# Population Biology and Interactions of Arbuscular Mycorrhizal Fungi and Their Benefits in Strawberry Cultivation

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No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Kent or any other University or Institute of learning.

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## Abbreviations

| | |
|---|---|
| Analysis of Variance | ANOVA |
| Arbuscular mycorrhizal fungi | AMF |
| Base pair | bp |
| Basic local alignment search tool | BLAST |
| Centimetre | cm |
| Cultivar | cv. |
| Day | d |
| Degrees Celsius | $^{o}$C |
| Deoxynucleotide triphosphate | dNTP |
| Deoxyribonucleic acid | DNA |
| *et alia* | *et al.* |
| European Bank of the Glomeromycota | BEG |
| Glomeromycota *in vitro* collection | INVAM |
| Gram | g |
| Hour | h |
| *Id est* ` | *i.e.* |
| International culture collection of the (vesicular) arbuscular mycorrhizal fungi | INVAM |
| Internal transcribed spacer | ITS |
| Large subunit | LSU |
| Litre | l |
| Microlitre | µl |
| Micrometre | µm |
| Millilitre | ml |

| | |
|---|---|
| Millimetre | mm |
| Millimolar | mM |
| Minute | min |
| Molar | M |
| Most probable number | MPN |
| Nanogram | ng |
| NCBI | National Centre for biotechnology information |
| Parts per million | ppm |
| Percent | % |
| Polymerase chain reaction | PCR |
| Power of hydrogen | pH ($^{-}$ log of $[H^{+}]$ |
| Quantitative polymerase chain reaction | qPCR |
| Regulated deficit irrigation | RDI |
| Restricted maximum likelihood | REML |
| Revolutions per minute | rpm |
| Ribonucleic acid | RNA |
| Root length colonisation | RLC |
| Second | s |
| Ultraviolet | UV |

## Abstract

The diversity of arbuscular mycorrhizal fungi (AMF) and relative abundance among species may affect their ecological impact. Species-specific primers for qPCR quantification of *Funneliformis geosporus* and *F.mosseae* DNA were developed to quantify their relative abundance for use in studying mixed inocula in roots of strawberry under different conditions of water stress. Co-occupation of the same root by both species was shown to commonly occur but the relative abundance of the two species varied with water stress. Greater root colonisation was observed microscopically under water stress but this increased colonisation was often accompanied with decreased amounts of fungal DNA in the root. *Funneliformis mosseae* tended to become more abundant under water stress relative to *F. geosporus*. There was significant correlation in the fungal colonisation measurements from the microscopic and qPCR methods under some conditions, but the nature of this relationship varied greatly with AMF inoculum and abiotic conditions.

Water stress experiments, undertaken with strawberry (*Fragaria x ananassa)* show a reduction of plant development when subjected to regulated deficit irrigation (RDI) conditions. The effect on growth of AMF colonisation and relative abundance of two co-occurring species of AMF, *F. geosporus* and *F. mosseae*, were determined in cultivated strawberry under conditions of water stress. Three AMF inoculation treatments (a single treatment either of *F. mosseae* BEG25, *F. geosporus* BEG11 or a 50:50 mixed inoculation treatment of both species) were compared to un-inoculated plants. This study demonstrated that in strawberry plants, under these experimental conditions, single species inoculation treatments gave similar benefits to the host as the mixed inoculation treatment regardless of irrigation regime, suggesting colonisation was of greater importance than mycorrhizal fungal species. The addition of AMF inocula to plants, subjected to reduced irrigation of up to 40%, restored plant growth to the same or higher values as the non-mycorrhizal, fully-watered plants. The water use efficiency of plants was greater under the RDI regime and in AMF-inoculated plants, but there were no significant differences between plants inoculated with the single or combined inoculum.

The occurrence of multiple variant sequences within the rRNA genes of the AMF is now widely accepted; however the mechanisms for this are not currently clear. This work investigated the effect of different culture conditions (*in vitro* and *in planta* culture) on sequence diversity and relative abundance of a culture of *Rhizophagus clarus*. Next generation sequencing, using the Illumina platform, generated three major sequence variants that were the most common sequence variants in all conditions. All other sequences grouped phylogenetically about these three major sequence types along with an outgroup of less common sequence types. This study demonstrated that changes in the frequency of dominant sequence variants had occurred when AMF are maintained for two years under different culture conditions.

# Chapter 1. Introduction

## 1.1 Introduction to AMF

Arbuscular mycorrhizal fungi (AMF) are one of the most widely distributed fungal groups on Earth, and are found in almost all ecosystems (Jansa *et al.*, 2002). They colonise the rhizosphere and can form mutualistic symbioses with more than 80% of vascular plant families. Arbuscular mycorrhizal fungi form arbuscules within the roots of plants that allow the transfer of nutrients between the fungus and the roots (Smith and Read, 1997). Mineral nutrients, mainly phosphorus, nitrogen and water are extracted from the soil via the extensive hyphal network and transferred to the plant improving plant health and growth (Smith *et al.*, 2010). Organic carbon compounds are transferred to the AMF in return. Arbuscular mycorrhizal fungi protect plants from pathogens (Borowicz, 2001; Ismail and Hijri, 2012; Ren *et al.*, 2013) and buffer against adverse environmental conditions. The external mycelium of AMF improves soil structure by formation of soil aggregates (Rilling, 2004; van der Heijden *et al.*, 2006). There are currently approximately 230 described species of AMF (Krüger et al., 2012), however this number is continually increasing and is likely to be an underestimation.

The Glomalean fungi are an ancient lineage of apparently asexual organisms. Recently some evidence of mating-related genes found in the newly published genome sequence suggest the possible existence of a cryptic sex-related process (Tisserant *et al.*, 2013) although this is still unclear. They are one of the few fungal groups to have a fossil record (Remy *et al.*, 1994), and they are thought to have facilitated the colonisation of land by ancient plants. Simon *et al.* (1993) estimated a date for the origin of AMF-like fungi of 353-462 Myr ago by studying phylogeny based on small subunit rRNA sequences. This estimate is consistent with both the fossil record and the estimated divergence of ancient land plants from aquatic ancestors.

Arbuscular mycorrhizal fungi are important in the maintenance of soil fertility. Infertile soils are an increasing concern to farmers globally. Low nutrient availability, reduced biological diversity and increased pathogen populations are generally the cause, and soil management commonly results in increased inputs of fertilisers and pesticides (which can aggravate the problem). The maintenance of a developed and diverse population of AMF, and other soil micro-organisms, is important in achieving sustainable agriculture (Helgason *et al.*, 1998; Jeffries and Barea, 2012), and as such these fungi may become increasingly fundamental to sustainable food security (Ceballos *et al.*, 2013).

It is inaccurate to study plant-soil interactions without considering AMF, as they constitute a considerable and important part of the microbial biomass in terrestrial ecosystems (Rosendahl, 2008). Arbuscular mycorrhizal fungi are considered to have an important role in sustaining plant communities, especially their structure and biodiversity (Sanders *et al.*, 1996). Increased diversity of AMF positively affects plant biodiversity, variability and productivity (van der Heijden *et al.*, 1998b; van der Heijden, 2002).

Young (2008) described AMF as "fiendishly difficult to study" and there are a number of reasons for this. AMF are obligate biotrophs and it is therefore difficult to study the functional dynamics of the fungi in either natural or laboratory-based investigations (Tisserant *et al.*, 1998). The culture of AMF using *in-vitro* methods (Cranenbrouck *et al.*, 2005) is now fairly developed for a limited number of species, but only in complex media and in the presence of transformed carrot root. There is limited variation in morphological characteristics in the spores of these fungi thus creating difficulties in identification and morphotyping, which requires a considerable knowledge of the morphological characteristics of a wide range of different species (Clapp *et al.*, 2001; Tisserant *et al.*, 1998). A further complication is the existence of multiple sequence variants even within single spores of many AMF. Intensive study of within-spore sequence variation, of commonly-targeted genomic regions such as the ITS region, within a single isolate can match the world-wide variation existing across a range

of isolates of the same species. This phenomenon is now well-described, if still controversial with respect to the origin of variation (Hijri and Sanders, 2005; Pawlowska and Taylor, 2004). The highly polymorphic nature of these organisms has meant that genomic information on AMF is still far behind other fungal species and many of the host plants that AMF associate with (Salvioli and Bonfante, 2013).

### 1.1.1 Taxonomy

Fungal studies require a consensus on taxonomy and species recognition (Rosendahl, 2008), however the taxonomy of AMF is still the subject of fierce debate. Most traditional taxonomy was based solely on morphological characteristics of spores (Walker *et al.*, 2007), however phylogeny based on the ribosomal RNA genes has become much more significant over the last few years (Young, 2012).

Arbuscular mycorrhizal fungi form the division Glomeromycota (Schussler *et al.*, 2001), but were originally placed in the order Glomales and the division Zygomycota. Recent genome sequencing information has suggested they may be closer to the Mucoromycotina within the paraphyletic Zygomycota than first realised (Lin *et al.*, 2014; Tisserant *et al.*, 2013), but for now the taxonomy remains remain unchanged. The Zygomycota shares a common ancestry with the Ascomycotan and the Basidiomycotan clades.

Morton and Redecker (2001) introduced the new genera, *Archaeospora* and *Paraglomus,* to the Glomeromycota to remove some of the polyphylogenic anomalies within *Glomus*. The taxonomy of AMF was questioned further when, using nearly full-length SSU rRNAgene sequences, Schwarzotte *et al.* (2001) confirmed that *Glomus,* the largest group, was polyphyletic. They suggested *Glomus* formed 2 major clades, described as *Glomus* group A and *Glomus* group B.

Recently other authors have significantly revised the taxonomy of this group. Oehl *et al.* (2011) summarised a number of recent findings, using rRNA sequence data and morphological characteristics, to suggest a reorganisation of the Glomeromycota with the addition of new genera. This work was only accepted in part by Redecker *et al.* (2013) who recently proposed another classification of the Glomeromycota. This latest classification of Glomeromycota was based on a consensus of regions spanning ribosomal RNA genes, 18S (SSU), ITS1-5.8S-ITS2 (ITS), and/or 28S (LSU). The phylogenetic reconstruction underlying this classification is shown in Figure 1.1 and is the currently the accepted taxonomic structure used by the majority of research groups and the curators of the 2 culture collections of International Bank of the Glomeromycota (IBG) and INVAM and the Glomeromycota *in vitro* collection (GINCO).

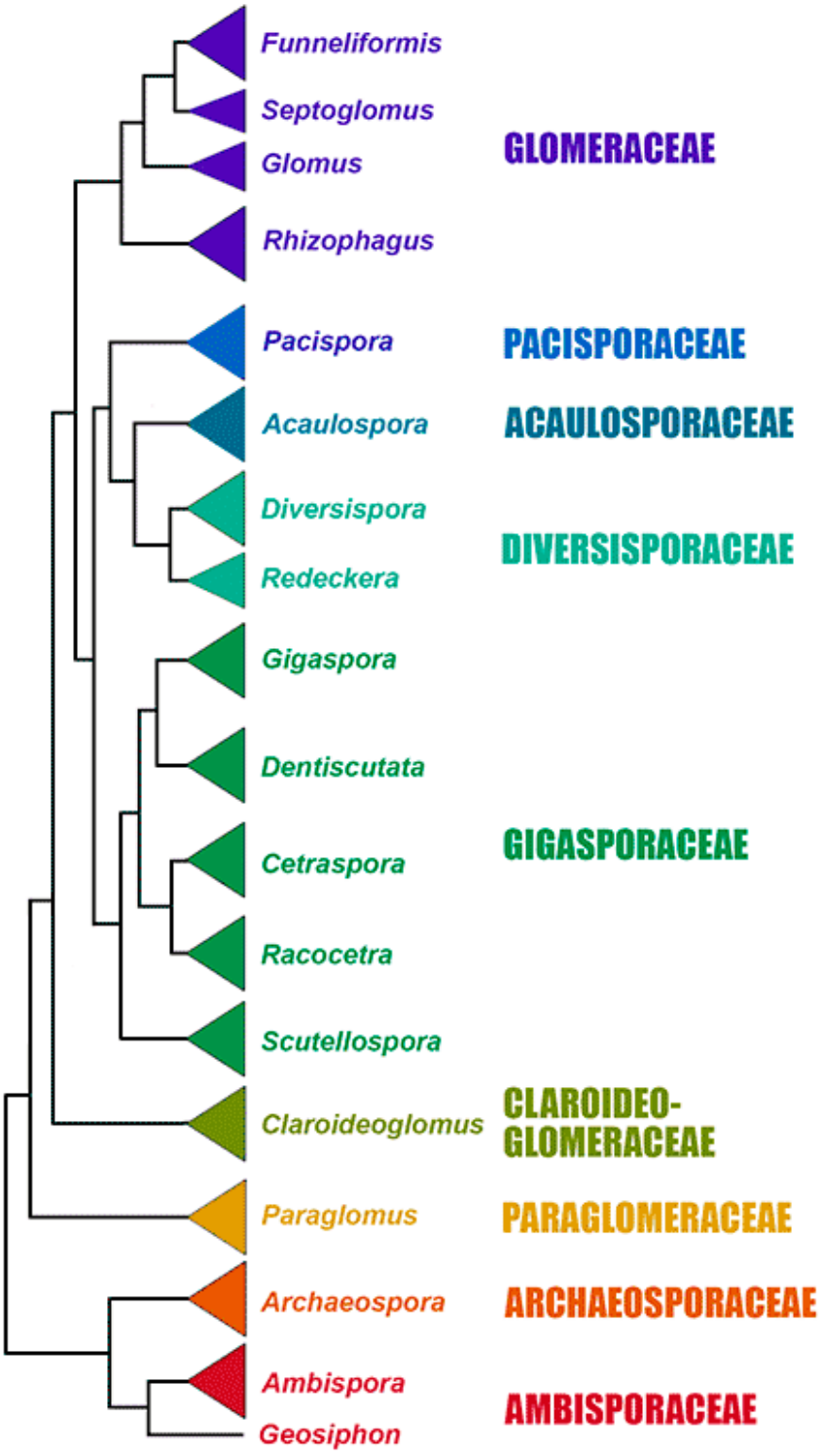



Figure 1.1. The currently accepted taxonomy of the Glomeromycota as outlined by INVAM. (www.invam.wvu.edu/the-fungi/classification)

Molecular analyses have shown that there is considerable genetic diversity within morphologically recognisable species in the Glomeromycota and most recent analyses cluster sequence data into 'species groups' or 'phylogenetic clusters'. Additionally there are a large number of isolates of AMF that have sequences registered in sequence databases but do not necessary also have morphotypes and vice versa with *in vivo* isolates held in culture collections (Öpik *et al.*, 2010).

### 1.1.2 Sequence diversity

Spores and hyphae of AMF are multi-nucleate, with estimates of the number of nuclei per spore varying between 800 and 2000 in species of *Glomus* (Burggraaf and Beringer, 1989; Clapp *et al.*, 2002). The genome size, ploidy level and number of chromosomes is not accurately known for most AMF (Sanders, 2002). However the long-awaited, recent publication of the sequence of the *R. irregularis* genome (Tisserant *et al.*, 2013), confirmed that *R. irregularis* is a 153-Mb haploid genome with a low level of genome polymorphism.

Previous investigations of the rRNA genes have highlighted a high level of genetic variability in the Glomeromycota (Clapp *et al.*, 2001). Currently the use of rRNA genes is the only viable molecular approach to discriminating different AMF species, as there is very little sequence data available for other areas of their genome (Jansa *et al.*, 2008). Ribosomal RNA genes are found in high copy number and contain both highly-conserved coding regions as well as internal transcribed spacers (Reddy *et al.*, 2005; Simon *et al.*, 1993; Wyss and Bonfante, 1993). Many groups (Antoniolli *et al.*, 2000; Clapp *et al.*, 2001; Hijri *et al.*, 1999; Hosny *et al.*, 1999; Koch *et al.*, 2006; Lanfranco *et al.*, 1999; Lloyd MacGilp *et al.*, 1996; Pringle *et al.*, 2000; Rodriguez *et al.*, 2001; Wyss and Bonfante, 1993) have reported that genetic heterogeneity is widespread within the Glomalean fungi. Thiéry *et al.* (2012) found sequence variation from point mutations and small indels in all regions of the full nuclear ribosomal operon, with the

highest degree of variation in the ITS regions, an intermediate level in the LSU and the lowest for the SSU. Multiple variants of ribosomal gene sequences have been found to exist within single spores of AMF (Clapp *et al.*, 2001; Hijri *et al.*, 1999; Kuhn *et al.*, 2001; Lloyd MacGilp *et al.*, 1996; Rodriguez *et al.*, 2001; Sanders, 1999; Thiéry *et al.*, 2012).

Kuhn *et al.* (2001) confirmed genetic heterogeneity also exists within single copy loci of functional genes. They examined the BiP (Binding immunoglobulin protein) gene, which encodes for a binding protein, and reported it to contain 15 variant sequences in a single isolate of *G. intraradices* (approximately equal to the variation observed in rRNA gene sequences in AMF). Since then sequence variation in functional genes has been observed by a number of different authors, Corradi *et al.* (2004) saw variation in the ß-tubulin gene in isolates of *G. intraradices* and Corradi and Sanders *et al.* (2006) showed variation in the P-type ATPase genes. Croll *et al.* (2008a; 2008b) and Mathimaran *et al.* (2008) looked at genetic markers at multiple loci including functional genes and saw that these were also variable within isolates of *G. intraradices*. The genome sequence of *R. irregularis* (Tisserant *et al.*, 2013) also showed multiple SNP's in many non-redundant virtual transcripts which confirmed sequence polymorphism in a number of genes and these authors suggested that polymorphism is widespread throughout the genome. Recent work has interestingly shown mitochondrial genes to contain less variation than the nuclear genes (Franz Lang and Hijri, 2009; Krak *et al.*, 2012; Sýkorová *et al.*, 2012), and as such these areas may become increasing important for taxonomic studies of the AMF.

There is much debate surrounding the nature of this genetic heterogeneity as it is unclear whether AMF are homo- or heterokaryotic. Kuhn *et al*. (2001) demonstrated, using fluorescent *in situ* hybridization, that individual spores contained a population of genetically different nuclei, i.e. that they are heterokaryotes. They also looked at the single copy BiP gene

and found variation, again suggesting heterokaryosis. However this was questioned in a later study (Pawlowska and Taylor, 2004) that showed rRNA gene sequence variants were contained within single nuclei of *G. etunicatum* based on the direct PCR amplification of rRNA gene sequences from micro-dissected nuclei. This latter result would suggest a homokaryotic structure. Recent studies (Angelard *et al.*, 2013; Boon *et al.*, 2013; Ehinger *et al.*, 2012) have demonstrated strong evidence of genome segregation at sporulation in single spore lines, suggesting that heterokaryosis is likely. Ehinger *et al.* (2012) examined four alleles of the Bg112 locus and showed that five sibling lines exhibited genetic and phenotypic differences.

Other recent data from single nucleus genome sequencing of *R. irregularis* (Lin *et al.*, 2014) showed a low level of polymorphism between nuclei but showed a highly divergent 45S rDNA repeat unit within a single nucleus. These authors show that 99.97% of the aligned genome sequence was identical between different nuclei and that inter-nuclei polymorphism was very high in ITS regions of the rRNA. However, a recent study by Tisserant *et al.* (2013), using whole genome comparisons of *R. irregularis* DAOM 197198, suggested some allelic variation among nuclei. Therefore the nature and definition of the AMF genomic structure remains unresolved.

The mechanism for AMF to maintain this genetic variation remains unclear. Anastomosis was reported to occur within 3 different species of AMF (*G. mosseae, G. intraradices* and *G. caledonium*) in pot-cultures (Giovannetti *et al.*, 1999). More recently anastomosis was demonstrated to be an essential mechanism of maintaining genetic variation (Boon *et al.*, 2013), as differential segregation of nuclei at sporulation led to a loss of intra-isolate variation that could only be overcome via anastomosis. Genome sequencing of *R. irregularis* has also identified mating-related genes, which may indicate the possibility of a process similar to sexual recombination (Tisserant *et al.*, 2013).

It is suggested that the high genetic diversity and 'redundancy' of alleles observed in these fungi buffers the effects of environmental changes, and can alter phenotypic traits in the absence of sexual recombination (Koch *et al.*, 2004; Riley and Corradi, 2013). Koch *et al.* (2006) suggest that genetically different isolates of AMF could exert different effects on plant growth and nutrition. Munkvold *et al.* (2004) showed that genetically different isolates of *G. intraradices* had different effects on plant growth and phosporus uptake. Tisserant *et al.* (2012) suggested that widespread sequence variation implies that it has functional importance.

## 1.2 Study of AMF communities

There is a need for adequate identification of AMF associating with different host plants in ecosystems, in order to study AMF communities, and this requires reliable methods (Croll *et al.*, 2008b). Field studies of arbuscular mycorrhizal communities have taken a range of approaches, either using ***indirect*** methods such as isolation of spores from soil or from trap cultures, or ***direct*** methods via isolation of fungal DNA from roots.

The use of morphological data and spore characteristics alone is not always adequate for the identification of these fungi. There are relatively few morphological characteristics that can be used for species delimitation in AMF and these are solely based on spore morphology such as size, shape, colour, basal structure, ornamentation and wall structure (Rosendahl *et al.*, 1994). Morphotyping requires a considerable knowledge of these morphological characteristics from a wide range of different species (Clapp *et al.*, 2001; Tisserant *et al.*, 1998), preferably from pot-cultured material. Other methods of identification have been introduced, which include immunological reactivity (antigen binding), lipid profiles, and isozyme profiling (Dodd *et al.*, 1996; Thingstrup and Rosendahl, 1994), however these methods only had limited success. In recent times the use of molecular techniques, especially

those based on PCR, has revolutionized the analysis of fungal populations and been used extensively to study and identify AMF (Clapp *et al.*, 2002). Many ecological studies looking at the community structure of AMF, once based on spore morphology, now utilise molecular techniques.

### 1.2.1 Indirect analysis of spore populations

Indirect analyses are based on spores isolated from soil and identified by morphology or sequencing. Prior to molecular analyses, there was no way to discriminate AMF structures inside roots, thus species diversity was measured by analysing spores in adjacent soils. This type of analysis records only spore presence, not significance in relation to root colonisation. Isolating spores from soils can be criticised as it reflects only the sporulation stage of the AMF life-cycle (a dormant or resting phase) rather than activity within a symbiosis seen when looking at hyphal abundance (Gamper *et al.*, 2008). Limited variation in morphological characteristics created difficulties in identification of AMF, and few people had the expertise to identify species accurately from spore samples collected directly from the field. Molecular tools now offer more objective methods of identification of isolated spores, providing the user has reasonable molecular expertise, and the methods are rapid, and may reveal taxa not easily detected by other methods.

### 1.2.2 Direct analysis using molecular tools

There is now an extensive international collection of studies where molecular techniques have been used to examine populations of AMF within roots. The direct isolation of DNA from plant roots reveals species which have colonised and, by inference, are significant in the symbiotic relationship. Initially these studies used simple PCR analyses, cloning and T-RFLP analyses to indicate the diversity of AMF within roots, but later studies used specific primer pairs to study richness of particular taxa.

Early work was typified by field studies by Clapp *et al.* (1995) and Helgason *et al.* (1998; 1999; 2002) who compared SSU sequence diversity of AMF found in a Yorkshire woodland to that in nearby arable soils. Eleven sequence groups were found in the woodland site, but only 6 were found in the arable soils (of which two were found at both sites). Over 90% of the sequences from the arable site resembled *Glomus mosseae* when aligned with the limited named accession sequences then available from GenBank. Arbuscular mycorrhizal fungi colonising *Hyacinthoides non-scripta* in mixed woodland in York was studied using the same approach (Helgason *et al.*, 1999). Sequence variation showed how roots were colonised by mixed communities of AMF and that relative abundance of *Glomus, Acaulospora* and *Scutellospora* within the root system varied through the season or with the local predominance of certain trees. The study was unusual in that the molecular data could be compared against an intensive morphological study of colonisation (Merryweather and Fitter, 1998a, b), which indicated a reasonable correlation between the two approaches. The molecular analysis of AMF communities has since progressed rapidly as sequences have accumulated within GenBank. For example, Husband *et al.* (2002) used SSU sequences to examine AMF communities colonising 3 plant species in a tropical forest. Thirty AMF types were detected, of which 17 had not been found earlier from temperate ecosystems.

Molecular analyses rely on primers that will detect all the target organisms and the widely-used NS31 and AM1 primer pair, based on the small subunit rRNA genes, will detect most of the members of the Glomeromycota (Redecker *et al.*, 2003); however some non-glomalian sequences may also be amplified from within the Ascomycota and Basidiomycota. Earlier primers varied in specificity, but Redecker (2000) designed SSU/ITS primers specific for all 5 families of the Glomeromycota, although some anomalies have since been reported, e.g. Wubet *et al.* (2003). Taxon-specific primers are useful when a measure of species richness is required but identification by sequence relies on a large and correct database. Bridge *et al.*

(2003) observed that up to 20% of the sequences available from public databases may be unreliable and Krüger *et al.* (2012) state that the annotation of sequence entries into databases is often inadequate or incorrect. Studies involving sequence examination and phylogeny need to include the use of outgroups in the phylogenetic analysis and a comparison to known sequences from databases (such as NCBI) to avoid inclusion of contaminant sequences (Clapp *et al.*, 2002). The choice of DNA region for primer design is also an important consideration. Krüger *et al.* (2012) state that the use of SSU and the ITS does not have high enough resolution to differentiate fully between closely related species and as such the LSU may be better for species-specific primer design.

There are still issues surrounding these molecular techniques, however, advances in techniques mean that they are now less costly and time consuming. Spores of AMF contain very little DNA (Stukenbrock and Rosendahl, 2005b), though with new molecular methodology much more analysis is now possible. Sequence heterogeneity has to be taken into account when using any molecular methodology with these fungi, from primer design through to phylogenetic studies. The overriding issue is the difficulty of knowing what this sequence diversity actually represents and the effect it has on individual isolates (Sanders, 2004a).

One drawback with molecular approaches is that rarer DNA templates may not be detected in a background of much more frequent templates. Taxon-specific primers can be used in a nested approach to detect these otherwise indistinguishable species. Gamper & Leuchtmann (2007) used this method to detect two species of AMF in grassland soils, whilst Stukenbrock & Rosendahl (2005a) used nested PCR and LSU clade-specific primers to examine relative abundance of sub-groups of *Glomus* within roots of plants in undisturbed coastal vegetation. They found different frequencies of the dominant types between plots and host plants

suggesting a patchy distribution of *Glomus* species in this undisturbed soil, and the formation of large mycelial networks that are associated with several plant species.

### 1.2.3 Quantification of AMF within roots

The molecular approaches discussed above indicate qualitative presence of the respective AMF but are not quantitative (Alkan *et al.*, 2004; Alkan *et al.*, 2006). More recently, real time or quantitative PCR (qPCR) methodology has been applied to AMF studies and initial results have shown the potential to move into a new phase of *quantification* of changes in abundance of individual taxa within roots over time.

Traditionally the only way of quantifying the amount of AMF colonisation in a root system was to undertake microscopic analysis using techniques such as the magnified intersect method (McGonigle *et al.*, 1990) or the Trouvelot method (Trouvelot *et al.*, 1986). Both of these methods involve the microscopic analysis of root fragments and observation of the amount of root colonised by hyphae. The disadvantage for using this type of analysis alone is that due to limited morphological features observed by microscopy it is impossible to detect which species are present in the root.

Different researchers have applied crude measures such as relative intensity of DGGE bands or frequency of occurrence in clones taken from multiply-sampled root fragments. Saito *et al.* (2004) used a detailed analysis of frequency of particular sequence types to give a 'semi-quantative' measure of the % root length colonised by particular groups of *Glomus* found in the roots of grassland plants. They acknowledged that this assumes that frequency of occurrence of sequences reflected the relative amount of DNA in the roots, and that this changes with the number of PCR cycles. Stukenbrock & Rosendahl (2005a) also compared

relative frequencies of sequences of *Glomus* sub-groups to assess the relative amounts of different phylogenetic clusters within plant roots from coastal grassland.

Standard PCR, although useful for the specific detection of fungi, is based on the "end-point" of the PCR reaction which does not allow for quantification (Filion *et al.*, 2003). Quantitative PCR (qPCR) has been used in the past in other areas of biology (Bustin *et al.*, 2009) such as medicine (Orlando *et al.*, 1998), and plant pathology (Bohm *et al.*, 1999). The advantage of quantitative PCR is that it uses fluorescence probes that are detected during the exponential phase of the PCR reaction, thus allowing for a quantitative measure of the PCR product (Heid *et al.*, 1996). These methods are now more widely used to quantitatively study AMF in natural environments (Gamper *et al.*, 2008). A quantitative measure of the relative amount of colonisation of the root system was developed to test whether a certain plant is preferentially colonised by a particular Glomalean taxon (Santos *et al.*, 2006). Similar methodology was also required to investigate how different proportions of co-occurring AMF affect the growth of their host plants under different environmental conditions (Robinson-Boyer *et al.*, 2009).

Alkan (2006) achieved a quantitative measure of co-colonisation by AMF within roots using qPCR in experimental systems using two species of *Glomus (G. intraradices/G. mosseae)*. Phosphorus and saline stress, choice of host plant and spatial distribution within the root all influenced the outcome of interactions between these two fungi. This demonstrated the potential of the methodology for quantification of individual AMF in mixed communities. One issue with using *G. intraradices* in this type of study is that it produces large amounts of intraradical spores (containing large amounts of DNA) and hence might give a disproportionate DNA signal compared to *G. mosseae* which sporulates external to the root. However, Jansa *et al.* (2008) used qPCR to show that roots in pots inoculated with mixtures

of AMF, including *G. intraradices, G. mosseae* and *G. claroideum*, were usually preferentially colonised by *G. mosseae*.

Similar limitations of the qPCR approach were discussed by Gamper e*t al*. (2008) when using a Taq-Man assay on 18S rDNA to quantify mycelial abundance of 5 taxa of AMF in mixed-species environments. The large contribution of spore DNA to the pool of DNA extracted from the samples under investigation was again highlighted as a potential problem as strong correlations were found for qPCR of DNA and spore numbers, but not between hyphal length and qPCR, possibly due to uneven fungal distribution within roots. Quantification of RNA as an alternative approach did not resolve this problem (Gamper *et al.*, 2008). Hence, those taxa which produce large numbers of spores or vesicles in the roots, will produce larger signals for qPCR and the results from biological and genetic quantification may not correlate.

Various authors have seen conflicting results when correlating the data from microscopic observations of root colonisation with data from the qPCR analysis. Alkan *et al.* (2004; 2006) demonstrated a positive correlation when they compared the qPCR assay with the traditional microscopic grid-line intersect method, and Isayenkov *et al.* (2004) also demonstrated a good correlation between visual observations and qPCR. However, Jansa *et al.* (2008) showed that there was no significant correlation between the traditional root colonisation assay (microscopy) and the qPCR assay. Pivato *et al.* (2007) found that abundance readings varied between the microscopic and qPCR assessment and they suggest that this may be due to variation in the number of nuclei in the fungal hyphae during development, or the number of rDNA sequences per nucleus. Until differences in quantities of DNA of AMF in a root system can be related to physical traits,such as spore of hyphal density, direct comparisons among species or isolates is meaningless (Krak *et al.*, 2012).

Another issue with using this technique is that currently the only targets available are the multiple copy rRNA genes (Jansa *et al.*, 2008). Sequence data are limited in other areas of the genome for AMF, thus designing primers is restricted to this area (Gamper *et al.*, 2008). Even in the small sub-unit rRNA region it is impossible to design specific primers for families or genera (Gamper *et al.*, 2008) although the large sub-unit gives better resolution for separating closely related taxa (Rosendahl, 2008). Jansa (2008) suggests the need to develop information on other genes, especially single copy genes.

The importance of designing molecular markers that adequately distinguish between taxa was considered by Thonar *et al.* (2012) who suggested that verification requires extensive cross-amplification assays, to ensure no amplification of other non-target sequences within the system of study. These authors suggested that this is the reason that qPCR has so far been restricted to simple model systems using only a small number of AMF isolates, and has only been used by a limited number of researchers.

Even though there are issues surrounding the use of the qPCR technique, it is a valuable method to give new insight into AMF and host plant interactions (Alkan *et al.*, 2006), and it is a rapid, specific and quantitative method requiring a minimal amount of plant and fungal material (Gamper *et al.*, 2008; Isayenkov *et al.*, 2004).

## 1.3 Host specificity and community analysis

The introduction of qPCR is a useful tool to enable the study of the community structure of AMF and as such community studies are no longer limited to looking at only species richness and not abundance. Many authors have demonstrated that plant communities, their structure diversity and productivity, are affected by the below-ground AMF community

(Grime *et al.*, 1987; van der Heijden *et al.*, 1998a). Van der Heijden *et al*. (1998a) reported that AMF are required to maintain a basic level of plant biodiversity and that the structure of plant communities varied significantly with treatments that included different AMF taxa. Most AMF species can colonise a range of host plants and therefore lack of absolute specificity has been assumed (Sanders, 2003), however species of AMF may differ in their effects on plant growth. Various studies have shown that there is a certain amount of host specificity within AMF. Vandenkoornhuyse *et al.* (2003) used T-RFLP profiling to examine AMF diversity from 89 root samples from 3 co-existing grass species in Scotland and found that host preferences were apparent. Scheublin *et al.* (2004) found 14 sequence groups in DNA profiles from non-legumes and legumes showing that different plants hosted different communities – and nodules hosted different communities than their parent roots. Sýkorová *et al.(2007)* compared 3 methods (molecular probes, trap plants and bait plants) to study diversity and showed that AMF which colonise greenhouse trap plants are not necessarily reflective of the AMF which colonise bait plants placed directly into field soils *in situ*. Alkan *et al.* (2006) showed that *G. mosseae* and *G. intraradices* both showed host preference, when studied using qPCR, and traditional microscopy.

Sanders (2004a) suggested that perhaps the relationship between AMF and their plant hosts is not so much due to the variation between species or morphotypes but more to do with the genetic differences between AMF, that is, whether inter-specific or intra-specific AMF diversity is of greater ecological importance. Munkvold *et al.* (2004) and Koch *et al.* (2006) showed that both intra-specific and intra-isolate differences lead to different responses in host plants. These authors suggest that a change in sequence diversity could lead to a change of effect and this may be more important when considering the impact of an AMF community on the plant hosts. Pivato *et al.* (2007) stress the importance of assessing not only the diversity of AMF species but also the abundance of representative AMF genotypes.

The multiple occupancy of a single root fragment by AMF from different genera or species is now commonly accepted (Alkan *et al.*, 2006; Reddy *et al.*, 2005; Van Tuinen *et al.*, 1998), although this co-occupation is still poorly understood (Alkan *et al.*, 2006; Krak *et al.*, 2012) and it is unclear if this relationship is competitive, synergistic or antagonistic. Van Tuinen *et al.* (1998) showed in pot experiments using nested PCR targeting the D1/D2 region of the LSU that *G. mosseae, G. intraradices, Gigaspora rosea* and *S. castanea* frequently co-existed within the same root fragment, where all 4 fungi were included as inoculum for leek and onion. Functional complementarity, or more than 1 AMF species colonising a root synergistically, has been described by a number of authors (Alkan *et al.*, 2006; Koide, 2000; Reddy *et al.*, 2005). Some data do not support functional complementarity (Edathil *et al.*, 1996; Hart *et al.*, 2013), showing that maximum benefit to the host plant can be achieved by a single effective AMF species. Koide (2000) suggest that certain AMF maybe more beneficial to some plant hosts than others. They suggest that the co-colonisation of 2 or more AMF species could enable a wider spectrum of benefits and is therefore more beneficial to the plant host. Jansa *et al.* (2008) demonstrated evidence for functional complementarity in a single host root system when examining *G. mosseae, G. claroideum* and *G. intraradices*. Wagg *et al.* (2011a) found a positive AM fungal richness-plant productivity relationship overall, however they found a range of AMF interactions ranging from facilitation to antagonism giving rise to positive and negative effects plant effects that were dependent on both abiotic conditions and host plant. Until the use of qPCR was adapted to studies involving AMF it was impossible to study the interaction of multiple AMF occupancy in roots. It is hoped that the increasing use of qPCR may provide a better quantitative understanding of the AMF-host symbiosis (Alkan *et al.*, 2006).

## 1.4 Mycorrhizal alleviation of water deficit stress

With changes in the world climate, water limitation is becoming an increasing concern for global crop productivity. Most plants benefit from mycorrihizal symbiosis through the

improvement of water status and uptake (Al-Karaki, 1998; Aliasgharzad *et al.*, 2006; Auge, 2001; Bolandnazar *et al.*, 2007; Koch *et al.*, 2006; Smith and Read, 1997). Bolandnazar *et al*. (2007) showed that the introduction all 3 of the AMF species studied (*G. versiforme, G. intraradices* and *G. etunicatum*) to onion plants improved the water use efficiency and gave higher yields even when longer irrigation intervals were applied, compared with non-mycorrhizal plants. Ruiz-Lozano *et al.* (1995) examined 7 different *Glomus* species and examined their effects on drought tolerance of lettuce plant. They found that these fungi had different traits that affected the drought resistance of host plants. Auge (2001) reviewed the relationships between AMF and plants in water-stressed environments, and concluded that drought only affected levels of root colonisation in about half of the reports examined and in these instances the level of root colonisation was increased rather than decreased. The level of AMF sporulation seemed to be reduced by extreme conditions, e.g. either chronically dry soils or permanently water-logged soils.

Several mechanisms were proposed to explain how the AMF symbiosis can alleviate plant drought stress (Jeffries and Barea, 2012), including: (1) direct uptake of water by the fungal hyphae from soil areas inaccessible to plant roots and the water transfer to the host plant, (2) a better osmotic status of AM plants which allows these plants to maintain a favourable gradient of water flow from soil into their roots, (3) the enhancement of plant gas exchange by the AM symbiosis, which maintains the correct stomatal opening and $CO_2$ assimilation, (4) an increase in the soil water retention properties through the formation of stable soil aggregates, (5) the stimulation of assimilative activities essential for plant growth such as nitrate reductase activity, which is strongly inhibited in plants by water deficit, (6) the protection of the host plant against the oxidative damage generated by drought, and (7) the activation of aquaporin expression. Smith *et al.* (2010) suggest that the direct uptake of water via hyphae is still controversial as an explanation for the drought tolerance effect of mycorrhiza and suggest that the alleviation of nutrient deficiency is highly important.

## 1.5 Mycorrhizal benefits to strawberry production

Strawberry is an important horticulture crop and within the UK and strawberries accounted for 62% of all soft fruit production in 2011 worth an estimated £242million (Farm Business Survey, 2010/2011, www.fbspartnership.co.uk).

A number of studies have reported the beneficial effects of mycorrhiza on strawberry plants: increased colour parameters and concentration of phenolic compounds (Castellanos-Morales *et al.*, 2010; Plenchette *et al.*, 1983), increased runner production (Niemi and Vestberg, 1992). Inoculation by commercial AMF inoculation has been shown to increase both growth (crowns, roots and leaf area) and tolerance to water stress in micro-propagated strawberry (Borkowska, 2002). However Borowicz (2010) showed no impact of colonisation by AMF on root and shoot fresh weights weight, even under drought conditions, whilst using inoculum obtained from prairie soils. (Hernandez-Sebastia *et al.*, 1999) showed how AMF colonisation prior to transplantation of micro-propagated strawberries helped with water stress tolerance during acclimatisation.

Currently 80% of UK strawberry production for supermarkets is grown under protection and primarily polythene tunnels (www.calu.bangor.ac.uk) requiring the use of water supplied by irrigation. Water abstraction rates in the major strawberry-growing regions are already unsustainable, and the demand for irrigation will rise in the near future. The Water Act 2003 was introduced to safeguard the environment and will place restrictions on future water use.

Global food security and sustainable agriculture has become high on the agenda for global governments and policy makers. The growing demands on land use, fertiliser and water availability all lead to an increasing concern about how future global demand is going to be met in the future (Fitter, 2012).

## 1.6 Summary and objectives

Indirect (spore isolation from soil) and direct (DNA isolation from roots) methods have been used successfully in assessing AMF diversity within an ecosystem. Provided they are done thoroughly, both approaches can reveal large numbers of taxa in a given ecosystem, but the community composition determined from spores can be different than those in roots (Wu *et al.*, 2007). Spore-based methods rely on identification of morphospecies and record simply the presence or absence of the spores regardless of functional significance. Spore numbers bear no relation to the amount of root colonised by the respective AMF. In contrast, direct molecular analyses reveal the diversity of fungi occupying the root and presumably contributing to the mycorrhizal effect on plant growth. However, the molecular 'species' may or may not correlate with traditional morphospecies and the gene tree approach to taxon recognition is sensitive to sampling bias (Rosendahl, 2008). Alternative molecular tools now exist to quantitatively analyse the effect of environment, management or inoculation of soils on more diverse AMF communities. Quantitative PCR can be used for simultaneous specific and quantitative investigations of particular taxa of AMF in roots and soils colonised by several taxa (Gamper *et al.*, 2008) providing the constraints outlined above are taken into consideration.

The efficiency of spore formation and root colonisation from spores or extraradical mycelia are determinants of the maintenance and expansion of AMF occupation in ecosystems (Wu *et al.*, 2007). It has been suggested (Isayenkov *et al.*, 2004) that quantification of hyphae, by

either microscopy or qPCR may not necessarily correspond to the degree of symbiotic activity. These authors state that the number of arbuscules, and therefore active sites of transfer are a more accurate measure of this symbiosis, however the fungi may change to either active transport forming arbuscules, storage vesicles or reproduction according to their current requirements (Hart *et al.*, 2013). It may, therefore be necessary to simultaneously examine a number of different parameters to fully understand the community structure of AMF. Quantification by genetic or microscopic approaches uses different biological units for measurement. As it is not clear which of these units is more significant; using a combination of methods may give a much more comprehensive understanding of AMF communities (Gamper *et al.*, 2008).

Arbuscular mycorrhizal fungi exert significant influences on ecosystem processes via a number of different mechanisms. Mycorrhizal associations are multi-functional and exhibit complementarity, assisting the plants in nutrient acquisition, water uptake, mediating carbon transfer and protection from pathogens. Intensification of agriculture and forestry is reducing the AMF diversity and compromising AMF function, suggesting that AMF are an important part of sustainable low-input agricultural systems. Below-ground biological systems need the same care as above-ground systems because AMF are essential in sustainable land management practices. It is important to know what species are put into the environment as well as monitoring their growth inside the roots; how the community structure is changing during the restoration period; which species are significant and at what time. The beneficial effects of AMF on plant growth have led to the development of AMF as bio-inoculants for forestry, agriculture and horticulture.

At present there is a lack of descriptive data on the community structure of AMF in different environments and in relation to the community structure of their host plant (Sanders, 2004a).

Current research focuses on AMF species diversity, ignoring species abundance. It is not clear whether relative abundance in fungal biomass is as important as the diversity in AMF species for their ecological functioning.

This project will focus on developing molecular tools based on rRNA genes to quantify mixed populations of AMF species within plant roots based on a qPCR system. The aim of this project is to use qPCR to analyse the relative abundance of AMF within a plant root and to examine the effects of changes in this relative abundance on the host plant growth, health and water uptake. This study aims to combine traditional methods with qPCR to fully understand the AMF communities within host root systems. Strawberry will be used as the plant system under investigation as it is a significant crop in the UK and there are mixed messages about the benefits of mycorrhizal colonisation. This will also provide the opportunity to study how different mycorrhizal fungi affect the tolerance of strawberry to the effects of drought stress, a common environmental constraint on strawberry production.

The methodology chosen for this study is dependent on genomic sequence diversity within individual AMF. There is little known about whether and how differences in environments and host plant species influence the genetic diversity present in AMF populations (Koch *et al.*, 2006). This study also provides the opportunity to examine the effect of different culture conditions on the sequence diversity abundance in the rRNA genes by using 2 distinct culture methods, *in planta* and *in vitro.* This work will lead to further understanding of the multi-copy rRNA genes that will be analysed in the qPCR system and if AMF adapt to environmental changes at a genetic level. The relationship between the diversity of sequences and functional characteristic needs to be addressed as this may have implications for the study of AMF in many areas (Kjoller and Rosendahl, 2001) e.g. for the development of inoculm, the

understanding of plant and AMF ecosystems and the successful restoration of plant communities.

## 1.7 Aims

The aims of this study were:

To develop a suitable molecular tool to quantify the relative abundance of different AMF species within a single root system and to use this to investigate the following null hypotheses:
The differences in the relative abundance of fungal species will have no effect on the host, e.g. plant health, water uptake and growth.
Environmental conditions, predominantly water stress, will have no effect on the composition of AMF.
There will be no effect from different culture conditions on the sequence diversity abundance in the rRNA genes.

# Chapter 2. General Methods

## 2.1 Plant and fungal material

### 2.1.1 Plants

#### 2.1.1.1 Micropropagated plants

Strawberry (*Fragaria x ananassa*) was chosen as a host plant for this study. To be certain of the absence of pre-existing mycorrhizal contamination, micro-propagated strawberry plants (cv. Everest) were purchased from Hargreaves Plants (UK) and established in SMR rooting agar (Appendix 1). These plants were incubated at 20$^{o}$C for 2 weeks with overhead lighting until the roots had developed sufficiently to be transplanted into Vermiculite in a propagation chamber. These plants were then weaned over a period of 4 weeks, at 20$^{o}$C with overhead lighting, with increasing ventilation to the chamber.

#### 2.1.1.2 Runner plants.

Pre-established strawberry plants (cv. Elsanta) were grown in a GroDome (Unigro, UK) set at 22$^{o}$C day/20$^{o}$C night with a 14 h day/10 h night cycle with supplementary lighting until runners were profuse. Individual runners were pinned down (using opened paper clips) into 2-litre pots containing Terragreen (Agsorb, Oil-dry Ltd, Wisbech, UK). Once these runner plants had established independently they were cut away from the mother plants and used for experiments after ca. two weeks of independent growth.

## 2.2 Fungal material

#### 2.2.1.1 Pure cultures

Pure cultures of four AMF species (*Funneliformis mosseae* BEG 25, *F.geosporus* BEG 11, *Rhizophagus intraradices* BEG 72, *Glomus microaggregatum* BEG 56), initially obtained as inoculum from Plantworks UK, Sittingbourne, Kent, UK (www.rootgrow.co.uk), were placed as a layer (10g) into 1-litre pots filled with Terragreen (Agsorb, Oil-dry Ltd, Wisbech, UK) and planted with onion (*Allium cepa*) and corn *(Zea mays*). These were grown in a GroDome (Unigro, UK) set at 22$^{o}$C day/ 20$^{o}$C night with a 16h day/8 h night cycle with supplementary lighting. Regular sampling and microscopic observation of these cultures (using wet sieving, see below) confirmed all species to be pure and producing ample spores after ca. 3 months growth. Fertigation was carried out using Vitafeed 102 (Vitax, Leicester, UK) at a rate of 1g/litre with approx. 25mls added to the top of every pot, once every 2 weeks.

#### 2.2.1.2 Bulk inoculum

The pure cultures of *F. mosseae* BEG 25 and *F. geosporus* BEG 11 were used to produce inoculum for this study. Core samples of 40ml were taken from the relevant pure pot culture and placed as a layer into two 3-litre pots filled with Terragreen (Agsorb, Oil-dry Ltd, Wisbech, UK) and planted with corn (*Zea mays*) and parsley (*Petroselinum crispum*). These were grown in a GroDome (Unigro, UK) set at 22$^{o}$C day/20$^{o}$C night with a 16h day/8h night cycle with supplementary lighting for ca. 3 months with regular sampling for purity and sporulation. A most probable number (MPN) test (Cochran, 1950) was performed on the inoculum in order to quantify the infectivity of the cultures (see 3.4), thus ensuring an equivalent colonising potential of the 2 species within the experiment. Fertigation was carried out using Vitafeed 102 (Vitax, Leicester, UK) at a rate of 1g/litre with approx. 25mls added to the top of every pot, once every 2 weeks.

## 2.3 Sample collection

### 2.3.1 Spore extraction, Wet sieving method

Protocols for spore extraction (Gerdemann and Nicolson, 1963) were adapted from the international Bank of Glomeromycota protocols (www.i-beg.eu). A borer was used to sample substrate from pot cultures. Each sample was thoroughly washed through 710µm and 45µm pore sieves with running water and the backwash contents of the 45 µm sieve transferred into a petri dish (Fig 2.1). Samples were then viewed under a stereomicroscope. Spores were collected using fine forceps and mounted onto slides to check for quality and purity or collected for molecular biology studies.

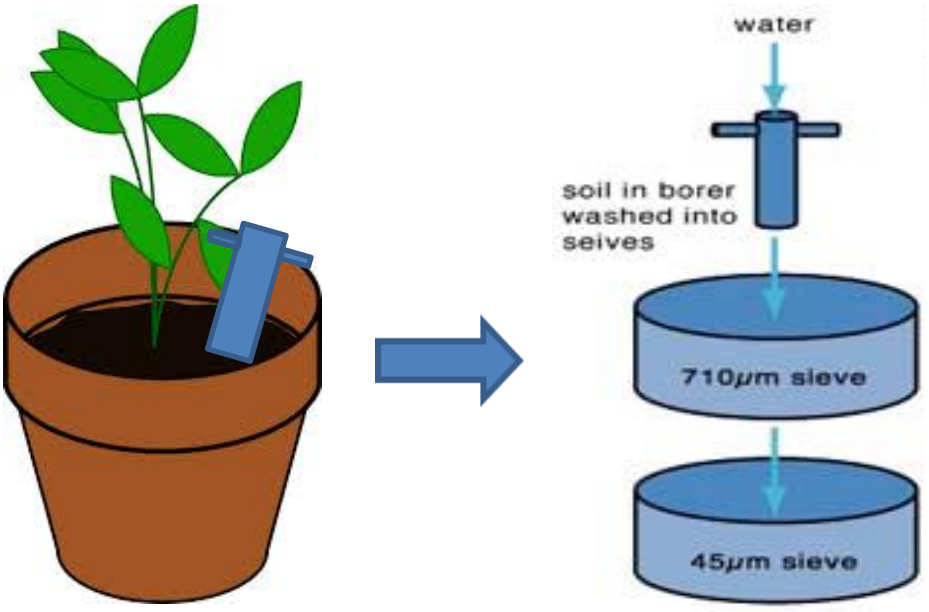



Figure 2.1. Wet sieving method (adapted from www.i-beg.eu)

### 2.3.2 Root collection.

At harvest, plants were removed from their pots and terragreen substrate carefully removed from around the root ball. Roots were washed in clean water before samples being removed for root staining or being frozen at -80$^{o}$C for molecular analysis.

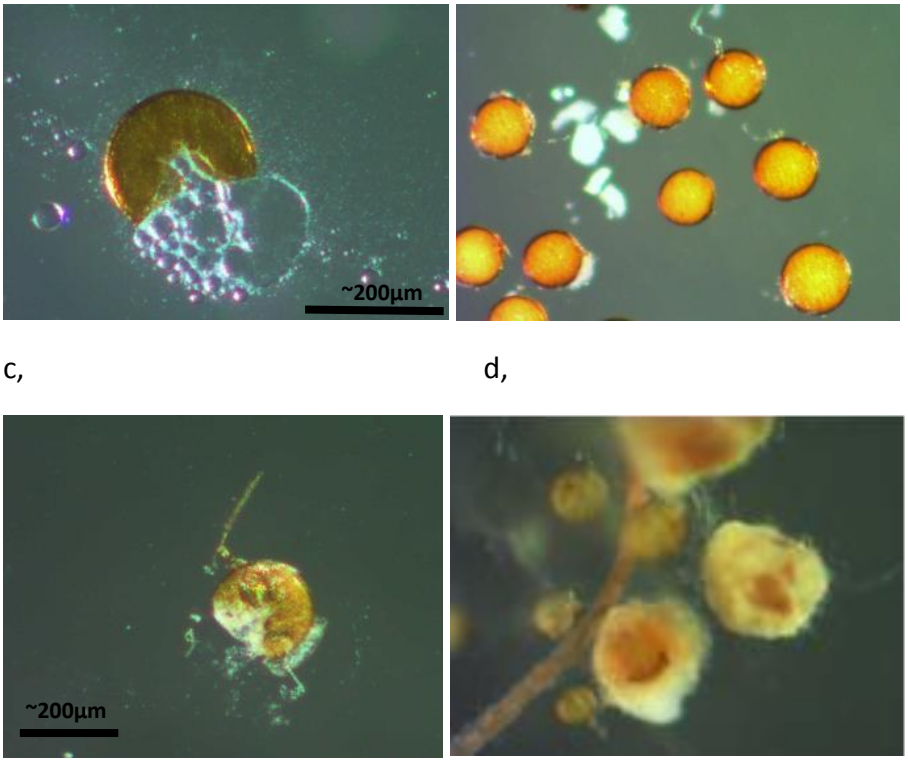
## 2.4 Isolate quality control and quantification

### 2.4.1 Spore identity

The identity and monospecificity of all the isolates were checked at regular intervals by observing the morphological characteristics of the spores, making sure that all isolates corresponded to their original species descriptions (www.invam.wvu.edu). Examination of the spores were carried out using a Olympus SZ11 stereomicroscope, at magnifications up to 80X and a Leitz Diaplan microscope with magnifications up to 400X.

All samples appeared to be pure, containing spores all of the same morphotype. Spores were identified to type species descriptions and only those that appeared healthy and full of lipids were considered to be viable and used for this study. Figure 2.2 shows *F. mosseae* and *F. geosporus* spores extracted from pure culture.

a, b,




c, d,

Figure 2.2. Spores under a stereomicroscope extracted from *F. geosporus* (a and b), and *F. mosseae* (c and d).

### 2.4.2 Trypan blue root staining of total mycelium

To observe the structures of AMF within the root, roots were cleaned and stained. The method described on International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi (INVAM) website (www.invam.caf.wvu.edu) was used for staining taken from Phillips and Hayman (1970). Approximately 0.5 g of randomly picked roots were placed into histocassettes (Simport, Canada) and then cleared in 2 % (w/v) KOH at 90 °C for one hour, before being rinsed three times with water. Cassettes containing roots were then incubated at 20 °C in 2 % (v/v) HCl for 30 min, transferred to trypan blue 0.05 % (w/v) in lactoglycerol (lactic acid, glycerol, water – 1 : 1 : 1) and incubated at 90 °C for one hour. Roots were destained by rinsing with 50% (v/v) glycerol.

### 2.4.3 Root length colonisation assessment

To quantify total length of roots colonised by AMF in a root sample, a grid-line intersect method of calculation was used (McGonigle *et al.*, 1990). Stained roots were mounted (using distilled water) on a slide and a cover-slip carefully positioned. Root cells were gently separated by applying slight pressure to the root. Slides were examined under a Leitz Diaplan microscope with magnifications up to 200X. Sections of root were recorded as either positive or negative for any mycorrhizal structures as they crossed an intersect line of an eyepiece gratitude. A minimum of 100 intersects were analysed for each root sample to give greater accuracy. Figure 2.3 shows the protocol.

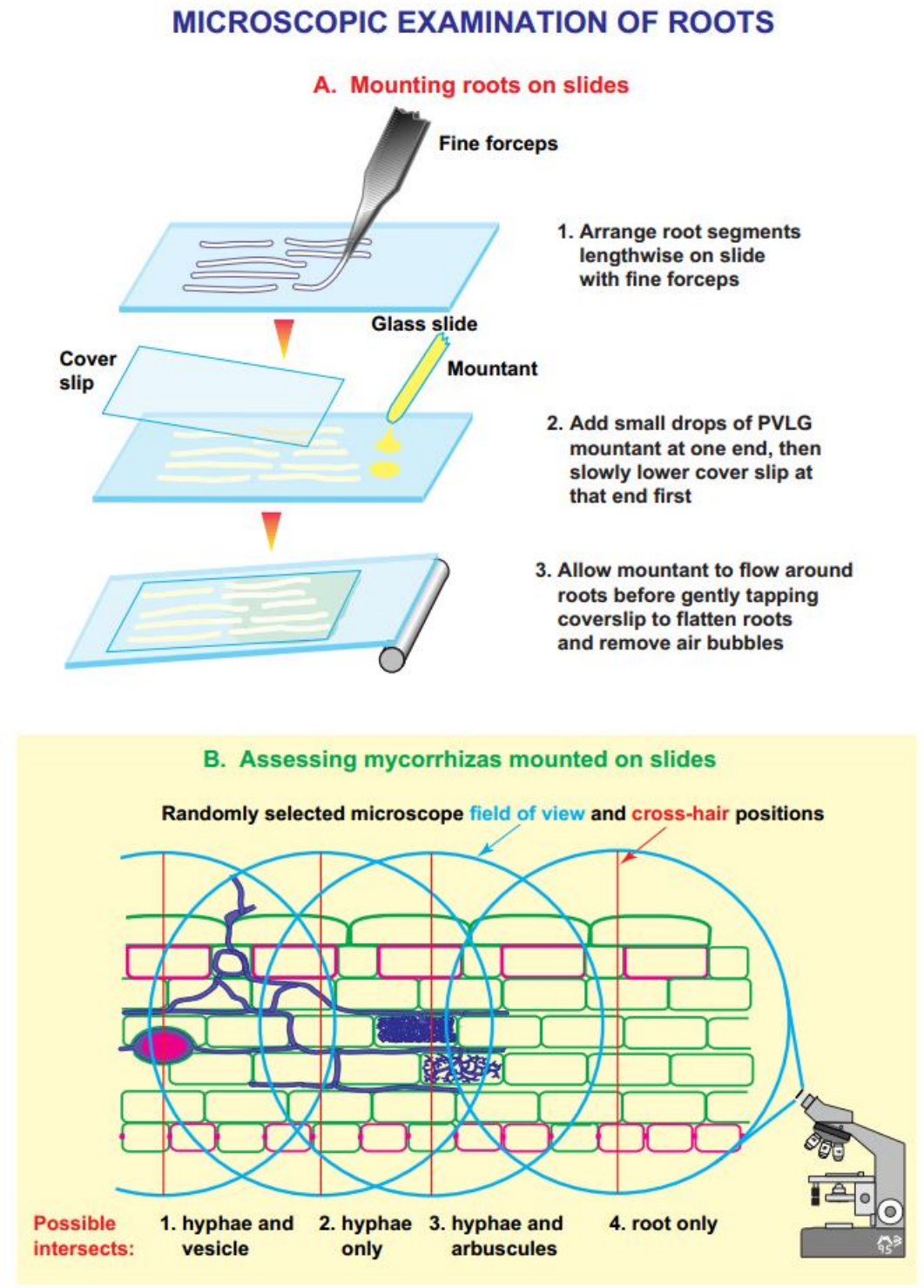



Figure 2.3. Method of calculation of root length colonisation (figure taken from www2.dijon.inra.fr/mychintec/Protocole/Image3.pdf)

### 2.4.4 Most probable number (MPN)

This test was used to determine the infectivity and estimate the number of propagules in the inoculum sample (Alexander, 1965; Cochran, 1950). The MPN method is a standard method used to test the infectivity of mycorrhizal inoculum (www.invam.wvu.edu).

Samples for testing were diluted using the attapulgite clay, Terragreen (Agsorb, Oil-dry Ltd, Wisbech, UK). Dilutions were 1/10, 1/100 and 1/1000. One volume of Rootgrow final product was thoroughly mixed with 9 volumes of the diluting substrate to give the 1/10 dilution and one part of this mixture was then mixed with 9 parts of the substrate to give the 1/100 dilution and finally diluted again to give the 1/1000 dilution. Five replicate pre-sterilised pots were used at each dilution and planted with three seeds of maize (*Zea mays*). These were grown in a GroDome (Unigro, UK) set at 22$^{o}$C day/20$^{o}$C night with a 14h day/night cycle with supplementary lighting. Maize roots were then harvested from each pot, placed into histocassettes, stained with trypan blue (see 3.2) and assessed microscopically for the presence of AMF structures. Based on the incidence of microscopic presence of AMF structure in the sampled roots, MPN was then estimated, using MPN tables (Cochran, 1950).

## 2.5 Spore sterilisation

Collected spores were sterilised before being used for molecular analysis or for setting up *in vitro* cultures. Spores were collected in a petri dish after wet-sieving and were selected using fine forceps and placed into a 1.5ml microcentrifuge tube. Two hundred µl of 2% (v/v) Chloramine T and 1 drop of Tween 80 were added to the spores and the contents left to stand on the bench for 10 mins. Spores were then centrifuged at 10,000 rpm for 2 mins and the liquid removed carefully by pipetting. This was repeated once and, then using centrifugation

and pipetting, spores were rinsed three times in sterile Milli Q water. Solutions of Streptomycin (200ppm) and Gentamycin (100ppm) were added to the spores after the last wash.

## 2.6 *In vitro* culture

### 2.6.1 Axenic production of AMF

A split-plate method (St-Arnaud *et al.*, 1996) was used to cultivate *Rhizophagus intraradices* MUCL 43194 and *R. clarus* MUCL 46238 (GINCO, Belgium). A split, two-chamber 90 mm Petri dish was filled in one compartment with medium M (Bécard and Fortin, 1988), (see Appendix 1) and inoculated with transformed chicory A4NH (*Cichorium intybus*) roots, (GINCO, Belgium). The proximal compartment was filled with medium M minus sucrose. The culture was incubated at 25 °C for 3-4 months in the dark before sub-culturing. Cultures were regularly checked for contamination, root growth and sporulation. To initiate new cultures, a 0.5 $cm^2$ section of agar with roots from the proximal compartment was cut and transferred onto a fresh plate. The same size section of solid media containing spores was taken from a distal compartment and placed near transferred roots. Plates were double-sealed with Parafilm (Sigma-Aldrich, UK). All manipulations were carried aseptically.

### 2.6.2 Extraction of spores from *in vitro* culture

To retrieve spores from the *in vitro* cultures, the phytagel containing culture was dissolved using citrate buffer (Appendix 1). The phytagel in the distal compartment was cut into 25 $mm^2$ sections, removed to a container and flooded with 0.01 M sodium citrate buffer. This was gently agitated for 30 mins and then washed though a 45µm mesh sieve. Spores were retrieved by backwashing onto a Petri plate (Cranenbrouck *et al.*, 2005)

## 2.7 General molecular methods

### 2.7.1 DNA extraction from roots and fungal spores

The Qiagen DNeasy mini kit (Qiagen, Hielden, Germany) was used to extract DNA from roots or spores, following manufacturers recommendations. Approximately 100 mg (wet weight) of roots were placed in liquid nitrogen for 5 mins before being disrupted using ball bearings (4 mm diameter) using a Qiagen Retsch Tissue lyser (Qiagen, Hielden, Germany) for 5 mins. Spores were crushed with a micro-pestle in 1.5 ml Eppendorf tubes containing Buffer AP1 (400 µl) and RNaseA (4 µl), as per the manufacturers recommendation.

### 2.7.2 Working solutions for PCR

Primers were received and stored (at -20$^{o}$C) as stock solutions at 100µM concentrations. Primers were diluted to 2µM for use in each standard PCR master mix (Appendix 1). dNTP's were received from Promega, UK, at 100 µM and stored at -20$^{o}$C as stock solutions. These were mixed and diluted to 2.5µM and stored at -20$^{o}$C for use. Each reaction was performed in a PTC-200 Peltier thermocycler (MJ Research, Watertown, MA, USA) according to the following protocol: initial denaturation cycle at 94$^{o}$C (3 min), followed by 35 cycles of 94$^{o}$C (30 sec), 50$^{o}$C (45 sec), 72$^{o}$C (1min), with a final extension of 72$^{o}$C (7 min)

### 2.7.3 Agarose gel electrophoresis

Agarose gel 2 % (w/v) in TBE buffer was dissolved by heating and left to cool to ca. 50$^{o}$C before being poured into a gel mould. DNA samples were mixed with Blue-Orange loading Dye (Promega, UK) for loading. Appropriate size DNA ladders were co-electrophoresed with the samples. Gels were stained in ethidium bromide solution (0.1 µg ml$^{-1}$) for 30 min and visualised by BioRad GelDoc XR (Bio-Rad, USA).

## 2.8 Irrigation

Irrigation was provided to strawberry plants using separate irrigation lines consisting of 13 mm wide LDPE pipe (City Irrigation Ltd, Bromley, UK) with 2 L $h^{-1}$ Netafim drippers (City Irrigation Ltd, Bromley). Stakes were attached to the drippers via 4 mm wide flexible PVC microtube (City Irrigation Ltd, Bromley, UK) and placed in the Terragreen substrate in the pots. Pots were covered with a plastic cover to reduce soil evaporation, see Fig 2.4.

Figure 2.4. Strawberry plant under controlled irrigation, showing irrigation dripper and plastic pot cover.

### 2.8.1 Regulated Deficit Irrigation (RDI)

RDI provides a method for producing a water stress on plants that is estimated by the amount of water they are using over a 24-hour period. This method was based on that reported by Grant *et al.* (2009). The plants under the well-watered treatment received 100% of the water

estimated to have been lost via evapotranspiration per degree hour (ETp) and the plants under the RDI treatment received 70% of water for the WW treatment in Exp 1 and 60% in Exp 2 & 3.

Evapotranspiration per degree hour (i.e. water loss) was calculated for a sample of 4 well-watered pots (containing a sample from each treatment and at different positions throughout the bench area). This was determined by weighing pots after irrigation and then again approximately 22 hours later prior to the next irrigation event, repeated weekly. An evaporimeter (Evaposensor, Skye Instruments Ltd., Powys, UK), positioned at the centre of the experiment, was recorded over the same time period to determine the accumulated degree hours ($^0$C h) between the 2 weight measurements. One degree hour is a difference in temperature of 1$^o$C between dry and wet artificial leaves on the Evaposensor. The accumulated degree h is a measure of evaporative demand therefore evapotranspiration per degree hour could be calculated for each plant.

The accumulated degree hour over 24 h periods were recorded, and multiplied by the average ETp values for the well-watered pots to determine the irrigation requirement and thus set the irrigation duration. The water limited plants received only 70% of this requirement in Experiment 1 and 60% of the requirement in Experiments 2 and 3. Irrigation was adjusted at least 3 times per week, however as conditions were fairly stable within the GroDome, little adjustment was required. Figure 2.5 shows the irrigation system in operation within the GroDome.


Figure 2.5. Irrigation system within the GrowDome at EMR. Four irrigations lines can be observed, 2 delivering the full water requirement of the plants and 2 delivering 70% of the water requirement.

# Chapter 3. Designing species-specific primers and optimisation of a qPCR protocol for use with strawberry roots

## 3.1 Introduction

The overall aims of this project were dependent upon the development of the molecular assay of quantifying DNA of individual AMF strains within strawberry roots. The results reported here are concerned with the method development required to achieve this objective.

Traditional PCR-based techniques have been extensively used to study and identify AMF (Clapp *et al.*, 2002) in a variety of environments, including the roots of plant. These molecular approaches indicate the qualitative presence of the respective AMF but are not usually quantitative. Until recently, the only way of quantifying the amount of AMF colonisation in a root system was to undertake microscopic analysis using techniques such as the magnified intersect method (McGonigle *et al.*, 1990) or the Trouvelot method (Trouvelot *et al.*, 1986). Both of these methods involve the microscopic analysis of root fragments and the quantification of the amount of root colonised by hyphae. The limited range of morphological features of the root-colonising structures of AMF observed by microscopy make it impossible to study the diversity of specific AMF taxa present (Merryweather and Fitter, 1998b). A real time or quantitative PCR (qPCR) approach allows for a quantitative and specific analysis of the relative abundance of individual AMF taxa within a root system and this methodology has recently been used successfully to study AMF (Gamper *et al.*, 2008; Jansa *et al.*, 2008; König *et al.*, 2010; Pivato *et al.*, 2007; Santos *et al.*, 2006; Thonar *et al.*, 2012; Wagg *et al.*, 2011a). The method is dependent on the design of target-specific primers.

In this project, there were two target taxa, *F. mosseae* BEG 25 and *F. geosporus* BEG 11. Species-specific primers were designed within the D2 region of the LSU of the rRNA gene,

against these two species, (Fig. 3.1). Both of these isolates have been extensively analysed in the D2 region using traditional cloning and Sanger sequencing techniques (Clapp *et al.*, 2001) with a number of sequence variants well documented in GenBank. The degree of resolution of the LSU is regarded to be high and was used to differentiate between closely related AMF species (Krüger *et al.*, 2012; Wagg *et al.*, 2011a).

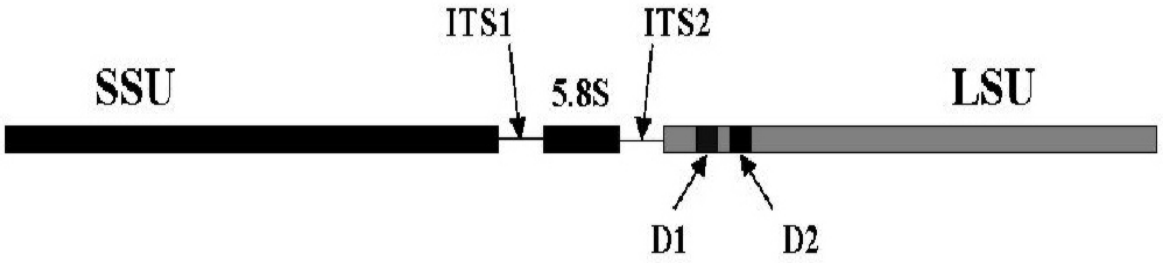



Figure 3.1. Diagram of the rRNA showing the position of the D2 region.

Previous investigations of the rRNA genes have highlighted a high level of genetic variability in the Glomeromycota, with multiple variants of sequences shown to occur within individual spores and isolates, as well as within and between species (Clapp *et al.*, 2001; Hijri *et al.*, 1999; Kuhn *et al.*, 2001; Lloyd MacGilp *et al.*, 1996; Rodriguez *et al.*, 2001; Sanders, 1999). When designing specific primers, careful consideration for sequence heterogeneity within taxa needs to be given to ensure good amplification from many sequence types from the target species and to avoid any non-specific amplification from sequence variants of non-target species. This has been discussed in a number of recent papers (Gamper *et al.*, 2010; Thonar *et al.*, 2012) which emphasise that the variation in rRNA regions should be considered in all primer design for AMF species.

The aims of this work were to design specific primers to *F. mosseae* BEG 25 and *F. geosporus* BEG 11 and to test their specificity against each other and against *G. microaggregatum, R.*

*irregularis* and *C. claroideum*. These specific primer pairs were then used to optimise and test a qPCR system, to allow the study of the relative abundance of these two isolates within a single strawberry root system.

## 3.2 Methods

### 3.2.1 Primer design

Primers were designed based on previously published sequences deposited in GenBank for *F. mosseae* BEG 25 AF304982 and *F. geosporus* BEG 11 AF305004. These sequences are both frequently observed sequence variants (Clapp *et al.*, 2001) of these isolates. Due to the variability among these sequences, alignment of a selection of sequences from each species was used to ensure that the newly designed primer would amplify from a range of the variable sequences from that species. Alignments also included sequences from the LSU rRNA sequences of other AMF taxa and strawberry (*Fragaria x ananassa*). For the alignment, variant D2 region sequences from *F. mosseae* BEG 25 (AF304982-AF304994) and *F. geosporus* BEG 11 (AF304995-AF305008), along with the sequences of *G. microaggregatum, R. irregularis* and *C. claroideum* were downloaded from GenBank. These sequences were aligned using Clustal Omega www.ebi.ac.uk/Tools/msa/clustalo, and trimmed using Jalview www.jalview.com to remove low quality end regions.

Primers were designed such that short PCR target lengths (~80-100bp) were amplified as is suggested for qPCR analysis and constructed using well-documented considerations for qPCR primer design (real time PCR: from theory to practise www.invitrogen.com). A number of primer pairs were initially designed and tested for their specificity by alignment. Any primer pairs that were shown to give good specificity using these methods were synthesised by Sigma-Aldrich (UK). Eleven different primer pairs for *F. mosseae* and 12 primer pairs for *F.*

*geosporus* were synthesised and tested. Primers were stored at -20$^0$C at 100µM concentration until required when they were diluted to 2µM for use.

### 3.2.2 Molecular primer testing

The *F. mosseae* and *F. geosporus* primer pairs were tested for specificity to their target species using standard PCR techniques. Specificity was also tested against *G. microaggregatum* and *R. irregularis*. Two hundred spores, from established pure pot culture of the four species of AMF, were collected by wet-sieving from routine stock culture pots, whilst samples of approximately 0.1 g of strawberry roots were obtained from the inoculated and uninoculated plants. This material was placed in liquid nitrogen. Spores were crushed using a micropestle and roots were disrupted with ball bearings using a Qiagen Retsch Tissue lyser (Qiagen, Hielden, Germany) for 5 min. The plant DNeasy mini Kit (Qiagen, Hielden, Germany) was used to extract pure genomic DNA from AMF spores, and from strawberry roots with or without AMF inoculation, following the manufacturer's protocol. Three extractions, each from a separate subsample of two hundred spores, were obtained for *F. mosseae* BEG 25 and *F. geosporus* BEG 11. Primers were tested for specificity against each of these extractions. ALF01 and NDL22 primer sets were used to confirm extractions from all four pure cultures were positive.

Originally the PCR protocol described by Clapp *et al.* (2001) was used to amplify this DNA, however amplification was weak and inconsistent. This was most likely due to the PCR products obtained by the Qiagen DNeasy system being of higher concentration than those extracted using phenol/chloroform extraction by Clapp *et al.* (2001). Extractions were diluted 1:10 using water (Sigma, UK) before the PCR and a more efficient PCR protocol was used with a reduced concentration of primer and dNTP. This protocol had been used successfully to amplify a range of plant pathogens from roots in previous studies at East Malling and was

tested against the AMF extractions. Consistent amplification with each of the species used was seen and as such this protocol was used for the remainder of the study.

The PCR parameters finally used were such that the reaction mixture (20 µl reaction) contained 2.0 µl GeneAMP 10X buffer II (Applied Biosystems, Cheshire, UK), 1.6 µl of $MgCl_2$ (25 mM), 1.6 µl dNTP (2.5 mM), 2.0 µl of each primer (2 µM), 0.1 µl AmpliTaq DNA polymerase (Applied Biosystems, Cheshire, UK), 8.7 µl of water (Sigma, UK) and 2.0 µl of DNA template. Each reaction was performed in a PTC-200 Peltier thermocycler (MJ Research, Watertown, MA, USA) according to the following protocol: initial denaturation cycle at 94$^{o}$C (3 min), followed by 35 cycles of 94$^{o}$C (30 sec), 50$^{o}$C (45 sec), 72$^{o}$C (1min), with a final extension of 72$^{o}$C (7 min). Amplified products were then visualized on a 2% agarose gel, stained with ethidium bromide (0.5µg/ml) and visualised under UV light.

### 3.2.3 Validation of the qPCR

To select the final primer pairs to be used for the remainder of the study, all primer pairs were subjected to testing and analysis using the qPCR method. Each of the primers designed for both species were used to amplify against a standard serial dilution of genomic DNA of *F. mosseae* BEG 25 and *F. geosporus* BEG 11 (extracted from spores using the methodology outlined above). Similarly non-inoculated strawberry root was extracted using the root extraction protocol as outlined above. To generate standard curves, concentrations of both the fungal and plant DNA extraction were first established using a NanoDrop 1000 spectrophotometer (Thermo Fisher scientific, UK). Purities were verified using a ratio of absorbance at 260 nm and 280 nm and concentrations found to be 4.0ng/µl for *F. mosseae*, 6.9 ng/µl for *F. geosporus* for the spore extracts and 4.6 ng/µl for the strawberry root extraction. These samples were diluted in a 10-fold serial dilution series and run on the qPCR system with the relevant primer pairs. This was used to select the most efficient primers in

the qPCR system and the most appropriate points on the calibration curve. The *F. mosseae* primer pairs used by Alkan *et al.* (2006) were also included as a control to check that the qPCR system was working. These were not used in the final study as alignment analysis showed them to only amplify infrequently found sequences in the *F. mosseae* BEG25 and they showed a low efficiency in the qPCR protocol finally chosen. Initially the qPCR protocol used by Alkan et al. (2006) was used but this was modified later to increase efficiency (see results).

QPCR reactions were set up in MicroAmp optical 96-well reaction plates (Applied Biosystems, Cheshire, UK), with a total reaction volume of 20µl per well. Each reaction contained 10 µl of Power SYBR green PCR master mix (Applied Biosystems, Cheshire, UK), 2µl of each primer (2µM), 2µl of PCR water (Sigma, UK), and 4 µl of DNA template. Each reaction was carried out in triplicate and each set included a non-template controls containing water (Sigma, UK) to check for potential contamination in the master mix. The qPCR reactions were run in a 7500 Real time PCR system (Applied Biosystems, Cheshire, UK) according to the following protocol: 50°C (2 min), initial denaturation at 95°C (10 min) followed by 40 cycles of 95°C (15 sec), 55°C (30 sec) and 72°C (34 sec), followed by elongation at 72°C (10 min), and a dissociation step was then added as a final step to allow the melting curves of the amplified products to be analysed.

Strawberry Ef1α (Elongation factor 1 α, an endogenous gene) was used as a normaliser in the qPCR, and its amplification in the strawberry root was achieved using the previously published primer pairs: FaEF1 - 5'-TGC TGT TGG AGT CAT CAA GAA TG-3' and FaEF1R - 5'-TTG GCT GCA GAC TTG GTC AC-3' (Carbone *et al.*, 2006).

### 3.2.4 Standard curve generation

Standard curves were generated from the dilution series of spore extracts from both the *F. mosseae* and the *F. geosporus* as above. A new 5-point standard curve was generated (in triplicate) on every plate used for assessment. Standard dilutions were originally made and then stored as aliquots at -20$^{o}$C to be used to calibrate each plate used for the samples. Each sample plate was analysed with a new standard curve to avoid plate and between run variations. Very similar slopes with good slope efficiencies and R2 values were observed on every run. If any sample was greatly different to the other replicates (as is possible from amplification error or well interference) it was discarded from the standard curve calculation during formulation.

### 3.2.5 Cross amplification analysis of qPCR

Cross-amplification experiments, as with standard PCR methodology, were also employed to assess the specificity of the qPCR assays. Quantitative PCR analysis was used to test both fungal-specific primers against spore DNA extractions from the pure pot cultures and target and non-target inoculated roots. Extraction of DNA from spores and roots was carried out as for standard PCR testing and qPCR protocols were as described above. Originally master mix and plates were prepared on the open bench, however the sensitivity of this technique is very high (especially when using *F. geosporus* primers). Amplification was frequently observed in the no template controls, most likely coming from air-borne spores. All preparation of master mix and plate loading was consequently moved into a laminar flow hood for sterility.

## 3.3 Results

### 3.3.1 Primer design

A total of 37 sequences were used to compare sequence variation within and between taxa. Alignment of these previously published sequences showed a good degree of variability

between sequences at ~100-170bp's into the D2 region, as defined by the ALF01 (Clapp *et al.*, 2001) and NDL22 (Van Tuinen *et al.*, 1998) primers used to amplify many of the sequences deposited in Gen Bank, and as such specific primers were designed about this variable region. Primers were designed using Primer-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Figure 3.2 shows the alignment with the final primer pairs chosen after the molecular testing had taken place. Good alignment of the chosen primers can be clearly seen with each target species. Some *F. mosseae* sequences have one or two base pair variants in some sequences, which could potentially lead to some primer mis-alignment in these variants. The 2 forward primers are not specific to their target species (The mossL14 primers are specific to *Funneliformis*, however geoLp3 primers do amplify other some other genera of AMF), however the reverse primers make the pairing specific. These primers were designed to be specific in this system, however to be used in a natural environment they would need further analysis.

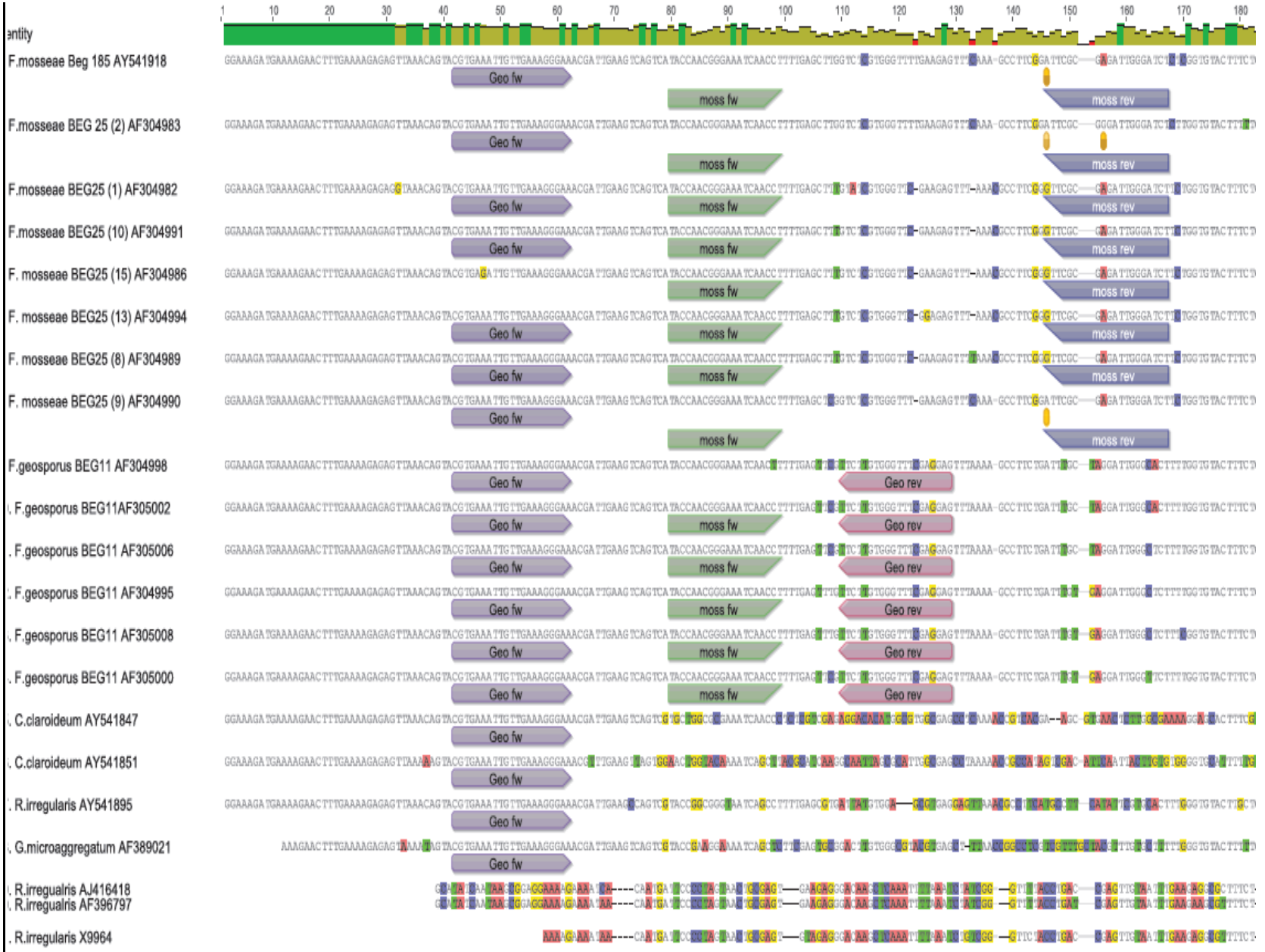

1 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180
entity
F.mosseae Beg 185 AY541918
F.mosseae BEG 25 (2) AF304983
F.mosseae BEG25 (1) AF304982
F.mosseae BEG25 (10) AF304991
F. mosseae BEG25 (15) AF304986
F. mosseae BEG25 (13) AF304994
F. mosseae BEG25 (8) AF304989
F. mosseae BEG25 (9) AF304990
F.geosporus BEG11 AF304998
. F.geosporus BEG11AF305002
. F.geosporus BEG11 AF305006
. F.geosporus BEG11 AF304995
. F.geosporus BEG11 AF305008
. F.geosporus BEG11 AF305000
. C.claroideum AY541847
. C.claroideum AY541851
. R.irregularis AY541895
. G.microaggregatum AF389021
. R.irregularis AJ416418
. R.irregularis AF396797
. R.irregularis X9964
Geo fw
moss fw
moss rev
Geo rev

Figure 3.2. Alignment showing a selection of the sequences included for primer design. Variant sequences from *F. mosseae* and *F. geosporus* were included in the analysis and marked primer pair sites are shown for both species specific primers (figure only shows primer pairs finally chosen after molecular testing). Yellow markers show potential base pair sites of mis-alignment between the reverse primer and a few *F. mosseae* sequence variants. Nucleotide base differences are represented in the alignment by a colour highlight, such that T green, A is red, C is blue and G is yellow. Produced using Geneious software (Geneious version (R6) created by Biomatters, www.geneious.com)

### 3.3.2 Molecular Primer testing

Good yields of DNA (~6-8ng/µl before dilution) were extracted from the spore extracts, which amplified successfully using the ALF01/NDL22 primer set and showing bands at ~460bp as expected for the D2 region, see Fig 3.3.

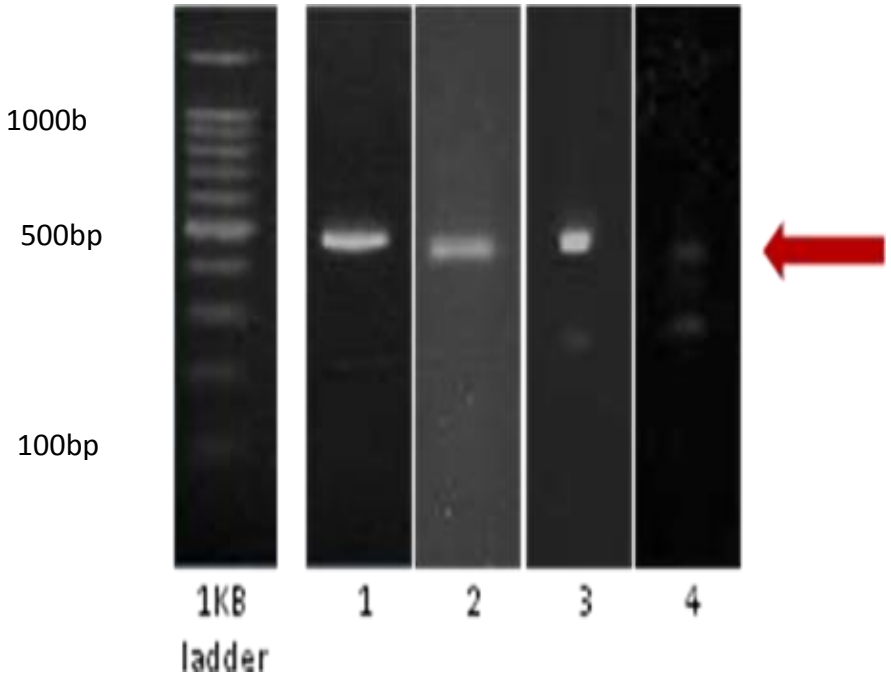



Figure 3.3. Positive bands of PCR extracts from spores taken from pure pot cultures amplified with the ALF01 and NDL22 primers, lane 1= *F. mosseae* BEG 25, lane 2= *F. geosporus* BEG 11, lane 3= *R. irregularis* BEG 72 and lane 4= *G. microaggregatum* BEG 56.

As shown in table 3.1, three possible primer sets for *F. mosseae* (marked *) showed good amplification products (ca. 88 bp) when tested against spore extracts of *F. mosseae* BEG 25, and no amplification products when tested against the spore extracts of *F. geosporus* BEG 11, *R. irregularis* or *G. microaggregatum*. Similarly three possible *F. geosporus*-specific primers (marked**) showed good and specific amplification (product size ca. 86 bp) of the spore extracts from *F. geosporus* BEG 11, but not *F. mosseae* BEG 25, *R. irregularis* or *G. microaggregatum*. Only these 6 primers were chosen for continued analysis as all other primer sets were not absolutely specific against non-target taxa.

| **Primer pair** | **Target species** | ***F.mosseae*** | ***F.geosporus*** | ***R. irregularis*** | ***G. microaggregatum*** |
|---|---|---|---|---|---|
| **pp1 *** | *F. mosseae* | ✓ | | | |
| **pp2** | *F. mosseae* | | | | |
| **pp3*** | *F. mosseae* | ✓ | | | |
| **pp4** | *F. mosseae* | ✓ | | ✓weak | |
| **pp5** | *F. mosseae* | ✓ | ✓weak | | |
| **pp6** | *F. mosseae* | | | | |
| **pp8** | *F. mosseae* | ✓ | ✓ | | |
| **pp9** | *F. mosseae* | ✓ | ✓weak | | |
| **pp10** | *F. mosseae* | ✓ | ✓weak | | |
| **pp13** | *F. mosseae* | ✓ | ✓weak | | |

| | | | | | |
|---|---|---|---|---|---|
| **pp14*** | *F. mosseae* | ✓ | | | |
| **pp1**** | *F. geosporus* | | ✓ | | |
| **pp2**** | *F. geosporus* | | ✓ | | |
| **pp3**** | *F. geosporus* | | ✓ | | |
| **pp4** | *F. geosporus* | | ✓ | ✓weak | |
| **pp5** | *F. geosporus* | ✓ | ✓ | | |
| **pp6** | *F. geosporus* | ✓ | ✓ | | |
| **pp7** | *F. geosporus* | ✓ | ✓ | | |
| **pp8** | *F. geosporus* | ✓weak | ✓ | | |
| **pp9** | *F. geosporus* | | ✓ | ✓weak | |
| **pp10** | *F. geosporus* | | ✓ | ✓weak | |
| **pp29** | *F. geosporus* | | ✓ | ✓weak | |
| **pp23** | *F. geosporus* | ✓weak | ✓ | | |

Table 3.1. Table showing specific amplification of each of the primers pairs shown by alignment to be specific and as such synthesised. Primers that were seen to be specific using molecular methods and chosen for further analysis are marked * for *F. mosseae* and ** for *F. geosporus.* Primers that were finally chosen for this study are marked in red.

All selected primer pairs only amplified the relevant species when tested against DNA extractions from strawberry roots inoculated with either *F. mosseae or F. geosporus* (as shown in Fig.3.4, only those primers finally used in the study are shown in this figure). Neither primer resulted in any amplification from uninoculated strawberry root.

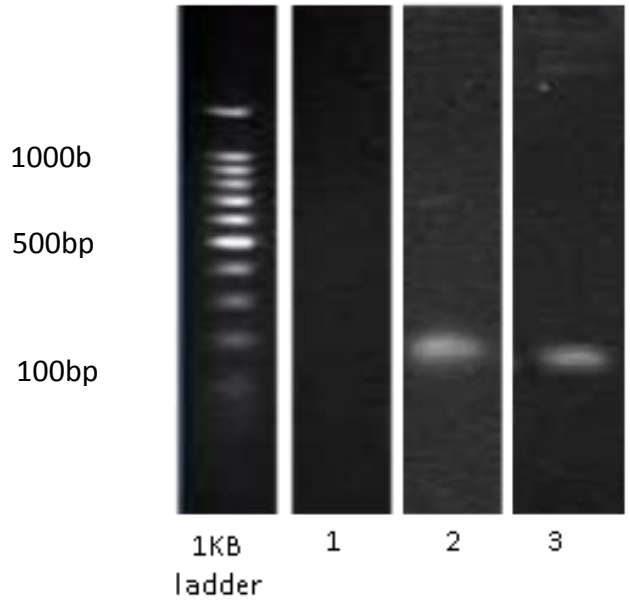



Figure 3.4. PCR amplicons using the primer sets finally chosen for both *F. mosseae* BEG 25 and *F. geosporus* BEG 11. Lane 1 = No amplification for un-colonised strawberry roots, with *F. mosseae* primer pair (No amplification was also shown for the *F. geosporus* primers, data not shown), Lane 2 = *F. mosseae* primers against root extractions inoculated with *F. mosseae* BEG 25, and lane 3 = *F. geosporus* primers against roots inoculated with *F. geosporus* BEG 11.

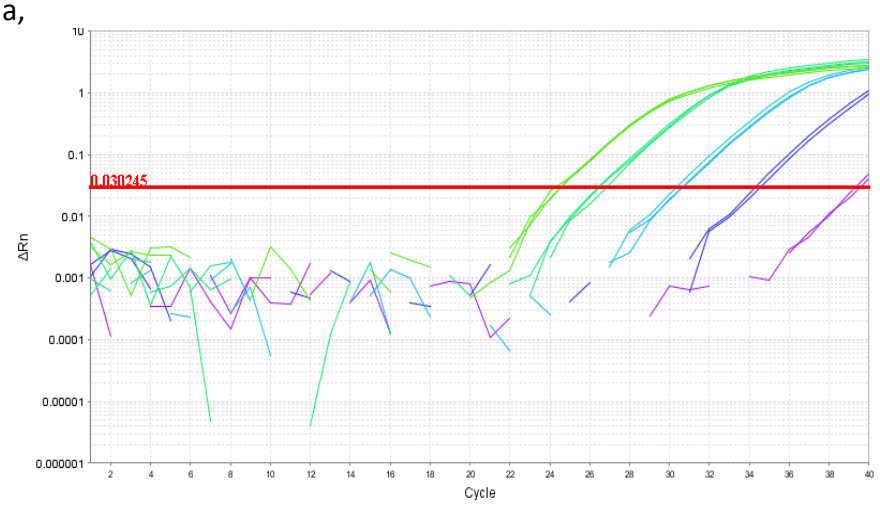
Primer pairs that were found to amplify target genes were further tested with Oligo Analyser (http://eu.idtdna.com/analyzer/applications/oligoanalyzer/) for the presence of dimerisation and secondary structure formation. Low ΔG values confirmed that primer sets for *F. mosseae* and *F. geosporus* were unlikely to form secondary structures, or heterodimer formations. Fungal primers had annealing temperatures ranging between 62 and 64°C, and the strawberry EF1α primer annealed at ~66°C.

### 3.3.3 Validation of qPCR

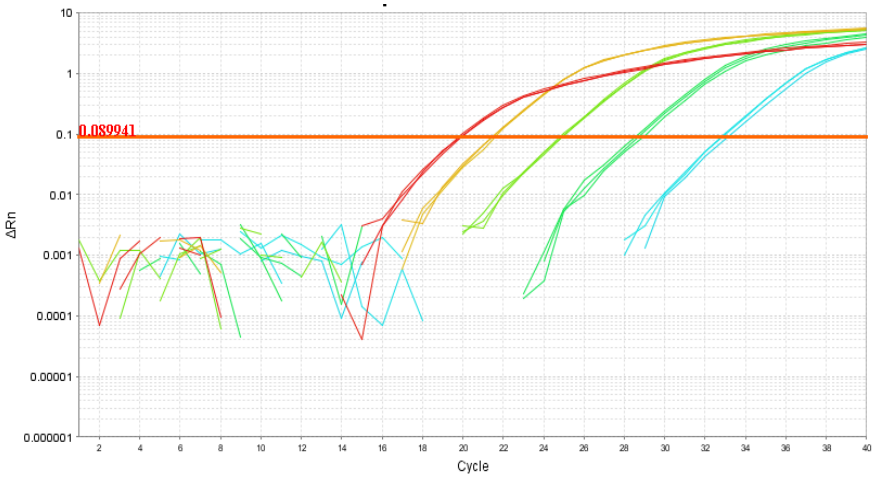
Originally the - protocol used by Alkan *et al.* (2006) was tested with the *F. mosseae* specific primers of these authors, however, this resulted in no amplification of the newly designed primer pairs . This protocol had an annealing temperature of 62$^{o}$C which was lowered to 55$^{o}$C (in-line with the standard PCR protocol). Good amplification was then seen in all 3 *F. mosseae* specific primers. The *F. geosporus* primers were only tested at the 55$^{o}$C annealing temperature as good amplification was obtained.

All primer primers showed amplification of target inoculated roots in the qPCR system. The final primer pairs chosen were: *F. mosseae* primers, mossL14fw- TACCAACGGGAAATCAACCT with mossL14rev- AGATCCCAATCTCGCGAAC and *F. geosporus* primers, geoLp3fw-CGTGAAATTGTTGAAAGGGAA with geoLp3rv-CTCCTCGAAACCCACAAGAA. These were selected as they showed very little background amplification, had evenly spaced well-formed amplification plots and a single clean peak observed in the dissociation curve, with no evidence of primer dimers. Fig. 3.6 shows the amplification plots and dissociation curves of the 2 primer pairs. The 2 chosen specific primer pairs also gave good linear detection from the standard dilutions and slope efficiency values suggesting good reaction efficiency, with reliable $R^2$ values (see Standard curve generation).

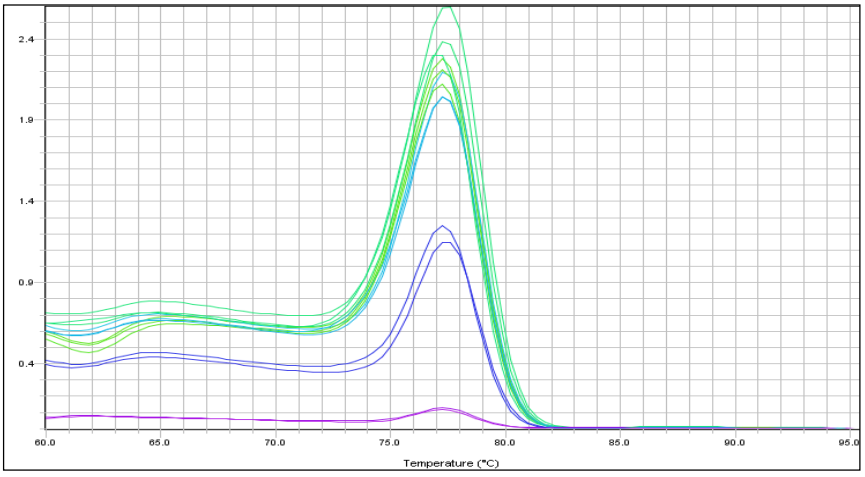
a,




b,




c,



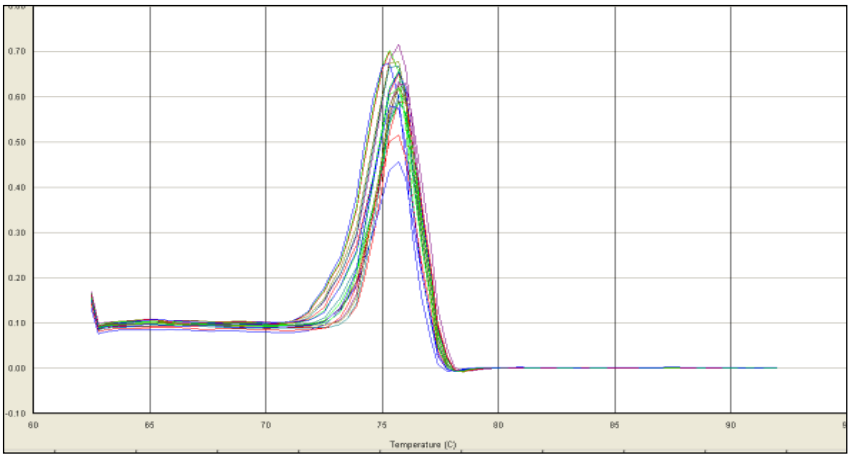



Figure 3.6. Amplification plots from primer pair mossL14 (a) and geoLp3 (b), showing the increase of fluorescent signal (ΔRn) over PCR cycle no., of samples from a serial dilution of spore extractions from *F. mosseae* and *F. geosporus* respectively. Amplification demonatrate even amplification from samples over the dilution range studied. Dissociation curves, showing change in fluorescence (ΔFΔT) vs temperature, of both primer pairs, mossL14 (c) and geoLp3 (d). These graphs show the analysis of the dissociation characteristic of double stranded DNA at melting, and as such indicate likely polymorphism and primer dimerisation.

The other *F. mosseae* specific primers, pp1 and 3, showed some evidence of primer dimers in analysis of the dissociation curves. In contrast, the dissociation curve for mossL14 showed no evidence (Fig. 3.7). The primer pair finally chosen was geoLp3 as this showed no evidence of any primer dimers, whereas pp1 and 2 showed very slight dimerization.

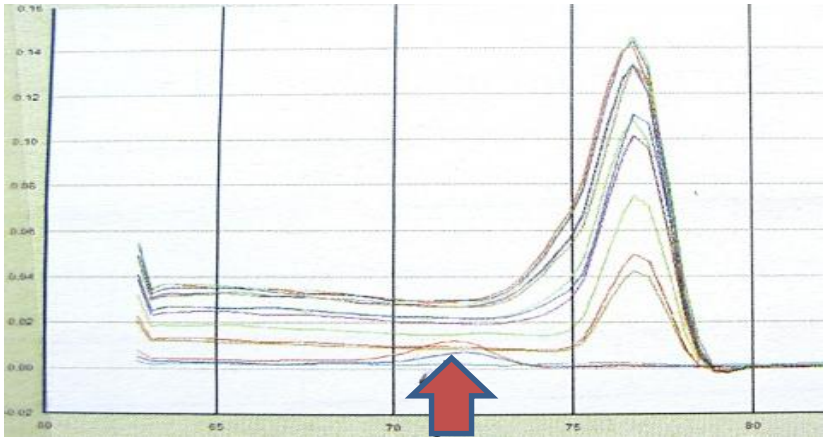
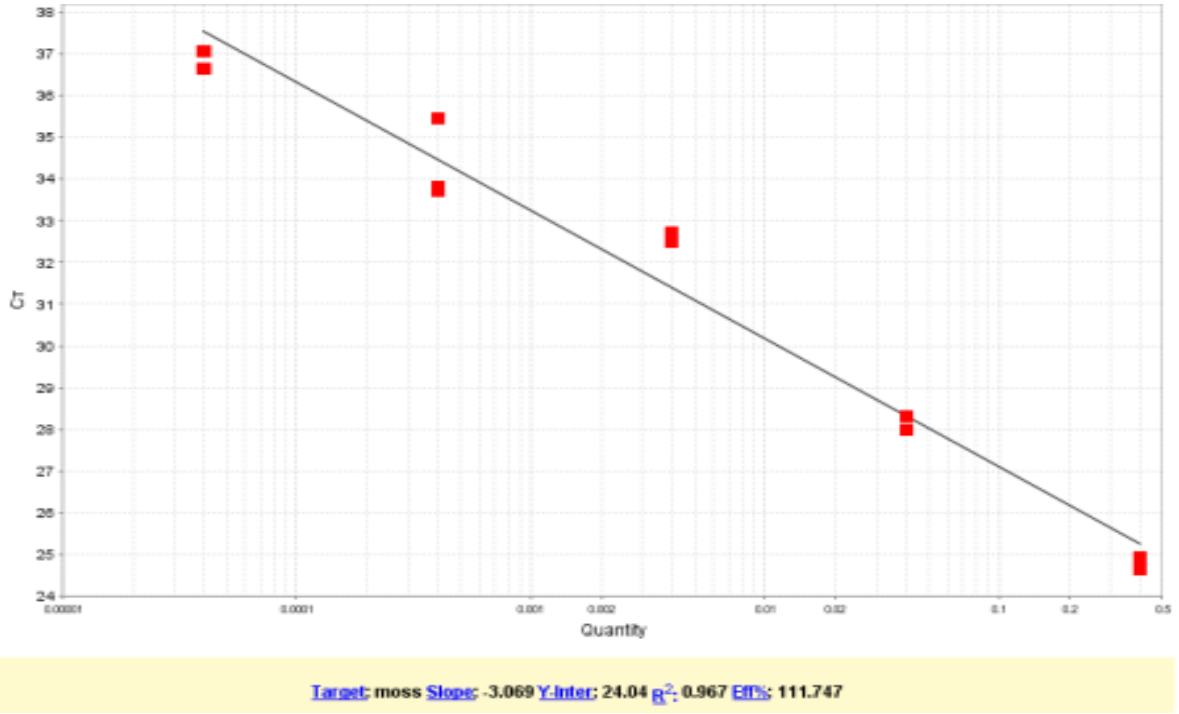



Figure 3.7. Dissociation curve showing possible primer dimer formation of *F. mosseae* specific pp1.

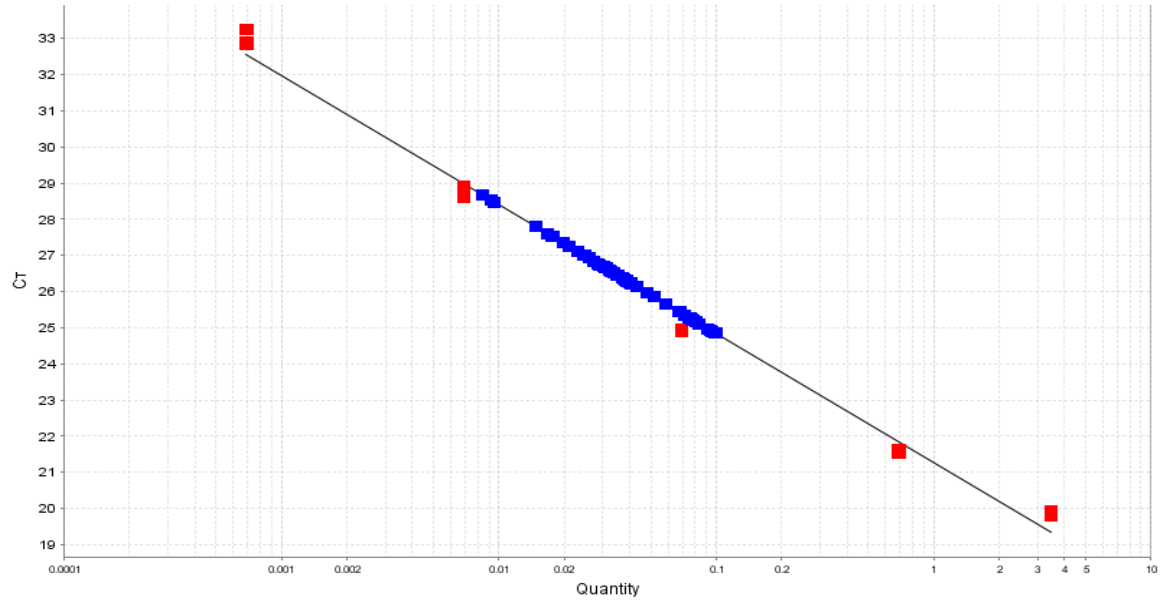
### 3.3.4 Standard curve generation

All specific primers gave a linear relationship of CT values with fungal DNA, see fig.3.8. The *F. mosseae* mossL14 primers gave a linear relationship of CT values with fungal DNA ($l_n$-transformed) in the test sample within the range of 4.0x $10^{-1}$ ng/µl to 4.0x$10^{-5}$ ng/µl fungal DNA. Similarly for the *F. geosporus* geoLp3 primers, this relationship was linear for the fungal DNA ($l_n$-transformed) in the range of 3.45 ng/µl to to 6.9x$10^{-4}$ ng/µl. $R^2$ values were between 0.97-0.98 for mossL14 primers and 0.99 for geoLp3 primers, indicating a high degree of reproducibility. The corresponding slope values of the two linear relationships ranged from -3.7 to -3.1, and from -3.4 to -3.6, respectively, indicating good PCR efficiencies at roughly 90-100% efficient. A similar linear relationship was also observed for strawberry Ef1α DNA (ln-transformed) in the range of 4.6 ng/µl to 4.6x$10^{-4}$ ng/µl with $R^2$ value of 0.99 and slope values ca. 3.5.

a,

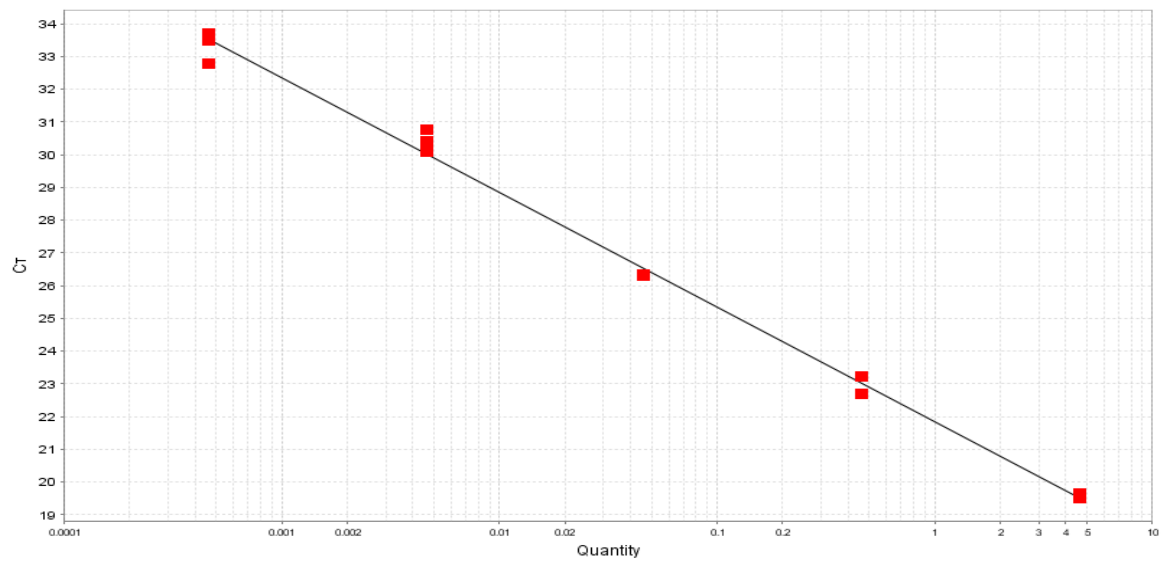


b,




Target: Geo Slope: -3.574 Y-Inter: 21.254 $R^2$: 0.992 Eff%: 90.454

C,




Target: EF1a Slope: -3.504 Y-Inter: 21.818 $R^2$: 0.995 Eff%: 92.928

Figure 3.8. Standard curves obtained by plotting CT value against log genomic DNA concentration showing the slopes from the 5 point serial dilutions of primer pair mossL14 (a), geoLp3 (b) and EF1α (c). Fig 3.8, b shows examples of extracted root samples from the study strawberry roots in the middle of the standard curve.

As part of the selection process for the finally chosen primer, standard curves were generated for all specific primers. Primers for *F. mosseae* that were not chosen gave slopes of pp1, slope 3.95, $R^2$ 0.98 and pp3 slope 3.8 and $R^2$ 0.95 and as such were less efficient than the mossL14. The *F. mosseae* primer pairs used by Alkan *et al.* (2006) showed a low efficiency in this system along with evidence of primer dimers on the dissociation curve and thus were also disregarded as potential candidates. Primers specific for *F. geosporus* all gave good slope values ranging between -3.3 to -3.1 with $R^2$ values of ca. 0.99. The final primer pair geoLp3 gave slightly less background amplification in the no template controls and no evidence of any primer dimers and as such selection was made based on these criteria.

### 3.3.5 Cross amplification analysis-qPCR

Amplification profiles of all of the primer sets gave positive amplification only in the presence of the target taxa, using either spore or root extractions, showing a clean single peak in the dissociation curve and no evidence of primer dimerization. Background amplification was rarely detectable or, even if detected, observed below cycle 35 in the no template controls, and thus disregarded (Bustin and Nolan, 2004). Likewise, this analysis was carried out with the EF1α primer pairs again using uninoculated and inoculated strawberry root extractions, and good qPCR profiles were again obtained. Table 3.2 shows the cycle threshold (CT) values and DNA quantities (as calculated from the standard curve) for both primer sets against target and non-target inoculated roots.

| Primer name | Target | CT | Qty | Mean Qty | StdDev Qty |
|---|---|---|---|---|---|
| **mossL14** | geo root | 37.03 | | | |
| **mossL14** | geo root | 38 | Disregarded above 35 cycles | | |
| **mossL14** | geo root | 37.39 | | | |
| **mossL14** | moss root | 26.5 | 1.07E-01 | 1.08E-01 | 3.28E-03 |
| **mossL14** | moss root | 26.42 | 1.12E-01 | 1.08E-01 | 3.28E-03 |
| **mossL14** | moss root | 26.5 | 1.06E-01 | 1.08E-01 | 3.28E-03 |
| **geoLp3** | geo root | 22.59 | 2.74 | 3.02 | 2.52E-01 |
| **geoLp3** | geo root | 22.37 | 3.23 | 3.02 | 2.52E-01 |
| **geoLp3** | geo root | 22.42 | 3.1 | 3.02 | 2.52E-01 |
| **geoLp3** | moss root | Undetermined | | | |
| **geoLp3** | moss root | 35.13 | Disregarded above 35 cycles | | |
| **geoLp3** | moss root | 39.03 | | | |

Table 3.2. Cross reactivity checking of primers using the qPCR system. Strawberry roots colonised with either *F. mosseae* (moss root) or *F. geosporus* (geo root) were amplified using both chosen specific primer pairs and ct value (threshold cycle) and the quantity of DNA recorded (ng). Only background amplification was recorded for both primers for non-specific amplification (seen at the same level as the NTC).

To determine that the fragments amplified from these 2 chosen primers were actually originating from the target species, amplified products were cloned and sequenced. Amplified standard PCR products from DNA extractions taken from inoculated strawberry roots using

each primer pair were cloned using a Topo TA cloning kit (Life Technologies, UK) following the manufacturer's recommendations, and sequenced (GATC biotech, Constance, Germany). DNA sequences were assessed for quality using Chromas version 1.42 (Griffith University, Australia), trimmed and compared with sequences deposited in GenBank using BLAST analysis (www.ncbi.nlm.nih.gov/**BLAST)**. BLAST analysis of returned sequences confirms that primer pair moss Cl14/13 amplified a sequence showing alignment to other sequences of *Glomus mosseae* LSU rRNA in Gen Bank, and that primer pair Geo1Fw and geo3rev amplified sequences that align with those of *Glomus geosporum* LSU rRNA.

### 3.3.6 Experimental assessment in roots using the qPCR system.

The validated qPCR technique was used successfully for the assessment of relative fungal DNA of both species. Amplification of the fungal DNA was achieved using the newly designed primer pairs, as above, and amplification of the strawberry root using EF1α primer pairs was recorded for each sample. Quantitative PCR parameters used were as stated above. Threshold cycle (CT) values for the fungal DNA and the strawberry root DNA were recorded for each sample and the amounts of fungal and strawberry DNA were estimated from the respective calibration curves. The final AMF value for each sample was expressed as the ratio between the estimated amounts of fungal DNA (ng/µl) and strawberry DNA (ng/µl).

Root samples from the experimental plants were extracted using the methods described above for the strawberry root extraction. DNA concentrations from these sample plants generally plotted in the middle of the standard curve for both primer pairs (as seen in Fig. 3.8, b for *F. geosporus*). Any sample that fell at either end of the range of standards or did not give a strong amplification was re-extracted from the root sample, and re-assessed.

## 3.4 Discussion

The newly-designed primer pairs, mossL14fw/rev and geoLp3fw/geoLp3rev, amplified DNA from their respective *F. mosseae* and *F. geosporus* targets. Rigorous cross-amplification evaluations showed that these two primer pairs were species-specific, resulting in amplification of target DNA in a mixed background and against plant genomic DNA. When designing these primers, careful consideration was given to sequence heterogeneity within taxa, ensuring good amplification from all sequence types from the target species (Gamper *et al.*, 2010; Thonar *et al.*, 2012). The same cross-amplification validation was applied using the qPCR methodology. Final primer sets were chosen based on initial qPCR validation that showed more efficient slopes combined with no evidence of primer dimer formation or background amplification.

Primers mossL14 and geoLp3 were designed to not only be specific for use in this system but are also specific against *G. microaggregatum, R. irregularis* and *C. claroidum* (tested by alignment in GenBank only as spore production in this culture was low). In future work these primers may be of use to further study interaction with these other species, however further cross amplification testing would be required if a qPCR system was required.

# Chapter 4. Effect of inoculation of drought-stressed strawberry with a mixed inoculum of arbuscular mycorrhizal fungi: qPCR studies of population dynamics of inoculum in roots

## 4.1 Introduction

Arbuscular mycorrhizal fungi form arbuscules within the roots of plants that allow the transfer of materials between the fungus and the plant (Smith and Read, 1997). Mineral nutrients and water are extracted from the soil via the extensive hyphal network and transferred to the plant improving plant health and growth (Fitter *et al.*, 2011; Ngwene *et al.*, 2013; Ruzicka *et al.*, 2012; Smith *et al.*, 2010). Organic carbon compounds are transferred to the AMF in return. In addition, AMF protect plants from pathogens (Borowicz, 2001; Ismail and Hijri, 2012; Ren *et al.*, 2013) and buffer against adverse environmental conditions, especially drought (Smith *et al.*, 2010). With changes in the world climate, water limitation is becoming an increasing concern for global crop productivity. As stated earlier plants under water stress apparently benefit from the mycorrhizal symbiosis through the improvement of water status and uptake. However, Auge (2001) reviewed a large number of studies and noted conflicting results when root colonisation was studied in response to drought treatment. Thus, drought alleviation is not always a consequence of mycorrhizal colonisation, and mycorrhizal colonisation may affect the plant tolerance to drought stress in different ways.

The co-occupation of a single root fragment by AMF from different genera or species is poorly understood (Alkan *et al.*, 2006; Krak *et al.*, 2012). It is unclear if the relationship between co-occurring AMF species is independent, competitive, synergistic or antagonistic (Wagg *et al.*, 2011a); indeed their relationship may vary with plant hosts and/or environmental conditions. It has been suggested recently that individual species occurring within the same root systems may employ different mechanisms or benefits to the plant thus to avoid inter-species

competition (Jansa *et al.*, 2008; Kiers *et al.*, 2011). Kiers et al. (2011) show how individual AM species could employ different strategies in providing resource allocation and benefit to a host, when multiple occupants are sharing an individual root system. Real time or quantitative PCR (qPCR) methodology has now been applied to AMF studies and initial results have led a new phase to quantify changes in abundance of specific individual taxa within roots (Gamper *et al.*, 2008; Jansa *et al.*, 2008; König *et al.*, 2010; Pivato *et al.*, 2007; Santos *et al.*, 2006; Thonar *et al.*, 2012; Wagg *et al.*, 2011a).

These recent studies have yielded conflicting results with respect to how well the root colonisation data from microscopic observations are correlated with the qPCR data. Some authors (Alkan *et al.*, 2004; 2006; Isayenkov *et al.*, 2004; Wagg *et al.*, 2011a) reported a positive correlation, however, Jansa *et al.* (2008) and Pivato *et al.* (2007) showed that there was no significant correlation. Gamper *et al.* (2008) found there to be a strong correlation between qPCR signal and spore numbers, however not between vital hyphal length and qPCR. The reason for the disparity in correlation of data from PCR-based methods and root colonisation assay remains unclear, and may include uneven fungal distribution within roots (Gamper *et al.*, 2008) and a variation in the number of nuclei in the fungal hyphae during development, or the number of rDNA sequences per nucleus (Pivato *et al.* (2007). For the first time, Shi *et al*. (2012) compared microscopic colonisation data directly to nested PCR data at individual plant level, and found that the two methods did not consistently correspond.

The qPCR technique is a valuable tool to give new insight into AMF and host plant interactions (Alkan *et al.*, 2006) and provides a rapid, specific and quantitative method requiring a minimal amount of plant and fungal material (Gamper *et al.*, 2008; Isayenkov *et al.*, 2004). The technique can be used to provide information about the beneficial role of different AMF within agroecosystems, for example in alleviating symptoms of drought stress. The strawberry

industry is one of the UK's major horticultural crops and drought stress is a significant factor determining productivity. Strawberry plants are highly responsive to AMF colonisation (Borkowska, 2002; Castellanos-Morales *et al.*, 2010), but no studies have attempted to understand the relative role of different AMF within the same root system in conferring the beneficial effect to strawberry plants.

The aim of this study was to develop a specific quantitative molecular tool (qPCR) to observe relative abundance of 2 co-occurring species of AMF, *Funneliformis geosporus* and *F. mosseae*, and then use the tool to study the effect of relative abundance of the two species on strawberry plants (*Fragaria x ananassa).* This chapter concerns (i) the development of a qPCR technique, (ii) the correlation between data obtained from microscopic root colonisation assay and the new qPCR methods, and (iii) the effect of water stress on AMF colonisation and relative abundance of the two AMF species. As only a single isolate was used from each of the two AMF species, the observed interaction could be equally described as between two isolates or between two species. For consistency, the results are described in terms of between-species interactions.

## 4.2 Materials and Methods

### 4.2.1 Fungal inoculum

Pure cultures of two AMF species (*F. mosseae* BEG 25, *F.geosporus* BEG 11) were initially obtained as inoculum from Plantworks UK, Sittingbourne, Kent, UK (www.rootgrow.co.uk) and cultured as described in Chapter 2, section 2.1.2. After three months, a most probable number (MPN) test (Cochran, 1950), as described in section 2.3.4, was performed to quantify the infectivity of the cultures, thus ensuring an equivalent colonising potential of the two species for experimentation. For the first experiment (Experiment 1) the MPN value for *F.*

*mosseae* was estimated to be 920,000 propagules/litre and *F. geosporus* 540,000 propagules/litre. The difference in these two estimates was within one order of magnitude. Given the inherent uncertainty of the MPN test, these two cultures were assumed to be equivalent in their colonising potential. Similarly, in Experiments 2 and 3 MPN values for both species were similar (> 1.6 million propagules/litre) and the colonising potentials were thus assumed to be equal.

### 4.2.2 Interaction of co-inoculation or single-species inoculum in relation to water stress

In total there were four AMF inoculation treatments: inoculation with the two individual species alone, mixed inoculation with both species, and an un-inoculated control. In the mixed inoculation treatment, both species were added at a rate of 50% of the single treatment. In addition to these four AMF treatments, water stress was the other factor studied. Two levels of irrigation were used: fully watered [WW] and water-stressed [Regulated Deficit Irrigation-RDI]. Thus, there were eight treatments covering the two experimental factors. Three repeat experiments (Experiments 1,2, and 3) were completed over time to study the effect of single and mixed inoculations on strawberry under WW and RDI irrigation regimes.

#### 4.2.2.1 Plant and AM inoculation

For Experiments 1 & 2, micro-propagated strawberry plants of cv. Everest (Hargreaves Plants, UK) were used ensure the absence of pre-existing mycorrhizal colonisation. Plants were established in rooting agar media and incubated at 20$^{o}$C for 2 weeks with overhead lighting until the roots had developed sufficiently to be transplanted into Vermiculite in a propagation chamber. These plants were weaned for 4 weeks at 20$^{o}$C with overhead lighting, with increasing ventilation to the chamber. Experiment 3 was established using the runner plants of cv. Elsanta to ensure that no exceptional results were obtained from the use of micro-propagated plants (Szczygiel *et al.*, 2002), and to aid plant survivability and increase fruit

production (which is lower in micropropagated plants). Runners of pre-established plants were pinned down into Terragreen (Agsorb, Oil-dry Ltd, Wisbech, UK) and grown with the mother plants until a root system had been established before being separated from mother plants. For each experiment, eighty 1-litre pots were filled with Terragreen with 20 pots per AMF treatment; each pot received a layer (10% of the total volume) of each of the following four treatments: *F. mosseae* BEG 25 inoculum; *F. geosporus* BEG11 inoculum; a mixture of the two species (50% total volume for each species) or Terragreen alone (uninoculated control). A single, young strawberry plant, in experiments 1 & 2 this was a micro-propagated plant, whereas in experiment 3 it was a young runner plant, was placed into each pot.

#### 4.2.2.2 Irrigation

The 80 strawberry plants were grown in fully-watered conditions for approx. 2 weeks in a GroDome compartment (Unigro, UK) set at 22$^{o}$C day/20$^{o}$C night with a 14 h day/10 h night cycle with supplementary lighting. When the plants were sufficiently established, water-stress (RDI) was applied to 40 of the 80 plants. Plants were placed in a lattice 4x4 block design with the four AMF treatments along the four irrigation lines of 13 mm wide LDPE pipe (City Irrigation Ltd, Bromley, UK). Two of the irrigation lines were randomly assigned to the WW treatment (plants receiving 100% of the water estimated to have been lost via evapotranspiration (ETp)) and the RDI treatment (plants receiving 70% of water for the WW treatment in Experiment 1 and 60% in Experiment 2 & 3). RDI conditions were reduced further in the experiments 2 and 3 to give an increased stress to the drought plants (RDI plants in Experiment 1 were not showing extensive signs of drought stress). Plants were irrigated via 2 L $h^{-1}$ Netafim drippers (City Irrigation Ltd, Bromley) linked to the irrigation lines; the drippers were of sufficient length to allow pots placed in a required position within a block. There were five replicate blocks of this 4x4 lattice design within each experiment.

In each experiment, four plants from each WW treatment (i.e. total 8 plants) were randomly selected (with the constraint that these plants were well dispersed within in the whole experimental plot) to estimate weight losses over a 24h period. ETp values were measured three times per week *in situ* using an ET sensor (Evaposensor, Skye Instruments Ltd., Powys, UK), and irrigation times for WW and RDI treatments were then adjusted from the average weight losses and ETp values (Grant *et al.*, 2009). Little adjustment was required due to the relatively stable conditions within the GroDome.

Plants were grown under these conditions for a further 10 weeks before harvesting. Fertigation was carried out using Vitafeed 102, N-P-K 18-0-36 (Vitax, Leicester, UK) at a rate of 1g $l^{-1}$ with approx. 25 ml added to the top of every pot, regardless of treatment regime, once every 2 weeks. Plant death occurred in some experiments, due to failure of initial plant establishment. At the end of the study Experiment 1 had 80 plants, Experiment 2 had only 52. In Experiment 3, all 80 plants survived to the end of the experiment but only 40 plants were used for studying the relationship between qPCR data and microscopic root colonisation; the other 40 plants were used for further plant growth/survival studies (Chapter 5).

#### 4.2.2.3 Root sample collection

Plants were harvested from each pot and the substrate carefully washed from the root mass. A sub-sample (ca. 2 g) of roots was retained for microscopic analysis and cleared with KOH before being stained using Trypan Blue. They were examined for AMF colonisation using the grid-line intersect method (McGonigle *et al.*, 1990); colonisation was expressed as percentage of the root colonised by AMF. A larger sample of washed root (ca. 10 g) washed in deionised water, cut into ca. 1 cm sections, placed into a sealed bag and stored at -80$^{o}$C until use for the qPCR analysis.

### 4.2.3 qPCR analysis

#### 4.2.3.1 Primer design and validation of qPCR

The plant DNeasy mini Kit (Qiagen, Hielden, Germany) was used to extract pure genomic DNA from AMF spores, and from strawberry roots with or without AMF, following the manufacturer's protocol (section 2.6.1). Species-specific primers were designed within the D2 region of the LSU of the rRNA gene, against *F. mosseae* BEG 25 and *F. geosporus* BEG 11, as described in section 3.2.1. The degree of resolution of the LSU is regarded to be high and was used to differentiate between closely related AMF species. A total of 37 sequences were used to compare sequence variation within and between taxa for primer design. Primers were designed using Primer-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and synthesised from Sigma-Aldrich (UK). A number of primer pairs were initially designed (at least 12 per species) and tested for their specificity by alignment with sequences from GenBank using BLAST (http://blast.ncbi.nlm.nih.gov) and Jalview (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Three other pure AMF isolates (*R. intraradices* BEG 72, *G. microaggregatum* BEG 56, and *C. claroideum* PW5) were included for evaluating primer specificity, as described in section 3.2.2.

Primer pairs that were found to amplify target genes were further tested with Oligo Analyser (http://eu.idtdna.com/analyzer/applications/oligoanalyzer/) for the presence of dimerisation and secondary structure formation. The chosen specific primers were then tested with the qPCR system to establish a calibration curve, as described in section 3.2.3. Both primer pairs were tested for specificity within the qPCR system against target and non-target samples of DNA extractions from strawberry root samples inoculated with either *F. mosseae, F. geosporus* or control, untreated samples and no significant amplification profiles were observed.

#### 4.2.3.2 Quantitative PCR protocols

A qPCR protocol was established for use with roots colonised with the two AMF species see section 3.2.3. Strawberry Ef1α (Elongation factor 1 α, an endogenous gene) was used as a normaliser in the qPCR, and its amplification in the strawberry root was achieved using the previously published primer pairs: FaEF1 - 5'-TGC TGT TGG AGT CAT CAA GAA TG-3' and FaEF1R - 5'-TTG GCT GCA GAC TTG GTC AC-3' (Carbone *et al.*, 2006).

The quantities of target sample DNA were compared against standard dilutions of spectrophotometrically quantified genomic DNA, extracted from 200 spores of *F. mosseae* BEG 25 and *F. geosporus* BEG 11 and against non- inoculated strawberry root DNA (section 3.2.3). A new standard curve was run on each from these stored pre-quantified aliquots in order to eliminate between run disparities. Threshold cycle (CT) values for the fungal DNA and the strawberry root DNA were recorded for each sample. The amounts of fungal and strawberry DNA were estimated from the respective calibration curves, and the final AMF value for each sample was expressed as the ratio between the estimated amounts of fungal DNA (ng/µl) and strawberry DNA (ng/µl).

### 4.2.4 Statistical analysis

Statistical analysis was carried out using GenStat 13 (VSN International, England). The results presented here are pooled from the three repeat experiments, in order to provide statistical robustness. A full REML analysis was used to assess how root colonisation is affected by AMF treatments (single vs mixed inoculations) and irrigation regimes. In the REML analysis, row, column or block effects originating from the experimental design and the 3 repeated experiments over time were treated as random factors. Initial REML analysis showed that there was no evidence of any row, or column, effects within a block in any of the analyses, or differences between the replicate water levels, so a simplified analysis was carried out on the

data adjusted for the difference for repeated experiments over time and blocks within each experiment (treating them as fixed effects).

The effect on microscopic root colonisation was assessed using a factorial treatment structure [Inoculation {mixed vs single (geo vs moss)} x (WW vs RDI)]. The effect on the qPCR data of AMF DNA (as the measure of fungal abundance) was also assessed using a factorial treatment structure {Species (geo vs moss) x (WW vs RDI) x (single vs mixed)}. qPCR data were ln-transformed to reduce variance heterogeneity. Significances of treatment effects were assessed using F-tests.

To compare the qPCR data with the microscopic colonisation assessment, simple regression analysis was performed with the irrigation included as a factor. Microscopic root colonisation data were related to qPCR data on the basis of individual plants in order to assess whether these two estimations of mycorrhizal abundance within a root system were correlated on an individual plant basis.

## 4.3 Results

### 4.3.1 Primer optimisation

Primers were designed for *F. mosseae* BEG 25 AF304982 and *F. geosporus* BEG 11 AF305004, although a selection of heterogenic sequences from each species was used during alignment to ensure good amplification. The *F. mosseae* primers (mossL14fw-

TACCAACGGGAAATCAACCT with mossL14rev- AGATCCCAATCTCGCGAAC) and *F. geosporus* primers (geoLp3fw- CGTGAAATTGTTGAAAGGGAA with geoLp3rv- CTCCTCGAAACCCACAAGAA) were designed for the D2 region of the LSU rRNA. Alignment with previously published sequences from GenBank and BLAST analysis confirmed that the chosen primer pairs showed good specificity to either the *F. mosseae* or the *F. geosporus* respectively and no alignment to sequences from any of the other three AMF species tested. Both primer pairs only amplified the relevant species when tested against DNA extractions from strawberry roots inoculated with either *F. mosseae or F. geosporus* or non-target AMF taxa, using both tradition PCR and qPCR methods (sections 3.3.2 and 3.3.5).

### 4.3.2 Microscopic results and effects of drought

A good level of colonisation was observed microscopically in all AMF-inoculated treatments, with colonisation levels ranging between 55 and 70%, and no mycorrhizal colonisation detected in the control plants (fig. 4.2). A difference in colonisation level was apparent between WW and RDI treatments with colonisation being significantly lower ($P< 0.001$) for the WW treatment than for the RDI treatment. There was an overall difference *(*$P<0.001$) among AMF treatments: *F. mosseae* showed a reduced colonisation compared to both the *F. geosporus* alone or the mixed inoculum treatments, this was however, only due to a reduction of *F. mosseae* in one out of the three replicate experiments. There was no significant interaction between the AMF treatment and the irrigation regime on the colonisation of strawberry roots by AMF.

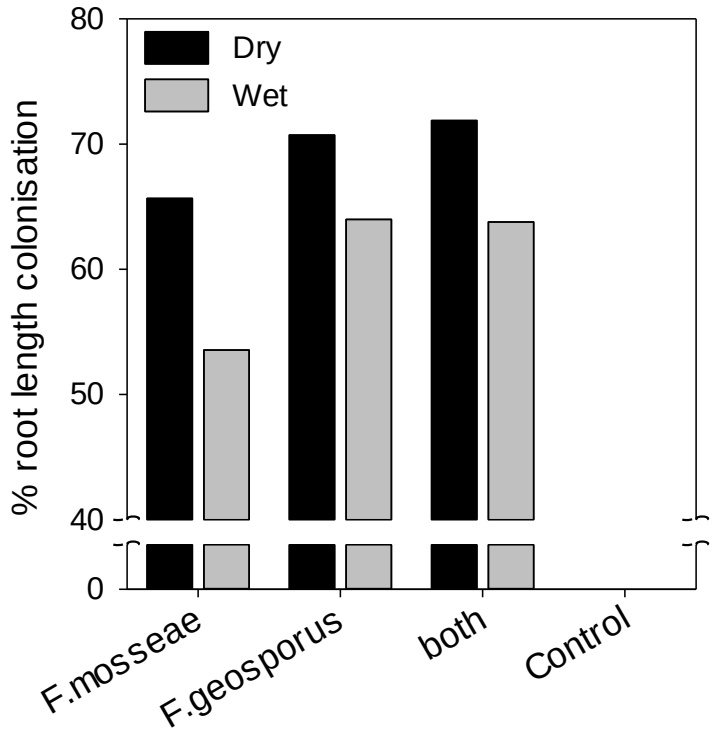



Figure 4.2. Predicted means of root length colonisation for each inoculum treatment and at wet and dry conditions. SED = 2.239 (av), 2.259 (max) and 2.207 (min).

### 4.3.3 Quantitative PCR

#### 4.3.3.1 Standard curve generation

Both specific primers gave a linear relationship of CT values with fungal DNA (ln-transformed) in test samples with good PCR efficiencies, see section 3.3.4. The qPCR analysis was used to test both fungal specific primers against spore DNA extractions from the pure pot cultures and inoculated roots. Amplification profiles of all of the primer sets gave positive amplification only in the presence of the target taxa, showing a clean single peak in the dissociation curve and no evidence of primer dimerization.

#### 4.3.3.2 Co-inoculation or single-species inoculation in relation to water stress

Results of the three replicate experiments were combined here to give robust statistical analysis. Although variation did occur between the experiments similar trends were observed and these differences were taken into account in the overall statistical analysis by treating replicate experiment as a random factor. The qPCR results show an effect of the WW and the RDI treatments; however, this effect varied between replicate experiments and was dependent on both the water regime and AMF species inoculated. Generally, as single-species inoculation, samples inoculated with *F. geosporus* gave a higher amount of fungal DNA than samples from plants inoculated with *F. mosseae*. In all experiments with mixed inoculum, qPCR results demonstrated that *F. mosseae* and *F. geosporus* co-existed within in a single root system, with both species being represented in reasonable proportions, i.e. neither species excluded the other to any significant degree (Fig.4.3). Amplified samples inoculated with both species fell approximately in the middle of the standard calibration curve.

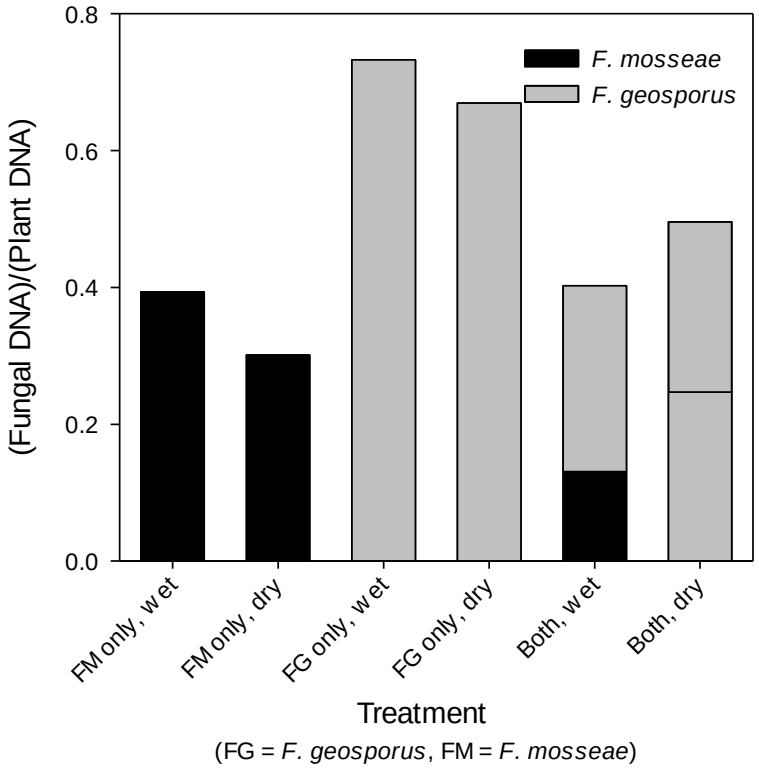



Figure 4.3. Average fungal DNA (as quantified by the qPCR method), expressed as fungal DNA (ng)/plant DNA (ng) for the three AMF inoculation treatments, *F. mosseae* BEG25 alone, *F.geosporus* BEG11 alone, or both the species co-inoculated at a rate of 50% each of the single treatment, at the well watered (WW) and dry (Regulated Deficit Irrigation, RDI) conditions (SED = 0.35).

There was an overall difference in qPCR results between the WW and RDI treatments, with a smaller amount of DNA recorded in the RDI treatment compared to the WW treatment. There was a significant interaction *(P*=0.033) between irrigation and inoculation treatment which showed a decrease of *F. geosporus* DNA in the RDI treatment but an increase of *F. mosseae* compared to the WW treatment. In addition, the interaction between AMF species, single vs mixed treatment, and the irrigation treatment was almost statistically significant *(P*=0.08). Either the same or a reduced amount of DNA was observed in the RDI treatment compared

to the WW treatment in all conditions with the exception of *F. mosseae* in the mixed inoculation where its DNA increased in the drought treatment (Fig.4. 4).

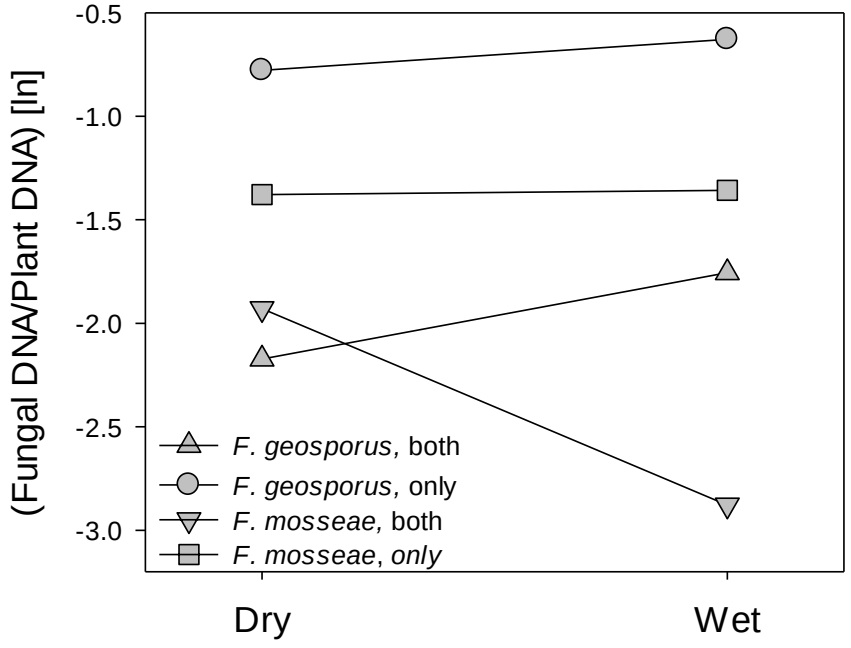



Figure 4.4. Mean ln (fungal ng/plant ng DNA) of the AMF species with the irrigation regime and single vs mixed AMF inoculation (s.e.d.: max = 0.351, min = 0.341 and average =0.348.

There was a significant difference between the single-species inoculation vs mixed species inoculation *(P*<0.001) in both *F. mosseae* and *F.geosporus* treatments. In the mixed species inoculation, both species were added at a rate of 50% of the single treatment so that a 50% reduction in the amount of either species would be expected in the mixed species inoculation. Overall the reduction in the amount of fungal DNA in the mixed species inoculation was more than 50%. The observed amount of *F. geosporus* DNA in the mixed inoculation treatment was less than 50% of that in the single inoculation, although not significantly so, for both WW and RDI treatments. The observed DNA for the *F. mosseae* in the mixed inoculation in the RDI

treatment was slightly above the 50%, but for the WW condition it was significantly lower than the 50% (Fig. 4.5).

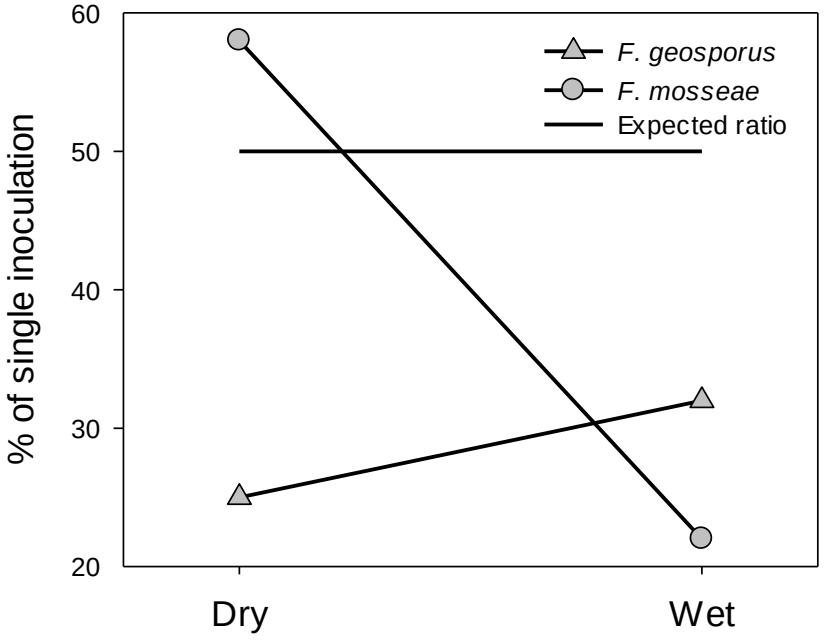



Figure 4.5. The level of *F. mosseae* and *F. geosporus* DNA when inoculated with both species as a percentage of the single-species inoculation under the two irrigation regimes (WW & RDI). The theoretical percentage is 50% (Standard error of mean in percent = 0.35)

### 4.3.4 Comparison of microscopic colonisation with qPCR data

The overall trends from the qPCR results do not agree with the overall trends from the microscopic colonisation data. In all treatments, the % root length colonisation as determined microscopically was higher in the RDI-treated plants, yet the qPCR data suggest that lower amounts of DNA were recovered from the RDI-treated plants.

Regression analysis, however, suggested a significant relationship between root colonisation and qPCR data but this relationship varied greatly with AMF species, and between single and mixed inoculations (Fig. 4.6). A strong positive correlation between the two types of data was observed when *F. geosporus* was assessed in the single-species inoculation (Fig. 4.6a). On the contrary, for *F. mosseae* there was a very weak negative relationship between the two types of data in the single-species inoculation (Fig. 4.6b). For two-species inoculation, the amount of *F. geosporus* DNA appeared to correlate negatively with the degree of root colonisation (Fig. 4.6c). On the other hand, the amount of *F. mosseae* DNA was positively correlated with the root colonisation (Fig. 4.6d). In the mixed inoculation, the ratio of *F. mosseae* DNA with *F. geosporus* DNA was positively related to the extent of root colonisation (Fig. 4.7). Overall the relationship between root colonisation and qPCR data is a positive one, when individual plants are plotted.

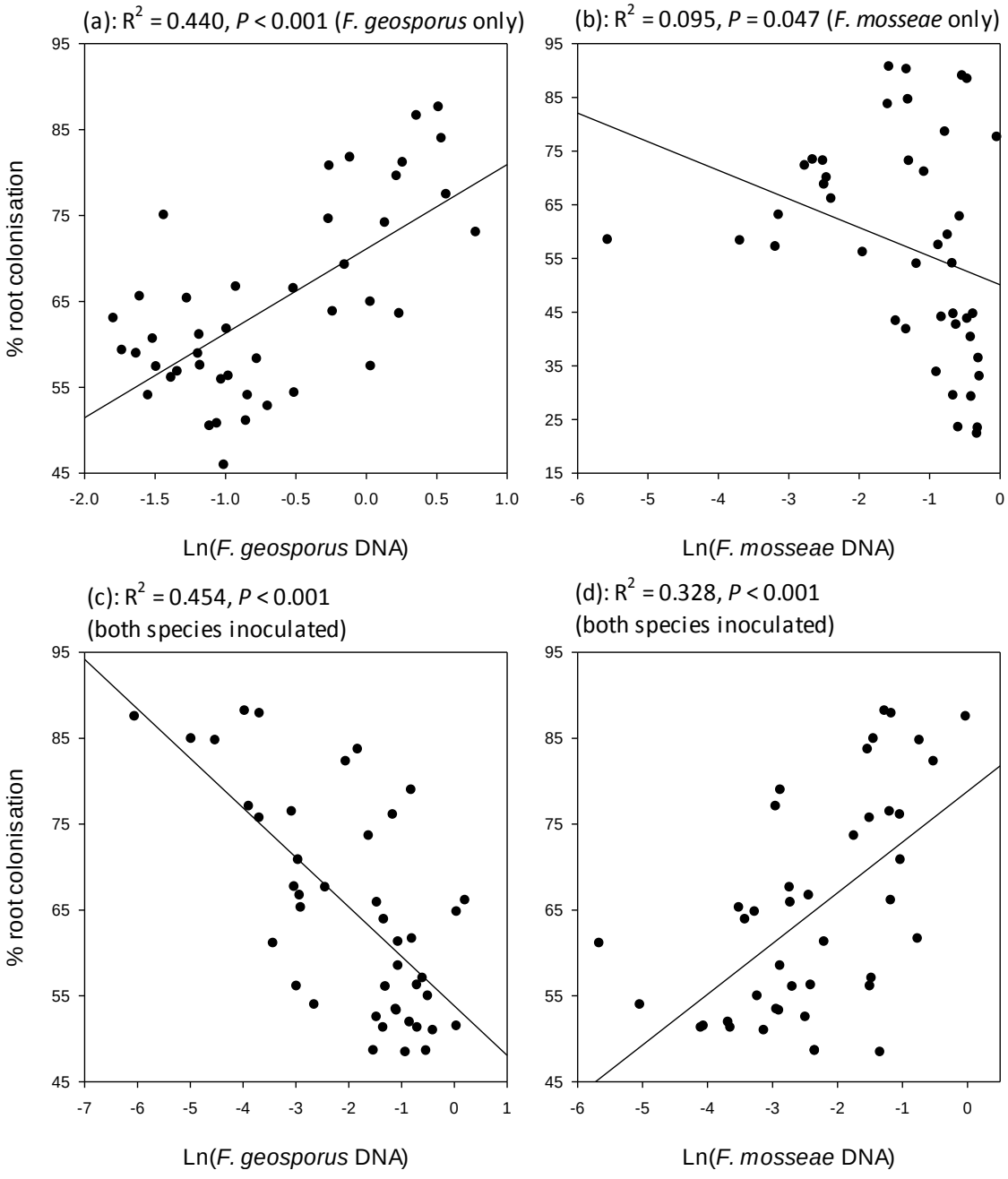



Figure 4.6. Percentage root colonisation by AMF plotted against the qPCR-quantified fungal DNA data for each individual AMF species in either single-species or two-species inoculation. The solid lines are fitted regression models describing their relationships.

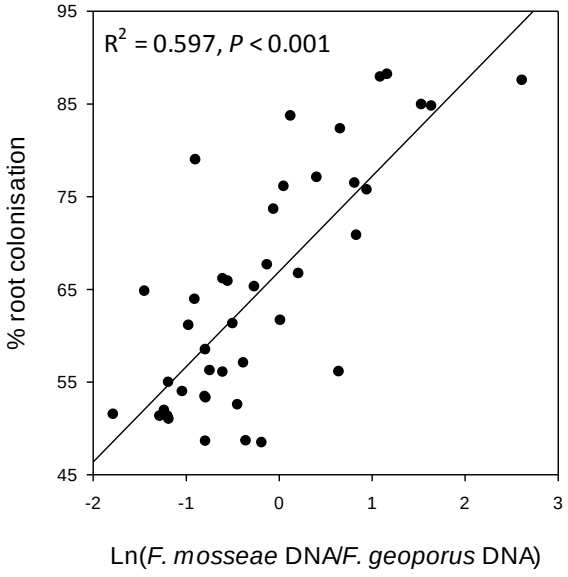



Figure 4.7. Percentage root colonisation plotted against the ln-transformed ratio of quantified fungal DNA (*F. mosseae/F. geosporus*) for the mixed inoculation treatment.

## 4.4 Discussion

### 4.4.1 qPCR methods

The species specific primers and qPCR protocol described in Chapter 3 were successfully applied to the present experiments to study interactions between two specific AMF species. The newly-designed primer pairs, mossL14fw/rev and GeoLp3fw/GeoLp3rev, amplified DNA from their respective *F. mosseae* and *F. geosporus* targets, resulting in amplification of target DNA in a mixed background and against plant genomic DNA. The amount of fungal DNA varied considerably across the three repeated experiments. This may reflect small temporal differences in conditions or in host plants that affect AMF colonisation, composition and structure (Dumbrell *et al.*, 2011). Generally *F. geosporus* gave a greater amplification signal than the *F. mosseae*, despite the fact that root colonisation data for the two species were similar. This might be due to differences in the copy numbers of the rRNA genes in the two

isolates or may reflect other differences in physiology. For example, hyphal abundance, and vesicle and arbuscule frequency may vary considerably between species even though total root length colonised might be similar (Gamper *et al.*, 2008). As a consequence, the amount of DNA present per unit root length might be a better indication of functional cytoplasm than the more traditional measure of % root length colonised. The initial colonising potential was estimated by the MPN test. Although this test is regarded as the best indication for inoculum efficacy, it is only a rough estimation.

### 4.4.2 Compatibility between species

Until recently the relationship between individual AMF species within a single root system was unclear, and there was a debate as to whether two species could share the same niche. This study demonstrates the coexistence of *F. mosseae* and *F. geosporus* within a single root system, consistent with other recent findings (Alkan *et al.*, 2006; Janoušková *et al.*, 2009; Jansa *et al.*, 2008; Kiers *et al.*, 2011; Reddy *et al.*, 2005; Van Tuinen *et al.*, 1998; Wagg *et al.*, 2011a). Of course, it is possible that other AMF strains or species may interact differently.

Overall, the colonising potential of the two species was similar when inoculated onto strawberry alone. Consistent strong amplification was recorded for both species in the mixed inoculation treatment and neither species was completely dominant, although overall qPCR amplification was reduced in the mixed inoculum treatment. This would suggest complementarity between these 2 species as reported previously (Janoušková *et al.*, 2009; Jansa *et al.*, 2008) for other AMF species. It has been suggested recently that individual species occurring within the same root systems may employ different mechanisms or benefits to the plant thus to avoid inter-species competition (Jansa *et al.*, 2008; Kiers *et al.*, 2011).

Although co-colonisation by both species did occur, there was some indication of competition between them. Overall the observed *F. geosporus* DNA abundance in the mixed inoculation was significantly less than expected in the both irrigation regimes, suggesting reduced colonising potential of *F. geosporus* in mixed inoculation. However, the extent of competition varied with experimental conditions and was not as strong as previously reported (Jansa *et al.*, 2008; Krak *et al.*, 2012). On the other hand, in the mixed inoculation the amount of *F. mosseae* DNA appeared to be less than expected only under the WW condition. This result agrees with previous studies in that the fungal abundance was frequently less in plants that received mixed AMF inoculum than their respective mono-inoculated counterparts (Jansa *et al.*, 2008; Krak *et al.*, 2012; Wagg *et al.*, 2011b).

### 4.4.3 The effect of drought on species abundance

Root colonisation by AMF in response to drought stress has been examined previously by a number of authors, primarily via traditional studies of root colonisation. There are no quantitative data showing differential effects on mixed populations of AMF within roots. Auge (2001) reviewed many years of research surrounding the relationships between AMF and plants in water-stressed environments. Drought only affected levels of root colonisation in about half of the published studies, and in these instances the level of root colonisation was increased rather than decreased, as was observed in strawberry roots here.

Present results showed a good level of mycorrhizal colonisation when examined microscopically, similar to levels of 55-70% root length colonised, as observed in other studies (Kiers *et al.*, 2011; Thonar *et al.*, 2012). All roots sampled from the water-stressed plants had a higher colonisation by AMF, suggesting that under water-stressed conditions plants become more dependent on the fungal symbiosis, which could be partly explained by the increased water use efficiency of AMF-colonised plants (Omirou *et al.*, 2013). However, it should be

noted that there was considerable variability in levels of colonisation under water-stressed conditions within replicate experiments, a phenomenon often reported previously (Auge, 2001).

In contrast to the microscopic observations, qPCR abundance data indicated a decreased level of *F. geosporus* in plants inoculated with this species with or without co-inoculation with *F. mosseae* under water stress. In contrast, *F. mosseae* had a higher abundance under water stress, primarily due to the increased DNA signal in the mixed inoculation under water stress. Thus, *F. mosseae* appears to become the more dominant species under water stress. Jansa *et al.* (2008) also found an isolate of *F. mosseae* to dominate, possibly due its rapid ability to develop hyphae and its increased colonisation rate. Wagg *et al.* (2011a) found that the dominance of *F. mosseae* was very much dependent on the host plant and soil conditions in a grass cover ecosystem.

In the treatments inoculated with a single AMF, water stress alone does not significantly influence fungal abundance of either species within inoculated roots, suggesting that it is the combination of co-occupying the same niche and water stress that leads to an increase of *F. mosseae* abundance within the root system. Thus, *F. mosseae* is likely to be less sensitive to water stress than *F.geosporus*. Omirou *et al*. (2013) also showed that water stress can lead to a reduction in native AMF diversity. Another possible explanation is that host plant preference for particular AMF species may vary with conditions and this might be regulated by differential supplies of host carbon to the 'preferred' species. This positive behaviour of the addition of a co-colonising isolate leading to the increase in activity of another has previously been reported (Alkan *et al.*, 2006; Hart *et al.*, 2013).

It is becoming increasingly evident that abiotic conditions are, to a large extent, responsible for the observed changes in relative abundance of AMF species within a root system. These include soil conditions (Hazard *et al.*, 2013; Wagg *et al.*, 2011a), phosphorus and saline stress (Alkan *et al.*, 2006), and temporal changes such as temperature and sunshine hours (Dumbrell *et al.*, 2011). Thus it is possible that even small changes to abiotic factors could cause a shift in the relative abundance of the two species in the present study, which may explain the observed variability in relative abundance in the three repeated experiments.

### 4.4.4 Comparison of the two quantification techniques

This study demonstrated a discrepancy between the microscopic method used to quantify the AMF within root systems and the new qPCR method, confirming previous studies (Jansa *et al.*, 2008; Shi *et al.*, 2012). This was particularly pronounced for plants subjected to water stress and highly dependent on the species present in the root. Under water stress conditions, microscopic quantification showed increased colonisation of roots, whereas qPCR analysis showed a relative decrease in *F. geosporus* DNA and an increase of *F. mosseae* DNA in the co-inoculated plants. A study showed that drought reduced the abundance of arbuscules and vesicles in strawberry roots (Borowicz, 2010); thus total root hyphal colonisation levels may well be high, but cytoplasm content low, leading to reduced qPCR signals.

This is the first study to compare the results from microscopic quantification of root colonisation with qPCR abundance data on the basis of individual plants and to assess how their relationship were influenced by single-species or co-inoculation and overall a positive relationship was shown between the two techniques. The extent of linear correlation varied greatly with AMF species and inoculum composition which may partially explain conflicting results among previous results (Alkan *et al.*, 2004; Hart *et al.*, 2013; Wagg *et al.*, 2011a). There was greater positive correlation between the two methods for *F. geosporus* than for *F.*

*mosseae*. Although it is very difficult to interpret this result it is possible this may be due to the differences in fungal structures between the two species (Alkan *et al.*, 2004; Kiers *et al.*, 2011). Further investigation is required to examine different species with differing vesicle and arbuscle densities within roots to assess the relationship between the two techniques.

It is still unclear how the strength of a qPCR signal relates to the vitality and biomass of any AMF species (Thonar *et al.*, 2012). This inaccuracy is potentially enhanced further by some of the innate issues and interpretation problems of the qPCR technique in general (Bustin and Nolan, 2004) and the small sample sizes used for molecular analysis (Gamper *et al.*, 2008; Shi *et al.*, 2012; Wagg *et al.*, 2011a). The difficulty lies in the fact that the extent and even the direction of this relationship depends on AMF species of interest, other AMF species present in the root system, and experimental conditions. Thus, we recommend that both methods should be used to give a complementary picture of the extent of AMF colonisation.

## 4.5 Conclusion

Using the new species-specific primers designed in Chapter 3, and a qPCR method, as well as the microscopic quantification of root colonisation, we studied how AMF colonisation of strawberry roots is affected by inoculation with one or two species under two irrigation regimes. Co-occupation of the same root by both species was shown to commonly occur but the relative abundance of the two species varied with water stress. Greater root colonisation was observed microscopically under water stress but this increased colonisation is often accompanied with decreased fungal DNA in the root. There was a significant relationship in the fungal colonisation measurements from the microscopic and qPCR methods under some conditions but the nature of this relationship varied greatly with AMF inoculum and abiotic conditions.

# Chapter 5. Inoculation of drought-stressed strawberry with a mixed inoculum of arbuscular mycorrhizal fungi: effects on plant tolerance to drought stress

## 5.1 Introduction

Colonisation by AMF conveys distinct competitive advantages to plants as the extraradical mycelium of the fungus allows the plant to access mineral nutrients and water way beyond the root system. Infertile soils are an increasing concern to farmers and plant producers globally. Low nutrient availability and reduced biological diversity are generally the cause, while current soil management practices in intensive agriculture commonly result in increased inputs of fertilisers and pesticides, which may further reduce microbial diversity in soil (Oehl *et al*., 2004; Verbruggen *et al*., 2010; Verbruggen *et al*., 2013).

The effects of drought are a particular challenge in many parts of the world and will increase with global climate change. Humans currently intercept c. 60% of all water run off following precipitation and use 80% of this for agriculture (Fitter, 2012). Even in areas with moderate-high annual rainfall, modern farming practices still necessitate efficient water management. The beneficial effect of AMF inoculation in strawberry is controversial. Some studies have reported the beneficial effects of mycorrhiza on strawberry plant growth and yield (Borkowska, 2002; Castellanos-Morales *et al.*, 2010; Hršelová *et al.*, 1989; Niemi and Vestberg, 1992; Stewart *et al.*, 2005; Vestberg, 1992) whilst others showed either limited (Garland and Schroeder-Moreno, 2011) or no beneficial effects (Vestberg *et al.*, 2004).

Functional complementarity, whereby more than one AMF species colonise a root synergistically, has been observed (Alkan *et al.*, 2006; Jansa *et al.*, 2008; Jin *et al.*, 2013; Koide,

2000; Reddy *et al.*, 2005). Wagg et al. (2011a) examined the complementarity and selection effects in an AMF community and found that these contributed up to an 82% and 85% improvement (respectively) in plant productivity above the average respective fungal species monoculture, though this relationship varied with plant species, AMF species combinations and abiotic conditions. These authors reported an overall positive relationship between fungal richness and plant productivity. However, other reports suggest that maximum benefit to the host plant can be achieved by a single effective AMF species (Edathil *et al.*, 1996; Hart *et al.*, 2013), with no additional benefit from a mix of species. In a study of field transplanted micro-propagated strawberry (Stewart *et al.*, 2005), treatments with a single inoculum or a mixed inoculum containing *Glomus intraradices*, *G. mosseae* and *G. etunicatum* (*sensu lato*) gave an equal benefit. However, Hršelová *et al*. (1989) showed that some AMF species, such as *G. caledonium* and *G. margarita* (*sensu lato*), were better than others in increasing strawberry plant biomass.

Colonisation of plants by AMF generally leads to increased plant development, although the magnitude of this effect is variable due to many factors, such as P-availability and AMF genera (Hoeksema *et al.*, 2010). A recent meta-analysis (Treseder, 2013), encompassing data from 67 published papers, suggested that increased AMF colonisation generally leads to an increased plant biomass and P content. There is a debate as to the relationship between colonisation level and plant growth benefit and there are several reports of negative growth responses of plants to AMF inoculation (Smith *et al.*, 2010).

The aim of this study was to determine the effects of two species of AMF, *Funneliformis geosporus* and *Funneliformis mosseae,* on the growth of cultivated strawberry (*Fragaria x ananassa)* and its tolerance to water stress. The relative effect of the two species, in a single or mixed inoculum, was determined both by root length colonisation (RLC) and the qPCR

method as described in Chapter 4. These two species co-occupied the root system of strawberry plants, but their relative abundance was affected by water stress. In this chapter the effects of single or combined AMF inoculation on plant growth and tolerance to water stress are reported. Most studies to date have examined plant growth in relation to average RLC across many replicate plants. In the present study, the relationship of AMF colonisation was plotted against the plant development characteristics of each plant within each treatment regime.

## 5.2 Methods

Three replicate experiments to study the effects of the relative abundance of AM fungal species on plant health, water uptake and growth were established. Data was combined for all three experiments to allow for statistical robustness with experiment replicate treated as an experimental factor in statistical analysis.

Much of the experimental details have been described previously (Chapter 4). Only key experimental procedures and experimental details specifically relevant to this chapter are described here.

### 5.2.1 Inoculum production and experimental design

Strawberry plants (*Fragaria x ananassa*) were kept at either well-watered (WW) or regulated deficit irrigation (RDI) conditions (plants receiving 70% of water for the WW treatment in Experiment 1 and 60% in Experiment 2 & 3) for 10 weeks. Plants were grown in a GroDome containment compartment under controlled conditions (22$^{o}$C day/20$^{o}$C night with a 14 h day/10 h night cycle with supplementary lighting). Four AMF inoculation treatments were established: inoculation with the two individual species alone, inoculation with both species,

and an un-inoculated control. In the mixed species inoculation, both species were added at a rate of 50% of the single-species inoculation.

Plants were placed in a randomised block design with up to five replicate blocks depending on experiments. Within each block was a 4x4 lattice design, four AMF treatment x four pipelines (two of which were randomly allocated to the WW or RDI treatment). By the end of 10 weeks all 80 plants survived in Experiment 1 and 3, and only 52 survived in Experiment 2. In Experiment 3, only 40 plants were used to assess plant growth (because of the nature of destructive sampling) and the other 40 plants were used to study plant survival under drought conditions (see below). All 80 plants in Experiment 3 were assessed for fruit weight, Soil Plant Analysis Development (SPAD) readings and water use efficiency (WUE) as these were assessed before the drought experiment started.

### 5.2.2 Water use measurements and plant survival study

During the last two weeks of each experiment, when plants were fully grown, WUE was recorded. Evapotranspiration was recorded for every plant on two separate occasions and WUE for each plant was determined by dividing the plant fresh weight at harvest by the average total water consumed (g) by each plant over a 24-hour period.

In Experiment 3, only 40 of the 80 plants were harvested from two replicate irrigation lines. The plants from the two remaining irrigation lines were firstly watered to full capacity over a 24h period, after which irrigation was completely removed and the plants were left to dry within the GroDome compartment. These plants were monitored daily to record the number of days post water shut-off that strawberry leaves reached 50% senescence and the date on which a plant died.

### 5.2.3 Plant growth measurements

Chlorophyll content readings were taken from one leaf of each plant, using a SPAD meter (Opti-science USA model CCM-200), which gives a measure of chlorophyll content/leaf area. This measurement was taken twice in the final two weeks of each experiment.

Fruit yield was recorded for each plant by picking and weighing fruit twice weekly on ripening. Fruit production only occurred in Experiment 2 and 3. As the emphasis for this current research was on the effect of AMF diversity/abundance on plant growth, pollinators were not artificially provided in the compartment, which affected fruit production. Furthermore, micro-propagated plants were used for the first two experiments and these respond differently to runner plants in fruit production (Szczygiel *et al.*, 2002). Hence, the yield data may not truly reflect the production potential under commercial conditions.

Plants were finally harvested from each pot and substrate was carefully washed from the root mass. Root and shoot lengths were measured along with shoot and root fresh weights for each plant harvested. A sample (ca. 2 g) of roots was cleared with KOH before being stained using Trypan Blue and assessed microscopically for RLC using the grid-line intersect method (McGonigle *et al.*, 1990). Colonisation was expressed as percentage of the root colonised by AMF.

### 5.2.4 Statistical analysis

All results presented for the plant growth effect analysis are pooled from the three repeat experiments, in order to provide statistical robustness. Standard errors of the differences (SED) given for all predicted means account for block and replicate experiments effects.

Statistical analysis was carried out using GenStat version 13 (VSN International, England). A REML (restricted maximum likelihood) analysis was used to assess how plant growth was affected by AMF treatments (control vs inoculated, and inoculated was further divided into moss vs geo vs both) and irrigation regimes. In the REML analysis, row, column or block effects originating from the experimental design, and three repeated experiments over time, were treated as random factors. Initial REML analysis showed that there was no evidence of any row, or column, effects within a block in any of the analyses, or differences between the replicate irrigation pipes. A simplified analysis was thus carried out on the data adjusted for the effects for experiments and blocks within experiments (i.e., they were treated as fixed effects). Data was ln-transformed to reduce variance heterogeneity. Significances of treatment effects were assessed using F-tests.

Multiple linear regression was used to study the relationship between RLC and plant growth measurements on a plant by plant basis with irrigation included as a factor to determine whether the observed effect on plant growth was due solely to root colonisation by AMF. Experiment and block were also included as additive factors.

## 5.3 Results

### 5.3.1 Plant growth effects

For all growth characters, both single inoculation treatments (*F. mosseae* or *F. geosporus*) and the mixed inoculation with both species gave similar benefit to strawberry plants as shown by REML analysis. The qPCR data presented previously (Chapter 4) confirmed that both species survived in the mixed inoculation and each species formed approx. 50% of the total DNA as the single inoculation (although this varied considerably with irrigation treatments).

The difference between the RDI and WW treatments was always significant for all plant growth characters (with the exception of root length) and AMF inoculation led to increased plant growth (Table 5.1). The effects of AMF inoculum and irrigation regimes on shoot and fruit fresh weights are illustrated in Figure 5.1.

| Plant growth measurements | AMF inoculation | | | Irrigation regime | | |
|---|---|---|---|---|---|---|
| | Control | Treated | s.e.d. | Dry | Wet | s.e.d. |
| Root FW (g) | 1.700 | 2.126 | 0.0622* | 1.821 | 2.005 | 0.0627* |
| Shoot FW (g) | 1.438 | 2.028 | 0.0629* | 1.596 | 1.869 | 0.0634* |
| Root length (cm) | 3.290 | 3.405 | 0.0212* | 3.363 | 3.332 | 0.0214 |
| Shoot height (cm) | 1.700 | 2.091 | 0.0358* | 1.820 | 1.971 | 0.0361** |
| Shoot/root FW (g) | -0.262 | -0.098 | 0.0381* | -0.225 | -0.135 | 0.0385** |
| Fruit weight (g) | 0.719 | 1.927 | 0.4628* | 1.085 | 1.562 | 0.4664* |

[a]: The symbol '*' and '**' indicate that the specific comparison is statistically significantly different from 0 at the level of 5% and 1%, respectively. The differences among three AMF-inoculated treatments were all very small and not statistically different. There was no evidence of interactions between treatments for growth measurements, except for root length where there was significant interaction with irrigation regime (*P*=0.017)

Table 5.1. Predicted means of strawberry growth measurements (ln-transformed) for comparisons among four AMF inoculations (including the un-inoculated control) and two irrigation regimes (fully watered [WW]– Wet; regulated deficit irrigation [RDI]- Dry

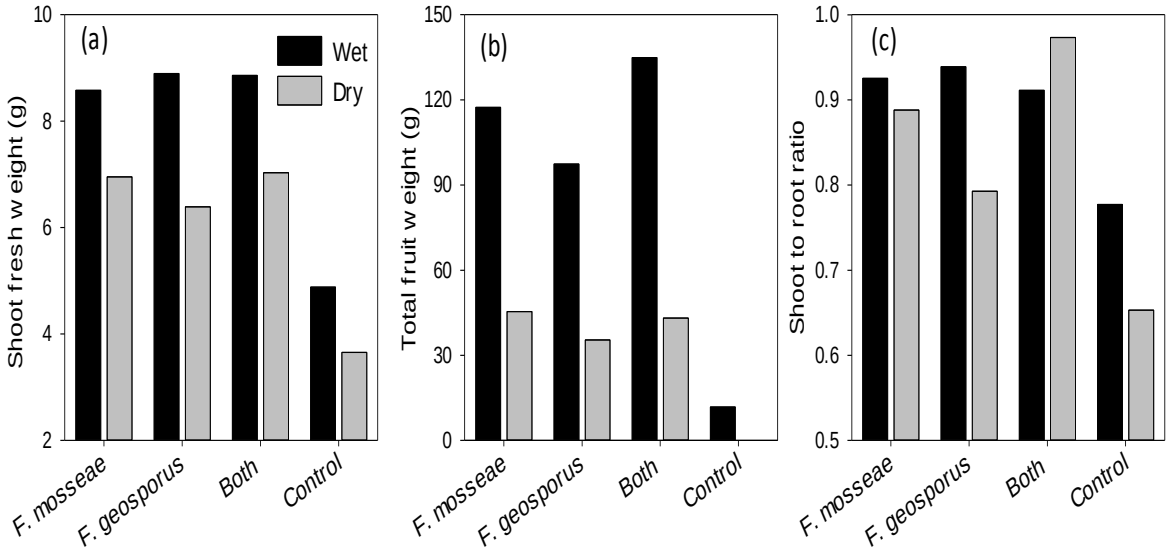



Figure 5.1. Histograms of (a) average shoot fresh weight (g), (b) average fruit weight (g) per plant and (c) average shoot to root ratio of fresh weight under regulated deficit irrigation (RDI - dry) and well-watered (WW - wet) conditions when inoculated with F. *mosseae* alone, or *F. geosporus* alone, or both (as a 50% mix) or not inoculated. The differences among three AMF-inoculated treatments were all very small and not statistically different. There was no evidence of interactions between AMF treatments with irrigation regime, except for root length where there was significant interaction (P=0.017). (Refer to table 5.1 for appropriate SED values)

An increase in fresh weight of shoot and roots resulted from inoculation of AMF and also from the WW irrigation treatment (Table 5.1). Inoculation with AMF and the WW treatment also led to an increase in shoot production relative to root production (Table 5.1 and Figure 5.1c). Although the RDI-treated plants were significantly smaller than those WW-treated, the control plants under the WW condition were still not as large as those AMF-inoculated but subjected to the RDI regime (Table 5.1). The shoot fresh weight of AMF-inoculated plants under the RDI regime was significantly greater ($P$<0.001) than that of the control plants under the WW regime and this difference was also observed for fruit yield ($P$<0.05). Therefore the effect of adding the AMF treatment (in any combination) can curtail the effect of up to 40%

water reduction on plant growth. Fruit fresh weight, although low, shows a large increase when AMF were present, with no fruit at all in the RDI uninoculated plants.

### 5.3.2 Water use efficiency and drought tolerance

The plants that were inoculated with AMF had greater ($P$<0.001) WUE than those un-inoculated control plants irrespective of the irrigation regime (Figure 5.2). However, there were no significant differences among the three AMF inoculation treatments. Those plants subjected to the RDI also had higher ($P$<0.001) WUE than those WW-treated plants regardless of AMF inoculation.

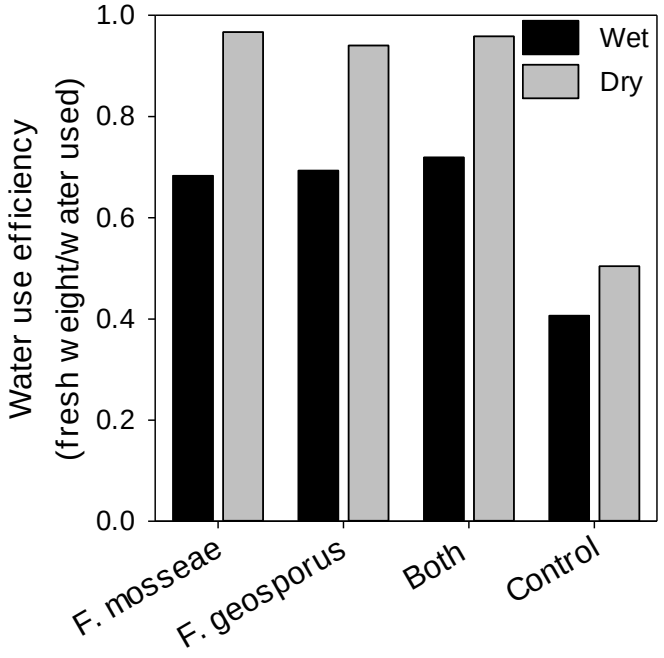



Figure 5.2. Water use efficiency (WUE, measured as plant fresh weight at harvest divided by water (g) used in a 24-h period) for AMF-inoculated and non-inoculated plants at the regulated deficit irrigation (RDI - dry) and well-watered (WW - wet) conditions. (Refer to Table 5.1 for appropriate SED values)

AMF inoculation significantly ($P$<0.001) increased plant survival when irrigation was completely terminated, irrespective of single or mixed inoculation. On average, inoculated plants took 3-6 days longer to reach 50% leaf senescence (visual estimation) and survived for 4-5 days longer than the control plants (Figure 5.3). Plants that were originally subjected to the RDI treatment also survived longer ($P$<0.001) than those plants that were initially subjected to the WW treatment. A similar trend was observed for plant survival to 50% senescence and the difference between inoculated and control treatments in the WW was greater than in the RDI condition ($P$=0.008).

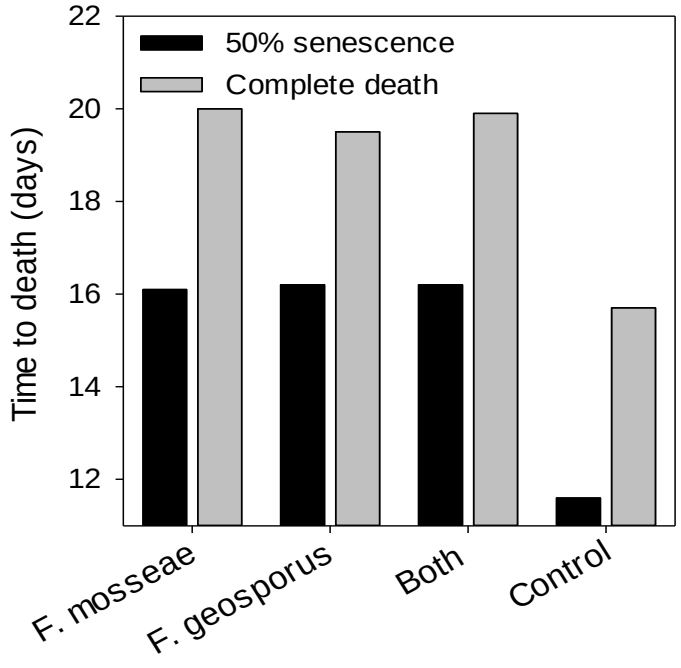



Figure 5.3. Average number of days to 50% leaf senescence or plant death for each AMF inoculation treatment when all plants were fully watered for a-24 h period before the irrigation was terminated. The SED for the comparison between the control and AMF inoculation was 0.480 and 0.347 for the time to 50% senescence and to death, respectively.

### 5.3.3 Quantitative relationship of root colonisation with plant growth

On the basis of individual plants, there is generally a positive regression between percentage of root colonised by AMF and plant growth characters except for the root length and fruit weight (Table 5.2). However, the exact relationship between RLC and plant growth characters varied greatly with inoculation treatment (AMF species, and single or mixed inoculation) and irrigation regimes. The difference in this linear relationship between the WW and RDI irrigation regimes was always significant regardless of the nature of AMF inoculation. Such a complex relationship is illustrated for shoot weight and shoot-root fresh weight ratio in Figure 5.4.

| Plant growth measurements | Regulated deficit irrigation (RDI – dry) | | | | | | Well-watered (WW – wet) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ***F. geosporus*** | | ***F. mosseae*** | | **Both** | | ***F. geosporus*** | | ***F. mosseae*** | | **Both** | |
| | **slope** | s.e. | **slope** | s.e. | **slope** | s.e. | **slope** | s.e. | **slope** | s.e. | **slope** | s.e. |
| Root FW (g) | **0.019** | 0.007 | **0.015** | 0.005 | **0.014** | 0.006 | **0.026** | 0.008 | **0.012** | 0.004 | **0.039** | 0.007 |
| Shoot FW (g) | **0.022** | 0.007 | **0.022** | 0.004 | **0.021** | 0.006 | **0.037** | 0.007 | **0.018** | 0.004 | **0.040** | 0.007 |
| Root length (cm) | 0.000 | 0.003 | 0.002 | 0.002 | 0.003 | 0.002 | 0.003 | 0.003 | 0.002 | 0.001 | -0.001 | 0.003 |
| Shoot height (cm) | **0.013** | 0.004 | **0.010** | 0.003 | **0.011** | 0.004 | **0.016** | 0.004 | **0.010** | 0.002 | **0.021** | 0.004 |
| Shoot/root FW | 0.003 | 0.005 | **0.007** | 0.003 | **0.008** | 0.004 | **0.010** | 0.005 | **0.006** | 0.002 | 0.001 | 0.005 |
| SPAD reading | **0.009** | 0.004 | 0.003 | 0.002 | **0.007** | 0.003 | **0.014** | 0.004 | **0.005** | 0.002 | **0.012** | 0.004 |
| Fruit weight (g) | **-0.116** | 0.063 | 0.018 | 0.039 | 0.031 | 0.049 | 0.019 | 0.053 | 0.016 | 0.053 | 0.002 | 0.046 |
| WUE | **0.018** | 0.007 | **0.017** | 0.005 | **0.018** | 0.007 | **0.028** | 0.008 | **0.014** | 0.004 | **0.038** | 0.007 |

Table 5 2. Slopes (and standard errors) in the fitted linear models relating plant growth characters (ln-transformed) to % root length colonisation (data were adjusted for experiments and blocks before regression). Linear models were not fitted to the control treatment since there was no root colonisation by AMF. Significant slopes are highlighted in bold.

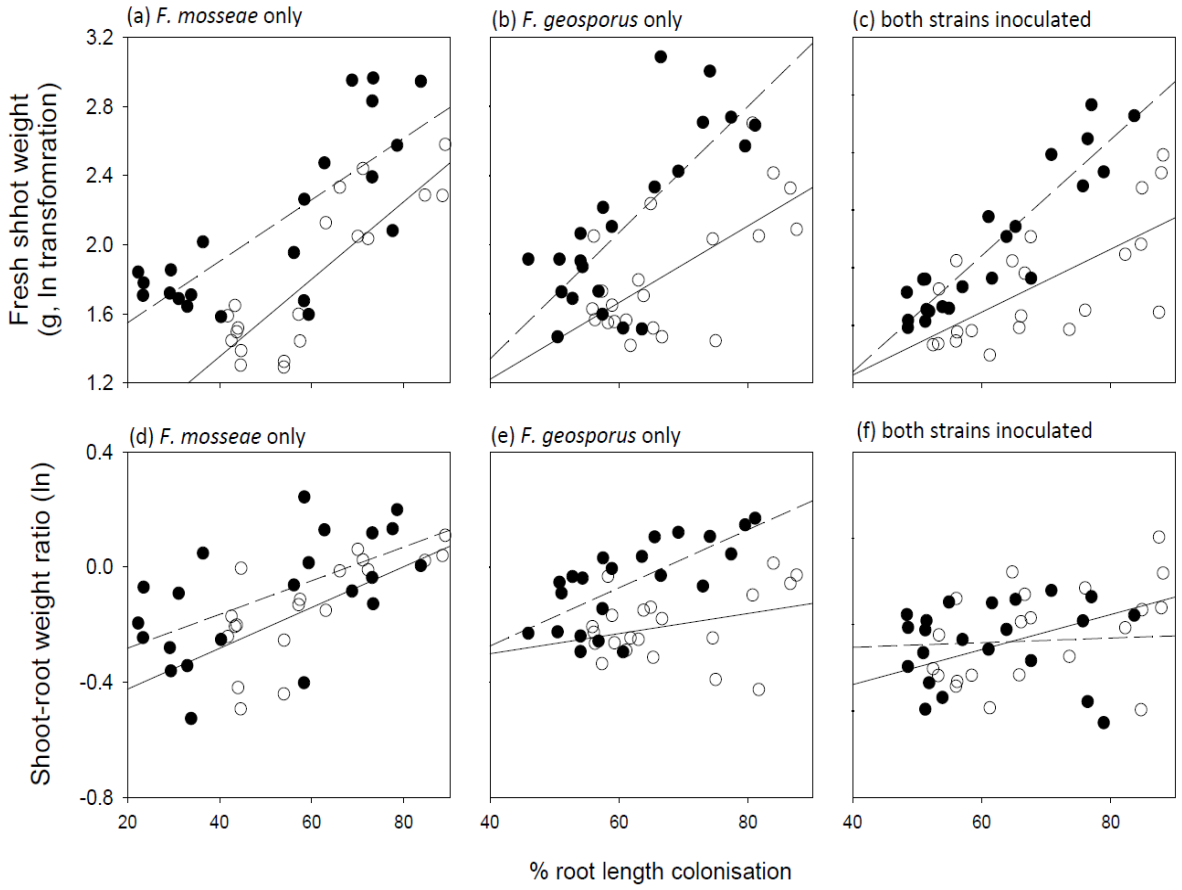



Figure 5.4. The relationship between (a-c) shoot fresh weight (ln-transformed) and (d-f) shoot to root fresh weight ratio (ln-transformed) of individual plants against % root length colonisation by AMF for individual AMF inoculation treatments. Data were adjusted for experiment and block effects. Solid and dashed lines are fitted linear models: one for well-watered (WW – wet, black dots) and the other for regulated deficit irrigation (RDI – dry, white dots) treatment, with the model parameter estimates given in Table 5.2. Each data point represents an individual plant.

## 5.4 Discussion

### 5.4.1 Mycorrhizal treatment benefits plant growth

Inoculation with AMF was beneficial to strawberry plant growth regardless of irrigation regimes. Past studies of the benefit of mycorrhizal inoculation with strawberry have had inconsistent results, ranging from no effects (Borowicz, 2010; Vestberg *et al.*, 2004) to positive

effects on strawberry biomass production (Hršelová *et al.*, 1989; Stewart *et al.*, 2005; Vestberg, 1992). In general, the presence of AMF/plant symbiosis often results in altered rates of water movement into, through and out of the host plants, with beneficial consequences for tissue hydration and plant physiology (Ruiz-Lozano, 2003). Consequently, the AMF symbiosis can protect crop plants against the detrimental effects of water deficit.

One of the main benefits of AMF symbiosis for plants is the increased uptake of P; thus the amount of available P in the substrate may affect the magnitude of AMF effects on plant growth. The present study focused on a low P fertigation regime, whereas current commercial strawberry production relies on fairly high P input. This may account for the more consistent benefit associated with AMF colonisation than in other studies that were conducted in non-nutrient limiting conditions (Garland and Schroeder-Moreno, 2011; Vestberg *et al.*, 2004). Nevertheless, AMF colonisation increased P uptake and biomass, even in a high P soil environment (Stewart *et al.*, 2005), depending on strawberry cultivar and AMF inoculum. Strains of commercially available inoculum were used in the present study , one of which was an isolate of *F. mosseae* shown to be a more favourable AMF species for strawberry than *G. intraradices* (*sensu lato*) and other unidentified *Glomus* species (Vestberg, 1992). Other studies used native inoculum (Borowicz, 2010), which may have had fewer infective propagules, or may be less effective, although native inoculum can be as beneficial as the commercially available *G. intraradices* (*sensu lato*) strain (Garland and Schroeder-Moreno, 2011). This study used a soil-free substrate for growing (Terragreen) which could also explain the positive effect of adding inoculum. Traditionally much strawberry production was conducted in either field soil or in peat-based substrates. The trend in UK strawberry production is to move into soil-less growing systems such as coir and production under protected growing conditions, whereby the use of AMF may benefit plant growth and yield.

Fruit yields in this study were low, due to the fact that micro-propagated plants were directly used and no insect pollinators were provided in the confined compartment (natural pollinators cannot enter the compartment since the facility is completely sealed for the purpose of controlled isolation). Nevertheless, there was a significant reduction in fruit yield under the RDI regime and the AMF-colonised plants had a higher yield than the non-mycorrhizal plants. Chavez and Ferrera-Cerrato (1990) showed that the effect of AMF inoculation on fruit yield of micro-propagated plants of four cultivars was highly variable depending on both the AMF species used and strawberry cultivar with both positive and negative effects obtained. One cultivar 'Tioga', however, produced low yields with delayed flowering and no fruit production in the controls but a significant yield when inoculated with either of two species of AMF. These authors suggested this effect may be due to increased phosphorus uptake, known to affect floral differentiation and growth, or that the AMF colonisation caused changes in hormonal and nutritional conditions.

An increased shoot production relative to root production was observed in the AMF-treated plants, consistent with previous results (Borkowska, 2002; Veresoglou *et al.*, 2012); there was a reduction in shoot to root ratio under the RDI conditions, compared to the WW regime. In general, there is a positive correlation between shoot to root ratio and % root colonisation by AMF, suggesting that AMF colonisation has greater effects on shoot production than on roots. Colonisation, by AMF may allow the plant to invest greater resource into shoot development. It is assumed that, as the symbiosis improves plant nutrition (Veresoglou *et al.*, 2012), a greater shoot area is available to provide a larger photosynthetic area and therefore increased energy for the plant to use for yield, without the need to increase root production.

Leaf chlorophyll content (SPAD reading) was found to be higher in the control plants than the AMF-inoculated plants. The SPAD value provides an indicator of the photo-synthetically active

light transmittance characteristics of the leaf (Richardson *et al.*, 2002), suggesting that the control plants photosynthesised more per unit area of leaf than mycorrhizal plants. Although the irrigation effect on SPAD readings was variable between treatments in the present study, Borkowska (2002) observed a change of chlorophyll fluorescence values in non-mycorrhizal plants under drought stress, however in mycorrhizal plants there was no change. This author suggested that AMF inoculation may have a role in protecting photochemical systems, even under stressed conditions, and thus colonised plants may require less apparatus to achieve the same photosynthetic capacity. These results differ from other published reports such as Augé *et al.* (1987) and Zhu *et al.* (2010) which could possibly be due to host species, or environmental conditions.

### 5.4.2 Plant growth effect and relationship to amount of colonisation

There is still debate as to the nature of the relationship between AMF root colonisation level and plant growth benefit, which may partially be due to the methodology used to measure colonisation or abundance of AMF. In the present study, we measured the abundance using both % root colonisation and a real-time PCR technique and demonstrated a positive relationship between the two measures (Chapter 4). This study demonstrated that the increase in plant growth was directly influenced by an increase in % root colonisation by AMF when individual plants were examined. Very few studies have examined RLC effects on an individual plant basis, although Gange and Ayres (1999) measured the response of individual plants to AMF colonisation, in order to study the response of plants at different levels of colonisation. They suggested that an increased benefit was given to the plant up to a point at which a plateau was achieved and then declined at higher colonisation, which contrasts with the findings reported here. Due to the absence of colonisation data in the range of 0-20, only a linear model (excluding control plants) was fitted in the present study, since the main purpose was to demonstrate the positive relationship rather than prediction. However, it could be argued that a curvilinear relationship may better describe the relationship if the

control plants (with zero colonisation) were included in the regression analysis. To illustrate this curvilinear relationship, a quadratic model was fitted to the shoot fresh weight data (Figure 5.5). The fitted quadratic models show that there is a point at which % colonisation level gives rise to a detectable increase in plant growth effects, generally at about 30-50% colonisation. In this study low levels of colonisation (< 20% RLC) were not observed and as such the response of strawberry to such low levels of AMF colonisation cannot be seen. However, low colonisation levels have shown some effects in other studies (Lekberg and Koide, 2005; Treseder, 2013) in a meta-analysis based on mean colonisation values. These low levels can occur in natural situations (Feldmann *et al.*, 2009; Lekberg and Koide, 2005). A dose response experiment would be required to show whether there is a threshold point and, if so, at what level of root colonisation.

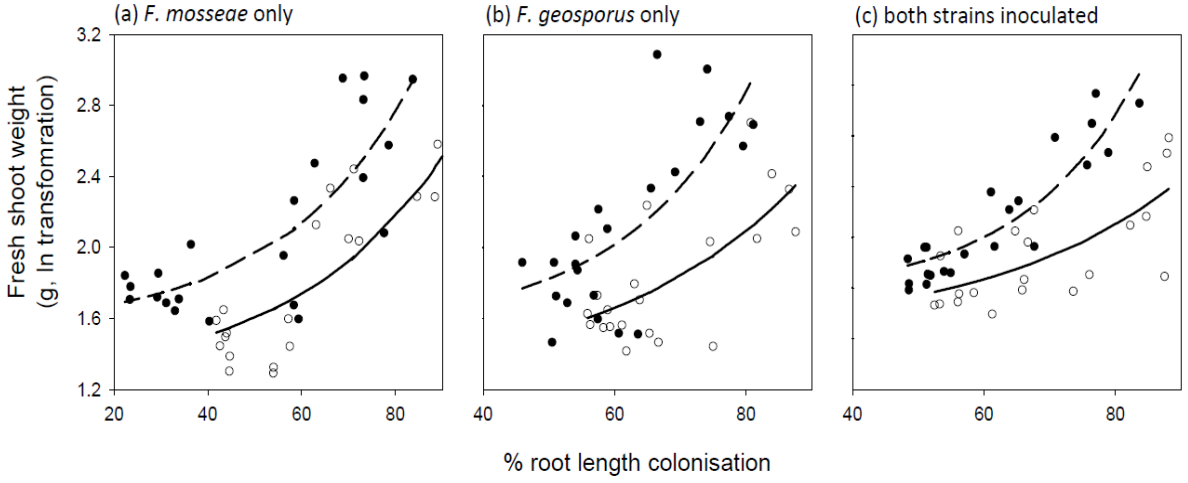



Figure 5.5. The quadratic models relating shoot fresh weight (ln-transformed) of individual plants to % root length colonisation (including the control treatment). Data were adjusted for experiment and block effects. Solid and dashed lines are fitted linear models for the regulated deficit irrigation (RDI - dry) and well-watered (WW - wet) treatment, respectively; the model parameter estimates are given in Table 5.2. Each data point represents an individual plant.

These results show that the addition of AMF was of greater importance to strawberry plant growth and yield than species diversity or abundance, however this could vary for different

combinations of AMF species and could be dependent on native population of AMF. In field-grown strawberry production future trials should be mindful about how the introduction of new species affects native populations, although in soil-less substrates it may be more beneficial.

### 5.4.3 Water use efficiency

AMF inoculation increased all growth characters measured under both WW and RDI conditions, regardless of single- or mixed-species inoculation, consistent with the recent meta-analysis of Jayne and Quigley (2014). Inoculation of AMF benefited plant growth equally at both irrigation regimes when compared to control plants. In the present study, the addition of AMF inocula to plants subjected to reduced irrigation of up to 40% restored plant growth back to the same or higher values as the non-mycorrhizal, fully-watered plants. Similar results have been observed for lettuce (Baslam and Goicoechea, 2012). Thus, using mycorrhizal inoculation in crop production may contribute to a reduced need for irrigation without compromising plant growth or yield. This effect may be partially explained by the increased AMF colonisation under the RDI regime, suggesting that under water-stressed conditions plants become more dependent on the fungal symbiosis.

The water use efficiency of plants was greater under the RDI regime than under the WW regime, consistent with previous findings (Grant *et al.*, 2010; Omirou *et al.*, 2013) and was greater in AMF-inoculated plants than the non-mycorrhizal plants, although there were no significant differences between plants inoculated with the single or combined inoculum. Inoculation with AMF is known to increase WUE in watermelon (Omirou *et al.*, 2013) and wild shrub species (Querejeta *et al.*, 2006) although this was variable with different plant and AMF combinations. This suggests that AMF colonisation not only increases water uptake but also results in the plant becoming more efficient in using available water (Kaya *et al.*, 2003; Omirou

*et al.*, 2013), possibly via mechanisms of increased stomatal conductance and transpiration (Auge, 2001), increased nutrient availability, and changes to aquaporin activation (Smith *et al.*, 2010). The most notable result of the increased WUE of plants colonised by AMF was the significant increase in the survival time of strawberry plants when water was not provided: mycorrhizal plants surviving on average 4.4 days longer than control plants. This result was consistent with previous findings (Borkowska, 2002), and increased survival again did not depend on the nature of AMF inoculation (species, and single vs mixed inoculation).

### 5.4.4 Single or combined inoculum

Arbuscular mycorrhizal fungi inoculation was shown to result in good colonisation of all the treated plants, with averages ranging between 30-60% of roots colonised across all treatments, similar to that recorded by Vestberg (1992). Furthermore, the qPCR data showed that these two species have similar colonising potential in the mixed inoculation, although varying to a certain extent with irrigation. Thus, it is reasonable to assume that both *F. mosseae* and *F. geosporus* in the mixed inoculation contribute to the observed effects on plant growth. It is likely that, as these two species both give similar beneficial effects to the plant in a mixed inoculation as in single-species inoculation, there is little competition between them. In this study, two closely related species of AMF were used, both in the same genus (Redecker *et al.*, 2013). Closely related species of AMF were shown to give greater plant growth effects and were better able to co-exist than more distant species (Roger *et al.*, 2013). Other authors, however, have found that more distantly related species are more likely to co-exist than phylogenetically similar species (Maherali and Klironomos, 2007; Maherali and Klironomos, 2012). Results seen here may vary more if different AMF species were used, or if host plants or environmental conditions were different.

This study demonstrated that in strawberry plants, under these experimental conditions, single species inoculation treatments gave similar benefits to the host as the mixed inoculation treatment. Although no difference was observed between the three AMF treatments, there were clear benefits in all plant growth characters for AMF-inoculated plants, suggesting that species diversity is not important as long as colonisation is present. This conclusion was further supported by the fact that there was a positive relationship between % root colonisation and the increase in plant growth based on individual plants, although such a relationship was influenced by AMF species and irrigation. Further studies are needed to demonstrate whether this conclusion is true to other host/AMF combinations, and other characters (e.g. disease tolerance).

The present results raise a question: to what extent is the AMF diversity important for crop production in an agricultural environment, in contrast to a natural environment where multiple occupancy may enable a plant to harness a wider spectrum of benefits (Alkan *et al.*, 2006)? The answer depends on many factors, including the taxonomic closeness of those AMF species, relative abundance of these AMF species, host species, soil characteristics etc. For example, *G. intraradices* became important only in one variety of micro-propagated strawberry under a high P environment (Stewart *et al.*, 2005; Wagg *et al.*, 2011a) observed a range of AMF interactions (ranging from positive to negative) that were highly variable depending on many factors such as AMF species, host plant species and abiotic factors in grassland species. It is likely that the inclusion of more than one species, as long as the mix of species was complementary, would be of advantage in a variable abiotic situation. Addition of either species in this study did not have a deleterious effect on the other and this has implications for use in mixed commercial inoculants.

# Chapter 6. The effect of culture conditions and host plant on AMF sequence diversity in an isolate of *R. clarus*

## 6.1 Introduction

Although genetic diversity within AMF is widely accepted, there is still limited understanding of how this diversity affects host plants and how environmental conditions affect such genetic diversity. The complex nature of these obligate symbiotic organisms is still hampering the study of how they interact with plants (Salvioli and Bonfante, 2013) at a physical and genetic level. Both intra-specific and intra-isolate genetic differences lead to different responses of AMF in host plants (Koch *et al.*, 2006; Munkvold *et al.*, 2004). Genetically different AMF, even of the same species, have different effects on plant growth (Koch *et al.,* (2006). These authors suggest that a change in sequence diversity could lead to a change of effect on host and this may be more important when considering the impact of an AMF community on the plant hosts. Sanders (2004a) suggested that perhaps the relationship between AMF and their plant hosts is not so much due to the variation between species or morphotypes but more to do with the genetic differences between AMF. The relative influence of inter-specific or intra-specific AMF diversity on hosts is of greater ecological importance.

Intra-specific variation in phenotypic traits has been shown to be induced by both abiotic and biotic factors (Behm and Kiers, 2014), but it is unclear if this variation is reflected at a genetic level. A better understanding of genetic variation would allow for a greater understanding of how AMF respond phenotypically to changes in environmental conditions (Behm and Kiers, 2014). Ehinger et al. (2012) showed that *R. irregularis* has a high natural genetic variation and that genetically different lines could be produced by establishing single spore culture lines. Genetically different variants on the Bg112 locus with novel phenotypes, from five sibling lines of clonal single spore lines were generated from a single spore culture. Segregated lines had

differential effects on plant growth (dry weight) of *Oryza sativa* and *Plantago lanceolata* (Angelard *et al.*, 2010). Angelard *et al.* (2013) also looked at single spore lines of *R. irregularis* in response to a shift in host plant species using *Daucus carota* and *Solanum tuberosum*. Using AFLP, these authors reported the genotypic change and phenotypic responses of segregated spore lines of *in vitro* AMF cultures on these two different hosts. They found differences in amplified fragment length polymorphism (AFLP) patterns (allele change) between the host plants, but these changes also varied between segregated lines (suggesting that the alleles were located on different nuclei). Again this genetic exchange significantly altered plant growth and phenotypic traits of the AMF (measured as a change in spore density) between different segregated lines. Both of these results suggest segregation of nuclei at spore formation resulting in a different complement of nucleotypes, which could potentially help AMF species to rapidly change phenotype under different environmental conditions.

Previous work has shown that sequence variants occur at different frequencies in different isolates of AMF (Clapp *et al.*, 2001; Oliveira *et al.*, 2010; Robinson, 2005; Rodriguez *et al.*, 2005). A possible explanation for the existence of these sequence variants and the variability in their frequency is that they could represent function variations and that this variation may not be selectively neutral (Sanders, 2004b). It could be hypothesised that different variants are expressed differentially under different environmental conditions. Oliveira *et al.* (2010) showed that an isolate of *G. geosporum* grown in two different substrate conditions (one being an inert zeolite substrate and the other being a highly alkaline anthropogenic sediment) showed changes in phenotypic traits such as germination rate and arbuscule abundance. Variation was also demonstrated at the genetic level. Robinson (2005) showed that the relative frequencies of some sequence variants changed under varying conditions of light intensity and host plant species. It is therefore important to determine how environmental and different culture conditions influence AMF sequence diversity or the frequency of occurrence of sequence variants.

Next generation sequencing (NGS) provides a new technology for relatively inexpensive production of large volumes of sequence data to provide fast and accurate genome information. The technique is equipment-driven with Illumina currently dominating the market (Metzker, 2010), along with the Roche 454 life sciences machine and Applied Biosystems SOLiD system (Salvioli and Bonfante, 2013). Since the introduction of the 454 in 2005, this technology has revolutionised microbial ecological studies (Mardis, 2011). NGS has been used in the field of mycorrhiza primarily for metagenomic studies to quantify species presence and fungal community structure within environments (Davison *et al.*, 2012; Lumini *et al.*, 2010; Öpik *et al.*, 2009). Although originally 454 sequencing had a limited allowance for replication, new NGS technologies allow amplicons to be recovered from environmental samples with a depth that is orders of magnitude greater than previously possible (Davison *et al.*, 2012). NGS is developing at a rapid pace with the Illumina technology now extending the lengths of reads available (Lindahl *et al.*, 2013) with version 3 chemistry offering paired-end reads up to 300bps. However, there are still many challenges and developments to be met with this technology, not least of which is the computing technology required to handle this amount of sequence data (Salvioli and Bonfante, 2013). Error control in NGS is an important consideration when analysing results. Boon *et al.* (2013) recommended a highly conservative approach in the sequences used for downstream analysis. Other issues include the financial expense of multiple replications and the statistical analysis being less mature (Salvioli and Bonfante, 2013).

In recent years the production of AMF by *in vitro* methods has developed significantly with some large inoculum production companies favouring this method over a more traditional vegetative *in planta* production method (Ijdo *et al.*, 2011). It is thought that mass production of AMF *in vitro* will potentially overcome the problem of using AMF inoculum in large scale agriculture, as it provides a more consistent inoculum quality, which is sterile and free from potentially harmful microorganisms, and with a lower labour cost. *In vitro* production yields

infective propagules that have low weight and volume, thus reducing transport costs. It has been shown recently to be economically viable to apply *in vitro* grown inoculum in the tropics for food production (Ceballos *et al.*, 2013). In addition, *in vitro* inoculum production may lead to AMF products that can be applied more widely in agriculture as it can be applied as a liquid via irrigation systems or applied onto a variety of carrier materials. The primary disadvantage of the *in vitro* inoculum production is that it is currently limited to only a few AMF species, namely those found in the *Rhizophagus* group (Ijdo *et al.*, 2011). Studies involving environmental changes in *in vitro* systems are limited by the fact that the root organ cultures have very limited growth and the required transformed root cultures (and nutrient requirements) are available only for a limited number of host plant species (Behm and Kiers, 2014).

In this chapter, the genomics of an isolate of *R. clarus* MUCL 46238 was compared in *in vitro* and *in planta* conditions. This isolate has been in long term *in vitro* culture and is widely available via Glomeromycota *in vitro* Collection (GINCO). The null hypothesis was that the method of cultivation of this strain would have no effect on the extent of sequence diversity. Relative changes in sequence diversity were quantified using NGS and resulting sequence data were subjected to further phylogenetic analysis.

## 6.2 Materials and Methods

### 6.2.1 Plant and fungal material

#### 6.2.1.1 Pure cultures

Cultures of five different AMF species (*F. mosseae* BEG 25, *F. geosporum* BEG 11, *R. irregularis* BEG 72 and BEG 145, *C. claroideum* PW5 and *G. microaggregatum* BEG 56) were established in Terragreen (Agsorb, Oil-dry Ltd, Wisbech, UK) in pot culture with onion (*Allium cepa*) and *Desmodium ovalifolium.* Fungal material was obtained as potential commercial inoculum from Plantworks UK, Sittingbourne, Kent, UK. Regular sampling of these cultures confirmed that all of the above species were established as pure, sporulating cultures.

#### 6.2.1.2 Establishment of in vitro culture

For consistency between the work carried out in Chapters 1-4, initial attempts were made to establish *F. mosseae* BEG25*, F. geosporus* BEG11 *R. irregularis* BEG72, *C. claroideum* PW5 and *G. microaggregatum* BEG 56 in an *in vitro* culture system. *In vitro* cultivation was established based on the methods published previously (Cranenbrouck *et al.*, 2005). Spores were isolated from the pure pot cultures and sterilised by placing in 2% Chloramine T with 1 drop of Tween 80 for 2 mins (twice), then rinsed 3 times with sterile deionised water, before being placed into a 200ppm Streptomycin (Sigma, UK) and 100ppm Gentamycin (Sigma, UK) solution. Spores were stored at 4$^{o}$C for 2 weeks to provide a period of cold shock in order to initiate germination. Spores were then transferred to Petri plates containing excised sections of transformed chicory or carrot roots, obtained from the GINCO (www.mycorrhiza.be/ginco-bel), on medium M (Bécard and Fortin, 1988) and incubated at 24$^{o}$C.

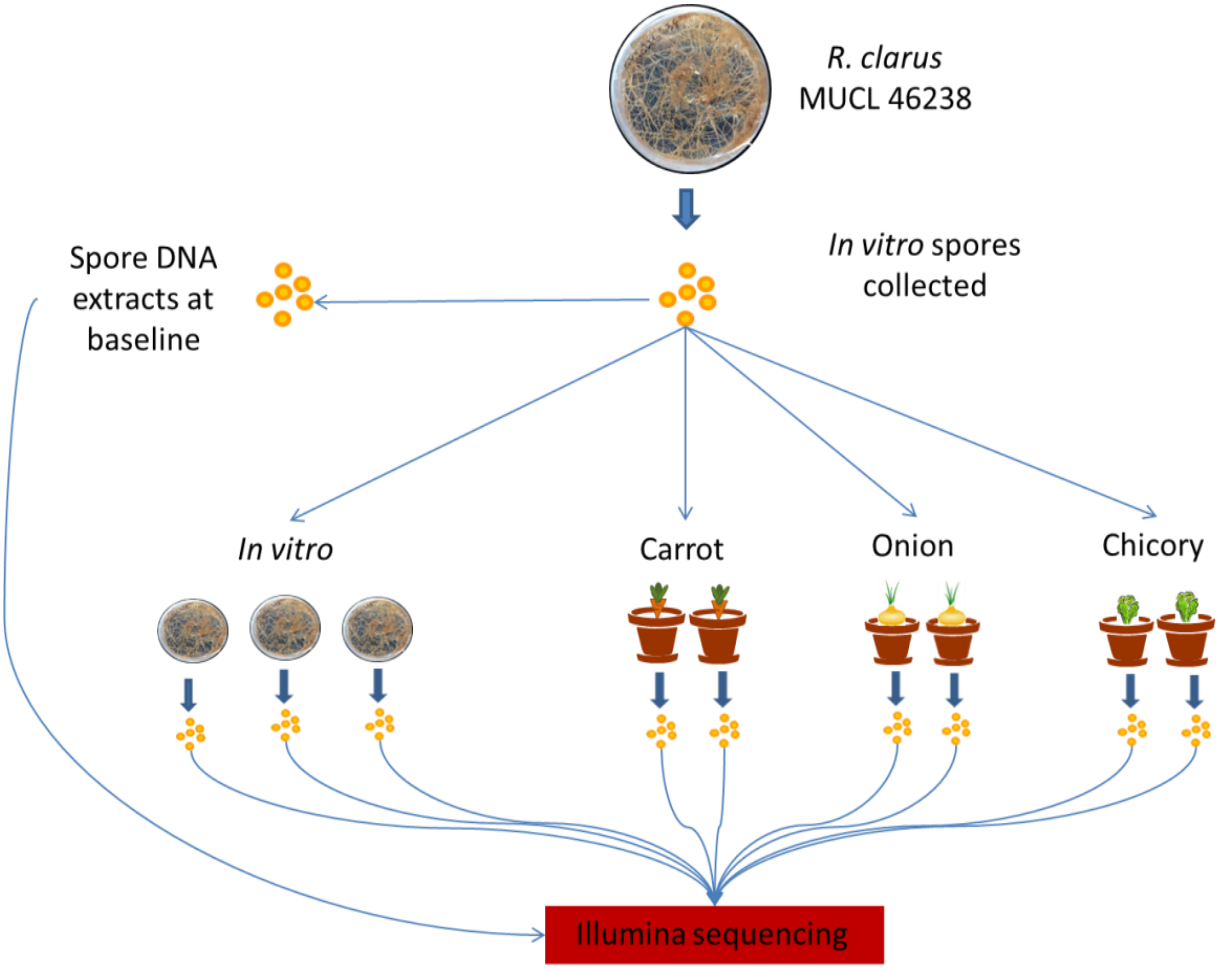
After a number of attempts were made to culture these isolates using *in vitro* methods, only *F. mosseae, F. geosporum* and *R. irregularis* were seen to germinate when plated onto transformed chicory roots. However, new mature spores were not produced in any of these cultures. The *in vitro* culture of many AMF is notoriously difficult especially for large spore *Glomus* species (personal communication Dr. Y Dalpe, GINCO) and continuous culture has only been observed in certain species (Cranenbrouck *et al.*, 2005). To enable the development of

this study, alternative isolates with known ability to grow in *in vitro* culture were obtained. Two *in vitro* cultures, *R. clarus* MUCL 46238 and *R. irregularis* MUCL 43194, were purchased from GINCO. These cultures were established following the protocol recommended by GINCO. The disks supplied from GINCO containing spores and hyphal fragments were placed directly onto Petri plates containing medium M with transformed chicory roots. Plates containing these cultures were incubated in the dark at 24$^{o}$C for 4 months.

When sporulation was prolific, samples of spores were used to initiate further *in vitro* cultures and also to establish *in planta* cultures. For the *in vitro* lines, sub-cultures containing ~200 spores were aseptically transferred onto fresh medium M along with root fragments. These plates were maintained in the dark at 24$^{o}$C for ~2 years, being sub-cultured routinely approximately every 4 months. To initiate the *in planta* cultures, spores from the same plate were extracted by dissolving the phytagel using sodium citrate (10mM) solution (Cranenbrouck *et al.*, 2005) and washing and collecting the spores on a fine sieve. Pots were filled with Terragreen and ~200 spores were transferred to each pot directly under seeds of onion (*Allium cepa* cv. Red Beard), carrot (*Daucus carota*, cv Early Nantes 5) or chicory (*Cichorium intybus*, cv Sugar Loaf). Seeds were germinated by placing an empty petri dish on top of the Terragreen. The plants were grown in a greenhouse with supplementary heat (20$^{o}$C) and light (16h day, 8h night cycle) for ~ 2years. Plants were watered according to their requirement and fed regularly with 1.4 g $L^{-1}$ Vitafeed 102 (Vitax Ltd., Leicester, UK).

Relative changes in sequence diversity over time were studied using NGS and phylogenetic analysis. Sub-samples of ~200 spores were retained for DNA extraction from the initial plate used to establish *in vitro* and *in planta* cultures to form a baseline estimation of the sequence diversity and relative abundance of sequence variants. After 2 years of subculture, spore samples from each of the 3 replicate *in vitro* plates and each of the two replicate *in planta*

cultures were collected and used for DNA extraction and sequencing. Only *R. clarus* was used for the downstream analysis. Culture of the *R. irregualris* slowed down over the 2-year period in *in vitro* culture and good replication of DNA extracts from replicate *in planta* culture was preferable in this study. Figure 6.1 shows a schematic representation of samples used in NGS.




| Treatment | AMF 1 Baseline | AMF 2 *in vitro* | AMF 3 *in vitro* | AMF 4 *in vitro* | AMF 5 Chicory | AMF 6 Chicory | AMF 7 Onion | AMF 8 Onion | AMF 9 Carrot | AMF 10 Carrot |
|---|---|---|---|---|---|---|---|---|---|---|

Figure 6.1. Schematic representation of the work flow of different culture conditions used for the NGS.

### 6.2.2 DNA extraction and selection of samples for NGS

The plant DNeasy mini Kit (Qiagen, Hielden, Germany) extraction kit has been shown to produce high yields of pure genomic DNA from AMF spores by the work in Chapters 3 & 4 and many previous studies. Hence, this kit was also used to extract pure genomic DNA from AMF spores for meta-genomic sequencing, following the manufacturer's protocol (with elution using 2 x 50µl of elution buffer). Two hundred spores from each of the 2-year-old cultures were collected, either by dissolving the phytagel in the *in vitro* cultures and washing the spores on a fine sieve with deionised water, or collection by wet-sieving from the established pot cultures and surface sterilising (as above). Spores were then crushed using a micropestle before DNA extraction.

Extracted DNA was amplified with ALF01 (Clapp *et al.*, 2001) and NDL22 (Van Tuinen *et al.*, 1998) primers, known to amplify the LSU rRNA D2 region of AMF species. PCR parameters have been optimised such that the reaction mixture (20µl reaction) contained 2.0µl GeneAMP 10X buffer II (Applied Biosystems, Cheshire, UK), 1.6µl of $MgCl_2$ (25 mM), 1.6µl dNTP (2.5mM), 2.0µl of each primer (2µM), 0.1µl AmpliTaq DNA polymerase (Applied Biosystems, Cheshire, UK), 8.7µl of water (Sigma, UK) and 2.0µl of DNA template. Each reaction was performed in a PTC-200 Peltier thermocycler (MJ Research, Watertown, MA, USA) according to the following protocol; initial denaturation cycle at 95$^{o}$C (3 min), followed by 53 cycles of 94$^{o}$C (30 sec), 50$^{o}$C (45 sec), 72$^{o}$C (1 min), with a final extension of 72$^{o}$C (7 min). Amplified products were then visualized using gel electrophoresis. Those samples showing a medium to strong band visualisation on electrophoresis were chosen for downstream Illumina analysis (Lindahl *et al.*, 2013).

### 6.2.3 Next Generation Sequencing

The recommended workflow protocols for metagenomic library preparations 'Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System' (http://res.illumina.com/documents/products/appnotes/16s-metagenomic-library-prep-guide.pdf) were followed with a few changes as outlined below.

For the amplicon PCR the primer pair ALF01 and NDL22, plus the primer set ITSI-F (5'CTT GGT CAT TTA GAG GAA GTA A) (Gardes and Bruns, 1993) and HC2 (5'ATA TGC TTA AGT TCA GCG GG) (Navajas *et al.*, 1994) were used. The AMF DNA extracts were then amplified in a 13 µl reaction mixture containing 1.25 µl of 10X buffer basic (Molezym GmbH and Co. Bremen Germany), 1 µl 25 mM $MgCL_2$ (Qiagen, Hielden, Germany), 1 µl dNTP (2.5 mM), 1.25 µl forward and reverse primers each (2µM), 0.05µl MolTaq basic DNA polymerase (Molezym GmbH and Co. Bremen Germany), 5.2µl of molecular biology reagent water (Sigma, UK) and 2µl of DNA (from spore extractions at ~1-2 ng $\mu l^{-1}$). Each reaction was performed in a Dyad MJ research thermocyler, according to the following protocol, 94°C (3 min) followed by 35 cycles of 94°C (30 sec), 55°C (45 sec), 72°C (1 min), reducing 0.5°C per cycle until 50°C, with a final extension of 72°C (5 min). PCR products were visualised by gel electrophoresis. This PCR was carried out in triplicate and pooled to reduce potential bias in amplification.

Following a PCR clean-up of the amplicon PCR using Agencourt AMPure XP beads (Beckman Coulter, USA), a barcoded template library was generated by PCR using the primer set ITSI-F/HC2 which were modified at the 5' end with adaptors, TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG - forward adaptor and GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA - reverse adaptor. The ITSI-F / HC2 primer set was chosen as it gave better amplification when combined with the barcode attachments used in the Illumina sequencing and as such only these were used for the remainder of this work. PCR amplification using these primers gave a

product of ~750bp, which is consistent with the target region of the rRNA genes including the end of the SSU, ITS1, 5.8S and the start of the LSU region plus the adaptor primers.

The adapted primers were then modified to include the identifying Illumina Nextera indices, unique to each sample, as described in table 6.1, by PCR as described in the manufacturer's protocol.

| Treatment | Nextera index 1 (i7) | Nextera index 2(i5) |
|---|---|---|
| **AMF 1 Baseline** | N704 | S505 |
| **AMF 2 *in vitro*** | | S506 |
| **AMF 3 *in vitro*** | | S507 |
| **AMF 4 *in vitro*** | | S508 |
| **AMF 5 Chicory** | N705 | S501 |
| **AMF 6 Chicory** | | S502 |
| **AMF 7 Onion** | | S503 |
| **AMF 8 Onion** | | S504 |
| **AMF 9 Carrot** | | S505 |
| **AMF 10 Carrot** | | S506 |

Table 6.1. Table showing Nextera reference indices added to each DNA sample. These unique identifiers allow for identification of each individual sample and allows de-multiplexing in downstream analysis. As such afer the Illumina sequencing has taken place each DNA sequence can be related back to its origin. Nextera indices are as standard in the manufactures protocol.

Following the index PCR clean-up step, using the Agencourt AMPure XP beads, as per the manufacturer's instructions, PCR products were qualitatively assessed using a Fragment Analyzer (Advanced analytical, Ames, IA, USA) using the High Sensitivity NGS fragment analysis Kit (Advanced analytical, Ames, IA, USA). PCR products were also quantitatively assessed using a Qubit 2.0 Fluorometer (Life Technologies, USA).

Treatment samples, containing environmentally different spore extracts, were pooled so as to be analysed on the same Illumina run to avoid run-quality bias. These samples were multiplexed with 56 additional fungal samples used for an unrelated study, to reduce the cost per sample. However, the unique DNA barcode indices allowed sequences from all samples to be de-multiplexed in subsequent processing. Samples were pooled ensuring each sample was equimolar. The final concentration of the pooled library was 4 nM. The amplicon library was denatured using 1 mM NaOH and diluted to 30 pM as per the nmanufacturer's protocol. The diluted and denatured amplicon library was then combined with a denatured PhiX library at an equimolar concentration at a rate of 20% to increase heterogeneity of the sample. These samples were then run on an Illumina MiSeq with 300bp paired end sequencing (version 3 chemistry).

### 6.2.4 Sequence analysis

Only sequences with a minimum length of 450 bps were retained for subsequent analysis. Sequence data were initially dealt with based on the QIIME analysis pipeline (Caporaso *et al.*, 2010): (1) data were first de-multiplexed into individual samples, (2) primers were removed from sequences, (3) operational taxonomic units (OTUs) were identified for each sequence against the UNITE fungal ITS database (Abarenkov *et al.*, 2010) at 97% similarity, (4) every unique sequence and its frequency in each sample was stored. Finally, a small utility programme was written in Delphi to (1) retrieve every unique sequence that has been

identified as AMF for all samples, (2) summarise the data over all samples as a frequency table: number of each AMF sequence in each sample.

Only OTUs that formed > 0.3% of the total returned *R. clarus* sequences were included in the sequence abundance analysis. This was to provide even sampling depths with the maximum number of sequences for analysis, as recommended to improve reliability (Lindahl *et al.*, 2013). Below this value (0.3%), sequence variants were more likely to arise through sequencing error. Above 0.3% of the total *R. clarus* sequences at each sample included in the analysis ranged from 6 *de novo* sequences at the lowest common sequence and up to 38787 at the highest. Each *de novo* sequence, belonging to AMF above the 0.3% threshold frequency, was aligned using Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo). Sequences were then trimmed to 256 bp using Jalview (www.jalview.com) to remove low quality end regions.

Phylogenetic analysis used PAUP (Swofford, 1999) and was carried out using both neighbour joining (Saitou and Nei, 1987) and maximum parsimony (Swofford, 1999). Maximum parsimony analysis was performed using heuristic search options with 100 bootstrap replications (Felsenstein, 1985) to produce a more conservative tree. Thirty-three sequences were included in the analysis, including an outgoup *Diversispora* HE863860, reference sequences for *R. clarus, R. irregularis* and a representative from each unique sequence type. Geneious software [Geneious version (R6), Biomatters, www.geneious.com] was used to run PAUP and also to edit trees. All sequences grouped into 4 main clades and many of the sequences varied by just a few bps. The 4 clades clustered about the 3 major sequence types with an additional outgroup. These clades were used to form the sequence groups for sequence abundance assessments, rather than individual sequences, to increase statistical power by avoiding too many sequence variants with very few occurrences across all the samples.

### 6.2.5 Statistical analysis

The frequency of occurrence of each sequence type group was analysed in relation to hosts. For statistical analysis, three samples were excluded because of very low sequencing coverage (reasons unknown): *In vitro* rep2 AM3, chicory rep 1 AM5 and carrot rep 1 AM9. REML was run on the remaining sequences with the outgroup removed. The outgroup sequence numbers were much lower than the 3 major groups and as such they were analysed separately in a second stage. Outgroup sequences were analysed using ANOVA on only this group and using GLM assuming the error distribution following a binomial distribution.

## 6.3 Results

### 6.3.1 Fungal cultures

Plants grew well over the 2-year period, and fungal cultures were checked microscopically for sporulation after ~1 and ~2 years. Sporulation was good for all treatments and cultures visually appeared to be pure. *In vitro* cultures established well from the baseline plates. The rate of sporulation of *R. irregularis* MUCL 43194, cultured on transformed carrot root slowed down over the 2-year period, however *R. clarus* MUCL 46238 (cultured on transformed chicory root) continued to sporulate well at each subculture. Multiple subcultures were initiated at each step of the same line from the original baseline sample, as not every plate re-initiated, and some were lost due to contamination or lack of re-generation.

### 6.3.2 DNA amplification

PCR amplification, using the NDL22/ALF01 primer pairs, of multiple spore extractions from the baseline samples and all treatment samples yielded bands of ~460 bps, consistent with AMF DNA (Rodriguez *et al.*, 2005), see fig. 6.2.

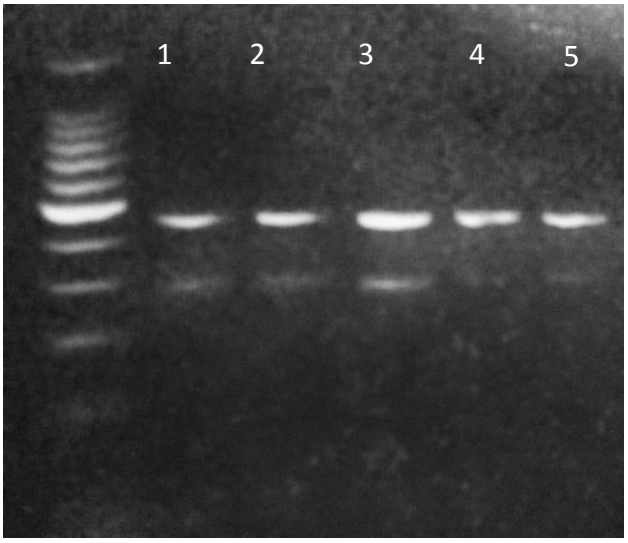



Figure 6.2. Gel showing 100 bp ladder, 1= carrot, 2= onion, 3= chicory, 4= *in vitro* chicory 1, and 5= *in vitro* chicory 2. Some slight contaminant band was found in these samples.

Two primer sets, ALF01/NDL22 and ITSI-F/ HC2, both modified with 5' adaptors were compared for successful amplification using the same spore extracts. The ITS1-F/ HC2 primer set gave better amplification and was thus used for the remainder of the Illumina workflow protocol. These gave a band of ~750 bps consistent with the expected combined length from of the ITS1, 5.8S, ITS2 and partial LSU read expected with the addition of the adaptor sequences. The fragment length of 585 bps was the expected length calculated from other isolates of *R. clarus* sequences deposited in GenBank.

### 6.3.3 Illumina results.

Three samples had very low sequencing coverage (reasons unknown): *In vitro* rep2 AM3, chicory rep 1 AM5 and carrot rep 1 AM9. For other samples, there were > 100000 raw reads for each sample. More than 90% of these sequences were of AMF. Sequences that did not align to *R. clarus* aligned predominantly to *R. irregularis*, unidentified *Glomeromycota* sequences or *Diversispora* sequences. The OTUs not aligning with *R. clarus* were excluded

from the frequency abundance analysis. The number of sequences with OTUs aligning with *R. clarus* returned from each treatment sample ranged between 34 and 101156 sequences. All treatment samples, included in the relative frequency analysis, had a sequence depth of >3000 sequences returned.

Of those OTUs that formed > 0.1% of the total *R. clarus* returned sequences, a total of six variants were obtained from the baseline sample with an average of 6.0 from the *in vitro* replicate samples, 7.0 from the chicory replicate samples, 8.5 from the onion replicate samples and 10.5 from the carrot replicate samples (see fig. 6.3). The frequency of these OTU sequence variants ranged between 0.1% and 45.8% of the total returned *R. clarus* sequences. Four samples had a large number of sequences with OTUs not aligning with *R.clarus*: AM5, AM6 and AM9 (which aligned using BLAST to *R. irregularis*) and AM 10 (which aligned using BLAST to *Diversispora*).

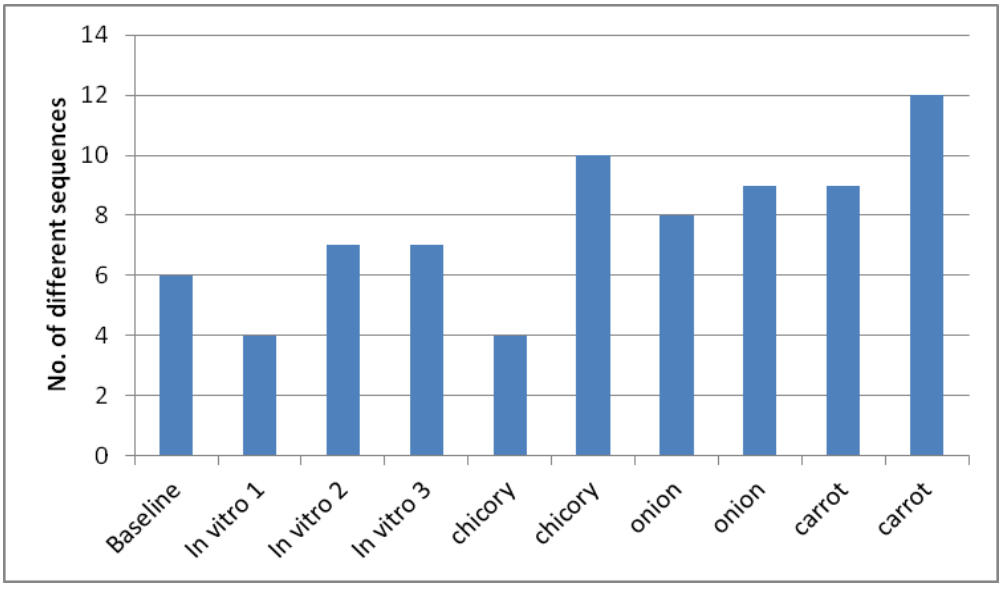



Figure 6.3. Graph showing total numbers different of returned sequences, with OTUs that formed >0.1% of the total *R. clarus,* for each culture condition analysed.

A threshold limit of numbers of sequences to be included in the frequency analysis was set such that only sequences with > 0.3% or higher of the retuned *R. clarus* sequences were included. *De novo* sequences above this threshold were correlated with the OTUs, and aligned in Clustal Omega. Of the variant sequences, three major sequence types were identified and these were found to be dominant in all samples.

The three major sequences obtained were checked against existing sequences in the NCBI database using BLAST (www.ncbi.nlm.nih.gov/index.html) and all found to have good homology to *R. clarus* in GenBank (this was added as a second check even though the OTUs were showing good alignment). Sample AM1 (generally the most common sequence type) was aligned with 99% sequence homology to *R. clarus* accession number FM865542 and mapped to the end of the 18S rRNA gene, ITS1 and the first 130 bps (out of 158 bps) the 5.8S rRNA gene. Variation with sequence type 1 (as shown in Fig 4 ) and of the other 2 major sequences, occurred predominantly within ITS 1 and at about 80 bps into the 5.8S rRNA gene. Sequence variation was predominantly found (when compared to the *R. clarus* FM865542) in the ITS 1 region at ~155-175 bps into the sequence read in a repetitive AT rich area and a second region at ~230 bps within the 5.8S. The alignment with the deposited *R. clarus* FM865542 shows that total read length between the primer sites for ITS1-F/HC2 in *R. clarus* is 585 bps. It was not possible to give longer reads using paired end Illumina sequencing, as this fragment plus the adaptor primer sequence exceeds the confidence limits at each end of 300 bps (i.e. total possible sequencing length including adaptor and primer sequences is 600 bps).

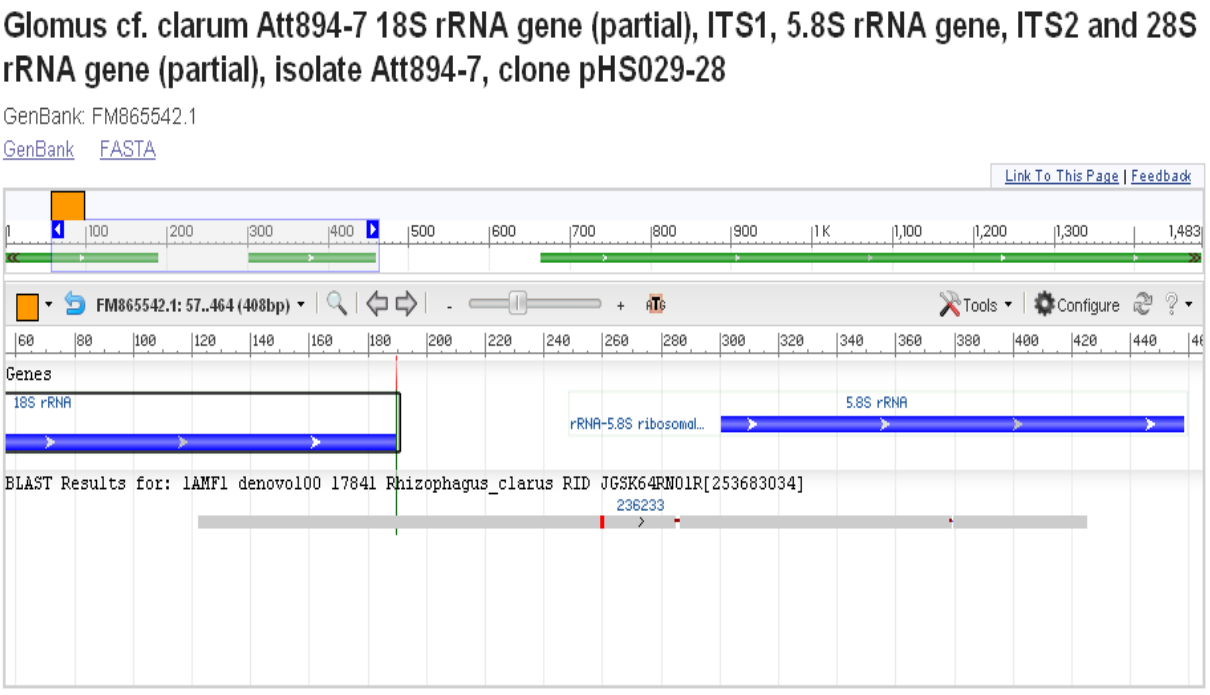
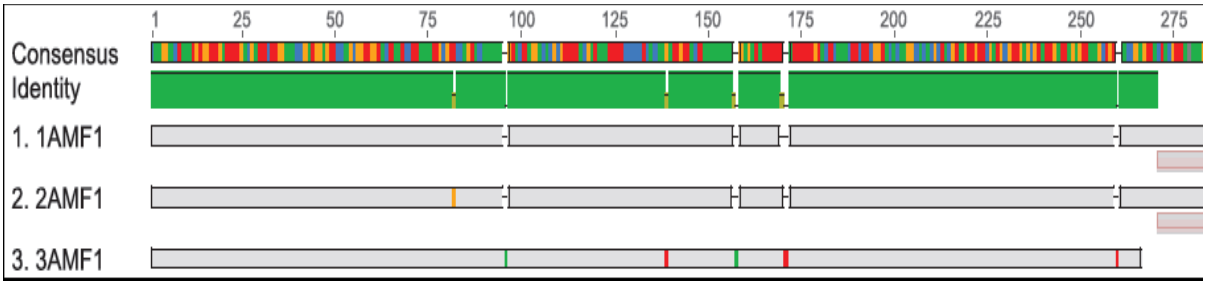



Figure 6.4. Blast alignment with accession number FM865542, showing location of the AMF1 sequence type read comparable to the rRNA gene regions. Blue bars represent the mapping of the gene, and shows the position of the 18S and 5.8S rRNA, and the grey bar shows the position of the sequence read. Red markers and > show locations of variability between AMF1 and the reference sequence.

Alignment of all sequences, from all of the culture conditions including the baseline treatment, showed 93.4-100% sequence identity between spore samples with up to 17 bp differences. Figures 6.5 a&b, show the alignment between the three major sequence variants.

a,




b,

```
3AMF1/1-265                          CTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAG
gi|253683034|emb|FM865542.1|/123-706 CTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAG
1AMF1/1-301                          CTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAG
2AMF1/1-301                          CTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAG

3AMF1/1-265                          GATCATTACCAATTTTAGCGAACCTGATCTTTTTTTGATCATGGTCTCGCGAAAATCGTA
gi|253683034|emb|FM865542.1|/123-706 GATCATTACCAATTTTAGCGAACCTGATCTTT-TTTGATCATGGTCTCGCGAAAATCGTA
1AMF1/1-301                          GATCATTACCAATTTTAGCGAACCTGATCTTT-TTTGATCATGGTCTCGCGAAAATCGTA
2AMF1/1-301                          GATCATTACCAATTTTAGCGAGCCTGATCTTT-TTTGATCATGGTCTCGCGAAAATCGTA

3AMF1/1-265                          TTTAAAACCCCCACTCTTATAAGAATCATTTTTTTTTTGTGTATAAAAAAATAAAAAAGA
gi|253683034|emb|FM865542.1|/123-706 TTTAAAACCCCCACTCTTATAAGAATCATTTTT-TTTTGTGTATAAAAAAATAAAAAAGA
1AMF1/1-301                          TTTAAAACCCCCACTCTTGTAAGAATCATTTTT-TTTTGTGTATA--AAAATAAAAAAGA
2AMF1/1-301                          TTTAAAACCCCCACTCTTGTAAGAATCATTTTT-TTTGTGTATAA--AAAATAAAAAAGA

3AMF1/1-265                          TCACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGTAGCGAAGTGCGAT
gi|253683034|emb|FM865542.1|/123-706 TCACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGTAGCGAAGTGCGAT
1AMF1/1-301                          TCACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGTAGCGAAGTGCGAT
2AMF1/1-301                          TCACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGTAGCGAAGTGCGAT

3AMF1/1-265                          AAGTAATGTGAATTGCAGAATTCCG----------------------------------
gi|253683034|emb|FM865542.1|/123-706 AAGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTTTGAACGCAAATTGCACTCT
1AMF1/1-301                          AAGTAATGTGAATTGCAGAT-TCCGTGAATCATCGAATCTTTGAACGCAAATTGCACTCT
2AMF1/1-301                          AAGTAATGTGAATTGCAGAT-TCCGTGAATCATCGAATTTTTGAACGCAAATTGCACTCT
```

Figure 6.5. a, shows alignment of the 3 major sequence types produced using Geneious version R6 (www.geneious.com), and b, shows a detailed alignment of the 3 major sequence types compared to the reference sequence. Coloured vertical lines on a, and red horizontal bars on b, represent areas of sequence variation.

### 6.3.4 Sequence analysis

#### 6.3.4.1 Phylogenetic assessment

In total 33 sequences were analysed (including examples of sequences not aligning to *R. clarus* from this analysis and representatives from other genera and species obtained from GenBank). In each sequence, 266 bps were assessed, of which 101 (38%) base positions were variable, and of these 20 (19.8%) were parsimony-uninformative. The neighbour-joining and maximum parsimony trees gave largely the same tree topologies (see fig. 6.6).

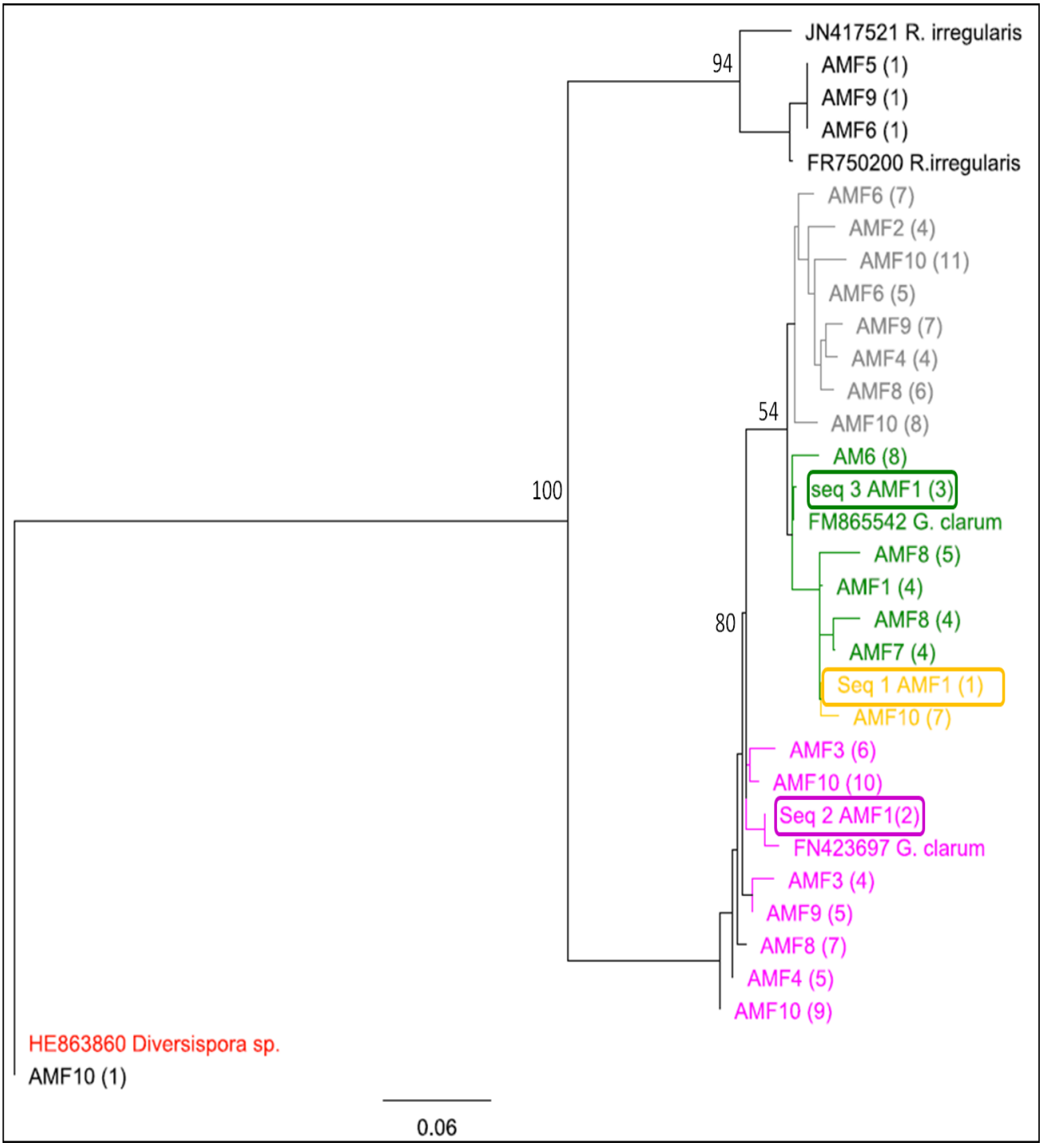



Figure 6.6. Neighbour-joining tree showing all unique sequence variants along with reference sequences of *R. clarus, R. irregularis* and *Diversispora*. Bootstrap values were obtained from maximum parsimony analysis (using 100 boostrap replications). The three major sequence types are highlighted in boxes and the four sequence groups by colour, such that sequence group 1 is coloured in yellow, group 2 in pink, group 3 in green and group 4 in grey. Tree produced using Geneious version R6 (www.geneious.com) with PAUP plugin.

The tree clearly shows four distinct clusters of sequence variants. The three major sequence types fell into 3 clades, along with other less frequent sequence types clustering tightly about them. A fourth group consisting of less frequent sequence types fell as a separate outgroup cluster. Only 1 sequence from this group originated from an *in vitro* spore sample and none of the less frequent types come from the baseline sample. The alignment from the sequences forming the four groups is shown in fig 6.7. Group 2 was more divergent from groups 1 and 3. The fourth outgroup appeared to have differences at the two main site of variation: at ~155 bps similar to group 1, and at ~230 bps similar to group 2.

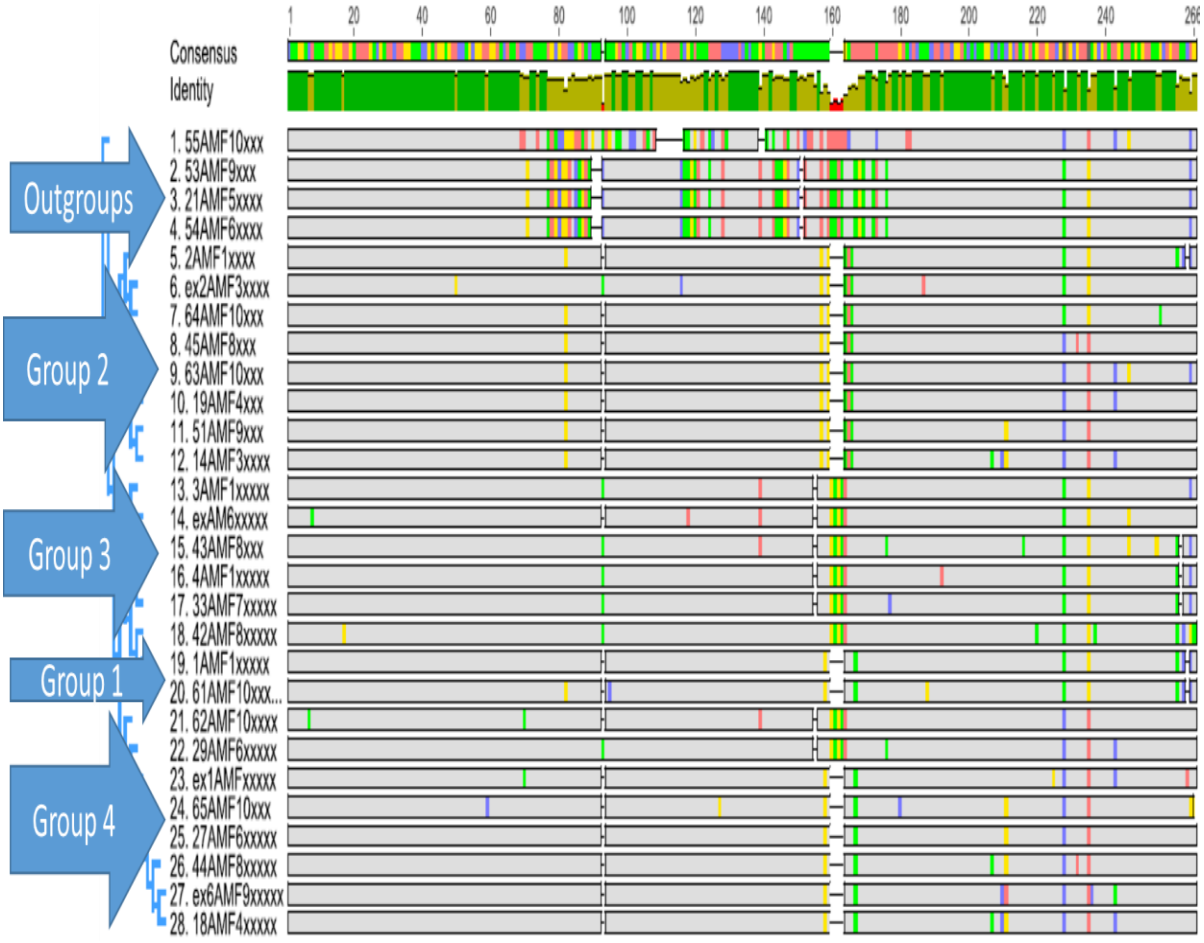



Figure 6.7. Alignment of all unique sequence variants, differences highlighted by gaps and coloured markers showing base pair changes. Alignment produced using Geneious version R6 (www.geneious.com). Nucleotide base differences are represented in the alignment by a colour highlight, such that T green, A is red, C is blue and G is yellow

### 6.3.4.2 Sequence Frequency

The frequency of sequence variants in the baseline sample was determined from the spores of the original *R. clarus in vitro* isolate and compared with those found in spores formed after 2 years of culture in either the *in vitro* or *in planta* culture with chicory, onion or carrot (Fig. 6.8).

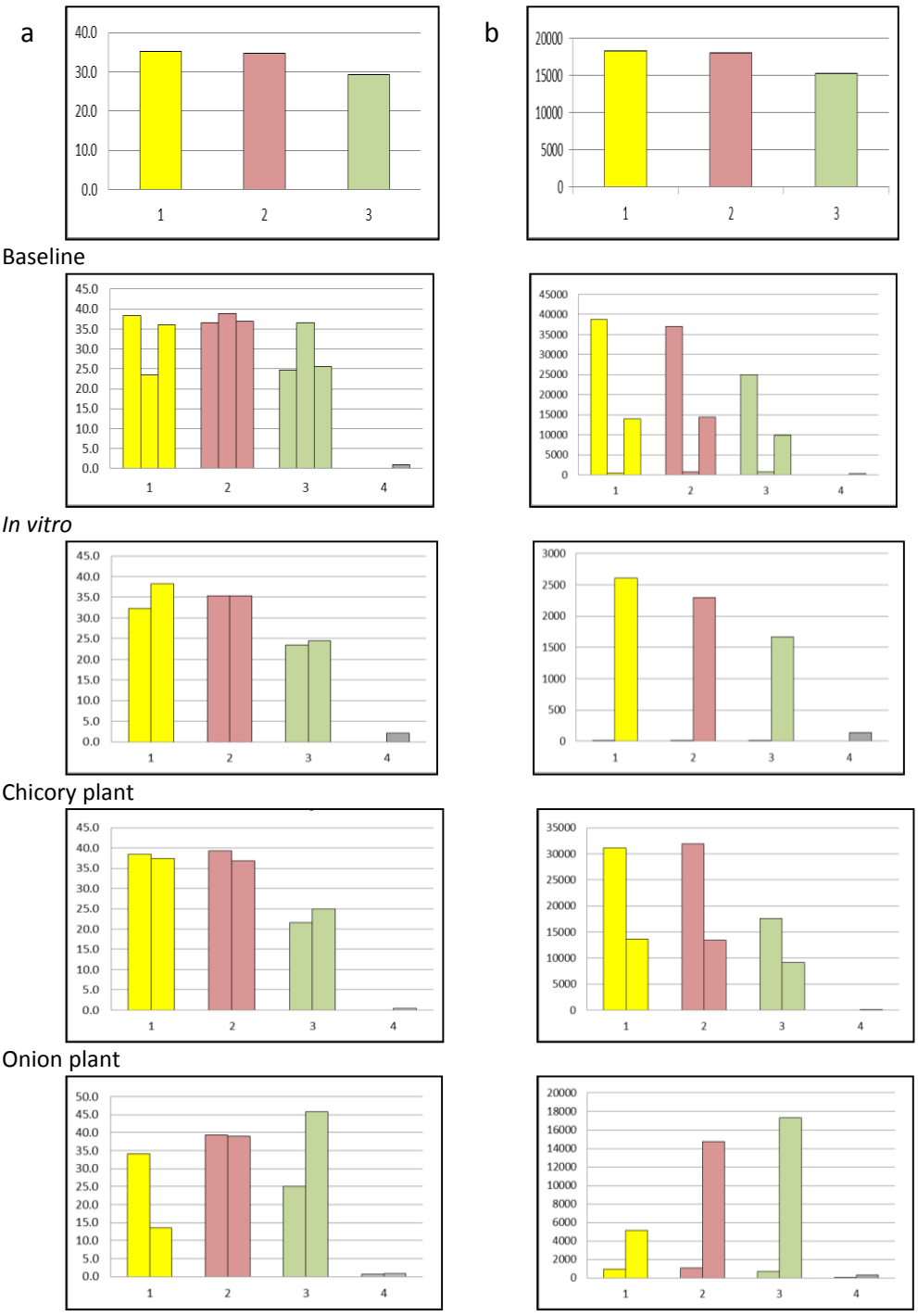



Figure 6.8. Graphs showing relative frequencies of sequences types (Phylogenetic groups 1-4) at each treatment condition expressed as either a as a) percentage of the total sequences forming >0.3% of the returned *R. clarus* sequences or b) actual numbers of sequences retuned for those sequences forming >0.3% of the total *R. clarus* (individual bars represent treatment replicates at each condition).

The graphs (Fig 6.8) show that the three major sequence groups were dominant in all spore samples analysed, however their relative frequencies differed among the culture treatment conditions. REML analysis revealed a significant interaction between sequence type and culture condition at $P < 0.001$ when only the three major sequence groups were analysed. Biological replicates at the same culture conditions gave similar trends in the frequency of the dominant sequences, with the only exceptions being when one biological replicate was of a low sequence depth (*In vitro* rep2 AM3, chicory rep 1 AM5 and carrot rep 1) and as such these were discarded from the analysis. At different culture conditions the three major sequence variants became either more or less frequent than the baseline (Fig 6.9). Only the carrot treatment condition was highly different from the other conditions: the frequencies of both the group 1 and 3 sequences differed significantly from those at the baseline. The group 2 sequence types were not significantly different from the baseline at any of the treatment conditions. In the onion treatment condition the frequency of the group 3 sequences was significantly lower than that of the baseline. In the chicory and the 2 years *in vitro* condition, the frequency of the group 3 sequences was lower than that the baseline, and the difference was close to statistically significant at the 5% level. However, the numbers of biological replicates in this study is low. The fourth outgroup was not present in significant numbers (i.e. in the top 0.1% of returned *R. clarus* sequences) in the baseline sequences. The outgroup sequences were found in very low numbers and no significant differences were found when analysed either in combination with the major sequences or alone with GLM.

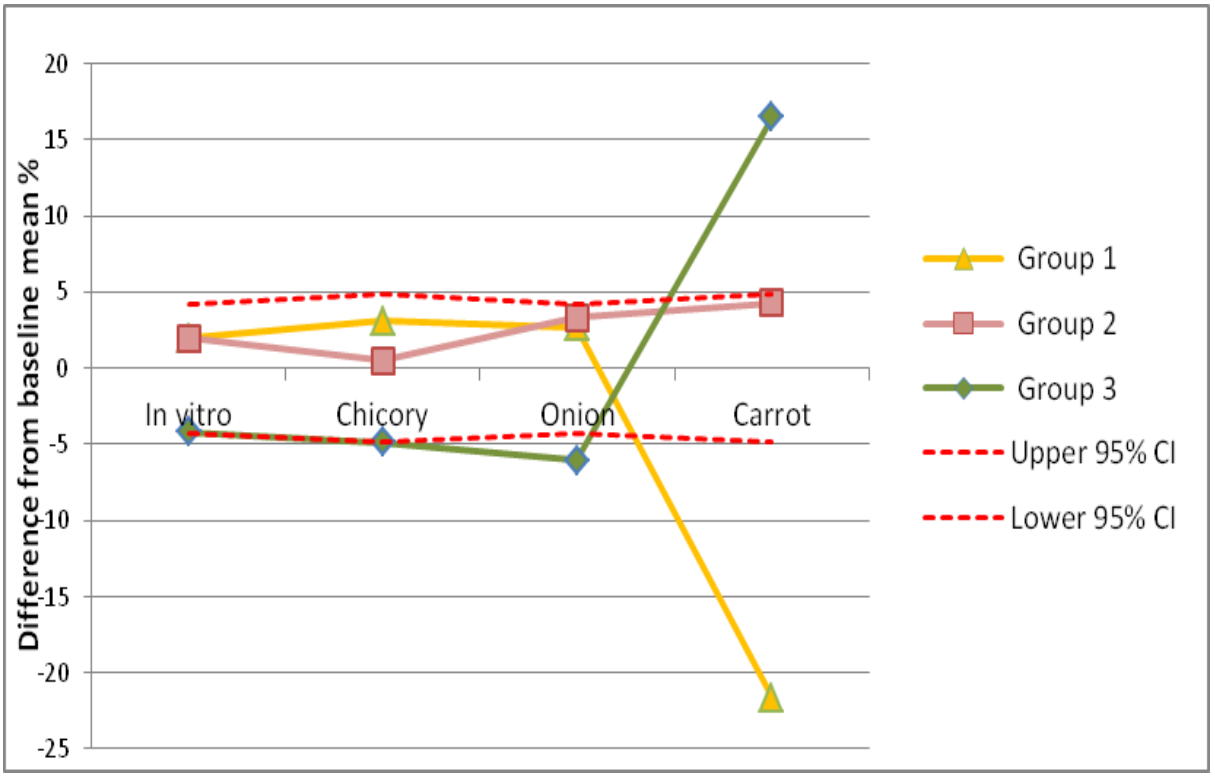



Figure 6.9. Comparison of each variant sequence group (phylogenetic groups 1-3, fig. 6.6) from the basline sequnce frequency of the *in vitro* culture at time point zero. The difference of the sequence frequencies at each treatment condition are compared to the baseline sequence relative frequencies calculated as predicted mean percentage of the total sequences forming >0.3% of the returned *R.clarus* sequences . Upper and lower limits taken from LSD values at 5% from each condition from REML analysis.

The graph below shows the sum total difference of all 3 sequence types from basline values expressed as predicted mean percentage of the total sequences forming >0.3% of the returned *R. clarus* sequences. It is possible to see that the *in vitro* and chicory culture conditions have the most similar total relative sequence distribution whereas onion and carrot are furthest away. This is particularly interesting as the *in vitro* is similar and *in vitro* host is chicory (Fig. 6.10).

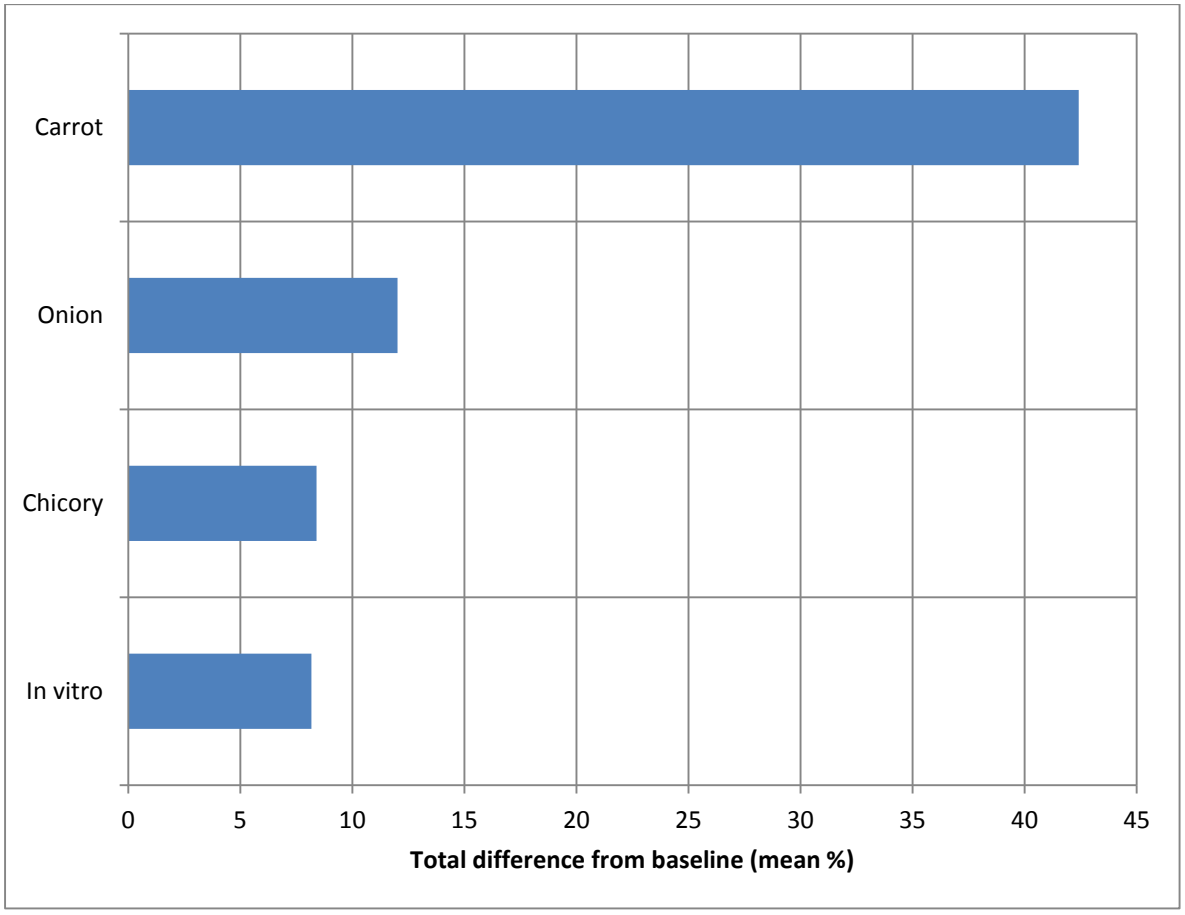



Figure 6.10. Graph showing the sum total difference of all 3 sequence types from basline values expressed as as predicted mean percentage of the total sequences forming >0.3% of the returned *R. clarus* sequences.

## 6.4 Discussion

The culture and production of AMF by *in vitro* methods is becomingly increasingly common and important, with limited knowledge of how this affects the phenotypic traits or genetics of these fungi (Behm and Kiers, 2014). The aim of this study was to understand how culture conditions affect sequence diversity. We have demonstrated that culture conditions, such as a change from *in vitro* culture to *in planta* culture, or a change of host plant species, can

potentially affect frequencies of sequence variants. This is the first study using NGS to assess sequence variation between *in vitro* and *in planta* culture conditions. The sequence frequencies found in *R. clarus* grown in three different plants were different from one another, suggesting host plant affects sequence frequencies. Indeed, the sequence diversity found in carrot was very different than that found on chicory and onion. These differences were also much greater than the relative differences between *R. clarus* grown on chicory *in vitro* or *in planta* suggesting that culture method has little effect on sequence frequency.

In this study we chose to use multiple spore line cultures and multiple spore extractions in an attempt to examine all genetic variation occurring. Behm and Kiers (2014) proposed that a single spore line would be preferable to examine phenotypic plasticity. Single spore isolates are time-consuming to establish, however, and recent studies examining genetic variation (Angelard *et al.*, 2013; Boon *et al.*, 2013; Ehinger *et al.*, 2012) have shown genetic segregation and between spore variation within single spore isolates. Both inter- and intra-spore variation within a single isolate of *Diversispora* was observed (Thiéry *et al.*, 2012), which led to the recommendation that multiple spores should be used to study sequence variation within isolates. Boon *et al.* (2013) shows that individual spores do not contain the same level of allelic diversity, and that there was a loss of total allelic diversity in individual spores. By using multiple spore cultures, and thus a pool from the population, it was anticipated that increased sequence variation occurring within each culture would be included, as would be the case under standard growth conditions. The rRNA genes are found in high copy number and were used here as they contain a high degree of variation (Simon *et al.*, 1993; Wyss and Bonfante, 1993) as well as covering more conserved rDNA genes, and they have good coverage in reference databases (Lindahl *et al.*, 2013). The ITS 1 region is likely to have a higher sequence diversity than the SSU or LSU (Thiéry *et al.*, 2012).

### 6.4.1 Success of the Illumina method

The NGS Illumina method of sequencing used in this study was successful in that it returned large numbers of sequences with OTUs that aligned with other *R. clarus* sequences in the UNITE fungal database and GenBank. A phylogenetic analysis was carried out to confirm taxonomic positioning of these sequences. The sequences obtained using the Illumina technology resulted in sequence polymorphism which is comparable to that found in many other studies involving the rRNA genes of a range of isolates of AMF (Clapp *et al.*, 2001; Kuhn *et al.*, 2001; Oliveira *et al.*, 2010; Rodriguez *et al.*, 2005) using traditional cloning and Sanger sequencing methods. A recent study using NGS demonstrated that pyrosequencing and Sanger sequencing amplified the same dominant sequences in the PLS gene of *G. etunicatum* (Boon *et al.*, 2013). This study shows that NGS technologies are a valuable addition to the repertoire of techniques and can enable a greater insight into these complex organisms. Vast numbers of sequences can be recovered, rapidly and fairly affordably, increasing the likelihood of capturing infrequent sequences, without a time-consuming cloning step (Öpik *et al.*, 2009). Earlier methods of NGS were very limited in their allowance for replication, however, with improving technologies that offer greater sequencing depth at a lower price (Schmidt *et al.*, 2013), NGS will feature significantly more in ecological research in the near future. The sequencing depth was generally good with > 3000 sequences with OTUs aligning with *R. clarus* for each sample from the biological replicates. Only three samples, AM3, AM5 and AM9, had poor sequences aligning to *R. clarus.* This level of sequencing depth is questionable for the validity of the frequencies of these low abundance sequences and they were discounted from statistical analysis. Smith and Peay (2014) reviewed the importance of depth of sequencing and the results of this study also showed that these biological replicates with low depth reads did not show such high repeatability.

### 6.4.2 Sequence polymorphism

Multiple sequence types were observed in the partial SSU, ITS 1 and the partial 5.8S region analysed in this study. These sequence variants were shown to occur with unequal frequencies, which is concurrent with previous studies that have carried out extensive sequencing of rRNA genes, using traditional Sanger sequencing (Clapp *et al.*, 2001; Oliveira *et al.*, 2010; Robinson, 2005; Rodriguez *et al.*, 2005). Prior to phylogenetic clustering, of those sequences that formed more than 0.1% of the OTUs returned for *R. clarus,* between 6 and 10 sequence variant OTUs were observed at each sample. This is in agreement with previous reports of sequence variation in rRNA genes in different AMF species. A greater number of sequence variants were obtained from those spores taken from the plant treatments. Most sequence polymorphism occurred in the ITS 1 and the 5.8S regions.

Three major sequence variants were observed and dominated all samples from every culture condition. Less frequent sequence types were seen to group phylogenetically in clades around these dominant sequences, with the exception of a fourth clade of less frequent sequences which formed an outgroup. This out-group appears to have derived before the divergence of sequence type 3 from sequence type 1. Sequence type 2 is likely to have diverged from the other sequence types first, as it was the most distant group. Sequences have low numbers of SNPs (sometimes as little as 2 bps) separating them within each clade. Due to the potential error rate in base calling likely with this technology, and especially in the highly repetitive ITS regions (Lindahl *et al.*, 2013; Schmidt *et al.*, 2013), it is unclear if these SNPs are due to error or true sequence polymorphism occurring within the genomes of these cultures occurring at individual culture conditions. Indeed, this is the main reason why we excluded those sequence variants with a very low frequencies (< 0.3%) from subsequent analysis. By analysing phylogenetic clades of sequences, rather than individual sequences, a more conservative approach to sequencing error is achieved, although, far greater sequence polymorphism may be present than in this analysis of sequence polymorphism frequency.

Analysis of the frequency of these four groups of sequences revealed that they did occur at different frequencies within the samples analysed, and that the frequency of dominant sequences may vary with the culture condition. After a 2-year period of growth at each condition, the frequencies of these four groups of sequences, in certain culture conditions, changed significantly from those initial frequencies in the baseline samples. The fourth outgroup of low frequency sequences did not occur in the spores taken from the baseline, and after 2 years no significant differences were found between treatment conditions between these low frequency sequences. Low numbers of biological replicates were used for this study, due to the economic limitations of NGS, and the sequencing of some of these replicates gave very low depths that required their removal from analysis. To fully confirm these results, further samples need to be analysed. This outgroup of sequences maybe an important divergence of sequences that was not detected in all samples due to its low number.

### 6.4.3 Mechanisms of phenotypic change

AMF colonise a wide range of hosts and habitats, and thus how they thrive and rapidly adapt to such a wide range of habitats is an important question, especially in a clonally-reproducing organism. Angelard *et al*. (2013) suggest that this is possibly achieved by separation of genetic information among different nucleotypes, with some nuclei favoured under certain host conditions enabling a potential for AMF to adapt within its lifetime.

The mechanism for phenotypic changes within isolates is still unclear and is out of the scope of this study. The genetic structure of AMF remains uncertain, and, without a definitive understanding of whether AMF are homo- or heterokaryotic, this question remains very difficult to answer. Behm and Kiers (2014), in their recent framework for assessing

intraspecific variation, suggested three possible mechanisms for phenotypic plasticity: either by genes producing different phenotypes accordingly from either the same or different nuclei with nuclear frequencies remaining unchanged, or local adaption whereby selection for different genes results in nuclei frequency changes.

From this work there is a potential that the frequency of individual sequence types may be preferentially selected, but the mechanism (or reason) for this is unknown. In a heterokaryotic system, selection for individual nuclei would be a possible method, however, single nucleus genome sequencing of *R. irregularis* suggests a homokaryotic organisation of nuclei (Lin *et al.*, 2014). It is likely that changes in the frequency of sequence types within a culture lineage may be the basis for phenotypic plasticity.

### 6.4.4 Adaption to environment

How AMF change in response to their environment is important for understanding their role in providing host benefit. Different isolates of the same species have been shown to differ in their phenotypic characteristics (Munkvold *et al.*, 2004). Isolates of AMF grown and produced in *in vitro* culture may well have different genetic and phenotypic traits from those grow *in planta* which need to be further investigated given the current trend for increasing *in vitro* production. This study suggests that there is a potential for plasticity within the *R. clarus* isolate to change with abiotic and biotic conditions. Angelard *et al.* (2013) observed a change in allele frequency as a rapid response to a change of host plant, however the change varied between segregated lines. These authors showed this change over a large number of alleles and that nucleotype frequency altered the phenotypic characteristic, such as spore density, in single spore lines.

It is likely that culturing these fungi under very specific laboratory culture (*in vitro* or *in planta*) will have an effect on the phenotype and the genetic drift of these fungi. *In vitro* culture entails a selection of spores at each sub-culture event, and as such an inconsistency between cultures may arise even from the same lines. This may prove problematic for consistency in studies, even of model fungi and for *in vitro* produced inoculum. Producers of AMF inoculum need to consider the need to maintain genetic diversity in production to enable isolates to adapt to their target environment. Clearly for mass production of these fungi this effect needs to be fully understood when considering large scale application of AMF inoculum.

In this study the *in vitro* line was different from the baseline after 2 years. This could be due to the selection of spores at each subculture event and thus a genetic drift could always be occurring in any continuous culture of AMF. Here, however, evidence is provided that a change in the method of culture or host species promotes an increased shift in the frequency of different sequence groups.

## 6.5 Conclusion

This study has identified, in one set of genes that are known to be highly variable, that changes occur in the frequency of dominant sequence variants, when AMF are cultured under different culture conditions or on different host plants. Host plants seem to have the greater influence. Further work is required to confirm the results and to fully understand the mechanisms that are responsible for this change and how these changes relate to phenotypic traits of these fungi.

With the recent sequencing of the *R. irregularis* genome (Tisserant *et al.*, 2013), a new source of genetic data is available to researchers of AMF, and with new technologies such as NGS,

some of the many unknowns about these ancient organisms may finally be answered. New NGS methods offer far higher resolution and magnitude of sequencing (Lindahl *et al.*, 2013), which will lead to far increased understanding of mycorrhizal genomics and fungal ecology. However there are issues with these methods, such as the sequencing error artefacts and the huge challenge to bioinformatics and computing methodologies (Wooley *et al.*, 2010).

# Chapter 7. Discussion

## 7.1 Overview

The aims of this study were:

To develop a suitable molecular tool to quantify the relative abundance of different AMF species within a single root system and to use this to investigate the following null hypotheses:
The differences in the relative abundance of fungal species will have no effect on the host, e.g. plant health, water uptake and growth.
Environmental conditions, predominantly water stress, will have no effect on the composition of AMF.
There will be no effect from different culture conditions on the sequence diversity abundance in the rRNA genes.

During the course of the investigation, a system to examine how different species interact within a single root system and under varying environmental conditions was developed. This system has been used to examine how these environmental conditions affect individual, and mixed species within a system and its effect on the host plant. By studying the genetic basis of how culture conditions and host plant changes can affect a single species at a genetic level, the importance of biotic and abiotic changes to this organism can be seen.

### 7.1.1 Development of a molecular tool to quantify the relative abundance of different AMF species within a single root system.

A successful method of reliably amplifying and quantifying 2 species of AMF in a single root system was developed (Chapter 3). Species-specific primers were designed for *F. mosseae* and *F. geosporus* that were also tested for specificity against a background of other species (*G.*

*microaggregatum, R. irregularis* and *C. claroideum*). With a carefully selected target region, and a large amount number of sequences publically available, it was possible to design primers for these two fairly closely related species that were usable within a qPCR system. This was the key to success for the overall project. The qPCR method used in this study enabled the interaction to be studied between two AMF species within a single strawberry root system and as such, the results from Chapters 3 and 4 demonstrate the benefit of using a specific qPCR method for such analysis. The qPCR method gave good, consistently reliable amplification and was a rapid method for the assessment of multiple samples.

This is the first study to our knowledge to compare the results from microscopic quantification analysis with qPCR analysis on an individual plant by plant basis, comparing the correlations of individual isolates in the separate treatments. This study clearly shows that the amount of correlation differed greatly according to individual species and their response in a single or mixed inoculation. Thonar *et al.* (2012) suggest that qPCR as a tool could potentially replace other traditional methods of estimates of root colonisation. The results presented here show that using qPCR alone to have a understanding of the complex interactions between species underground is not a comprehensive method and that further work is required to fully understand how qPCR data relate, not only to traditional estimates of root colonisation, but also to the vitality and physical traits of AMF structures within the root system (Krak *et al.*, 2012). It is likely that no single method will give a full insight into complexities of a below ground ecosystem.

### 7.1.2 The effects of environmental conditions on the composition and relative abundance of AMF.

Many studies have looked at species diversity in ecosystems and the effects and benefits of single AMF species to plants. New techniques such as qPCR now make it possible to study the relative abundance of individual species and how these affect plant hosts. It is becoming increasingly evident that abiotic conditions are, to a large extent, responsible for observed changes in relative abundance of AMF species within a root system. These include soil conditions (Hazard *et al.*, 2013; Wagg *et al.*, 2011a), phosphorus and saline stress (Alkan *et al.*, 2006), and temporal changes such as temperature and sunshine hours (Dumbrell *et al.*, 2011).

This work (Chapter 4) used the qPCR system, developed in Chapter 3, to show that both *F. mosseae* and *F. geosporus* colonised the roots of strawberry plants. Co-occupation of the same root by both species was shown to commonly occur in the ~50:50 ratio that was applied as inoculum, and as such little competitive effect between the two species was shown**.** The results presented here, however, show that a change of environmental conditions (i.e. drought stress) affects the relative abundance of the two species in the mixed inoculum treatment. *Funneliformis mosseae* became more abundant only under water-stressed conditions, relative to *F. geosporus,* suggesting that *F.mosseae* may be more favoured, and thus more beneficial to the host under water stress. These results show that even small changes to abiotic factors can cause a shift in relative abundance of these two species. Wagg *et al.* (2011a) also demonstrated that individual AM fungal species could demonstrate both complementarity and antagonism when put in different abiotic situations and by the addition of extra species.

With the widespread use of qPCR techniques researchers have shown different interactions between isolates and species, however, as shown here these interactions may change under different environmental conditions and that the AMF/host relationship is fluid. Many studies

only examine the benefit of single species to plants under controlled conditions. The present study demonstrates the need, when studying the effects of AMF, to consider a range of environmental situations and species combinations to have a clear picture of the effect to host plants.

### 7.1.3 The effect of the relative abundance of fungal species on host parameters.

The results (Chapter 5) showed that inoculation with either *F. mosseae* BEG25, *F. geosporus* BEG11 gave equal benefit to the host, showing increased plant growth, yield and tolerance to water. Treatment with a mixed inoculation (regardless of the relative abundance of species present) did not show any increased benefit (or deleterious) effect in strawberry regardless of the irrigation regime, even though the finding in Chapter 3 showed that the relative abundance of *F. mosseae* and *F. geosporus* changed under varying environmental factors. These results suggest that AMF colonisation is of greater importance than species present and their relative abundance. However it should be noted that this is only for these species studied and may be different for other species, on other host plants, especially if those species were more taxonomically distant. The increased dominance of *F. mosseae* under the mixed inoculum and water-stressed conditions did not have a significant impact on the physical traits measured here.

This study demonstrated a benefit of using AMF in strawberry production, providing increased yield and growth. It also showed that AMF inoculation of plants subjected to a reduced irrigation of up to 40% restored plant growth to the same or higher values as the non-mycorrhizal, fully-watered plants. The water use efficiency of plants inoculated with AMF was significantly higher than the control plants suggesting that the benefit from AMF is not only concerned with direct uptake of water via hyphae. Smith *et al.* (2010) suggest that other benefits of the AMF symbiosis help with this drought tolerance such as by increasing plant

nutrition and health and affecting soil structure via hyphal spread and production or by regulating aquaporin expression. These results are important for crop production given that water is a key resource that is becoming limited and expensive in horticulture and agriculture. The 2030 Water Resources Group predicts that industrial use of water will almost double by 2030, and with global change, the frequency and severity of global scale drought will increase (Fitter, 2012).

In Chapter 5, an increase in plant growth was shown to be directly influenced by an increase in % root colonisation by AMF, which was not dependent on the inoculation treatment (single species or mixed) when individual plants were examined. These results are important as they show that AMF inoculation is of greater importance to strawberry growth and drought tolerance than AMF species abundance. It was also shown that AMF species can be synergistic within a single root system and that an increased spectrum of benefits may be gained if certain environmental situations arise.

Therefore the addition of more species in an inoculum may be of greater benefit than single species alone as different species may become more favourable only under certain conditions. If mixed species inocula are used, however, it is important to examine the interactions between other species to be sure that there are no deleterious effects. Kiers *et al.* (2011) have recently shown that different species may both survive together by employing different mechanisms to benefit the plant in different ways and as such multiple species occupancy of plants may provide a wider spectrum of benefits than colonisation by a single isolate (Alkan *et al.*, 2006).

### 7.1.4 The effect of different culture conditions on sequence diversity in the rRNA genes.

Next generation sequencing was successfully used to analyse multiple sequences within a culture of *Rhizophagus clarus* (Chapter 6). The occurrence of multiple variant sequences within the rRNA genes of the AMF is now widely accepted and the results from Illumina sequencing showed a similar level of sequence variation to that found in traditional cloning and Sanger sequencing. The NGS carried out in this study generated three major sequence variants that were the most common sequence variants in all conditions. Other sequences grouped phylogenetically about these three major sequence types along with one outgroup of less common sequence types. By maintaining this isolate for two years under different culture conditions of *in vitro* (with chicory roots) and *in planta* (with chicory, onion, or carrot), changes in the frequency of dominant sequence variants occurred. Thus culture condition and host plant changes do have some effect on the relative abundance of multiple sequences in AMF.

Although the occurrence of multiple sequences is now widely accepted, the mechanisms behind these changes remain unclear. Until recently there was little information on what affected these sequences and if a change occurred at a genetic level (sequence diversity abundance) in response to environmental or host changes. Recent work (Ehinger *et al.*, 2012) found differences to occur between single spore lines and Angelard *et al.* (2013) showed that a change of host plant to effected ALFP patterns in single spores lines of *R. iregularis*. Here, for the first time, it has been shown that culture condition and host plant can affect the sequence diversity and relative frequency of sequence types. This work is important as it demonstrates that isolates of AMF can change under different conditions and in long term culture at a genotypic level, which in turn is likely to have an effect on the phenotype of the fungi. We showed a rapid genetic change in response to a particular host environment condition which must be an important consideration in both the study and use of AMF. This rapid change in genetic structure provides a mechanism whereby a potentially asexual organism can remain strong in rapidly changing environments, and it is likely that these

organisms have adapted these unique strategies to facilitate their survival. Here only one species as a single culture was used, however the results in Chapter 4 showed the interactions between species are important. The metagenomic techniques used here could be readily applied to looking at the interactions between AMF species and other hosts.

## 7.2 Impact on AMF research

This work has shown how molecular tools can be used successfully in studying AMF and their genomic structure. The use of qPCR has become more widely used for the study of AMF during the course of this project and as such will lead to greater advances in the understanding of AMF species interactions with their hosts and environments. Advances in platform chemistries and reduction of costs involved in NGS allow this technique to be employed in a wide range of uses on the quest to understand AMF community diversity, interactions, environmental changes and genomics.

The results in Chapters 4 and 5 demonstrate that environmental conditions can affect the interaction between species. In natural systems it is very rare that single species would be found alone. Studies using mixed communities should be considered where possible as this reflects a more realistic situation, and these interactions may have important implications to individual species. These results show that overall colonisation levels and abundance of species can be more important than species diversity, and as such should be considered in future studies. For studies looking at best application methods for field trials it is important to study which combinations of species are synergistic to provide enhanced host benefit, and the effect that any applied species are likely to have on native populations of AMF.

Chapter 6 demonstrates sequence heterogeneity in relation to culture condition and host. In this project sequence variants were carefully taken into consideration for primer design,

however this is not always the case. Here it was shown that dominance of sequence types can change according to different situations and if primers do not amplify all sequence variants then this could significantly affect studies examining a quantitative estimate of abundance. These NGS results give a further understanding of the multi-copy rRNA genes and that these multiple sequences need to be considered in quantitative assessments of AMF.

## 7.3 Implications for inoculum production

This study showed the need for a better understanding of the complex interactions of these fungi with different hosts and different environments in order to implement the best methods for application in large scale crop production and for the production of commercial inoculum. Alkan *et al.* (2006) suggest that, under natural conditions, multiple occupancy of roots would be of greater benefit to a plant than a single AMF isolate and suggested that a combined approach may lead to the plant harnessing a wider spectrum of benefits. In this study there was no deleterious effect to either species (*F.mosseae* or *F. geosporus*) by having the other present. As some abiotic conditions (such as drought in this study) may favour certain species, the application of more than one species could potentially be more beneficial to the plant. If selectivity is occurring, an increased AMF species richness will raise the possibility that one of these species will be more effective in a given situation.

The large scale production of AMF inoculum has recently become more commonly achieved using *in vitro* methods. This has many advantages for inoculum production, both economically and for the contaminant-free manner in which it is produced. This type of production however is limited to only a very few species, primarily *R. irregularis*. As such most of these inoculum products only contain single species. This move to a less diverse inoculum, and the introduction of a single species dominance, needs to be better understood for the future use, to ensure the best method for introduction of these fungi into crop production.

This study found *F. mosseae* colonised a greater proportion of the root from the mixed inoculum under drought conditions. As isolates and species can be favoured under specific conditions, these results demonstrated the importance of growing isolates individually for large scale inoculum production, thus preventing the dominance of one single isolate in any mixed species product.

The method of cultivation was shown here to affect the relative sequence abundance within a single AMF. Producers need to be aware that any long term continuous production methods could alter these fungi at a genomic level. A good level of diversity needs to be maintained in production cultures, and as such keeping isolates for many years in the same conditions/host plants, or in long term *in vitro* culture may genetically alter these isolates. Angelard *et al.* (2013) showed genetic drift happens in any subculture line with spore selection taking place, and as such any multispore culture is likely to change over time. This genetic change needs to be examined in relation to phenotypes.

## 7.4 Applied application of AMF into horticulture and agriculture systems

The green revolution dramatically increased global crop production and lead to intensive farming practises that are highly dependent on synthetic NPK fertilisers and pesticides. This level of high intensity agriculture is no longer sustainable primarily due to energy costs of N fertilisers and the decreasing supplies of P (Cordell *et al.*, 2009), along with a decreasing armoury of pesticides (due to EU and global legislation) and water limitation. The importance of finding new ways of meeting future food requirements is now evident on a global scale. In this study, the importance of AMF inoculation has been shown both the growth and yield of an economically important crop, strawberry, and its beneficial effect in improving tolerance

to water stress. Inoculation with any combination of AMF was shown here to significantly help plants to tolerate water stress including complete drought conditions, such as irrigation failure.

Mycorrhizal fungi could provide an important contribution to sustainable agriculture and horticulture; however a significant change in the perception of growers and governmental policy makers is required to enable this change to happen. The work carried out in this study demonstrates that the complex nature of AMF has to be carefully considered in order to apply AMF in large scale horticulture and agriculture. The fluid nature of the interactions between these species and their changing genomic structure has implications for the use of AMF and their large scale production. What is true in one situation between a plant and its host may well not be the case in another situation with changes in biotic and abiotic factors. As Smith *et al.* (2010) highlight, it may not be a simple case of AMF providing the plant with nutrients and water, as there are many other benefits to the plant that come from engaging in this symbiosis, such as tolerance to pathogens and the effects on soil structure.

Temporal changes should be taken into account when studying AMF in field situations. This study shows that a change of condition can affect the amount of colonisation and relative abundance of species present. Therefore if isolates and native field populations are being tracked using molecular techniques, single time point studies are not a true reflection of the dynamics of the interaction.

When introducing new species into field situations the effect that these will have on the native populations of AMF need to be considered. Here the effect of combining 2 species, *F. mosseae* and *F. geosporus,* did not affect the relative abundance of either species until an

environmental effect (drought stress) was present. Other species, however, may have different effects and interactions and the effect of commercially produced inoculum may adversely affect native populations. There is recent research to suggest that mixed species AMF inoculants promoted plant growth without affecting subsequent non-resident inoculants (Jin *et al.*, 2013), but this needs to be considered in studies involving a number of biotic and abiotic treatments .

## 7.5 Future work

We have developed a molecular method that allows the study of the relative abundance of different AMF species on strawberry. This protocol could easily be modified to investigate the interaction between other species of AMF and other cultivars of strawberry, in different growing conditions or with different stresses such as pathogen or pest attack. The methods used here could be applied to other important horticulture crops. As only two species of AMF were included in the interaction studies here, further work should include a greater number of species, including those that are less similar phylogenetically. The primers designed have also been tested for specificity against *R. irregularis* and *G. microaggregatum* (and by alignment with *C. claroideum*) and as such these could be used for a future study. Both of the species used in this study conferred a similar effect on the plant, however, it would be interesting to include a more 'cooperative' or 'effective' species such as *R. irregularis* (Kiers *et al.*, 2011).

Assessment of AMF rarely includes how fungal activity changes over time (Dumbrell *et al.*, 2011; Shi *et al.*, 2012), and most studies are therefore only a snapshot of any situation. In this study, like many others, only a single analysis of the interactions taking place was carried out at the end point of the experiment. Temporal variations in rates and abundance of colonisation occur in different AMF situations can occur and given the disparity between the

two quantification techniques (traditional RLC and qPCR) these interactions are likely to occur throughout any experiment. It is likely that species that are important in early colonisation are not always important as the plant grows and abiotic conditions change. It would be interesting to look at the dynamics within the strawberry root, and how they change over time, and future studies should include time course sampling, over a longer term and in strawberry into a second season of growth to observe the longer term interaction between these species.

The work on the effect of culture conditions on multiple sequences is very much the start of investigations needed to understand the influence of environment on the genomic structure of AMF. Initially the treatment replicates that gave very low numbers of returned sequences need to be re-run or other replicated DNA extractions from those conditions need to be sequenced using the Illumina method. Experiments designed using greater numbers of replicates is now possible due to the increasing availability and decreasing costs of NGS technology. The changes seen in multiple sequences may lead to phenotypic changes in cultures and should be analysed for trait and gene expression variation within isolates (Behm and Kiers, 2014). Possible effects may include physiological traits such as spore density, arbuscule density and extent of intra- and extraradical hyphae. A time-point experiment may also provide clues as to the speed that these changes take place to give a better indication of the possible mechanisms for this adaption.

It may be of interest to study sequence variation change in other regions of the genome such as functional genes. With the advances in genome knowledge now available from the *R. irregularis* genome project, it may be possible to identify genes that have specific functions for AMF growth. This assessment of sequence variation change could then target functionally

important genes involved with certain traits to test levels of sequence diversity and gene expression and how these change with according to environment.

# Chapter 8. References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# Appendix 1, General recipes.

## SMR rooting agar

Murashige and Skoog media (Murashige and Skoog, 1962)

For 1 litre:

| | |
|---|---|
| 4.41g | M & S basal salt mixture (Sigma Aldrich, UK) |
| 8.0mls | Indole-3-butyric acid (Sigma Aldrich, UK) |
| 1.2mls | Gibberellic acid (Sigma Aldrich, UK) |
| 30g | Sucrose |
| 7.5g | Agar (Oxoid 3, Oxoid, UK) |

pH to 5.6

## Medium M (Becard and Fortin, 1988).

Stock solutions are made and stored as stated

| Stock solutions | Ingredients | Molarity (mM) | Quantity ($gl^{-1}$) | Additional information |
|---|---|---|---|---|
| **$Ca(NO_3)_2$** | $Ca(NO_3)_2$ | 175.61 | 28.8 | Stored at 4 °C for up to 3 months |
| **Macro-elements** | $MgSO_4 . 7H_2O$ | 297 | 73.1 | Stored at 4 °C for up to 3 months |
| | $KNO_3$ | 79 | 8 | |
| | KCl | 87.2 | 6.5 | |
| | $KH_2PO_4$ | 3.53 | 0.48 | |
| **Micro-elements** | $MnCl_2 . 4H_2O$ | 3.03 | 0.6 | Stored at 4 °C for up to 3 months |
| | $ZnSO_4 . 7H_2O$ | 0.923 | 0.265 | |
| | $H_3BO_3$ | 2.42 | 0.15 | |
| | $CuSO_4 . 5H_2O$ | 0.052 | 0.013 | |
| | $Na_2MoO_4 . 2H_2O$ | 0.001 | 0.00024 | |
| **KI** | KI | 0.452 | 0.075 | Stored at 4 °C for up to 3 months |
| **NaFeEDTA** | $FeSO_4 . 7H_2O$ | 11.021 | 2.785 | Heated to 60 °C until dissolved, stored at 4 °C for up to 3 months |
| | $Na_2EDTA$ | 10 | 3.725 | |
| **Vitamins** | Glycine | | 0.3 | Stored at -20 °C for up to 6 months |
| | Thiamine hydrochloride | | 0.01 | |
| | Pyridoxine | | 0.05 | |
| | Nicotinic acid | | 0.05 | |
| | Myo-inositol | | 5 | |

Medium M is made as required.

| Stock solutions | Concentration (%) | Additional information |
|---|---|---|
| **$Ca(NO_3)_2$** | 16.9 | pH 5.5 adjusted with KOH before adding the Phytagel |
| **Macro-elements** | 16.9 | |
| **Micro-elements** | 16.9 | |
| **KI** | 16.9 | Omit addition of sucrose to hyphal distal compartment |
| **NaFeEDTA** | 2 | |
| **Vitamins** | 8.4 | |
| **Sucrose** | 16.9 | |
| **Phytagel** | 5.1 | |

**Citrate buffer**

9.0 ml of 0.1 M Citric Acid

41.0 ml of 0.1M NaCitrate, dihydrate

bring final volume to 500 ml with glass distilled water

pH to 6.0 with 5N NaOH

**General PCR protocol**

Each 20 µl reaction contained;

2.0 µl GeneAMP 10X buffer II (Applied Biosystems, Cheshire, UK)

1.6 µl of $MgCl_2$ (25 mM)

1.6 µl dNTP (2.5 mM)

2.0 µl of each primer (2 µM)

0.1 µl AmpliTaq DNA polymerase (Applied Biosystems, Cheshire, UK)

8.7 µl of water (Sigma, UK)

2.0 µl of DNA template.

# Appendix 2, publication

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Mini-review

# Shifting the balance from qualitative to quantitative analysis of arbuscular mycorrhizal communities in field soils

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ABSTRACT

Population studies of arbuscular mycorrhizal fungi (AMF) have traditionally been achieved by indirect analyses of soil-borne spore populations. These studies are not necessarily reflective of populations of AMF within the roots. Advances in molecular biology have revolutionized the analysis of fungal populations colonizing roots and forming mycorrhizas. Initially these studies were qualitative and reported presence or absence of particular AMF species in soils or in roots for comparison between different environments. More recently, the methodology has developed for direct quantification of AMF within roots. Quantitative PCR provides the means to study spatial distribution and individual quantification of AMF in mixed communities over time. In this review, we discuss the progress and application of indirect, direct and finally quantitative methodologies for studying arbuscular mycorrhizal communities. We conclude that the molecular tools now exist to quantitatively analyse the effect of environment, management or inoculation of soils on AMF communities within roots.



## Introduction

Arbuscular mycorrhizal fungi (AMF), phylum *Glomeromycota*, form one of the most common and oldest symbiotic associations on Earth. They can colonize over 80 % of terrestrial plants (Smith & Read 1997). Among the different types of mycorrhiza, arbuscular mycorrhizas (AM) are characterised by intracellular arbuscules in the cortex of the root (Fig 1).

Arbuscular mycorrhizas are geographically ubiquitous (Reddy *et al.* 2005) and improve plant growth and nutrient uptake, especially phosphorus absorption (Smith & Read 1997) and, in some cases, nitrogen transfer (Govindarajulu *et al.* 2005). They protect plants from pathogens (Borowicz 2001) and buffer against adverse environmental conditions. The external mycelium of AMF improves soil structure by formation of soil aggregates (Rilling 2004; Van der Heijden *et al.* 2006). Increased diversity of AMF positively affects plant biodiversity, variability and productivity (Van der Heijden *et al.* 1998b; O'Connor *et al.* 2002; Van der Heijden 2002). Van der Heijden *et al.* (1998a) reported that AMF are required to maintain a basic level of plant biodiversity and that the structure of plant communities varied significantly with treatments that included different AMF taxa. Reduction from four AMF strains to one within a model ecosystem led to a decrease in biomass of several plant species suggesting AMF communities are important ecosystem components and need to be protected. The mechanism by which plant diversity and species composition is regulated is unknown, but is a key issue to successful management for conservation and restoration of natural ecosystems. Below-ground biological

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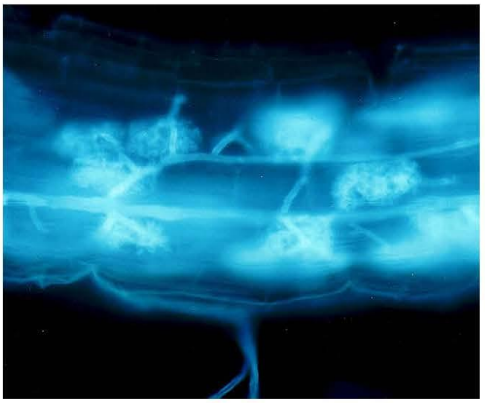

**Fig 1 – Arbuscules and mycelium of *Glomus coronatum* BEG49 fluoresce inside maize roots when stained by calcofluor white.**

systems need the same care as above-ground systems because AM are essential in sustainable land management practices. It is important to know what species are put into the environment as well as monitoring their growth inside the roots; how the community structure is changing during the restoration period; which species are significant and at what time. The beneficial effects of AM fungi on plant growth have led to the development of AMF as bioinoculants for forestry, agriculture and horticulture.

Field studies of arbuscular mycorrhizal communities have taken a range of approaches, either using *indirect* methods such as isolation of spores from soil or from trap cultures, or *direct* methods *via* isolation of fungal DNA from roots (Table 1). Isolating spores from soils can be criticised as it reflects the sporulation stage of the AMF life cycle (a dormant phase) rather than activity within a symbiosis. However, the method is rapid, and it may reveal taxa not easily detected by molecular methods providing the user has reasonable taxonomic expertise. Intensive soil sampling, including the use of successive trap culturing, takes much longer but reveals significantly greater diversity than a 'one-off' spore extraction. Molecular biology has now revolutionized the analysis of fungal populations colonizing the roots and forming mycorrhizas (Clapp *et al.* 2002a). Results indicate that AMF present in soils as spores may be rarely found in roots of native plants in field surveys or *vice versa* reflecting the fact that not all AMF present as spores form active mycorrhizas and many taxa present in the roots may not be sporulating. Molecular analyses, however, rely on primers that will detect all the target organisms, and the widely-used AM1 primer for example, only detects members of the Glomerales and Diversisporales. Although earlier primers varied in specificity, Redecker (2000) designed SSU/ITS primers specific for all 5 families of the Glomeromycota, although some anomalies have been reported (e.g. Wubet *et al.* 2003). Taxon-specific primers are useful when a measure of species richness is required but identification by sequence relies on a large and correct database and mis-identifications can occur, as in the case of *Acaulospora colossica* (Pringle *et al.* 2003), if the database is not large enough or is not interrogated appropriately (Clapp *et al.* 2002b). A further complication is the existence of multiple sequence variants within single spores of many AMF; intensive study of within-spore sequence variation of commonly-targeted genomic regions, such as the ITS region, within a single isolate can match the world-wide variation existing across a range of isolates of the same species. This phenomenon is now well-described, if still controversial with respect to the origin of variation (Pawlowska & Taylor 2004; Hijri & Sanders 2005). Molecular analyses have shown that there is considerable genetic diversity within morphologically recognisable species within the Glomeromycota, and a recent authoritative review suggests that there is currently no formalised operational species concept in this group (Rosendahl 2008). Most recent analyses cluster sequence data into 'species groups' or 'phylogenetic clusters'. These molecular approaches indicate qualitative presence of the respective AMF but are not quantitative. More recently, real time or quantitative PCR (q-PCR) methodology has been applied to AMF studies and initial results have shown the potential to move into a new phase of *quantification* of changes in abundance of individual taxa within roots over time.

## Analysis of spore populations

These studies are based on spores isolated from soil and identified by morphology or sequencing. They record spore presence, not significance in relation to root colonisation. Many such studies have been reported and recent examples are summarised in Table 1. Gai *et al.* (2006) reviewed 20 years of work in China to report a total of 104 species from 9 genera from spore isolations or trap cultures from many soils. These results reflect the world-wide situation in that the frequency of occurrence and abundance of *Glomus* spores were much higher than other genera, with *Glomus mosseae* being reported from over 90 % of sites investigated. Molecular studies confirm a similar situation with regards to root occupancy of cultivated soils.

Soil treatments may influence community analyses based on spores. Franke-Snyder *et al.* (2001) compared spore diversity in a conventional *versus* two low-input farming systems and showed little difference in the community make-up of 15 fungal species identified. In contrast, Jansa *et al.* (2002) showed *Glomus* spp. increased in tilled soils, with 5 genera (17 species) being found using sequence-based identification of DNA extracted from spores from trap cultures. The trap plant species had a significant effect on the community make-up, a potential drawback with this methodology.

An intensive study of diversity in several ecosystems in Central Europe found 41 morphospecies by direct isolation of spores and an additional 4 morphospecies from trap cultures (Oehl *et al.* 2003). Some species were widespread, others more specialised. Increased land usage was correlated with decreased diversity, shifting to those species which colonised roots slowly but sporulated rapidly. Cousins *et al.* (2003) compared 20 urban sampling sites in an arid USA ecosystem to show how previous land use influenced the AMF community. Diversity was lower at sites currently or previously used

**Table 1 – Examples of studies of AMF communities using spore isolation or molecular detection in a range of ecosystems**

| Authors | Indirect/ direct | Method[a] | Target gene | Notes |
|---|---|---|---|---|
| Franke-Snyder *et al.* (2001) | I | S, T | – | 15 species: agricultural soils, USA |
| Dodd *et al.* (2002) | I | S, T | – | 12 species; virgin soil, UK |
| Jansa *et al.* (2002) | I | S, T | ITS | 17 species; agricultural soil, Switzerland |
| Cousins *et al.* (2003) | I | S, T | – | 18 species, 5 genera; urban soils, USA |
| Oehl *et al.* (2003) | I | S, T | – | 45 species; grasslands/arable soils, Central Europe |
| Mathimaran *et al.* (2005) | I | S, T | ITS | 8 *Glomus* species, 1 *Paraglomus*; agricultural soil, Switzerland |
| Oliveira *et al.* (2005) | I | S, T | – | 6 *Glomus* spp.; alkaline waste sediment, Portugal |
| Beuchamp *et al.* (2006) | I | S, T | – | 30 morphospecies, 14 *Glomus*, 11 *Acaulospora*, 3 *Entrophospora*, 1 *Paraglomus*, 1 *Archaeospora* ; *Populus/Salix*, Arizona |
| Schalamuk *et al.* (2006) | I | S | – | 24 species; wheat, Argentina |
| Li *et al.* (2007) | I | S | – | 47 species; 3 fields, China |
| Wang *et al.* (2008) | I | S, T | – | 20 species; 90 soils, China |
| Whitcomb & Stutz (2007) | I | S, T | – | 12+ species; shrub/tree-planted soil, Arizona |
| Helgason *et al.* (1999) | D | R | SSU | 3 genera, 8 sequence groups; bluebell, England |
| Rosendahl & Kjøller (2001) | D | R | LSU | *Glomus*-specific; pea, Denmark |
| Helgason *et al.* (2002) | D | R | SSU | 3 genera, 11 sequence groups; mixed woodland plants, England |
| Vandenkoynhuyse *et al.* (2002) | D | R | SSU | 24 groups; two grass species, Scotland |
| Husband *et al.* (2002b) | D | R | SSU | 30 sequence groups; tropical forest seedlings Panama |
| Moora *et al.* (2004) | D | R | SSU | 20 sequence groups, 4 AMF genera; *Pulsatilla*; Estonia |
| Jansa *et al.* (2003) | D | R | LSU primers specific for certain *Glomus* sp. or *Gigaspora* or *Scutellospora in toto* | *G. claroideum, intraradices, mosseae*; *Gigaspora*, *Scutellospora* |
| Heinemeyer *et al.* (2003) | D | R | SSU | 10 sequence types; native grassland, UK |
| Wubet *et al.* (2003) | D, I | R | ITS | 20 Glomeraceae, 1 Diversisporaceae, 1 Archaeosporaceae; *Prunus*, Ethiopia |
| Rosendahl & Stukenbrock (2004) | D | R | LSU specific *Glomus* clade | 11 clusters from *Glomus*; undisturbed soil, Denmark. Notes multiple sequences |
| Gollote *et al.* (2004) | D | R | D1, D2 LSU generic | *Glomus*, *Scutellospora* and *Acaulospora*; Grass monocultures following undisturbed community |
| Scheublin *et al.* (2004) | D | R | SSU | 15 sequence types, 11 unascribed |
| Wirsel (2004) | D | R | SSU | 21 sequence types; *Phragmites*, Germany |
| Aluhu *et al.* (2006) | I/D | S, T, R | LSU | 10 genotypes; *Hedera/Rubus*, sand dune, Japan; compares trap cultures against molecular study |
| DeBellis & Widden (2006) | D | R | SSU | 10 phylotypes, mainly *Glomus*; *Clintonia*, mixed forest, Canada |
| Renker *et al.* (2005) | D | S, T, R | ITS | 6 *Glomus* spp.; several hosts, polluted soil, Germany |
| Renker *et al.* (2006) | D | R | ITS | 10 species, single site, *Plantago*, Germany |
| Börstler *et al.* (2006) | D | R | ITS | 19 species, all genera except *Scutellospora* and *Pacispora*; 2 grassland sites, Germany |
| Santos *et al.* (2006) | D | R | SSU | 10 groups by DGGE, 7 *Glomus*, 2 *Scutellospora*, 1 *Diversispora*; *Festuca/Achillea*, Sweden |
| Vallino *et al.* (2006) | D | R | SSU | 14 groups, mainly *Glomus*; *Solidago gigantea*, Italy |
| Wubet *et al.* (2006) | D, I | R | SSU | 16 *Glomus*, 3 *Diversispora*, 1 *Archaeospora*; *Podocarpus*, Ethiopia |
| Porras-Alfaro *et al.* (2007) | D | R | SSU | 7 *Glomus* phylotypes; *Bouteloua*, semiarid grassland, USA |
| Wu *et al.* (2007) | D, I | S, R | LSU | 17 clades, 3 families; Mount Fuji |

a S – spore isolation, T – trap cultures, R – root samples extracted for DNA.

for agriculture, rather than in those that were former desert. Different species predominated with different land uses. Direct spore isolations underestimated species diversity by 3.2 species per sampling site relative to trap cultures showing that the former method is inadequate. Oehl *et al.* (2004) compared the effects of conventional or organic farming on AMF diversity finding spore abundance and diversity higher in the latter. The AMF community also differed between the farming systems with *Glomus* species common in both, but *Acaulospora* and *Scutellospora* spores more abundant in organic systems. Increased diversity was also found by Hirji *et al.* (2006) in 5 agricultural field sites of differing management intensity. In addition to fungi from *Glomus* group A, common in agricultural soils, they also found *Scutellospora*, *Paraglomus* and *Acaulospora*. Molecular analyses of species diversity correlated well with spore analyses. Low-input agriculture involving crop rotation was thought to provide better conditions to preserve AMF diversity.

Some soils seem to contain only *Glomus* spores, especially soils under stress, whereas others have a diversity of genera. For example, Oliveira *et al.* (2005) found 6 species of *Glomus* in a highly alkaline anthropogenic waste sediment in Portugal. Mathimaran *et al.* (2005) found 8 *Glomus* species in an agricultural soil by direct extraction of spores, but *Paraglomus occultum* was additionally found in trap cultures. *Microaggregatum/Glomus intraradices* formed almost 50 % of the

spores isolated at some sampling times. The trap plant species again had a strong effect on the community of spores isolated. In contrast, Schalamuk et al. (2006) found spores of at least 24 species belonging to 6 genera of AMF in non-tilled and tilled wheat soils in Argentina. In this study, tilling or fertilisation did not seem to affect the diversity of spores present, instead spore communities were affected by wheat phenology. An intensive study of 3 adjacent fields with different cultivation histories in Southwest China revealed surprisingly high diversity, with 47 species of AMF identified from field-collected spores (31 *Glomus*, 8 *Acaulospora*, 6 *Scutellospora*, 1 *Entrophospora* and 1 *Gigaspora*). Habitat was presumed to have a greater effect on community make-up than host preference (Li et al. 2007). Wang et al. (2008) analysed 90 agricultural soils in the same region and found 30 Glomalean species (20 *Glomus*, 4 *Acaulospora*, 3 *Scutellospora*, 1 *Ambispora*, 1 *Archaeospora* and 1 *Paraglomus*) again suggesting high diversity despite intensive management.

The effectiveness of spore isolation and trap culturing was demonstrated effectively by Beauchamp et al. (2006) who found spores from 30 morphospecies of AMF in soils from a mixed riparian stand of *Populus* and *Salix* in Arizona. All genera other than *Scutellospora* and *Gigaspora* were found, both of which are large-spored genera. Small-spored *Glomus* species dominated the spore counts, as often occurs in desert soils. Whitcomb & Stutz (2007) also used trap cultures to find 12 species of AMF in two planted plots in Arizona They investigated how sampling effort reflected species richness, and showed that 15 samples was sufficient to detect 70–80 % of species present in each plot. Spore studies such as these suggested to them that AMF communities are highly variable on a local scale, but this may reflect sporulation activity rather than level of root colonisation and symbiotic significance. They also concluded that a limited number of species would have remained undetected using this approach. Sýkorová et al. (2007) compared 3 methods to study diversity and showed that AMF which colonise greenhouse trap plants are not necessarily reflective of the AMF which colonise bait plants placed directly into field soils *in situ*. Only *G. intraradices* was found in high frequency using all three methods (molecular probes, trap plants and bait plants).

In summary, spore analyses can provide an effective assay of the AMF community present as viable propagules in the soil, providing sampling is intensive and involves successive trap cultures. Trap culture plants can affect the diversity recorded, which correlates with field observations that crop monoculture reduces diversity. In a mixed, natural ecosystem it is likely that different AM taxa colonise different plant roots at different stages in community development, and an effective trap culture regime will capture most of this diversity over a prolonged assay period. Build-up of diversity can be rapid, as Dodd et al. (2002) showed by isolating spores of 12 species of AMF from a 6-year old reclamation platform associated with the building of the Channel tunnel.

## Direct analysis using molecular tools

Direct isolation of DNA from plant roots reveals species which have colonised and, by inference, are significant in the symbiotic relationship. Initially these studies used simpler PCR analyses, cloning and T-RFLP analyses to indicate the diversity of AMF within roots, but later studies used specific primer pairs to study richness of particular taxa.

Early progress within pot experiments using nested PCR targeting the D1/D2 region of the LSU discriminated *G. mosseae*, *Glomus intradices*, *Gigaspora rosea* and *Scutellospora castanea* where all 4 fungi were included as inoculum for leek and onion (Van Tuinen et al. 1998). All 4 fungi frequently co-existed within the same root fragment, but *G. mosseae* dominated. This approach was used in microcosms to show that colonisation of *Medicago* roots by *G. mosseae* was reduced uniformally by sewage sludges, relative to two other co-inoculated AMF (Jacquot et al. 2000). Field studies were initiated by Clapp et al. (1995) and extended by Helgason et al. (1998; 1999; 2002) to compare SSU sequence diversity of AMF found in a Yorkshire woodland to that in nearby arable soils. In total, 15 sequence groups were given generic designations (Glo 1–10, Acau 1–4 and Scut 1). Eleven of these were found in the woodland site, but only 6 were found in the arable soils (of which two were found at both sites). Over 90 % of the sequences ('Glo1') from the arable site resembled *G. mosseae* when clustered with the limited named accession sequences then available from GenBank. Diversity of AMF colonising *Hyacinthoides non-scripta* in mixed woodland in York was studied using the same approach (Helgason et al. 1999). Sequence variation was used to show how roots were colonised by mixed communities of AMF and that relative abundance of *Glomus*, *Acaulospora* and *Scutellospora* within the root system varied through the season or with the local predominance of certain trees. Eight of the AMF sequence groups were found in roots of this woodland plant. The study was unusual in that the molecular data could be compared against an intensive morphological study of colonisation by these 3 genera (Merryweather & Fitter 1998a, b) such that reasonable correlation between the two approaches was confirmed. Further work in arable soils near York showed much lower diversity, with *Glomus* species predominating (Daniell et al. 2001), whereas more intensive study of 5 plant species at the woodland site (Helgason et al. 2002) revealed a further 5 sequence types bringing the total to 13. Most of these sequence groups have now been assigned to species where close sequence matches were available from pot cultures or database accessions. In associated glasshouse trials, one of the trap cultures closely related to Glo9, assigned to *Glomus hoi*, consistently occupied a large proportion of root systems, and outperformed three other AMF from the site in improving P uptake and growth of 4 out of the 5 plants. However, in field conditions, Glo9 was almost exclusively associated with *Acer pseudoplatanus*.

The molecular analysis of AMF communities has since progressed rapidly as sequences accumulate within GenBank. For example, Husband et al. (2002a) used SSU sequences to show that AMF communities colonising seedlings of *Tetragastris panamensis* in a tropical forest shifted in composition over a 3-year study. Eighteen AMF types were detected, of which 16 were from *Glomus* which is unusual for an undisturbed ecosystem. Only two types, both *G. mosseae*, could be ascribed to known species within sequence databases. In a wider study of 3 plant species in the same ecosystem

(Husband et al. 2002b), a total of 30 AMF types were detected, of which 17 had not been found earlier from temperate ecosystems. Heinemeyer et al. (2003) detected 10 sequences from Glomalean fungi in plant roots from a native grassland and showed that shading reduced colonization by AMF, most notably in one *Glomus* species which was correlated with a lower abundance of *Trifolium repens*. Seasonal shifts in community make-up were noted with a significant increase in occurrence of sequence Glo1 in Summer, although the authors comment that AMF clone frequency does not necessarily correlate with the proportion of root colonised, nor are the most abundant fungi in the roots the most ecologically significant. Vandenkoornhuyse et al. (2002) found 24 groups of AMF SSU sequence groups after intensively sampling two plant species in Scotland. They then used T-RFLP profiling (Vandenkoornhuyse et al. 2003) to examine AMF diversity from 89 root samples from 3 co-existing grass species in the same ecosystem. Results suggested that host preferences were apparent and that soil treatment could modify AM community structure. T-RFLP profiling of the LSU was compared with spore extractions by Wu et al. (2007) to examine AMF community structure in desert soils on Mount Fuji. Seventeen clades of AMF were found, but those found by spore sampling were inconsistent with those found in the roots by T-RFLP. Six clades were found on the basis of spores, 6 were found only in roots and 5 were found using both methods.

Species- or genus-specific primers have been used by other workers to study species-presence. Rosendahl & Kjøller (2001) used selective *Glomus*-specific primers to show that sequence information derived from pea roots grown in a Danish field correlated well with 17 *Glomus* isolates held in culture, of which 5 came from the same field. Jansa et al. (2003) used specific primers to show soil tillage affected the community structure of AMF in maize. Colonization by *Scutellospora* was greatly reduced in tilled soils whereas *Glomus* species were common in tilled and non-tilled soils. Rosendahl & Stukenbrock (2004) studied *Hieracium pilosella* in undisturbed, mixed vegetation and detected 11 phylogenetic clusters from *Glomus* using primers targeted at the *G. mosseae/caledonium/intraradices* clade; most represented unknown species. *G. intraradices* showed higher sequence diversity than other species and intense sampling of sequences from a single root fragment containing *G. intraradices* revealed no sequence variation, indicative of a single individual in each root fragment. Wirsel (2004) also studied a single plant, *Phragmites australis*, in a wetland ecosystem. The use of a less specific SSU analysis revealed 21 phylotypes across a range of genera – which also indicates that individual plant species may host many AMF. Primers specific for *Acaulospora longula* and *G. mosseae* were used to show that there was no preference in either the fungi or the plant for the symbiotic partnership (Geue & Hock 2004). Wubet et al. (2003) used ITS variability to detect sequences from 20 Glomeraceae types and one type each in Diversisporaceae and Archaeosporaceae in roots of *Prunus africana* in Ethiopian forests. The AM community differed between the two sites studied, and only 3 spore types were found in adjacent soil using traps. The sequence similarity of 20 of the AMF types to sequences in GenBank was too low to relate them to known species, but two types were assigned to *Glomus etunicatum* and the *G. mosseae* complex. The ITS region was also targeted by Renker et al. (2005) to identify AMF in roots of 21 plants or from spores in trap cultures from soil collected at a polluted site in Germany. Six *Glomus* species groups were identified from sequences, 2 from roots and 4 from spores. There was no overlap in sequences found in roots and in spores. Renker et al. (2006) then analysed 50 root samples from *Plantago* growing at a single site in Germany finding 10 *Glomus* species representing all groups within this genus. No other species were detected. As observed by Rosendahl & Stukenbrock (2004), multiple cloning from single root pieces gave no evidence of multiple occupancy. In contrast, all genera other than *Scutellospora* and *Pacispora* were represented in 19 ITS species groups found by Börstler et al. (2006) in two mountain meadows with differing management strategies. Species composition, but not necessarily species richness, was related to the plant diversity at the two study sites.

Scheublin et al. (2004) found 14 sequence groups in DNA profiles from non-legumes and legumes showing that different plants hosted different communities – and nodules hosted different communities than their parent roots. Moora et al. (2004) detected 12 and 8 SSU sequence groups, from 4 genera, in roots of two species of *Pulsatilla* (one rare, one common) in forest or grasslands respectively. Groups showed site specificity but no differences were noted between the two hosts. The grassland community supported seedling growth at an order of magnitude greater than that of the forest inoculum (Moora et al. 2004). The more common *Pulsatilla pratensis* performed better with grassland inoculum than the rarer *P. patens*, while the latter grew slightly better with forest inoculum, suggesting that the presence of AM fungi that are more beneficial to the common species may influence the differential distribution of the two plants. Öpik et al. (2006) reviewed 26 publications on natural populations of root-colonising AMF identified using rDNA sequences, 95 distinct taxa had been reported, 49 of which were recorded from two or more study sites. The lowest diversity occurred in arable fields and polluted soils (i.e. anthropogenic sites). Some taxa were ubiquitous, with the commonest types being the *G. intraradices/Glomus fasciculatum* complex, *G. mosseae* and '*Glomus* sp. UY125'.

Ahulu et al. (2006) compared communities from co-occuring *Hedera* and *Rubus* species and found similar communities in each (despite the fact that paris- and arum-type arbuscules were formed in these respective species). Plant trap cultures and spore analyses suggested that AMF populations were highly divergent but molecular analysis of roots showed both species to be colonised by 8 genotypes (4 of which could be associated with particular species from 3 genera). Both species contained a single genotype not found in the other. *Scutellospora erythropa* was the most frequently detected genotype in root fragments from *Hedera*, whilst three genotypes were predominant in *Rubus* roots. Santos et al. (2006) examined SSU profiles from roots of *Festuca* and *Achillea* growing in semi-natural grassland with a fertilisation gradient. Seven *Glomus* groups, 2 *Scutellospora* groups and 1 *Diversispora* group were found with no plant specificity except in rare phylotypes. Five of the *Glomus* groups accounted for 93 % of the sequences encountered with up to 3 groups occurring in the same root sample. Diversity decreased with increased levels of N in the soil. Wubet et al. (2006) used SSU analyses to identify 20

sequence types of AMF in roots of *Podocarpus falcatus* in dry Afromontane forest in Ethiopia and, when comparing these results with those from other trees sampled in the same region, concluded that AMF communities were host–plant specific.

Molecular analyses have also been used to assess how rapidly re-establishment of an AMF community occurs in an uncontaminated soil used 25 years earlier to cover an ex-chemical site (Vallino *et al.* 2006). SSU analyses showed reasonable AMF diversity in roots of *Solidago gigantea*, with 14 AMF types, dominated as expected by *Glomus* types.

In contrast, low diversity (7 *Glomus* types only, the commonest close to *G. intraradices/fasciculatum*) was found in poorly colonised *Bouteloua* roots (Porras-Alfaro *et al.* 2007) in a semiarid grassland subjected to long-term N fertilization. Diversity was highest in the N-amended plots.

One drawback with molecular approaches is that rarer DNA templates may not be detected in a background of much more frequent templates. Taxon-specific primers can be used in a nested approach to detect these otherwise indistinguishable species. Gamper & Leuchtmann (2007) used this method to detect two species of AMF in grassland soils, whilst Stukenbrock & Rosendahl (2005) used nested PCR and LSU clade-specific primers to examine relative abundance of subgroups of *Glomus* within roots of plants in undisturbed coastal vegetation. They found different frequencies of the dominant types between plots and host plants suggesting a patchy distribution of *Glomus* species in this undisturbed soil, but also the formation of large mycelial networks that are associated with several plant species. The dominant phylogenetic cluster was found in all plant species in all plots sampled.

## Quantification of AMF within roots

Most molecular approaches used to date characterize the AMF community in terms of presence only – there is no direct quantitative measure of relative abundance of a particular individual within the root system. Thus these methodologies do not necessarily relate to the actual amounts of root colonisation or of the ecological significance of the taxa amplified. Crude measures have been applied such as relative intensity of DGGE bands or frequency of occurrence in clones taken from multiply-sampled root fragments. A quantitative measure of relative amount of colonisation of the root system is needed to test whether a certain plant is preferentially colonised by a particular Glomalean taxon (Santos *et al.* 2006). Similar methodology will also be needed to investigate how different proportions of mixed AMF colonies affect the growth of their host plants under different environmental conditions. Competitive PCR was initially suggested as having potential (Edwards *et al.* 1997), but was not progressed. Saito *et al.* (2004) used a detailed analysis of frequency of particular sequence types to give a 'semiquantative' measure of the % root length colonised by particular groups of *Glomus* found in the roots of grassland plants. They acknowledged that this assumes that frequency of occurrence of sequences reflects the relative amount of DNA in the roots, and that this changes with the number of PCR cycles. Stukenbrock & Rosendahl (2005) also compared relative frequencies of sequences of *Glomus* sub-groups to assess the relative amounts of different phylogenetic clusters within plant roots from coastal grassland.

More direct quantitative measures of co-colonisation by AMF within roots has recently been achieved using q-PCR (Alkan *et al.* 2004; 2006) in experimental systems using two species of *Glomus* (*G. intraradices/G. mosseae*). Phosphorus and saline stress, choice of host plant and spatial distribution within the root all influenced the outcome of interactions between these two fungi. This demonstrated the potential of the methodology for studying individual quantification of AMF in mixed communities. *G. intraradices* produces large amounts of intraradical spores (containing large amounts of DNA) and hence might give a disproportionate DNA signal compared to *G. mosseae* which sporulates external to the root. This did not seem to be a problem, however, when Jansa *et al.* (2008) used q-PCR to show roots in pots inoculated with mixtures of AMF, including *G. intraradices*, were usually dominated by *G. mosseae*. Similar limitations of the q-PCR approach were discussed by Gamper *et al.* (2008) when developing new assays to quantify mycelial abundance of 5 taxa of AMF in mixed-species environments. The large contribution of spore DNA to the pool of DNA extracted from the samples under investigation was again highlighted as a potential problem as strong correlations were found for q-PCR of DNA and spore numbers, but not between vital hyphal length and q-PCR. Quantification of RNA as an alternative approach did not resolve this problem (Gamper *et al.* 2008). Hence, those taxa which produce large numbers of spores, or produce large numbers of vesicles in the roots, will produce larger signals for q-PCR and the results from biological versus genetic quantification may not correlate.

## Conclusion

Indirect (spore isolation from soil) and direct (DNA isolation from roots) methods have been used successfully in assessing AMF diversity within an ecosystem. Provided they are done thoroughly, both approaches can reveal large numbers of taxa in a given ecosystem (Table 1), but the community composition determined from spores can be different than those in roots (Wu *et al.* 2007). Spore-based methods rely on identification of morphospecies and record simply the presence or absence of the spores regardless of functional significance. Simple extraction of spores directly from the soil is inadequate, and sequential trap cultures must be used to record the full range of species present and to encourage initially non-sporulating fungi to reveal themselves. Spore numbers bear no relation to the amount of root colonised by the respective AMF. In contrast, direct molecular analyses reveal the diversity of fungi occupying the root and presumably contributing to the mycorrhizal effect on plant growth. However, the molecular 'species' may or may not correlate with traditional morphospecies and the gene tree approach to taxon recognition is sensitive to sampling bias (Rosendahl, 2008).

The ribosomal genes remain the only target with sufficient database depth to conduct discriminative studies at a taxon-specific level – the ITS and SSU regions offering widespread cover using single primer sets. However, some groups may still not be covered, and broadening the primers to cover less

specific sequences means some non-Glomalean sequences may be amplified, or that the primers become genus-specific rather than 'species-specific'. New SSU primers have recently been described (Lee *et al.* 2008) which should amplify all published AMF sequences except those of *Archaeospora trappei*. Gamper *et al.* (2008) suggest that there needs to be enlargement of the sequence databases to include as many phylogenetic groups as possible for future development of molecular tools. Variability within the LSU is greater and is useful to develop clade-specific primers but not for primers with widespread cover across the range of AMF. The ribosomal genes are multi-copy and thus need to be cloned such that any quantitative measure has been lost in most community studies of AMF. Only where sequencing can be applied directly to PCR products from small root samples from intensively sampled material can the results be used quantitatively, as Rosendahl and Stukenbrock (2004) demonstrated when studying a specific *Glomus* clade in coastal grasslands.

Alternative molecular tools now exist to quantitatively analyse the effect of environment, management or inoculation of soils on more diverse AMF communities. Q-PCR can be used for simultaneous specific and quantitative investigations of particular taxa of AMF in roots and soils colonised by several taxa (Gamper *et al.* 2008) providing the constraints outlined above are taken into consideration. In addition, new techniques of high throughput sequencing (e.g. pyrosequencing) are rapidly developing (Roesch *et al.* 2007) but have not yet been applied to AMF. For bacterial community analysis, this approach is already replacing community fingerprinting methods and clone libraries (Jonasson *et al.* 2002). Microsatellite (simple sequence repeats) analysis is also starting to impact as a tool to discriminate individual strains within species complexes (Mathimaran *et al.* 2008; Croll *et al.* 2008). These results will be key to understanding how ecosystem management affects potential sustainability *via* indirect effects on AMF populations below ground. Understanding mycorrhizal ecology remains a key component of ecosystem management.

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# Appendix 3, publication

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ORIGINAL PAPER

# Inoculation of drought-stressed strawberry with a mixed inoculum of two arbuscular mycorrhizal fungi: effects on population dynamics of fungal species in roots and consequential plant tolerance to water deficiency

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**Abstract** The effect of inoculation with two arbuscular mycorrhizal fungi (AMF) on growth and drought tolerance of cultivated strawberry (*Fragaria* × *ananassa*) was studied. Three treatments (a single treatment either of *Funneliformis mosseae* BEG25, *Funneliformis geosporus* BEG11 or a 50:50 mixed inoculation treatment of both species) were compared to uninoculated plants. Species-specific primers for qPCR quantification of *F. geosporus* and *F. mosseae* DNA were developed to quantify the relative abundance of each fungus in roots of strawberry under different conditions of water stress. Co-occupation of the same root by both species was shown to commonly occur, but their relative abundance varied with water stress (reduced irrigation of up to 40 %). Greater root colonisation was observed microscopically under water stress, but this increased colonisation was often accompanied with decreased amounts of fungal DNA in the root. *F. mosseae* tended to become more abundant under water stress relative to *F. geosporus*. There was significant correlation in the fungal colonisation measurements from the microscopic and qPCR methods under some conditions, but the nature of this relationship varied greatly with AMF inoculum and abiotic conditions. Single-species inoculation treatments gave similar benefits to the host to the mixed inoculation treatment regardless of irrigation regime; here, amount of colonisation was of greater importance than functional diversity. The addition of AMF inocula to plants subjected to reduced irrigation restored plant growth to the same or higher values as the non-mycorrhizal, fully-watered plants. The water use efficiency of plants was greater under the regulated deficit irrigation (RDI) regime and in AMF-inoculated plants, but there were no significant differences between plants inoculated with the single or combined inoculum. This study demonstrated that the increase in plant growth was directly influenced by an increase in root colonisation by AMF when individual plants were examined.

**Keywords** Arbuscular mycorrhiza · Co-occurring fungal species · Competition · Strawberry · Water stress · Water use efficiency

**Electronic supplementary material** The online version of this article (doi:10.1007/s00572-014-0603-6) contains supplementary material, which is available to authorized users.

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## Introduction

It is well known that arbuscular mycorrhizal fungi (AMF) improve plant nutrient uptake, protect plants from pathogens (Borowicz 2001; Ismail and Hijri 2012; Ren et al. 2013) and buffer against adverse environmental conditions, especially drought (Smith et al. 2010). With changes in the world climate, water limitation is becoming an increasing concern for crop productivity. Humans currently intercept ca. 60 % of all water run-off following precipitation and use 80 % of this for agriculture (Fitter 2012). It is thought that most plants benefit from mycorrhizal symbiosis under water stress conditions through the improvement of water status and uptake (Al-Karaki 1998; Aliasgharzad et al. 2006; Auge 2001; Bolandnazar et al. 2007; Koch et al. 2006; Omirou et al. 2013; Smith et al. 2010; Smith and Read 1997; Wu et al. 2013). However, Auge (2004) has reviewed a large number of studies and showed that drought only affected levels of root

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colonisation in about half of the reports examined, and in these instances, the level of root colonisation was increased rather than decreased. In addition, it was concluded that drought stress alleviation is not always a consequence of mycorrhizal colonisation but that colonisation may affect the plant tolerance to drought stress in different ways.

Even in areas with moderate-high annual rainfall, modern farming practices still necessitate efficient water management. For example, 80 % of UK strawberry (*Fragaria* × *ananassa*) production for supermarkets is currently grown under protection systems, primarily in polythene tunnels (http://www.calu.bangor.ac.uk/Technical%20leaflets/020203Protected%20strawberry%20production.pdf), requiring the use of irrigation. Nevertheless, field-grown strawberry plants still form a significant proportion of the commercial strawberry crop and these plants are often exposed to conditions of water stress. Water abstraction rates in the major strawberry growing regions of the UK are already unsustainable, and the demand for irrigation water will rise in the near future.

The beneficial effect of AMF inoculation on strawberry is controversial. Some studies have reported the beneficial effects of mycorrhiza on strawberry plant growth and yield (Borkowska 2002; Castellanos-Morales et al. 2010; Fan et al. 2011; Hršelová et al. 1989; Niemi and Vestberg 1992; Stewart et al. 2005; Vestberg 1992) whilst others showed either limited (Garland and Schroeder-Moreno 2011) or no beneficial effects (Vestberg et al. 2004). Borowicz (2010) examined the effect of AMF inoculation on water stress in wild strawberry (*Fragaria virginiana* Duchesne) and reported no strong effects of AMF on strawberry tolerance to drought. Yin et al. (2010) showed that inoculation of AMF in drought-stressed strawberry plants increased the enzymes associated with the plant's protective system to water stress.

Most AMF species can colonise a range of host plants, and a lack of host specificity has been assumed (Sanders 2003). Nevertheless, individual AMF species may differ in their effects on plant growth. The multiple occupancy of a single root fragment by AMF from different genera or species is now widely accepted (Alkan et al. 2006; Reddy et al. 2005; Van Tuinen et al. 1998; Wagg et al. 2011a), although this co-occupation is currently poorly understood (Alkan et al. 2006; Krak et al. 2012), and it remains unclear if the relationship is competitive, synergistic or antagonistic; indeed, their relationship may vary with plant hosts and/or environmental conditions (Wagg et al. 2011a).

Functional complementarity, whereby more than one AMF species colonise a root synergistically, has been observed (Alkan et al. 2006; Jansa et al. 2008; Jin et al. 2013; Koide 2000; Reddy et al. 2005). For example, Koide (2000) suggested that the co-colonisation by two or more AMF species could enable a wider spectrum of advantages and therefore be more beneficial to plants. Wagg et al. (2011a) examined the complementarity and selection effects in an AMF community and found that co-occupied roots showed 82 and 85 % improvements, respectively, in plant productivity above the average respective fungal species in monoculture, though this relationship varied with plant species, AMF species combinations and abiotic conditions. These authors reported an overall positive relationship between fungal community richness and plant productivity. However, other reports suggest that maximum benefit to a host plant can be achieved by a single effective AMF species (Edathil et al. 1996; Hart et al. 2013), with no additional benefit from a mix of species. It has been suggested recently that individual species occurring within the same root system may confer different benefits to the plant, thus avoiding inter-species competition (Jansa et al. 2008; Kiers et al. 2011). Kiers et al. (2011) showed how individual AMF species could employ different strategies in providing resource allocation and benefit to a host, when multiple occupants share an individual root system. In a study of field-transplanted, micropropagated strawberry (Stewart et al. 2005), treatments with a single inoculum or a mixed inoculum containing *Glomus intraradices*, *Glomus mosseae* and *Glomus etunicatum* (sensu lato) gave equal benefit. However, Hršelová et al. (1989) showed that some AMF species, such as *Gigaspora caledonium* and *Gigaspora margarita* (sensu lato), were better than others in increasing strawberry plant biomass.

This paper reports the effect of single or combined inoculation with *Funneliformis geosporus* and *Funneliformis mosseae* on the growth and tolerance to water stress of cultivated strawberry (*Fragaria* × *ananassa*). Strawberry plants can be highly responsive to AMF colonisation (Borkowska 2002; Castellanos-Morales et al. 2010), but no studies have attempted to understand the relative role of different AMF within the same root system in conferring beneficial effects. The relative abundance of the two fungi within root systems was estimated by qPCR to determine the effect on plant growth, how they interacted and how drought stress would affect their relative abundance. The relationship of AMF colonisation with plant growth was also determined on the basis of individual plants, and plant growth characters and root colonisation were assessed on each plant within each treatment regime. A single isolate was used from each of the two AMF species; the observed interaction could thus be described as between two isolates or between two species. For consistency, the results are described in terms of between-species interactions.

## Methods

Three successive experiments were carried out on the effects of the relative abundance of the two AMF species on plant health, water uptake and growth. Inevitably, there were variations in environment and experimental conditions over time.

Such inter-experiment differences were fully taken into account in statistical analysis of the data where experiment repeat was treated as a block factor.

### Fungal inoculum

Pure cultures of two AMF species (*F. mosseae* BEG25, *F. geosporus* BEG11) were initially obtained as inoculum from Plantworks UK, Sittingbourne, Kent, UK (www.rootgrow.co.uk). Core samples of 40 ml were taken from the original inoculum and placed as a layer into two 3-l pots filled with Terragreen (Agsorb, Oil-dry Ltd., Wisbech, UK), planted with corn (*Zea mays*) and onion (*Allium cepa*), and grown in a GroDome (Unigro, UK) set at 22 °C day/20 °C night with a 14-h day/10-h night cycle with supplementary lighting. After 3 months, a most probable number (MPN) test (Cochran 1950) was performed to quantify the infectivity of the cultures, thus ensuring an equivalent colonising potential of the two species for experimentation. For the first experiment (experiment 1), the MPN value for *F. mosseae* was estimated to be 920,000 propagules/l and *F. geosporus* 540,000 propagules/l. The difference in these two estimates was within one order of magnitude, and as such, these two cultures were assumed to be equivalent in their colonising potential. Similarly, in experiments 2 and 3, MPN values for both species were similar (>1.6 million propagules/l) and the colonising potentials were again assumed to be equal.

### Experimental design

In total, there were four AMF inoculation treatments: inoculation with the two individual species alone, mixed inoculation with both species, and an uninoculated control. In the mixed inoculation treatment, both species were added at a rate of 50 % of the single treatment. In addition, water stress was studied; two levels of irrigation were used: fully watered [WW] and water-stressed [regulated deficit irrigation (RDI)]. Thus, eight treatments covered the two experimental factors. Three repeat experiments were performed to study the effect of single and mixed inoculations on strawberry under WW and RDI irrigation regimes.

### Plant and AMF inoculation

Different cultivars and different propagation techniques were used to cover a range of situations in strawberry production. For experiments 1 and 2, micropropagated strawberry plants cv. Everest (Hargreaves Plants, UK) were used to ensure the absence of pre-existing mycorrhizal colonisation. Plants were established in rooting agar media and incubated at 20 °C for 2 weeks with overhead lighting until the roots had developed sufficiently to be transplanted into vermiculite in a propagation chamber. Plants were then weaned for 4 weeks at 20 °C with overhead lighting, with increasing ventilation to the chamber. Experiment 3 was established using the runner plants of cv. Elsanta to ensure that results were not affected by the use of micropropagated plants (Szczygiel et al. 2002), to aid plant survival and to increase fruit production (which is lower in micropropagated plants). Runners of pre-established plants were pinned down into Terragreen (Agsorb, Oil-dry Ltd., Wisbech, UK) and grown with the mother plants until a root system had been established before being separated from mother plants. For each experiment, 1-l pots were filled with Terragreen and 20 pots were used per AMF treatment; each pot received a layer (10 % of the total volume) of *F. mosseae* BEG25 inoculum, *F. geosporus* BEG11 inoculum, a mixture of the two species (50 % total volume for each species), or Terragreen alone (uninoculated control). A single, young strawberry plant (experiments 1 and 2—a micropropagated plant, experiment 3—a young runner plant) was placed into each pot.

### Irrigation

Strawberry plants were grown in fully watered conditions for approx. 2 weeks in a GroDome compartment (Unigro, UK) set at 22 °C day/20 °C night with a 14-h day/10-h night cycle and supplementary lighting. When the plants were sufficiently established, water stress (RDI) was applied to half of the 80 plants. Plants were placed in a 4×4 lattice block design with the four AMF treatments along the four irrigation lines of 13-mm wide LDPE pipe [City Irrigation Ltd., Bromley, UK). Two of the irrigation lines were randomly assigned to the WW treatment (plants receiving 100 % of the water estimated to have been lost via evapotranspiration (ETp)] and the RDI treatment (plants receiving 70 % of water for the WW treatment in experiment 1 and 60 % in experiments 2 and 3). RDI conditions were reduced further in the second and third experiments to give an increased stress to the drought plants (RDI plants in experiment 1 were not showing significant signs of drought stress). Plants were irrigated via 2 l/h Netafim drippers (City Irrigation Ltd., Bromley) linked to the irrigation lines; the drippers were of sufficient length to allow pots to be placed in required positions within a block. There were five replicate blocks of this 4×4 lattice design within each experiment.

In each experiment, eight plants from each inoculation regime (WW treatment only) were randomly selected (with the constraint that these plants were well dispersed within the whole experimental plot) to estimate weight losses over a 24-h period. ETp values were measured three times per week in situ using an ET sensor (Evaposensor, Skye Instruments Ltd., Powys, UK), and irrigation times for WW and RDI treatments were then adjusted from the average weight losses and ETp values (Grant et al. 2009). Little adjustment was required due to the relatively stable conditions within the GroDome.

Plants were grown under these conditions for a further 10 weeks before harvesting. Fertigation was carried out using Vitafeed 102, N-P-K 18-0-36 (Vitax, Leicester, UK) at a rate of 1 g/l with approx. 25 ml added to the top of every pot, regardless of treatment regime, once every 2 weeks. Plant death occurred in some experiments, due to failure of initial plant establishment. Experiment 1 consisted of 80 plants, and experiment 2 had only 52. In experiment 3, 40 plants were used to assess plant growth, qPCR analysis and root length colonisation (RLC) (because of the nature of destructive sampling), and the other 40 plants were used to study plant survival under drought conditions (see below). All 80 plants in experiment 3 were assessed for fruit weight, soil plant analysis development (SPAD) readings and water use efficiency (WUE) as these were assessed before the drought experiment started.

## Primer design and validation of qPCR

Species-specific primers were designed within the D2 region of the LSU ribosomal RNA (rRNA) gene against *F. mosseae* BEG25 and *F. geosporus* BEG11. The degree of resolution of the LSU is regarded to be high and can be used to differentiate between closely related AMF species (Krüger et al. 2012; Wagg et al. 2011a). Primers for qPCR were designed from previously published sequences deposited in GenBank, including sequence variants from *F. mosseae* BEG25 (AF304982-AF304994) and *F. geosporus* BEG11 (AF304995-AF305008). Due to the variability among these sequences, alignment of a selection of sequences from each species was used to ensure that the newly designed primer would amplify from all the variable sequences from that species. A total of 37 sequences were used to compare sequence variation within and between taxa for primer design. The *F. mosseae* primers (mossL14fw-TACCAACGGGAAATCAACCT with mossL14rev-AGATCCCAATCTCGCGAAC) and *F. geosporus* primers (geoLp3fw-CGTGAAATTGTTGAAAGGGAA with geoLp3rv-CTCCTCGAAACCCACAAGAA) were designed for the D2 region of the LSU rRNA using Primer-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and synthesised from Sigma-Aldrich (UK). Both primer pairs were aligned with a sample of previously published sequences (Supplementary material 1) and primers optimised (Supplementary material 2).

## Quantitative PCR protocols

DNA extraction and qPCR protocols were established for use with roots colonised with the two AMF species (Supplementary materials 2 and 3). The quantities of target sample DNA were compared against standard dilutions of spectrophotometrically quantified genomic DNA, extracted from 200 spores of *F. mosseae* BEG25 and *F. geosporus* BEG11 and against uninoculated strawberry root DNA. Concentrations of the fungal and plant DNA extracts were established using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, UK). DNA purities were verified using a 260:280-nm absorbance ratio and concentrations estimated to be as follows: *F. mosseae* 4.0 ng/μl, *F. geosporus* 6.9 ng/μl and strawberry root 4.6 ng/μl. Samples were serially diluted and qPCR performed with appropriate primer pairs. Aliquots of each serial dilution were stored at −20 °C for use to generate calibration curves for each plate of experimental samples. A new standard curve was run for each qPCR using these pre-quantified aliquots in order to eliminate between run disparities.

Threshold cycle (CT) values for fungal and strawberry root DNA were recorded for each sample. Amounts of fungal and strawberry DNA were estimated from respective calibration curves, and the final AMF value for each sample was expressed as the ratio between the estimated amounts of fungal DNA (ng/μl) and strawberry DNA (ng/μl).

## Plant growth measurements

Chlorophyll content readings were taken from one leaf per plant, using a SPAD meter (Opti-Sciences, USA, model CCM-200), which gives a measure of chlorophyll content/leaf area. This measurement was taken twice in the final 2 weeks of each experiment. Fruit yield was recorded for each plant by picking and weighing fruit twice weekly on ripening. Fruit production only occurred in experiments 2 and 3. As the emphasis was on the effect of AMF diversity/abundance on plant growth, pollinators were not artificially provided in the compartment, which affected fruit production.

Plants were harvested from each pot and the substrate carefully washed from the root mass. Root length and shoot height (crown to highest leaf tip) were measured along with shoot and root fresh weights for each plant harvested. A subsample (ca. 2 g) of roots was cleared with 2 % (*w*/*v*) KOH before being stained using 0.05 % (*w*/*v*) trypan blue in lactoglycerol (modified from Phillips and Hayman 1970). AMF colonisation was examined using the grid-line intersect method (McGonigle et al. 1990) and expressed as percentage of the root colonised by AMF. Another root sample (ca. 10 g) was washed in deionised water, cut into ca. 1-cm sections, placed into a sealed bag and stored at −80 °C until use for qPCR analysis.

## Water use measurements and plant survival

WUE was recorded during the last 2 weeks of each experiment, when plants were fully grown. Evapotranspiration was

recorded for every plant on two separate occasions, and WUE for each plant was determined by dividing the plant fresh weight at harvest by the average total water consumed (g) by each plant over a 24-h period.

In experiment 3, only 40 of the 80 plants were harvested from two replicate irrigation lines. The plants from the two remaining irrigation lines were firstly watered to full capacity over a 24-h period, after which irrigation was completely removed and the plants were left to dry within the GroDome compartment. These plants were monitored daily to record the number of days post water shut-off at which strawberry leaves reached 50 % senescence and the date on which a plant died.

### Statistical analysis

Statistical analysis was carried out using GenStat version 13 (VSN International, England). As the focus was on the effect of AMF inoculation and irrigation treatments on plant development (rather than variability among experiments), each repeat experiment was treated as a block factor over time in all statistical analysis. All treatment results were hence summarised over the three repeat experiments.

A restricted maximum likelihood (REML) analysis was used to assess how plant growth and AMF colonisation (% RLC and qPCR data) were affected by AMF treatments and irrigation regimes. In the REML analysis, row, column or block effects originating from the experimental design and three repeated experiments over time were all treated as random factors. AMF (control vs inoculated; inoculated further divided into [moss vs geo], and [mixed vs single]) and irrigation (WW vs RDI) were treated as fixed factors. Initial REML analysis showed that there was no evidence of any row or column effects within a block in any of the analyses or differences between the replicate irrigation pipes. A simplified analysis was thus carried out on the data adjusted for the effects for experiments and blocks within experiments (i.e. they were treated as fixed effects). All data, except RLC, was ln-transformed to reduce variance heterogeneity. Significances of treatment effects were assessed using $F$ tests.

To compare qPCR data with microscopic colonisation assessments, simple regression analysis was performed with the irrigation included as a factor. Microscopic root colonisation data were related to qPCR data on the basis of individual plants in order to assess whether these two estimations of mycorrhizal abundance within a root system are correlated on an individual plant basis. Both repeat experiment and block within experiment were also included as factors. Similarly, regression analysis was used to determine the relationship of plant growth with microscopic colonisation.

## Results

### Effects of drought on root colonisation

Root colonisation was observed microscopically in all AMF-inoculated treatments, with levels ranging between 55 and 70 %, and no mycorrhizal colonisation was detected in control uninoculated plants. A difference in colonisation was apparent between WW and RDI treatments with levels being significantly lower ($P<0.001$) for the WW treatment than for the RDI treatment. There was an overall difference ($P<0.001$) among AMF treatments: *F. mosseae* showed a lower colonisation compared to *F. geosporus* alone or the mixed inoculum treatments; this was due to a reduction in *F. mosseae* colonisation in one out of the three replicate experiments (Fig. 1). There was no significant interaction between AMF treatment and irrigation regime on mycorrhizal colonisation of the strawberry roots.

### Comparison of qPCR fungal detection after co-inoculation or single-species inoculation in relation to water stress

The qPCR data (calibration curve establishment given in Supplementary material 4) show an effect of the WW and RDI treatments on the amount of fungal DNA in mycorrhizal strawberry roots. However, this effect varied between replicate experiments and was dependent on both the water regime

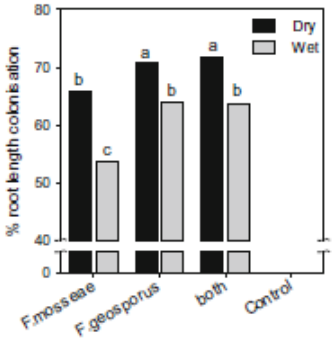


**Fig. 1** Strawberry root length colonisation (RLC) means for each inoculum treatment under wet and dry conditions. *Letters above each bar* indicate results of pairwise comparisons based on the least significant difference (LSD) test at the level of 5 % [standard error of differences (SED)= 2.239 (ave), 2.259 (max) and 2.207 (min)]: treatments sharing at least one common letter do not differ significantly from each other. RLC was significantly ($P<0.01$) greater for the 'dry' than for the 'wet' treatment (SED=1.304)

and the AMF species inoculated. Generally, in single-species inoculations, samples inoculated with *F. geosporus* gave a higher amount of fungal DNA than samples from plants inoculated with *F. mosseae*. In all experiments with mixed inoculum, qPCR results demonstrated that *F. mosseae* and *F. geosporus* co-existed within in a single root system, with neither species excluding the other to any significant degree (Fig. 2).

There was an overall difference in qPCR values between the two irrigation regimes, with a lower amount of DNA detected in the RDI treatment compared to the WW treatment. There was a significant interaction ($P$=0.033) between irrigation regime and AMF inoculation treatment, which showed a decrease of *F. geosporus* DNA in the RDI treatment but an increase for *F. mosseae*, compared to the WW treatment. When all inoculation conditions were considered, either the same or a reduced amount of DNA was recorded in the RDI treatment compared to the WW treatment, except for *F. mosseae* in the mixed inoculation where it increased in the drought treatment compared to the WW. The interaction between AMF species, single versus mixed treatment, and the irrigation treatment was almost significant ($P$=0.08).

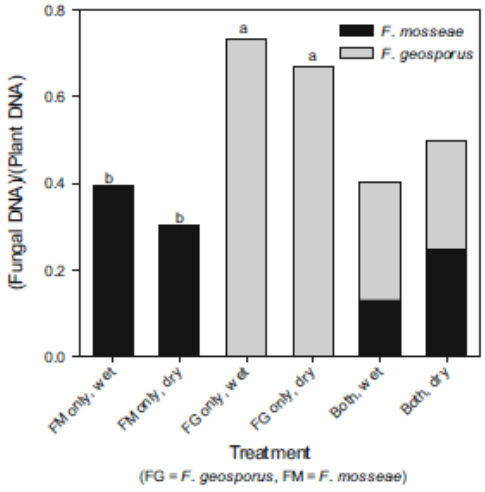


**Fig. 2** Average fungal DNA (quantified by qPCR), expressed as nanogram fungal DNA/nanogram plant DNA for the three AMF inoculation treatments, *F. mosseae* BEG25 alone, *F. geosporus* BEG11 alone or both species co-inoculated at a rate of 50 % each of the single treatment, under well-watered (wet) and dry conditions. *Letters above each bar* indicate results of pairwise comparison based on the least significant difference (LSD) test at the level of 5 % [average standard error of differences (SED)=0.35 on the ln scale]: treatments sharing at least one common letter do not differ significantly from each other. Overall fungal DNA for each species in co-inoculation did not significantly differ from 50 % of the corresponding single-species inoculation

There was a significant difference in DNA amounts between single-species and mixed-species inoculation ($P$<0.001). In the mixed-species inoculation, both species were added at a rate of 50 % of the single inoculation treatment so that a 50 % reduction in the amount of either species would be expected in the mixed species inoculation versus the single inoculation treatment. Overall, however, the amount of fungal DNA in the mixed-species inoculation was lower than the 50 % estimated ratio for both species at the two irrigation regimes, except for *F. mosseae* in the RDI treatment where it was slightly greater than the expected 50 %.

## Fungal colonisation estimated using a microscope compared with qPCR data

The overall trends from the qPCR results do not agree with the overall trends from the microscopically determined root colonisation data. In all treatments, the percentage RLC was higher in the RDI-treated plants, but the qPCR data indicated that lower amounts of DNA were recovered from the RDI-treated plants. Regression analysis based on individual plants (Fig. 3), however, suggested a significant relationship between root colonisation and qPCR data, but this relationship varied with the AMF species and between single and mixed inoculations. A strong positive correlation between the two types of data was observed for *F. geosporus* in the single-species inoculation ($r$=0.66, $P$<0.001). In contrast, for

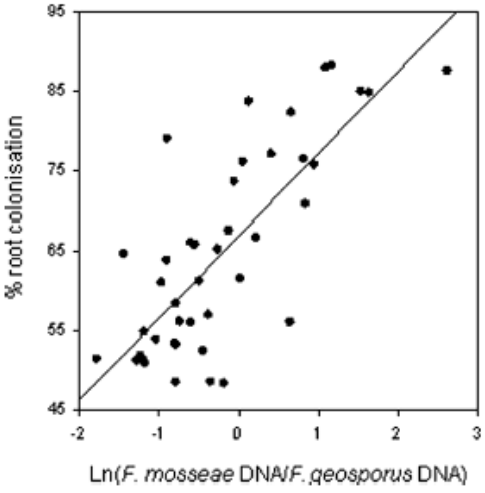


**Fig. 3** Percentage strawberry root colonisation by AMF plotted against the ln-transformed qPCR-quantified fungal DNA data (*F. mosseae*/*F. geosporus*) for the mixed inoculation treatment. The *solid line* is a fitted regression model describing the relationship. *Each point* represents a single experimental plant

*F. mosseae*, there was a weak negative relationship between the two types of data in the single-species inoculation ($r$=−0.31, $P$=0.047). In the mixed inoculation, the ratio of *F. mosseae* DNA with *F. geosporus* DNA was positively related to the extent of root colonisation. Overall, the relationship between root colonisation and qPCR data can be considered a positive one.

## Effects on plant growth

For all growth parameters, both single (*F. mosseae* or *F. geosporus*) and mixed inoculation (both AMF) species treatments gave similar benefits to strawberry plants, as shown by REML analysis. The difference between the RDI and WW treatments was always significant for all plant growth characters (with the exception of root length) and AMF inoculation led to increased plant growth (Table 1). The effects of AMF inoculum and irrigation regimes on shoot and fruit fresh weights are illustrated in Fig. 4.

Inoculation of both the AMF and the WW irrigation treatment resulted in higher values for shoot and root fresh weight (Table 1). Both treatments also led to higher shoot production relative to root production (Table 1, Fig. 4c). RDI-treated plants were significantly smaller than WW-treated ones, and control plants under the WW condition were smaller than those inoculated with AMF but subjected to the RDI regime (Table 1). Shoot fresh weight of AMF-inoculated plants under the RDI regime was significantly greater ($P$<0.001) than that of control plants under the WW regime, and this difference was also observed for fruit yield ($P$<0.05). Fruit fresh weight, although low, was much higher in AMF-inoculated plants, with no fruit at all in the uninoculated plants in the RDI treatment.

## Water use efficiency and drought tolerance

Plants inoculated with AMF had greater WUE ($P$<0.001) than uninoculated control plants, irrespective of the irrigation regime (Fig. 5), and there were no significant differences among the three AMF inoculation treatments. Plants subjected to the RDI also had higher WUE ($P$<0.001) than WW-treated plants, regardless of AMF inoculation.

Inoculation with AMF significantly ($P$<0.001) increased plant survival when irrigation was completely terminated, irrespective of whether the plants had single or mixed inoculation. On average, inoculated plants took 3–6 days longer to reach 50 % leaf senescence and survived for 4–5 days longer than control plants (Fig. 6). Plants that were originally subjected to the RDI treatment also survived longer ($P$<0.001) than those that were initially subjected to the WW treatment, regardless of whether they received inoculation. A similar trend was observed for plant survival to 50 % senescence, and the difference between inoculated and control treatments in the WW was greater than in the RDI condition ($P$=0.008).

## Quantitative relationship between root colonisation and plant growth

On the basis of individual plants, there was generally a positive correlation between percentage root colonised by AMF and plant growth parameters, except for root length and fruit weight (Table 2). However, the exact relationship between RLC and plant growth characters varied greatly with inoculation treatment (AMF species, single or mixed inoculation) and irrigation regimes. There was a significant difference in this linear relationship between the WW and RDI irrigation regimes, regardless of the nature of AMF inoculation. The relationship between percentage root colonised and shoot

**Table 1** Means of strawberry growth measurements (ln-transformed) for four AMF treatments (including the uninoculated control) and two irrigation regimes (fully watered—wet; regulated deficit irrigation—dry)

| Plant growth measurements | AMF inoculation | | | Irrigation regime | | |
|---|---|---|---|---|---|---|
| | Control | Treated | SED | Dry | Wet | SED |
| Root FW (g) | 1.700 | 2.126* | 0.0622 | 1.821 | 2.005* | 0.0627 |
| Shoot FW (g) | 1.438 | 2.028* | 0.0629 | 1.596 | 1.869* | 0.0634 |
| Root length (cm) | 3.290 | 3.405* | 0.0212 | 3.363 | 3.332 | 0.0214 |
| Shoot height (cm) | 1.700 | 2.091* | 0.0358 | 1.820 | 1.971** | 0.0361 |
| Shoot/root FW (g) | −0.262 | −0.098* | 0.0381 | −0.225 | −0.135** | 0.0385 |
| SPAD | 3.733 | 3.637** | 0.0298 | 3.716 | 3.655** | 0.0300 |
| Fruit weight (g) | 0.719 | 1.927* | 0.4628 | 1.085 | 1.562* | 0.4664 |

The symbols '*' and '**' indicate statistically significantly differences between treated and control strawberry plants. Parameters are statistically significantly different between wet and the dry treatments at the level of 5 and 1 %, respectively. Differences among the three AMF inoculation treatments were not statistically different. There was no evidence of interactions between AMF treatments with irrigation regime, except for root length (significant interaction $P$=0.017)

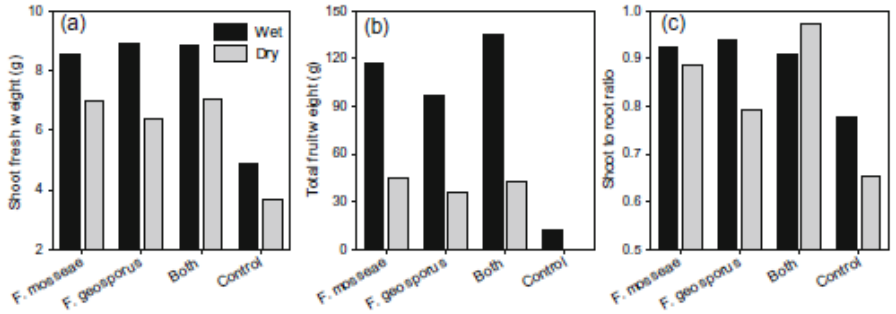


**Fig. 4** Histograms of **a** average shoot fresh weight (g), **b** average fruit weight (g) per plant and **c** average shoot to root ratio of fresh weight under regulated deficit irrigation (dry) and well-watered (wet) conditions of strawberry inoculated with *F. mosseae* alone, *F. geosporus* alone, both (as a 50 % mix) or not inoculated. Differences between the three AMF-inoculated treatments were very small under the same irrigation regime and not statistically different; all inoculated treatments had greater shoot fresh weight, fruit weight and shoot to root ratio than the uninoculated control. Average standard error of differences (SED) on the ln scale was 0.110, 0.495, and 0.066 for shoot fresh weight, fruit weight and shoot to root ratio, respectively. Overall, the 'wet' regime resulted in greater values than the 'dry' regime for all the variables (corresponding SED=0.063, 0.466 and 0.038), and there were no interactions between the irrigation and AMF treatments

weight or shoot-root fresh weight ratio is illustrated in Supplementary material 5.

## Discussion

The present study has shown that inoculation with one or two species of AMF is important for strawberry growth and yield and improves tolerance to drought stress. Using qPCR, based on species-specific primers for *F. geosporus* and *F. mosseae* DNA and microscopic quantification of root colonisation, we have studied how AMF colonisation of strawberry roots is affected by inoculation with one or two species under two irrigation regimes. Co-occupation of the same root by both AMF species commonly occurred, but the relative abundance of each varied with water stress. Increase in plant growth was directly influenced by an increase in percentage root colonisation when individual plants were examined, suggesting that for these two AMF, root colonisation was of greater importance than species diversity or their relative abundance.

Past studies concerning the benefit of mycorrhizal inoculation of strawberry plants have given inconsistent results, ranging from no effects (Borowicz 2010; Vestberg et al.

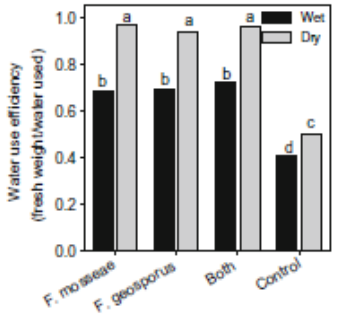


**Fig. 5** Water use efficiency, measured as plant fresh weight at harvest divided by water (g) used in a 24-h period, for AMF-inoculated and uninoculated strawberry plants at the regulated deficit irrigation (dry) and well-watered (wet) conditions. *Letters above each bar* indicate results of pairwise comparison based on the least significant difference (LSD) test at the level of 5 % [standard error of differences (SED) on the ln scale= 0.125]: treatments sharing at least one common letter do not differ significantly from each other. Water use efficiency was significantly ($P<0.01$) greater for the 'dry' than for the 'wet' treatment (SED=0.072)

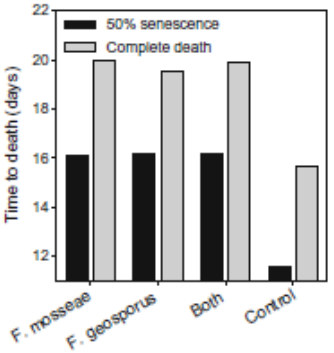


**Fig. 6** Average number of days to 50 % leaf senescence or death of strawberry plants for each AMF inoculation treatment when all plants were fully watered for a 24-h period before the irrigation was terminated. There were no significant differences among three AMF treatments (SED= 0.614 and 0.432 for the time to 50 % senesce and death, respectively); overall, AMF-inoculated plants survived longer ($P<0.01$) (SED= 0.480 and 0.347 for the time to 50 % senesce and death, respectively) than uninoculated controls

**Table 2** Slopes and standard errors (SE) in the fitted linear models relating strawberry growth parameters (ln-transformed) to percentage root length colonisation (data were adjusted for experiments and blocks before regression)

| Plant growth measurements | Regulated deficit irrigation (RDI—dry) | | | | | | Well-watered (WW—wet) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *F. geosporus* | | *F. mosseae* | | Both | | *F. geosporus* | | *F. mosseae* | | Both | |
| | Slope | SE | Slope | SE | Slope | SE | Slope | SE | Slope | SE | Slope | SE |
| Root FW (g) | **0.019** | 0.007 | **0.015** | 0.005 | **0.014** | 0.006 | **0.026** | 0.008 | **0.012** | 0.004 | **0.039** | 0.007 |
| Shoot FW (g) | **0.022** | 0.007 | **0.022** | 0.004 | **0.021** | 0.006 | **0.037** | 0.007 | **0.018** | 0.004 | **0.040** | 0.007 |
| Root length (cm) | 0.000 | 0.003 | 0.002 | 0.002 | 0.003 | 0.002 | 0.003 | 0.003 | 0.002 | 0.001 | −0.001 | 0.003 |
| Shoot height (cm) | **0.013** | 0.004 | **0.010** | 0.003 | **0.011** | 0.004 | **0.016** | 0.004 | **0.010** | 0.002 | **0.021** | 0.004 |
| Shoot/root FW | 0.003 | 0.005 | **0.007** | 0.003 | **0.008** | 0.004 | **0.010** | 0.005 | **0.006** | 0.002 | 0.001 | 0.005 |
| SPAD reading | **0.009** | 0.004 | 0.003 | 0.002 | **0.007** | 0.003 | **0.014** | 0.004 | **0.005** | 0.002 | **0.012** | 0.004 |
| Fruit weight (g) | **−0.116** | 0.063 | 0.018 | 0.039 | 0.031 | 0.049 | 0.019 | 0.053 | 0.016 | 0.053 | 0.002 | 0.046 |
| WUE | **0.018** | 0.007 | **0.017** | 0.005 | **0.018** | 0.007 | **0.028** | 0.008 | **0.014** | 0.004 | **0.038** | 0.007 |

Linear models were not fitted to the control treatment since there was no root colonisation by AMF. Significant slopes are highlighted in bold

2004) to positive effects on strawberry biomass production (Hršelová et al. 1989; Stewart et al. 2005; Vestberg 1992). In general, the presence of an AMF/plant symbiosis often results in altered rates of water movement into, through and out of the host plants, with beneficial consequences for tissue hydration and plant physiology (Ruiz-Lozano 2003). Consequently, the AMF symbiosis can protect crop plants against the detrimental effects of water deficit. Several mechanisms have been proposed to explain how the AM symbiosis can alleviate plant drought stress, including physical, nutritional and cellular effects (Jeffries and Barea 2012). Inoculation with AMF increased all growth characters of strawberry plants measured under both WW and RDI conditions, regardless of single- or mixed-species inoculation, which is consistent with the recent meta-analysis of Jayne and Quigley (2014). Inoculation with AMF benefited strawberry plant growth equally under both irrigation regimes when compared to control plants. The addition of AMF inocula (in any combination) to plants subjected to reduced irrigation of up to 40 % restored plant growth back to the same or higher values as the non-mycorrhizal, fully watered plants. Similar results have been observed for lettuce (Baslam and Goicoechea 2012). Thus, using mycorrhizal inoculation in crop production may contribute to a reduced need for irrigation without compromising plant growth or yield. This effect may be partially explained by increased AMF colonisation under the RDI regime, suggesting that under water-stressed conditions, strawberry plants become more dependent on the fungal symbiosis.

Water use efficiency of the strawberry plants was greater under the RDI regime than under the WW regime, which is consistent with previous findings (Grant et al. 2010; Omirou et al. 2013) and was greater in AMF-inoculated plants (single or combined inoculum) than non-mycorrhizal plants. Inoculation with AMF has been reported to increase WUE, for example, in watermelon (Omirou et al. 2013) and wild shrub species (Querejeta et al. 2006), although effects varied with different plant and AMF combinations. This suggests that AMF colonisation not only increases water uptake but also results in the plant becoming more efficient in using available water (Kaya et al. 2003; Omirou et al. 2013), possibly via mechanisms of increased stomatal conductance and transpiration (Auge 2001), increased nutrient availability and/or changes in aquaporin activation (Smith et al. 2010). The most notable consequence of increased WUE in the AMF-colonised strawberry plants was the significant increase in their survival time when water was not provided: mycorrhizal plants survived on average 4.4 days longer than control plants. This result is consistent with previous findings (Borkowska 2002), and again increased survival did not depend on the nature of AMF inoculation (single- or mixed-species inoculation).

One of the main benefits of AMF symbiosis for plants is increased P uptake; thus, the amount of available P in the substrate may affect the magnitude of AMF effects on plant growth. Lisek et al. (2012) demonstrated a negative impact of mineral fertilisers (NPK) on AMF taxa present in strawberry roots. The present study focused on a low P fertigation regime, whereas current commercial strawberry production relies on fairly high P input. This may account for the more consistent benefit associated with AMF colonisation than in other studies that were conducted in non-nutrient limiting conditions (Garland and Schroeder-Moreno 2011; Vestberg et al. 2004). Nevertheless, AMF colonisation has been reported to increase P uptake and biomass, even in a high P soil environment, depending on strawberry cultivar and AMF inoculum (Stewart et al. 2005). Santos-González et al. (2011) found higher colonisation levels in four different strawberry cultivars in a high P soil than in a low P soil. Strains of commercially available inoculum were used in the present study, one of which was an isolate of *F. mosseae*, a species reported to be

more favourable for strawberry growth than *G. intraradices* (sensu lato) and other unidentified *Glomus* species (Vestberg 1992). Other studies have used native inoculum (Borowicz 2010), which may have fewer infective propagules or may be less effective, although some studies have shown that native inoculum can be as beneficial as a commercially available AMF such as *G. intraradices* (sensu lato) (Garland and Schroeder-Moreno 2011). In the present study, the use of a soil-free substrate (Terragreen) no doubt contributed to the positive response of strawberry to the addition of single species or mixed inoculum, due to the lack of background AMF. Traditionally, strawberry production has been conducted in either field soil or in peat-based substrates, but the trend in UK strawberry production is to move to soil-less growing systems such as coir, and production under protected growing conditions, whereby the use of AMF maybe of increased benefit for plant growth and yield. It should be noted that the uninoculated controls were grown in Terragreen alone and were not supplemented with an AMF-free microbial sieving of the original inocula. Thus, it is possible that bacteria associated with the inoculum may, in part, also have contributed to the observed effects of inoculation. However, given the positive correlation between RLC and growth, it is likely that the AMF are responsible for the effects.

Fruit yields in this study were low and fruit production itself only occurred in experiments 2 and 3. Insect pollinators were not provided in the confined compartment (natural pollinators cannot enter the compartment since the facility is completely sealed for the purpose of controlled isolation). Furthermore, micropropagated plants were used for the first two experiments and these respond differently to runner plants in terms of fruit production (Szczygiel et al. 2002). Hence, the yield data may not truly reflect the production potential under commercial conditions. Nevertheless, there was a significant reduction in fruit yield under the RDI regime where AMF-colonised plants had higher yields than non-mycorrhizal plants. Chavez and Ferrera-Cerrato (1990) showed that the effect of AMF inoculation on fruit yield of micropropagated plants of four strawberry cultivars was highly variable depending on both the AMF species and strawberry cultivar used, with both positive and negative effects obtained. The increased shoot production relative to root production in the AMF-treated strawberry plants is consistent with previous results (Borkowska 2002; Veresoglou et al. 2012). However, there was a reduction in shoot to root ratios under the RDI conditions, compared to the WW regime. It is assumed that, as colonisation by AMF improves plant nutrition (Veresoglou et al. 2012), a greater shoot area was available to provide a larger photosynthetic area for the plant to use for yield, without the need to increase root production.

There is still debate as to the nature of the relationship between root colonisation levels by AMF and plant growth benefit, which may partially be due to the methodology used to measure colonisation or abundance of AMF. In the present study, an increase in plant growth was directly influenced by an increase in percentage RLC by the AMF when individual plants were examined. Very few studies have examined RLC effects on an individual plant basis, although Gange and Ayres (1999) measured the response of individual plants to AMF colonisation, in order to study the response of plants at different levels of colonisation. They suggested that an increased benefit was given to the plant up to a point at which a plateau was achieved and then declined at higher colonisation, which contrasts with the findings reported here. Due to the absence of colonisation data in the range of 0–20 % RLC, only a linear model (excluding control plants) was fitted, since the main purpose was to demonstrate the positive relationship rather than prediction. However, a curvilinear relationship may better describe the relationship if the control plants (with zero colonisation) were included in the regression analysis. For instance, when a quadratic model was fitted to the shoot fresh weight data (results not shown), the fitted quadratic models indicated that a point exists at which the percentage colonisation level gives rise to a detectable increase in strawberry growth effects, generally at about 30–50 % colonisation. However, low colonisation levels have been shown to have some growth effects in a meta-analysis based on mean colonisation values (Lekberg and Koide 2005; Treseder 2013). These low levels can occur in natural situations (Feldmann et al. 2009; Lekberg and Koide 2005).

The present study also confirmed previous reports of a discrepancy between microscopic and qPCR methods to quantify AMF within root systems. Whilst some authors (Alkan et al. 2004, 2006; Isayenkov et al. 2004; Wagg et al. 2011a) have reported a positive correlation, Jansa et al. (2008) and Pivato et al. (2007) showed that there was no significant correlation between these methods. Gamper et al. (2008) found a strong correlation between qPCR values and spore numbers but not between vital hyphal length and qPCR. The reason for the disparity between data from PCR-based methods and root colonisation estimates remains unclear. It may include uneven fungal distribution within roots (Gamper et al. 2008), a variation in the number of nuclei in the fungal hyphae during development or the number of rDNA sequences per nucleus (Pivato et al. 2007). Under the water-stressed conditions in the present study, microscopic quantification showed an increase in colonisation of strawberry roots by AMF, whereas qPCR analysis showed a relative decrease in *F. geosporus* and an increase in *F. mosseae* DNA in co-inoculated plants.

Inoculation with AMF resulted in good root colonisation of all the treated strawberry plants, with averages ranging between 55 and 70 % RLC across treatments, similar to that recorded by Vestberg (1992). Furthermore, qPCR data indicated that *F. mosseae* and *F. geosporus* have similar colonising potential in the mixed inoculation, although varying to a

certain extent with irrigation. Thus, it is reasonable to assume that both AMF species in the mixed inoculation contribute to the observed effects on plant growth, and there is little competition between them, consistent with previous findings (Janoušková et al. 2009; Jansa et al. 2008). The two isolates of AMF used were from the same genus (Redecker et al. 2013). One previous study has shown that closely related AMF species give greater plant growth effects and better co-exist than more distant species (Roger et al. 2013), but Maherali and Klironomos (2012) found that more distantly related species are more likely to co-exist than phylogenetically similar species. Although co-colonisation of strawberry roots by both *F. mosseae* and *F. geosporus* did occur, there was some indication of competition between them as the abundance of *F. geosporus* DNA in the mixed inoculation was significantly less than expected in both irrigation regimes. However, the extent of competition varied with experimental conditions and was not as strong as previously reported (Jansa et al. 2008; Krak et al. 2012). The amount of *F. mosseae* DNA also appeared to be less than expected under the WW condition. These results agree with previous studies where fungal abundance was frequently less in plants that received mixed AMF inoculum than their respective mono-inoculated counterparts (Jansa et al. 2008; Krak et al. 2012; Wagg et al. 2011b).

Root colonisation by AMF in response to drought stress has been examined previously by a number of authors, primarily using staining methods and microscopy. There are no quantitative data concerning differential effects of drought on mixed populations of AMF within roots. Results from the present study show mycorrhizal colonisation levels in strawberry plants of 55–70 % RCL when examined microscopically, as observed in other studies (Kiers et al. 2011; Thonar et al. 2012). All roots from the water-stressed strawberry plants had a higher colonisation by AMF, suggesting that under water-stressed conditions, plants become more dependent on the consequential increase in water use efficiency (Omirou et al. 2013). However, it should be noted that there was considerable variability in levels of colonisation under water-stressed conditions within replicate experiments, as reported previously (Auge 2001). In contrast to the microscopic observations, qPCR abundance data indicated that *F. mosseae* became the more dominant species under water stress. Jansa et al. (2008) made similar observations with an isolate of *F. mosseae*, possibly due its rapid ability to develop hyphae and increase colonisation rate. Wagg et al. (2011a) found that the dominance of *F. mosseae* was very much dependent on the host plant and soil conditions in a grass cover ecosystem. In the treatments inoculated with a single AMF, water stress alone does not significantly influence fungal abundance of either *F. mosseae* or *F. geosporus* within mycorrhizal strawberry roots, suggesting that it is the combination of co-occupying the same niche and water stress that leads to an increase of *F. mosseae* abundance. Thus, *F. mosseae* is likely to be less sensitive to water stress than *F. geosporus*. Omirou et al. (2013) showed that water stress can lead to a reduction also in native AMF diversity. Another possible explanation is that host plant preference for particular AMF species may vary with conditions and this might be regulated by differential supplies of host C to the 'preferred' species (Kiers et al. 2011).

The present study demonstrated that in strawberry plants, under the given experimental conditions, single AMF species inoculation can give similar benefits to the host as a mixed inoculum, i.e. the addition of any AMF inoculum (single or mixed) was of greater importance for strawberry plant growth and yield than species diversity or abundance. This conclusion is supported by the fact that there was a positive relationship between percentage RLC and plant growth increase based on individual plants, although such a relationship was influenced by AMF species and irrigation. Further studies are needed to demonstrate whether this conclusion is true for other host/AMF combinations, native AMF populations and other parameters (e.g. disease tolerance). When introducing AMF into field-grown strawberry production, it will be necessary to further understand how an introduced new species or isolate affects native populations (Janoušková et al. 2013), although with the significant trend of strawberry production moving to soil-less substrates (Takeda 1999), the use of AMF inoculation may give greater benefits.

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