhodes & Gerdemann, 1975; Smith & Smith, 2011; Cavagnaro *et al.*, 2015). Aside from P uptake, AMF are capable of directly transporting other compounds to the plant, including water and nitrogen (N) (Dietz *et al.*, 2011; Hacquard *et al.*, 2013; Hodge & Storer, 2014).

The mechanism by which H_2O is taken up by AMF hyphae is mediated by fungal aquaporin (AQP) channels present in the hyphal cell walls (Giovannetti *et al.*, 2014). Six AQP from two AMF species, *Glomus intradices* (syn *Rhizophagus intraradices,* Aroca *et al.*, 2009; Li *et al.*, 2013b) and *R. clarus* (Kikuchi *et al.*, 2016) have been identified to date and primarily control hyphal supply of water to plants (Li *et al.*, 2013a; Calvo-Polanco *et al.*, 2014), but have also been linked to the facilitation of N and P transport through fungal hyphae (Kikuchi *et al.*, 2016). AMF colonisation can also have a significant impact on the expression of certain plant AQP genes, the directional response of which is dependent on plant and fungal species combinations and application of biotic and abiotic stresses (Uehlein *et al.*, 2007; Armada *et al.*, 2015).

Silicon (Si) uptake by plants is a multistage process, initially achieved by uptake of silicic acid ($Si(OH)_4$) from the soil through an AQP channel, a ubiquitous membrane protein in plants (Ma *et al.*, 2006; Maurel *et al.*, 2015). Colonisation by AMF has been shown to stimulate alterations in AQP gene regulation and channel gating in all five AQP groups, including the NIP sub-family, to which the Si channel, Lsi1 belongs (Uehlein *et al.*, 2007; Giovannetti *et al.*, 2012). There has been some indications that AMF colonisation may improve Si concentrations within plants under different conditions (Yost & Fox, 1982; Kothari *et al.*, 1990; Hammer *et al.*, 2011). Within the last few years several studies have directly measured Si concentrations in plants with an arbuscular mycorrhizal (AM) symbiosis, although the mechanism for improved uptake is still unknown (Garg & Bhandari, 2015; Oye Anda *et al.*, 2016; Garg & Singh, 2017; Frew *et al.*, 2017b).

The ability of AMF to improve plant Si concentration has significant implications for understanding species interactions and plant defence in natural environments as well as informing sustainable agricultural practice. Si has been shown to have a number of functional roles in plants (Cooke & Leishman, 2011; Cooke *et al.*, 2016), but one of the most important is as a defence against herbivores (Reynolds *et al.*, 2009; Hartley & DeGabriel, 2016) and pathogens (van Bockhaven *et al.*, 2013). Deployment of these defences depends in part on the availability of $SiOH_4$ in the soil (Garbuzov *et al.*, 2011) which is in turn affected by numerous environmental factors, such as soil microbial (Alfredsson *et al.*, 2016) and faunal (Bityutskii *et al.*, 2016) communities, pH (Gocke *et al.*, 2013), and litter degradation (Carey & Fulweiler, 2016). The use of AMF as a method to improve Si concentration is now being considered, particularly as Si does not decrease the ability of AMF to colonise plants as previously thought (Garg & Bhandari, 2015; Maurel *et al.*, 2015).

This existing work on the impact of AMF colonisation on Si uptake has been carried out exclusively in agricultural species, but native grasses are also Si accumulators (Massey *et al.*, 2007a) and if AMF can increase plant Si accumulation in natural grasslands, there are important implications for plant-herbivore interactions (Hartley & DeGabriel, 2016). In this paper we present the results of the first experiment testing how Si uptake of the native grass *Brachypodium sylvaticum* Huds.(Beauv.) changes with AMF colonisation and Si addition. It was hypothesised that Si and AMF addition will independently improve Si uptake and foliar concentration, and that these effects will be additive, in line with previous studies. A secondary hypothesis that foliar P concentration will improve with colonisation by AMF was also tested by comparing the Si and P concentration and growth parameters of *B. sylvaticum* plants grown in a fully factorial randomised block design experiment.

3.2 Materials and methods

The methodology used in this chapter follows that detailed in Chapter 2 Section 2.2.2. Based on the positive colonisation results of the *Brachypodium sylvaticum* plants, this chapter focusses on further analysis of this species alone. In summary, there were four treatments (AMF-/Si-, AMF-/Si+, AMF+/Si-, AMF+/Si+): addition of live (AMF+) or twice sterilised single species AMF inoculum (AMF-) and addition of Si solution (Si+) or an equivalent volume of deionised water (dH_2O) (Si-), resulting in four treatments. Each replicated 7 times resulting in 28 *B. sylvaticum* plants grown. These were grown in a randomised block design interspersed with the other three species documented in Chapter 2 (Section 2.2.2). Statistical analyses are as described in Chapter 2, Section 2.2.2. The data fit a normal distribution and homogeneity of variance, and therefore two-way ANOVA and correlations were able to be carried out.

3.3 Results

3.3.1 *Plant growth and colonisation*

Plants of the AMF- treatments showed no presence of AMF structures under microscopic analysis, but both AMF+ treatments had a substantial level of root length colonised (RLC) by AMF structures and no detrimental effect of Si application on RLC was observed (Table 4). Root biomass was significantly affected by AMF colonisation, decreasing on average to 11% of the total plant biomass, compared to 30% in non-colonised plants (Table 4). Specific root length can be used as a proxy for root structural characteristics, with higher values indicating a finer root system (more length (m) per g), and lower values representing a root system formed primarily of coarser or thicker roots (less length (m) per g). Colonisation by AMF increases specific root length relative to non-colonised treatments, demonstrating that AMF have a significant impact on root structural characteristics leading to the development of a root system dominated by fine roots (Table 4). While the addition of AMF led to a significant reduction in the size of several root characteristics measured, this was not directly correlated with the presence of AMF structures in the root. Total root length colonisation was not significantly correlated to root length ($t_{(10)}$=-1.91, p=0.09), specific root length ($t_{(10)}$=-0.27, p=0.79) or root biomass ($t_{(10)}$=-1.81, p=0.10).

While leaf and root biomass decreased significantly in the AMF+ treatments, compared to AMF- controls ($F_{(1,22)}$=7.06, p=0.01; $F_{(1,22)}$=49.29, p<0.01 respectively), stem biomass did not ($F_{(1,22)}$=1.96, p=0.18, Table 1). Despite the dramatic decrease in root biomass in AMF+ treatments, shoot biomass was not reduced to the same extent. The addition of Si solution in combination with AMF significantly increased leaf biomass and leaf water content, but no other growth parameters (Table 4).

3.3.2 *Foliar Si and P content*

Plants colonised by AMF had significantly lower foliar Si concentration than non-colonised counterparts ($F_{(1,22)}$=18.28, p<0.01), while the Si+ treatment increased concentration relative to Si- controls ($F_{(1,22)}$=9.29, p<0.01) (Figure 11a). No significant interaction effect was shown between the AMF and Si treatments ($F_{(1,22)}$=1.96, p=0.18). Leaf P concentrations, showed a pattern similar to Si, with colonisation leading to a significant decrease in concentration ($F_{(1,22)}$=82.78, p<0.01), and Si treatments causing it to increase ($F_{(1,22)}$=22.51, p<0.01) (Figure 11c). However, unlike for the Si concentrations, there was a significant interactive effect ($F_{(1,22)}$=6.04, p=0.02) of the AMF and Si treatments. Overall, the AMF-/Si+ treatments had the highest Si and P concentrations, and AMF+/Si- had the lowest, reflecting the impacts of the treatments on root biomass.

Due to the significantly decreased root fractions of AMF colonised plants, uptake efficiency of roots was calculated (foliar Si or P content (mg)/root length (cm)). Plants colonised by AMF had greater Si and P uptake efficiency than non-colonised treatments (Figure 11b, d). The addition of AMF was the sole factor leading to a more efficient uptake of Si ($F_{(1,22)}$=14.34, p<0.01) and P ($F_{(1,22)}$=17.97, p<0.01). There was no significant effect of Si supplementation on Si ($F_{(1,22)}$=1.33, p=0.26) and P ($F_{(1,22)}$=1.39, p=0.25) uptake, nor was there a significant interaction effect between AMF and Si treatments (Si: $F_{(1,22)}$=0.003, p=0.96; P: $F_{(1,22)}$=0.004, p=0.95).

Total leaf Si content was positively correlated to increases in root length in colonised plants ($t_{(10)}$=4.10, p<0.01), but no such relationship was observed in non-colonised plants ($t_{(11)}$=1.13, p=0.28) (Figure 12a). The same pattern was observed for total leaf P, but the relationship in the AMF+ treatments was stronger ($t_{(10)}$=5.70, p<0.01), and weaker for AMF- treatments ($t_{(11)}$=0.19, p=0.85) compared to the Si data (Figure 12b). The correlations in Figure 12a and 2b show clear segregation of treatments in terms of AMF addition, but also of Si addition. The total root length colonised by AMF was found to be an important factor in determining the relationship between root length and total foliar P content ($F_{(1,17)}$=12.99, p<0.01), but there was no interaction detected between these factors for the Si data ($F_{(1,17)}$=1.64, p=0.22). These relationships mirror the uptake efficiencies represented in Figure 11b and d, which provides evidence of improved nutrient uptake efficiency in AMF treated plants.

Table 4: Treatment effect of silicon (Si) addition and arbuscular mycorrhizal fungi (AMF) on fungal root length colonisation (RLC, %), dry weight biomass of plant fractions (g; leaf, stem and root), leaf water content (g) and specific root length (m/g) of *Brachypodium sylvaticum* plants.

Treatment		**(n)**	**RLC (%)**	**Dry weight biomass (g)**			**Leaf water content (g)**	**Specific root length (m/g)**
AMF	Si			Leaf	Stem	Root		
-	-	7	0^{a} (± 0)	1.43^{b} (± 0.07)	0.64^{a} (± 0.02)	0.80^{b} (± 0.07)	2.96^{b} (±0.23)	32.72^{a} (± 1.69)
-	+	6	0^{a} (± 0)	1.36^{ab} (± 0.08)	0.65^{a} (± 0.04)	0.94^{b} (±0.15)	3.07^{b} (±0.13)	31.43^{a} (± 4.21)
+	-	7	33.14^{b} (± 2.88)	0.86^{a} (± 0.16)	0.48^{a} (± 0.08)	0.19^{a} (±0.11)	1.74^{a} (±0.38)	52.56^{b} (± 6.65)
+	+	6	35.67^{b} (± 7.15)	1.21^{ab} (± 0.21)	0.63^{a} (± 0.11)	0.20^{a} (±0.07)	2.35^{ab} (±0.42)	39.48^{b} (± 6.22)

Symbols represent Si/AMF absent (-) Si/AMF present (+). Numerical values presented represent the mean ± standard error. Mean values followed by different letters within the same column indicate statistical differences where $p<0.05$ assessed by Tukey HSD post-hoc test.

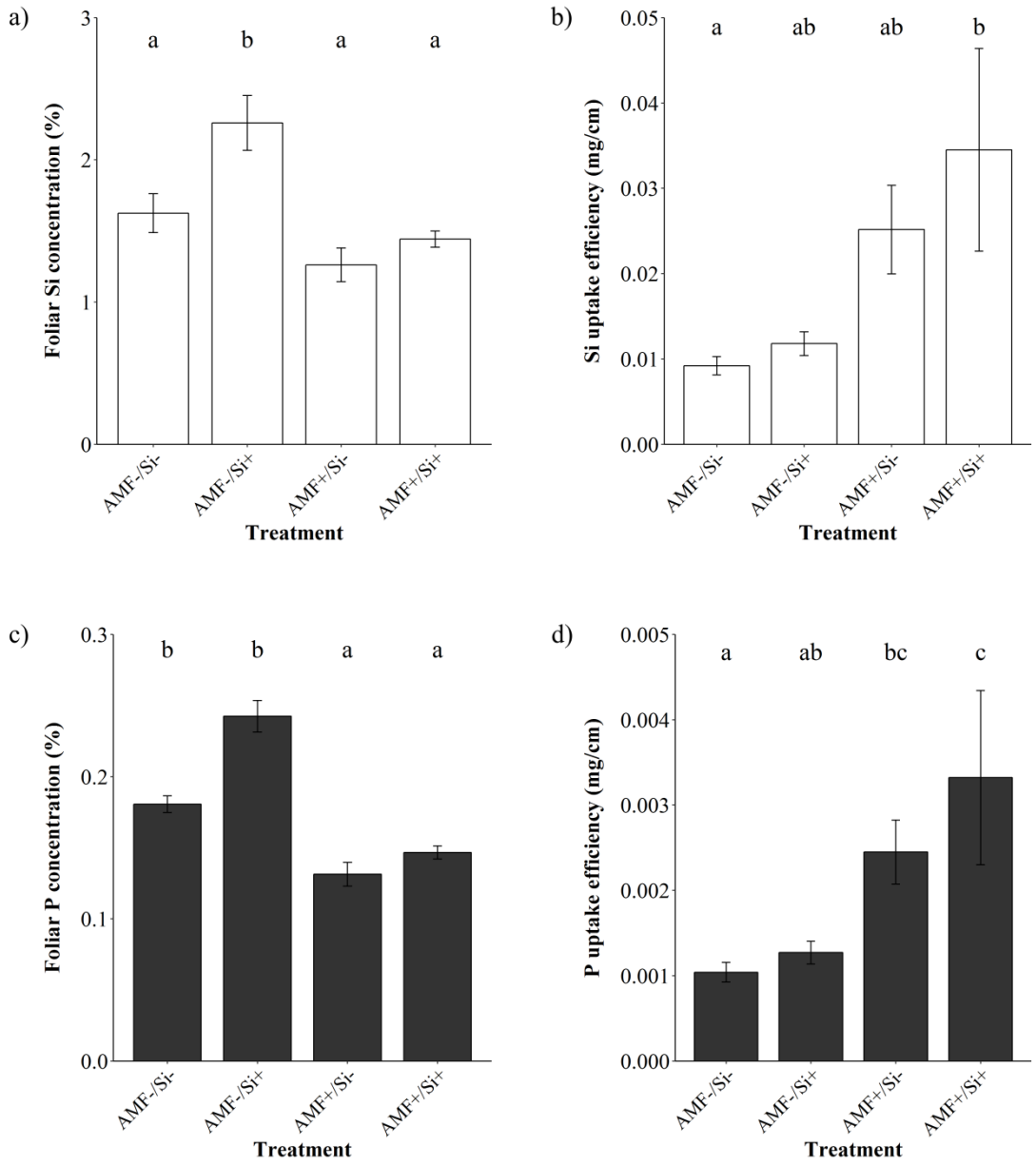

Figure 11: Treatment effect of silicon (Si-/Si+) and arbuscular mycorrhizal fungi (AMF-/AMF+) on foliar Si (a) and P (c) concentration (elemental % dry weight), and Si (b) and P (d) uptake efficiency (elemental uptake per unit root length, as calculated by total foliar elemental content/root length (cm)). Bars represent mean values of each treatment, and error bars represent standard error.

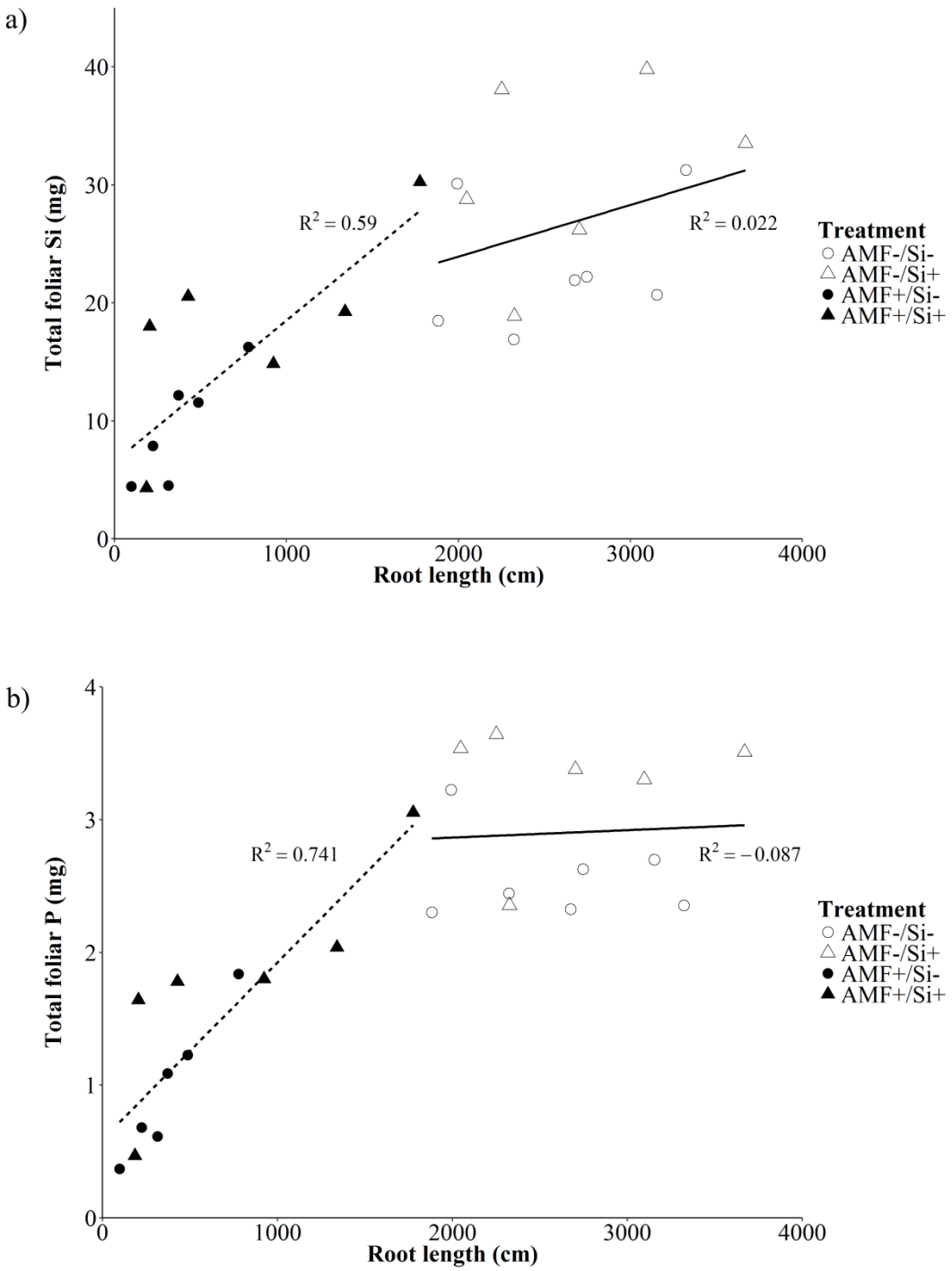

Figure 12: Treatment effect of silicon (Si-/Si+) and arbuscular mycorrhizal fungi (AMF-/AMF+) on the relationship between root length and total foliar Si (a) and P (b). Empty symbols represent non-colonised (AMF-) treatments, and filled symbols represent colonised plants (AMF+), triangles represent treatments receiving supplementary silicon (Si+) and circles represent the treatment that received a deionised water control (Si -).

3.4 Discussion

The data presented in this paper show the significant impact that symbiotic root fungi can have on plant growth, root architecture, and nutrient status. Plants colonised by AMF showed a significant reduction in root biomass compared to non-colonised plants, but were capable of supporting larger relative aboveground biomass, suggesting that the presence of the AMF in the roots led to higher root function efficiency. This was corroborated with the nutrient uptake data that showed that per centimetre of root, AMF treated plants were two to three times more efficient at taking up Si and P compared to non-colonised counterparts. The hypothesis of this study was that AMF and Si application in tandem would lead to the highest overall Si concentration. Si addition alone did improve both Si and P concentration, and in tandem with AMF addition did lead to some small improvements, compared to the non-Si supplemented treatment. However, accommodating for plant biomass differences, the AMF treatment was the most significant stimulus for improved nutrient uptake efficiency. This is important in regards to alternative methods for improving Si uptake in plants in the field, whereby the addition of AMF can yield multiple benefits in terms of improved nutrition (Leigh *et al.*, 2009; Smith *et al.*, 2011), defence (Pozo *et al.*, 2009; Hartley & Gange, 2009) and Si supply without costly Si supplementation.

The most striking data from this paper are the strong correlation of shoot nutrient content and root length in AMF colonised plants; a relationship that is lacking in non-colonised plants (Figure 12a,b). This strong relationship in colonised plants demonstrates that increases in root length are directly linked to total leaf Si and P content. This is supported by the data in Figure 11b and d, whereby per unit length of root, AMF plants are more efficient at nutrient uptake. Total root length colonised was not found to correlate with root length, nor nutrient concentration or uptake. However, it was a significant factor in the relationship between total foliar P content and root length, which was not the case in the total foliar Si concentration. The fact that the presence of AMF structures in the root is important for P, but not for Si, does suggest some functional segregation, and different mechanisms for the improvement of uptake of both nutrients. The idea that total root length colonised is not indicative of symbiont function has been noted previously (Smith *et al.*, 2004). This strongly suggests that the presence of AMF in the root is stimulating change that is not related to abundance of fungal structures in the root, but rather functional symbiont activity. This therefore raises questions about the mechanisms by which Si uptake is improved in colonised plants.

AMF are capable of supplying a variety of resources from the soil to the plant through the ERM. This is a well-established mechanism for P, N and water transport, where transporters and channels have been identified in AMF hyphae and at the peri-arbuscular membrane (PAM),

where plant and fungus meet within the root and exchange products necessary for the successful symbiosis (Leigh *et al.*, 2009; Smith *et al.*, 2011; Kikuchi *et al.*, 2016). AQP channels in the ERM have been implicated in the success of N and P uptake by mycorrhizal fungi, primarily through their role in facilitating water flow through the hyphae, a process that is stimulated by plant transpiration (Kikuchi *et al.*, 2016). Transpiration is also the driving force for the transport of $SiOH_4$ from root to leaf tissues (Ma & Yamaji, 2006). However, the polymerisation and deposition of silica within the leaf is carried out by another, currently unknown, but likely biological process in the apoplast involving proteins, peptides, or sugars condensing the soluble $SiOH_4$ (Kumar *et al.*, 2017a). The data showing an improvement in foliar water content as a result of joint AMF and Si addition may support this, as Si uptake was also more efficient in the AMF+/Si+ treatments (Table 4, Figure 11). Further data looking at ERM length and activity of known AMF AQP genes will clarify the role of AMF in direct supply of Si. Results of other studies on AMF and Si have speculated that there may be a direct transfer of Si through the fungal ERM and that Si enters the plant through the arbuscules and PAM (Oye Anda *et al.*, 2016). This is also supported by the work of Hammer *et al* (2011) who were able to measure the accumulation of Si in AMF spores and hyphae under salt stress conditions, indicating that there is some mechanism for AMF hyphae to assimilate Si. The differences in rates of uptake of Si and P in mycorrhizal plants may therefore be due to differences in plant demand for each of these elements, water uptake or the varying availability of these nutrients in the soil.

An alternative mechanism for improved nutrient uptake efficiency in AMF colonised plants is that the colonisation by AMF is stimulating improved plant uptake of resources. The evidence is conclusive on direct uptake of P, N and water by AMF hyphae, but the differences in patterns of Si and P uptake in the data may suggest that Si is not taken up in the same manner. Total root length colonisation was important in the relationship between P content and root length, but this was not the case for Si content. Colonisation of plants by AMF leads to highly complex transcriptional responses by plants; colonisation has been shown to up- and down-regulate a multitude of plant genes, including those coding for AQP channels, both under stress and non-stress conditions (Ouziad *et al.*, 2006; Uehlein *et al.*, 2007; Bárzana *et al.*, 2015; Chitarra *et al.*, 2016). There are examples of AQP genes coding for channels in the NIP family, to which the Si channel Lsi1 belongs, being stimulated by AMF colonisation, providing a potential mechanism for Si uptake to improve in colonised plants (Giovannetti *et al.*, 2012). In this study specific root length was significantly increased in colonised plants, which supports the hypothesis that AMF stimulate plant-based Si uptake (Table 4). This translates to an increase of fine roots thereby creating a greater root surface area and enabling access to smaller soil pores. A network of finer roots confers the ability to access water and solute resources inaccessible to coarser roots, which may be reflected in the higher relative leaf water content in AMF colonised plants (Table 4). However, an abundance of fine roots does not necessarily lead to an increase in the abundance

of nutrient transporters in the root system. The Si AQP channel, Lsi1 that controls the initial entry of $SiOH_4$ into the root is located in mature root regions of lateral and main roots, farther than 10mm from root tips, and not present in root hairs (Ma *et al.*, 2011). So combined with an overall decrease in root size and increase in fine roots, AMF stimulated changes in Si uptake may be increasing the efficiency of transporters but unlikely to be stimulating changes in root structure that favours an increase in Si channel abundance. More detailed root structural analysis and monitoring of Si AQP (*Lsi1*) gene activity in the roots will enable this hypothesis to be answered.

The main confounding factor in this study has been the substantial size difference between colonised and non-colonised plants (Table 4). Unusually, foliar P content also decreased with colonisation by AMF. As the results indicate, P content is intrinsically linked with root length and unlike similar studies colonisation by AMF significantly decreased root length and biomass (van der Heijden, 2004; Garg & Singh, 2017). Reports of decreased biomass in colonised plants are not uncommon, and are attributed to the fungus being a carbon drain when the plant has adequate nutrient supply (Johnson et al., 1997; Smith & Smith, 2011). In this study, plants were grown in a soil medium, rather than nutrient limiting media (often sand and attapulgite clay) commonly used in mycorrhizal studies, which may explain why non-mycorrhizal plants show higher levels of P as it was not limiting in these conditions. Yield penalties have been observed in *Brachypodium distachyon,* where colonisation by certain AMF led to decreased biomass and P relative to controls (Hong *et al.*, 2012). Biomass gains for *Brachypodium* plants are highest in part-shaded conditions, so potentially the glasshouse conditions were unsuitable for the growth of these plants (Füzy *et al.*, 2014). The results of this study suggest the plant may be allocating less growth to the roots as the fungal hyphal network is efficiently increasing the surface area of absorption of the root system. It could be argued that due to their smaller size, the colonised plants may be at a different developmental stage to the non-colonised plants, and that the AMF are having a parasitic effect. With the current data is it difficult to determine developmental markers by disentangling them from the growth data taken, but this something that should be considered in future studies.

This work is the first demonstration of improved Si uptake in non-crop species colonised by AMF. The results of this study indicate that AMF colonisation leads to dramatic changes in root size and architecture which can improve Si and P uptake, and while P uptake is related to total root length colonisation in the root, Si uptake is not. AMF colonised plants had significantly decreased root biomass but aboveground biomass was not decreased to the same extent suggesting that the AMF were acting to support the root system. Under glasshouse conditions used in this study, there is a clear growth penalty associated with being mycorrhizal, but in a natural system, mycorrhizal plants would likely have a competitive advantage over non-

mycorrhizal equivalents, where growth resources could be allocated to above ground growth without detriment to the belowground system. It is currently unclear what the mechanisms behind the improved uptake efficiency of Si and P by mycorrhizal plants are, however we have outlined two testable hypotheses: a) AMF colonisation stimulates activity of plant genes encoding Si AQP channels (perhaps related to improved water supply and transpiration), and b) AMF hyphae directly take up Si and transport it to the plant, in a similar mechanism to P and N. Improved plant Si uptake in mycorrhizal plants is supported not only by this work but previous studies, and these findings open up new opportunities to improve plant nutrition using natural systems.

4 How does mycorrhizal community affect Si and P content of *Brachypodium sylvaticum* plants?

4.1 Introduction

Rhizosphere organisms improve plant survival by improving availability and supply of nutrients, and through enhancing natural resistance to biotic and abiotic stresses. The arbuscular mycorrhizal fungi (AMF, Phylum Glomeromycota) are one of the most widespread and ancient symbiotic associations (Parniske, 2008; Davison *et al.*, 2011). AMF are obligate biotrophs that form specialised, highly branched structures in plant root cortical cells called arbuscules, from which the fungus exchanges limiting nutrients from the soil for photosynthetic carbon (Smith & Read, 2010). While AMF are the most widely recognised plant growth promoting fungi, there are a multitude of interactions between microbes in the rhizosphere, and with their plant hosts that contribute to improving plant resilience to biotic and abiotic stresses (Berg, 2009).

Although the majority of land plants are capable of forming a symbiosis with AMF, the environmental conditions are not always conducive to their formation (Smith & Read, 2010). In high intensity agricultural systems, plants are typically supplied with a volume of nutrients sufficient for their growth throughout the growing season, therefore reducing the reliance on AMF associations (Jensen & Jakobsen, 1980; Koide, 1985; Santos *et al.*, 2006). Additional processes such as monoculture production, applications of pesticides and repeated tilling of soil all lead to depauperate fungal soil communities (Helgason *et al.*, 1998; Daniell *et al.*, 2001; Bowles *et al.*, 2016). There is significant evidence for improved ecosystem function with high species diversity, and this is predicted to be due to an increase in variety of functional traits within the community (Maherali & Klironomos, 2007). For example, plants can be colonised by multiple AMF species, but there is a substantial range in the carbon cost and nutrient supply of these fungi, leading to the acknowledgement of a 'mutualism-parasitism' continuum among AMF (Johnson *et al.*, 1997; Munkvold *et al.*, 2004; Helgason & Fitter, 2009). The success and efficacy of the symbiosis is dependent on a multitude of factors determined by the identity of the plant and fungus as well as the abiotic environment and developmental stage (Johnson *et al.*, 1997; van der Heijden & Kuyper, 2001).

Intra-specific diversity can also have significant implications on plant growth and nutrition, and potentially leads to higher levels of functional diversity than interspecific diversity (Munkvold *et al.*, 2004). Different isolates of the same AMF species can alter plant growth and nutrition with varied outcomes, even if the isolates originate from the same community (Koch *et al.*, 2006; de Novais *et al.*, 2014). It is predicted that high levels of intraspecific variation in AMF species tolerant of soil disturbance may compensate for the lack of species diversity in highly managed and disturbed environments (Munkvold *et al.*, 2004). Furthermore, plants colonised by

AMF also demonstrate increased recruitment of plant growth promoting bacteria compared to non-mycorrhizal plants, and the efficacy and specificity of recruitment depends on the AMF species colonising the plant (Sood, 2003; Cameron *et al.*, 2013). However, evidence shows that a similar mechanism exists for a direct recruitment of plant pathogenic bacteria and fungi to the plant rhizosphere, a mechanism irrespective of plant P status (Scheffknecht *et al.*, 2006; Maherali & Klironomos, 2007).

Plant colonising fungi also contribute to a range of other benefits for plants, both directly and indirectly. For example, colonisation leads to complex effects on the regulation of a variety of plant genes including those involved in plant water relations and defence. Similarly, the result and direction of these responses depends on multiple factors including specific combinations of plant and fungal species and the application of stress (Uehlein *et al.*, 2007). Aside from their benefits on plant productivity, soil microbes, and particularly AMF perform a variety of additional useful functions within the soil profile, such as improving soil particle aggregation (Rillig & Mummey, 2006), reduction of nutrient leaching (Cavagnaro *et al.*, 2015), carbon sequestration (Wilson *et al.*, 2009) and limiting the release of nitrous oxide (N_2O) from the soil produced by denitrification (Bender *et al.*, 2014).

In order to study the mechanisms and investigate specific plant responses to fungal root colonisers, the effects of single species on plant responses are typically examined in isolation. Single species stock or 'trap' cultures are produced by growing mycorrhizal responsive plants with a single species of mycorrhizal fungi to generate a large number of spores and root fragments as an inoculum source (Mosse, 1962). However, the long durations of growth and sub-culturing can lead to the introduction of fungal contaminants (Chapter 2, Section 2.3). Over-reliance of studies on the effect of AMF in insolation from other beneficial soil fungi, in single-species and single-isolate additions on plant growth and nutrition has been repeatedly highlighted in the literature and results in a bias towards easily cultivatable species (van der Heijden & Scheublin, 2007; de Novais *et al.*, 2014). Comparisons between field and artificial AMF species assemblies demonstrate that similar richness can be achieved artificially, and plant benefits can be mirrored, but requires two or three different families of the Glomeromycota to be present, while most studies focus on the Glomeraceae alone (Maherali & Klironomos, 2007). Artificial species assemblages can also not bear any real comparison to field conditions. In one study where *Rhizophagus irregularis* was added into natural species mixtures, *R. irregularis* became the dominant root coloniser at a detriment to plant growth and overall decreased extraradical mycelia (Symanczik *et al.*, 2015). Using inocula derived from agricultural and natural soils will enable a comparison to be made on how species in the field interact and affect plant growth under controlled conditions. This introduces the limitation of adding a high degree of complexity into the system, but will ultimately create a more realistic evaluation on how

natural microbial communities affect the growth of plants and relate controlled and targeted experiments carried out in glasshouses to real-world environments.

In the majority of published studies, there is a tendency for colonisation data presented to focus purely on the abundance of AMF structures within the root, and to not record other fungi that are present within the root, or indeed to check the identity of colonising fungi in a robust way (Chapter 2, Section 2.3). The ability to amplify and sequence all fungal DNA using high-throughput or next-generation sequencing (NGS) techniques has yet to be applied to the sequencing of plant roots in glasshouse conditions using traditional inoculum additions of root fragments and spores, but its use here will evaluate the true diversity of fungi present in these systems and whether this is a useful and cost-effective technique to use in such systems.

The aim of this study was to evaluate the plant growth and nutritional response of *Brachypodium sylvaticum* plants to different fungal inocula sources. NGS techniques were applied to highlight differences in the microbial communities and relate these to alterations in plant growth and nutrition, and to evaluate the effectiveness of NGS in an artificial environment. Using natural inocula sources from commercially available stocks, agriculture, and woodland we created varying fungal communities at different levels of disturbance. We predicted that:

1. Plants will have distinct fungal communities associated with each treatment.
2. Plant growth and nutrition will be improved with colonisation of AMF and other symbiotic fungi, with higher foliar P and Si concentration compared to controls.
3. Plants treated with inocula derived from an undisturbed (i.e. non-agricultural) site will have higher diversity of root colonising fungi.

4.2 Materials and methods

4.2.1 *Preparation of biological materials*

Fungal inocula were obtained from soil or substrates collected from four sources predicted to have varying fungal diversity: woodland grass, an agricultural field margin, an agricultural pasture, and a single species AMF stock pot. The woodland sample was obtained from Hetchell Woods, a site of special scientific interest (SSSI) close to Leeds (SE 376424). The pasture (SE442423) and field margin (SE441423) samples were taken from the Leeds University field research unit, close to Bramham, Leeds. A vegetated soil patch of approximately 30x30x10 cm was collected from each of these sites. The single species stock pot was *Plantago lanceolata* plants colonised by single AMF species in a 1:1 sand and attapulgite clay substrate, the identity of the fungal colonisers (predominately *F. mosseae*) in the root fragments were checked through clone libraries and sanger sequencing as described in Chapter 2 (Section 2.3.3). Throughout this paper, the treatments will be referred to as Control, *F. mosseae*, Field Margin, Pasture and Hetchell.

500ml of substrate was repeatedly washed with tap water through a sieve stack until the flow-through was clear. The sieve stack had 5 sieve sizes, starting with 2mm diameter mesh to prevent large debris to 600µm through to 50µm. Spores were collected by backwashing from each sieve <2mm with 100ml of dH_2O, the backwash from each sieve was mixed together and 20ml of the spore backwash mixture was added to each experimental pot. Control pots received 20ml of dH_2O. Bacterial filtrates were added to pots to control for the effect of non-fungal rhizosphere organisms. 500ml of substrate was mixed with 1L of water and filtered through 10 layers of muslin, followed by Whatman No.1 filter paper (pore size 11µm) until filtrate was clear. 20ml bacterial filtrate from the original source site was added to the respective treatment pots. Control pots received 20ml of filtrate mixed from the other treatments. Plant roots and spore washes were added to pots in combination to ensure a full range of fungal propagules was applied to capture the full diversity within the inocula (Varela-Cervero *et al.*, 2015).

4.2.2 *Experimental set-up and harvest*

Treatments were replicated eight times, (n=40), based on results of an *a priori* power analysis (α=0.05, effect size=0.8 (based on pilot data), power = 0.95). Half-litre volume pots were filled with twice-autoclaved sieved soil (<2mm) which was allowed to rest 3 days between autoclave events and after the final autoclave. Plants were grown in glasshouse conditions (16:8h, 20:18°C day:night) in a randomised block design. Nine seeds were sowed and later thinned to leave one plant per pot. Plants received 50ml of $NaSiO_3.9H_2O$ every 4 weeks, to ensure Si was not limiting.

Plants were harvested 10 weeks after seedling emergence. Roots were removed intact, rinsed and scanned using an Epson Perfection V700 photo scanner and image data analysed using WinRhizo (Regent Instruments Inc., Quebec City, Canada) to obtain root characteristics. Roots were then cut into 1cm pieces and randomly sub-sampled for root staining for mycorrhizal colonisation analysis and stored in 40% ethanol until staining. Remaining root, leaf and stem were dried separately at 70°C for 72 hours. Root dry weights (DW) were estimated to account for staining samples ((remaining root fresh weight (FW) + root FW of AMF subsample) / remaining root FW) x remaining root DW).

Si and P analysis followed the same protocol as described in Section 2.2.2; Materials and methods; I. Root staining and root length colonisation of AMF and non-AMF was measured according to the same protocol as described in Section 2.2.2; Materials and methods; III.

4.2.3 *Illumina Miseq sequencing*

DNA was extracted and amplified from dried plant roots according to the protocol described in Chapter 2, Section 2.4. Samples were randomly split across two different sequencing runs, due to operational costs and availability. From 40 samples, 37 generated PCR products. Of these samples 27 generated products from both the ITS1F-ITS4 (ITS region) and NS31-AM1 (AM region) amplicons, 7 and 3 samples only amplified at the ITS and AM regions respectively. This was not related to the sequencing run which samples were placed. Sequence products were trimmed, de-multiplexed and quality filtered using QIIME v.1.9.1 (Caporaso *et al.*, 2010). ITS and AM OTUs were generated and sorted using USEARCH (v.8,Edgar, 2010) using the general fungal UNITE (v7.1, Kõljalg *et al.*, 2005; Abarenkov *et al.*, 2010) and Glomeromycota specific MaarjAM (v.0.8.1, Öpik *et al.*, 2010) databases respectively, at a 97% similarity threshold**.** OTU sequences were also aligned using the EMBL-EBI online Clustal Omega tool (Goujon *et al.*, 2010), which generates a simple distance phylogeny and a percentage identity matrix. OTUs that had high similarity (>90% for ITS, >95% for AM amplicons) were highlighted and compared with sequences in the NCBI-BlastN database (search excluded environmental samples). Multiple OTUs were collapsed into a single record, and read counts aggregated if the Blast outputs led to the same taxonomic outcome at high confidence. Taxonomic attributes of OTUs previously assigned as 'unidentified' or 'unassigned' were edited to reflect the findings of the BlastN search and non-fungal OTUs were removed. Following this rare (<0.001% of total reads) OTUs were also filtered out. Trophic guilds were assigned to group OTUs into ecologically meaningful categories using FunGuild (Nguyen *et al.*, 2016). Species names used throughout reflect the current accepted species names as published in the *IndexFungorum* database, where these have changed recently is documented in Table 5.

4.2.4 *Statistical analysis*

All percentage data (for Si and P, and fungal colonisation) was arcsine square root transformed prior to statistical analyses. Statistical tests and production of graphs was carried out in RStudio version 3.1.2 (R Development Core Team, 2011; RStudio Team, 2016). Plant growth, Si & P and alpha diversity data were tested for normality and homogeneity of variances following the methods outlined in Chapter 2 Section 2.2.2. One-way analysis of variance (ANOVA) was carried out on mixed-effect linear models where the fixed effects were the fungal community treatments and Si treatments. Correlations of root length data and fungal abundances were performed on transformed data using the Pearson product-moment correlation, calculated using the stats:cor.test function in R. Significant differences in fungal guild related between treatments were assessed using multivariate analysis of variance (MANOVA).

For the MiSeq data, to normalise the OTU table values for statistical analysis the python package normalize_table.py using the CSS algorithm was implemented as suggested by (Paulson *et al.*, 2013; McMurdie *et al.*, 2014) an alternative to rarefying. Fungal α-diversity and richness was calculated on the normalised OTU tables using the Shannon Diversity index, and the Chao1 estimation respectively, ANOVA tests were implemented to determine significant differences. Differences in fungal community composition between treatments were determined by performing a PERMANOVA (R function vegan:adonis) on a Bray-Curtis dissimilarity matrix of the CSS normalised OTU abundance tables, incorporating block as a random factor.

4.3 Results

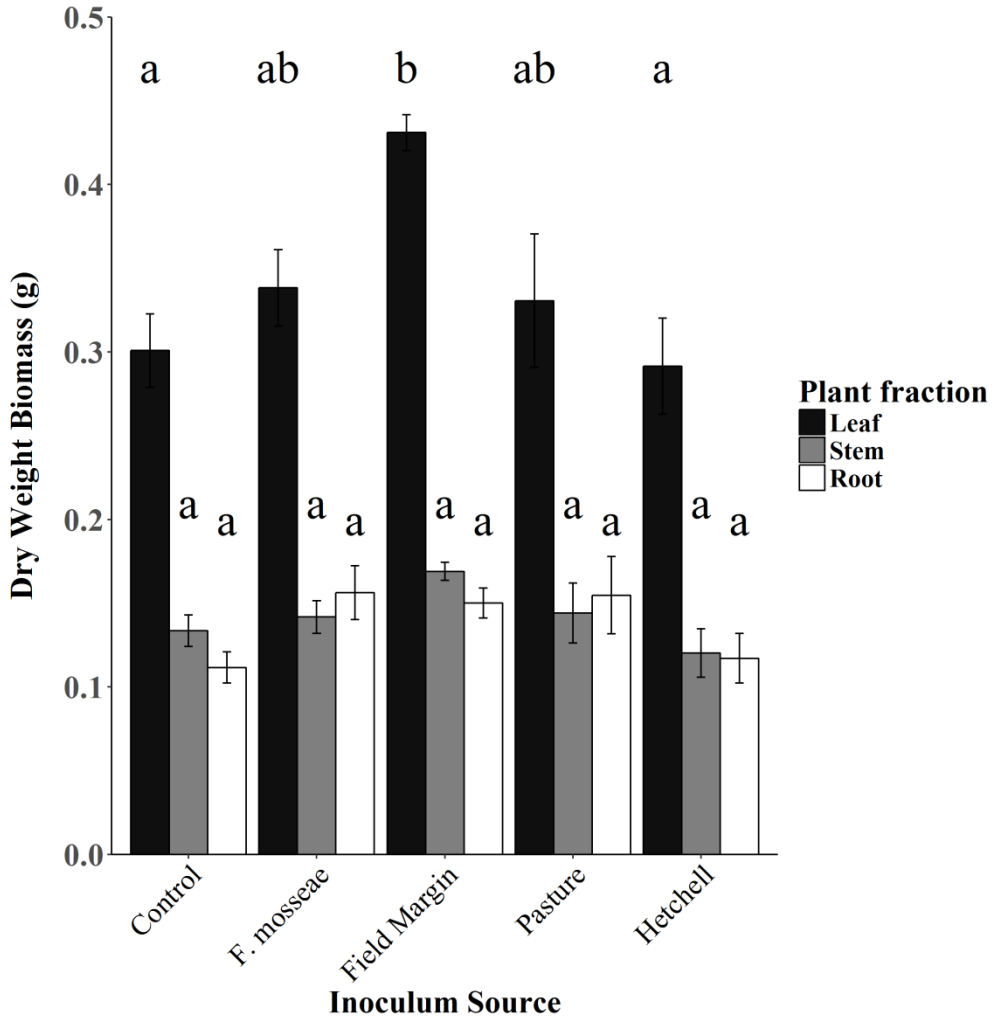

Figure 13: Dry weight biomass (g) of *Brachypodium sylvaticum* plants grown with different inocula. Biomass is shown within different plant fractions, leaf (black bars), stem (grey bars) and root (white bars). Error bars represent standard error, n=8 for all treatments.

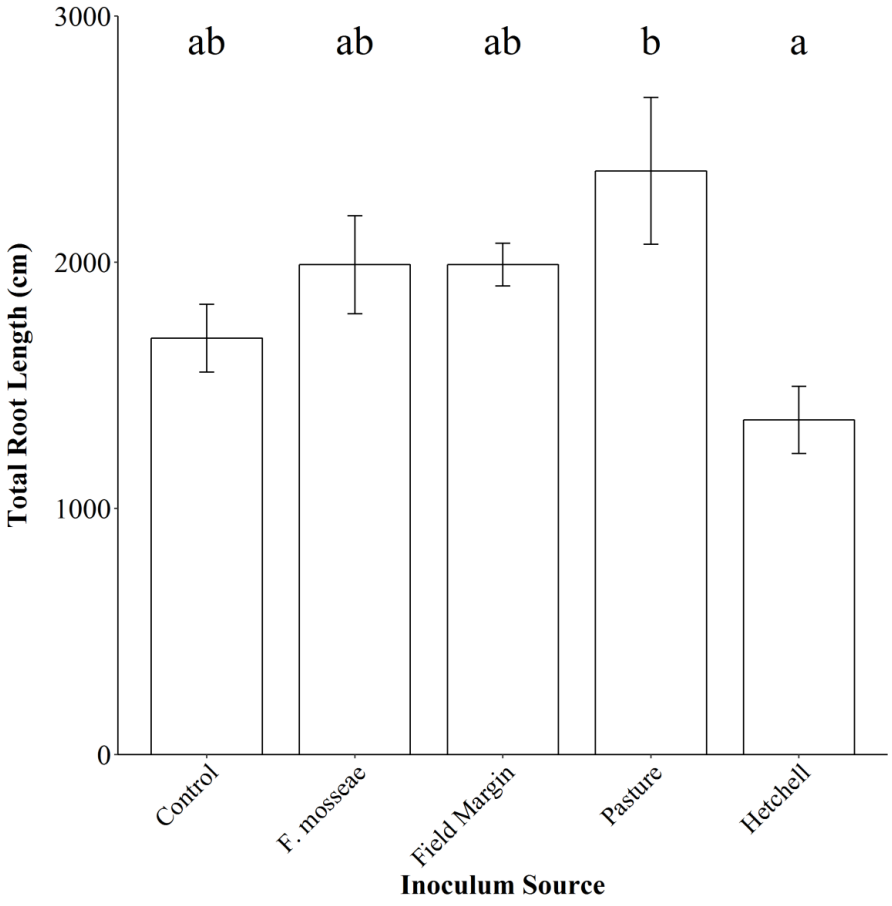

Figure 14: Total Root Length (cm) of *Brachypodium sylvaticum* plants grown under the varying inoculum treatments as determined by WinRhizo. Sample size, n=8 for all treatments and error bars represent standard error.

4.3.1 *Plant growth and colonisation*

Total plant biomass differed significantly between treatments ($F_{(4,35)}$=3.25, p=0.02), primarily driven by differences in leaf biomass ($F_{(4,35)}$=4.34, p<0.01), where Field Margin inoculated plants had significantly higher leaf biomass than Hetchell and Control treatments (Figure 13). Stem and root biomass did not differ significantly between treatments (stem: $F_{(4,35)}$=2.16, p=0.09, root: $F_{(4,35)}$=2.01, p=0.11). However, total root length did vary significantly between treatments ($F_{(4,35)}$=4.11, p<0.01, Figure 14). The results of a MANOVA test demonstrate that this difference was purely driven by differences in fine root length ($F_{(4,35)}$=4.46, p<0.01), where the plants receiving the inoculum from Hetchell woods had significantly lower length of fine roots than plants treated with the Pasture inoculum (Tukey p=0.002, Figure 14). Root length was not found to be significantly correlated with AMF or non-AMF root length colonisation (AMF (excluding control treatments): $t_{(30)}$=2.01, p=0.52, Non-AMF: $t_{(38)}$=-0.12, p=0.90). Plants grown with the inoculum from the Pasture field and Hetchell woods showed relatively similar

levels of colonisation by AMF and non-AMF fungi, but showed significant differences in root length characteristics (Figure 14, Figure 15).

All treatments receiving a fungal inoculum exhibited relatively low levels of colonisation by AMF (< 10%), but in line with other experiments (Table 4) and no mycorrhizal structures identified in the control treatments (Figure 15). Statistical analysis on the fungal inoculum treatments (excluding the control) showed a weak difference ($F_{(3,28)}$=3.04, p=0.05) between levels of mycorrhizal colonisation, with the single species *F.mosseae* treatment containing the highest abundance of AMF structures within the root (Figure 15). Non-AMF colonisation was higher in all treatments than the AMF colonisation, including the single species *F. mosseae* treatment, but there were significant differences in the non-AMF colonisation ($F_{(3,28)}$=3.77, p=0.03) between treatments also, with Field Margin demonstrating the highest concentration of non-AMF colonisation at around 28% (Figure 15).

4.3.1 *Foliar Si and P content*

There were significant differences in foliar Si concentration values between treatments ($F_{(4,35)}$=3.16, p=0.03, Figure 16a) but not for foliar P concentration ($F_{(4,35)}$=1.46, p=0.24, Figure 16c). Due to variations in root length between treatments (Figure 14) Si and P uptake per unit root length was calculated. This measure can be considered a measure of uptake efficiency, with higher values indicating more nutrient uptake per unit of root length. Differences in Si uptake between treatments were accentuated when accounting for root length differences ($F_{(4,35)}$=7.48, p<0.01), results from a *post-hoc* test showed that plants receiving the Pasture inoculum demonstrated significantly lower Si uptake efficiency compared to other all treatments (Tukey p<0.05) except for the control treatment (Figure 16b). Similar results were observed in the P uptake by root length, where plant uptake efficiency of P was significantly affected by inoculum addition ($F_{(4,35)}$=5.69, p<0.01) but to a lesser extent than Si uptake. Similarly, the results of the post-hoc test illustrated that plants receiving the Pasture inoculum were significantly different from plants treated with the *F. mosseae* single species, Hetchell and Field Margin inocula (Tukey, p<0.05, Figure 16d). Overall, Si content and uptake was more variable across treatments than P content and uptake.

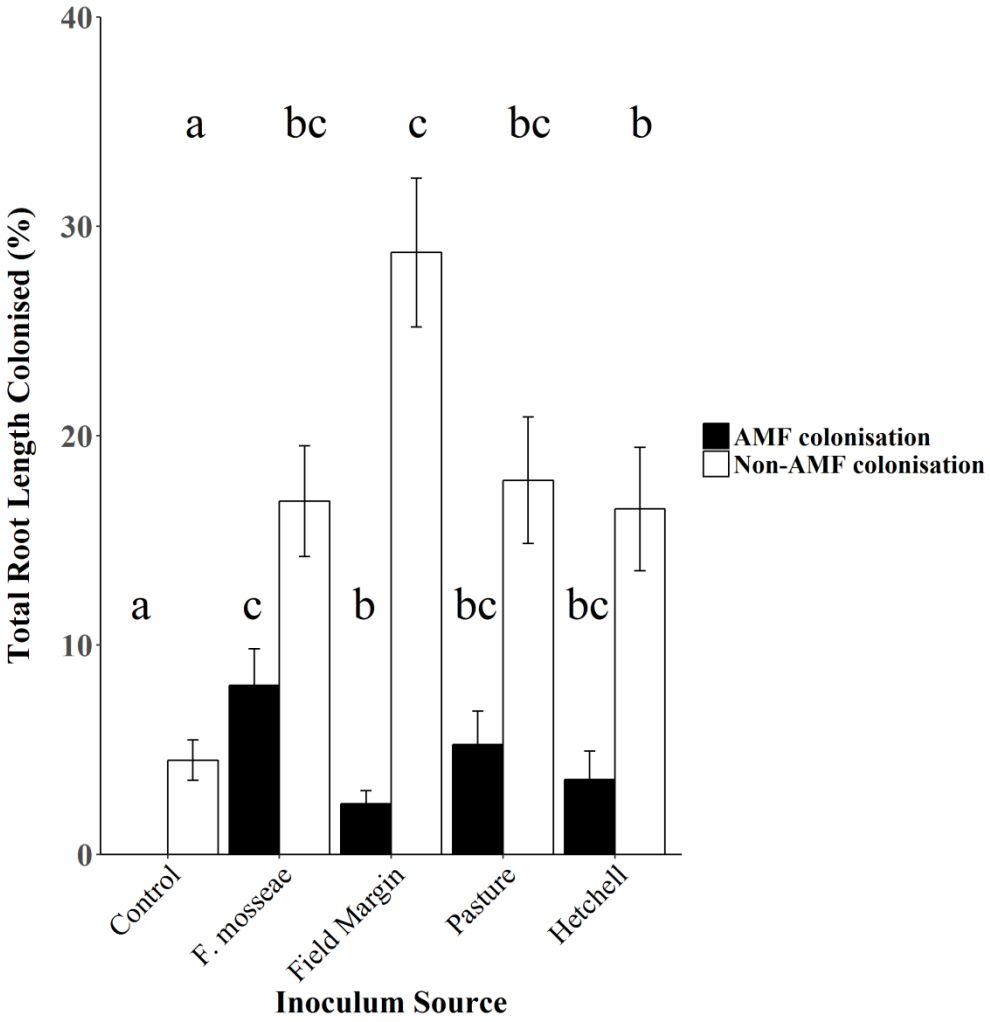

Figure 15: Total root length colonised (%) of *Brachypodium sylvaticum* roots by AMF and non-AMF fungal structures for each inoculum treatment. Sample size, n=8 for all treatments and error bars represent standard error.

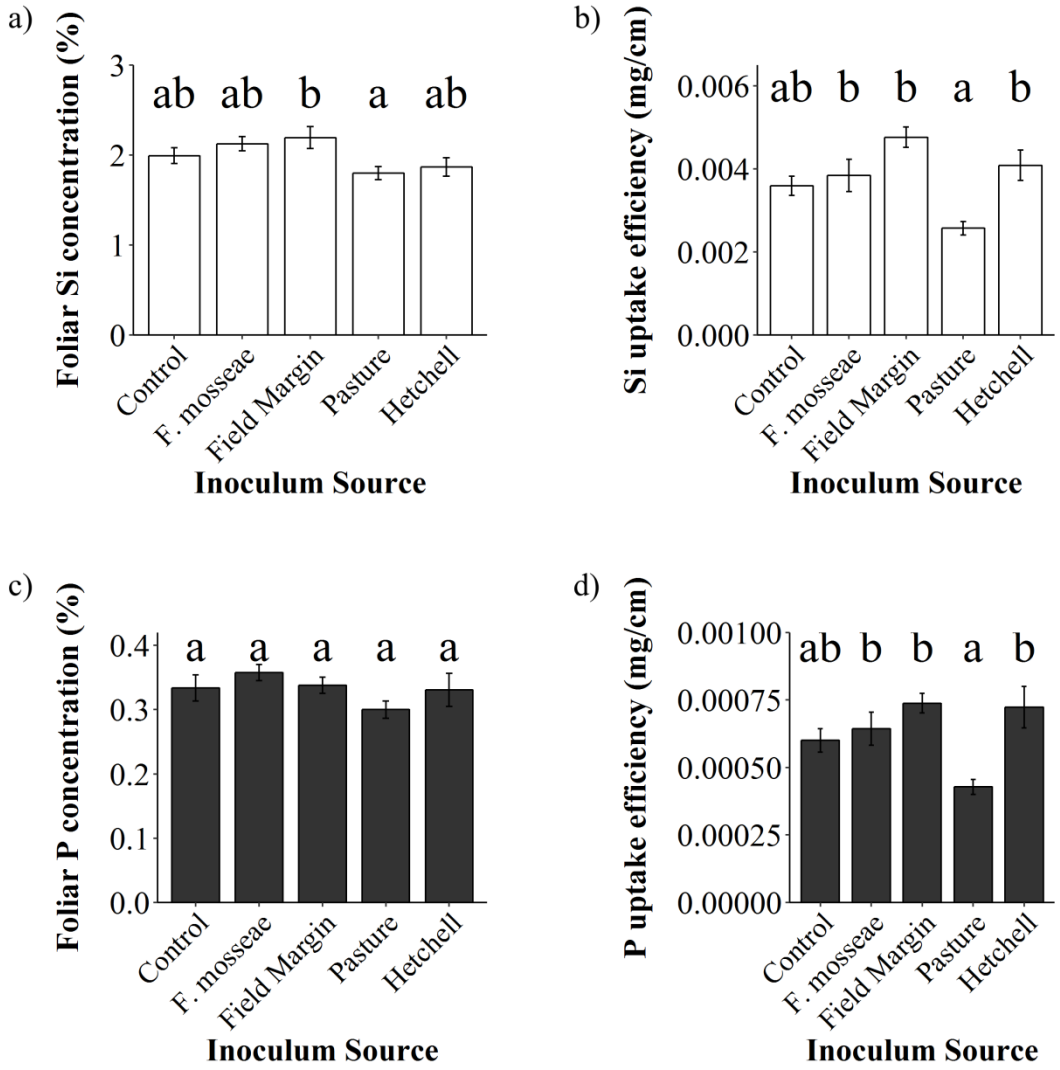

Figure 16: Foliar Si (a) and P (c) elemental concentration of dry weight leaf biomass in *Brachypodium sylvaticum* plants grown with different inoculum treatments. Si (b) and P (d) uptake by plant root length. Sample size, n=8 for all treatments and error bars represent standard error.

4.3.2 *Fungal Diversity*

OTU tables were generated for the AM (NS31-AM1) and ITS (ITS1F-ITS4) amplified regions. Initial sequence reads after the split library stage (Step 6, Table 3) were 1,855,103 sequences for the ITS amplicon and 1,305,286 sequences for the AM amplicon. Extensive filtering and cross-referencing with sequence databases was required and a high proportion of amplified sequences were non-fungal or very rare and subsequently removed or collapsed into one OTU due to high sequence similarity. After this, sequence reads for the ITS amplicon totalled 1,077,443 and 324,830 in the AM dataset. This corresponded to 94 OTUs decreasing to 36 in the ITS region after the removal of plant and non-fungal sequences and 83 OTUs generated using USEARCH decreasing to 3 post-filtering for the AM region after the removal of plant, non-fungal and non-AMF sequences. Consequently, after removal of non-fungal and rare OTUs, some samples had

too few reads (<100) to be kept in analyses and were also removed to avoid skewing the data where other samples typically possessed several thousand sequence reads in total. This resulted in a decrease in sample size in the ITS subset from 34 samples to 24, and from 30 samples to 7 in the AM subset. This lead to an uneven depth of sampling across the treatments. For the AM subset there were no samples from the Control or Field Margin groups and fewer than three samples from the Field Margin, Pasture and Hetchell groups so further statistical analysis on this dataset was not possible.

There was, nevertheless, an apparent trend in the distribution of OTUs within the AM subset samples, with plant receiving inoculum from the single species inoculum (*F. mosseae*) predominately having high abundance of *Rhizophagus irregularis* (VTX00114), while the other treatments showed greater abundance of two different Glomus OTUs (both VTX00105). Interestingly, the visual analysis of fungal colonisation using staining and microscopy techniques did yield broadly similar results to the sequencing outputs. Field Margin samples had the lowest observed colonisation by AMF visually, and also showed no amplification of AMF sequences (Figure 15). Similarly, the single species *F. mosseae* treatment showed the highest level of AMF colonisation, and had the highest average value of reads across the AMF OTUs amplified (~1570), although none of these corresponded to *F. mosseae* isolates. Pasture treatments also showed higher colonisation by AMF in the visual analysis compared to Hetchell treatments, and this again was seen in the average number of AMF read counts for each treatment (10358, and 4 respectively). Control treatments did amplify some Glomeromycotan OTUs, but across all replicates this was only 3 reads.

Diversity metrics

Shannon diversity metrics were used for α-diversity. Values were found to not be significantly different, suggesting overall diversity of each treatment was similar ($F_{(4,12)}$=0.89, p=0.50). There was also no significant block effect ($F_{(7,12)}$=1.61, p=0.22). Analysis on ß-diversity indicated that significant differences between fungal communities were evident across different treatments (PERMANOVA: $F_{(4,23)}$=1.266, p=0.048). This difference demonstrates that the Pasture treatment is somewhat distinctive in community composition compared to the other treatments, despite some overlap (Figure 17). To investigate the functional characteristics of the fungi within each treatment, fungal trophic guild composition was analysed (Figure 18). A MANOVA test indicated there were significant differences between guild composition between treatments (*Wilks' Λ*= 0.07, $F_{(4,19)}$=3.17, p=0.02), a result driven by differences in saprotroph abundance across treatments ($F_{(4,19)}$=3.16, p=0.04). Control treatments had significantly higher numbers of saprophytic fungi compared to plants grown with Hetchell woods derived inoculum (Tukey p=0.024). No other significant differences in guild contribution between treatments, due to the high variability in the composition of fungi within treatments (Figure 17).

OTU taxonomy and guild (as determined by analysis through FunGuild) is detailed in Table 5. The most widespread OTUs ('Core OTUs'), occurring in nearly all samples and representing ~60% of the total reads corresponded to *Olpidiaster brassicae* (47% of all reads, present in all samples) and *Serendipita indica* (12% of all reads, present in 21 samples). An intermediate grouping ('intermediate OTUs'), covered 33% of all reads and corresponds to 11 OTUs, found in 1 - 13 samples. This intermediate grouping contains OTUs from across all fungal phyla, including two Glomeromycetes (AMF), and is predominately made up of saprophytic and symbiotic fungi. The remaining 24 OTUs ('rare OTUs') make up 7% of the total reads, and are found in 1 – 11 samples, this group contains a large number of Sordariomycetes (Ascomycota) and is broadly dominated by saprophytic and pathogenic fungi.

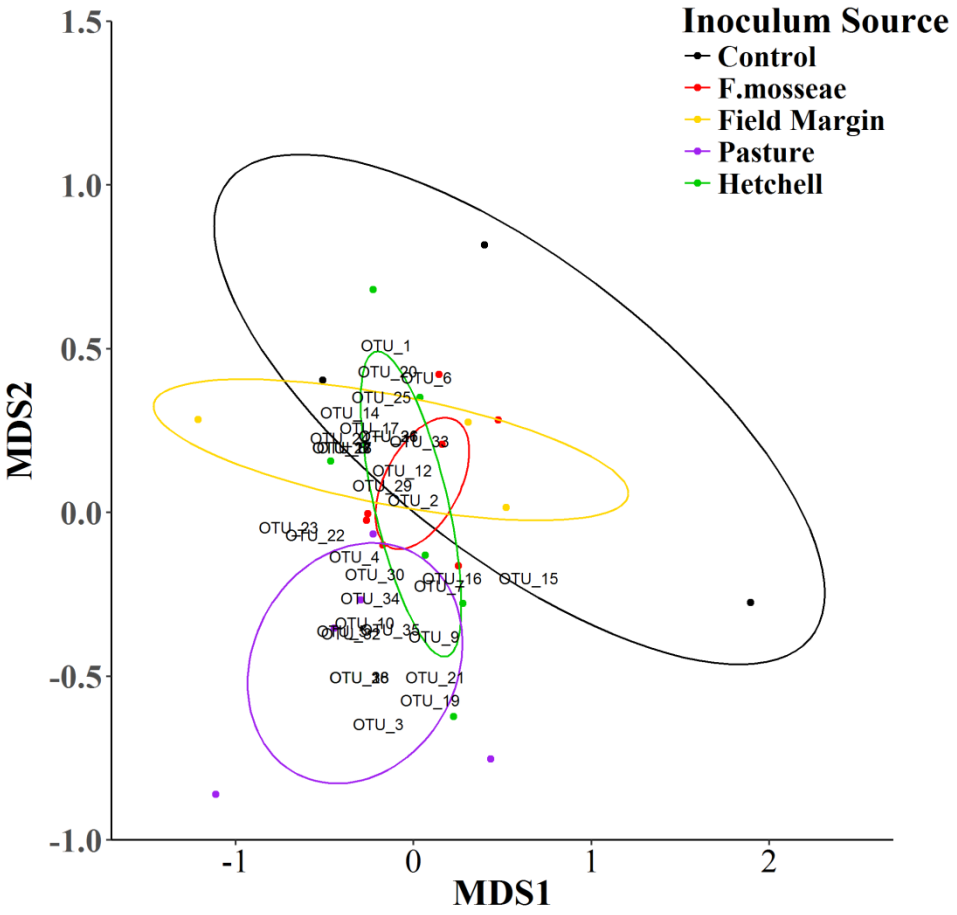

Figure 17: Non-metric multi-dimensional scaling (NMDS) plot generated using Bray-Curtis dissimilarities matrix of a normalised OTU table. Dots represent individual samples. Ellipses represent standard error of the (weighted) average of scores. Inoculum treatments are overlaid on the ordination represented by the coloured ellipses which represent the standard error of the weighted averages of scores. Control (black, n=3), single species inoculum *F. mosseae* (red, n=7), and inocula derived from Field Margin (yellow, n=3), Pasture field (purple, n=5) and Hetchell woods (green, n=6).

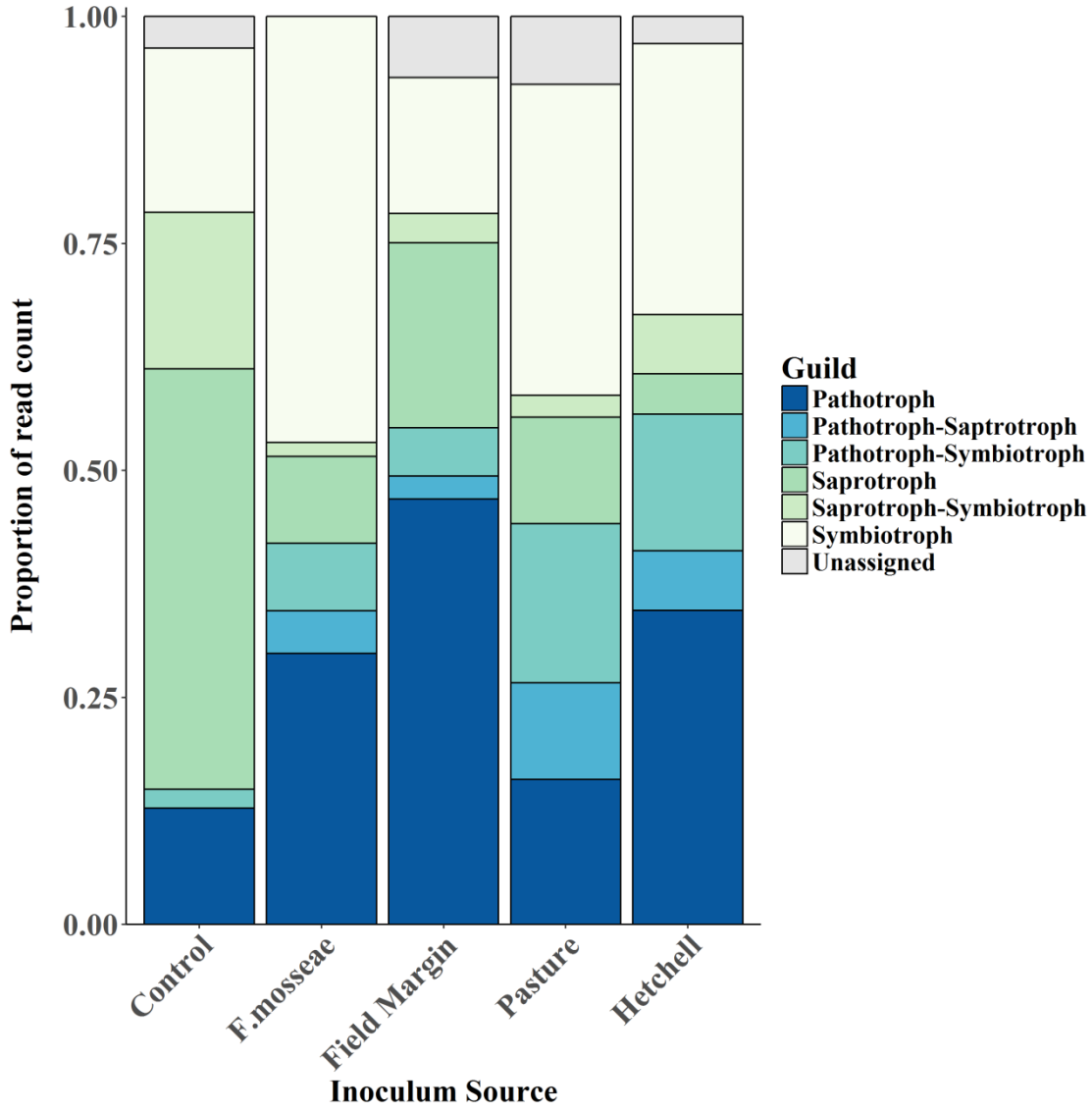

Figure 18: Mean relative abundance of fungal sequences within plant roots grown under experimental conditions. Sequences generated from Illumina sequencing of the ITS1f-ITS4 region and represented by trophic guild (assigned by FunGuild) for each of the inoculum treatments; Control (n=3), single species inoculum *F. mosseae* (n=7), and inocula derived from Field Margin (n=3), Pasture field (n=5) and Hetchell woods (n=6).

Table 5: Identity and read information for all 39 operational taxonomic units (OTUs) generated in this study. Taxonomy was checked against NCBI Blast and MaarjAM databases, QC = Query Coverage, ID = Maximum identity, Accession= Accession reference number for closest hit, VT= Virtual Taxon generated by the MaarjAM database. No. reads is a total read count value across all samples that contained this OTU (No. samples). Guilds marked with a star are most probable assignments based on the literature.

OTU	No. reads	No. samples	Blast assigned taxonomy	Species synonymy and isolate information	QC	ID	Accession/ VT*	Phylum	Trophic mode
ITS region (ITS1F-ITS4)									
1	506384	24	*Olpidiaster brassicae*	*Olpidium brassicae*	100	99	AB205207	Chytridiomycete	Pathotroph
2	132698	21	*Serendipita indica*	*Piriformospora indica*	100	99	KC176326	Basidiomycete	Symbiotroph
3	84056	13	*Cladosporium herbarum*		100	100	KX611004	Ascomycete	Pathotroph-Symbiotroph
4	53715	9	*Coprinopsis lagopus*	*Coprinus lagopus*	100	100	JN943127	Basidiomycete	Saprotroph
5	51372	10	*Rhizophagus intraradices*	Clone U3.2,*Glomus intraradices*	100	98	AJ567352	Glomeromycete	Symbiotroph
6	48133	3	*Rhizopus arrhizus*	*Rhizopus oryzae*	100	100	KX922855	Zygomycete	Saprotroph
7	35378	8	*Vishniacozyma victoriae*	*Cryptococcus victoriae*	100	100	KY105839	Basidiomycete	Saprotroph-Symbiotroph
8	27825	1	*Meliniomyces sp.*		100	94	EF093175	Ascomycete	Saprotroph-Symbiotroph
9	25020	7	*Buckleyzyma phyllomatis*	*Sporobolomyces phyllomatis*	100	96	KY101786	Basidiomycete	Pathotroph
10	14633	4	*Vermiconia calcicola*		100	100	KP791762	Ascomycete	Saprotroph
11	12918	1	*Talaromyces diversus*	*Penicillium diversum*	100	95	KJ775702	Ascomycete	Saprotroph
12	10856	8	*Rhizophagus irregularis*	DAOM229456, *Glomus irregulare*	100	100	HF968929	Glomeromycete	Symbiotroph
13	10488	1	*Clitocybe sp.*		100	99	KJ680990	Basidiomycete	Saprotroph
14	9104	4	*Humicola sp.*		100	100	KJ528986	Ascomycete	Saprotroph
15	7409	1	*Baeospora myosura*		100	100	JF907779	Basidiomycete	Saprotroph

OTU	No. reads	No. samples	Blast assigned taxonomy	Species synonymy and isolate information	QC	ID	Accession/ VT*	Phylum	Trophic mode
16	6750	3	*Myrmecridium schulzeri*		100	99	EU543253	Ascomycete	Saprotroph
17	6435	6	*Fusarium oxysporum*		100	100	KX655587	Ascomycete	Pathotroph
18	5886	1	*Entophylctis sp.*		54	99	AY997049	Chytridiomycete	Unassigned
19	5471	4	*Fusarium sporotrichioides*		100	100	KY081692	Ascomycete	Pathotroph-Saprotroph
20	4319	2	Order Chytridales		47	99	EF432822	Chytridiomycete	Unassigned
21	3662	2	*Operculomyces laminatus*		47	95	NR119590	Chytridiomycete	Unassigned
22	2980	2	*Chaetomium piluliferum*		100	100	KF915989	Ascomycete	Saprotroph
23	2463	11	*Microdochium bolleyi*		100	100	KY365586	Ascomycete	Pathotroph-Symbiotroph
24	2428	2	*Plectosphaerella cucumerina*		100	99	KY468524	Ascomycete	Pathotroph
25	2352	3	*Schizothecium glutinans*	*Podospora glutinans*	100	99	AY615207	Ascomycete	Saprotroph
26	1714	1	*Mucor circinelloides*		100	100	KY933391	Zygomycete	Saprotroph
27	760	1	Family Lasiosphaeriaceae		100	99	KT948028	Ascomycete	Unassigned
28	564	1	*Unidentified fungal endophyte*		98	99	FN392300	Unidentified	Unassigned
29	452	2	*Latorua caligans*	*Torula caligans*	100	99	FJ478093	Ascomycete	Pathotroph-Saprotroph
30	337	3	*Rhizophagus intraradices*	Clone M31.17, *Glomusintraradices*	100	99	FJ769310	Glomeromycete	Symbiotroph
31	302	1	*Chaetomium globosum*		100	100	KX421416	Ascomycete	Saprotroph
32	187	2	*Dokmaia sp.*		100	97	GU973777	Ascomycete	Saprotroph*
33	133	1	*Rhizophagus irregularis*	Clone 15.8m, *Glomus irregulare*	100	99	JF820477	Glomeromycete	Symbiotroph
34	111	2	*Ambispora sp.*	Clone 2.8	45	90	FN820281	Glomeromycete	Symbiotroph
35	87	2	*Naganishia uzbekistanensis*	*Cryptococcus liquefaciens*	100	100	KY104336	Basidiomycete	Pathotroph
36	61	1	*Ascochyta sp.*		100	100	AF520641	Ascomycete	Pathotroph

OTU	No. reads	No. samples	Blast assigned taxonomy	Species synonymy and isolate information	QC	ID	Accession/ VT*	Phylum	Trophic mode
AMF region (NS31-AM1)									
1	89562	7	*Rhizophagus intraradices*	BEG 121	100	100	AJ536822/ VTX00105	Glomeromycete	Symbiotroph
2	65997	7	*Rhizophagus irregularis*	DAOM229456	100	99	HF968850/ VTX00114	Glomeromycete	Symbiotroph
3	7594	4	*Glomus* sp.	Glo 10	100	98	AY129570/ VTX00105	Glomeromycete	Symbiotroph

4.3.3 *Relationships between fungal diversity and plant growth parameters*

As noted in earlier sections, there were significant differences among treatments in terms of certain measurements, particularly root length, leaf biomass and Si and P content and uptake. To investigate this further in relation to the fungal diversity in the root, a correlation matrix was created to visualise relationships between these parameters (Figure 19). Correlations that were significant post-bonferroni correction (circles with a white X), had correlation values exceeding r=0.7 and a p value of <0.05 (Figure 19). *Vermiconia calcicola* and *Ambispora* sp. Showed the highest correlation (r=0.97, p<0.01) co-occurring in a Hetchell (Block 3) and Pasture (Block 4) sample at high frequencies. Similarly, *Fusarium sporotrichioides* and *Operculomyces laminatus* were highly correlated (r=0.93, p<0.01) and found co-occurring at high read count in a single sample Pasture (Block 5). *Copinopsis lagopus* and *R. intraradices* (OTU5), were found to be correlated (r=0.86, p<0.01) and co-occurring at similar abundances across several *F. mosseae*, Pasture and Hetchell treatments, both were also found to be present in a control sample (Block 6), indicating potential sample contamination. Other significant correlations between OTUs included *Plectospharella cucumerina* and *Schizothecium glutinans* (r=0.78, p<0.01) co-occurring in Field Margin and Hetchell (both Block 1) samples and between *S. glutinans* and *Latorua caligans*, although these two were only co-occuring in one sample, Hetchell (Block 1).

There were no significant correlations between any OTUs and physiological parameters after the correction; however, some interesting trends should be highlighted. Only two OTUs showed any relationship with Si and P (Figure 19). OTU 32, representing an unknown species of the *Dokmaia* genus, present in a Pasture (Block 4) and Hetchell (Block 1) sample demonstrated a weak negative correlation with Si (r=-0.46, p>0.05) and P (r=-0.39, p>0.05) content in plants. *Buckleyzyma phyllomatis* (OTU 9) was shown to be weakly positively correlated with P content (r=0.52, p>0.05). *S. glutinans,* which correlated significantly with other OTUs also demonstrated a weak negative correlation with root area (r=-0.41, p>0.05). *Chaetomium piluliferum* and *S. indica* also showed an antagonistic relationship (r=-0.44, p>0.05), where the two samples that *C. piluliferum* was present in (Field Margin, Block 1; Pasture, Block 4) showed no reads, or very low read counts (3 hits) of *S. indica* respectively. Other circles showing weak positive correlations largely represent co-occurrence in certain samples.

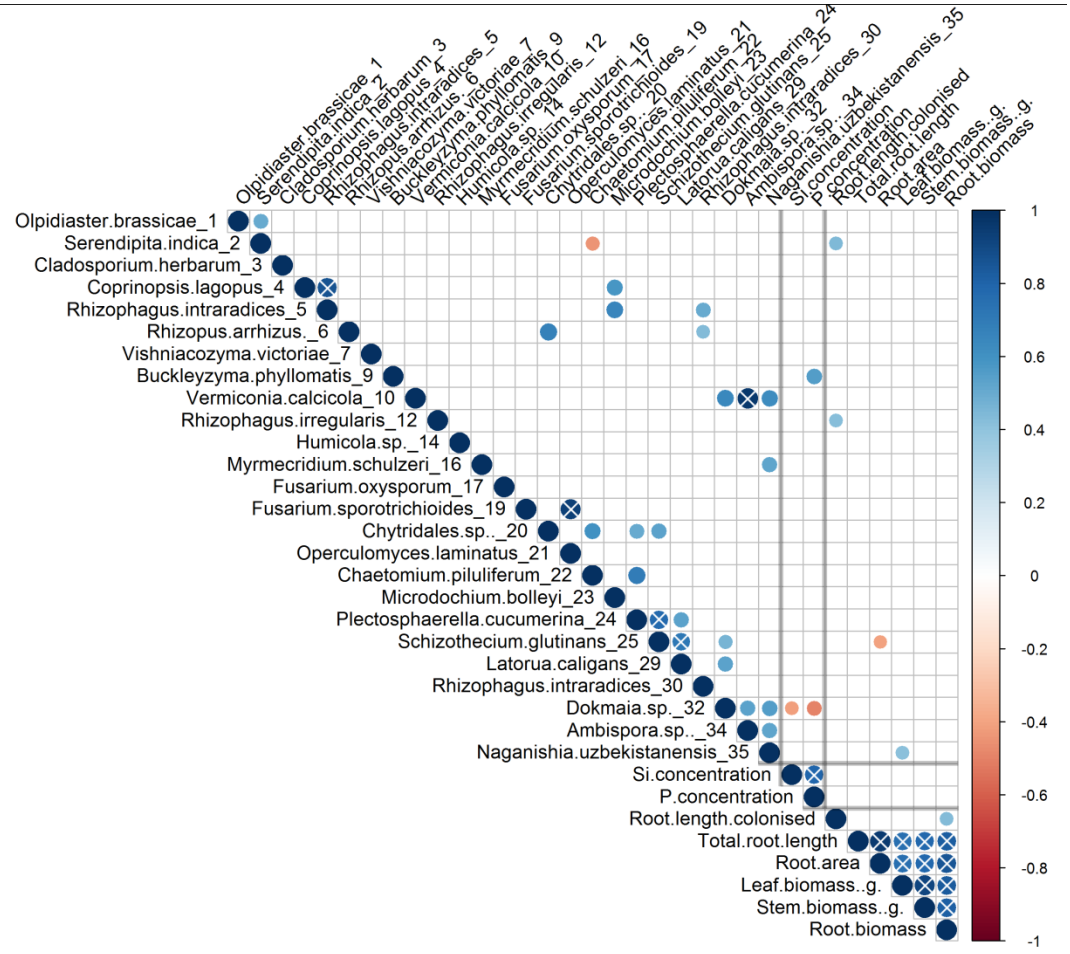

Figure 19: Correlations between OTUs (excluding those that occur in 1 sample only) and key plant measures, generated using the Pearson method. Si and P concentration relate to foliar concentrations (%) that have undergone arscine transformation prior to analysis. The value for root length colonised has also been arcsine transformed. Larger, darker circles represent stronger correlations, blue indicates a positive correlation, red represents a negative correlation. Circles present on the graph represent a significant correlation prior to a Bonferroni correction for multiple comparisons; circles marked with a white X indicate a significant correlation post correction.

The following section briefly comments on the species of interest and particularly those found to have significant correlations in the above analysis.

Olpidiaster brassicae (OTU 1) - Pathotroph

O. brassicae showed the highest occurrence in this experiment, occurring in all samples. This species has a broad host range, infecting many crop species as well as weeds that can act as reservoirs (Hartwright *et al.*, 2010). The combination of this broad host range and a particularly long soil spore viability period of twenty years make this a very difficult pathogen to eradicate (Campbell, 1985). It is not clear whether all plants that are infected by this species show symptoms of infection, no visible infection signs were noted in this experiment.

Serendipita indica (OTU 2) - Symbiotroph

The root endophyte *S. indica* (syn *Piriformospora indica*) was identified in 21 out of 24 samples. However, most read counts were low (<200), with the exception of the *F. mosseae* treatment, where read counts for all replicates were within the 10,000 range. This species has been seen before during investigation of single species stock pot cultures of mycorrhizal fungi (Chapter 2, Section 2.3.3). This Basidiomycete fungus belongs to the novel family Serendipitaceae (Weiß *et al.*, 2016), and has been identified as a model mutualistic fungus due to its ability to be cultured axenically and colonise the model plant species *Arabidopsis thaliana* (Peškan-Berghöfer *et al.*, 2004). This species has also been linked to improvements in plant nutrition, including P (Yadav *et al.*, 2010; Ghanem *et al.*, 2014), and resistance to disease (Schäfer *et al.*, 2009), herbivore attack (Cosme *et al.*, 2016), and abiotic (Waller *et al.*, 2005; Mohd *et al.*, 2017) stresses, conferring similar benefits as AMF (Nath *et al.*, 2016). Despite performing similar roles, there does not appear to be any competitive exclusion between AMF and S. *indica,* due to distinct colonisation patterns and niche partitioning (Schäfer *et al.*, 2009; Newsham, 2011). *S. indica* typically forms within root hairs and requires cell-death dependent colonisation, whereas AMF colonise differentiated and physiologically active cortical cells close to the vascular bundle (Deshmukh *et al.*, 2006).

Glomeromycota (OTUs 5, 12, 30, 33, 34) - Symbiotroph

From the 36 OTUs generated with the ITS region, 5 were identified as belonging to the phylum Glomeromycota, compared to the AM region, which identified only three OTUs. The only treatment that showed no presence of arbuscular mycorrhizal sequences was the field margin treatment. Control samples did show presence of Glomeromycota OTUs in two out of the three samples, but only a single figure number of reads across all samples. The plants which received *F. mosseae* single species inocula contained three different OTUs corresponding to *R. irregularis* (two separate isolates) and *R. intraradices*, neither of which were the intended 'single species' that the inoculum was determined to contain (Chapter 2, Section 2.3.3). In fact, no OTUs recorded corresponded to *F. mosseae* in this experiment.

Cladosporium herbarum (OTU 3) - Pathotroph-Symbiotroph

Reads from this species were recorded in 13 samples, but most showed only a few reads and there was no amplification in control treatments. Four samples did show high read counts however, one replicate from the Field Margin treatment (Block 1: 22,021 reads), two replicates from the Pasture treatment (Block 2:39284; Block 4: 92) and one replicate from the Hetchell treatment (Block 7: 22634). This species is of particular interest due to its ability to solubilise P from the soil, and improve AMF colonisation (Singh & Kapoor, 1998). However, there was no correlation shown between this species and any AMF or P uptake. Interestingly, this species (or

rather species complex (Schubert *et al.*, 2007)) is said to be one of the most common fungi globally, frequently found to be growing on decomposing leaves but also found as an endophyte, and frequently isolated from the environment (Bensch *et al.*, 2012).

Buckleyzyma phyllomatis (OTU 9) - Pathotroph

This basidiomycete yeast was the only OTU to show a positive relationship with P concentration (Figure 19). The literature on this species is very limited, and there is no record of it being isolated from plants within the UK. This OTU had 96% similarity with records of *B. phyllomatis*.

Vermiconia calcicola (OTU 10) - Saprotroph

This OTU was strongly amplified in 4 samples, and was found to be strongly correlated with OTU 34, which has been assigned as a species from the Genus *Ambispora* with low confidence due to the low query coverage. Interestingly *V. calcicola* is a newly identified, extremotolerant species has previously only been shown to colonise stone (Isola *et al.*, 2016). While this is unusual, the results of the BlastN search consistently had high matches for members of the *Vermiconia* genus and other fungi regularly isolated from monuments and stone.

Fusarium sporotrichioides (OTU 19) - Pathotroph-Saprotroph

This species was found across 4 samples. The fungus *F. sporotrichioides* is defined as a pathotroph, and is widely studied due to its capability of producing a potent mycotoxin (trichothecenes), which causes disease symptoms in a large range of plant species (Hohn & Beremand, 1989; Cosic *et al.*, 2012; Moya-Elizondo *et al.*, 2013; Ivanova *et al.*, 2016). *F. sporotrichioides* was significantly correlated to *Operculomyces laminatus* (OTU 21) due to their co-occurrence in a single Pasture sample. This recently described chytrid species was present in one sample (Pasture, Block 5), but unfortunately there is very little information about the biology of *O. laminatus* (Powell *et al.*, 2011).

Plectosphaerella cucumerina (OTU 24) - Pathotroph

This Ascomycete species was positively correlated with *Schizothecium glutinans*, occurring in the Hetchell and Field Margin samples. This species employs a variety of feeding mechanisms, as it can survive in the soil saprophytically (Palm *et al.*, 1995), cause plant disease as a necrotrophic fungus (Pétriacq *et al.*, 2016), and act as a biocontrol agent as a nematophagous fungus (Atkins *et al.*, 2003)

Schizothecium glutinans (OTU 25) - Saprotroph

This 'dung-loving' (coprophilous) fungus, also known as *Podospora glutinans* was amplified in 3 samples, from Field Margin and Hetchell treatments. While current literature refers to this as a coprophilous species, there are records of it colonising plant roots also. Kwaśna *et al* (2008) also recorded this species in their analysis of tree roots (*Betula*, *Fagus* and *Larix*), matching the same accession (AY615207) as in this study. Interestingly, the same authors carried out a study in wheat roots, and also recovered sequences of *P. glutinans*, matching the same record on the NCBI database and again a first recording for this species in wheat (Kwaśna *et al.*, 2010). It has been noted that the Podospora-Schizothecium genus complex is in need of complete revision (Cai *et al.*, 2005).

Chaetomium species (OTU 22, 31) - Saprotroph

While not demonstrating a significant correlation, *C. piluliferum* (OTU 22) was the only OTU to demonstrate any negative relationship with another OTU (*S. indica*, OTU 2). Members of this genus are particularly interesting however, and demonstrate a source of varied metabolites (presumably deriving from their role as saprophytes) which had led them to be highlighted as a potential biocontrol agent (Zhang *et al.*, 2012). *C. globosum* (OTU 31) for example has recently been demonstrated to have a direct negative impact on *F. sporotrichioides* (Jiang *et al.*, 2017). There were no incidences of samples containing either of the *Fusarium* species (OTU 17, 19) also containing either of the *Chaetomium* species (OTU 22, 31) present in this study.

Microdochium bolleyi (OTU 23) - Pathotroph-Symbiotroph

A dark septate endophyte (DSE), defined as an opportunistic pathogen and frequently isolated from plant roots, particularly grasses and Arabidopsis (Newsham, 2011; Mandyam *et al.*, 2013) was found in low levels throughout 11 out of 24 treatments, and was a noticeable outlier. The role of DSE in plants is not clear, and this species has been shown to have a range of impacts on plants from parasitic to mutualistic, and in certain conditions have been referred to as 'surrogate mycorrhizas' although there is no consensus on the mechanism for exchange of resources between these endophytes and the plant host (Haselwandter & Read, 1982; Bledsoe *et al.*, 1990; Jumpponen, 2001).

Dokmaia sp. (OTU 32) - Saprotroph

This OTU occurred in two samples, and was shown to be weakly, negatively correlated with foliar Si and P contents. There is very little in the literature regarding this genus, but it has typically been isolated from decaying organic matter (Promputtha *et al.*, 2003; Ezeokoli *et al.*, 2016)

4.4 Discussion

Overall, the addition of different inocula had significant impacts on the growth and nutrient uptake of *B. sylvaticum* plants, as well as the fungal species isolated from their roots. Conditions for plant growth and set up were identical for all treatments in this study, with the exception of the root and spore filtrates added as inoculum, so it is highly probable that the observed differences in plant response are caused by the fungal communities in the roots.

NGS techniques were applied here to analyse the diversity of fungi within the roots. Of the 40 replicates used in this experiment, only 24 demonstrated successful amplification of fungi and were subsequently used in analysis. There are two reasons for why this technique was not successful in amplifying products from all samples: 1) the primer pairs were not selective enough to be able to exclude plant DNA and 2) fungal colonisation was sufficiently low to not be amplified during the sequencing process. In the absence of high fungal abundance, plant sequences were amplified preferentially and were subsequently discarded. The use of this method did however highlight the presence of rarer fungi that may have otherwise been overlooked through other conventional methods of amplification and analysis, particularly in cloning and visual analyses of colonisation. It may be the case that other samples which lacked the highly abundant OTUs (OTU 1 and 2) may have contained interesting diversity at low abundance that was not picked up through this technique, particularly as rarer OTUs were filtered out for the purposes of normalising the dataset. The results of the NGS analysis have yielded interesting results, but there are a number of shared OTUs between treatments that suggest that the treatments do not have distinct communities, despite the clear differences in the plant growth and colonisation results. It is noted that due to the reduced dataset of 24 samples, repeated analysis of some of the initial plant growth data did not yield significant differences between treatments and these are commented on throughout the discussion.

It was hypothesised that the application of different inoculation treatments would result in distinct fungal communities isolated from the plant roots. Overall, the application of the different inoculum treatments did lead to treatments having significant differences in community assemblages (Section 4.3.2, *Diversity metrics*). However, this relationship appeared to be driven by differences between the Control and Pasture treatments, while the single species *F. mosseae*, Field Margin and Hetchell treatments showed significant overlap (Figure 17). Distinct communities, with highly individual OTUs assemblages were not evident, and there was a high incidence of co-occurrence of many OTUs across samples, particularly of *O. brassicae* which was present in all samples (Table 5). However, analysis of the functional guilds also highlighted some key differences between the treatments, particularly where Control treatments contained a significantly higher proportion of saprophytic fungi compared to the other treatments. This is most likely due to the addition of the autoclaved roots as a control of

the addition of live roots as an inoculum source in the other treatments. This is an interesting result as it implies that administrating a sterilised treatment to control for the addition of roots in other treatments is actually causing other guilds of fungi to colonise the plant roots. In essence this creates another treatment category rather than a base-line comparison for the other treatments. The single species *F. mosseae* treatment also showed interesting diversity results. Both the visual microscopy analysis and the NGS data show that non-AMF species colonised the plants within the *F.mosseae* treatment at a higher rate than the target AMF species. *S. indica* was able to successfully colonise all seven replicates analysed, whereas AMF were only present in four of these samples. This presents an issue when attempting to disentangle which effects are being caused by each species, when both are very similar functionally.

It was also hypothesised that both plant growth and nutrition would be improved with higher levels of AMF colonisation. Plants grown with inoculum from the Pasture field and Hetchell woods showed relatively similar levels of colonisation by AMF and non-AMF fungi, both in the root staining analysis and in the sequence analysis (Section 4.3.2). Interestingly, plants within the Pasture treatment were predominately colonised by two isolates of *R. intraradices* (OTU 5, 30), while plants of the *F. mosseae* treatment were almost exclusively colonised by *R. irregularis* (OTU 12), and not *F. mosseae*. In the one *F. mosseae* replicate (Block 2) that was colonised at very low levels by *R. intraradices* (OTU 5), there was no colonisation by *R. irregularis* (OTU 12). This effect was only seen in one treatment but it may be an indication of competitive exclusion, as it has been noted previously in the literature, *R. irregularis* frequently becomes dominant in mixed species inoculums to the detriment of other AMF (Symanczik *et al.*, 2015).

The treatments had a significant effect on plant growth. For biomass, dry leaf mass was very variable, with plants grown with the Field Margin inocula being significantly larger than Control and Hetchell treatments. Interestingly, Field Margin treated plants showed the lowest level of AMF colonisation, and growth suppression by AMF has been noted previously in glasshouse experiments using *B. sylvaticum* (Chapter 3, Section 3.3.1). While Pasture and Hetchell plants had a similar biomass across all plant fractions, Pasture plants had significantly higher root length compared to Hetchell plants, demonstrating differences in root structure between the treatments. Fine root length is crucial to nutrient and water uptake (McCully, 1999) and has been shown to decrease with mycorrhizal colonisation in monocots (Kothari *et al.*, 1990; Gutjahr *et al.*, 2009, 2015). This was not observed in this study. Overall, Pasture had the second highest level of colonisation by AMF, behind plants growth with the single species inoculum *F. mosseae*. Neither of these plants demonstrated reductions in fine root length compared to controls and treatments lacking in AMF colonisation. Howver, it should be noted that statistical analysis using the 24 samples that produced usable data from the MiSeq, revealed

that the significant differences between root length were no longer present ($F_{(4,19)}$=1.67, p=0.20). Alternatively, other species colonising the plant roots may be causing changes in plant root growth. There was a weak negative relationship between OTU 25 (*S. glutinans*) and total root length highlighted in the correlation analysis, and this OTU was present at high levels in a Hetchell sample (Block 1), which was the treatment that had the lowest fine root length. However, this OTU was also present at much higher levels in the highly diverse Field Margin sample (Block 1) which did not exhibit growth suppression of fine roots.

Foliar Si and P content and concentration were strongly linked to the physical characteristics of the plants, Si concentration was significantly different across treatments, but P concentration was not. Accounting for the differences in root length there was high variability in the efficacy of different treatments in Si and P uptake. For example, plant receiving the Hetchell woods inoculum yielded similar Si and P concentrations as the Pasture samples despite having similar leaf biomass and significantly lower length of fine roots (Figure 16b and d). Two OTUs were highlighted in the correlation analysis as being weakly related to Si and P uptake, primarily OTU 32 (*Dokmaia* sp.) was shown to be negatively related to the Si and P contents of the plants but only occurs in one sample at high frequencies (Pasture, Block 4). It would be expected that higher levels of colonisation by AMF would improve the uptake efficiency of P, and potentially also Si as has been shown previously (Chapter 3, Section 3.3.2). If this were the case, we would expect to see the plants grown with *F. mosseae* inoculum and the Pasture inoculum to outperform plants from the other treatments. This clearly is not the case with regards to the Pasture treatment, and the *F. mosseae* treatment is not significantly different to the control or even the Field Margin treatment which also showed low to no AMF colonisation. However, in plant roots colonised by a variety of fungi, the specific combinations of these may be more important for function rather than individual species.

It was unexpected that plants receiving the Hetchell woods inoculum would show such low colonisation by AMF, considering the presence of *Brachypodium* species at the source site. It was predicted that inocula from Hetchell woods would contain a fungal community is already adapted to colonising the roots of *Brachypodium* plants, and would therefore resemble a distinct community, distinguishable from the other treatments. However, there was only 1 occurrence of a single OTU (OTU 29, *Latorua caligans*) which was found exclusively in a Hetchell woods replicate. Evidently the Pasture treatment was the most effective natural inocula in delivering AMF colonisation, however this treatment yielded the poorest nutrient uptake results, including surprisingly for P uptake, it is difficult to attribute this to any specific fungus within the pasture treatment due to the variability of the data, but it is important to show that presence of AMF in the roots does not always lead to improved nutrient uptake. By using a natural inoculum from an agricultural field, which contains a frequently used laboratory species of AMF, we can

demonstrate that applying a single species AMF inoculum in a glasshouse environment will not yield the same results in a field setting.

Overall, the addition of different fungal inocula to *B. sylvaticum* has led to interesting differences in the growth and nutrient uptake of these plants. There were distinctly different responses in particular between the Pasture field treatment and the Hetchell woods treatments. The results of the NGS analysis have highlighted some interesting relationships between the OTUs and have shown that the plants grown with inoculum from the Pasture and Control treatment led to a distinct fungal community colonising these plants. Interestingly, the effect of supplying a sterilised version of the treatment to the Control plants actually acted as a treatment in itself, leading to a higher percentage of saprophytic fungi colonising the roots of these plants. The single species AMF treatment also yielded unintended results, with *S. indica* another plant growth promoting fungus outperforming the target AMF species. The consequences of this could be important for interpreting plant responses, as high colonisation by this fungus in conjunction with AMF would make it difficult to disentangle the contributions of each species. Overall, this experiment has demonstrated the importance of analysing the fungal diversity of treatments, even within control and single-species treatments. These results highlight the importance of accurately recording of root fungal colonisation of non-target species in the results of glasshouse experiments. Fungal communities in different environments clearly have the potential to have significant impact plant physiology and nutrition and future work should focus on identifying the functional contributions of these root colonising fungi when present in plant roots.

5 Community dynamics of fungi colonising *Brachypodium sylvaticum* roots and effects on foliar silicon and phosphorus concentrations

5.1 Introduction

Diverse fungal communities are important for plant growth and survival, and the interactions between microbes that colonise plant roots are as important as the identity of the individual fungi that colonise them. There are a number of interactions that can happen between microbes within the rhizosphere, and these can be competitive and antagonistic, mutualistic and passive, or mutually beneficial (Johansson *et al.*, 2004; Philippot *et al.*, 2013). It is these interactions that make the study of plants and microbes in-situ so important. Artificially generated plant and fungal combinations can be unrepresentative for plant function in the natural environment due to the difficulty of culturing certain species (Helgason *et al.*, 2002). Studying the fungal community within plant roots in the field at a fine scale has largely been made possible through the use of novel molecular sequencing methods. These enable accurate identification of fungal colonising species which is not possible to do at such high resolution with morphological analyses (Taylor *et al.*, 2017). This has particular advantages for investigating the effect of soil heterogeneity on soil microbial communities, particularly of root colonising fungi.

The heterogeneity of the microbial community in soil is driven by a number of abiotic and biotic factors that dictate the dispersal capabilities of these fungi. Soil conditions are also prone to change within short distances, due in part to the underlying geology of the region, the vegetation overstory and external influences caused by fauna (Concostrina-Zubiri *et al.*, 2013; Steven *et al.*, 2013; Bender *et al.*, 2016). Changes in soil abiotic conditions, particularly pH, are important in determining nutrient and solute availability. For example, silicon, an important defence element in grasses, is taken up by the plant in the form of silicic acid ($Si(OH)_4$), which is available in the soil at concentrations of 0.1-0.6mM (Yost & Fox, 1982; Epstein, 1999). Soluble Si is most available in slightly acidic soils (pH4-7), at pH 9.8 silicic acid disassociates to form $H_3SiOH_4^-$ which adsorbs to aluminium (Al) and iron (Fe) hydrous oxides (Golubev *et al.*, 2006; Haynes, 2014). Plant available phosphate availability however, follows the opposite trend, with plant available P decreasing in acidic soils as inorganic P binds to Al_3^+ which is present at higher concentrations in the soil at acidic pH (<5.5) (Kluber *et al.*, 2012; Jiao *et al.*, 2016). Plant growth is dependent on high levels of available P, which is usually limiting due to the low mobility of P in the soil profile, but exacerbated by reduced pH in acidic conditions.

Arbuscular mycorrhizal fungi (AMF) are plant root symbionts that substantially increase the supply of P to plants in limiting conditions, and receive carbon from plants in return (Smith & Read, 2010). AMF have the potential to improve plant P uptake in acidic conditions, by

immobilising Al_3^+, although AMF do vary in their ability to tolerate extreme pH values (van Aarle *et al.*, 2002; Aguilera *et al.*, 2011). Fungal communities (Dumbrell *et al.*, 2010a), mycelial biomass (van Aarle *et al.*, 2002) and root colonisation (Coughlan *et al.*, 2000) have been shown to be significantly affected by pH. Soil pH has repeatedly been shown to be the overarching deterministic factor in affecting the growth and communities of AMF, outweighing the impact of other variables measured due to its substantial effect on other soil properties (Dumbrell *et al.*, 2010a; Kluber *et al.*, 2012; Carrino-Kyker *et al.*, 2016).

Hetchell Woods is a site of special scientific interest (SSSI), located near Leeds, UK (53°52'36.54"N, 1°25'45.94"W). The area is unique in comprising an area where soil pH varies dramatically over a distance of a few metres, which results in dramatic changes in plant and microbial communities (Dumbrell *et al.*, 2010). A series of studies have been published on the AMF colonising plant roots at Hetchell Woods, making the AMF community at this site one of the most extensively catalogued in Europe (Dumbrell *et al.*, 2010a,b, 2011). Previous studies at this location have demonstrated how abiotic soil factors, particularly pH are capable of influencing the mycorrhizal community (Dumbrell *et al.*, 2010a). They have also demonstrated how AMF communities change throughout the year (Dumbrell *et al.*, 2011). Over winter months where carbon supply from plants is low, diversity and evenness of AMF communities in plant roots is high, while as the supply of carbon rapidly increases in spring months the dominance of individual, opportunistic species increases, thereby reducing evenness in these communities (Dumbrell *et al.*, 2010b, 2011). Temporal variation has also been shown to be significant in pasture and arable settings, with effects of season having a stronger deterministic effect on AMF communities compared to large scale fertiliser inputs (Hazard *et al.*, 2014).

Although extensive work has been carried out at Hetchell Woods, what remains to be studied is whether the patterns in AMF community structure are also applicable to non-AMF root colonising fungi across a pH gradient and how replicable the effects are between years, given the variation across seasons. In the Dumbrell *et al* (2010b, 2011) papers the authors observed that the AMF taxa that become dominant in plant roots in spring and summer months are not consistent, suggesting that the successful dominant taxa are opportunists in the right place at the right time. If this is the case we would perhaps expect to see variability in the identity of the dominant AMF species between years. This trend may be applicable to other groups of root colonising fungi, which share similar life history traits with mycorrhizal fungi. This study will link changes in microbial root colonising communities to the foliar Si and P concentrations of *Brachypodium sylvaticum* plants in their natural environment, across a pH gradient. It was predicted that:

1. In increasingly alkaline conditions, silicic acid availability in the soil will decrease, while P availability will increase, leading to decreased foliar Si concentrations and increased foliar P concentrations.
2. Fungal colonisation will be lower in very acidic soil, and microbial communities and richness will change in accordance with changes across the transect distance, related to changes in the pH profile.
3. Dominant AMF root colonisers will differ between the two sampling years, and it will be determined whether similar trends are observed in the non-AMF fungal colonisers.

5.2 Materials and methods

5.2.1 *Sampling protocol*

Samples were collected in the summer of 2015 (6th August) and 2016 (6th July) along a 10m transect with increasing distance from the limestone outcrop. Historical weather records were obtained for the Met Office stations Church Fenton (~12miles from Hetchell Wood) for long term records (1981-2010). Data from Bramham station (~3.5 miles from Hetchell Wood) was used for records during the sampling period as Church Fenton ceased to function in 2013 and Bramham records were inaccessible for long-term historical data. According to the work carried out by Dumbrell *et al.* (2010a) the transect is predicted to cover a change of pH from ~4.5 to 7.5. Three separate *B. sylvaticum* plants were collected within each 1m^2 for replication along the transect along with soil samples from the immediate area below the extracted plants. Plants and soil were placed in plastic zip-lock bags, transported back to the University of York and stored at 4°C until processing which was completed within 3 days.

Plants were separated into leaf, stem and root fractions. Roots for DNA extraction and staining were only used if connected to the *B. sylvaticum* plants to ensure these results corresponded to the foliar nutrient analyses. Soil was sieved (<2mm) and tweezers were used to remove root fragments to prepare the soil for pH measurements. Soil and plant material were dried at 70°C for 3days to a week according to the methods of (Dumbrell *et al.*, 2010a). Soil pH was measured using 0.01M $CaCl_2$ (soil:solution ratio of 1:5 (w:v)) on dried soil. Root samples for each sample were divided; half was stored in 40% ethanol until mycorrhizal colonisation analysis and half dried at 70℃ for DNA extraction. For full mycorrhizal count procedure see Section 2.2.2; Materials and methods; III. DNA was extracted, amplified and sequenced according to the protocols detailed in Chapter 2, Section 2.4. Samples were randomly split across two different sequencing runs, due to operational costs and availability.

5.2.2 *Statistical analysis*

All percentage data (for Si and P, and fungal colonisation) was arcsine square root transformed prior to statistical analyses, but original concentration values are used for the production of

graphs. Statistical analysis and graph production was carried out using Rstudio running R version 3.1.3 (R Development Core Team, 2011; RStudio Team, 2016). Root colonisation, nutrient (Si and P) and pH data was checked for a normal distribution and homogeneity of variance following the methods outlined in Chapter 2, Section 2.2.2. In the case where data did not fit a normal distribution or had unequal variances, a Kruskal-Wallis test was used in the stead of a one-way ANOVA. Post-hoc tests were used where appropriate; the stats::TukeyHSD was used following ANOVA tests, while the FSA::dunnTest was used with the Bonferroni method following Kruskal-Wallis tests. Statistical tests were performed to examine whether distance along the transect and year of sampling had an effect on root colonisation, nutrient content and pH data and whether the distance factor was more significant in one year than another. This was tested using 'Distance' along the transect and 'Year' of sampling as two independent variables, and testing for interaction effects.

For sequence data analysis, fungal α-diversity and richness was calculated on normalised OTU tables (Chapter 2, Table 3, Step 22) using the alpha_diversity.py to calculate Shannon Diversity index, and the Chao1 estimation. The Kruskal-Wallis test was used to determine significant differences between alpha diversity metrics. Differences in fungal community composition were determined by performing a PERMANOVA (R function vegan::adonis) on a Bray-Curtis dissimilarity matrix of the CSS normalised OTU abundance tables (Chapter 2, Table 3, Step 21). A non-metric multidimensional scaling (NMDS) plot was also generated using the Bray-Curtis dissimilarity matrix using the vegan:MetaMDS function. To identify specific taxa that differed as a result of sampling year, the differential_abundance.py package was implemented to test for significant differences in abundance of specific OTUs between the two years. A similar test that allows comparisons between more than two groups, compare_categories.py, was used to test whether there were any taxa present in a single location, or at particularly high abundance in a single location along the transect. To compare the OTUs sequenced in this study to those in the Dumbrell *et al.*(2011) AMF amplicon sequences were aligned using MEGA v6.06 (Tamura *et al.*, 2013) and a Neighbour-Joining tree with five hundred bootstrap replications was generated, also using the MEGA program.

5.3 Results

5.3.1 *pH*

Kruskal-wallis non-parametric tests were used to determine differences in pH values across the dataset. pH did not change significantly over the distance of the transect in either year (2015: $H_{(9)}$=15.41, p=0.08; 2016: $H_{(9)}$=15.79, p=0.07) and there were no significant differences in pH between the two years ($H_{(1)}$=3.10, p=0.08). Although there was no change in pH across the transect, values for soil pH were more variable in the first 5 metres, closer to the limestone

outcrop and pH in 2016 was more variable than in 2015 (Figure 20). Due to the lack of pH gradient, rather than treating pH as a deterministic or independent variable in statistical tests, pH was incorporated into linear models as a random variable. These models were compared with simpler models without the random term and models were selected based on lowest Akaike Information Criterion (AIC) values, indicating a more representative model of the data (Akaike, 1973).

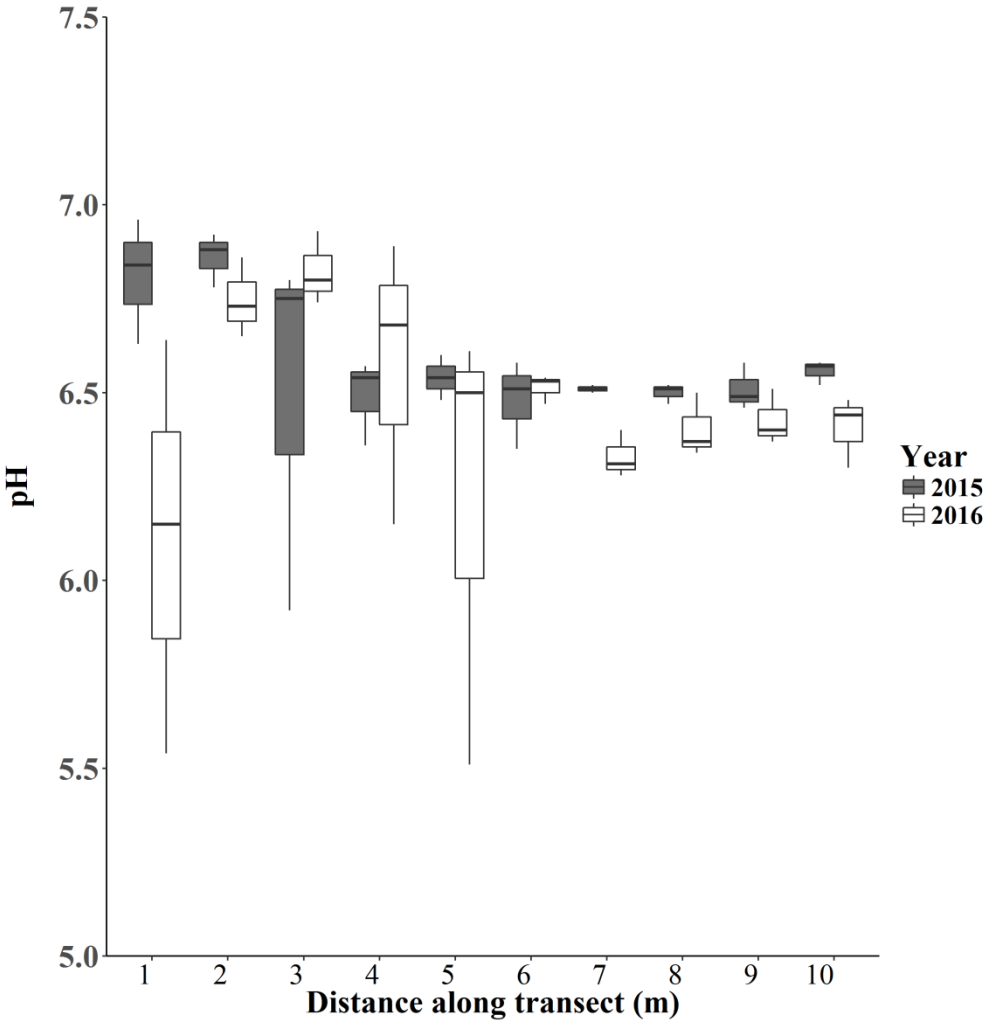

Figure 20: Change in soil pH across the transect distance for the two sampling years. Shaded boxes represent samples from 2015, while the white boxes represent samples taken in 2016. The heavy line within the box represents the median value, with the upper and lower hinges showing the 75th and 25th quartile respectively. The whiskers extend to 1.5 times the interquartile range.

Table 6: Monthly records from the Bramham and Church Fenton Meteorological Office weather stations. Average values for June and July from 1981-2010 at the Church Fenton station are shown alongside individual records Bramham station for the week prior to sampling in 2015 and 2016, prior to and during sample collection.

Church Fenton 1981-2010 average		**Average temperature (°C)**		**Total average rainfall**	
Month		Minimum	Maximum	**(mm)**	
June		9.6	18.8	54.8	
July		11.8	21.2	50.2	
August		11.7	20.8	57.9	
Bramham 2015-2016 average		Average temperature (°C)		Rainfall (mm)	
Month	Year	Minimum	Maximum	Total	Average
June	2015	7.8	19.0	28	0.9
	2016	10.1	18.8	55.4	1.8
July	2015	10.5	20.3	86.0	2.8
	2016	12.1	21.6	30.4	1.0
August	2015	11.2	21.3	124.6	4.0
	2016	12.5	21.3	86.8	2.9
Bramham station weekly average					
w/c 31st July 2015		10.1	21.8	3.0	0.4
w/c 30th June 2016		9.0	19.1	8.8	1.3

5.3.2 *Root length colonisation*

Total root length colonised was determined by microscopic analysis of stained fungal structures in the root, separated into arbuscular mycorrhizal structures (AMC) and 'other' fungal structures or non-mycorrhizal colonisation (NMC). AMC was significantly different across the transect ($F_{(9,39)}$=3.55, p<0.01) and between years ($F_{(1,39)}$=8.08, p=0.007). This relationship was also apparent in NMC across the transect ($F_{(9,39)}$=2.36, p=0.03) and between years ($F_{(1,39)}$=4.34, p=0.05), but the relationship was much weaker than for AMC. Overall AMC decreased in 2016, while NMC increased (Figure 21). The highest recorded value for AMC in both 2015 and 2016 appeared to be at the 1m distance, closest to the limestone outcrop (Figure 21). Overall fungal root length colonisation did not change between the two years ($F_{(1,39)}$=0.181, p=0.672), but the distance did become a significant factor when analysing the two years together ($F_{(9,39)}$=3.539, p=0.003) and this seemed to be driven by the very low colonisation at 5m along the transect.. There was no significant interaction between distance and year for either AMC ($F_{(9,39)}$=0.76, p=0.65) or NMC ($F_{(9,39)}$=0.97, p=0.48).

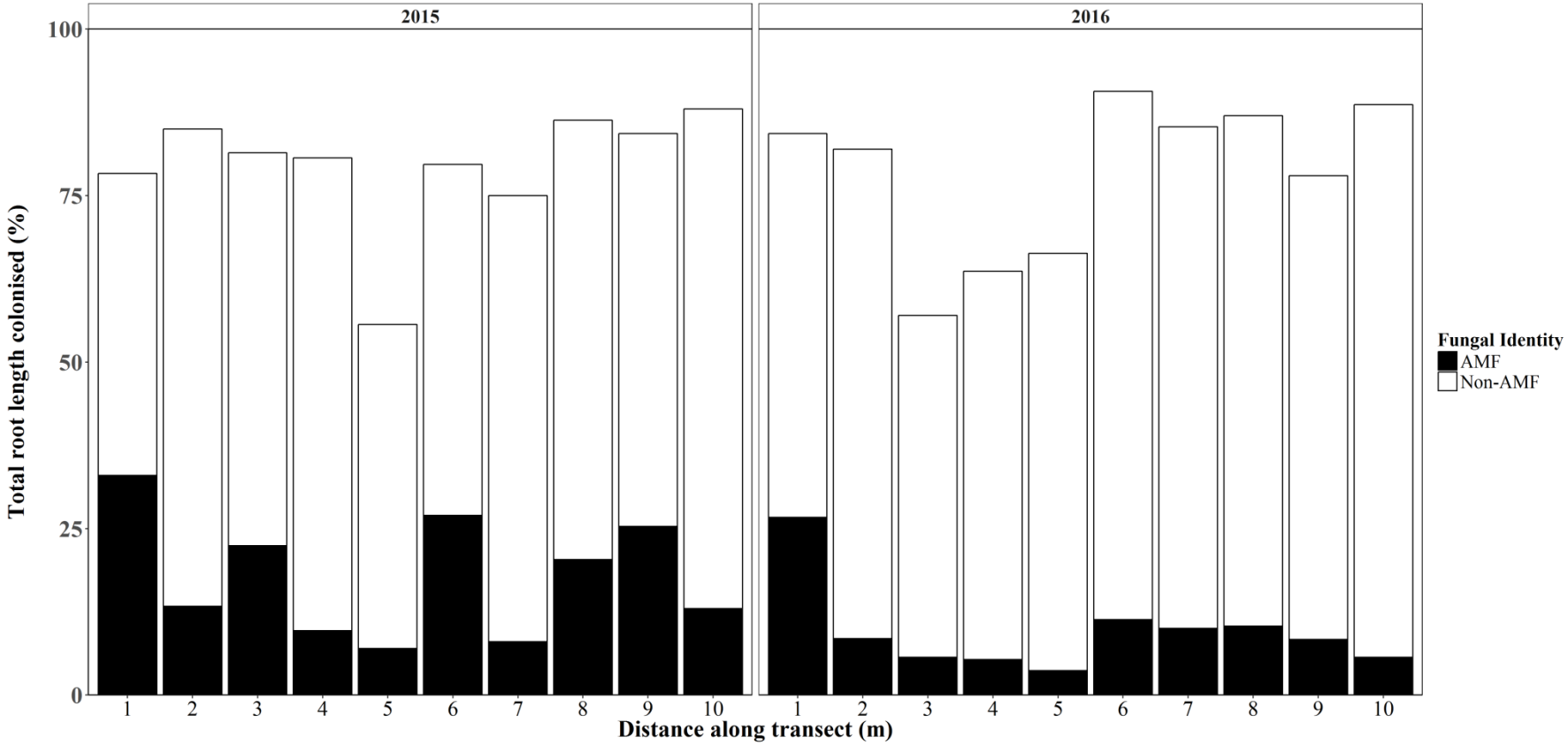

Figure 21: Total root length colonised (%) by fungal structures of *Brachypodium sylvaticum* plants as determined by staining and counting method. Samples were collected. along a 10 metre transect during two years. The left panel shows colonisation in plant roots collected during 2015, and the right panel shows colonisation in root of plants collected in 2016. Black bars represent arbuscular mycorrhizal fungal (AMF) colonisation, and the white bars represent non-AMF colonisation.

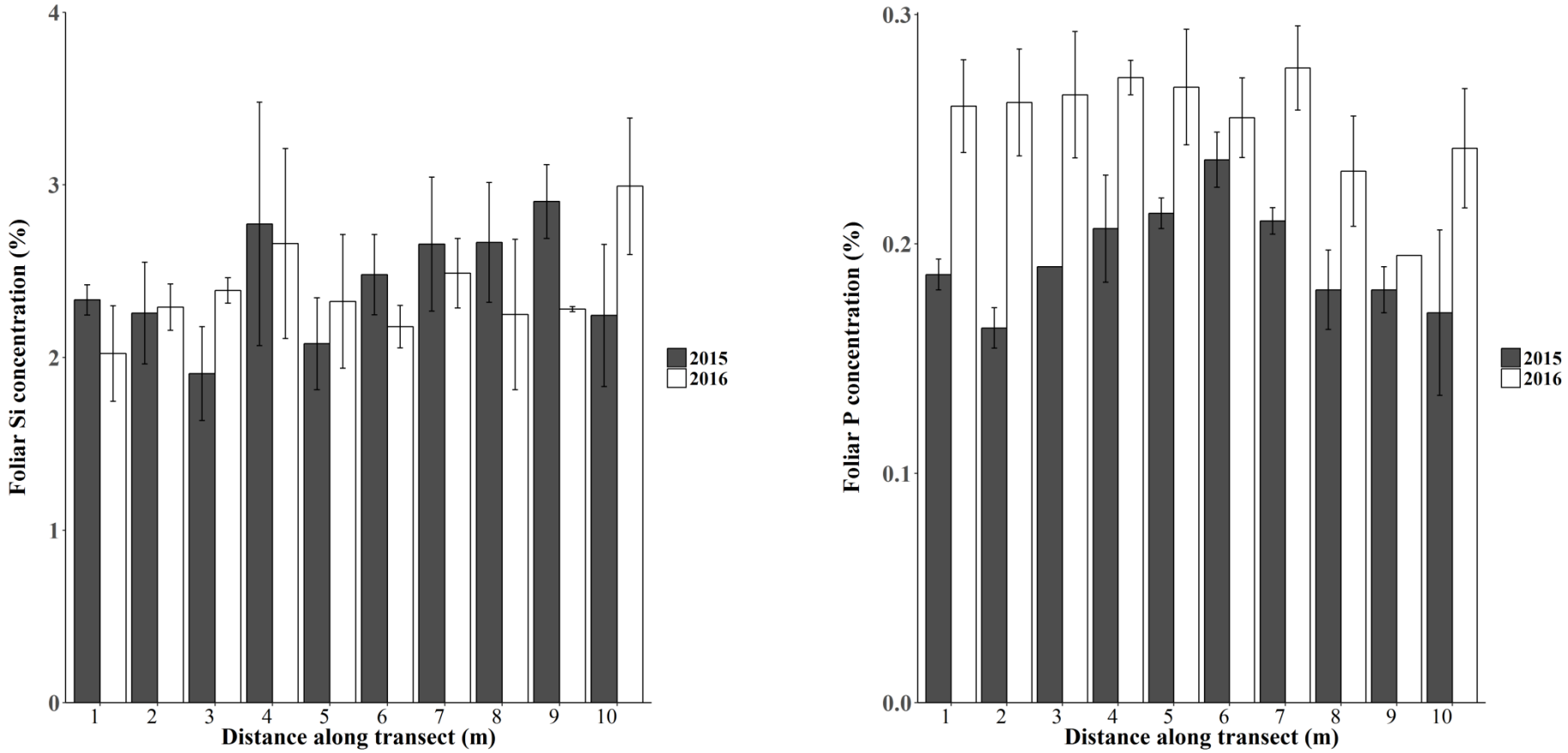

Figure 22: Foliar Si and P concentration (% of leaf dry matter) of *Brachypodium sylvaticum* plants across a two year period. Shaded bars represent samples from 2015, and white bars represent samples collected in 2016. Error bars represent standard error.

5.3.3 *Si and P*

Foliar Si concentration did not change over the distance of the transect ($F_{(9,38)}$=0.78, p=0.64), nor did it differ between years ($F_{(1,38)}$=0.02, p=0.89) and there was no interaction between distance and year ($F_{(9,38)}$=0.84, p=0.58). Foliar P concentration was more variable and did vary somewhat across the transect, but not significantly ($F_{(9,38)}$=2.08, p= 0.057) but values were significantly different between years ($F_{(1, 38)}$=47.51, p<0.001) and there was no interactive effect between distance and year ($F_{(9, 38)}$=0.88, p=0.55). No significant correlation between total root length colonisation by AMF and foliar P concentration was evident (Pearson's $t_{(55)}$=-1.91, p=0.06) although there was a slight negative trend. However, there was a significant, negative correlation between AMF colonisation and foliar Si concentration ($t_{(55)}$=-2.08, p=0.042).

5.3.4 *Fungal diversity*

Two samples for the ITS amplicon and three samples for the AMF amplicon did not produce enough PCR product to be carried forward for sequencing. In the preliminary sequence preparation steps, several samples did not produce usable sequences for the generation of OTU tables. This was not related to the sequencing run which samples were placed. Efforts were made to recover samples by lowering the quality thresholds during the split_library.py stage, but several samples still did not generate useable sequences for the generation of OTU tables. OTU tables were generated separately for the AMF (NS31-AM1) and ITS (ITS1F-ITS4) amplified regions. Extensive filtering and cross-referencing with sequence databases was required and a high proportion of amplified sequences were non-fungal or very rare and subsequently removed or collapsed into one OTU due to high sequence similarity. This resulted in 193 OTUs generated using QIIME decreasing to 15 post-filtering for the AM region after the removal of plant and non-AMF sequences, and 331 decreasing to 109 in the ITS region after the removal of plant and non-fungal sequences and collapsing highly similar sequences (>99% similarity) as determined by a sequence alignment using the EMBL-EBI ClustalW. Consequently, after removal of non-fungal and rare OTUs, some samples had too few reads (<100) to be kept in analyses and were also removed. This resulted in a decrease in sample size in the ITS subset from 58 samples to 33, and from 57 samples to 15 in the AM subset. OTUs were re-labelled post filtering and the assigned numbers reflect decreasing abundance. Therefore OTU 1 will allocated to the most abundant taxa in the analysis, while OTU 109 and OTU 15 will represent the taxa with the lowest abundance in the ITS and AMF amplicon, respectively. The filtering led to an uneven depth of sampling across amplicons and the transect, and between years. This is detailed in the appropriate tables and figures.

Of the 109 OTUs generated by the ITS amplicon, 36 OTUs represented 90% of the total sequences across all samples (Table 7). Overall the analysis shows that the root colonising fungal community is diverse and not dominated by a few high abundance species. The most

frequently isolated taxon was OTU1 *Itersonillia perplexans*, this plant pathogen was represented in 13 samples and accounted for 10% of all reads. This was also the most dominant OTU sequenced from the 2015 samples, accounting for 14.41% of all reads, slightly less than twice that of the next most abundant OTUs at ~8%. Two OTUs had similarly high read counts in the samples collected in 2016, OTUs 7 and 8, which both accounted for ~11% of all reads individually (Table 7). For the AMF amplicon, there were only 15 OTUs in total (Table 8). The AMF primer pair was more successful in amplifying AMF species than the ITS primer pair, which amplified four AMF sequences corresponding to *Rhizophagus irregularis* (OTU 15) and three unidentified *Glomus* species (OTUs 26, 42, 94). The dominant AMF amplicon taxon changed depending on the sampling year, with OTU 1 *Glomus* sp. (VTX00122) being the dominant taxon in 2015 accounting for 39% of all sequence reads. In 2016 however, results show a more even community, with three OTUs (1, 3 (VTX00163), and 5 (VTX00342)) sharing similar shares of the overall sequence reads (25-28%). AMF amplicon sequencing was only successful in four samples in 2016, two of which (at 1m and 7m along the transect) showed colonisation dominance by OTU 1, while the other two had a higher overall diversity and richness (Table 8).

Species richness (Chao1) and alpha diversity (Shannon index) were higher in 2016 compared to 2015, in both amplicons (Table 9). The Chao1 estimate of species richness did not show any significant differences across the distances measured for the ITS amplicon (Kruskal-Wallis: $H_{(9)}$=4.91 p=0.84), but did indicate that there were differences between years ($H_{(1)}$=13.60, p<0.001,Table 9). Shannon-diversity index, a measure of the diversity within groups also showed no significant differences in diversity of root colonising fungi across the distance of the transect ($H_{(9)}$=5.38 p=0.80) but there was a significant difference between years ($H_{(1)}$=12.98, p<0.001). For the AMF amplicon, there were no significant differences between the diversity of AMF colonising plant roots across the transect distance ($H_{(6)}$=4.617, p=0.594) or between years ($H_{(1)}$=0.017, p=0.896). Species richness of AMF also did not change across the transect ($H_{(6)}$=4.953, p=0.550) or between years ($H_{(1)}$=0.017, p=0.895).

Table 7: The most abundant taxa (representing 90% of total sequences) or operational taxonomic units (OTUs) of the ITS region amplified by the ITS1F-ITS4 primer pair. Sample counts and proportion of sequence reads (%) are shown for all samples, as well as separately for 2015 and 2016 samples. Taxonomy was assigned through NCBI blast and trophic guild is assigned using FunGuild.

OTU	Sample count[a]	%[b]	2015		2016		Taxonomic assignment (synonymous taxonomy)	QC[c]	ID[d]	Accession	Phylum	Trophic guild
			Count	%	Count	%						
1	13	10.64	12	14.41	1	0.01	*Itersonilia_perplexans*	100	100	KU563636	Basidiomycota	Pathotroph
2	11	8.56	4	8.81	7	7.84	*Phallus_impudicus*	100	92	KU516101	Basidiomycota	Saprotroph
3	9	6.16	7	8.27	2	0.21	*Olpidiaster brassicae (syn. Olpidium_brassicae)*	98	96	AB205213	Chytridiomycota	Pathotroph
4	10	6.01	9	4.89	1	9.16	*Penicillium_chrysogenum*	100	100	KU947079	Ascomycota	Pathotroph
5	8	5.34	6	7.22	2	0.01	*Cladosporium* sp.	100	100	KY921924	Ascomycota	Pathotroph-Symbiotroph
6	8	3.79	6	5.10	2	0.10	*Neoascochyta* sp.	98	100	KT389526	Ascomycota	-
7	8	3.68	4	0.93	4	11.44	*Sebacina_cystidiata*	100	100	JQ665511	Basidiomycota	Symbiotroph
8	7	3.67	2	1.01	5	11.19	*Ophiosphaerella* sp.	100	97	KT692575	Ascomycota	Saprotroph
9	22	3.21	12	4.20	10	0.43	*Vishniacozyma victoriae (syn Cryptococcus_victoriae)*	100	100	KY105837	Basidiomycota	Saprotroph-Symbiotroph
10	7	3.20	7	4.34	0	0.00	*Heterobasidion_annosum*	100	100	KU645332	Basidiomycota	Saprotroph
11	19	3.18	10	3.23	9	3.02	*Myrmecridium_schulzeri*	100	100	KC989072	Ascomycota	Saprotroph
12	3	2.86	1	3.87	2	0.01	*Naganishia albida (syn. Cryptococcus_albidus)*	100	100	KY102595	Basidiomycota	Saprotroph-Symbiotroph
13	3	2.84	3	3.84	0	0.00	*Hymenoscyphus herbarum (syn. Calycina_herbarum)*	100	97	JN033407	Ascomycota	Saprotroph
14	5	2.46	1	2.14	4	3.36	Ascomycota sp.	97	98	GQ996144	Ascomycota	-
15	9	2.34	7	3.15	2	0.06	*Rhizophagus_irregularis (syn. Glomus irregulare)*	100	100	HF968934	Glomeromycota	Symbiotroph
16	3	2.13	3	2.89	0	0.00	*Psathyrella_spadiceogrisea*	98	97	DQ389682	Basidiomycota	Saprotroph

17	7	1.62	3	0.44	4	4.96	*Serendipita_herbamans*	100	100	NR_144842	Basidiomycota	Symbiotroph
18	1	1.50	1	2.03	0	0.00	*Phomatospora_biseriata*	100	96	KX549454	Ascomycota	Saprotroph
19	2	1.44	0	0.00	2	5.51	*Hymenoscyphus* sp.	97	97	KT268833	Ascomycota	Saprotroph
20	5	1.27	4	1.71	1	0.00	*Hemimycena_gracilis*	95	98	DQ490623	Basidiomycota	Saprotroph
21	6	1.25	0	0.00	6	4.78	*Cadophora* sp.	100	99	KX610415	Ascomycota	Symbiotroph
22	5	1.21	1	0.00	4	4.61	*Cyathicula* sp.	100	100	KC989059	Ascomycota	Saprotroph
23	2	1.03	2	1.39	0	0.00	*Psathyrella_lutensis*	100	99	FN396145	Basidiomycota	Saprotroph
24	5	1.00	3	0.86	2	1.40	*Rhizoctonia* sp.	100	99	JQ859888	Basidiomycota	-
25	7	0.95	3	0.66	4	1.78	*Geminibasidiaceae*	97	95	NR_111878	Basidiomycota	-
26	6	0.93	4	1.22	2	0.12	*Glomus* sp.	100	96	AJ504638	Glomeromycota	Symbiotroph
27	2	0.93	1	0.91	1	0.99	*Mycena* sp.	100	86	JF908444	Basidiomycota	Pathotroph-Saprotroph
28	3	0.89	0	0.00	3	3.41	Helotiales sp.	100	97	KX438326	Ascomycota	-
29	5	0.88	1	0.51	4	1.93	*Exophiala* sp.	100	95	KX610445	Ascomycota	Saprotroph
30	3	0.81	2	1.10	1	0.02	*Coprinellus_micaceus*	100	100	KX449450	Basidiomycota	Saprotroph
31	4	0.77	0	0.00	4	2.93	*Darksidea* sp.	100	87	KT270207	Ascomycota	-
32	4	0.74	2	1.00	2	0.01	Helotiales sp.	100	93	KX438326	Ascomycota	-
33	2	0.74	1	0.06	1	2.68	Corticiaceae sp.	100	85	AB8318854	Basidiomycota	-
34	1	0.69	1	0.94	0	0.00	*Coprinopsis_lagopus*	100	100	JN943127	Basidiomycota	Saprotroph
35	3	0.69	3	0.93	0	0.00	*Aspergillus_flavus*	100	100	MF120213	Ascomycota	Pathotroph-Saprotroph
36	3	0.69	1	0.53	2	1.14	Mycena sp.	100	81	JF908409	Basidiomycota	Pathotroph-Saprotroph

[a] Sample count is the number of samples (out of 33) that contained sequences for this OTU.

[b] The number of reads this OTU represents out of the total number of reads across all samples.

[c] Query coverage (QC, %) of the submitted sequence against the top hit on BLAST.

[d] The maximum identity (ID, %) of matched nucleotides of the submitted sequence against the top hit on BLAST.

Table 8: Details of all operational taxonomic units (OTUs) amplified by the NS31-AM1 Illumina primer pair. Total read counts across all samples ('Sample count', n=15) and isolation frequency (%) detailing how often the OTU was amplified in different samples. This is replicated for each sampling year, 2015 and 2016. Taxonomy was assigned using the MaarjAM database, or NCBI Blast (in bold), whichever had the highest query coverage and identity. Virtual taxa from the MaarjAM database were included regardless of database used.

OTU ID	Sample count[a]	%[b]	2015		2016		Assigned taxonomy (isolate information)	Virtual taxon	QC[c]	ID[d]	Accession number
			Count	%	Count	%					
1	12	36.80	8	39	4	25	*Glomus* sp.	VTX00122	100	100	HF568332
2	7	22.79	5	24	2	14	*Rhizophagus irregularis* (DAOM229456)	VTX00114	**100**	**100**	**HF968850**
3	5	15.09	3	13	2	28	*Glomus* sp.	VTX00163	100	100	LN621058
4	5	9.11	3	10	2	1	*Glomus* sp.	VTX00072	100	100	LN621104
5	5	8.46	3	5	2	25	*Glomus* sp.	VTX00342	100	93.3	LN619907
6	4	4.68	2	5	2	2	*Glomus* sp.	VTX00153	100	100	LN620070
7	3	2.22	1	2	2	1	*Glomus* sp.	VTX00214	100	100	FR728571
8	5	0.47	3	0	2	2	*Rhizophagus irregularis*	VTX00247	**100**	**99**	**FJ009612**
9	1	0.09	0	0	1	1	*Claroideoglomus lamellosum*	VTX00193	**99**	**99**	**KU136434**
10	2	0.08	0	0	2	1	*Glomus* sp.	VTX00122	99.6	100	LN620004
11	1	0.08	0	0	1	1	*Glomus* sp.	VTX00129	100	100	LN620841
12	2	0.06	0	0	2	0	*Claroideoglomus* sp.	VTX00057	**100**	**100**	**HF568164**
13	1	0.03	0	0	1	0	*Funneliformis mosseae*	VTX00067	**100**	**100**	**KU136407**
14	2	0.02	1	0	1	0	*Funneliformis constrictum*	VTX00064	**100**	**100**	**KU136431**
15	1	0.02	0	0	1	0	*Archaeospora trappei*	VTX00245	**100**	**97**	**AJ006800**

[a] Sample count is the number of samples (total n=15, 2015 n=11, 2016 n=4) that contained sequences for this OTU.

[b] The number of reads this OTU represents out of the total number of reads across all samples.

[c] Query coverage (QC, %) of the submitted sequence against the top hit on BLAST.

[d] The maximum identity (ID, %) of matched nucleotides of the submitted sequence against the top hit on BLAST.

Table 9: Alpha diversity metrics Chao1 and Shannon-Diversity Index across the two sampling years, 2015 and 2016 for the ITS and AMF amplicon. Different letters indicate a significant difference between values within each amplicon.

Amplicon	Sampling Year	Sample size	Chao1 Species richness	Shannon diversity
ITS	2015	26	13.54[a]	3.59[a]
	2016	10	26.54[b]	4.31[b]
AMF	2015	11	5.32[a]	0.57[a]
	2016	4	7.00[a]	1.04[a]

Beta Diversity

With the use of the vegan::adonis PERMANOVA permutation test on the ITS amplicon Bray-Curtis dissimilarity matrix, the effect of the independent variables 'Distance' along transect, 'Year' of sampling and pH on sample communities was assessed. Both Year and Distance generated significant effects independently, the most significant factor accounting for 34.5% of the total variation ($F_{(1,35)}$=3.082, p=0.001) was the year of sampling, followed by Distance accounting for 7.7% of the total variation ($F_{(9,35)}$=1.638, p=0.003). pH was not a significant factor accounting for only 2.41% of the total variation ($F_{(1,35)}$=1.030, p=0.442). Convergent solutions for the NMDS using the ITS amplicon Bray-Curtis dissimilarity matrix were found at 5 attempts, with a stress value of 0.26. Figure 23 displays the clear separation of communities of the ITS samples by year and distance between data points demonstrates their similarity, so data points that are close together have similar community structures. The length of the arrows represents the strength of the correlation between the factor and the ordination, and the direction indicates the direction in which this variable changes most rapidly. This demonstrates that changes in the foliar P concentration changes most rapidly in the direction with the 2016 samples (Figure 23).

The differential_abundance.py that was used to test differences in OTU abundance between years highlighted 21 OTUs that varied in abundance significantly between the two years after a post-hoc multiple comparison correction (Table 10). With the exception of OTU 11, an unidentified Ascomycete, all OTUs that showed significant differences between years were higher in abundance in 2016 compared to 2015. The results of the compare_categories.py test to highlight differences between OTU abundance across the distance of the transect did not show any significant differences (data not shown).

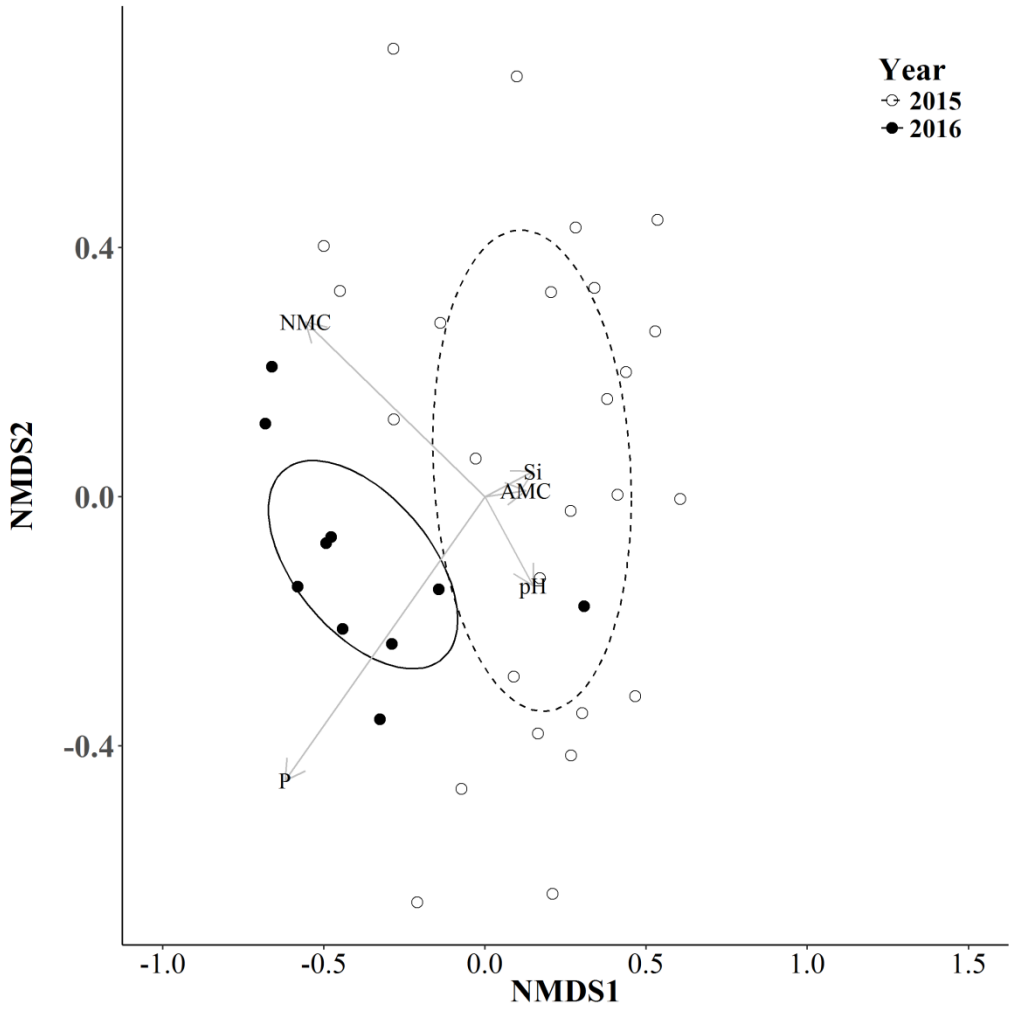

Figure 23: Non-metric multidimensional scaling plot performed on a bray-curtis simmilarity matrix generated from a normalised OTU table. Shaded circles and solid ellipse represent communities from 2016 (n=10), and the non-shaded circles and dashed ellipse represent samples from 2015 (n=26). The length of arrows for the NMC (non-mycorrhizal root colonisation), AMC (arbuscular mycorrhizal root colonisation), Si (foliar silicon concentration), P (foliar phosphorus concentration) and pH demonstrate the strength of correlation between this factor and the ordination.

The OTUs that had significantly different abundance between the two sampling years (Table 10) were plotted on a correlation matrix to explore whether these OTUs showed a relationship with other parameters measured. Particularly to identify if there was any relationship between OTU abundance and foliar P concentration, which was significantly lower in plants sampled in 2016 (Figure 22, Figure 24). This analysis was performed on a reduced dataset that only used samples where the NGS data was successful (Section 5.3.3). P concentration was shown to be weakly, negatively correlated to pH in the multiple correlation analyses, although not significantly after post-hoc multiple comparison corrections. Looking to the complete dataset,

this relationship was explored further. The weak correlation was found between P concentration and pH ($t_{(56)}$=-2.38, p=0.021), indicating that at the higher recorded pH values (6.5-7) P concentration was lower than a more acidic values, although it appears this relationship may be being driven by low sample numbers at low pH values, although these values did not exceed Cook's distance and so remained in the analysis. Analysing 2015 and 2016 records separately, there was no demonstrable trend or relationship shown between pH and foliar P concentration (2015: $t_{(28)}$=-1.65, p=0.11; 2016: $t_{(26)}$=0.63, p=0.53). pH also appeared to have a weak negative effects on three OTUs in this analysis, all of these showed a small positive effect on the P concentration.

Table 10: OTUs (Operational taxonomic units) that differed significantly in abundance between the two sampling years; 2015 and 2016. Calculated using the Qiime differential_abundance.py package, p-values displayed are adjusted using the Bonferroni correction for multiple comparisons.

OTU	Isolation frequency		Mean read count		Adjusted *P* value	Species ID	Phylum	Trophic mode
	2015	2016	2015	2016				
21	1	6	1	9448	<0.001	*Cadophora* sp.	Ascomycota	Symbiotroph
22	4	5	5	9126	0.005	*Cyathicula* sp.	Ascomycota	Pathotroph-Saprotroph
63	0	4	0	710	0.016	*Apodus deciduus*	Ascomycota	Saprotroph
28	2	5	2	6739	0.016	Helotiales sp.	Ascomycota	-
31	0	5	0	5799	0.016	*Darksidea* sp.	Ascomycota	Symbiotroph
14	3	5	11979	6639	0.023	Ascomycota sp.	Ascomycota	-
46	1	5	1	3157	0.028	*Neonectria* sp.	Ascomycota	Pathotroph
57	1	5	1	1425	0.033	Ascomycota sp.	Ascomycota	-
66	0	2	0	586	0.033	*Amanita* sp.	Basidiomycota	Saprotroph
54	0	2	0	1588	0.033	*Microdochium* sp.	Ascomycota	Pathotroph-Symbiotroph
50	1	3	1	1880	0.033	*Sporobolomyces roseus*	Basidiomycota	Pathotroph
8	3	5	5623	22134	0.038	*Ophiosphaerella* sp.	Ascomycota	Saprotroph
81	0	2	0	178	0.039	Pezizaceae sp.	Ascomycota	-
7	6	6	5200	22630	0.039	*Sebacina cystidiata*	Basidiomycota	Symbiotroph
88	0	2	0	94	0.039	*Epicoccum nigrum*	Ascomycota	Pathotroph
29	6	5	2833	3817	0.039	*Exophiala* sp.	Ascomycota	Saprotroph
68	1	2	1	498	0.039	*Mortierella gamsii*	Zygomycota	Saprotroph
94	0	2	0	70	0.039	*Glomus* sp.	Glomeromycota	Symbiotroph
19	2	7	2	10896	0.039	Hymenoscyphus sp.	Ascomycota	Saprotroph-
97	0	2	0	59	0.039	*Solicoccozyma aeria* (aka *Cryptococcus aerius*)	Basidiomycota	Saprotroph
70	0	2	0	391	0.052	*Dictyosporium* sp.	Ascomycota	Saprotroph

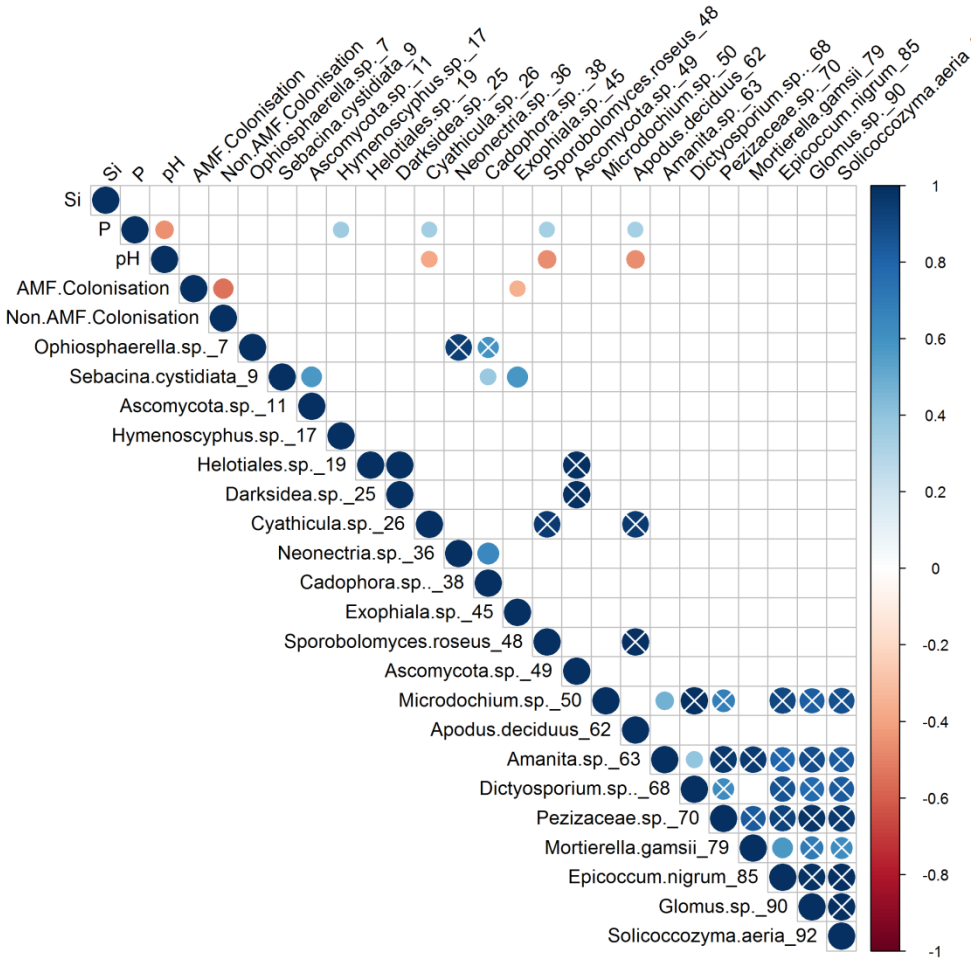

Figure 24: Correlations between OTUs (operational taxonomic units) that differ significantly between sampling years (2015-2016) and key plant measures, generated using the Pearson method. Si and P relate to foliar silicon and phosphorus concentrations (%), respectively, which have undergone arscine transformation prior to analysis. The value for root length colonisation of AMF (arbuscular mycorrhizal fungi) and non-AMF has also been arcsine transformed. Larger, darker circles represent stronger correlations, blue indicates a positive correlation, red represents negative correlation. Circles present on the graph represent a significant correlation prior to a Bonferroni correction for multiple comparisons; circles marked with a white X indicate a significant correlation post correction.

5.3.5 *Comparisons with other studies on Hetchell Woods*

The ITS and AMF amplicon sequencing results of this study were compared to the results obtained in the glasshouse experiment in Chapter 4 to compare how effective the natural inoculum was at providing fungi to colonise plants within a controlled glasshouse experiment. The inoculum for Chapter 4 was collected during November 2015, so the taxa overlap may not be entirely representative. There were six taxa within the ITS amplicon identified that were

represented in both studies, and one shared AMF amplicon OTU (Table 11). In the glasshouse experiment in Chapter 4 (Section 4.3.2) 18 ITS and 3 AMF amplicon OTUs were isolated from plants grown with the Hetchell Woods generated inoculum while this study produced 109 OTUs from the ITS amplicon and 15 OTUs from the AMF amplicon across the two years studied.

Table 11: Shared Operational Taxonomic Units (OTUs) between the glasshouse experiment in Chapter 4, and this study for both ITS and AMF amplicons.

Chapter 4			**This study (Chapter 5)**		
OTU	Taxonomy	Accession	OTU	Taxonomy	Accession
ITS amplicon					
3	*Olpidiaster brassicae*	AB205207	**1**	*Olpidiaster brassicae*	AB205213
2	*Serendipita indica*	KC176326	**95**	*Serendipita indica*	KC176326
3	*Cladosporium herbarum*	KX611004	**5**	*Cladosporium* sp.	KY921924
4	*Coprinopsis lagopus*	JN943127	**34**	*Coprinopsis lagopus*	JN943127
7	*Vishniacozyma victoriae*	KY105839	**9**	*Vishniacozyma victoriae*	KY105837
12	*Rhizophagus irregularis*	HF968929	**15**	*Rhizophagus irregularis*	HF968934
AMF amplicon					
2	*Rhizophagus irregularis* (DAOM 229456)	HF968850/ VTX00114	**2**	*Rhizophagus irregularis* (DAOM 229456)	HF968850/ VTX00114

The AMF amplicon results of this study were compared to the AMF OTUs generated in the Hetchell Woods study by Dumbrell *et al* (2011, Table S3) to assess whether any species are consistently isolated from the plant roots at this site. The findings of the Dumbrell *et al* (2011) paper demonstrated that there were significant differences in AMF communities across various seasons, so for this comparison only the OTUs that were amplified from the two sampling events in July were used to ensure complementarity with this study. This study used the primer pair NS31-AM1 to sequence the 'AMF region', while Dumbrell et al (2011) used the NS31-WANDA primer pair, which has significant overlap with the primer pair used in this study. Unlike in the comparison of OTUs to Chapter 4, there were no identical accessions (Table 11, Figure 25). Seven OTUs (OTUs 1,2,4,6,7,10,14) clustered closely and shared a virtual taxon ID (VTX) with sequences published in the Dumbrell *et al* (2011) paper (Table 8, Figure 25).

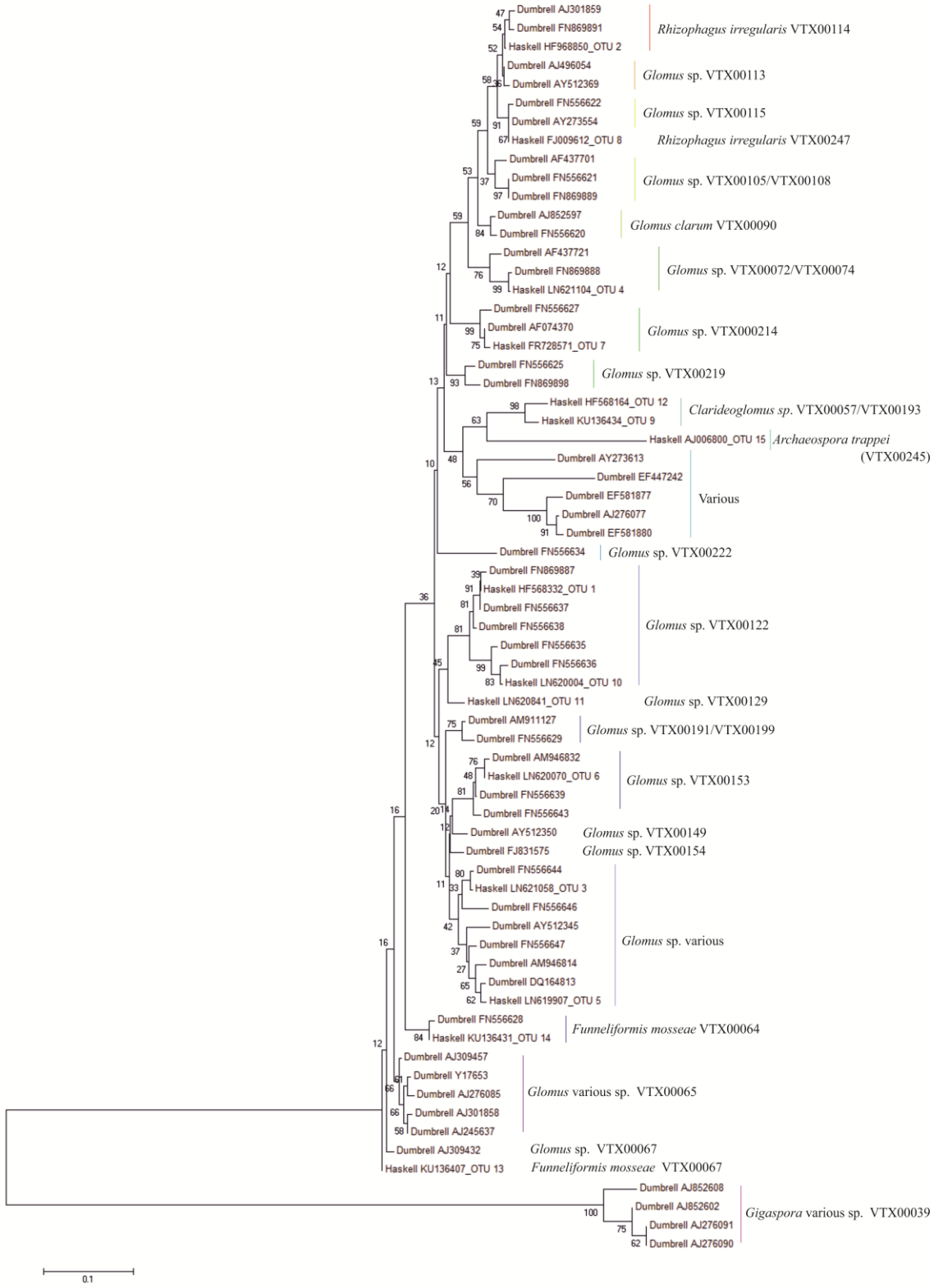

Figure 25: Neighbour-joining tree displaying relationships between sequences from this study (Haskell_AccessionNumber_OTUnumber) and the 52 OTUs that were present in samples collected in July from the Dumbrell *et al.* (2011) study (Dumbrell_AccessionNumber). Clusters are annotated on the right hand side with BLAST assigned taxonomy and MaarjAM virtual taxon numbers (VTX). Where annotation states 'various' this indicates 3+ different taxa/VTX represented within a

single cluster, 'various sp.' indicates 3+ different species while 'sp.' refers to unknown species. Bootstrap values indicate support for nodes.

5.4 Discussion

The results of this study have foremost demonstrated the importance of repeated sampling in natural environments. Many of the measured variables differed significantly between the two sampling years, most striking was the difference in foliar P concentration, with values increasing significantly in 2016 (Figure 22). Colonisation of plant roots by AMF and non-AMF also varied between years based on the microscopy results (Figure 21), and a number of OTUs were present in plant roots at higher abundances in 2016 compared to 2015 (Table 10). Comparatively, distance along the transect did not appear to be as crucial as sampling time in determining fungal communities colonising roots and nutrition of *B. sylvaticum* plants. This was not what was predicted in the original hypotheses, as it was also predicted that the distance would cover a strong pH gradient based on previous work in the area. In this study there was no evidence of a pH gradient across the transect although pH values were much more variable at distances close to the limestone outcrop (Figure 20).

The primary hypothesis of this experiment was that at lower pH, silicic acid would be present at higher concentration in the soil profile and therefore lead to higher foliar Si concentrations. Across the limited pH profile found in this study, there was no evidence of foliar Si concentration being affected by pH. Interestingly there did appear to be a small interaction between pH and P concentration, although not in the direction hypothesised, with lower values at the higher, more neutral pH values (6.5-7), compared to the lower, more acidic pH values. This is a weak relationship and may be driven by unequal sample sizes of pH readings across the samples. It was predicted that P availability would be affected by changes in pH, but no significant difference in pH was detected betweeen years (Figure 20).

The relationship between pH and P may be influenced by the interaction of fungal taxa colonising plant roots. Both Si and P foliar concentrations showed a weak negative relationship with AMC, but only the relationship with Si was significant. The absence of a relationship is unusual, but the observation of a negative trend is unexpected given the widely documented benefits of AMC on plant P and Si content (Smith & Smith, 2012; Oye Anda *et al.*, 2016; Garg & Singh, 2017). There were no AMF OTUs that were shown to interact with P or Si in the correlation analysis. However, three OTUs from the ITS amplicon that were significantly different between the two sampling years were shown to be negatively affected by pH, and positively correlated to foliar P concentration, although these correlations were not significant

after applying a post-hoc correction (Figure 24). OTU 26 (*Cyathicula* sp.), OTU 48 (*Sporobolomyces roseus*) and OTU 62 (*Apodus deciduus*) were all shown to be positively related to P concentration, and negatively related to pH (Figure 24). None of these OTUs were classed as plant symbionts, but rather a mixture of saprophytes and pathogens. The OTU that was identified as an unidentified *Cyathicula* species was found in samples collected in 2015 and 2016, but only at high abundance in the latter (Table 10). Species within this genus are saprophytic fungi and teleomorphs, or the sexual stage, of species in the *Chalara* genus, members of which are causative agents of ash dieback (*C. fraxinea*) (Carpenter & Dumont, 1978; Gams & Philippi, 1992). *A. deciduus,* a saprophytic fungus in the order Sordariales was found exclusively in root samples collected in 2016, in four of the ten samples in this sampling group (Cai *et al.*, 2006). *S. roseus* is a Basidiomycotan yeast frequently isolated from grass leaves and seed heads that is predicted to be antagonistic to common plant pathogenic fungi (Bashi & Fokkema, 1977; Hertz *et al.*, 2016). There were no records of this fungus being isolated from plant roots, but the taxonomy was assigned with high confidence with a 100% query coverage and identity with NCBI BLAST.

Given the low frequency of isolation of these OTUs within the sampling periods and the weak correlations it is unlikely that they are the causative agents of the improvements of foliar P concentration witnessed. However, the presence of these taxa in the soil and along with other fungi that do not colonise plant roots may have an impact on the P cycling within the soil. Microbial activity is crucial in the turnover of P and maintaining concentrations of biologically available P in the soil profile (Stewart & Tiessen, 1987; Crews & Brookes, 2014). Minimal changes in pH have the potential to determine soil microbial communities which may in turn have an indirect impact on foliar P concentration (van Aarle *et al.*, 2002; Zhalnina *et al.*, 2015).

A significant difference between foliar P was demonstrated between the two sampling periods, but this does not appear to be directly driven by pH, which did not vary significantly between years. Inorganic (P_i) P is the primary form available for plant uptake, yet the availability of P_i is negatively correlated with soil moisture (Magid & Nielsen, 1992). Microbial P on the other hand increases with soil moisture, and it has been proposed that microbes and plants may compete for available P in the soil (Liebisch *et al.*, 2014). Rainfall records between the two sampling years were very different (Table 6). The 2015 samples were taken at the beginning of August, a particularly dry period with only 3mm of rain in the week prior to sampling, while July 2015 overall was a wetter month than average (Table 6). Samples for 2016 were collected in early July which followed an average rainfall in June and a drier than average July although decreased rainfall occurred largely after sampling (data not shown). The foliar P concentration measured is not an ephemeral P solution but P incorporated into the leaf cells, so the immediate

weather may not be the best indication of the plant uptake. The drier conditions in the months prior to sampling in 2016 may be the reason for the increased P concentration in these plants.

It was also hypothesised that fungal colonisation would be lower in acidic soil, and while root AMC and NMC varied significantly across the transect, there was no evidence of this being related to pH. The sequencing data also revealed that fungal communities were different over the transect distance, although there were no individual OTUs that were unique to particular distances along the transect. While AMC indicated that the amount of the root colonised by AMF was variable across the transect distance and decreased in 2016, this was not matched by the sequencing data which demonstrated that only one AMF OTU differed significantly between years and the abundance increased in 2016, albeit at low sequence read depth (70 reads). NMC increased in 2016 according to the microscopic analysis, and the sequencing results appear to support this statement, with non-AMF OTUs dominating the table of OTUs that differed significantly between years (Table 10).

The results for the AMF amplicon support the third hypothesis, along with previous findings on AMF communities studied at Hetchell Woods and as part of a wider meta-analysis (Dumbrell *et al.*, 2010b). This study suggested that AMF communities colonising plant roots are dominated by a single AMF taxon and that the identity of this dominant taxon is variable. It was hypothesised that this would lead to different AMF taxa dominating the colonisation in plant roots in different years, and based on the share of sequence reads this is supported by the data collected in this study (Table 8). However, while roots collected in 2015 were clearly dominated by a single OTU (OTU 1), the 2016 results show a more even community, with three OTUs (1,3,5) sharing similar overall sequence reads. AMF amplicon sequencing was only successful in four samples in 2016, two of which (at 1m and 7m along the transect) showed colonisation dominance by OTU 1, while the other two had a higher overall diversity and richness. This suggests that the scale at which AMF are capable of becoming dominant in plant roots occurs is very small, potentially at a plant-by-plant basis, rather than a universal dominance in a particular location. The results from 2015 support this, as replicates collected within the same metre show dominance by different OTUs, reflected in the 'sample count' column in Table 8 which illustrates that OTUs are not uniformly represented across samples. For example in 2015 at 9m along the transect, one plant sampled was exclusively colonised by OTU2 *Rhizophagus irregularis*, while another was dominated by OTU1, an unidentified *Glomus* species. This is further supported by the findings from the colonisation analyses which was conducted on the full dataset, whereby at each metre along the transect AMC varied with no clear trend across the transect. Regrettably, the sample sizes for the sequencing data in this study were unequal across the transect and between years due to poor quality sequencing runs, so this cannot be examined in further detail.

Of the total 70 OTUs that were recorded in the Dumbrell *et al* (2011) survey, during the two July sampling events 52 OTUs (35 and 36 OTUs at each sampling date within July) were isolated. Fifteen AMF OTUs were observed in the current study (Table 8), seven of which matched those found in the Dumbrell *et al* (2011) study (Figure 25). The lower abundance of taxa captured by this analysis may be due to the fact that this study focussed on one plant species across a smaller sampling area, and a different primer pair and sequencing methodology. Alternatively, the differences may be attributed to the temporal separation of sampling at this site as the Dumbrell *et al* (2011) study sampled in 2007-8. The results of this present study have demonstrated the differences in microbial community between two years, so it would not be unreasonable to expect shifts in the AMF community composition between this study and the previous survey in 2007-8. It is difficult to speculate on how significant the effect of time is on soil and root colonising fungi due to the lack of research on this subject, particularly in monitoring community dynamics in natural systems without experimental manipulations (Wilson *et al.*, 2009; Rousk *et al.*, 2011).

The comparison of OTUs isolated in this study compared to Chapter 4 showed approximately 80% lower OTU richness in the glasshouse experiment, indicating that the inoculum did not accurately recreate the natural community of Hetchell Woods. However, it did demonstrate that the inoculum was successful in introducing certain species from Hetchell Woods that were capable of successfully colonising plant roots (Table 11). This may be related to the findings mentioned earlier, that opportunistic fungi colonise plant roots rapidly and through competitive exclusion prevent further colonisation. The inability of natural AMF species to perform well in artificial, glasshouse systems has been noted previously (Helgason *et al.*, 2002). This may therefore indicate that a similar dominance process occurs in glasshouses, where plants grown from seed, or with no previous exposure to fungal inoculants, are rapidly colonised by a fungus at close proximity and subsequently less likely to form diverse AMF communities due to antagonistic behaviours of microbes within the roots.

There are a number of additional possible reasons for the lack of significant overlap between this study and Chapter 4, primarily the fact that the inoculum for the glasshouse experiment was collected in November 2015. The effect of season on the performance and presence of AMF in particular has been fairly well studied and diversity and community evenness of root colonising fungi has been observed to be higher in the winter season, before varied, opportunistic species proliferate and dominate during the spring and summer months (Dumbrell *et al.*, 2011; Hazard *et al.*, 2014). A comparison of the OTUs generated by roots collected in November 2007 from the Dumbrell *et al* (2011) to the OTUs of roots in the July sampling period revealed that the November samples had lower diversity than in July (17 OTUs compared to 35-36), and that there were no OTUs unique to the November samples. This may indicate that the lower number

of OTUs generated in the Chapter 4 analysis could be due to there being a lower overall pool of fungi present to colonise roots. The inclusion of a spore filtrate in the experimental set-up was intended to overcome this, but root fragments provide a more rapidly colonising source of fungi. The root fragments included in the inoculum for Chapter 4 originated from a variety of different plant species, rather than just *B. sylvaticum*, which could also be leading to a lack of overlap in the sequences obtained from these two experiments.

Overall, this study has shown interesting results on a variety of levels, primarily that repeated sampling is important in observing how natural systems operate. AMC and NMC varied significantly between years, as did the abundance of a range of fungal taxa and foliar P concentration. Secondly, root AMF communities in natural systems, and potentially in glasshouse systems also, are largely typified by the dominance of a single AMF taxon which supports previous findings (Dumbrell *et al.*, 2010b). This study focussed on a single plant species in a natural environment, and results suggest that the spatial scales that dictate fungal communities in roots is very small, potentially on a plant-by-plant basis. This was not strictly the case for non-AMF colonising fungi, which did not show a particular dominance by a single taxon, presumably due to the variety of niches occupied in plant roots by different fungi. Finally, we were able to show that natural spore filtrates and root fragments were capable of introducing natural fungal species into experimental pots in glasshouses, but that a significant loss in diversity was observed and that glasshouse contaminants are still capable of outcompeting natural inoculants. All of this is crucial in understanding the role of the soil biotic component on plant production and soil abiotic processes. Further studies are required that sample natural systems without experimental manipulation to understand natural variability in soil microbial communities.

6 General Discussion

The primary hypothesis of this study was that colonisation by AMF would improve Si and P uptake and foliar concentration, to contribute novel information to the rapidly growing field of AMF related improvements in Si uptake. The results of Chapter 3 demonstrate that colonisation by AMF is capable of significantly altering root growth, Si and P uptake and concentrations. The findings of this chapter suggested that colonisation by AMF was important in the relationship between Si uptake and root length, demonstrating that incremental increases in root length are strongly related to foliar Si and P content, but only in colonised plants. However, the results of Chapter 4 are a contrast to this. The presence of AMF within the roots, as determined by the visual and sequencing analyses, was not found to be related to Si or P uptake. That said, there were significant differences in Si and P concentration and uptake due to the different treatments and microbial communities, but from the data obtained it was difficult to draw any major conclusions in relation to causative agents. In the field setting of Chapter 5, there was a negative relationship observed between AMF colonising plant roots and Si and P concentration, but it was not possible to collect information on plant size (especially roots) in order to investigate the uptake rates of Si and P. This relationship is unusual but a similar decrease in shoot P concentration was observed in Chapter 3 (Figure 16), due to the smaller size of AM plants.

The two potential mechanisms for AMF improved Si uptake are that the colonisation of AMF improves the ability of plants to take up Si, either through changes in root structure or AQP gene expression and modulation. The second mechanism is that AMF hyphae in the ERM assimilate silicic acid and transport it, either passively or actively to the plant in a similar mechanism to P, N and water (Hammer *et al.*, 2011; Kikuchi *et al.*, 2016). Interestingly the results of the glasshouse experiments in Chapter 3 and 4 show a remarkably similar pattern in Si and P uptake and deposition in plants across the treatments, which may suggest a similar uptake mechanism (Figure 11, Figure 16). However, the results of the field study in Chapter 5 may suggest otherwise. Comparing the Si and P concentrations within the same year illustrates that Si concentration does not show the same pattern as P concentration (Figure 22). Further to this, P concentration increased significantly from 2015 to 2016, but no significant change was observed in the Si concentration data. The plants grown in the glasshouse experiments evidently were grown in controlled conditions with no application of stress or damage. The uptake of Si plants is a passive process initially, but transport from root to shoot is active, and deposition of Si is regulated by processes that occur in foliar tissues (Kumar *et al.*, 2017a). The Si uptake was strongly correlated with root length in AMF colonised plants only (Chapter3) and co-occurred with an improvement in plant water status, which suggests that plant transpiration is a driving force for the uptake. AMF are known to improve the water status of plants and the uptake and

transfer of water to plants through AQP in the ERM has been shown to facilitate the transport of N and P through the ERM also. Uptake of Si through plant AQP is also shown to be driven by the transpiration stream. While this study has provided additional evidence for the role of AMF in Si uptake and nutrition in plants, further work is required to determine the mechanism.

Si uptake is inducible when plants become stressed or damaged and the uptake of Si is beneficial to plant growth and defence, yet Si is still classed as a non-essential element (van Bockhaven *et al.*, 2013; Hartley & DeGabriel, 2016). Contrastingly P is regarded as the most important, and frequently limiting plant nutrient (Smith *et al.*, 2011), hence the regulation of uptake of each of these elements is likely to be very different. In the controlled glasshouse conditions the foliar Si and P concentration was similar, but in the natural environment when competition for P is high and availability is patchy and ephemeral, there was no overlap in the patterns of Si and P concentration. Comparisons for uptake between the glasshouse and field studies are not possible as root length measurements were not possible due to the difficulty of extracting full root systems from field environments. This does not lead to a clear assumption about whether AMF enable plants to assimilate Si directly or indirectly, as P concentration did not appear to be related to the presence of AMF either. The results from Chapter 5 suggest that rainfall may be related to P availability, and as Si uptake is principally controlled through transpiration (Ma *et al.*, 2007; Kumar *et al.*, 2017a), investigations into how water availability affects the uptake of Si and P under controlled conditions and in the field would be of interest. Unsurprisingly, it appears that glasshouse studies are not capable of replicating natural conditions even with the use of natural inocula.

The use of NGS methods to assess fungal diversity in Chapters 4 and 5 generated mixed results. The main limitation has been the loss of samples during and after the sequence processing. The reasons for this may be related to low quality template DNA, although quantities were checked at various stages pre and post-PCR prior to submission and were adjusted to meet the requirements of the MiSeq platform. Alternatively, the primer sets used may not be ideal for sequencing root colonising fungi or rather are not specific enough. The NS31-AM1 pair should exclude plant sequences but still a large number of plant sequences had to be removed from AMF amplicon samples from both Chapter 4 and 5. The primer pair ITS1F-ITS4 is also a large fragment covering both ITS1 and ITS2, and similar studies have instead preferentially used the shorter region ITS2 only (Granzow *et al.*, 2017). The issue of amplification of non-target DNA is an issue as low abundance organisms may be shadowed during the base calling stage of the MiSeq run. For Chapter 5, samples were split between two MiSeq plates due to space sharing with other lab members, and the quality of the initial run was much poorer than the second. This resulted in most sequences from the first run being discarded at the split library stage, even at a lower quality threshold. Several studies have been published that sample soil and roots from

glasshouse experiments to assess the fungal communities using the MiSeq platform (Edwards *et al.*, 2015; Granzow *et al.*, 2017). However, to date there have been none that are carried out in autoclaved soil. The low diversity seen in the samples of Chapter 4 may be due to this, which may explain the high number of plant reads that were amplified, due to the low abundance of fungal DNA, coupled with a high prevalence of plant DNA.

Despite the limitations, the sequencing results did provide interesting information about the fungal communities that emerge in glasshouse experiments. The application of the same sampling methodology for Chapter 4 and Chapter 5 enabled comparisons to be drawn about the efficacy of natural inocula in colonising plants in controlled conditions. It allowed parallels to be drawn between the data of Chapters 4 and 5 as well as against existing data from publications (Dumbrell *et al.*, 2011). Alternative methodologies such as bacterial cloning and Sanger sequencing are also costly and time-consuming, and likely have lower coverage than the MiSeq method.

The third hypothesis related to assessing the methods used across different experiments in this study and how replicable the results from glasshouse to field study could be. The single species inoculum used in Chapter 4 had previously been assessed using bacterial cloning and Sanger sequencing to determine the identity of the fungal root colonisers. The results of the mycorrhizal stock pot analysis (Chapter 2, Section 2.3) revealed that the majority of sequences were identified as *Funneliformis mosseae*, despite the original stock pot culture species being defined as *Glomus intraradices* (syn *Rhizophagus intraradices*). No OTUs in Chapter 4 corresponded to *F. mosseae*, but all AMF sequences from the roots of plants grown with the *F. mosseae* single species inocula corresponded to *Rhizophagus irregularis* which was observed once in the initial screen (Chapter 2, Section 2.3). This raises interesting questions about the conditions by which fungi colonise plants under experimental conditions. As noted in Chapter 5, the colonisation of plant roots by AMF is opportunistic and often results in a dominant species excluding others (Dumbrell *et al.*, 2010b). This dominance of root colonisation has been noted previously for *R. irregularis* in experimental conditions, where this species outcompeted native AMF species (Symanczik *et al.*, 2015). In documenting the inoculum composition prior to the experiment, and comparing it to the species isolated in the plant roots as a result of the addition of the inoculum it highlights the importance of understanding the mechanisms of AMF colonisation. In addition, *Serendipita indica* (syn. *Piriformospora indica*), a common plant growth promoting root symbiont was present at the highest read counts within the *F. mosseae* treatment and in the stock pot screen in Chapter 2 (Section 2.3). Although there is no competitive exclusion between AMF and *S. indica*, there is likely to be some division of plant carbon to each partner and it will be difficult to disentangle the beneficial effects on plant growth between these two functionally similar organisms (Schäfer *et al.*, 2009; Newsham, 2011).

The addition of autoclaved inoculum is a common and established practice in the set-up of glasshouse experiments to control for the potential addition of any nutrients from the roots or substrate (Gerdemann, 1965; Grümberg *et al.*, 2015). However, the result of the community analyses in Chapter 4 demonstrates that the inclusion of autoclaved roots may be having an unexpected effect by introducing saprophytic fungi into the pot environment (Figure 18). The methods used in this experiment subjected the roots to a double autoclaving with a resting period of three days in-between and after each autoclave. This is intended to kill any organisms that are stimulated to grow by the autoclave process, but may indicate that spores present in inocula may be capable of surviving the process and grow in the duration of the experiment. It may be that these species do not colonise plant roots but are present and active in the substrate during the experiment which may have an effect on nutrient availability. This is an important consideration for any inoculum addition experiment, as evidence shows that different species and treatments in glasshouse conditions leads to dominance of certain taxa, despite the same starting inocula, although in this case the inocula was sourced from the growing medium (unsterilised soil) (Granzow *et al.*, 2017).

6.1 Conclusion

Overall, this project has successfully shown that AMF are capable of improving the Si and P uptake of a non-crop species under controlled glasshouse conditions (Chapter 3). In addition, the application of NGS methods to verify root colonising communities has highlighted some potential oversights in the current techniques used in artificial glasshouse experimental manipulations using AMF inocula. Verifying the microbial communities in stock pot cultures and control treatments, which is often overlooked in glasshouse experiments using single species inocula, is particularly important as identification by staining and microscopy is open to error, differences between scorers, and it is challenging to discriminate between colonising taxa within the root. The comparisons of AMF communities between Chapters 3, 4, and 5 have supported previous findings that suggest that colonisation of roots results in an uneven AMF community, with a single AMF species as the dominant coloniser. This may be the reason why certain species are difficult to culture in controlled conditions (Helgason *et al.*, 2002). Adding diverse microbial inocula to experiments can make interpretation of results more difficult, and yet still does not generate communities that are representative of natural communities, particularly as the controlled conditions will preferentially select for certain species adapted to the temperature and moisture regimes applied. If mechanisms for the uptake of Si in AMF are to be determined, then culture experiments where roots are colonised by a single species or isolate are the most logical forward step (Voets *et al.*, 2005, 2009). Utilising these systems and producing a split-pot style experiment under sterile conditions will be the most valuable in

producing informative results on the mechanisms for improved mycorrhizal uptake of Si(Figure 26).

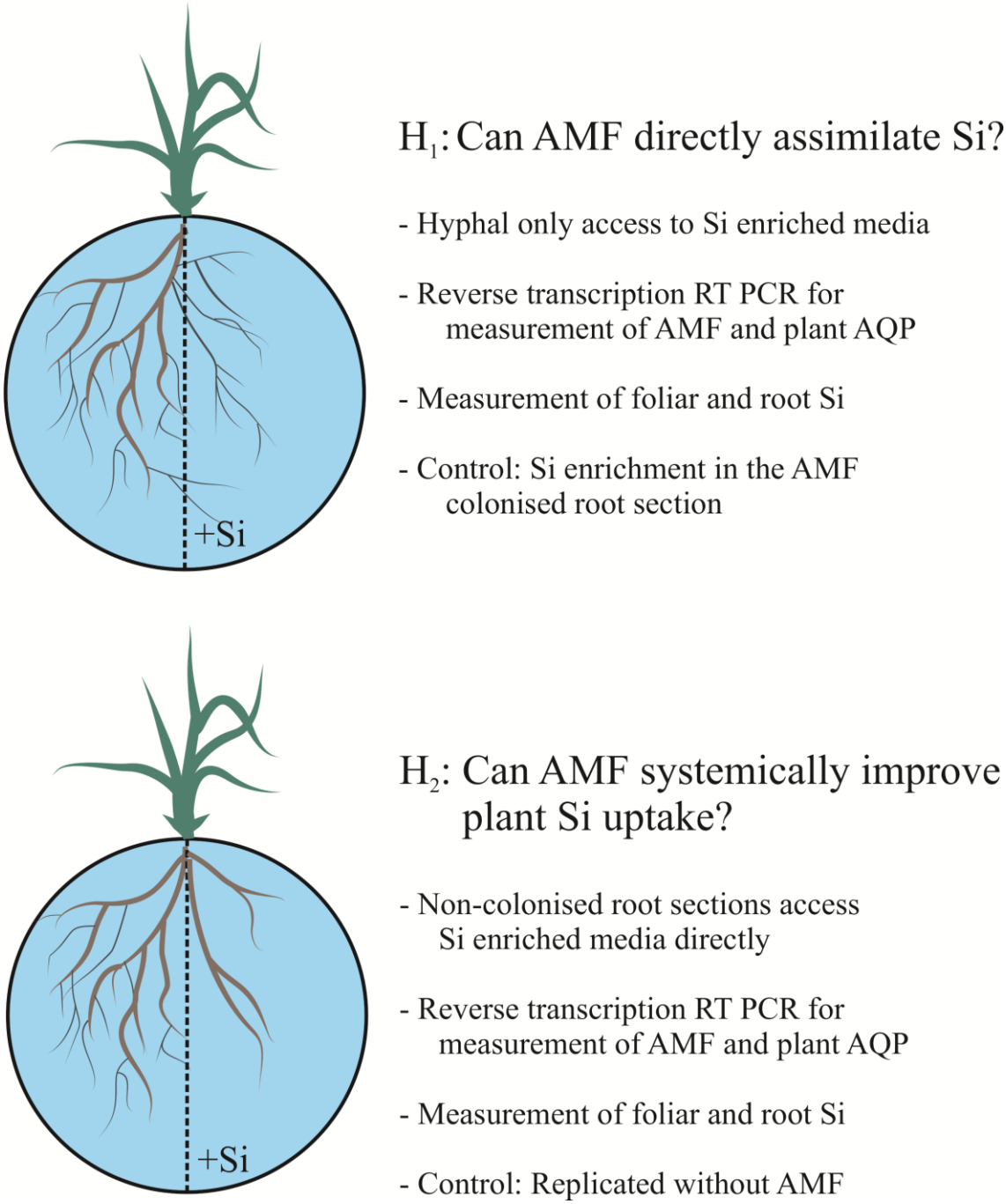

Figure 26: Proposed future experiments and hypotheses to test the mechanisms of improved Si uptake seen in plants colonised by arbuscular mycorrhizal fungus. Plantlets will be grown in petri-dishes (represented by the blue circles) in Si enriched, or basic agarose media with a single species/isolate arbuscular mycorrhizal fungus. Hyphae and/or roots will be permitted to grow over a dividing section to access different resources.

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