# Potential for Growth Enhancement by Arbuscular Mycorrhizal Fungi in Potato

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# Abstract

Arbuscular mycorrhizal fungi (AMF) are important in natural and agricultural ecosystems to provide and enhance nutrient uptake, particularly phosphorus. Potato is one of the most important food crops globally in terms of total production and a phosphorus-demanding plant, which grows in symbiosis with AMF. This field research investigates the influence of crop management practices (fertiliser inputs and crop protection measures) and potato variety on species abundance, the diversity and function of AMF in potato. The suitability of mycorrhizal inoculants for growth enhancement in potato under specific management conditions in the glasshouse was also conducted. Traditional root staining by ink and vinegar method was used to quantify AMF colonisation along with three molecular techniques to measure AMF species diversity and level of infection, including terminal restriction fragment length polymorphism (T-RFLP), using AMF-specific primers FLR3 and FLR4 for LSU rDNA amplification, Illumina MiSeq Next Generation Sequencing (NGS) using universal fungal primers ITS1f and ITS2 for ITS region amplification and qPCR using AMF-specific primers AMG1F and AM1 for SSU rDNA amplification. All three molecular techniques were successful in nuclear ribosomal DNA region amplification.

The results show that organic crop protection management significantly enhances AMF colonisation and the genetic diversity of the AMF community. The use of NPK fertiliser along with conventional pesticides decreased AMF colonisation, the quantity of the AMF gene, and diversity of the AMF community. The use of triple superphosphate in the field showed a negative effect on AMF colonisation, the quantity of the AMF gene, and the genetic diversity of the AMF community. The study did not demonstrate a strong relationship between AMF colonisation and phosphorus uptake or potato tuber yield. NGS results showed that potato colonised by native populations of AMF in the field were dominated by *Paraglomus*. Potato variety had no significant effect on AMF abundance or the diversity of the fungal community, but phosphorus uptake and potato tuber yield were significantly affected. In the glasshouse experiment, *Rhizophagus* was the only genus detected in inoculated plants, but colonisation was not correlated with phosphorus uptake and potato tuber yield. This study demonstrated that natural populations of AMF could effectively colonise potato in the field across a range of varieties but did not show any agronomic benefit from higher levels of AMF colonisation, even under low P conditions.

## Declaration

I declare that this thesis has been generated by myself. This thesis has not been submitted as part of any degree. All sources of the information have been acknowledged explicitly by means of referencing.

Salisa Suchitwarasan

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# Nomenclature

## Abbreviations

| | |
|---|---|
| **AMF** | Arbuscular mycorrhizal fungi |
| **ANOVA** | Analysis of Variance |
| **bp** | Base pair |
| **DNA** | Deoxyribonucleic acid |
| **HCl** | Hydrochloric acid |
| **ITS** | Internal transcribed spacer |
| **KOH** | Potassium hydroxide |
| **LSU** | Large subunit |
| **T-RFLP** | Terminal Restriction Fragment Length Polymorphism |
| **NGS** | Next generation sequencing |
| **ASVs** | Amplicon sequence variants |
| **PCR** | Polymerase chain reaction |
| **rDNA** | Ribosomal deoxyribonucleic acid |
| **dsDNA** | Double-stranded DNA |
| **nrDNA** | Nuclear ribosomal DNA |
| **mtDNA** | Mitochondrial DNA |
| **SSU** | Small subunit |
| **TAE** | Tris-acetate-EDTA |
| **UV** | Ultraviolet |
| **DCA** | Detrended correspondence analysis |

**Symbols**

| | |
|---|---|
| **°C** | Degree celsius |
| **µL** | Microlitre |
| **µm** | Micrometre |
| **cm** | Centimetre |
| **$cm^2$** | Centimetre square |
| **g** | Gram |
| **kg** | Kilogram |
| **m** | Metre |
| **mg $g^{-1}$ DW** | Milligram per gram dry weight |
| **mL** | Millilitre |
| **ng $\mu L^{-1}$** | Nanogram per microlitre |
| **nm** | Nanometre |
| **vol/vol** | Volume per unit volume |
| **wt/vol** | Weight per unit volume |
| **t $ha^{-1}$** | Tonnes per hectare |
| **Da** | Daltons |
| **Gbp** | Giga base pairs |

# CHAPTER 1. Introduction

## 1.1 General introduction

Potato (*Solanum tuberosum*) is one of the most important food crops, following rice and wheat in terms of global consumption, with a production of 374 million metric tons worldwide (Devaux *et al.*, 2014). Potato can be produced in all continents except Antarctica (Li *et al.*, 2019). The Food and Agriculture Organization (FAO) recommended that potato is important in food supply for a growing population and increasing demand for food in the future (Devaux *et al.*, 2014). However, potato production is expensive with high fertiliser and pesticide requirements, which lead to environmental pollution (Chifetete and Dames, 2020). The different types of microorganisms have increasingly been found to be advantageous as bio-fertilisers.

Mycorrhiza is the symbiotic relationship between fungi and plants (Barman *et al.*, 2016). Arbuscular mycorrhizal fungi (AMF) are the type of mycorrhiza which are obligate root symbionts capable of colonising the roots of most terrestrial plant species (around 80% of families) (Smith and Read, 2008). AMF are considered as natural bio-fertilisers because they can provide the host plant with water, nutrients, and pathogen protection (Berruti *et al.*, 2016). AMF are mainly important in natural and agricultural ecosystems because they have the potential to increase the plant uptake of nutrients and water by the spread of their mycelia into the rhizosphere and also within root cortex cells (Mosse *et al.*, 1981). AMF are also present in most agroecosystems and colonised roots of many agricultural crops (Lekberg *et al.*, 2008) such as potatoes, maize, wheat, rice, sunflower, millet, cassava, bananas, flax, yams, soybeans, and coffee (Jansa *et al.*, 2006; Baum *et al.*, 2015). All AMF form an intimate contact between hyphae and plant cells in an interface region where nutrient exchange occurs. Moreover, AMF can protect plants from pathogen and drought stress (Oyewole *et al.*, 2017) and are considered as agents that are not harmful to the environment and which can increase plant growth and crop yields (Newsham *et al.*, 1995).

AMF are particularly significant for plant survival and growth when the soil has low levels of plant-available nutrients, especially phosphorus (P) (Graham *et al.*, 1991b; George, 2000; Roy-Bolduc and Hijri, 2011) because they increase the absorptive surface area of the uptake system. Most research on the function of AMF has focused on P uptake, especially in

conditions of low P availability (Daniell *et al.*, 2001; van de Wiel *et al.*, 2016). Phosphorus is an essential nutrient for many cellular elements in plants. Regardless of the abundance of total P in the soil, the form of P that a plant directly accesses is negatively charged orthophosphate ions in the form of $H_2PO_4^-$ or $HPO_4^{2-}$ (Smith *et al.*, 2011), which is usually present only in low levels because of its precipitation and transformation into inorganic forms (Raghothama, 1999). The most important advantage that AMF provides to a host plant is enhancing P uptake, and plant P status is usually the significant controlling parameter in the plant-fungal association (Thompson, 1987; Graham, 2000; Smith and Read, 2010).

Potato is a P-demanding plant (Dechassa *et al.*, 2003), which grows in symbiosis with AMF (Bhattarai and Mishra, 1984). Rosen *et al.* (2014) reported that P deficiency in potato crops can increase the severity of many important diseases, for example, common scab (*Streptomyces scabies*), verticillium wilt (*Verticillium dahlia* and *Verticillium albo-atrum*), and late blight (*Phytophthora infestans*). P deficiency can also lower crop yields. Therefore, the significance of AMF and potato associations and their management is of considerable agricultural interest. There are many studies which have reported positive effects on potato growth from inoculation with AMF in field experiments and also glasshouse experiments (McArthur and Knowles, 1993; Duffy and Cassells, 2000; Amaya-Carpio *et al.*, 2009; Lone *et al.*, 2015; Manck-Götzenberger and Requena, 2016; Yooyongwech *et al.*, 2016; Astover *et al.*, 2018; Sakha *et al.*, 2019).

AMF have low specificity for the host plant and plant variety (Lee *et al.*, 2013; Campos *et al.*, 2018). However, AMF can present significant selectivity in their relationship with different plant species (Helgason and Fitter, 2009). Some AMF and host association can be more advantageous than others (Bever, 2002; Walder *et al.*, 2012). Little research has been done to study the specific association between AMF and potato varieties. Therefore, it is important to investigate the relationship between AMF and potato variety.

### 1.2 Thesis scope and structure

This thesis was conducted to study the relationship between AMF and potato in the field and glasshouse under different crop management practices and for different potato varieties. The overall aim of this study was to identify the optimal crop management practice and potato variety combinations to promote AM fungal associations in potato production. The impact of phosphorus source and availability on AMF abundance and diversity was studied in two field

experiments, and the potential of AMF inoculants to promote the growth of potatoes was studied in a glasshouse experiment.

In the first experiment (Chapter 3), archived potato root samples from an investigation conducted in 2011 (NUE-CROPS project) were used to assess the impacts of potato variety and different fertility and crop protection regimes on the abundance and diversity of AMF using traditional root staining methods and molecular techniques. Samples from this experiment were also used to identify the optimal DNA extraction method for getting high yields and good quality of DNA; the method developed was used as a standard protocol in the other two experiments.

The second experiment (Chapter 4) consisted of two years of potato trials (2017 and 2018) conducted in the field. Sixteen potato varieties were planted in the plots with and without phosphorus fertiliser. Root samples were collected, and the abundance and the diversity of AMF was assessed using traditional root staining and molecular techniques. Agronomic data, including P concentration, total P uptake, tuber yield, and P use efficiency, were also analysed to determine the impacts of AMF on crop nutritional status and development. The two highest yielding potato varieties from the 2017 potato trial were selected to study in the next glasshouse experiment.

The third experiment (Chapter 5) was a glasshouse potato trial that was conducted in 2018. Two potato varieties were used to test the efficacy of two AMF inoculants. Root samples were collected at three potato growth stages, and the abundance and the diversity of AMF was assessed using traditional root staining and molecular techniques. Agronomic data, including P concentration, total P uptake, above-ground plant biomass, and tuber yield, were also analysed to assess the impacts of AMF on crop nutritional status and development.

### 1.3 Research hypothesis

According to the information obtained from the literature review, many reports showed that AMF established symbiotic associations with many plants, including potato. Previous studies have shown that potato crops can be colonised widely by AMF. Several researches reported that crop management practices influence the abundance and diversity of AMF. Understanding their abundance and diversity in root systems is therefore a prerequisite for effective crop management. It is also important to study the abundance and diversity of AMF

in different potato varieties and different management practices to better understand how potato production systems can be designed for optimal AMF function. Most studies on the function of AMF have focused on phosphorus uptake, especially in conditions of low P availability. In this thesis, therefore, the hypotheses were as follows:

1. Conventional crop management practice will decrease the abundance and diversity of AMF in the potato field.

2. There will be an interaction between crop management practice and potato variety, which will result in different effects on the abundance and diversity of AMF depending on the crop management practice and variety.

3. Colonisation by indigenous AMF or AMF inoculants will enhance the growth of potato in the field and glasshouse, respectively.

### 1.4 Objectives

Each experiment has a single overall objective. The specific objectives of each experiment are described in each chapter. The general objectives of this thesis were as follows:

1. To investigate the impact of crop management practices and potato variety on the abundance and diversity of AMF in potato (Chapter 3).

2. To understand how level of soil available P and potato variety interact to affect the abundance and diversity of AMF and how AMF impact the agronomic performance of the crop (Chapter 4).

3. To evaluate the function of AMF inoculants for growth enhancement in potato under specific management conditions (Chapter 5).

# CHAPTER 2. Literature review-State of the art on the role of arbuscular mycorrhizal fungi in agricultural systems and methods for the study

## 2.1 Statement of the problem

Potato is grown in over 100 countries all around the world as a staple food crop and is the main vegetable that is consumed worldwide (DeFauw *et al.*, 2012). It is composed of carbohydrates; approximately 75% of the total dry weight is in the form of starch (Birch *et al.*, 2012). Potato tubers are also rich in many nutrients, for instance, vitamin C, vitamin B6, vitamin B1, and folate. Potato is also an important source of potassium, iron, and magnesium (Birch *et al.*, 2012). More than a billion people in the world consume potato, and the Food and Agriculture Organisation (FAO) of the United Nations considers this vegetable critical for food security in a world burdened not only by unpredictability in the food supply but also increased population growth, food demands and hunger rates (Devaux *et al.*, 2014). By declaring 2008 the International Year of the Potato, the United Nations acknowledged the importance of potato in reducing poverty and contributing to global food security (Wijesinha-Bettoni and Mouillé, 2019).

The world population is expected to reach 9.1 billion by 2050 (DeFauw *et al.*, 2012; Rodriguez and Sanders, 2015), which means that food production should be substantially increased. Globally, potato cultivation and potato consumption has increased, particularly in developing countries because of easy cultivation (Devaux *et al.*, 2014); potato can be grown on limited land and in harsh climates (Wijesinha-Bettoni and Mouillé, 2019). According to the FAO (2009), potato takes less time for planting, generating higher yields than other major crops. Consequently, potato is considered an important food security crop that can support the world food supply into the future. Therefore, research and development in potato science is an important activity to resolve poverty and hunger issues in the developing world (Devaux *et al.*, 2014).

### The need to improve nutrient use efficiency in potato crops

Potato production requires high nutrient and chemical fertiliser inputs, especially nitrogen and phosphorus (Wu *et al.*, 2013; Fernandes and Soratto, 2016; Hailu *et al.*, 2017) because of the short development cycle and high yields attained. Moreover, potato crops are particularly inefficient in accessing soil P, especially in low P availability soils because their roots are

sparse and shallow (Yamaguchi, 2002; Hopkins *et al.*, 2014; Thornton *et al.*, 2014). P is an essential macronutrient in the soil, which is critical for plant growth and helps the plant make up about 0.2% of its dry weight (Smith *et al.*, 2011). P also supports various functions in plant metabolism; it is a structural component of nucleic acids, phospholipids, and adenosine triphosphate in the living cells (Hailu *et al.*, 2017).

P is one of the most difficult soil nutrients for plants to obtain (Smith *et al.*, 2011; Mokrani *et al.*, 2018). P is taken up from the soil in the form of orthophosphate ions (Drechsler *et al.*, 2018). These phosphate ions may be present at very low levels in soil systems because they immobilise rapidly and form insoluble complexes with cations, especially aluminum and iron, under the acid conditions and calcium in alkaline soils. (Vance *et al.*, 2003; Lopez-Arredondo *et al.*, 2014; Wacker-Fester *et al.*, 2019). This results in a very narrow range of soil pH where P is readily available since these low solubility complexes form with aluminum and iron (pH < 6.0) or calcium (pH > 6.5) (Davenport *et al.*, 2005).

In addition to an agronomic need to improve crop's ability to access soil P, there is also a sustainability issue as fertiliser P comes from mined sources (rock phosphate) and thus is a non-renewable resource and expected to become insufficient in the future (Vance *et al.*, 2003; Cordell *et al.*, 2009; van de Wiel *et al.*, 2016). Improving P use efficiency can be addressed with different approaches, for example, recover and reuse phosphate, and reduce the use of phosphate fertilisers (Roy-Bolduc and Hijri, 2011). In addition, soil microorganisms, including arbuscular mycorrhizal fungi, are another biological strategy to improve P use efficiency (Schütz *et al.*, 2018).

Potato is a P-demanding plant and is not tolerant of low available P in soil (Dechassa *et al.*, 2003). P is required in particularly high amounts for this crop compared to others because P plays an essential role in starch metabolism (Fernandes and Soratto, 2016; Koch *et al.*, 2019). Potato requires P in large amounts needed for the phosphorylation of starch during the tuber bulking stage, especially in the early growth stages (Fernandes and Soratto, 2016). Sufficient soil P is necessary for producing high potato tuber yields and quality, P increases the number of potato tubers and tuber size (Alva *et al.*, 2011; Fernandes and Soratto, 2016). Consequently, there is a need to develop agricultural systems that more efficiently use the P present in the soil (Richardson and Simpson, 2011).

## 2.2 Arbuscular mycorrhizal fungi (AMF)

In the natural environment, various microorganisms in soil and the rhizosphere can enhance plant P acquisition by solubilisation and mineralisation of inorganic and organic P. There has been growing interest in understanding the role of these microorganisms in improving P availability in soils and plant health and production in general with a view to develop more sustainable agriculture (Richardson and Simpson, 2011). Such research is relevant for increasing the efficiency of crop production systems for the developing world and also for developing countries where access to mineral fertilisers is restricted (Sánchez, 2010).

AMF are obligate root symbionts and form symbiotic associations with a wide variety of host plants (Bücking *et al.*, 2012). The range of their hosts is about 80% of all terrestrial plant species (Smith and Read, 2008; Berruti *et al.*, 2016). AMF are one type of soil microorganism often considered important for increasing P uptake and plant growth (Smith *et al.*, 2003). This is because P plays an essential role in the arbuscular mycorrhizal symbiosis (Douds *et al.*, 2007). The extraradical hypha of AMF acts as an extended root and takes up phosphate, nitrogen, sulfur, and minor elements from the soil (Mensah *et al.*, 2015). It has been shown that plants with arbuscular mycorrhizal symbioses have enhanced P uptake and increased growth compared to controls without AMF symbioses in low P soils. This was illustrated in a study by Smith and Read (2010), who showed that P contents in AMF colonised roots can be 3 to 5 times higher than in non-colonised roots.

Franciszek Kamienski (1881) was the first to discover the association of a fungus and plant (the roots of pinesap; *Monotropa hypopitys* L). However, the term “Mycorrhiza” (meaning is fungus-root) for this symbiotic association was actually first coined by Frank (1885). Most mycorrhizas are mutualistic associations (Brundrett, 2004), whereby the interaction is characterised by an exchange of resources across the mycorrhizal interface. The fungus provides the host plant with macronutrients: phosphorus, nitrogen, potassium, magnesium, sulfur, and micronutrients: iron, manganese, copper, zinc (Ruytinx *et al.*, 2019); it also increases the abiotic (heavy metals, drought, salinity) and biotic (root pathogens) stress resistance (Bücking *et al.*, 2012). As a response, host plants transfer the photosynthetically fixed carbon in the form of sugars and lipids to the fungus (Frew, 2019). Based on fossil evidence, mycorrhizae are thought to have colonised the early land plant roots 400 to 450 million years ago during the Devonian period.

Regarding the relationship of fungus to root cells, mycorrhizae are classified into two groups; ectomycorrhiza and endomycorrhiza (Janerette, 1991). Ectomycorrhizal fungi (ECM) form on many important plants (oak, pine, hemlock, spruce, chestnut, walnut, birch, willow, and eucalyptus) with host-specific associations or the preference of ECM for particular host plants (Newton and Haigh, 1998). ECM (Figure 2. 1) produce a mantle sheath (a dense layer of fungal hyphae) around roots and a hartig net (fungal hyphae present between the epidermal and cortical host cells between root cells) (Roy *et al.*, 2020). Endomycorrhizal or arbuscular mycorrhizal fungi (AMF) are associated with most land plants (Roy *et al.*, 2020). AMF have three important components; spores, the structures within and between the root cells, and extraradical hyphae (Smith and Read, 2008). The extraradical hyphae spread into the rhizosphere and thereby enhance the absorption of nutrients and water from the soil through the arbuscules (Govindarajulu *et al.*, 2005; Karandashov and Bucher, 2005; Roy *et al.*, 2020)

AMF hyphae can penetrate into the host cells and construct fungal structures in host cortex cells. Figure 2. 1 illustrates the main structural differences between AMF and ectomycorrhizal fungi compared with the structure of a non-mycorrhizal association. AMF produce a structure called an “arbuscule” (hypha branched structure) in cortical host cells. Some AMF form fungal storage organs called “vesicles” that are also present in the host cortex.

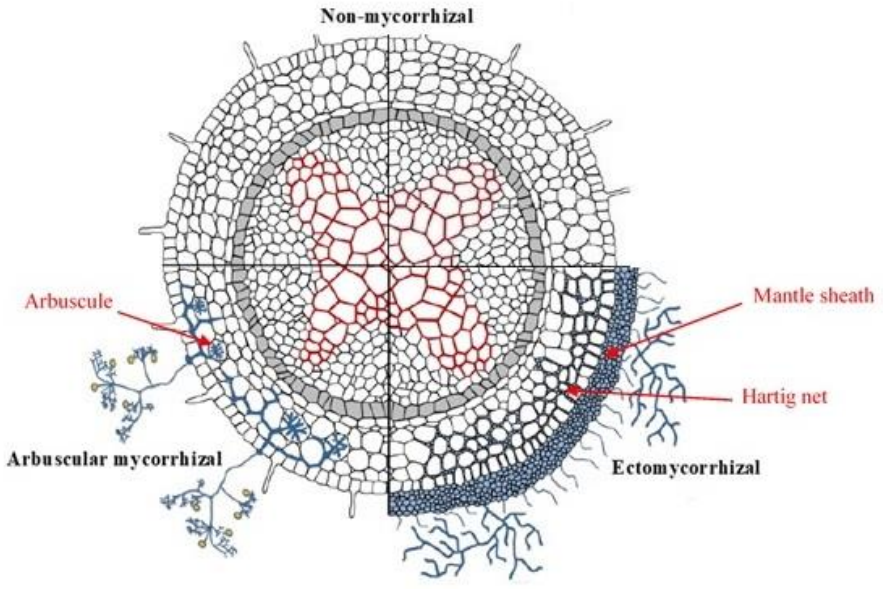



**Figure 2. 1** Structural characteristics of different types of mycorrhizal fungi compared with non-mycorrhizal in plant roots (arbuscular mycorrhizal and ectomycorrhizal, adapted from Bücking *et al.* (2012)).

### *2.2.1 Taxonomy and classification of AMF*

AMF may have been first described by Nägeli (1842) (Koide and Mosse, 2004). The subsequent history of the taxonomy and systematics of AMF can be separated into four periods. The initial period is termed AMF discovery, which spanned from 1845 to 1974 (Stürmer, 2012). In this period, AMF were mainly characterised based on the morphological characteristics of the sporocarp-forming species (a sporocarp is a structure that produces spores). The main method to extract sporocarps and non-sporocarpic spores from soils is wet sieving (Stürmer, 2012). This method was developed by Gerdemann and Nicolson (1963), and Gerdemann, in collaboration with Trappe later produced a seminal publication entitled "The Endogonaceae in the Pacific Northwest" (Gerdemann and Trappe, 1974). This seminal classification work described 30 species of arbuscular mycorrhiza and provided a basis for systematic knowledge during the years to follow (Stürmer, 2012).

The second period is known as the alpha taxonomy period (1975–1989), in which many new species were reported. All descriptions were based on spore morphology using spore sub-cellular structures, which are the most distinctly different features between species. Walker (1983) proposed a "murograph,"; a new description of species based on the distinct types of walls formed. Wall types are identified by phenotype in intact or broken spores. The groups represent aggregations of different wall types when a spore is broken. A murograph consists of a graphic representation of the different wall types and groups found in a spore. During this period, there were important AMF identification keys produced based on spore morphology, i.e., a synoptic key by Trappe (1982), the dichotomous key of Hall and Fish (1979), and keys for groups of AMF species developed by Koske and Walker (1985). An important publication, "Manual for the identification of vesicular arbuscular mycorrhizal fungi," was then published by Schenck and Perez (1988) that collected all summary species descriptions together. All keys are now out of print; however, some laboratories are still using them as an addition for AMF species identification (Kehri *et al.*, 2018).

The third period is the cladistics period from 1990 to 2000. This period introduced a new classification of AMF that includes information on the species genetic code as well as the traditional approach of using spore and phenotypic information (Stürmer, 2012). These classifications are characterised by; (I) phenotypic characters, (II) description of new taxa based on fossil records, (III) spore development and structure and, (IV) genetic characters. One of the most important developments during this period was the use of small subunit

(SSU) gene sequencing to clarify evolutionary associations among taxa within the order Glomerales (Stürmer, 2012).

Most recently, we have entered the phylogenetic synthesis period (2001 to present). This period is characterised by a new classification system based on genetic sequences of the SSU rRNA genes combined with phenotypic characteristics (Stürmer, 2012). The most important outcome of this period is from Schüßler *et al.* (2001). These authors proposed a new phylum known as Glomeromycota, which is a coherent monophyletic group of AMF within the kingdom Fungi. The classification of this phylum is based on SSU rRNA genes sequences. As a consequence, AMF were removed from the phylum Zygomycota. The phylum Glomeromycota consists of four orders; Archaeosporales, Diversisporales, Glomerales, and Paraglomerales (Redecker *et al.*, 2013; Redecker and Schüßler, 2014). However, Oehl *et al.* (2011) accepts five orders in the phylum Glomeromycota; Archaeosporales, Diversisporales, Gigasporales, Glomerales, and Paraglomerales. The order Gigasporales, which others include within the Diversisporales.

More recently, Spatafora *et al.* (2016), based on multigene phylogenetic analyses of genomes, has reclassified the AMF into the subphylum Glomeromycotina within the new phylum named Mucoromycota (Table 2. 1). The same four orders have been placed in the class Glomeromycetes, which includes 25 genera (Begum *et al.*, 2019). In this new subphylum Glomeromycotina, sexual reproduction is unknown and asexual reproduction occurs by producing azygospores or chlamydospores.

**Table 2. 1** Classification of AMF in the Phylum Mucoromycota (adapted from Spatafora *et al.* (2016)).

| | |
|---|---|
| Phylum | Mucoromycota |
| Subphylum | Glomeromycotina |
| Class | Glomeromycetes |
| Order | Archaeosporales |
| | Diversisporales |
| | Glomerales |
| | Paraglomerales |

### *2.2.2 Morphology, structural characteristics, and functions of AMF*

AMF were originally identified as a separate taxonomic group based on morphology, including intraradical and extraradical structures. The intraradical structures are formed inside the host cell roots, i.e., arbuscules, vesicles, spores, and hyphae. The extraradical structures are formed outside the host cell roots, found in the soils, i.e., hyphae and spores for some species (Kehri *et al.*, 2018). The information on these structures is provided in the following sections.

- **Hyphae**

There are two types of AMF hyphae: intraradical and extraradical. The intraradical hyphae (IH) are the infection unit (colonisation) within the roots of the host plant (Souza, 2015). IH are able to transfer water, nutrients, and metabolites from the surrounding soil to the roots of the host plant. The extraradical hyphae (EH) can penetrate and spread into soils and form an external network. EH can expand the plant's effective rhizosphere. EH are able to enhance nutrient uptake and the movement of nutrients from the EH to the host plant through the IH. EH have an additional role as reproductive organs that are able to produce the new spores in soil (Souza, 2015).

- **Arbuscules**

The AMF fungi can form arbuscules in the cortex of root cells, which leads to the name of this fungal group. An arbuscule is a tree-like shaped structure (Figure 2. 2), which is surrounded by the plasmalemma membrane of the host cells. This area is a symbiotic region within which metabolites (nutrients, amino acids, and sugar) are exchanged between fungi and plants (Bothe *et al.*, 2010; Gaude *et al.*, 2015).

- **Vesicles**

Many AM fungal species form vesicles, which are for the storage of energy in the form of lipids (Van Diepen *et al.*, 2007). Vesicles are globose or oval structures, as shown in Figure 2. 2 (Brundrett *et al.*, 1996). Vesicles are responsible for the maintenance and growth of the fungus after the stoppage of root metabolic function (Souza, 2015). In the past, the AMF were named vesicular-arbuscular mycorrhiza (VAM); however, it is now known that not all these

fungi form vesicle structures; consequently, the term arbuscular mycorrhizal fungi (AMF) is now used (Bothe *et al.*, 2010).

- **Spores**

Spores are reproductive structures formed both outside and inside the root cells (Bothe *et al.*, 2010). All AM fungal spores are asexual, formed singly, or in groups. Spores are borne terminally, laterally, or intercalary (occurring between differentiated tissues) on specialised hyphae (Gerdemann and Trappe, 1974). Many AMF species produce spores directly in soil or roots, but some species can produce them in a group (sporocarp), which for the AMF are aggregated spores with a definite enclosing peridium wall (Souza, 2015). Spores are structures produced for survival, resistance to harsh environmental conditions, and responsible for the dispersal and establishment of AMF (Varga *et al.*, 2015).

### *2.2.3 The life cycle of the AMF*

The life cycle of the AMF is graphically represented in Figure 2. 2. All structures, including the hyphae and spores, are found below ground. The life cycle starts with step 1, new spore production. AMF spores are produced on the hyphae termini. Step 2; dispersed AMF spores are germinated into hyphae (Bothe *et al.*, 2010), which can then colonise (step 3) and penetrate suitable plant root cells. The hyphae in this stage are called “appressorium.” Arbuscules are formed within the cortical cells of host plants where the nutrients are exchanged between the plant and the fungus. As mentioned above, some AMF species form storage structures called vesicles within the cortical cells. Following colonisation, the fungus produces new extraradical hyphae in the soil that will take up nutrients and transport them back to the plant. To complete the cycle, the fungus produces new spores that can germinate and colonise new host plants (Bücking *et al.*, 2012).

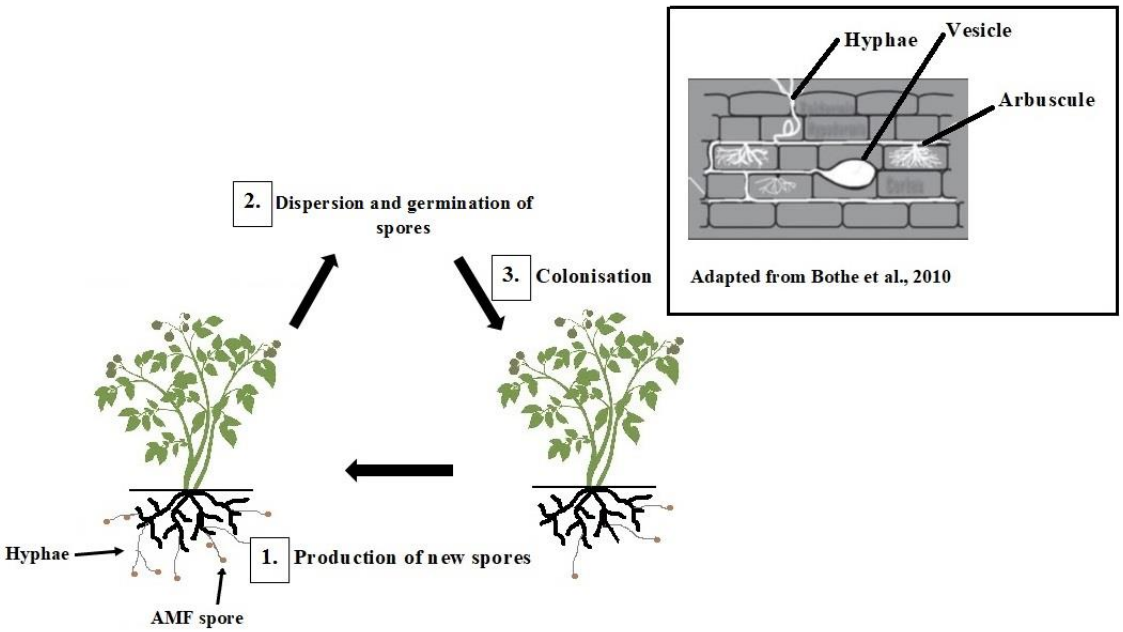



**Figure 2. 2** Life cycle of arbuscular mycorrhizal fungi.

### *2.2.4 Biogeography of AMF*

Since the eighteenth century, biologists have researched the geographic distribution of plant and animal diversity. It has only been relatively recently that the geographic distributions (biogeography) of microorganisms have been investigated (Martiny *et al.*, 2006). The occurrence of AMF living in association with plants in most parts of the world has now been documented (Smith and Read, 2008). AMF are now known to be among the most common soil microorganisms found in almost all terrestrial ecosystems from; tropical and temperate forests, grasslands to deserts, and agroecosystems (Brundrett, 1991). Öpik *et al.* (2006) reviewed and analysed the data from 26 publications that reported the occurrence of natural symbioses between AMF and host plants identified by using the rDNA region. The results showed that a high number of host plant species associated with a lower number of AM fungal taxa and there were differences between fungal communities of broad habitat types. High AM fungal richness was found in tropical forests.

Chaudhary *et al.* (2008) proposed a model for Glomeromycota biogeography based on three broad classes of factors controlling species distribution: abiotic external forces (e.g., climate, latitude), external biotic forces (e.g., plant community composition, specific interactions), and the intrinsic properties of the AMF (e.g., vulnerability to local extinction, dispersal ability).

Comprehension of the biogeography of AMF has expanded in the last two decades by using molecular methods (Stürmer *et al.*, 2018) enabled by DNA-based identification in the 1990s and advancement to high-throughput methods in the late 2000s. These methods have helped to clarify the mechanisms and processes of the AMF community and population ecology (Tedersoo, 2017).

The first biogeography database of AMF was produced by Stürmer *et al.* (2018). The database provides information on AM fungal species distributions mainly based on spore characteristic data and some DNA sequencing data. They describe the broad distribution patterns for class, orders, families, and genera in relation to continents, biogeographical realms, and climate zones. Species patterns are generally related to latitude, positions of ancient continents, and across biogeographical realms and biomes. The database consists of 7105 records gained from the international culture collection of (vesicular) arbuscular mycorrhizal fungi (INVAM) and international culture collection of Glomeromycota (CICG), and descriptions of AM fungal species in publications from 1960 to 2012. The results showed that the AM fungal distribution of families differed among four climate zones (tropical, subtropical, temperate, and boreal/austral) and seven continents (North America, South America, Africa, Europe, Asia, Antarctica, and Oceania (Australasia and Oceania). However, all genera appear to be cosmopolitan, meaning that they were found in at least four of seven continents. In terms of AMF species richness, the largest number of species was found in Asia, followed by South and North America.

In addition, the MaarjAM database from Öpik *et al.* (2010) also presents extensive AMF biogeographic records. It is an open-access database (http://maarjam.botany.ut.ee). This database is based mostly on small subunit (SSU) rRNA gene sequence occurrences, consisting of 2447 records. The MaarjAM database contains individual records of phylogroup occurrences organised by location and host plant species. The database includes two types of data, which are the ecological and taxonomic study of Glomeromycota. The database presents the AM fungal sequence data associated with metadata such as host plant, location, climatic zone, biome, etc. The database provides a reference dataset for the identification of AMF and presents the information about their host plant association, geographical distribution, and occurrence in different climatic regions and habitats.

## 2.3 Approaches to studying AMF in natural and managed systems

All AMF are endophytic obligate biotrophic fungi, which are very difficult to culture in synthetic media (Kehri *et al.*, 2018). AMF need the roots of a living host to colonise and complete their life cycle. This limits the approaches available to identify and study AMF present in natural and managed systems. The approaches and methods which have been developed to address this challenge are described below.

### *2.3.1 Morphological approaches to study AMF diversity*

This traditional method is mainly based on microscopic assessments of spore morphology, including shape, size, colour, ornamentation, wall layers, subtending hypha (hyphal attachment), and hyphal septation (Walker, 1983; Stürmer and Morton, 1997). AMF spore diameters are between 20-500 µm (Trappe, 1982; Schenck and Perez, 1990). These spores can be extracted from field soils and pot culture by a wet sieving and decanting method (Gerdemann and Nicolson, 1963). In this method, soil suspensions containing spores are first passed through various sieves with different mesh sizes (30-500 µm) (Muleta *et al.*, 2007). The larger particles of organic matter are removed by coarse mesh sizes. Following sieving, AMF spores can be collected by various decanting methods. For example, by using gelatin columns (Mosse and Jones, 1968), or 2M sucrose solution (Smith and Skipper, 1979) with centrifuging at 2000 rpm for 10 minutes, or the floatation-bubbling method using 50% aqueous glycerol solutions (Furlan and Fortin, 1975).

AMF spores are often collected after the extraction process by manually picking using a needle and micropipette and mounted on a glass slide. The mountant used can be polyvinyl lacto glycerol (PVLG) (Omar *et al.*, 1978) or PVLG with Melzer's reagent. The slides prepared can be stored for years.

The following spore characteristics are considered when describing AMF spore morphology. Illustratively, (Figure 2. 3) the arbuscular mycorrhizal spores are manifest in different shapes, sizes, and colours. Some species show surface ornamentation, wall, and hyphal attachments.

- **Shape and Size**

For the ease of AMF species identification, Trappe (1982) proposed the synoptic key to classify this fungal group by spore morphology. This key used the following terms to describe

the various shapes of spores from globose (spherical), reniform (kidney-shaped), ellipsoid (egg-shaped), obovoid (inversely ovoid), irregular and narrow clavate (club-shaped) to broadly clavate.

- **Colour**

The determination of colour is very difficult because spore colour can change for different ages of the same genus (Kehri *et al.*, 2018). Trappe (1982) reported that spore colour might vary from white to grey, yellow, brown, orange to red, violet, and to nearly black.

- **Surface ornamentation**

The surface of AMF spores at a young age is smooth while at maturity; they may be smooth, roughened, pitted, warty, covered with spines, or have the outer layer sloughing away (Berch and Koske, 1986; Błaszkowski *et al.*, 2001). Nonetheless, the pattern of surface ornamentation in spores is highly consistent within the same species and therefore is useful for classification purposes (Kehri *et al.*, 2018).

- **Wall**

Walker (1983) proposed a murograph, which is a graphic representation to describe the different wall types and groups in broken spores (Kehri *et al.*, 2018). The wall types such as unit wall (single layer), laminated wall (several layers), evanescent wall (single layer or several layers that break down when spore matured), and the membranous wall (the very thin wall that often wrinkles).

- **Hyphal attachment**

Some species form attachment hyphae, which are attached to the spore from which they arose. The kind of hyphal attachment is considered as an essential parameter to study AMF morphology (Gerdemann and Bakshi, 1976). The shape and colour of the hyphal attachment are used to classify AMF into groups (Palenzuela *et al.*, 2013).

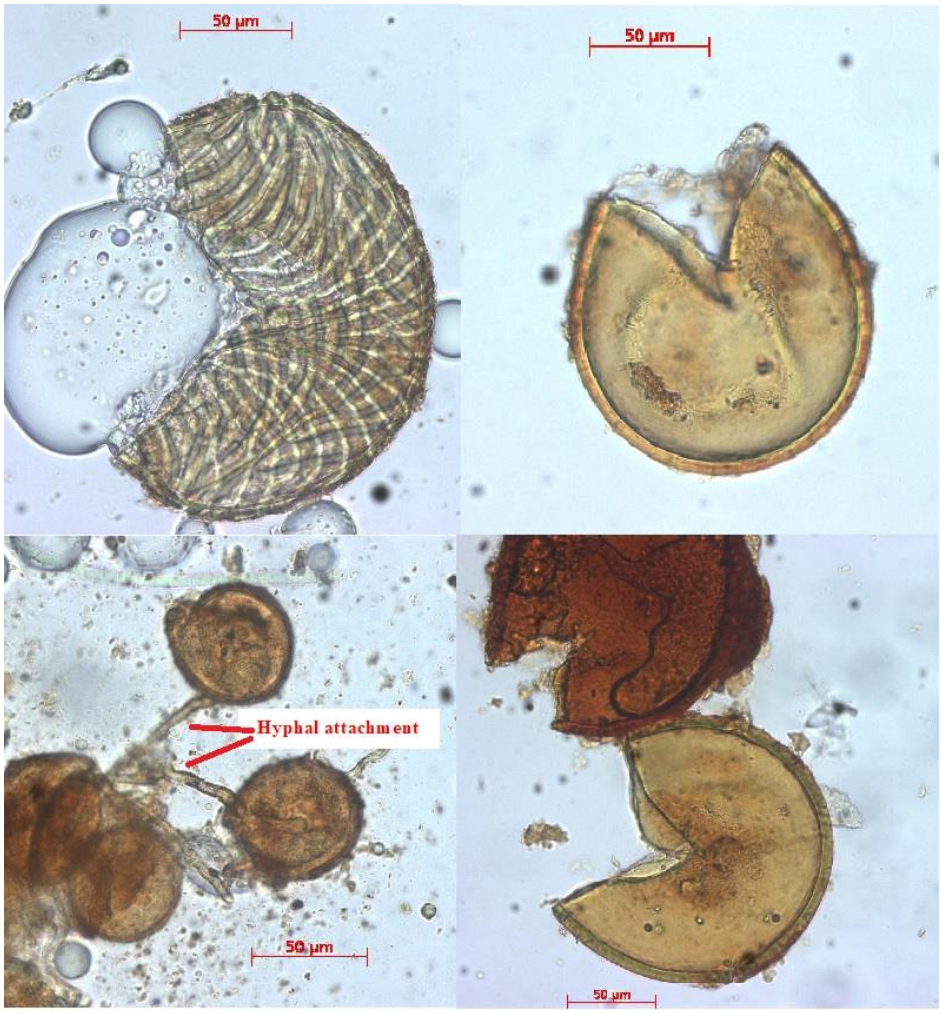



**Figure 2. 3** Various AMF spores extracted from the soil by wet sieving and decanting method (Suwanarit *et al.*, 2007).

### *2.3.2 Microscopic techniques to study AMF associations in roots*

AMF associations refer to the symbiotic associations between fungi in the phylum Glomeromycota and higher plant roots. The intraradical structures can be assessed to understand this association (Merryweather and Fitter, 1998) and are used to quantify the degree of colonisation by this fungal group in the root system (Biermann and Linderman, 1981). The percentage colonisation by AMF can be estimated based on the presence or absence of the intraradical structures in host roots (Biermann and Linderman, 1981). Arbuscules, vesicles, and intraradical hyphae can be viewed by staining to observe root colonisation under a compound light microscope. Consequently, various staining dyes have been developed, including trypan blue in lactophenol (Phillips and Hayman, 1970), cotton blue, and Sudan IV in lactophenol (Nicolson, 1959), and acid fuchsin (Gerdemann, 1955).

However, some chemicals should not be used for health and safety reasons (Vierheilig *et al.*, 1998), such as trypan blue, which is listed by the International Agency for Research on Cancer as a possible carcinogen (Cancer, 1975). Contact may cause irritation, gastrointestinal discomfort, and vomiting. Long term contact may induce retinal damage (Veckeneer *et al.*, 2001). Besides, acid fuchsin is a suspected carcinogen (Combes and Haveland-Smith, 1982). For these reasons, Vierheilig *et al.* (1998) proposed an alternative method for root staining using ink and vinegar. This method provides a simple, inexpensive, and safe technique.

### *2.3.3 Molecular approaches for AMF studies*

Molecular techniques have been developed and applied to study the community composition, alpha and beta diversity (community structure and assemblage between samples), taxonomy, and to quantify populations of AMF in soils and roots (Clapp *et al.*, 1995; Helgason *et al.*, 1998; Ma *et al.*, 2005; Taylor *et al.*, 2017). Colonised root fragments, AMF spores from soils, and bulk soils are used to extract nucleic acids in these studies. Ribosomal DNA (rDNA) is a well-established molecular marker for characterisation of AMF (Clapp *et al.*, 2002). The rDNA consists of conserved genes for the small subunit (18S SSU), 5.8S, and large subunit (LSU) regions of the eukaryotic ribosome. These sequences are separated by non-functional spacer regions called Internal Transcribed Spacer region 1 (ITS1) and 2 (ITS2). Targeted sequences from the SSU, LSU, ITS1, and ITS2 regions can be applied alone or in combination and are essential tools for identifying both phylogenetic relationships (DNA taxonomy) and species identification (DNA barcoding) (Van Tuinen *et al.*, 1998a; Schüßler, 1999; Redecker, 2000; Raja *et al.*, 2017). The details of using nucleic acids to study AMF are described in the following sections.

#### Sample preservation

This step is very important before starting molecular approaches. The aim is to keep an accurate representation of fungal communities (Rubin *et al.*, 2013) because these can change in composition over hours and days if not sufficiently preserved once removed from the natural environment. Besides, to avoid DNA degradation, the time from sampling to sample preservation should be kept to a minimum (Dighton and White, 2017). Samples from natural and managed systems, i.e., colonised roots and soils, can be stored frozen, dried (by air-drying, oven drying, silica gel drying, and freeze-drying), or mixed with a liquid preservative (such as RNAlater®; Thermo-Scientific, Waltham, Massachusetts or LifeGuard™ Soil

Preservation Solution; MOBIO Laboratories, Inc., Carlsbad, California). Immediate freezing of samples in liquid nitrogen and transfer to a -80 $^{o}$C freezer is considered adequate and common practice to preserve all nucleic acids (Dighton and White, 2017).

**DNA extraction**

Various methods are available for DNA extraction, such as CTAB (Cetyltrimethylammonium bromide) protocol, phenol-chloroform, trizol, and kit-based nucleic acid extractions (Dighton and White, 2017). While there is no standard protocol for DNA extraction, all DNA extraction protocols are normally based on the same basic process (Lindahl *et al.*, 2013) according to the following steps;

1) disruption of tissues by mechanical methods. This can include physical cell disruption by bead beating to maximise nucleic acid yields from samples (Dighton and White, 2017),

2) solubilisation of cell membranes by detergents under high salt concentrations for the release of nucleic acids into the solution and prevention of its electrostatic binding to contaminants,

3) removal of solid residues by filtration or centrifugation,

4) two options for precipitation of nucleic acids; a selective binding agent of nucleic acids to a solid matrix or pelleting by centrifugation,

5) removal of contaminants (protein, polysaccharides or phenolics) which can act as PCR inhibitors by additional precipitation and ethanol washes (Taylor *et al.*, 2014) or using a clean-up kit system, and,

6) final elution/solubilisation of purified nucleic acids.

DNA extraction protocols may sometimes be modified to add extra cleaning steps to minimise contamination by PCR inhibitors or tissue disrupting processes to maximise DNA yield; however, regardless of these modifications, it is essential that all samples within a study should be treated in the same way to avoid biases (Tedersoo *et al.*, 2010). High nucleic acid yields of good quality are desirable for community analysis (Dighton and White, 2017).

Most of the steps listed above have been incorporated into off-the-shelf extraction kits that are convenient and have been optimised for the specific sample types such as soil to produce a

high yield and good quality of DNA (Lekang *et al.*, 2015). DNA extraction kits from MoBio (https://mobio.com/) and Qiagen (www.qiagen.com) are widely considered as standard for the extraction of nucleic acids (Dighton and White, 2017). The kits designed for soil DNA extraction are generally used for roots because of the presence of compounds from the soil in root samples and the need to remove these during the DNA extraction process.

**DNA amplification by polymerase chain reaction (PCR)**

PCR amplification is a powerful method to produce multiple copies of a target region of DNA for molecular research of AMF. The PCR based approach has enabled the linking of phenotypic data (spore morphology) with genotypic data (DNA sequences) (Taylor *et al.*, 2017). PCR with specific primers (short nucleic acid sequences) can be applied to amplify genes from known AMF species in root tissues (Van Tuinen *et al.*, 1998a), which is a powerful method employed for ecological studies in the natural environments (Bridge, 1998). The advancement of specific primers targeting individual taxa has facilitated studies on well-described taxa of soil microorganisms; however, several unknown AMF can colonise roots in the field, so this specificity may not be suitable for field studies. Hence, general AMF primers for amplifying fragments of genes coding for SSU rDNA, ITS regions, and LSU rDNA, which target a broad range of the AMF, have been developed and applied (Kjøller and Rosendahl, 2000a). Targeting these regions associated with ribosomal genes allows for identification and discrimination to the genus and species level after sequencing.

To multiply the limited quantity of AMF DNA often obtained from the colonised roots, soils, and spores, a nested PCR approach can be applied (Kjøller and Rosendahl, 2000a). The nested PCR approach comprises two PCR steps for amplification (Saito *et al.*, 2001), where a universal set of primers is used in the first amplification and more specific primers for the second PCR step (Kjøller and Rosendahl, 2000a; Rosendahl and Matzen, 2008). This allows enhancing the specificity of the PCR reaction by reducing the non-specific binding and increases the quantity of template DNA for further community analysis, e.g., T-RFLP and NG sequencing (Yourno, 1992; Van Tuinen *et al.*, 1998a)

**DNA fingerprinting techniques**

Besides PCR-sequencing based approaches (see below), various low resolution (fingerprinting) PCR-based methods have been used to study AMF community diversity, i.e., restriction fragment length polymorphism (RFLP), single-stranded conformation

polymorphism (SCCP), terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) (Liang *et al.*, 2008). These are described in more detail below.

- **Denaturing Gradient Gel Electrophoresis (DGGE)**

The first use of DGGE to study microbial communities was by Muyzer *et al.* (1993). Since then, DGGE has been a powerful technique for the culture-independent detection and characterisation of fungal populations in plants and soils. DGGE is a technique to separate short to medium-sized DNA fragments on the basis of single nucleotide polymorphisms. This molecular technique is a fingerprinting method for complex microbial communities of environmental samples (Marschner, 2007; Strathdee and Free, 2013). After DNA extraction and PCR, DGGE can be applied by electrophoresis of amplicon DNA on acrylamide gels with gradients of denaturing chemical (urea and formamide) concentrations. Double-stranded PCR products of the same length but differing nucleotide compositions are separated in the denaturing gradient gel (Green *et al.*, 2010). The DNA fragments migrate through the gel from a low to a high denaturant concentration and form a band when they reach the concentration of denaturants at which they partially denature and are no longer able to migrate through the gel. A key component of this approach is the inclusion of a GC-rich addition (clamp) introduced into the amplified PCR products by the use of modified PCR primers, which ensures only partial denaturing of the amplicon fragment. The results are a banding pattern that varies with community composition. Sequences with a high GC content move further through the gel than GC poor sequences.

DGGE has become one of the most applied techniques to study the community structure of uncultured microorganisms (Muyzer and Smalla, 1998). DGGE has been successfully used for analysing the community structure of AMF in various habitats (Ma *et al.*, 2005; Liang *et al.*, 2008). It is a rapid and inexpensive method to assess complex communities of target organisms. However, it is estimated that microbial community analysis by DGGE can only detect 1–2% of the microbial diversity, which represents only the dominant species present in an environmental sample (Muyzer *et al.*, 1993). Moreover, community complexity may be underestimated as one band may contain the sequences of several species.

- **Terminal Restriction Fragment Length Polymorphism (T-RFLP)**

T- RFLP was first developed by Liu *et al.* (1997) and involves PCR amplification using a fluorescent-labelled primer with subsequent restriction digestion to visualise sequence variation of DNA samples. The data obtained are based on the electrophoresis of different sizes of the fragments of PCR amplicons (terminal restriction fragments; TRFs product) that contain the labelled primer. TRFs are detected as electropherogram "peaks." The variation in the presence and the location of cutting sites results in different species having terminal fragments of different lengths (Dickie and FitzJohn, 2007). TRFs community fingerprints for samples analysed can be compared in terms of diversity (richness, evenness) and community structure through analysis of relative abundances of TRFs (Watanarojanaporn et al., 2014). When combined with cloning and Sanger sequencing, it can be used for identifying organisms in targeted samples. However, in recent years, it is less popular because of the rapid growth of low-cost NG sequencing services that provide both community structure and species composition (taxonomic) data. Dickie and FitzJohn (2007) recommended that T-RFLP is more sensitive than DGGE for fungi, even though obtaining sequences from samples can be more easily conducted with DGGE (Ma *et al.*, 2005). Moreover, T-RFLP has essential advantages in cost (Widmer *et al.*, 2006).

**Molecular techniques for species identification**

- **Cloning and Sanger sequencing for community composition studies**

Identifying AMF in natural systems is complicated because these microorganisms live in a host plant (Smith *et al.*, 2008). In the past, identification was based on spore morphology, but more recently, DNA-based methods targeting the fungal structures in roots and soils have replaced this visual approach (Öpik and Davison, 2016). The Sanger sequencing method was developed by Sanger *et al.* (1977) to sequence DNA by chain termination and fragmentation techniques; DNA is amplified with labelled and unlabelled nucleotides and run through an automatic sequencer. Sanger sequencing approaches have been used to identify AMF in environmental samples since the 1990s, with studies typically generating sequencing depth (number of sequences per sample) in the tens to hundreds and tens to thousands of sequences (Öpik *et al.*, 2008). Recently this approach has become less popular due to the low number of sequences obtained and also the high cost of sequencing relative to new technologies in next-generation sequencing (Gorzelak *et al.*, 2012).

- **Next generation sequencing (NGS) methods for AMF community composition studies**

NGS refers to high-throughput DNA sequencing technology developed after Sanger sequencing (Kulski, 2016). From a community analysis perspective, NGS is different from the cloning-sequencing approach facilitated by the Sanger technique in that it provides a massively parallel sequence analysis of single genes from multiple samples at a much-reduced cost. Alternatively, using a shotgun sequencing approach, NGS technologies can produce large amounts (millions to billions) of DNA reads (Barba *et al.*, 2014) per instrument run with the ability to deliver fast, inexpensive, and accurate genome information.

The first generation of NGS technologies was developed in 2000 by the Massively Parallel Signature Sequencing (MPSS), Lynx Therapeutics (USA) Company. The company was later taken over by Illumina (Barba *et al.*, 2014). MPSS worked by counting all mRNA molecules in the cell sample or cell tissue. All genes were analysed by sorting out the number of mRNAs from each gene relative to the total number of molecules in the sample (Reinartz *et al.*, 2002).

Then, in 2005, 454 pyrosequencing emerged from the 454 Life Sciences company (Branford, CT, USA) (now Roche). This sequencing method allowed sequencing depth of environmental samples to increase to hundreds to thousands of sequences per DNA sample generating around 200,000 reads of 110 bp and reducing costs 6-fold compared to Sanger sequencing (Barba *et al.*, 2014; Vasar *et al.*, 2017). However, from a community analysis perspective, the size of the recovered DNA fragments reduced the taxonomic resolution making it difficult to compare the results from different studies and identification methods (Tedersoo *et al.*, 2010).

The Solexa/Illumina sequencing platform was released in 2007, superseding 454 pyrosequencing. Illumina has developed the HiSeq platform series, consisting of HiSeq® 2500, HiSeq 2000, HiSeq 1500, and HiSeq 1000 sequencing platforms that vary in cluster generations, run times, paired-end reads, and maximum reads length (Shokralla *et al.*, 2012). The Illumina sequencers generated much larger numbers of reads than 454 sequencings; however, the reads produced were only 35 bp long (Van Dijk *et al.*, 2014). These short sequences are sufficient for doing genomic sequencing where the fragments can be pieced together to reconstruct the entire genome but are problematic for community analysis approaches which use single gene amplification followed by taxonomic assignment during pipeline analysis.

In 2010, Ion Torrent (now Life Technologies) presented the Personal Genome Machine (PGM). This system was developed by the founder of 454 sequencing technology (Van Dijk *et al.*, 2014). The difference was that the PGM used semiconductor detection technology (not optical detection) and had a lower cost with a higher speed of detection using a smaller instrument. The PGM approach initially generated sequences up to 270 Mbp of sequence (Van Dijk *et al.*, 2014) with 150-250 nt (nucleotide) reads (Harvey *et al.*, 2019).

Illumina released MiSeq in 2011, a platform that shared most technologies with the HiSeq series. It generates 1.5 Gbp per run in around 10 hours (Barba *et al.*, 2014) with up to 300 bp paired reads (Van Dijk *et al.*, 2014; Jeon *et al.*, 2015). Illumina MiSeq generates shorter reads but achieves deeper sequencing compared to 454 pyrosequencing (Schmidt *et al.*, 2013). In addition, this approach has a lower error rate than 454 pyrosequencing with lower cost (Shokralla *et al.*, 2012; Schmidt *et al.*, 2013; Vasar *et al.*, 2017). Schmidt *et al.* (2013) found that paired-end Illumina MiSeq was suitable for fungal community assessment in environmental samples, and Illumina sequencing has been applied to study the species composition of AMF communities (Cui *et al.*, 2016; Vasar *et al.*, 2017; Egan *et al.*, 2018).

Since NGS or high throughput sequencing (HTS) approaches have been developed and applied in conjunction with advances in bioinformatics, knowledge of the biology, ecology, and biodiversity of AMF associations has increased (Balestrini and Lumini, 2018). This technique is now the preferred method for various areas of AM fungal research. For instance, Xu *et al.* (2017) used Illumina Miseq to compare the community structure of AMF in common wild rice (natural wetland) and cultivated rice fields using a nested PCR amplification approach (primers; ITS1F/ITS2R and AMFLR3/AMFLR4). They found 38 OTUs of Glomeromycota distributed in nine genera within the four orders of AMF; the diversity of AMF community in the natural wetland was higher than cultivated rice field. Cui *et al.* (2016) studied the effect of three land reclamation stages (since 2011, 2007, and 1979) on AMF community structure and diversity in coastal saline-alkaline fields in China using Illumina sequencing. They detected a change in AMF community structure and a decrease in AMF diversity since 1979.

**Molecular quantification of AMF**

Previously, the quantification of AMF relied on either spore counts (Oehl *et al.*, 2005) or root colonisation (Vierheilig *et al.*, 2005) enumerated by microscopy, biochemical measurements

by phospholipid fatty acids of AMF (the structural elements of membrane lipids) (Olsson *et al.*, 1999) or detection of root ergosterol (AM fungal biomass determined indirectly by the concentration of root ergosterol) (Hart and Reader, 2002). These methods provided general information about the abundance of the total AMF community under different conditions. However, they did not provide fine-scale taxonomically resolved quantification. Many primers have been reported for AMF detection (Simon *et al.*, 1993; Van Tuinen *et al.*, 1998a; Redecker, 2000), but they produced qualitative data with quantitative determination of the target AMF remaining an obstacle. As recently as 1997, only one publication had reported the quantification of *Glomus mosseae* in a host plant by means of PCR product ratio (Edwards *et al.*, 1997).

After the turn of the last century, the advancement of quantitative real-time PCR techniques allowed improvements in both detection and quantification of genes targeting AMF (Alkan *et al.*, 2004). qPCR is now a widespread technique with a high potential to process large numbers of samples with accurate quantitative information (Taylor *et al.*, 2017). qPCR uses fluorescent probes or fluorescent primers incorporated into target amplicon DNA to detect the PCR products and identify the number of thermal cycles required to reach the exponential phase of the amplification process. This number is directly correlated with the number of DNA sequence targets in the original sample, and absolute abundances can be derived by calibrating against known target sequence standards. This approach removes the non-linear amplification limitations that characterise the endpoint detection methods used in normal PCR. The first successful application of qPCR for AMF quantification in plant roots was reported by Alkan *et al.* (2004). They used species-specific primers to quantify *Rhizophagus irregularis* (formerly *Glomus intraradices*) in host tissues of tomato and *Medicago.* The authors found that qPCR was faster than microscopic methods such as root staining. Several other projects then used qPCR for studies of AMF (Alkan *et al.*, 2006; Janoušková *et al.*, 2017; Mohammed *et al.*, 2017; Bender *et al.*, 2019). For example, Corradi *et al.* (2007) used qPCR to quantify *Glomus intraradices* using primers targeting four different genomic regions (BiP, 18S, 5.8S, and 25S genes). Labonova *et al.* (2018) used qPCR to detect and quantify the genomic DNA of *Rhizophagus irregularis* in grapevines by the specific primers that were designed by Alkan (Alkan *et al.*, 2006). Voříšková *et al.* (2017) quantified four AMF isolates (*R. irregularis, Claroideoglomus claroideum, Gigaspora margarita,* and *Funneliformis mosseae*) by qPCR using markers in nrDNA and mtDNA in a greenhouse experiment with *Medicago truncatula* as the host plant. They found that all AMF were quantified by qPCR

targeted to both markers, the copy numbers of nrDNA and mtDNA were closely associated. The research challenges for qPCR include the design of qPCR markers that consistently (Thonar *et al.*, 2012). The markers require wide cross-specificity to target all species of AMF while at the same time not amplifying DNA from non-target fungi or other organisms. Therefore, qPCR quantification has been mostly limited to measure AMF abundance in a simplified system.

**AMF primers for PCR amplification**

Several primers have been published for AMF-targeted PCR amplification in various parts of the rDNA region, i.e., SSU, LSU, and ITS regions (Jansa *et al.*, 2003; Lekberg *et al.*, 2012; Jansa *et al.*, 2014; Řezáčová *et al.*, 2016). The most commonly used primer combination is AM1 (Helgason *et al.*, 1998) and NS31 (Simon *et al.*, 1992). Table 2. 1 illustrates some common primer sets for the molecular analysis of fungi and AMF (Taylor *et al.*, 2017). NS31 amplifies all eukaryotic species, and AM1 is specific to AMF; however, AM1 does not amplify the orders Archaeosporales and Paraglomerales (Schüßler *et al.*, 2001). Newer specific primers (AML1 and AML2) targeting the same region have subsequently been generated. Both primers can amplify sequence targets from all major orders of AMF (Lee *et al.*, 2008). The primers AM2 and AM3, designed by Santos-González *et al.* (2007) based on a modification of AM1 by increasing taxon coverage, were developed to amplify *Diversisporaceae* species. In addition, several AMF specific primers have been designed for the identification of the AMF by targeting the highly variable ITS region.

**Table 2. 2** Primer sets for molecular analysis of general fungi and AMF (Taylor *et al.*, 2017).

| Primer Set | Reference | Target Region | Notes |
|---|---|---|---|
| NS1, NS2, NS4, NS5, NS8, ITS1, ITS4 | White *et al.* (1990) | SSU, ITS | Universal eukaryote primers for nested PCR |
| NS31 | Simon *et al.* (1992) | SSU | Universal eukaryote primers |
| LR1, NDL22 | Van Tuinen *et al.* (1998b) | LSU | Universal eukaryote primers |
| AM1 | Helgason *et al.* (1998) | SSU | Can amplify other groups of fungi, limited coverage of Paraglomeraceae |
| FLR2 | Trouvelot *et al.* (1999) | LSU | Paired with LR1 for fungi only |
| SSU-Glom1, LSU-Glom1 | Renker *et al.* (2003) | ITS | Primary PCR with restriction digest before secondary amplification, using universal ITS primers for AMF (Glomeromycota) |
| FLR3, FLR4 | Gollotte *et al.* (2004) | LSU | AM fungal-specific primers |
| AMV4.5NF, AMV4.5NR | Saito *et al.* (2004) | SSU | AM fungal-specific primers |
| AMDGR | Sato *et al.* (2005) | SSU | AM fungal-specific reverse primers with improved coverage |
| AM2, AM3 | Santos-González *et al.* (2007) | SSU | Variants of AM1 that increase taxon coverage |
| AML1, AML2 | Lee *et al.* (2008) | SSU | Longer fragment than NS31-AM1, improved AMF taxon coverage, amplifies some plants |
| SSUmAf, SSUmCf, LSUmBr, LSUmAr | Krüger *et al.* (2009) | SSU, ITS, LSU | Composite primer mixtures for high-taxon coverage |
| Glo454 | Lekberg *et al.* (2012) | LSU | Combined with NDL22 for 454-pyrosequencing |
| intra, clar, moss, gig, scut | Thonar *et al.* (2012) | LSU | AM fungal-specific primers and hydrolysis probes |
| WANDA | Dumbrell *et al.* (2010) | SSU | Combined with NS31 10 bp toward 5' end from AM1, produces a shorter fragment for 454-pyrosequencing |

## 2.4 AMF in agriculture

The promotion of beneficial soil microorganisms is being considered as a method to improve plant productiveness and the sustainability of agricultural ecosystems (Verbruggen *et al.*, 2013). Lorenz Hiltner, the German agronomist, first defined the rhizosphere in 1904 (Stefan *et al.,* 2012) as the 'soil compartment influenced by the root' (Smalla *et al.,* 2006). Since then, many research studies have identified that the soil attached to the root system is a region of microbial abundance and activity because of the presence of root exudates. Many studies have shown that the microbial communities within the rhizosphere can positively affect plant growth (Amballa and Bhumi, 2016). Microbial attachment to and propagation on roots is generally described as root colonisation (Chin-A-Woeng and Lugtenberg, 2008). It should be noted that not all interactions are positive and that root colonisation is an essential factor in plant pathogenesis of soil-borne microorganisms as well as the role it plays in advantageous interactions, i.e., microbiological control, phytostimulation, phytoremediation, and biofertilisation.

AMF are common components of the soil microbial community inhabiting the rhizosphere. The first report of a positive effect of AMF inoculant on plants was published by Asai (1944) during World War II in Japan. Asai showed that mycorrhizal plants grew up faster than non-mycorrhizal plants (Koide and Mosse, 2004). In 1957, Mosse reported the study of AMF symbiosis in Europe. The research showed that AMF (*Glomus mosseae*) improved the growth of apple seedlings in sterile soil. Research on the capability of AMF in agriculture followed on with discoveries in the 1950s, 1960s, and 1970s. For instance, AMF have been shown to possess the potential to increase P uptake and promote the growth of plants (Koide and Mosse, 2004). More generally, AMF play essential functions in ecosystems, including nutrient uptake and transfer. AMF can be considered as performing the role of "biofertiliser and bioprotector" for sustainable agriculture (Köhl *et al.*, 2016; Teotia *et al.*, 2017).

Many research studies have been conducted in the greenhouse (Xavier and Germida, 1997) and field experiments where inoculation of AMF has been successful with the inoculated AMF colonising the roots and promoting plant growth (Kahiluoto and Vestberg, 1998; Al-Karaki, 2002; Al-Karaki *et al.*, 2004; Douds Jr *et al.*, 2005). Enhancing the AMF-plant symbiosis has been suggested as an important way of increasing food security and agricultural sustainability (Wilkinson *et al.*, 2019). Microbial symbionts of plants and microorganisms are considered particularly crucial for improving global crop yields, especially in settings with

limited resources. This is because AMF perform an essential function of mobilising and solubilising mineral nutrients from what can be considered unavailable organic substrates, mineral particles, and rock surfaces in the soil (Teotia *et al.*, 2017). Therefore, the significance of this symbiotic association and its management is of considerable agricultural interest.

### *2.4.1 Key functions of AMF in agricultural soils*

- **Plant nutrient uptake**

AMF can significantly enhance plant nutrient uptake (Begum *et al.*, 2019). Several research studies had illustrated the increased nutrient uptake of various macronutrients and micronutrients when AMF established symbiotic associations with host plants, under in a both field and glasshouse conditions. For instance, in a pot experiment under greenhouse conditions, AMF inoculant associations positively increased the concentrations of P, N, and Fe in the plant *Pelargonium graveolens* L. under drought stress (Amiri *et al.*, 2017). In addition, *Euonymus japonica* inoculated with *Glomus iranicum* var. *tenuihypharum* showed improved levels of P, Ca, and K under salinity stress (Gómez-Bellot *et al.*, 2015). It is thought that AMF enhance the plant's uptake of nutrients because the AM fungal hyphae greatly increase the volume of soil that can be accessed for nutrients, especially for plant species with poor root and root hair development (van de Wiel *et al.*, 2016). Consequently, the surface area for the absorption of nutrients is increased (Priyadharsini and Muthukumar, 2015). The most important nutrient that AMF can improve uptake for many plants is P (Smith *et al.*, 2011).

Plants take up P from the soil as inorganic orthophosphate (Pi) in the form of either $H_2PO_4^-$ or $HPO_4^{2-}$ (Shen *et al.*, 2011) by two pathways (Smith *et al.*, 2011); the first is the plant pathway via epidermal cells of root hairs and the second is the mycorrhizal pathway via extraradical hyphae. From Figure 2. 4, the direct uptake pathway from plants involves plant Pi transporters (membrane proteins) in roots that are located in the epidermal cells of root hairs (Bücking and Kafle, 2015). P is often limited in the soil by low mobility, causing a rapid development of depletion zones in the rhizosphere (Bücking *et al.*, 2012). Consequently, uptake by the plant pathway is often limited. The mycorrhizal pathway can uptake P by fungal Pi transporters in extraradical hyphae. Initially, solubilisation of phosphate from insoluble phosphates to the soluble form by lowering the pH may be facilitated by acid phosphatase enzymes in the hyphal tips of AMF (Lone *et al.* (2017). Then, P is transported through hyphae from

extraradical hyphae (ERH) to intraradical hyphae (IRH) by mycorrhiza-inducible plant Pi transporters in the periarbuscular membranes in the mycorrhizal interface region (Smith and Read, 2010). The ERH can absorb P in the soil beyond the depletion zones; hence the efficiency of P uptake has been enhanced. Several reports have shown the positive effect of AMF on P uptake, for example, the pot experiment of Zhang *et al.* (2019). They studied the synergistic effects of AMF inoculant (a mixture of four AMF species of *Glomus*) and biochar on growth and nutrient uptake of *Medicago sativa* in cadmium-contaminated soils. Their results showed that the combination of AMF inoculant and biochar significantly promoted N and P contents in shoots, N and P uptake and reduced cadmium uptake. Frew (2019) reported inoculation with a single species of *Rhizophagus irregularis* or a mixture of four AMF species (*Claroideoglomus etunicatum, Funneliformis coronatum, F. mosseae,* and *Rhizophagus irregularis*) can enhance the growth (aboveground biomass) and P concentration of *Hordeum vulgare* and *Sorghum bicolor* compared to an uninoculated control.

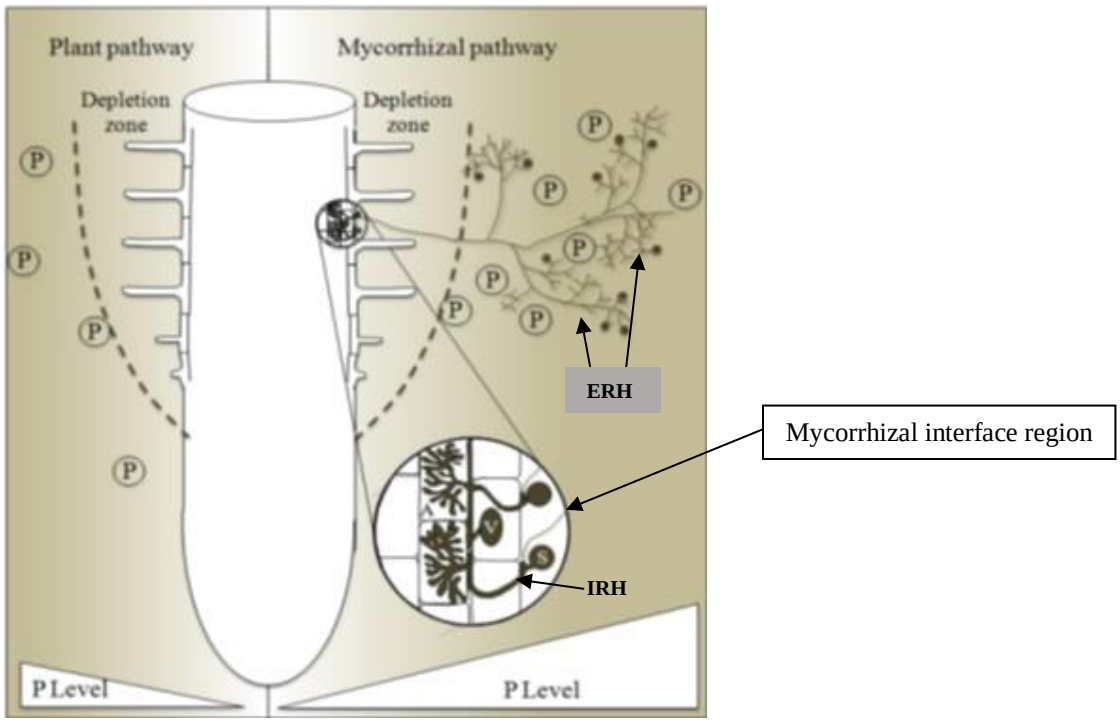



**Figure 2. 4** Two pathways of plant P uptake showing the plant pathway (left side) with inorganic P uptake limited to the depletion zone, and the mycorrhizal pathway (right side) showing the extraradical hyphae (ERH) extending the volume of soil accessed for inorganic P uptake and intraradical hyphae (IRH) inside the host cell (Bücking *et al.*, 2012).

- **AMF and plant diseases**

AMF can enhance host plant resistance to diseases caused by soil-borne pathogenic microorganisms and root pathogens (Graham, 2001; Lone *et al.*, 2017). There are many research studies on AMF as biological control agents against plant pathogens (Xavier and Boyetchko, 2004; Maherali and Klironomos, 2007; Yuan *et al.*, 2016; Rahman *et al.*, 2017; Bagy *et al.*, 2019). Some studies have investigated the interactions of AMF and phytopathogenic nematodes (Hol and Cook, 2005; Schouteden *et al.*, 2015; Tchabi *et al.*, 2016) and viruses (Miozzi *et al.*, 2019). AMF may also play a role in inhibiting plant pathogenic fungi (Liang *et al.*, 2015; Song *et al.*, 2015b). Many important plant pathogenic fungi, for example, *Phytophthora*, *Fusarium*, *Aphanomyces,* and *Sclerotium* have been reported to be controlled by AMF (Vigo *et al.*, 2000).

Control of plant pathogens may be through AMF colonisation inducing the production of secondary compounds in the host plants (Lone *et al.*, 2017). Secondary compounds have a positive effect on plant survival and include phytoalexins (antimicrobial substances) and pathogen-related proteins (antimicrobial and antifungal properties) (Verpoorte *et al.*, 2002; Cabral *et al.*, 2019). Devi and Reddy (2002) showed that levels of the phenolic compound (ortho-dihydric phenolic acid) was significantly increased in groundnuts when inoculated with AMF and the symbiotic N-fixing bacteria, *Rhizobium,* compared to non-inoculated plants. These phenolic compounds in plants play a significant role in host defense mechanisms against diseases. Silva *et al.* (2008) showed that *Zingiber officinale* (Ginger) that was inoculated with a mixed culture of AMF (*Scutellospora heterogama* SCT120E, *Gigaspora decipiens* SCT304A, *Acaulospora koskei* SCT400A, *Entrophospora*) developed oleoresin production. Oleoresin is an essential oil produced by plants that has a suppressing effect on the soil-borne plant pathogen *Pythium aphanidermatum (*Tabin *et al.* (2009))

Another mechanism of disease suppression could be through competition for resources. Wehner *et al.* (2010) and Schouteden *et al.* (2015) both hypothesised that AMF inhibit plant pathogens by competitive interactions for space or nutrients. This is because AMF and pathogens utilise common resources within the root for their growth; increasing the richness of AMF may result in more extreme competition with these plant pathogens.

Finally, AMF potentially enhance plant tolerance to pathogens by increasing nutrient and water uptake and hence the plant's ability to tolerate pathogens.

- **Enhancement of soil structure**

Soil structure is an important property that may also be positively affected by AMF. AMF and plant roots, by their interaction, are involved with soil aggregate stability, even if the mechanism is not known (Wu *et al.*, 2008). It is supposed that AMF colonisation may improve the soil structure by producing glomalin (Rillig, 2004), a glycoprotein. Wright and Upadhyaya (1999) reported that glomalin is correlated with soil aggregate stability and helps the soil to maintain water content (Begum *et al.*, 2019) by improving the soil water holding capacity. According to Holátko *et al.* (2021), they found that soil organic matter extraction is comprised of a variety of compounds with including some of non-AMF origin.

Consequently, the term glomalin-related soil proteins (GRSP) was used. GRSP concentrations in the soil have been correlated with AMF, but not always. This is because not all AMF species produce the same amount of GRSP per unit of their biomass. There were no significant correlation results, and even a negative correlation result has been observed (Holátko *et al.*, 2021). GRSP may not originate from AMF, for example, proteins from bacteria and some soil microorganisms, especially the microorganisms associated with AMF hyphae. The GRSP chemical structure is still unclear, and more work is needed to establish the best extraction and purification method for determining AMF biomass.

### *2.4.2 The application of AMF in agriculture*

In cropping systems, AMF can be managed using two main approaches. The first is by promoting associations using an inoculant of selected AMF strains, and the second is by adopting agricultural practices that encourage associations with indigenous (native) AMF (Roy-Bolduc and Hijri, 2011). A problem with using AMF inoculant is its cost of production on a large-scale because they are obligate symbionts (live inside the plant roots); they cannot be cultivated in the culture medium. The native populations are well adapted to local conditions making their promotion an attractive option, although they might have lower capable colonisation than selected strains. Therefore, to maintain the native AMF by the management of agricultural practices to support their symbiosis is a strategy that could be further developed in cropping systems.

Nowadays, AMF inoculants have been produced and commercialised by several companies using single species or a mixed species of AMF, with the number of new companies

increasing (Adeyemi *et al.*, 2020). Several methods have been tested to produce the AMF inoculants, such as in vitro cultivation, aeroponics, hydroponics, "pot cultures," and "on-farm" (IJdo *et al.*, 2011). Generally, inoculants are propagated on a host plant in the field or nursery by placing AM fungal propagules (roots colonised with AMF, fungal hyphae, and spores) in a carrier (sand, inorganic clay, vermiculite, perlite, peat, zeolite, etc.) (Gianinazzi and Vosátka, 2004). Plants such as maize (*Zea mays* L.), onion and leek (*Allium* spp.), and Bahia grass (*Paspalum notatum* Flugge) are commonly used for inoculant production (IJdo *et al.*, 2011). After that, the crops were harvested and root were removed. Root were kept in the closed plastic containers and stored at 22°C, the roots should be dried as rapidly as possible to minimise the growth of other microorganisms (Habte and Osorio, 2001).

Several experiments have demonstrated the successful use of AMF inoculants in many crops both in the field and in the glasshouse (Corkidi *et al.*, 2004; Jin *et al.*, 2013a; Cely *et al.*, 2016; Keller-Pearson *et al.*, 2020). However, in some cases, AMF inoculants fail, which could be due to many factors, for example, host compatibility, environment, and the competition with the native AMF (Berruti *et al.*, 2016; Cely *et al.*, 2016). Therefore, the assessment of the effects of commercial inoculants on crops is necessary (Adeyemi *et al.*, 2020).

## 2.5 Management practices to enhance AMF functions in soils

### *2.5.1 Impact of crop management strategies on AMF function in soils*

AMF are considered to be a key group of soil microorganisms to monitor and develop the relationships between management practices, soil properties, and crop performance in agricultural production systems (Lehman *et al.*, 2019). To understand the effects of different management practices on the AM fungal symbiosis is an essential factor for farm production and the sustainable use of soil (Manoharan *et al.*, 2017). Gosling *et al.* (2006) illustrated that in general, agricultural practices have a negative impact on the AMF association, both by directly killing or damaging the AMF and/or indirectly by creating conditions that are unsuitable to AMF colonisation. These practices include high levels of fertilisation, intensive tillage, fallow and mono-cropping, which have been shown to have a negative effect on the numbers of spores, spore diversity or root colonisation, species abundance, and species

diversity (Helgason *et al.*, 1998; Menéndez *et al.*, 2001; Jansa *et al.*, 2006; Köhl *et al.*, 2016; Lehman *et al.*, 2019).

Several studies have reported the adverse effect of mineral NPK fertiliser application on AMF (Galvez *et al.*, 2001; Oehl *et al.*, 2004; Gryndler *et al.*, 2006; Jansa *et al.*, 2006). On the other hand, organic fertilisers such as cattle manure and green manure may increase spores and hyphal density in soils (Douds Jr *et al.*, 1997; Gryndler *et al.*, 2001).

Using mineral P fertiliser can have a significant negative impact on AMF associations (De Clerck *et al.*, 2003; Kogelmann *et al.*, 2004). Jansa *et al.* (2006) reported that the negative effect had been observed under different soils and climate conditions with applications of $P \geq 50$ kg $ha^{-1}$. AMF spore density in soil and AMF colonisation in roots were reduced with increasing in P fertilisation of many crops (Kahiluoto *et al.*, 2001). In contrast, AMF colonisation was highest at the sites with lowest soil P. High P availability has been shown to inhibit the establishment and persistence of AMF symbiosis (Mosse, 1973a; Menge *et al.*, 1978; Balzergue *et al.*, 2013). The mechanism to explain P inhibition of AMF-plant association formation was investigated in a P-deficient soil (Graham *et al.*, 1981). Under low P availability, the membrane phospholipids of the plant were thin due to an absence of P for their construction. This led to increased root membrane permeability and increased root exudation (amino acid and carbohydrate) to support the activities of AMF (Jasper *et al.*, 1979; Graham *et al.*, 1991a). As a result of this exudation, the AMF infection levels increased. On the other hand, in soil with high P availability resulting in high membrane phospholipids, plants accumulate less-soluble carbohydrates in their root. Accordingly, root exudations were not in high enough quantities to maintain the infection process (Jansa *et al.*, 2006). Ratnayake *et al.* (1978) also indicated that the formation of AMF was associated with a membrane-mediated decrease in the root exudation. Therefore, AMF promotion at low soil P levels is associated with higher amounts of root exudates due to a P induced decrease in membrane phospholipid levels.

Jansa *et al.* (2006) reviewed that the effects of pesticides on AMF are very variable depending on the AMF species, crop, pesticide type, application rate, timing, and environmental conditions. Several pesticides can cause significant reductions in AMF root colonisation and sporulation (Abd-Alla *et al.*, 2000; Ipsilantis *et al.*, 2012; Hage-Ahmed *et al.*, 2019). In contrast, some pesticides, for example, nematicides, have had a positive effect on AMF associations. Gosling *et al.* (2006) showed that the nematicide Fenamiphos (ethyl, 4-

methylthio-*m*-tolyl, and iso-propylphosphoramidate) increased fungal colonisation, crop dry weight, and P uptake in cotton.

In terms of fungicides, the effects on AMF colonisation, sporulation, and development are very variable, possibly due to differences in host plants, types of fungicides and their modes of action, the soil environment, and application methods (Jin *et al.*, 2013b). For example, metalaxyl-based fungicides were found to suppress AMF in oranges (*Citrus sinensis* L.) (Carrenho *et al.*, 2000) and leeks (*Allium porrum* L.) (Hernández-Dorrego and Mestre-Parés, 2010). In contrast, metalaxyl-based fungicides promoted AMF in maize (*Zea mays* L.) (Burrows and Ahmed, 2007). Fungicides may affect AMF directly through the soils or indirectly through systemic responses in the plants. AM hyphae in soils could be expected to be more sensitive to the direct effects of fungicides than hyphae in host roots (Kjøller and Rosendahl, 2000b).

Herbicides are applied at pre-seeding, pre-emergence, or post-emergence directly on the surface or incorporated into the soils (Hage-Ahmed *et al.*, 2019; Rosner *et al.*, 2019). Herbicides have shown a neutral or positive effect on AMF. For example, the leaf-absorbed herbicide glyphosate did not affect AMF spore production and hyphal growth when applied at recommended rates in the field (Malty *et al.*, 2006; Pasaribu *et al.*, 2011). Rosner *et al.* (2019) showed that the herbicide MCPB (4-4chloro-2-methylphenoxy butyric acid) did not affect AMF in a pea crop. But there are some examples of herbicides negatively affecting AMF colonisation, spores, and hyphal density in the soil; for example, Prometryn (triazines) and Acetochlor (chloroacetamide) negatively affected *Glomus etunicatum* colonisation in a carrot field (Li *et al.*, 2013).

Tillage can negatively affect AMF colonisation and community composition, potentially because of the disturbance of the extraradical AM fungal networks (Castillo *et al.*, 2006; Manoharan *et al.*, 2017). Whereas in no-tillage systems, the fungal networks in the soil from the previous season can colonise the roots of new season plants.

### *2.5.2 Plant genotype impacts on AMF function in soils*

The plant species is the primary factor determining spore production, colonisation level, community structure, and diversity of AMF (Van der Heijden *et al.*, 1998; Liu and Wang, 2003; Liu, 2010), although many AMF have a broad host range of plant species. Plants with a

poor root system to facilitate the uptake of nutrients in soils have traditionally been considered to have high levels of AMF colonisation. The glasshouse experiment of Schweiger *et al.* (1995) also indicated that plant species with shorter, less regular root hairs responded most to AMF inoculation (*Glomus* sp.). This is because these plants that have limited ability to absorb nutrients. Therefore, plants may benefit from AM fungal hyphae, which increase the surface area available to absorb nutrients, especially P (Bates and Lynch, 2001; Raven and Edwards, 2001). However, a meta-analysis of research studies by Maherali (2014) reported no relationship between AMF colonisation and fine root traits. Martín-Robles *et al.* (2018) also indicated that the major driver of plant and AMF symbiosis is from environmental variation, especially P availability, not from the plant root system.

Plant genotypes have been presented to differ both in their ability to form mycorrhizal symbiosis and the benefit that they obtain from mycorrhizal colonisation (Yang *et al.*, 2010). However, the plant genotypes (i.e., different plant species and different crop cultivars) seem to have an impact on the diversity of AMF (Song *et al.*, 2015a). Several studies mention that there is little selective pressure on either of the symbiotic partners to evolve a high degree of specificity, plant species can associate with a broad range of AMF species and that AMF species can also associate with a broad range of plants species (Harley and Smith, 1983; Clapp *et al.*, 1995; Helgason and Fitter, 2005; Bharadwaj *et al.*, 2007). The host plants can affect the AMF community directly by regulating carbon distribution to roots, production of secondary metabolites, or the change of soil environment. These properties of different effectiveness and infectiveness may be under the genetic control of the host and the AMF, or a complex interaction of their symbiotic with soil and environmental factors (Sylvia *et al.*, 2003).

### 2.6 Potato variety

Potato (*Solanum tuberosum*) belongs to the family Solanaceae. There are two main subspecies of *Solanum tuberosum*: *andigena* or *Andean*; and *tuberosum* or *Chilean* (Zaheer and Akhtar, 2016). There are 337 potato varieties (or cultivars) listed in the UK Agriculture and Horticulture Development Board (AHDB) database (http://varieties.ahdb.org.uk/varieties). The database of the European potato cultivars is reported in https://www.europotato.org/varieties, this database comprises of 3807 potato varieties. There are about 5000 potato varieties worldwide (Zaheer and Akhtar, 2016). The origin of the potato

is in the Andean mountain region of South America. Three thousand potato varieties are found in only the Andes, mainly in Ecuador, Chile, Bolivia, Colombia, and Peru (Hijmans and Spooner, 2001). The first tubers were brought from South America to Europe by Spanish explorers in the late 16th-century (Eichhorn and Winterhalter, 2005). Since then, potato breeding of high yielding, tasty varieties, and disease resistance was popularised.

### 2.7 The role of AMF in the potato systems

The first successful potato field experiment with AMF (*Glomus macrocarpus*) was reported by Black and Tinker (1977); they found that AMF can colonise the potato roots of the variety "Pentland Javelin" based on the percentage of root length colonised. This was followed by several reports using different AMF inoculants and different potato varieties and different P availabilities (Douds *et al.*, 2007). Many studies have presented that AMF inoculants benefit potato production in a greenhouse (McArthur and Knowles, 1993; Brewer and Larkin, 2005) and the native AMF benefit potato production in field experiments (Douds Jr *et al.*, 2005; Douds *et al.*, 2007; Hijri, 2016; Alaux *et al.*, 2018).

Potatoes can form associations with a broad range of AMF species. Deja-Sikora *et al.* (2020) reported that well-established species of AMF in potato root are *Rhizophagus intraradices* (formerly *Glomus intraradices*), *Rhizophagus irregularis* (formerly *Glomus irregulare*), *Funneliformis mosseae* (formerly *Glomus mosseae*), or *Gigaspora* sp. However, other AMF species have also been detected in potato roots, for example, *Claroideoglomus* sp., *Acaulospora* sp., *Ambispora* sp., *Scutellospora* sp., etc. (Senés-Guerrero *et al.*, 2014; Pathak *et al.*, 2017; Sakha *et al.*, 2019).

Many studies showed the enhancement of potato growth by different AMF species and for different potato varieties. For example, in the two-year field experiments of Ngakou *et al.* (2006) in Cameroon, they found that the potato variety "Cipira" was moderate to highly arbuscular mycorrhizal dependent. The potato yield was not significantly increased by *Gigaspora* sp. in the first year but significantly increased in the second year when compared to the control with no AMF. The two-year field experiments of Douds *et al.* (2007) demonstrated that two commercial AMF inoculants; (i) *Glomus intraradices*, and (ii) mixed culture of AMF species *Glomus etunicatum, Glomus mosseae, Glomus claroideum*, and *Gigaspora gigantea* increased the tuber yield compared to a control with no AMF, even though soil available P was high (the amounts of fertiliser applied from 713 to 787 kg of

$P_2O_5$ $ha^{-1}$). Bharadwaj *et al.* (2008) conducted a greenhouse experiment that showed that the AMF inoculant *Glomus mosseae* increased the dry shoot weight and root weight of the potato variety "King Edward" compared with control plants, although this difference was not statistically significant.

In addition, AMF may improve the productivity of potatoes by increasing pathogen resistance (Pathak *et al.*, 2017), especially to late blight (caused by the fungi *Phytophthora infestans*), which is the most significant disease of potatoes worldwide (Speiser *et al.*, 2006; Gallou *et al.*, 2011; Alaux *et al.*, 2018; Kumar *et al.*, 2018). Many studies illustrated that AMF can improve disease resistance to other potato plant pathogens. For instance, reducing blackleg disease and tuber soft rot caused by bacteria *Pectobacterium carotovora* (Bagy *et al.*, 2019), and symptoms from Potato virus Y (PVY) (Miozzi *et al.*, 2019), root rot, crown rot, or stem canker caused by fungi *Rhizoctonia solani* (Yao *et al.*, 2002). The proposed mechanisms for how AMF provide pathogen protection include competition for the resources, and AMF stimulated host immune responses (Holland *et al.*, 2019) (see section 2.4.1).

There are few studies exploring host specificity of AM fungal species to potato cultivars (Cesaro *et al.*, 2008; Öpik *et al.*, 2009), although Öpik *et al.* (2009) mentioned that AMF have very low partner specificity. The results of the AMF and potato variety studies are varied based on AMF species, local varieties, locations, and especially P availability (Minemba *et al.*, 2019). The large scale field trial results from Hijri (2016) showed that AMF (*Rhizophagus irregularis*) increased the tuber yield significantly, but found no differences among potato varieties for AMF colonisation. However, Buysens *et al.* (2017) found that AMF (*Rhizophagus irregularis*) root colonisation was different between potato varieties (Charlotte, Nicola, and Bintje). Hannula *et al.* (2010) studied the impact of potato variety, including a genetically modified variety, on the community composition of three fungal phyla; Glomeromycota, Ascomycota, and Basidiomycota in soils; their results showed that there was no significant difference between the genetically modified variety and its parental variety for all fungal community groups.

# CHAPTER 3. The impact of crop management practices on the abundance and diversity of arbuscular mycorrhizal fungi in different potato varieties grown under different fertilities and crop protection regimes

## 3.1 Introduction

Crop management practices are manipulated as part of many agroecosystems to enhance the productivity of the soil, for instance, pesticide application, fertiliser application, tillage, monocultures or crop rotations, and water supply (Jansa *et al.*, 2006). In this chapter, the use of mineral fertilisers and contrasting crop protection practices (organic and conventional) and interactions with potato variety was the focus of the study.

Pesticides are agrochemicals used in conventional crop protection against plant pathogens and weeds and include fungicides, herbicides, insecticides, nematicides, and bactericides (Wang *et al.*, 2019). More than 7.4 million tons of pesticides were applied worldwide in 2017 (http://www.fao.org/faostat). Most pesticides eventually enter the soil, and some of the organic chemicals of which they are made (for example, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and petroleum hydrocarbons), are considered carcinogens, mutagens, and teratogens (Wang *et al.*, 2019).

Fertilisers are essential components in agriculture for improving nutrient availability, soil properties, and crop yield (Qin *et al.*, 2015). The organic fertiliser is made of organic materials, i.e., green manure crops, animal manure, crop residues, etc. (Kumar *et al.*, 2005). Inorganic fertiliser or mineral fertiliser is applied to compensate for nutrient losses under conventional management. It usually contains minerals or synthetic compounds of macronutrients (N, P, K, S, Mg, Ca). N is a major mineral fertiliser due to its importance in agricultural systems (Geisseler and Scow, 2014; Whitcomb *et al.*, 2014). The nutrient content in organic fertiliser varies considerably among source materials; N and P content is substantially lower than inorganic fertilisers (Green, 2015). Organic fertilisers enhance soil quality with regard to increasing microbial biomass and diversity (Manoharan *et al.*, 2017), whereas inorganic fertilisers directly improve crop productivity by supplying readily available nutrients that are easy for the crop to access. . However, long-term inorganic fertiliser applications can cause deterioration of soil biodiversity, soil quality, and ecosystem functioning (Boddington and Dodd, 2000; Qin *et al.*, 2015; Srivastava *et al.*, 2017).

The worldwide demand for potato consumption and production has increased along with recent global population growth (Jahanzad *et al.*, 2017; Mahanty *et al.*, 2017). Fertiliser and pesticide use is particularly necessary because potato crops generally have a higher nutrient demand, especially for NPK (Fernandes and Soratto, 2016). Also, potato varieties are affected by several pests (insects and nematodes), diseases (plant pathogenic fungi), and viruses (Pawelzik and Möller, 2014). For these reasons, enormous volumes of chemicals are used in potato agriculture with the inevitable environmental damage that happens as a consequence (Mahanty *et al.*, 2017) such as polluting air, water, and soil (Anaya, 1999; Srivastava *et al.*, 2017) because of accumulated chemicals (Mahanty *et al.*, 2017). Consequently, many researchers have tried to develop alternative management practices to reduce chemical use to preserve environmental quality and achieve sustainable use of natural resources (Nurbaity *et al.*, 2016; Oliveira *et al.*, 2016; Hage-Ahmed *et al.*, 2019; Keller-Pearson *et al.*, 2020).

The promotion of conditions that support beneficial soil microorganisms, including plant-growth-promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF), is one useful alternative strategy for sustainable soil fertility management. These microbes improve plant growth, crop productivity, plant health, and nutrient and water uptake without threatening the environment (Altomare and Tringovska, 2011). In particular, the beneficial rhizosphere microorganisms such as AMF (Jeffries *et al.*, 2003) are important components of all ecosystems (Srivastava *et al.*, 2017) and have the potential to sustain the productivity of agroecosystems and maintain the fertility of soils. The use of pesticides and fertilisers may contaminate the soil and negatively affect AMF communities (Ramos-Zapata *et al.*, 2012). Therefore, it is essential to study the impact of pesticides and fertilisers on AMF communities, especially under large-scale field conditions, to understand management practices that promote AMF colonisation (Kurle and Pfleger, 1996).

In addition, the decrease of arbuscular mycorrhizal propagules in conventional agricultural systems that use chemicals (fertilisers, herbicides, and fungicides) is a valuable indicator of the health of the plant-soil system. Hence, AMF can be considered as an important monitoring tool when assessing the environmental impact of old and new management practices (as discussed above) used in agriculture (Giovannetti *et al.*, 2006; Manoharan *et al.*, 2017; Srivastava *et al.*, 2017). According to the literature review, several studies have shown the enhancement of potato growth by AMF in different potato varieties, under both greenhouse

and field conditions, with no specificity between the variety and AMF. Thus, potato variety is also an essential factor for further investigation in this study.

Previously, the diversity of AMF in field settings was evaluated by fungal spore identification and classification into different groups (Jansa *et al.,* 2002), but the numbers of these spores may bear little relation to the level of fungal colonisation of crop roots (Mafaziya and Madawala, 2015). Subsequently, conventional polymerase chain reaction (PCR) techniques have been widely used to detect the presence of AMF within a single root system in various environments (Clapp *et al.,* 2003; Alkan *et al.,* 2006). This molecular approach can crudely indicate the population density of AMF but is not absolutely quantitative. Until now, total AMF colonisation in a root system has been absolutely quantified only using root staining and microscopic analysis techniques, for example, the magnified intersect method to assess the degree of colonisation (McGonigle *et al.,* 1990) in roots stained by ink and vinegar (Vierheilig *et al.,* 1998) or by trypan blue (Phillips and Hayman, 1970). These methods require the microscopic analysis of root segments and quantifying the total proportion of roots colonised by fungal structures. However, the limitation of the morphological study of the root-colonising AMF structures observed by microscopy is that it does not provide information about the species diversity of the specific fungi present (Merryweather and Fitter, 1998).

Advances in molecular techniques now make it feasible for quantitative and specific analysis of the relative abundance of individual AMF taxa within a root system by using real-time or quantitative PCR (qPCR) approaches (Clapp *et al.,* 2003; Thonar *et al.,* 2012; Fuentes *et al.,* 2016; Voříšková *et al.,* 2017). In addition, analysis of terminal restriction fragment length polymorphisms (T-RFLP) is a technique to analyse the diversity of amplified ribosomal RNA genes derived from environmental samples (Watanarojanaporn *et al.,* 2014) and has been used to study AMF community composition in ecology (Vandenkoornhuyse *et al.,* 2003; Dickie and FitzJohn, 2007). This technique is a recognised molecular community profiling method used to separate microbial taxa based on the sequence variability of a particular taxonomic marker gene. Most recently, next-generation sequencing (NGS), which is a high throughput DNA sequencing technology, has been recognised as a useful tool to study the diversity of AM fungal communities also by identification of the specific species present (Schmidt *et al.,* 2013).

This chapter reports on results from the study of potato root samples collected in 2011 as part of the NUE-CROPS project (Rempelos *et al.*, 2013). All root samples had been stored in the freezer at -80$^{o}$C to preserve all nucleic acids (Dighton and White, 2017), which leads to no known treatment effects. The aim of the project was to explore the role of crop management practices and potato variety in reducing the environmental impact of crop production while maintaining or improving yield and quality levels. Rempelos *et al.* (2013) have previously reported the agronomic results for all treatments in the trial; however, in this current study, a subset of the treatments was selected for a more detailed analysis of AMF colonisation and diversity in roots using the variety of analysis techniques discussed above. The research question in this chapter is to study the effect of conventional pesticides and mineral fertilisers on AMF populations in different potato varieties.

### 3.2 Aim and objectives

The overall aim of this study was to investigate the impact of potato variety, fertiliser, and crop management practice on AMF root colonisation and community structure, as well as fungal species abundance and the diversity in potato roots. Specific objectives were:

1. To determine the effect of conventional pesticides and mineral fertilisers on AMF abundance and the diversity of the native fungal community in potato roots.

2. To assess the impact of potato variety on AMF abundance and the diversity of the native fungal community in potato roots.

3. To investigate the interaction between potato variety and crop management practices on AMF abundance and the diversity of the native fungal community in potato roots.

In addition, this study was used to compare the efficacy of two methods of quantifying the abundance of AMF in roots, namely: a standard root staining method using an ink-vinegar solution and a qPCR method using AMF-specific primers. Two approaches for assessing AMF community diversity were also compared, namely: T-RFLP using AMF-specific primers and an NGS approach using general fungal primers.

## 3.3 Materials and Methods

### *3.3.1 Field experimental set up*

The NUE-CROPS project was conducted in 2011 at Newcastle University's Nafferton Experimental Farm, Northumberland, the U.K. The experiment was started in May 2011. There were six potato varieties used (Agria, Cara, Fontane, Nicola, Sante, and Sarpo Mira). The experimental factors included: 6 potato varieties; 5 fertility treatments (control, compost and mineral NPK each at two rates; 125, 250 kg $ha^{-1}$); 2 crop protection (CP) treatments (conventional-the standard rates of pesticides; herbicide and fungicide and organic-weed control (tillage) and disease control (Cu-based fungicides)) replicated four times in the field (total = 240 plots).

Figure 3. 1 shows the details of one block in the NUE-CROPS potato trial in 2011. Fertility treatment is the main plot (shown in five different colours). Each fertility treatment was separated into two crop protection treatments; the sub-plots with conventional pesticides are shown with stippling. Six potato varieties were planted across each fertility management main plot and are shown as V1 to V6.

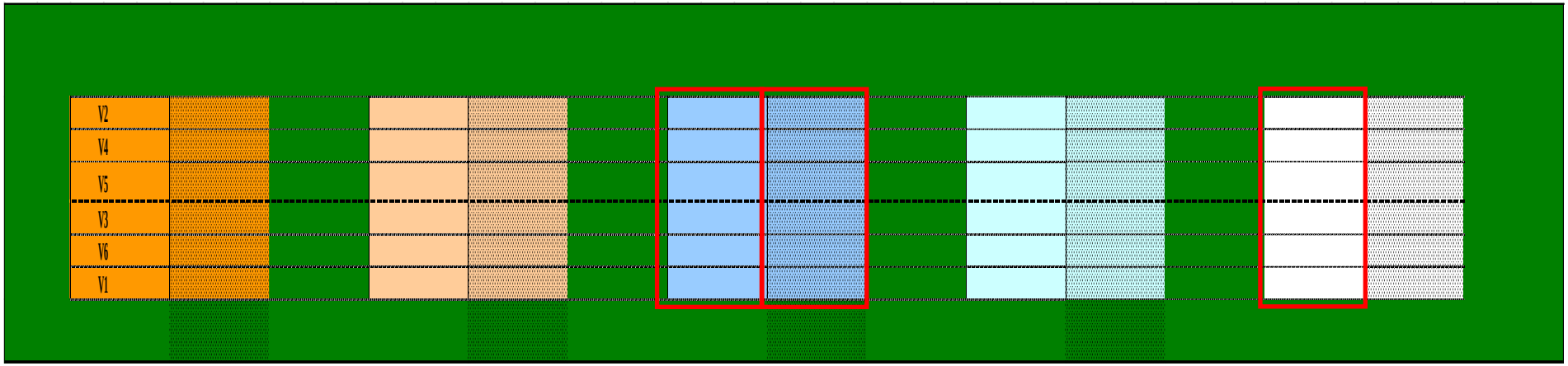
According to Table 3. 1, compost (250 kg N $ha^{-1}$ full rate plots, 85 kg N $ha^{-1}$ half-rate plots) was applied for organic fertility treatment, whereas mineral NPK was used for conventional fertility treatment. Mineral NPK additions comprised of ammonium nitrate (250 kg N $ha^{-1}$ full rate plots, 85 kg N $ha^{-1}$ half-rate plots), triple superphosphate (134 kg $P_2O_5$ $ha^{-1}$), and KCl (300 kg $K_2O$ $ha^{-1}$). Compost and mineral NPK were applied before potato planting. Conventional crop protection treatments were treated with Linuron (3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea, 1.2 L $ha^{-1}$; herbicide) for weed control, Fubol gold (metalaxyl-M and mancozeb, 1.9 L $ha^{-1}$) and Shirlan (fluazinam, 300 mL $ha^{-1}$) were applied for blight control. An organically approved fungicide; Headland Copper (256 g Cu $L^{-1}$; 3 L $ha^{-1}$), was used for blight control and tillage for weed control in the organic crop protection plots. The dates for each pesticide application are shown in Table 3. 1.

Potato plant samples were collected in July 2011. Two plants were randomly collected from each plot. Roots were separated from the other below-ground plant parts using sterilised scissors washed clean of soil by gently washing with water above a sieve (4 mm), then placed

in foil and immediately frozen in liquid nitrogen and stored in the freezer at -80$^{o}$C until analysis (Rempelos *et al.*, 2013).

**Table 3. 1** Field trial agronomic management.

| Date | Treatments | Details |
|---|---|---|
| March 2011 | Organic fertilisation | Compost application |
| April 2011 | Conventional fertilisation | Fertilised with mineral NPK |
| May 2011 | All | Planting |
| May 2011 | Conventional CP (weed control) | Herbicide application with Linuron |
| | Organic CP (weed control) | Mechanical cultivation (tillage) |
| June 2011 | Conventional CP | Fungicide application with Fubol gold |
| | Organic CP | Fungicide application with Headland Copper |
| | Conventional CP | Fungicide application with Shirlan |
| | Organic CP | Fungicide application with Headland Copper |
| July 2011 | All | Plant/Tuber/Root assessments |
| July 2011 | Conventional CP | Fungicide application with Fubol gold |
| | Organic CP | Fungicide application with Headland Copper |
| | Conventional CP | Fungicide application with Shirlan |
| | Organic CP | Fungicide application with Headland Copper |
| | Conventional CP | Fungicide application with Fubol gold |
| | Organic CP | Fungicide application with Headland Copper |
| August 2011 | Conventional CP | Fungicide application with Shirlan |
| | Organic CP | Fungicide application with Headland Copper |
| | Conventional CP | Fungicide application with Fubol gold |
| | Organic CP | Fungicide application with Headland Copper |
| September 2011 | All | Harvesting |




| Potato variety |
|---|
| V1 Agria |
| V2 Cara |
| V3 Sarpo Mira |
| V4 Fontane |
| V5 Nicola |
| V6 Sante |

| Fertility treatment | | |
|---|---|---|
| | Control | no input |
| | Compost | 125 |
| | Compost | 250 |
| | Mineral NPK | 125 |
| | Mineral NPK | 250 |

| Crop protection treatment |
|---|
| Conventional |
| Organic |

**Figure 3. 1** Diagram illustrating the layout of one block in the NUE-CROPS potato trial, 2011. The plots used in this study are outlined in red.

### *3.3.2 Sample selection*

For this study, archived root samples from three treatments of the NUE-CROPS potato experiment were selected (Table 3. 3 and Figure 3. 1). The control treatment with no fertility inputs and organic crop protection served as a low-input control. This treatment was considered the most deficient in phosphorus and, therefore, most likely to develop mycorrhizal associations (Mosse, 1973a; Mosse, 1973b; Graham *et al.*, 1981). The control was compared with the high rate of mineral NPK fertiliser (250 kg N $ha^{-1}$) under both organic and conventional crop protection practices. Soil test results before the application of the treatments for the field are shown in Table 3. 2. Based on the Nutrient Management Guide (RB209), the P index is 3, and the K index is 2 (AHDB, 2020).

**Table 3. 2** Soil test results of NUE-CROPS potato trial, 2011 (Mean±SE).

| **Mineral** | **mg $kg^{-1}$** |
|---|---|
| Soil P | 26.30±1.68 |
| Soil K | 129.10±5.17 |

**Table 3. 3** Treatments included in this study.

| **Treatment name in this study** | **Fertility** | **Crop protection regime** |
|---|---|---|
| 1. Control | No mineral fertiliser | Organic |
| 2. NPK with conventional crop protection (CCP) | The high rate of mineral NPK (250 kg N $ha^{-1}$) | Conventional |
| 3. NPK with organic crop protection (OCP) | The high rate of mineral NPK (250 kg N $ha^{-1}$) | Organic |

All three treatments are shown in Table 3. 3. There were six potato varieties (Agria, Cara, Fontane, Nicola, Sante, and Sarpo Mira) in each treatment with four replicate plots for each. Thus, 72 potato root samples (6 potato varieties x 3 treatments x 4 replicates= 72 root samples) were selected to study.

### *3.3.3 Experimental planning for analysis*

Techniques used for potato root analysis are shown in Figure 3. 2. Potato roots were analysed to assess AMF colonisation in the roots to reflect both the level of AMF abundance and the AMF community structure and genera composition. Three molecular techniques (qPCR, T-RFLP, and Illumina MiSeq next-generation sequencing) and the traditional method of root staining were used in the study.

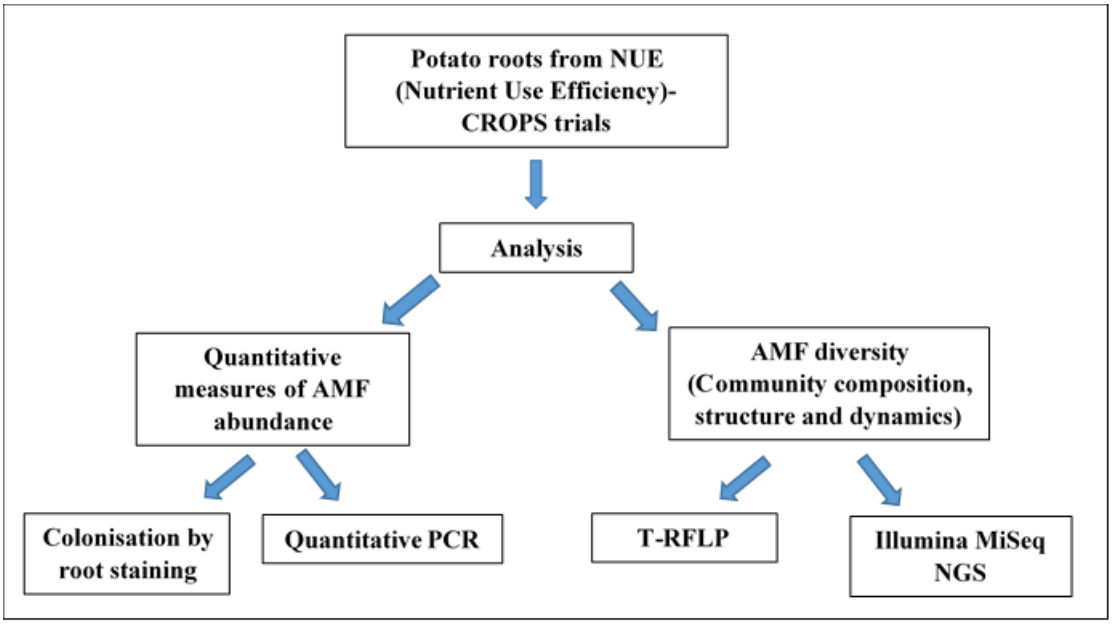



**Figure 3. 2** Techniques used for potato root analysis in the study.

### *3.3.4 AMF colonisation by root staining (Ink and vinegar method)*

#### Root preparing

Roots were defrosted, sub-samples were cut into pieces of 1.5-2.0 cm in length for approximately 50 pieces and put in 20 mL plastic scintillation vials.

#### Root staining

Following the protocol of the "Research institute of organic agriculture" (FiBL), Frick, Switzerland, modified from Vierheilig and Piché (1998), a KOH solution (10 mL of 10% (wt/vol)) was added to each vial. Vials were then placed in a water bath set at 80$^{o}$C for 30 min. KOH was then removed, and the roots were rinsed three times with 10 mL deionised water. The roots were then acidified with 10 mL of a 1% (vol/vol) HCl solution and allowed to stand at room temperature for 30 min. HCl was removed, and the roots were rinsed with 10

mL deionised water, as described above. The roots were then covered with 10 mL of a 5% ink-vinegar solution prepared by mixing black Parker ink (Parker Quink™) with household vinegar (5% acetic acid) and heating in an 80$^{o}$C water bath for 30 min. The roots were separated and rinsed with 10 mL deionised water, as described above. They were then covered with a 50% glycerine solution (prepared by mixing glycerine (87%) with deionised water) until an assessment of root colonisation. Roots can be stored in this form at room temperature for weeks to months.

**Slide preparation**

For each root sample stained, fine roots were selected using forceps, 25 root segments were placed parallel to each other on a glass slide perpendicular to the long axis of the slide (Figure 3. 3A). A few droplets of 50% glycerine solution were then added, and a coverslip was gently pressed on to the roots. The assemblage was then sealed with nail polish on all four sides of the coverslip. Slides prepared in this way can be stored at room temperature for a year.

**Assessing AMF root colonisation**

The magnified intersection method was used to assess root colonisation (McGonigle *et al.*, 1990). Briefly, slides were placed under a light compound microscope (Leica DMLB microscope) with an overall magnification of 400x (40x objective lens with 10x eyepiece magnification) (Figure 3. 3B). The roots were then surveyed systematically by moving the slide on the microscope stage concerning the eyepiece integrated micrometer gridlines aligned to the orientation of the roots on the slide. One hundred microscopic fields of view were assessed for each slide (sample), i.e., four transect lines which crossed the 25 root sections in a perpendicular direction. The presence of AMF structures (hypha, vesicles, and arbuscules) in each microscopic field was scored as 1 to 5 according to the "Research institute of organic agriculture" (FiBL) procedure.

Score 1= empty root (no infection; cannot see any AMF structures)

Score 2= root presents with hyphae only

Score 3= root presents with hyphae and arbuscules

Score 4= root presents with hyphae and vesicles

Score 5= root presents with all AMF structures (hyphae, vesicles, and arbuscules)

Sample AMF colonisation expressed as a percentage was then calculated using the following equation.

$$\% \text{ AMF colonisation} = n2+n3+n4+n5$$

where n2 represents the total number of score 2, n3 represents the total number of score 3, n4 represents the total number of score 4, and 5 represents the total number of score 5.

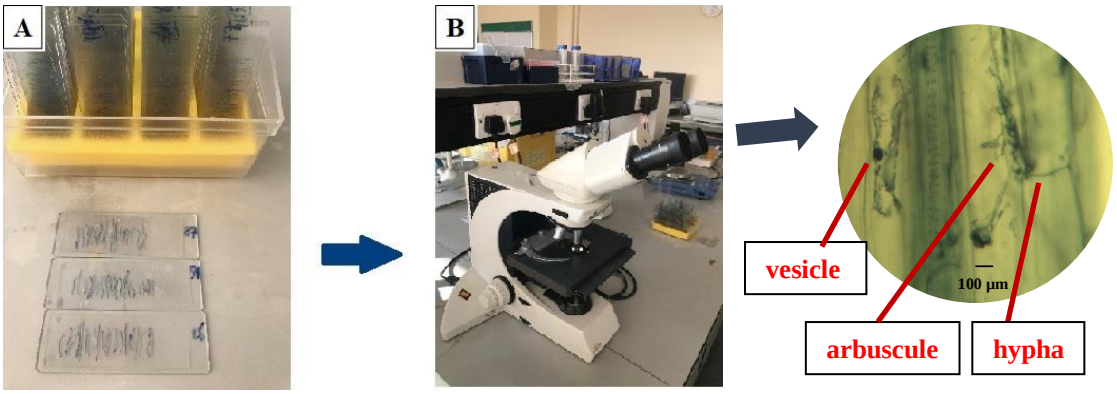



**Figure 3. 3** Slide preparation and root colonisation assessment by microscope.

**Statistical analysis of root colonisation data**

The percentages of AMF colonisation from all samples were subjected to two-way ANOVA with "Crop management practice (treatment)" and "Potato variety" as the main factors. The data were checked for normal distribution of the residuals using the qqnorm command; if the residuals were not normally distributed, then the data was transformed; square root data transformation was employed. A linear mixed-effects (lme) model was used to produce ANOVA P-values for main effects and all interactions with the nested structure of the split-plot design reflected in the random error term:block/treatment (Pinheiro and Bates, 2000) using the nlme package in the R programming language (RCore, 2016). When ANOVAs indicated a significant treatment effect ($p<0.05$), differences between individual means were analysed using Tukey's honestly significant difference (HSD) post hoc test in the multcomp package in R. A 95% significance level ($p<0.05$) was applied to discriminate the means. Where the interaction between treatment and variety was significant, the data was subset by treatment and a separate one-way ANOVA for the variety effect was conducted at each level of the three treatments.

### *3.3.5 Evaluation and optimisation of the nucleic acid extraction procedure*

A small study (six potato root samples) was conducted to find the optimal extraction method for getting a high yield and good quality of DNA from the potato roots for application of this optimised protocol in the next two experimental chapters as a standard protocol. Root samples were selected at random for this study. All six root samples had been stored in the freezer at -80$^{o}$C since 2011.

Three methods were tested using the same DNA extraction kit with a slight difference in the sample preparation before DNA extraction i.e.

**Method 1.** Extraction with a FastDNA SPIN kit for soil (MP Biomedicals, 101 Inc., La Jolla, CA, USA) following manufacturer's protocol and using frozen root samples with no pre-treatment.

**Method 2.** Extraction with a FastDNA SPIN kit for soil (MP Biomedicals, 101 Inc., La Jolla, CA, USA) following the manufacturer's protocol using frozen roots that had been milled before DNA extraction. Frozen root samples were milled to a powder in 2 mL microcentrifuge tubes with one sterile 5 mm stainless steel bead per tube. Tubes were placed into the TissueLyser Adapter Set 2x24 (QIAGEN) and ground for 1 min at 27 Hz.

**Method 3.** Extraction with a FastDNA SPIN kit for soil (MP Biomedicals, 101 Inc., La Jolla, CA, USA) following the manufacturer's protocol using freeze-dried and milled root samples before DNA extraction. Frozen root samples were dried in a freeze dryer (SP Scientific VirTis freeze dryer, 24DX48 GPFD) with the following conditions: -30$^{o}$C at 200mTorr for 34 hours; then the temperature is ramped up to 20$^{o}$C over 100 min (at 100mTorr) and held at these temperature and pressure conditions for 2 hours, before storage at 50mTorr until the samples are removed. The freeze-dried roots were then milled as described above.

**Nucleic acid quantification and purity checked**

The nucleic acid yield from all samples was quantified using a Nanodrop 8000 spectrophotometer (Thermo Scientific, Wilmington, USA). The purity of each sample was determined by measuring the 260 nm:280 nm absorbance ratios. A 260 nm:280 nm absorbance ratio of less than 1.8 indicates the presence of contaminants (proteins and phenols) which absorb light at 280 nm.

**Statistical analysis of samples for optimisation of the DNA extraction**

The nucleic acid yield (DNA concentration) is reported as ng $\mu L^{-1}$. The data were subjected to one-way ANOVA with "Extraction method" as the treatment factor and each root sample treated as a replicate (n=6) using the nlme package in R. When the treatment effect was significant ($p<0.05$), differences between individual extraction method means were analysed using Tukey's honestly significant difference post hoc test as described in 3.3.4. A 95% significance level ($p<0.05$) was applied to discriminate the means.

### *3.3.6 DNA extraction for all root samples, negative control, and positive control*

DNA was extracted from the potato root samples using method 3 (Extraction with a FastDNA SPIN kit for soil (MP Biomedicals, 101 Inc., La Jolla, CA, USA) following the manufacturer's protocol using freeze-dried and milled root samples before DNA extraction) as described in section 3.3.5.

Negative and positive controls were also extracted using the same extraction kit (FastDNA SPIN kit for soil, MP Biomedicals, 101 Inc., La Jolla, CA, USA) following the manufacturer's protocol. Distilled water was used for the negative distilled water control, and the commercial AMF inoculant (Rootgrow$^{TM}$ mycorrhizal fungi, Plantworks Ltd) was a positive control.

The quality of the DNA was checked using a Nanodrop 8000 spectrophotometer, as described in 3.3.5.

### *3.3.7 Quantification of AMF abundances using qPCR*

A subset of DNA samples with low and high levels of AMF colonisation identified by root staining was chosen for analysis. In this chapter, the potato variety Sarpo Mira (highest colonisation) and Sante (lowest colonisation) from all three crop management treatments were selected for qPCR. This analysis was conducted at the Research Institute of Organic Agriculture (FiBL) in November 2018.

**qPCR standard preparation**

A plasmid-based calibration standard of an AMF model species, in this case, *Rhizoglomus irregulare* (synonyms*; Rhizophagus irregularis* (Schüßler and Walker, 2010)), was cloned in a plasmid (gene sequence as shown in Table 3. 4). This plasmid standard was prepared by the technical staff at FiBL using the following steps (White *et al.,* 1990; Lee *et al.*, 2008). AMF spores were extracted from soil by the wet sieving and decantation method (Daniels and Skipper, 1982). Under a microscope, a single spore of AMF was selected and transferred to a 0.2 mL PCR tube using a pipette, and the spore was crushed using a pipette tip. DNA was then extracted by adding 2 µL of 0.25 M NaOH to the crushed spore. The mixture was then centrifuged, spun down and the tubes were incubated in a T3 Thermocycler (Biometra GmbH, Goettingen, Germany) at 95°C for 2 min. Then, 1 µL of 0.5 M Tris HCl (pH 8.0) and 2 µL of 0.25 M HCl was added to the extract and the mixture was incubated as described above at 95°C for 2 min.

The SSU rDNA gene fragment was amplified by nested PCR using universal fungal PCR primers NS1/NS4 in the first PCR step and specific AMF primers AML1/AML2 in the second PCR step. The PCR programme for both PCR steps was as follows: 95ºC for 5 min, 95ºC for 1 min, and 50ºC for 1 min (30 cycles), followed by 5 min at 72ºC. The sequence of these primers is provided in Table 3. 5. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) following the manufacturer's instructions. DNA was quantified using the Eppendorf BioSpectrometer®. The purified PCR product was ligated into the cloning vector and transformed by cloning into the competent cells.

The plasmid standard was prepared by Plasmid Miniprep Kit 1 (peqGOLD, VWR) following the manufacturer's instructions. Plasmid quantification was calculated as follows (Thonar *et al.*, 2012); plasmid DNA concentration (K in g $L^{-1}$) is measured using Qbit and calculated as copy numbers (NC in copies $L^{-1}$), considering the size of the plasmid (the amplicon length, L in bp), the molecular weight of the inserted dsDNA (660 Da $bp^{-1}$) and Avogadro's constant (Na, $6.022E^{+23}$).

$$NC = \frac{K \times Na}{660 \times L}$$

**Table 3. 4** Gene sequence of *Rhizoglomus irregulare.*

| | |
|---|---|
| Cloned PCR fragment of SSU region by AML1 and AML 2 primer pair | ATCAACTTTCCGATGGTAGGATAGAGGCCTACCATGGTGGTAACGGGTAACGG<br>TGTTAGGGCACGACACCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGG<br>ATGGCAGCAGGCGCGCAAATTACCCAATCCCGACACGGGGAGGTAGTGACAATA<br>AATAACAATACGGGGTTCTTTCGGATCTCGTAATTGGAATGAGTACAATTTAAATC<br>TCTTAACGAGGAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCC<br>AGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTTGTAGTTAAATTTC<br>GGGGTTAGTAGGTTGGTCATGCCTCCGGTATGTACTGATCTCACTGATTCCTCCTT<br>CCTTATGAACCGTAATGCCATTAATTTGGTGTTGCGGGGAATTTGGACTGTTACTT<br>TGAAAAAATTAGAGTGTTTAAAGCAAGCTAACGCTTGAATACATTAGCATGGAATA<br>ATGAAATAGGACGTTCGATCCTATTTTGTTGGTTTCTAGGATTGACGTAATGATTA<br>ATAGGGATAGTTGGGGGCATTTAGTATTCAATTGTCAGAGGTGAAATTCTTGGATTT<br>ATTGAAGACTAACTACTGCGAAAGCATTTGCCAAGGATGTTTTCATTAATCAAGAA<br>CGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACT<br>ATGCCGACTAGGGATCGGATGATGTTAATTTTTTAATGACTCATTCGGCGCCTTAC<br>GGGAAACCAAAGTGTTTGGGTTC |

**Table 3. 5** The primers used for the amplification of DNA from *Rhizoglomus irregulare* for qPCR standards.

| Primer | Primer sequence (5'-Sequence-3') | Direction | References |
|---|---|---|---|
| NS1 | GTAGTCATATGCTTGTCTC | Forward | Nam–Seok *et al.* (2006) |
| NS4 | TTCCGTCAATTCCTTTAAG | Reverse | Nam–Seok *et al.* (2006) |
| AML1 | ATCAACTTTCGATGGTAGGATAGA | Forward | Lee *et al.* (2008) |
| AML2 | GAACCCAAACACTTTGGTTTCC | Reverse | Lee *et al.* (2008) |

**Quantification of the target gene**

DNA extracts from samples were diluted (1:10) prior to PCR amplification. SYBR Green-based qPCR assays with plasmid standards were used for AMF SSU gene fragment quantification. qPCR reactions were set up in 96-well reaction plates. The primer pair AMG1F and AM1 (Salvioli *et al.*, 2008; Hewins *et al.*, 2015) were used to amplify at a region of the 5' end of SSU rDNA sequence (Table 3. 6). The target positions of the primer pairs used for the SSU rDNA amplification n are shown in Figure 3. 4. Each 15 µL reaction mix contained 0.4 µL of each primer (10µM), 5.2 µL of $ddH_2O$, 7.5 µL of SYBR Green Supermix (Bio-Rad) (1x), and 1.5 µL of diluted DNA template (1:10). The PCR programme was as follows: 95ºC for 3 min, 95ºC for 10 s, and 62ºC for 15 s (40 cycles), followed by 20 s at 72ºC. Plasmid standard dilutions were prepared to give standards containing $10^2$, $10^3$, $10^4$, and $10^5$ copies of the SSU rDNA gene target per µL. Negative controls containing water were

used to check for potential contamination in the master mix. All samples, including standards and negative controls, were amplified in three replicate qPCR reactions using a Bio-RAD C1000 Touch™ Thermal Cycler. At the end of the runs, AMF SSU concentrations in the templates were carefully recorded and used for the final calculation.

**Table 3. 6** The primers used for the amplification of the target DNA.

| Primer | Primer sequence (5'-Sequence-3') | Direction | References |
|---|---|---|---|
| AMG1F | ATAGGGATAGTTGGGGGCAT | Forward | Hewins *et al.* (2015) |
| AM1 | GTTTCCCGTAAGGCGCCGAA | Reverse | Salvioli *et al.* (2008) |

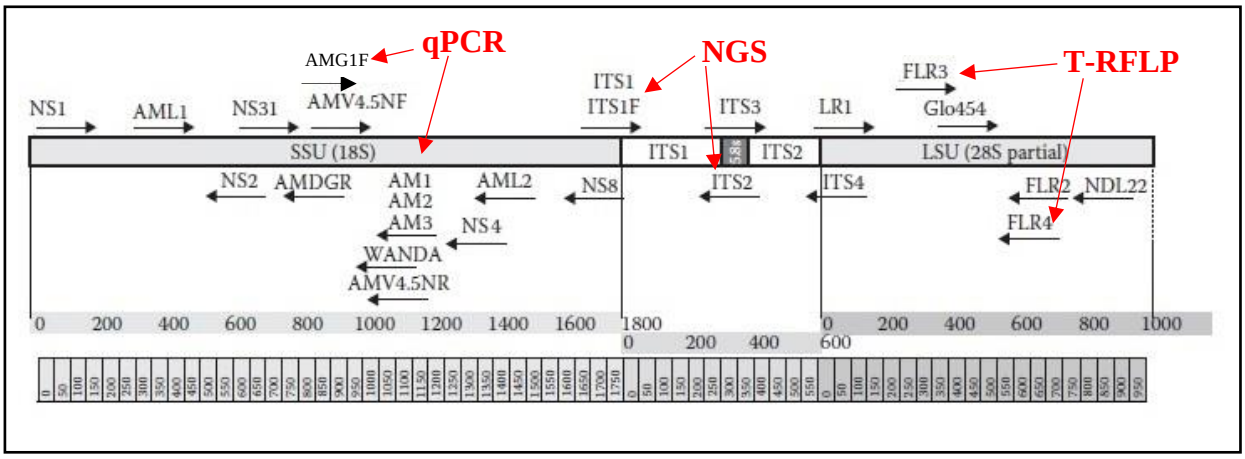



**Figure 3. 4** Schematic representation of the ribosomal RNA coding regions targeted by various primers developed for analysing AMF (adapted from Taylor *et al.* (2017)).

**Standard curve generation and gene copy number assessment**

The gene copy number in the root samples (in copies per root dry weight) was determined by comparing the quantification cycle (Cq) in the sample reactions to a standard curve using the CFX Manager™ software, version 3.1 (Bio-Rad). The template plasmid containing the target DNA sequences were applied to produce a standard curve as an external standard. Plasmids were quantified, and copy numbers were calculated as mentioned above (section 3.3.7). From $10^2$ to $10^5$ copies of the serially diluted cloned target DNA were included in each run. The Cq was determined for each run, such that the reaction efficiency and $r^2$ of the standard curve were optimised. The number of target DNA sequences present in each PCR mixture was calculated by comparing the crossing points of the samples with those of the standards.

The abundance of AMF in the roots (in copies per root dry weight) was calculated as follows (Thonar *et al.*, 2012);

$$\text{The abundance of AMF in the root sample} = \frac{(\text{AMF SSU concentration in the template}) \times EV \times DF1}{\text{Root dry weight} \times RF}$$

where EV represents elution volume of the root DNA extract (in this study, EV=60 µL), DF1 represents the dilution factor (in this study, DF1=10), root dry weight of each sample was recorded when DNA was extracted, and RF represents the recovery factor (in this study, RF=1).

**Statistical analysis**

The abundance of AMF (gene copy number) from all root samples were subjected to two-way ANOVA with "Crop management practice (treatment)" and "Potato variety" as factors as described for root colonisation data in section 3.3.4.

### *3.3.8 Comparison of AMF abundance measured by root staining and qPCR using AMF-specific primers*

To compare the AMF abundance data determined with microscopic colonisation assessment by root staining or qPCR, a Pearson's product-moment correlation was performed. This analysis was carried out using the devtools package in the R programming language.

### *3.3.9 Terminal restriction fragment length polymorphism method (T-RFLP)*

T-RFLP was performed by targeting a larger subunit (LSU) of AMF rDNA with specific primers.

**Nested PCR and agarose gel electrophoresis**

Nested PCR amplifications were conducted with two sets of primers (Mummey and Rillig, 2007). All primer sequences are given in Table 3. 7. The primer pair LR1 and FLR2 (Trouvelot *et al.*, 1999), which target all the fungi, were used for the first round of PCR amplification to amplify the 5′ end of LSU of the rDNA sequence. Each 10-µL reaction mix contained: 0.25 µL of each primer; 8.5 µL of PCR MegaMix (Clent Life Science Company); and 1 µL of diluted DNA (1:100). The PCR programme was as follows: 93ºC for 1 min, 58ºC

for 1 min and 72ºC for 1 min (35 cycles), followed by 10 min at 72ºC (Gollotte *et al.*, 2004). The target positions of these primers used for the LSU rDNA amplification are indicated in Figure 3. 4. The resulting PCR product from the first round was diluted tenfold. It was then used as a template for the second round of PCR using the AMF-specific primers FLR3 and FLR4 to amplify a smaller section of the 5′ end of LSU rDNA sequence under the same PCR conditions. FLR3 was fluorescently labelled at the 5′ end of the oligonucleotide with carboxyfluorescein (FAM). PCR products from both rounds were visualised after electrophoresis on a 1% agarose gel (stained with ethidium bromide) in TAE buffer under ultraviolet light.

**Table 3. 7** Primer sequences used for nested PCR.

| Primer | Primer sequence (5′ -Sequence-3′) | Direction | Reference |
|---|---|---|---|
| LR1 | GCATATCAATAAGCGGAGGA | Forward | Trouvelot *et al.* (1999) |
| FLR2 | GTCGTTTAAAGCCATTACGTC | Reverse | Trouvelot *et al.* (1999) |
| FLR3 | TTGAAAGGGAAACGATTGAAGT | Forward | Gollotte *et al.* (2004) |
| FLR4 | TACGTCAACATCCTTAACGAA | Reverse | Gollotte *et al.* (2004) |

**PCR amplicon purification**

PCR products from 2nd round PCR amplification reactions using the FLR3 and FLR4 primers were purified using a ChargeSwitch™ PCR Clean-Up Kit (ThermoFisher Company) following the manufacturer's protocol.

**Restriction enzyme digestion of AMF PCR amplicons**

Purified PCR products were then digested with the restriction enzyme TaqI. Each digestion contained 10 µL of pooled PCR product and 3 µL of TaqI in the manufacturer's recommended buffer. Digestions were incubated for 4 h at 65ºC, followed by enzyme heat inactivation at 94ºC for 10 min.

**Data analysis**

For each sample, terminal restriction fragments (TRFs) were identified using an ABI 3730 DNA sequencer (Applied Biosystems, California, USA) with 6-carboxyfluorescein (6-FAM) as a standard (DBS Genomics, Durham University). GeneMapper (ABI) was used to identify

and quantify the fluorescently labelled TRFs and generated a peak area for each identified peak. Individual peaks on each T-RFLP profile were represented as operational taxonomic units (OTU). Each OTU was considered analogous to a species for diversity analysis (Cooper *et al.*, 2011). A fixed bin width of 5 bp was selected for stable peak identification. Data were processed to yield peak relative fluorescence values by dividing the fluorescence of each bin by the sum of the total fluorescence for the sample. Peaks representing less than 1% of the total fluorescence of each sample were removed.

The Shannon's diversity index (H′) was calculated using the following equation (Cooper *et al.*, 2011):

$$H' = -\sum_{i=1}^{S} (Pi \log Pi)$$

where S represents the total number of fragments in each sample, *P* represents the relative abundance of a given fragment size (relative fluorescence for the data generated from T-RFLP). Band richness (S) and evenness (E) (E, calculated as Shannon's diversity index divided by the log10 of the number of fragments) were also calculated.

Differences in AMF community structure were assessed by detrended correspondence analysis (DCA) of the relative fluorescence data matrix using the vegan (Oksanen et al., 2018) and MASS (Oksanen *et al.*, 2015) packages in R. Crop management practice (treatment) and potato variety were set as environmental variables, and fragment lengths were set as species data. Samples were subjected to Detrended Correspondence Analysis (DCA) with non-transformed data. Ordination plots were drawn using R.

### *3.3.10 AMF community and diversity analysis using Illumina MiSeq NGS*

AMF community and diversity analysis were achieved using general fungal ITS gene amplification primers (ITS1f and ITS2; general primers for the fungi). Details of the primers used and PCR conditions are described in Smith and Peay (2014). The target positions of the primer pairs used for the ITS rDNA amplification are shown in Figure 3. 4 and sequence Table 3. 8. Sequencing was conducted by NU-OMICS (Northumbria University, UK) (https://www.northumbria.ac.uk/business-services/engage-with-us/research/nu-omics/illumina/) based on the Schloss wet-lab MiSeq standard operating procedure; SOP).

**Table 3. 8** The primers used for PCR amplification.

| Primer | Primer sequence (5′-Sequence-3′) | Direction | References |
|---|---|---|---|
| ITS1f | CTTGGTCATTTAGAGGAAGTAA | Forward | Op De Beeck *et al.* (2014) |
| ITS2 | GCTGCGTTCTTCATCGATGC | Reverse | Op De Beeck *et al.* (2014) |

**Bioinformatic processing of Illumina MiSeq output**

The NGS data was processed through a pipeline provided by Dr. Peter Leary, Experimental Scientific Officer, Bioinformatics Support Unit, Faculty of Medical Sciences, Newcastle University. Raw reads of each of the individual samples received from the Illumina MiSeq sequencer comprised of forward and reverse sequences were in the form of FASTQ files. Raw FASTQ files were demultiplexed, trimmed, and quality-filtered using the DADA2 package in R. The Qiime2 (Quantitative Insights Into Microbial Ecology 2) package in R was used to cluster sequences at a 97% sequence similarity against the UNITE dynamic database (https://unite.ut.ee/) (Berlanas *et al.*, 2019). The taxonomic information in the UNITE dynamic database depends on the NCBI Taxonomy classification (Stefani *et al.*, 2020). Relative abundances for taxonomic groups were calculated for various levels of classifications (from phylum level down to genus) (Kõljalg *et al.*, 2013). Reads were then clustered into amplicon sequence variants (ASVs) for each sample. The ASV tables were used as inputs for the subsequent analyses.

**Data analysis**

**General composition of general fungi and AMF community**

From the ASVs table, fungal taxonomic data were normalised as a proportion of the total number of fungal and AM fungal sequences in the library prior to analysis (McGee *et al.*, 2017). The fractional abundance of individual taxa (phylum, family, and genus) within each community was calculated by dividing the number of sequences in different taxonomic groups (phylum, family, and genus) by the number of total fungal and AM fungal sequences in the library obtained for that sample.

The composition of AMF community analysis was analysed as follows: the fractional abundance of different AMF genera from all samples were subjected to two-way ANOVA with “Treatment” and “Potato variety,” and the interaction between these two factors using

the nlme package in R. A linear mixed-effects (lme) model was used with the nested structure of the split-plot design reflected in the random error term:block/treatment (Pinheiro and Bates, 2000). When ANOVAs indicated a significant treatment effect ($p<0.05$), differences between individual means were analysed using Tukey's honestly significant difference (HSD) post hoc test in the multcomp package in R. A 95% significance level ($p<0.05$) was applied to discriminate the means. Where the interaction between treatment and potato variety was significant, the data was subset by treatment and a separate one-way ANOVA conducted at each level of the three treatments.

**Diversity analysis**

AMF diversity was estimated by the number of ASVs representing species richness (observed species) (Stefani *et al.*, 2020). The diversity was calculated using the phyloseq and vegan packages in R (Nilsen *et al.*, 2020). The observed species and Shannon's diversity index (H') were analysed and plotted in phyloseq. Species evenness was calculated by dividing Shannon's diversity index by the $\log_n$ of observed species (Nilsen *et al.*, 2020). The differences in alpha diversity between groups were evaluated using the Kruskal-Wallis test (McTaggart *et al.*, 2019).

**Taxonomic composition analysis**

A stacked bar plot of relative abundance in each sample was presented at the genus level in the plot.

## 3.4 Results

### *3.4.1 AMF colonisation by root staining*

**Evaluation of root staining method**

The ink and vinegar method successfully stained structures in the roots associated with AMF colonisation, although archived roots had been kept in the freezer at -80$^{o}$C since 2011. All AMF structures (arbuscules, vesicles, and hyphae) were stained with black ink and were clearly visible enough to assess the root colonisation using a compound light microscope (Leica DMLB microscope) at 400x magnification. Figure 3. 5 illustrates the AMF colonised roots compared with roots with no AMF colonisation.

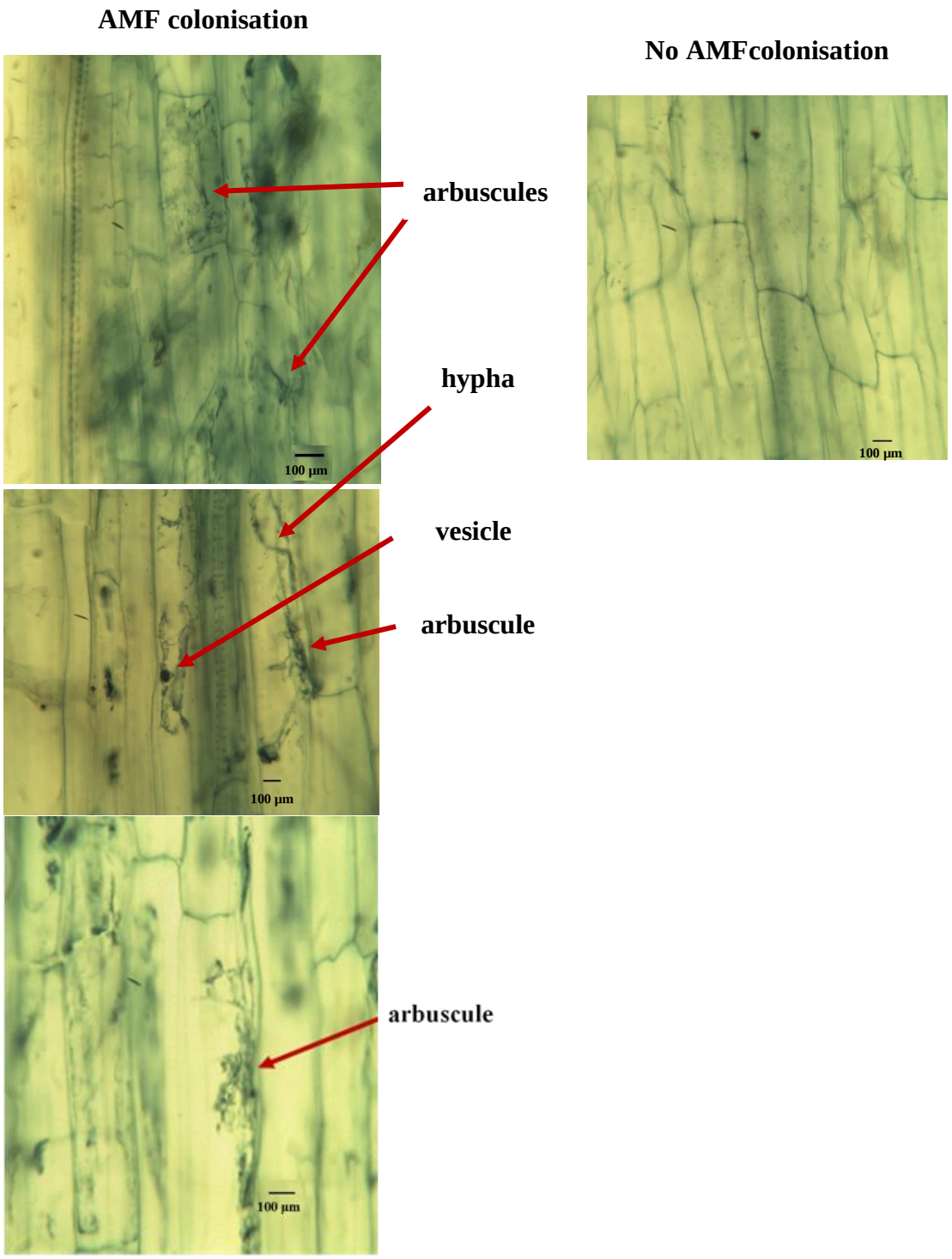



**Figure 3. 5** AMF colonised roots stained with an ink-vinegar solution showing key structures (left images) compared with roots showing no AMF colonisation (right image).

**Effects on AMF colonisation**

Crop management practices had a significant effect on the percentage of AMF colonisation in potato roots (Table 3. 9). The control treatment with no fertiliser amendment or conventional pesticides showed the highest AMF colonisation, whereas the colonisation in the NPK fertility treatment with CCP (conventional crop protection) was significantly higher than the NPK fertility treatment with OCP (organic crop protection).

In contrast to crop management, potato variety had no significant effect on AMF colonisation. Among the potato varieties, the highest AMF colonisation on average across the three management treatments was in the Sarpo Mira variety that had 52.17% colonisation. The lowest was in the Sante variety at 42.08% colonisation. These two potato varieties in all three management treatments were selected to study the absolute AMF abundance using qPCR. There was no significant interaction between treatment and potato variety for AMF colonisation.

**Table 3. 9** Effect of each crop management practice (Treatment) and potato variety (VA) on the percentage of AMF colonisation and each AMF structure observed in the roots by the root staining method (Main effect means±SE, ANOVA p-values, n=69). Means followed by the same letter within the same column and experimental factor are not significantly different at the level of $p<0.05$.

| Factor | AMF colonisation (%) | AMF structure (%) | | |
|---|---|---|---|---|
| | | Hyphae | Arbuscule | Vesicle |
| **Crop management practice (Treatment)** | | | | |
| 1: Control | 72.57±4.10 a | 72.57±4.10 a | 36.90±3.18 a | 5.00±1.29 a |
| 2: NPK with CCP | 41.17±2.71 b | 41.17+2.71 b | 27.75+2.30 b | 1.25±0.33 b |
| 3: NPK with OCP | 28.54±2.80 c | 28.54±2.80 c | 19.38±2.20 c | 1.17±0.10 b |
| **Potato variety (VA)** | | | | |
| Agria | 46.33±7.89 | 46.33±7.89 | 26.08±4.46 bc | 3.83±2.33 |
| Cara | 50.09±5.53 | 50.09±5.53 | 34.82±3.78 a | 1.64±0.59 |
| Sarpo Mira | 52.17±6.34 | 52.17±6.34 | 32.83±3.49 ab | 2.25±0.60 |
| Fontane | 45.00±7.69 | 45.00±7.69 | 27.27±4.65 ac | 2.27±0.71 |
| Nicola | 42.18±6.69 | 42.18±6.69 | 22.64±3.43 c | 0.73±0.63 |
| Sante | 42.08±8.17 | 42.08±8.17 | 22.25±4.14 c | 1.25±0.74 |
| **Anova P-values** | | | | |
| Treatment | **<0.0001** | **<0.0001** | **0.0002** | **0.0188** |
| VA | 0.1802 | 0.1802 | **0.0192** | 0.1744 |
| Treatment x VA | 0.1653 | 0.1653 | 0.3841 | 0.0732 |

**Identification of the dominant AMF structure in the roots assessed by the root staining method**

Crop management practices had a significant effect on the percentages of all three AMF structures observed in potato roots (Table 3. 9). The control treatment with no fertiliser or conventional pesticides showed the highest hyphae, arbuscule, and vesicle levels, whereas all three AMF structures were lowest in the NPK fertility treatment with OCP. Hyphae were the numerically dominant AMF structures found in the roots of all three crop management practices and all potato varieties. Very few vesicles were detected (5% or less), with the control treatment having the highest number of vesicles.

Potato variety had a significant effect on the percentage of arbuscule colonisation. The potato variety Cara showed the highest presence of arbuscules at 34.82%, while both Nicola and Sante had the lowest numbers of arbuscules present. Potato variety did not significantly affect the percentage of hyphae or vesicles present. There was no significant interaction between treatment and potato variety for AMF structure.

### *3.4.2 Evaluation and optimisation of the nucleic acid extraction procedure*

**Nucleic acid quantification and purity check**

DNA extraction method had a significant effect on the amount of DNA recovered based on the concentrations analysed (Table 3. 10). The nucleic acid yield (DNA concentration) data checked by a Nanodrop 8000 spectrophotometer showed that the highest DNA concentration (147.38 ng $\mu L^{-1}$) was obtained using DNA extraction method 3, which employed the FastDNA SPIN kit for soil with freeze-dried, milled samples and which followed the manufacturer's protocol.

DNA extracts evaluated by the ratio of absorbances at 260 nm:280 nm had values of 1.80-1.83, indicating that all DNA extracts were of good quality, i.e., not contaminated with protein or phenolic contaminants (Table 3. 10).

**Table 3. 10** Effect of each DNA extraction method on DNA concentration (nucleic acid yield) and the absorbance ratios (Main effect means±SE, ANOVA p-values, n=6). Means followed by the same letter within the same column are not significantly different at the level of $p<0.05$.

| Factor | DNA concentration (ng $\mu L^{-1}$) | Absorbance at 260 nm:280 nm |
|---|---|---|
| **Method** | | |
| 1. FastDNA SPIN kit for soil (frozen root samples) | 34.13±4.78 b | 1.80±0.005 |
| 2. FastDNA SPIN kit for soil (frozen root samples+milled) | 47.63±6.13 b | 1.83±0.005 |
| 3. FastDNA SPIN kit for soil (frozen root samples+freeze dried+milled) | 147.38±14.24 a | 1.81±0.003 |
| **ANOVA p-values** | | |
| Method | **<0.0001** | 0.053 |

Based on these results, the DNA extraction method 3 using the FastDNA SPIN kit for soil with samples previously freeze-dried and milled before extraction was selected to extract the DNA for all samples in all three experimental chapters in this thesis.

### *3.4.3 Quantification of AMF in roots using qPCR*

#### Standard curve and amplification curve generation

A qPCR technique was used successfully for AMF quantification in some DNA samples using the AMF-specific primers AMG1F and AM1 that target the SSU rDNA gene. According to Figure 3. 6, there was a linear relationship between Cq values (Quantification cycle) and the log of the starting quantity for standards ($R^2$= 0.994). The DNA concentrations of most samples fell between $10^2$ and $10^5$ gene copies per µL with a few below $10^2$ gene copies per µL.

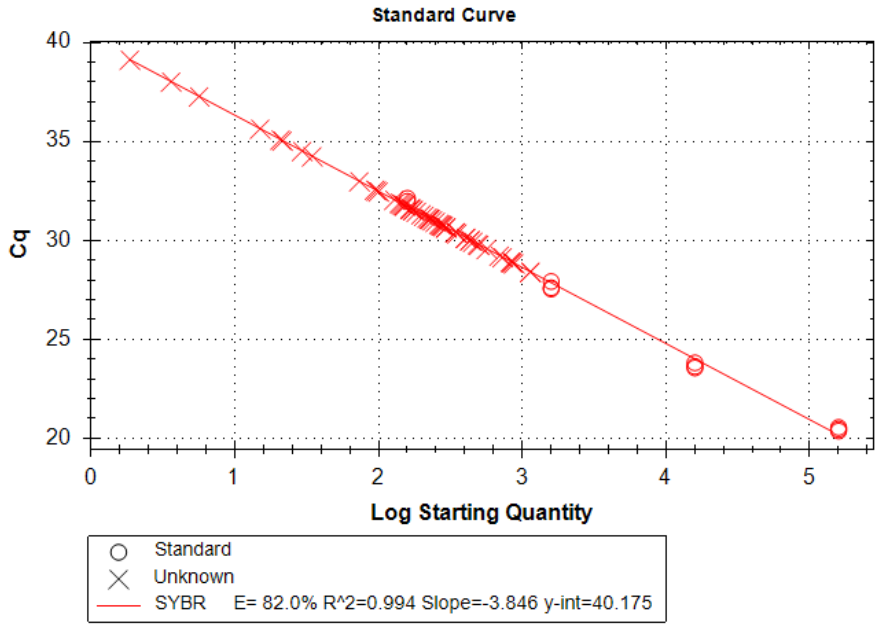



**Figure 3. 6** Standard curve obtained by plotting Cq value (Quantification cycle) against log starting quantity (genomic DNA concentration). Individual samples included in the study are indicated as "unknown" in the figure legend. E=amplification efficiency.

According to Figure 3. 7, some samples were not successfully amplified. Samples show the melting curve (Figure 3. 7A) and the amplification curve (Figure 3. 7B) below the negative control.

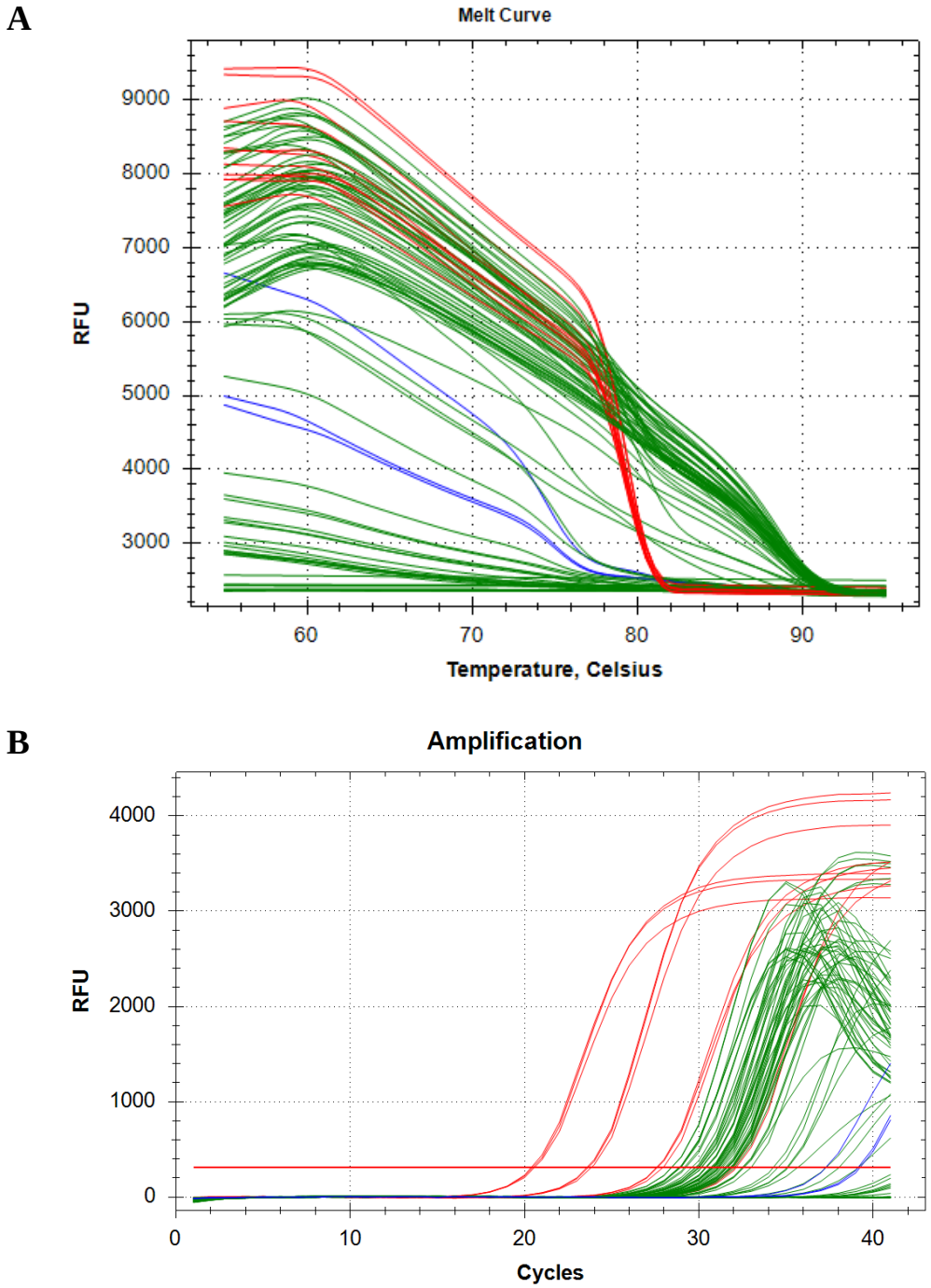



**Figure 3. 7** qPCR melting curve (A) and amplification curve (B) obtained by plotting temperature (°C) and cycles against relative fluorescence units (RFU). The red lines show standards, green lines show samples, and blue lines show the negative control.

**AMF gene abundance**

DNA samples from two potato varieties (Sarpo Mira and Sante) at all three levels of treatment were selected for DNA quantification using qPCR. These were selected to illustrate contrasting levels of AMF colonisation as Sarpo Mira is the potato variety that showed the highest AMF colonisation by root staining, whereas Sante had the lowest AMF colonisation based on root staining analysis. The results of gene copy number (GCN) from Figure 3. 8 and Table 3. 11 showed that gene copy number in the control treatment for both potato varieties was the highest. The lowest gene copy numbers were measured in the NPK treatment with CCP. As shown in Table 3. 11, there was no statistically significant difference among treatments or potato varieties.

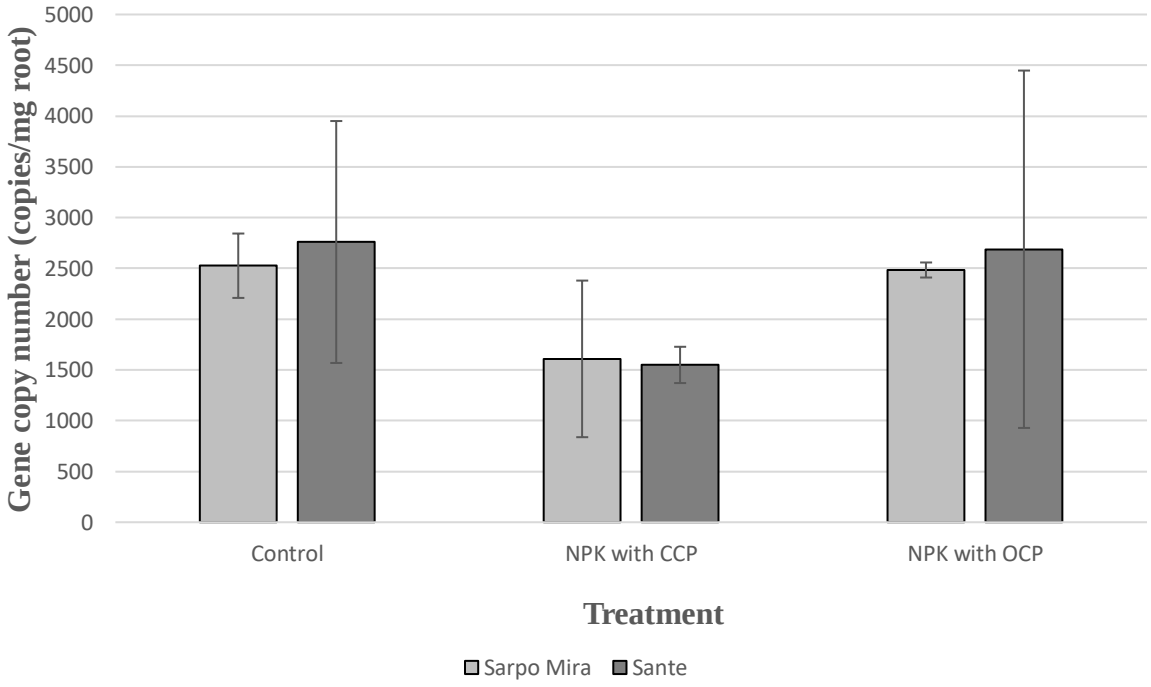



**Figure 3. 8** AMF gene copy number (gene copies per mg of the freeze-dried root) in three treatments (control, NPK with CCP, and NPK with OCP) for two potato varieties (Sarpo Mira and Sante). Values represent the interaction means, n=24. Error bars±SE.

**Table 3. 11** Effect of each crop management practice (Treatment) and potato variety (VA) on AMF gene copy number (gene copies per mg of the freeze-dried root) (Main effect means±SE, ANOVA p-values, n=24).

| Factor | Gene copy number (copies/mg root) |
|---|---|
| **Crop management practice (Treatment)** | |
| 1: Control | 2271.07±597.71 |
| 2: NPK with CCP | 1322.21±435.97 |
| 3: NPK with OCP | 2246.73±748.19 |
| **Potato variety (VA)** | |
| Sarpo Mira | 1827.84±342.60 |
| Sante | 2161.29±691.65 |
| **ANOVA p-values** | |
| Treatment | 0.5638 |
| VA | 0.6286 |
| Treatment x VA | 0.7231 |

### *3.4.4 Comparison of root colonisation by root staining with qPCR*

There was no correlation between AMF colonisation as assessed by root staining and gene copy number derived from qPCR, as shown by the scatterplot (r=0.053; p=0.82) (Figure 3. 9).

Sarpo Mira was the variety with the highest AMF colonisation using the root staining method, and Sante had the lowest colonisation. However, from the qPCR results, gene copy numbers for Sante were numerically higher than Sarpo Mira, but there was no statistically significant difference (Table 3. 11).

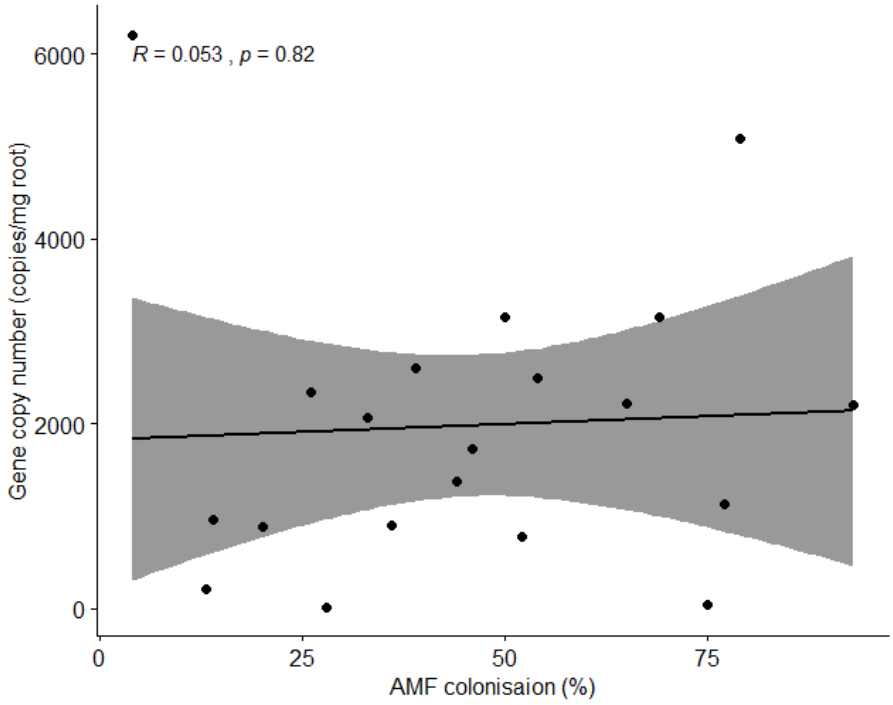



**Figure 3. 9** The percentage of AMF colonisation plotted against the AMF gene copy number for each root sample. (Pearson's product-moment correlation, r=0.053; p=0.82).

### *3.4.5 AMF community structure*

#### Nested PCR amplification

An initial run of the first-round PCR products on an agarose gel showed that most samples presented a band at ~600 bp in length (Figure 3. 10), indicating successful amplification of LSU rDNA.

The results of agarose gel electrophoresis for second-round PCR by using the AMF-specific primers FLR3 and FLR4 showed that most samples had a band present at ~300 bp (Figure 3. 11), including the positive control lane, indicating successful amplification of LSU rDNA. There was no band visible in the negative control lane.

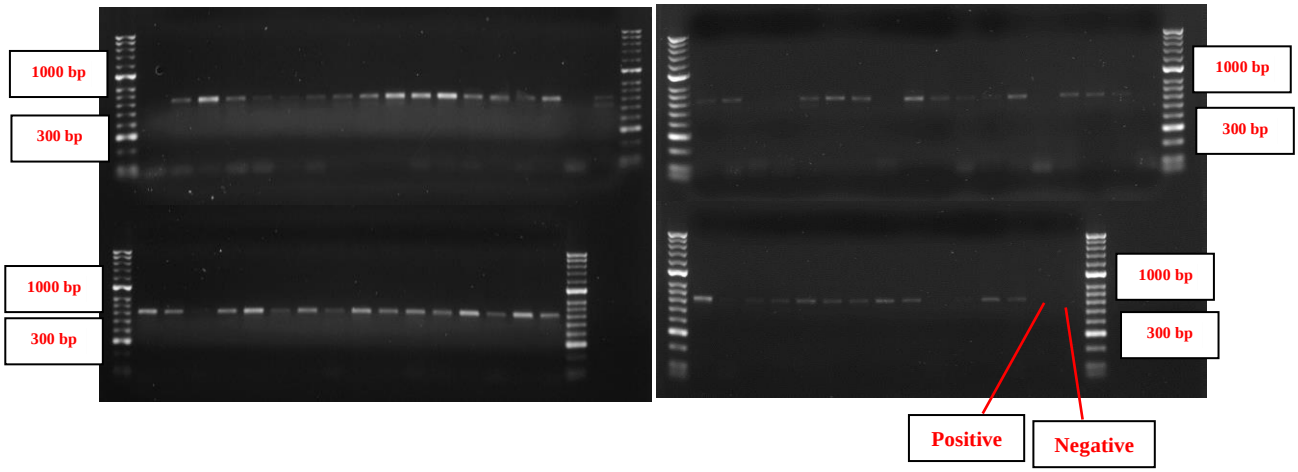



**Figure 3. 10** Agarose gel image of all AMF samples from the first-round PCR products.

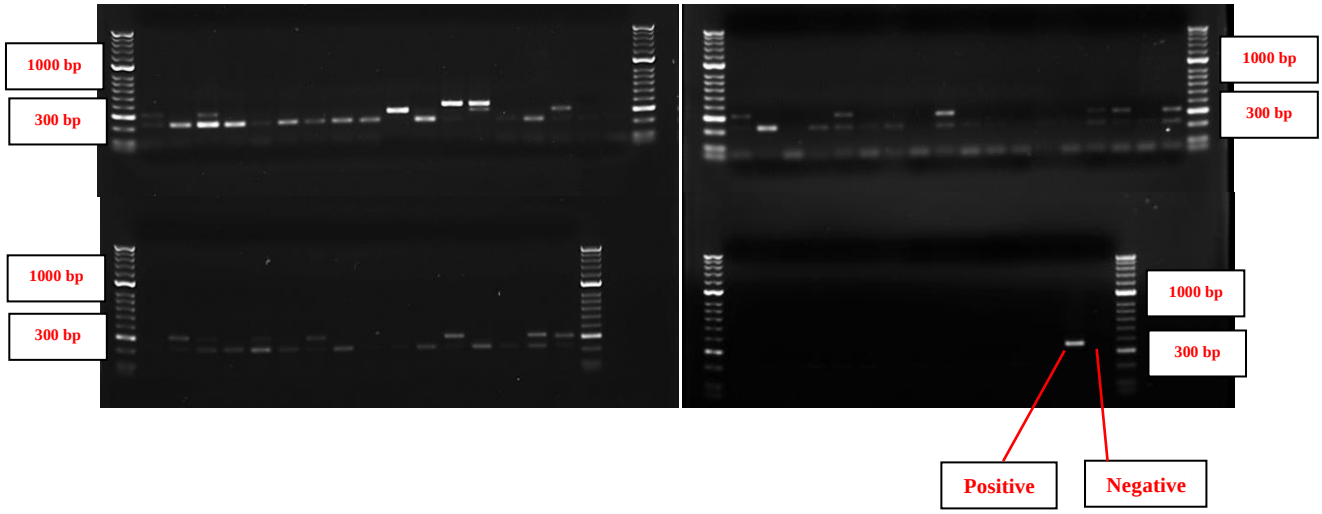



**Figure 3. 11** Agarose gel image of all AMF samples from the second-round PCR products.

**AMF community analysis**

The individual peaks on each T-RFLP profile were assumed to represent a separate operational taxonomic unit (OTU) with 29 OTUs identified in total. There were no significant effects of crop management practices or potato varieties on Shannon's diversity index, richness, or species evenness values (Table 3. 12).

**Table 3. 12** Effect of each crop management practice (Treatment) and potato variety (VA) on Shannon's diversity index (H'), richness (S), and species evenness (E') determined from the AMF T-RFLP profiles (Main effect means±SE, ANOVA p-values, n=65).

| Factor | AMF diversity indices | | |
|---|---|---|---|
| | **H′** | **S** | **E′** |
| **Crop management practice (Treatment)** | | | |
| 1: Control | 0.55±0.07 | 11.59±1.27 | 0.56±0.07 |
| 2: NPK with CCP | 0.66±0.07 | 9.58±1.26 | 0.71±0.06 |
| 3: NPK with OCP | 0.69±0.06 | 11.17±1.27 | 0.69±0.05 |
| **Potato variety (VA)** | | | |
| Agria | 0.73±0.08 | 10.33±1.57 | 0.78±0.08 |
| Cara | 0.54±0.12 | 9.00±1.95 | 0.55±0.10 |
| Sarpo Mira | 0.62±0.07 | 10.92±1.51 | 0.66±0.08 |
| Fontane | 0.66±0.11 | 9.80±2.09 | 0.68±0.09 |
| Nicola | 0.70±0.08 | 13.64±2.03 | 0.69±0.07 |
| Sante | 0.62±0.09 | 10.33±1.66 | 0.66±0.09 |
| **ANOVA p-values** | | | |
| Treatment | 0.3544 | 0.5901 | 0.3583 |
| VA | 0.7858 | 0.2621 | 0.6661 |
| Treatment x VA | 0.4099 | 0.0655 | 0.2261 |

**The detrended correspondence analysis (DCA) result**

DCA-derived sample scores for the first two ordination axes were plotted for both factors, i.e., crop management and potato variety (Figure 3. 12). The plots show that there is no clear grouping due to treatment or variety. This was confirmed by analysis of the sample scores for each TRF using ANOVA, which showed no significant effects of the treatments or the potato varieties.

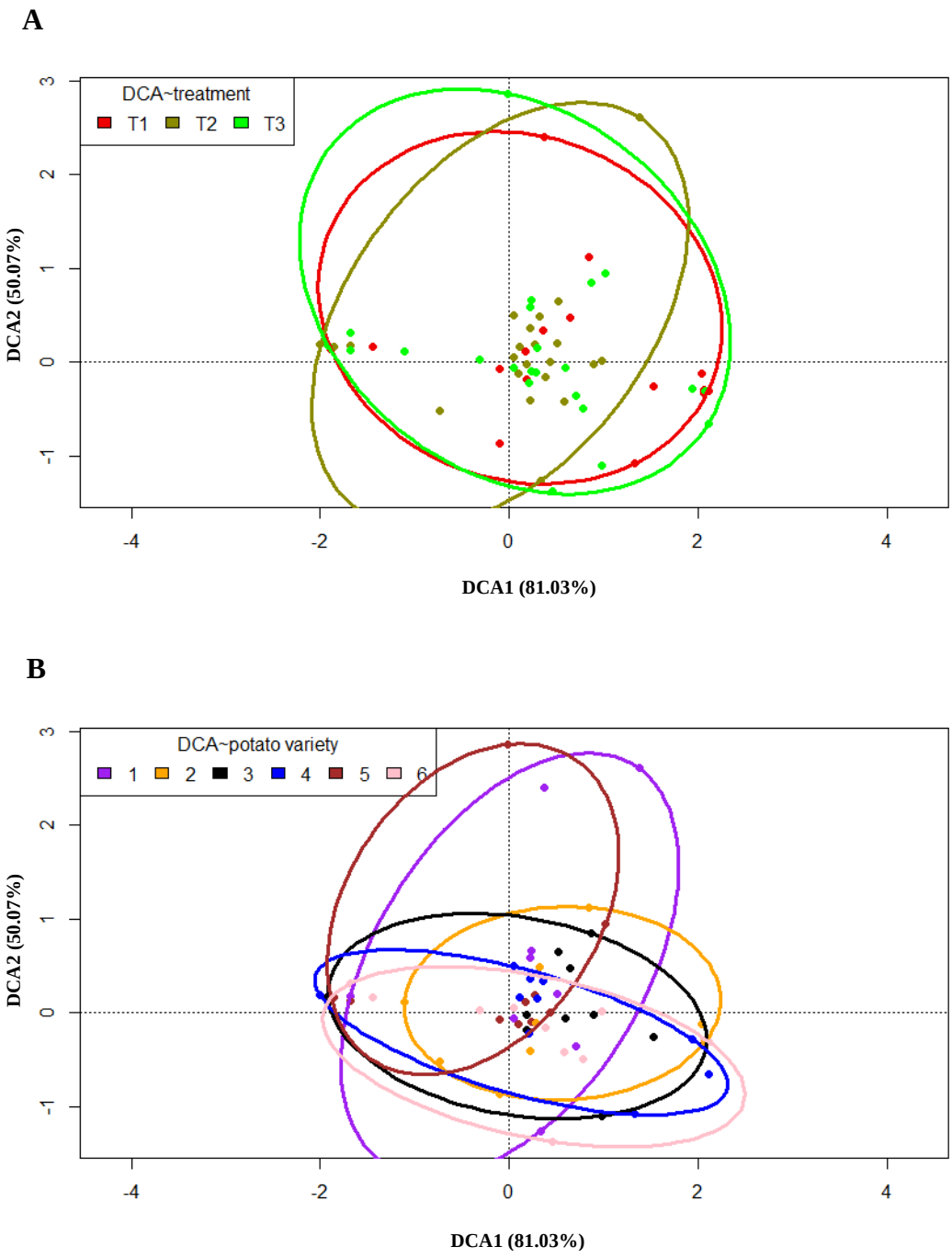



**Figure 3. 12** Detrended correspondence analysis (DCA) of the potato root AMF community based on T-RFLP. The DCA was based on the relative abundances of the OTUs. Points represent individual root samples (n=65). A) Samples identified by treatment; T1=Control, T2=NPK with CCP, T3=NPK with OCP, and B) Samples identified by potato variety; 1=Agria, 2=Cara, 3=Sarpo Mira, 4=Fontane, 5=Nicola, 6=Sante.

### *3.4.6 AMF diversity and community analysis*

As shown in Table 3. 13, fungal sequences were found in all samples from all three treatments. AMF sequences were found in all 17 samples from the control treatment, whereas only 16 samples contained AMF sequences in both the NPK with CCP and NPK with OCP treatments.

**Table 3. 13** The number of samples in each treatment for NGS analysis.

| Treatment | Number of samples sent for NGS | Number of samples with general fungal sequences | Number of samples with AMF sequences |
|---|---|---|---|
| 1. Control | 17 | 17 | 17 |
| 2. NPK with CCP | 24 | 24 | 16 |
| 3. NPK with OCP | 24 | 24 | 16 |

**General sequences composition**

**General composition of the fungal community**

A total of 918,115 sequences and 2,200 ASVs were obtained using ITS primers. The number of ASVs and sequence library abundances in each phylum of the Kingdom Fungi are shown in Table 3. 14. There were eight phyla (Ascomycota, Basidiomycota, Glomeromycota, Mortierellomycota, Rozellomycota, Olpidiomycota, Chytridiomycota, and Mucoromycota) and 1 group of unclassified fungi identified. Phylum Ascomycota was the main fungi in this study with 1,067 ASVs and 308,185 sequences, whereas there were 82 ASVs of phylum Glomeromycota (AMF) with 2,063 sequences. The percentage of ASVs in each phylum is presented in Figure 3. 13. The highest percentage of ASVs was in the phylum Ascomycota at 48.50%, while the phylum Glomeromycota (AMF) had only3.73% of the ASVs.

**Table 3. 14** The number of ASVs and sequence library abundance in each phylum of Kingdom Fungi.

| Phylum | Number of ASVs | Sequence library abundance |
|---|---|---|
| 1. Ascomycota | 1067 | 308185 |
| 2. Basidiomycota | 608 | 264338 |
| 3. Unclassified fungi | 251 | 46877 |
| 4. Glomeromycota | 82 | 2063 |
| 5. Mortierellomycota | 75 | 284429 |
| 6. Rozellomycota | 59 | 692 |
| 7. Olpidiomycota | 53 | 11425 |
| 8. Chytridiomycota | 4 | 102 |
| 9. Mucoromycota | 1 | 4 |
| **Total** | **2200** | **918115** |

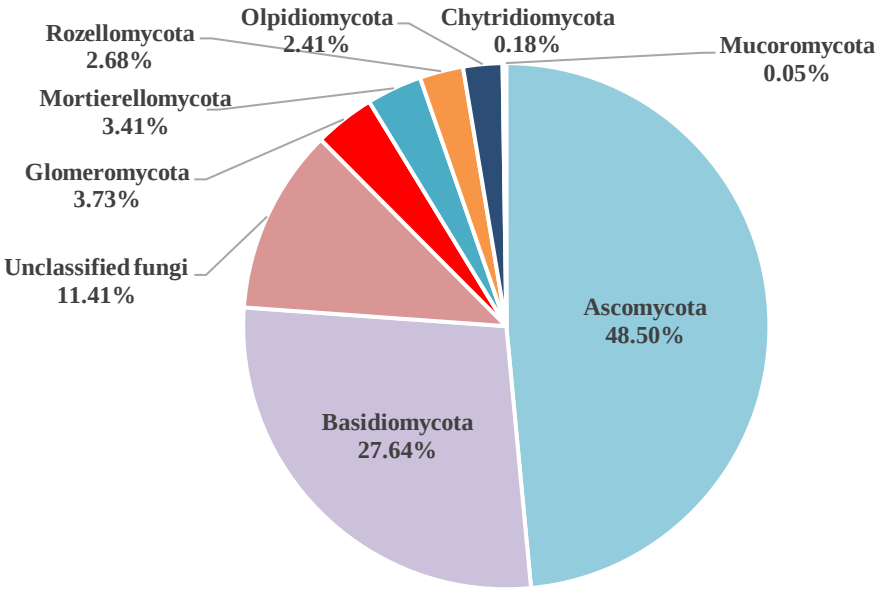



**Figure 3. 13** The percentage of ASVs in each phylum of Kingdom Fungi found in potato roots from three treatments (control, NPK with CCP, and NPK with OCP) in this study.

In terms of fractional abundance (Figure 3. 14), the highest fractional abundance is the phylum Ascomycota, whereas the fractional abundance of phylum Glomeromycota is less than 0.05.

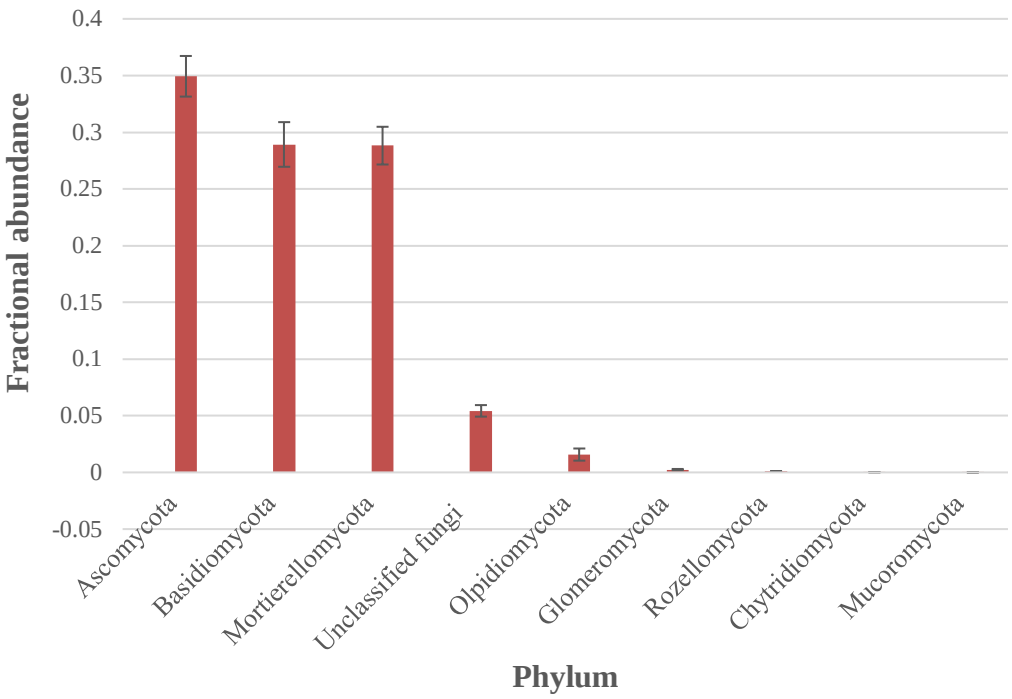



**Figure 3. 14** The composition of the fungal community (expressed as a fraction of the total sequences within the Kingdom Fungi) in potato roots. Values represent the means of all samples, n=65. Error bars±SE.

**General composition of the AMF community**

According to Table 3. 15, from a total of 82 ASVs and 2,063 sequences of phylum Glomeromycota, there are comprised of 5 families (Paraglomeraceae, Glomeraceae, Archaeosporaceae, Ambisporaceae, and Diversisporaceae), six genera (*Paraglomus*, *Glomus*, *Funneliformis*, *Archaeospora*, *Ambispora*, and *Diversispora*), unclassified Paraglomerales and unclassified Glomeraceae. The main genus is *Paraglomus* in the family Paraglomeraceae with 47 ASVs and 1,591 sequences. *Paraglomus* in the family Paraglomeraceae represents more than 50% of the ASVs in this study (Figure 3. 15).

According to fractional abundance from Figure 3. 16, *Paraglomus* also has the highest fractional abundance compared with another AMF genera.

**Table 3. 15** The number of ASVs and sequence library abundance in each family and genus of phylum Glomeromycota.

| Family | Genus | Number of ASVs | Total number of ASVs | Sequence library abundance |
|---|---|---|---|---|
| 1. Paraglomeraceae | *Paraglomus* | 47 | 47 | 1591 |
| 2. Glomeraceae | *Glomus* | 5 | 13 | 51 |
| | *Funneliformis* | 4 | | 10 |
| | Unclassified Glomeraceae | 4 | | 110 |
| 3. Archaeosporaceae | *Archaeospora* | 11 | 11 | 168 |
| 4. Ambisporaceae | *Ambispora* | 8 | 8 | 79 |
| 5. Diversisporaceae | *Diversispora* | 2 | 2 | 4 |
| 6. Unclassified Paraglomerales | | 1 | 1 | 50 |
| **Total** | | | **82** | **2063** |

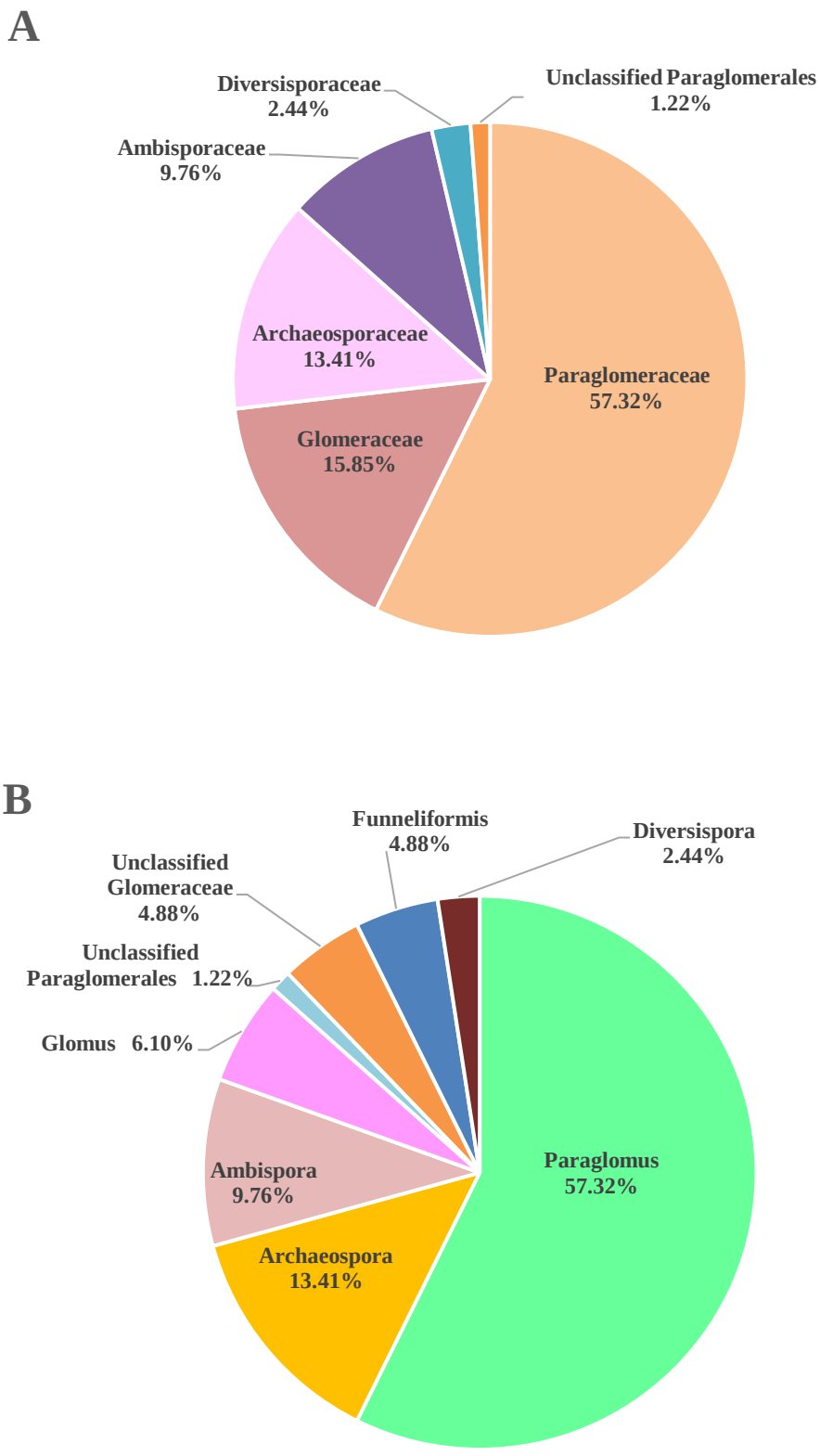



**Figure 3. 15** The percentage of ASVs in each family (A) and genus (B) of phylum Glomeromycota found in potato roots from three treatments (control, NPK with CCP, and NPK with OCP) in this study.

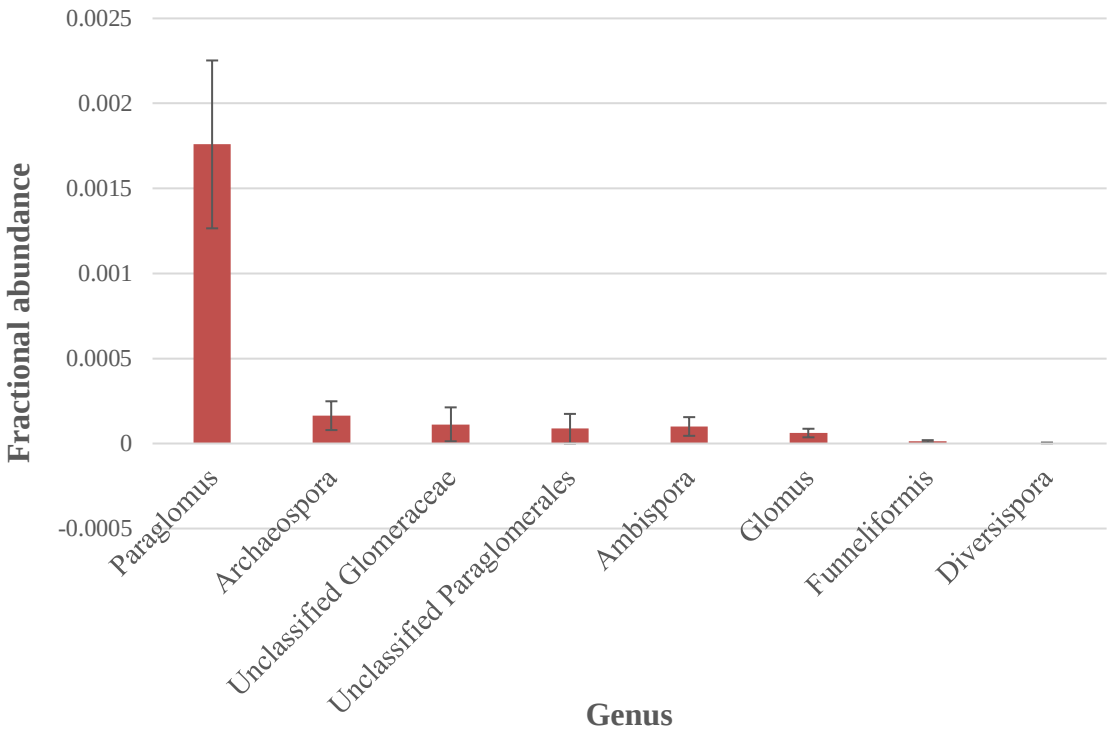



**Figure 3. 16** The distribution of genera in the Phylum Glomeromycota for all potato root samples (expressed as a fraction of the total sequences within the Kingdom Fungi). Values represent the means of all samples, n=65. Error bars±SE.

**The composition of the AMF community**

The control treatment had a significantly higher fractional abundance of *Paraglomus* than the other two treatments (Table 3. 16). While not statistically significant, the control treatment with no fertiliser amendment or conventional pesticides had numerically higher fractional abundances for the other AMF genera compared to the other two treatments

Potato variety had no significant effect on the fractional abundance for any AMF genera. *Paraglomus* is also the highest fractional abundance in all potato varieties compared with the other AMF genera. There was no significant interaction between treatment and potato variety for the fractional abundance of all AMF genera.

The fractional abundance of each AMF genus of the entire AM fungal sequences (Table 3. 17) showed that crop management practice and potato variety had no significant effect on the fractional abundance of all AMF genera in potato root. However, *Paraglomus* is the dominant AMF in all treatments and all potato varieties. There was no significant interaction between treatment and potato variety for the fractional abundance of all AMF genera.

**Table 3. 16** Effect of each crop management practice (Treatment) and potato variety (VA) on the fractional abundance of each AMF genus of the total fungal sequences (Main effect means$x10^{-5}$±SE$x10^{-5}$, ANOVA p-values, n=65). Means followed by the same letter within the same column and experimental factor are not significantly different at the level of $p<0.05$.

| Factor | Fractional abundance | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | ***Paraglomus*** | ***Archaeospora*** | **Unclassified Glomeraceae** | **Unclassified Paraglomerales** | ***Ambispora*** | ***Glomus*** | ***Funneliformis*** | ***Diversispora*** |
| **Crop management practice (Treatment)** | | | | | | | | |
| 1: Control | 415±170 a | 47±31 | 39±38 | 33±33 | 33±20 | 8±4 | 3±2 | 0.7±0.7 |
| 2: NPK with CCP | 56±16 b | 8±4 | 2±2 | 0±0 | 3±2 | 7±5 | 2±1 | 0.4±0.4 |
| 3: NPK with OCP | 126±36 b | 3±3 | 0±0 | 0±0 | 0±0 | 5±4 | 0±0 | 0±0 |
| **Potato variety (VA)** | | | | | | | | |
| Agria | 74±34 | 3±3 | 0±0 | 0±0 | 0±0 | 0±0 | 2±2 | 0±0 |
| Cara | 297±217 | 54±46 | 59±59 | 0±0 | 9±6 | 13±10 | 3±3 | 0±0 |
| Sarpo Mira | 345±162 | 23±16 | 6±5 | 47±47 | 32±28 | 20±9 | 0±0 | 0.8±0.8 |
| Fontane | 47±14 | 3±3 | 1±1 | 0±0 | 2±2 | 0±0 | 1±1 | 0±0 |
| Nicola | 125±52 | 10±7 | 0±0 | 0±0 | 0±0 | 0±0 | 2±2 | 0±0 |
| Sante | 126±45 | 2±2 | 0±0 | 0±0 | 13±8 | 1±1 | 0±0 | 1±1 |
| **ANOVA p-values** | | | | | | | | |
| Treatment | **0.0241** | 0.1391 | 0.2252 | 0.3255 | 0.0932 | 0.8047 | 0.4118 | 0.5611 |
| VA | 0.3844 | 0.4831 | 0.5309 | 0.5812 | 0.5176 | 0.0604 | 0.6729 | 0.6555 |
| Treatment x VA | 06170 | 0.4684 | 0.2965 | 0.6154 | 0.6853 | 0.9810 | 0.2797 | 0.4438 |

**Table 3. 17** Effect of each crop management practice (Treatment) and potato variety (VA) on the fractional abundance of each AMF genus of the total AM fungal sequences (Main effect means±SE, ANOVA p-values, n=65).

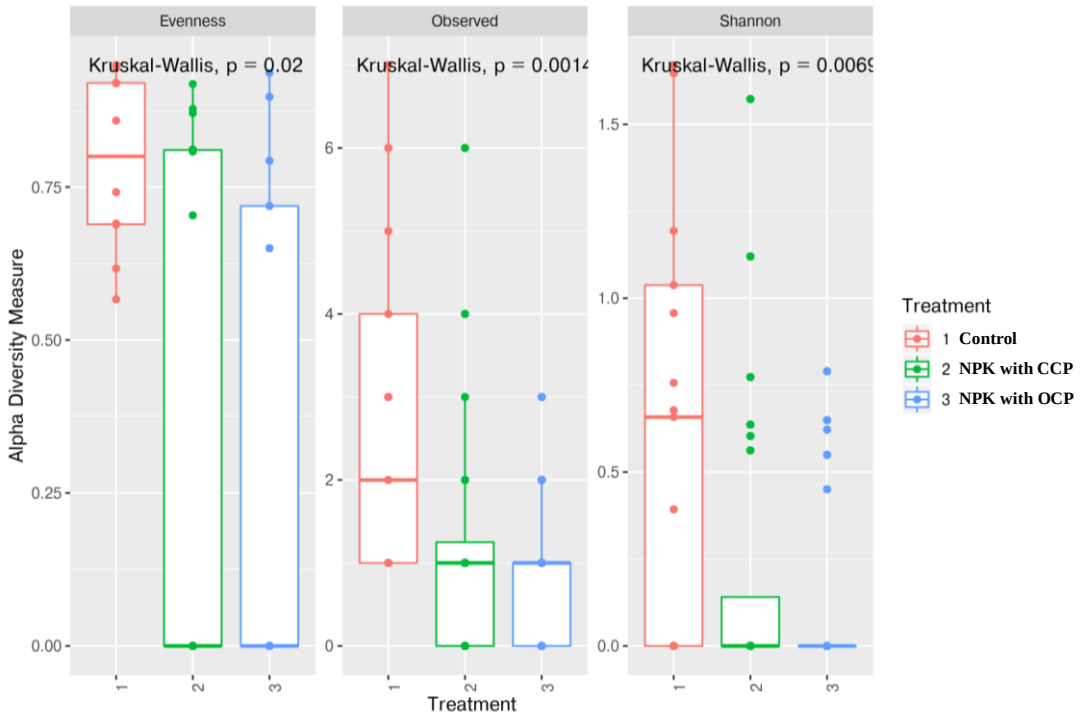
| Factor | Fractional abundance | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | ***Paraglomus*** | ***Archaeospora*** | **Unclassified Glomeraceae** | **Unclassified Paraglomeromycetes** | ***Ambispora*** | ***Glomus*** | ***Funneliformis*** | ***Diversispora*** |
| **Crop management practice (Treatment)** | | | | | | | | |
| 1: Control | 0.778±0.066 | 0.069±0.035 | 0.016±0.011 | 0.011±0.011 | 0.107±0.061 | 0.003±0.0002 | 0.013±0.009 | 0.002±0.002 |
| 2: NPK with conventional crop protection | 0.533±0.092 | 0.081±0.044 | 0.007±0.007 | 0.000±0.000 | 0.010±0.007 | 0.021±0.013 | 0.013±0.011 | 0.001±0.001 |
| 3: NPK with organic crop protection | 0.609±0.099 | 0.042±0.042 | 0.000±0.000 | 0.000±0.000 | 0.000±0.000 | 0.016±0.015 | 0.000±0.000 | 0.000±0.000 |
| **Potato variety** | | | | | | | | |
| Agria | 0.639±0.162 | 0.111±0.111 | 0.000±0.000 | 0.000±0.000 | 0.000±0.000 | 0.000±0.000 | 0.028±0.028 | 0.000±0.000 |
| Cara | 0.634±0.132 | 0.109±0.090 | 0.016±0.016 | 0.000±0.000 | 0.026±0.018 | 0.027±0.026 | 0.006±0.006 | 0.000±0.000 |
| Sarpo Mira | 0.653±0.109 | 0.072±0.046 | 0.018±0.014 | 0.016±0.016 | 0.020±0.013 | 0.052±0.030 | 0.000±0.000 | 0.002±0.002 |
| Fontane | 0.660±0.149 | 0.017±0.017 | 0.006±0.006 | 0.000±0.000 | 0.009±0.009 | 0.000±0.000 | 0.009±0.009 | 0.000±0.000 |
| Nicola | 0.573±0.140 | 0.051±0.034 | 0.000±0.000 | 0.000±0.000 | 0.000±0.000 | 0.000±0.000 | 0.012±0.012 | 0.000±0.000 |
| Sante | 0.597±0.132 | 0.028±0.028 | 0.000±0.000 | 0.000±0.000 | 0.120±0.086 | 0.003±0.003 | 0.000±0.000 | 0.003±0.003 |
| **ANOVA p-values** | | | | | | | | |
| Treatment | 0.3525 | 0.7041 | 0.3168 | 0.3255 | 0.1062 | 0.6443 | 0.5066 | 0.5444 |
| Variety | 0.9935 | 0.7590 | 0.5945 | 0.5812 | 0.2501 | 0.1857 | 0.4941 | 0.6596 |
| Treatment x Variety | 0.9843 | 0.5303 | 0.4060 | 0.6154 | 0.1073 | 0.8534 | 0.4806 | 0.4474 |

**AMF diversity**

Figure 3. 17A shows that there were significant effects from crop management practices on Shannon's diversity index and species evenness. The highest species richness and evenness were measured in the control treatment. However, there were very few AMF species "observed" in the samples according to the graph, which indicates the total numbers of ASVs for each of the treatments; the median number of AMF species in the control treatment was 2, with median values of approximately one genus for the other two treatments, and some samples with no AMF species detected at all.

For the potato variety analysis shown in Figure 3. 17B, the results illustrated no significant varietal effects on Shannon's diversity index, species richness, and species evenness. As reported for the treatment factor, there were not many AMF species found in the samples, although Sarpo Mira had as many as 6 ASVs present in at least one sample.

**A: Treatment**




**B: Potato variety**

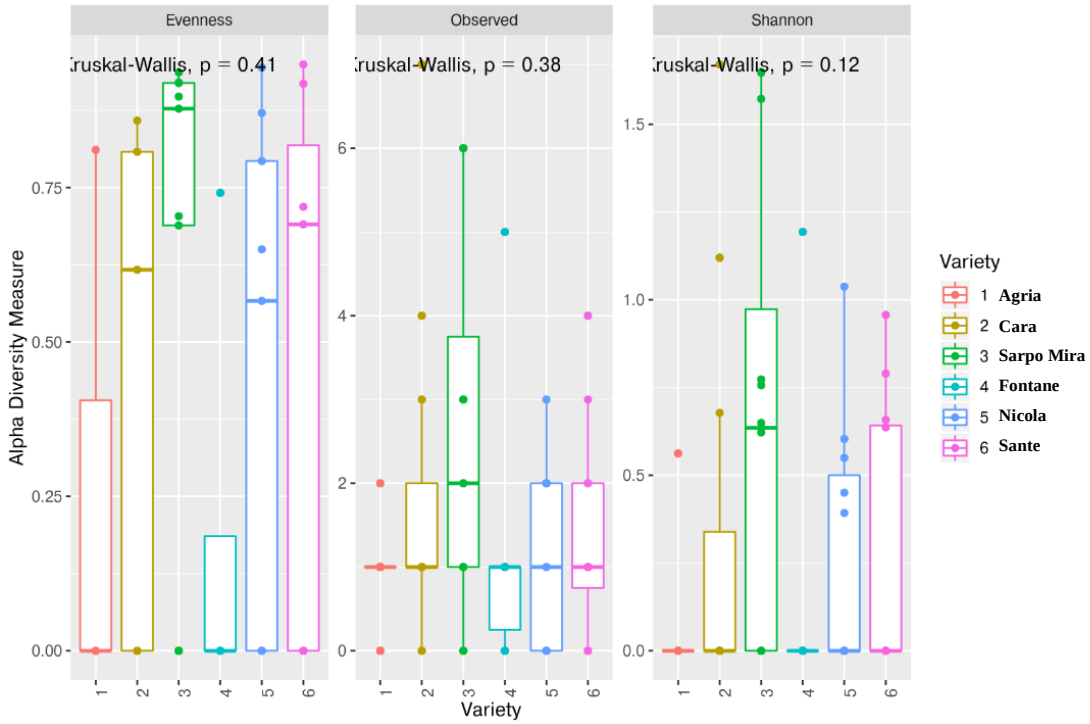



**Figure 3. 17** Boxplots showing evenness, number of ASVs (observed), and Shannon's diversity index (H'). Treatment and potato variety plotted against the alpha diversity measure. A) Samples grouped by treatment and B) Samples grouped by potato variety.

**AMF taxonomic composition**

Taxa bar plots of relative abundances of AMF genera-related sequences identified in amplicon libraries for the phylum Glomeromycota obtained from three treatments, and six potato varieties are shown in Figure 3. 18. The taxonomic diversity analysis of treatment (Figure 3. 18A) indicates that the main AMF genus presents in all three treatments is *Paraglomus* (blue taxa bars). In addition to the *Paraglomus,* various other genera in this phylum (*Ambispora*, *Archaeospora*, *Diversispora*, *Funneliformis*, and *Glomus*) were found in different proportions for each treatment. All samples in the control treatment had some AMF genera present with *Paraglomus* and *Archeospora* common in several samples and one sample with only *Ambispora* present. In contrast, in treatments NPK with CCP and OCP in eight samples, no AM fungal genera were detected.

Figure 3. 18B shows that the main genus of most samples from the root extracts of the six potato varieties is also *Paraglomus* (blue taxa bars), as observed for treatments. However, for some samples from each potato variety, no AM fungal genera were detected at all.

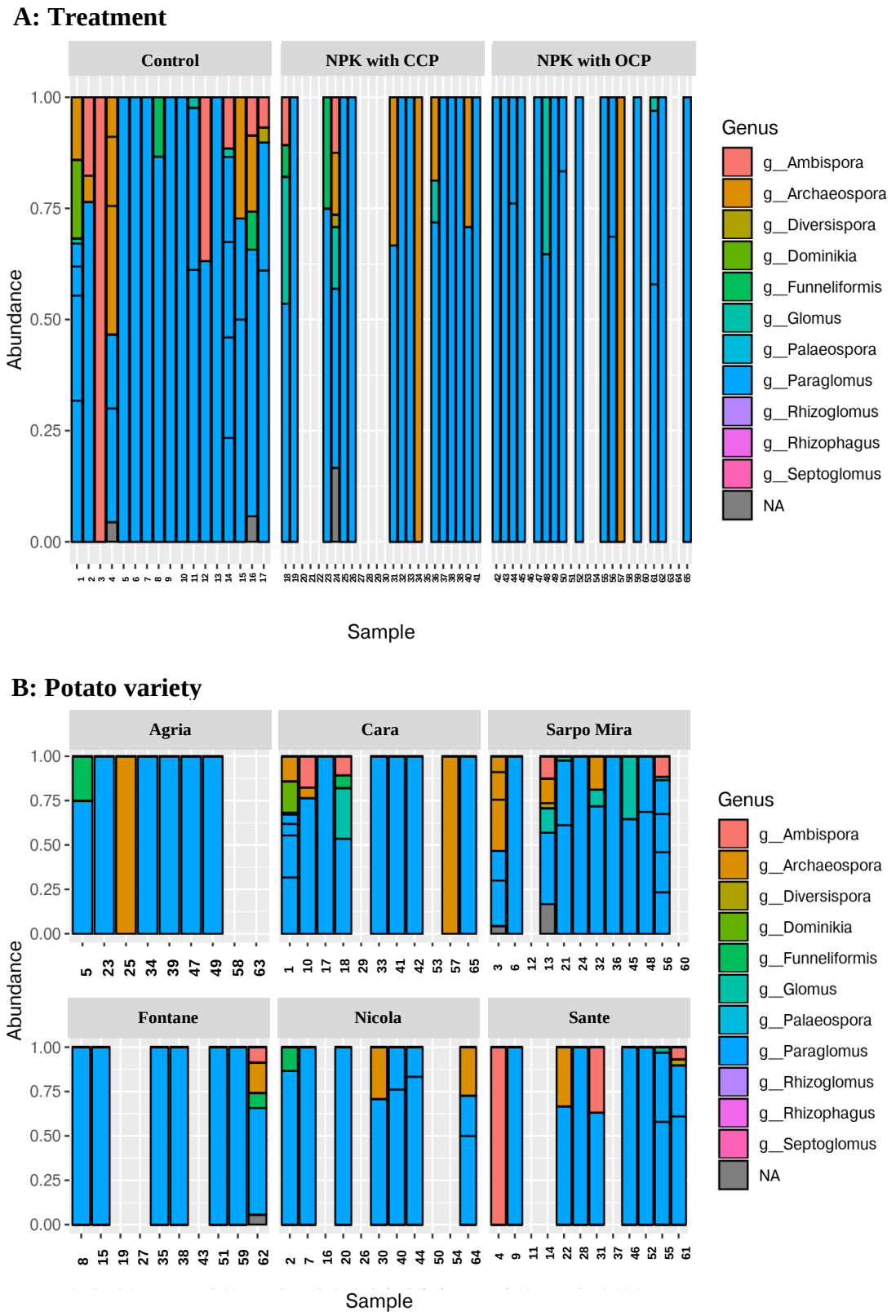



**Figure 3. 18** Taxa bar plots of the relative abundances of AMF genera-related sequences identified in amplicon libraries obtained from A: Treatments and B: Potato variety. Potato root samples plotted against the relative abundance.

## 3.5 Discussion

### *3.5.1 Methods to study the abundance and diversity of AMF in this study*

This study was used to compare the efficacy of two methods of quantifying the abundance of AMF in roots, namely: a standard root staining method using the ink-vinegar solution, and a qPCR method using AMF-specific primers. Two approaches for assessing AMF community diversity were also compared, namely: T-RFLP using AMF-specific primers and an NGS approach using general fungal primers. According to Table 3. 18, the root staining method clearly provided the most discrimination between the three treatments. The qPCR and T-RFLP did not show any differences between the treatments, but NGS was able to detect the large fertiliser effect but did not for conventional pesticides. Reductions in AMF abundance were detected using the root staining method, and declines in AMF diversity were detected by NGS when fertiliser was used compared to the control. AMF abundance assessed by the root staining method was increased when conventional pesticides were used compared to organic crop protection.

**Table 3. 18** Summary of effects of mineral fertilisers and conventional pesticides on abundance and diversity of AMF from four techniques used in this study.

| Factor | AMF abundance | | AMF diversity | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Evenness | | Richness | | Shannon's diversity index | |
| | Root staining | qPCR | T-RFLP | NGS | T-RFLP | NGS | T-RFLP | NGS |
| Use of mineral fertilisers (Trt 1 vs. Trt 3) | ↓ | ns | ns | ↓ | ns | ↓ | ns | ↓ |
| Use of conventional pesticides (Trt 2 vs. Trt 3) | ↑ | ns | ns | ns | ns | ns | ns | ns |

ns=not significant, indicated by p-value

arrows represent the level variation; increased expression (upward position) and reduced expression (downward position)

The specific primer sets for T-RFLP and qPCR in this study have been used in several studies (Gollotte *et al.*, 2004; Verbruggen *et al.*, 2012; Wetzel *et al.*, 2014; Krishnamoorthy *et al.*, 2015; Řezáčová *et al.*, 2016; García *et al.*, 2017). However, it seems that these primers cannot amplify the main genus *Paraglomus* that was found in most potato roots according to NGS results. In the case of T-RFLP, Wetzel *et al.* (2014) reviewed the limitations of the primers FLR3 and FLR4 and reported that they are not able to amplify sequences of AMF in the families Diversisporaceae, Archaeosporaceae, and Paraglomaceae. Manoharan *et al.* (2017) also mentioned the biased amplification of the FLR3/FLR4 primer pair within families Diversisporales and Paraglomerales, and that this primer set preferentially amplifies within the Glomerales. Besides, Gollotte *et al.* (2004) reported that the primer FLR4 was found to have multiple mismatches to *Archaeospora*, and this genus was found in many samples in this study. Gamper *et al.* (2009) illustrated that the primer pair FLR3/FLR4 showed poor binding site matching for the members of the family Diversisporaceae, which were present in this study and, therefore, may not have been amplified in the T-RFLP analysis.

In terms of the qPCR, Hewins *et al.* (2015) also used the same primers as this study (AMG1F/AM1) to study the AMF community in *Allium tricoccum* Ait. (wild leek). They found that these primers targeted *Glomus*, *Funneliformis*, and *Acaulospora* but not *Paraglomus*, which was the dominant genus in this study. This was also confirmed by checking both qPCR primers (AMG1F/AM1) against the National Center for Biotechnology Information (NCBI) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi), which showed that both primers cannot amplify the genus *Paraglomus.*

Meanwhile, *Paraglomus* is one of the target genera that ITS primers can amplify; several studies were reported. Interestingly, Gosling *et al.* (2014) used the ITS primers to study the distribution of the AMF order Paraglomerales from arable and horticultural farms, including potato farms in England. They used the molecular methods PCR and T-RFLP to investigate, and they found that *Paraglomus* were detected in most samples. However, they used the soil samples in their study, not roots.

The results of this study would have been more meaningful if the ITS primers for ITS region amplification had been applied in all molecular techniques in this study. If these primers (ITS1 and ITS2) had been used for qPCR and T-RFLP, then *Paraglomus* would have been amplified. The most generally used length is the ITS2 (Kryukov *et al.*, 2020). This region was also selected to amplify by NGS in this study. This region can provide sufficient identification

at the AMF species level in all root samples. Moreover, the ITS2 region provides a higher taxonomic resolution than the LSU or SSU region (Kryukov *et al.*, 2020). Consequently, the qPCR and T-RFLP approaches can be selected to study the abundance and diversity of AMF using ITS primers. Several qPCR and T-RFLP studies have used ITS primers to target the ITS rDNA region for amplification and successfully for AMF abundance and diversity studies (Dickie and FitzJohn, 2007; Kennedy *et al.*, 2007; Kurth *et al.*, 2013; Rodríguez-Yon *et al.*, 2021).

NGS was useful in this study. This method clearly differentiated the fertiliser effect on AMF diversity, which was not apparent using T-RFLP. NGS is a very powerful technique; NGS can identify a large number of AMF community composition in the root system. This sequencing technique is a generally used and helpful for AMF community composition analysis (Higo *et al.*, 2020). Nowadays, the study of AMF by using molecular approaches generally faces problems. However, in this study NGS with universal ITS primers provided an effective approach for understanding the diversity of the AMF community.

When comparing the results of two quantification methods, there was no correlation between qPCR and AMF colonisation by root staining. This is reviewed by Gamper *et al.* (2008), who suggested that the results of these two approaches are not comparable for determining AMF abundance. The qPCR can accurately quantify AMF in nucleic acids but it is poorly correlated with visual measures by a microscope; this could have been caused by differences in the number of target gene copies among members of the quantified AMF community. This study seems that the qPCR results showed lower AMF abundance than the microscopy technique. Another factor in this study could be that *Paraglomus*, which was the dominant species of AMF based on NGS, was not amplified by qPCR specific primers, whereas the microscopy quantified AMF structures (e.g., hyphae, arbuscules and vesicles) of all AMF species. However, Bodenhausen *et al.* (2021) suggested that a qPCR technique should be selected to compare with the traditional microscopy technique because it takes less time for qPCR machine running and analysing.

### *3.5.2 Effects of fertiliser on the abundance and diversity of AMF*

The root colonisation and NGS results indicated a reduction in AMF function and diversity when a high rate of mineral NPK fertiliser was used. There are many reports which have shown a negative fertiliser effect on AMF, as observed in this study. Gosling *et al.* (2006)

reported that soluble P fertilisers are disruptive to AMF communities and species diversity and that low input systems such as organic farming are ordinarily more favorable to AMF (Plenchette *et al.*, 2005). Oehl *et al.* (2004) showed that the AMF diversities of *Acaulospora* spp. and *Scutellospora* spp. decreased in long-term conventional management plots with high inputs of mineral fertiliser and AMF spore abundance was also negatively affected by fertilisers in the wheat field when compared to another treatment. Smith and Read (2010) reported that AMF activities are sensitive to fertilisation; they showed a negative correlation between high levels of P and N fertilisers and AMF root colonisation. Wu *et al.* (2011) also mentioned the long-term heavy application of mineral N under field conditions reduced the AMF colonisation of wheat roots and spore abundance in China. Furthermore, some studies have reported that the high-input of chemical nutrients in conventional farming systems negatively affects AMF (Verbruggen and Kiers, 2010; Prosser *et al.*, 2015). In terms of potato studies, several researchers have also reported a negative fertiliser effect on AMF, as shown in this study.

In nutrient-rich conditions, for example, when NPK fertilisers are added, plants can directly uptake enough nutrients from the soil without an AMF association. Consequently, the symbiosis of plants and AMF gradually decreases (Lin *et al.*, 2012). High nutrient inputs can lead to high P concentrations in plant tissue, which can reduce the soluble carbohydrates in the root exudates which AMF require for their growth, thus decreasing AM fungal diversity and species richness (Bhadalung *et al.*, 2005) (Mäder *et al.*, 2000; Astover *et al.*, 2018). On the other hand, in low nutrient input systems, AMF colonisation is promoted due to plant benefits from the fungi, such as increased nutrient uptake when nutrients availabilities are at low concentrations (Astover *et al.*, 2018; Loit *et al.*, 2018).

### *3.5.3 Effects of pesticides on the abundance and diversity of AMF*

The highest abundance and diversity were present in the control treatment, which used an organically approved fungicide: Headland Copper (copper oxychloride) for late blight control and tillage for weed control. Although this study did not have a without organic pesticide treatment to compare, it is clear that the organic pesticide used in this study may positively affect AMF symbiosis. In terms of organic fungicide, some reports showed the neutral and positive effect of Copper-based fungicide such as copper oxychloride, which is the same as this study on AMF symbiosis. Hage-Ahmed *et al.* (2019) reported that AMF root colonisation

was not affected by copper when applied to the soil. Copper seems compatible with AMF since AMF can tolerate Cu-contaminated environments (Ferrol *et al.*, 2009). AMF have been stated to occur vastly in Cu-contaminated soils (Wang *et al.*, 2007). AMF can release glomalin (protein secreted) into the soil and reduce the effects of Cu-contaminated soil.

The conventional pesticides used for conventional crop protection in this study included Linuron (3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea) for weed control and Fubol gold (metalaxyl-M and mancozeb) and Shirlan (fluazinam) for blight control. From the literature, the impact of pesticides on AMF varies with the type of chemical and dosage (Abd-Alla *et al.*, 2000; Samarbakhsh *et al.*, 2009; Buysens *et al.*, 2015; De Novais *et al.*, 2019), mode of action and AM fungal species (Kjøller and Rosendahl, 2000b; Jin *et al.*, 2013b). In this study, compared to organic pesticides, conventional pesticides positively affected the abundance of AMF as assessed by root colonisation (no significant pesticide effects were found for the T-RFLP or NGS results); however, this was in combination with NPK fertilisers application.

Herbicide application and the interaction with AMF can be varied (Chifetete and Dames, 2020). Herbicides typically show a neutral and positive effect on AMF. It has been indicated that some herbicides affect AMF colonisation within only the first few days but becoming balanced after a few weeks (Hage-Ahmed *et al.*, 2019), while the herbicides in this study were applied in the first month after planting. It seems that herbicides did not negatively affect AMF in this trial. Tillage may not decrease the abundance and diversity of AMF but can change the AMF community. The impact of tillage on the AMF community is still unsuccessfully understood, especially in the organic crop protection system (Säle *et al.*, 2015).

This study suggests that either organic pesticides or conventional pesticides are recommended to use in potato fields. Both pesticides should be applied in the field under without NPK fertiliser application for maximum AMF colonisation. This study did not have a without organic pesticide treatment to compare; however, the control treatment applied with organic pesticides: Headland Copper (copper oxychloride) and tillage to show the highest AMF abundance and AMF diversity. Meanwhile, the treatment with conventional pesticides is also had a positive effect on AMF abundance. In terms of organic fungicide and conventional fungicides for late blight control, one reason that the fungicides did not affect AMF may be due to the fact that AMF are soil microorganisms, and all fungicides in this study were sprayed onto the leaves of the potato plants, not directly applied to the soil. There are some

reports which have shown that fungicides can have neutral or positive effects on AMF symbiosis (Samarbakhsh *et al.*, 2009; Hernández-Dorrego and Mestre-Parés, 2010).

### *3.5.4 Impact of potato variety on the abundance and diversity of AMF*

The results of this study showed that potato variety has no impact on AMF abundance and diversity. However, the potato variety "Sarpo Mira" had the highest AMF colonisation (Table 3. 9), species richness, evenness, and species diversity (Figure 3. 17) compared to other potato varieties. The lack of significance for potato variety may be due to the low replication. After using the G Power analysis programme for checking, the replication of this study should be 4 to detect a significant treatment effect. In addition, the potato variety "Cara" had the highest number of arbuscules, but there were no differences in overall colonisation (Table 3. 9). Both "Sarpo Mira" and "Cara" seemed to have much higher fractional abundances of *Paraglomus* than the other varieties, although not significant (Table 3. 16). This may indicate that both potato varieties are suited to form an association with indigenous AMF in the field. Interestingly, Buysens *et al.* (2016) found that in the field experiment, the inoculant of AMF (*Rhizophagus irregularis* MUCL 41833) and *Trichoderma harzianum* MUCL 29707 increased the yield and the number of tubers of the potato variety "Sarpo Mira", however, it is the different from this study, they used the co-inoculants of AMF and other fungi.

Hannula *et al.* (2010) studied the AM fungal community under the different potato varieties in the Netherlands. They used T-RFLP to study the AMF community in six potato varieties (Modena, Karnico, Aveka, Aventra, Désirée, and Premiere) using the same primers as in this study (LR1/FLR2 and FLR3/FLR4). They found that potato variety did not affect the AMF community and no specificity between potato variety and AMF. They mentioned that the main factors affecting fungal communities are abiotic factors, such as agricultural management, soil properties, pH, nutrients, and water in soils. The reports from Öpik *et al.* (2009) and Santos-González *et al.* (2011) have also shown that the main factor determining AMF and plant associations is the soil properties and management regimes, not variety-specific. The main genus colonising potatoes in this study was *Paraglomus.* This genus is widespread in managed agricultural soils in England, including those growing potatoes (Gosling *et al.*, 2014). Stephan *et al.* (2019) also found that AMF in the Paraglomeraceae family can establish a symbiosis in potato roots of the variety "Kennebec."

AMF have very low partner specificity in plant species and plant variety (Cesaro *et al.*, 2008; Öpik *et al.*, 2009; Campos *et al.*, 2018). Plant species and variety can associate with a broad range of AMF species, and AMF species can also associate with a wide range of plants species and cultivars. Plants with a poor root system (i.e., shorter, less regular root hairs) to facilitate the uptake of nutrients in soils have traditionally been considered to have high levels of AMF colonisation. This is because their roots have a limited ability to absorb nutrients. Hence, they may benefit from fungal hyphae to increase the absorption of the nutrients, especially P. In terms of potato variety, there are few studies exploring host specificity of AMF to potato varieties. This study showed that in the Newcastle University study fields, the dominant genus in the potato roots was *Paraglomus*. This genus is perhaps preferential for potato in this field. Potato variety had no impact on AMF abundance and diversity. There are some reports that showed differences among potato varieties for the association of AMF, for example, Superior (Douds *et al.*, 2007), Bintje (Gallou *et al.*, 2011), Jyoti and TPS (Lone *et al.*, 2015), and, Charlotte, Nicola, and Bintje (Buysens *et al.*, 2017); however, these studies did not include the same varieties as this study.

### 3.6 Conclusion

Crop management practice affected the abundance and diversity of AMF in this study. The strongest results show that crop management practice with no mineral fertiliser and no conventional pesticides increased AMF colonisation; in contrast, the use of mineral fertiliser along with organic pesticides decreased AMF colonisation. Potato varieties in this study did not affect the abundance and diversity of AMF.

# CHAPTER 4. The impact of phosphorus on the abundance and diversity of arbuscular mycorrhizal fungi from a two-year (2017 and 2018) low-P potato trial

## 4.1 Introduction

Potatoes are considered to have inefficient phosphorus uptake due to their shallow root systems (Liu *et al.*, 2018). Since phosphorus (P) in the soils is often limited, sustainable methods to enhance P uptake are needed to preserve potato crop productivity. Wahid *et al.* (2019) and Peine *et al.* (2019) reported that P availability is most important to plant productivity and soil fertility after nitrogen. In agroecosystems, P demand is generally satisfied by fertilisation with highly soluble P; however, the sources of P are declining (Saia *et al.*, 2019). To maintain P availability for crop productivity, large amounts of P fertilisers are applied to P-deficient soils. However, frequent use of these mineral fertilisers can damage soil microbial communities (Geisseler and Scow, 2014). In sustainable agriculture, the use of beneficial soil microorganisms as bio-fertilisers to improve the nutrients supplied and enhance crop productivity is the alternative for reducing fertiliser inputs (Johansson *et al.*, 2004; Davies Jr *et al.*, 2005; Wahid *et al.*, 2016).

AMF are native to agricultural soils, and their associations are omnipresent in nature (Mei *et al.*, 2019). AMF generally provide host plants with access to soil resources, especially P (Frew, 2019); at the same time, carbohydrates from host plants are transferred to the AMF (Balestrini and Lumini, 2018). The symbiosis with AMF is known to increase the uptake of low availability P in the soils by plant roots. Positive effects of AMF colonisation on potatoes have been reported, for instance, increased P uptake (Balemi and Schenk, 2009; Roy-Bolduc and Hijri, 2011; Liu *et al.*, 2018), improved growth and yield (McArthur and Knowles, 1993; Duffy and Cassells, 2000; Ngakou *et al.*, 2006; Hijri, 2016), and enhanced protection of host plants against root pathogens (Sipahioglu *et al.*, 2009; Ismail *et al.*, 2013).

In terms of promoting AMF in agricultural fields, AMF can be managed using two main approaches. The first is through modifying the indigenous population of AMF by the addition of inoculum from selected AMF strains to the soil and the second is through adopting agricultural practices that promote indigenous AMF (Roy-Bolduc and Hijri, 2011). The major limitation of using AMF inoculum is their poor persistence in natural soils. This can be because of inappropriate host selection, unsuitable environmental conditions, and competition

with other native fungi (Setiadi, 2002; Cely *et al.*, 2016); also, the cost of inoculant production for field-scale application is high.

The diversity and function of AMF in agricultural fields are affected by differences in management approaches, particularly P inputs (Astover *et al.*, 2018). High P input from mineral fertiliser has a negative impact on AMF diversity and species richness; this condition causes a decrease in host plant resource allocation to AMF. On the other hand, the low P input promotes AMF colonisation because the host plant increases the allocated resource to AMF (Astover *et al.*, 2018). Root colonisation is significantly up-regulated when the soil Olsen-P is below a critical level (10 mg $kg^{-1}$) (Liu *et al.*, 2016). Many studies have reported high levels of AMF colonisation in potato roots in different potato varieties under low P inputs (Davies *et al.*, 2005; Davies Jr *et al.*, 2005; Douds *et al.*, 2007; Astover *et al.*, 2018).

Therefore, this study was designed to determine the effect of P availability (P inputs from mineral fertiliser) on AMF colonisation and their diversity in potato. As shown in Chapter 3, mineral NPK fertiliser negatively affected AMF abundance and diversity in a potato field experiment with a high P availability (P index=3). In contrast, potato variety did not show any differences in AMF abundance and diversity. As an expansion on that experiment, this chapter focused on the role of soil P availability, comparing AMF function in unfertilised plots with a low P index to plots where triple superphosphate was used to increase P availability in a wider range of varieties of potato. The experiment also explored the impacts of the treatments on agronomic parameters.

### 4.2 Aim and Objectives

The experiment was set up primarily to understand the interaction between P supply and potato variety. The overall aim of this study is to explore how P supply and potato variety interacted to affect AMF associations and how AMF impact the agronomic performance of the crop. Specific objectives were:

1. To investigate the effect of P availability on AMF abundance and the diversity of the native fungal community in potato roots from a two-year field experiment.

2. To determine the impact of potato variety on AMF abundance and the diversity of the native fungal community in potato roots from a two-year field experiment.

3. To investigate the interaction between P availability and potato variety on AMF abundance and the diversity of the native fungal community in potato roots from a two-year field experiment.

4. To explore the relationship between AMF colonisation and agronomic performance of potatoes, including tissue P concentration, total P uptake, tuber yield, and P use efficiency.

## 4.3 Materials and Methods

### *4.3.1 Experimental setup*

#### Potato germplasm multiplication trial

A potato germplasm multiplication trial was conducted in April 2016 at Nafferton farm before starting the two-year (2017 and 2018) low-P potato trials. Plots were planted with 300 potato varieties in two replicates under two management systems (organic crop protection with compost and conventional crop protection with NPK fertiliser). The primary objective of this trial was to screen these varieties to select a subset for the "low-P" potato trials in 2017 and 2018.

From the 2016 conventional trial results, eight potato varieties were selected from the top 20 highest yielding group (Cramond, Estima, Fortyfold, Lady Balfour, Pentland Crown, Shelagh, Sheriff, and Stamina); this group is the high yield potential group, and eight from the 20 lowest yielding varieties (Alpha, Anya, Darwina, Golden Wonder, Libertas, Markies, Romano and Sassy), this group is the low yield potential group for the low-P potato trials of 2017 and 2018.

Tubers for these varieties from the conventionally managed plots in the 2016 trial were selected to plant in the 2017 low-P trials. The tubers harvested from the low-P 2017 trial were saved for planting in the 2018 trials.

#### The "low-P" potato trials

The field experiments were conducted over two years (2017 and 2018) to identify the effect of P availability on AMF infection (hereafter referred to as low-P potato trials). Plots were selected from an experimental area that had previously been part of a fertility input x variety

trial. P indices in this subset of plots were 1 and 0 as of winter 2017 (see Table 4. 1 for initial soil conditions).

**Table 4. 1** The initial soil test results of all plots in this study.

| Plot number | Treatment | Replication | P (mg $L^{-1}$) | Index |
|---|---|---|---|---|
| 16 | with P | 1 | 12.40 | 1 |
| 17 | without P | 1 | 9.60 | 1 |
| 27 | with P | 2 | 8.80 | 0 |
| 28 | without P | 2 | 8.80 | 0 |
| 35 | with P | 3 | 9.20 | 1 |
| 31 | without P | 3 | 8.40 | 0 |

The "low-P" potato trials used six plots from the Quarry field trial (16, 17, 27, 28, 31, and 35), which represented low P plots in three replicates (Table 4. 1). Plots 16, 27, and 35 had previously received high applications of compost, so were selected as the “with P” plots. Plots 17, 28, and 31 had previously received low applications of compost and were selected as the “without P” plots. The original plot size was 24 m x 24 m. In 2017 half of the plot (12 m x 24 m) was used for potato planting while the other half was left as a grass/clover ley. In 2018 the grass/clover ley area was used for the potato trials. Each year, a subsection of the plot 4 x 24 m was used as the main plot (with/without P factor). Each main plot was divided into 16 variety sub-plots (1 x 6 m). In each sub-plot, 18 tubers of a single potato variety were planted with a spacing of 30 cm between tubers, and the rows were 100 cm apart. Buffer rows of Sarpo Mira, Cara, and Desiree were planted in the remaining areas of the potato half of the plot in each year. Figure 4. 1 shows an example of the layout in one main plot with buffer rows. Potatoes were planted on April 28$^{th}$ and harvested on October 9$^{th}$ in 2017 and planted on May 3$^{rd}$ and harvested on September 28$^{th}$ in 2018 (Table 4. 2). The total number of potato plots in each year of the study was 96 (16 potato varieties x 2 fertility treatments x 3 replicates).

All six main plots received N fertiliser at a rate of 180 kg N $ha^{-1}$ (529 kg ammonium nitrate $ha^{-1}$), representing a high rate of N to ensure no N deficiency in the plots. K was also applied to all plots at a rate of 330 kg $K_2O$ $ha^{-1}$ (550 kg KCl $ha^{-1}$) based on recommended rates from the

Fertiliser Manual (RB209) for a K index of 1 (AHDB, 2020). P was applied to the "with P" plots only amounting to 250 kg $P_2O_5$ $ha^{-1}$ (556 kg triple superphosphate $ha^{-1}$) to reflect recommendations for soils with a P index of 0 (AHDB, 2020).

According to Table 4. 2, mineral NPK was applied prior to potato planting. Fungicides used in this study were applied in the fields after root sampling for laboratory studies. In both years, Shirlan (300 mL $ha^{-1}$) and Mancozeb (1.7 kg $ha^{-1}$) were applied as fungicides. Reglone (3L $ha^{-1}$) was applied as herbicides. The dates for each pesticide application are shown in Table 4. 2.

**Table 4. 2** Field trial agronomic management in the "low-P" potato trials.

| 2017 | 2018 | Treatment | Details |
|---|---|---|---|
| 27 April | 27 April | All | Fertilised with mineral NK |
| 27 April | 27 April | with P | Fertilised with mineral P |
| 28 April | 3 May | All | Planting |
| 5 May | 21 May | All | Herbicide application Pendimethalin; Linuron |
| 21 May | 20 June | All | Herbicide application Laser |
| 27 July | 18 July | All | Root collecting for assessment |
| 9 August | 7 August | All | Fungicide application with Shirlan |
| 15 August | 10 August | All | Plant collecting for assessment |
| 17 August | 17 August | All | Fungicide application with Mancozeb |
| - | 10 September | All | Fungicide application with Shirlan |
| 17 August | 10 September | All | Crop desiccation with Reglone |
| 9 October | 28 September | All | Harvesting |

Table 4. 3 illustrates the conditions of air temperature, relative humidity, and total rainfall in the two-year "low-P" potato trials. The data are shown for each month during potato growth. Air temperature and relative humidity in each month of both years were not very different. In contrast, the total rainfall in each month was varied between years.

**Table 4. 3** The average monthly air temperature and relative humidity, and total monthly rainfall of two-year "low-P" potato trials.

| Month | Air Temperature (°C) | | Relative Humidity (RH) (%) | | Total rainfall (mm) | |
|---|---|---|---|---|---|---|
| | **2017** | **2018** | **2017** | **2018** | **2017** | **2018** |
| April | 7.95 | 8.11 | 84.04 | 89.65 | 14.80 | 67.60 |
| May | 11.90 | 11.99 | 85.51 | 84.32 | 19.80 | 31.00 |
| June | 14.43 | 12.83 | 85.68 | 75.89 | 127.20 | 38.60 |
| July | 14.42 | 16.94 | 87.17 | 80.21 | 68.40 | 25.20 |
| August | 14.66 | 15.29 | 83.74 | 86.80 | 31.60 | 108.60 |
| September | 12.35 | 12.38 | 91.71 | 94.43 | 84.40 | 53.00 |
| 1-10 October | 12.25 | 10.93 | 90.05 | 89.75 | 9.00 | 15.00 |

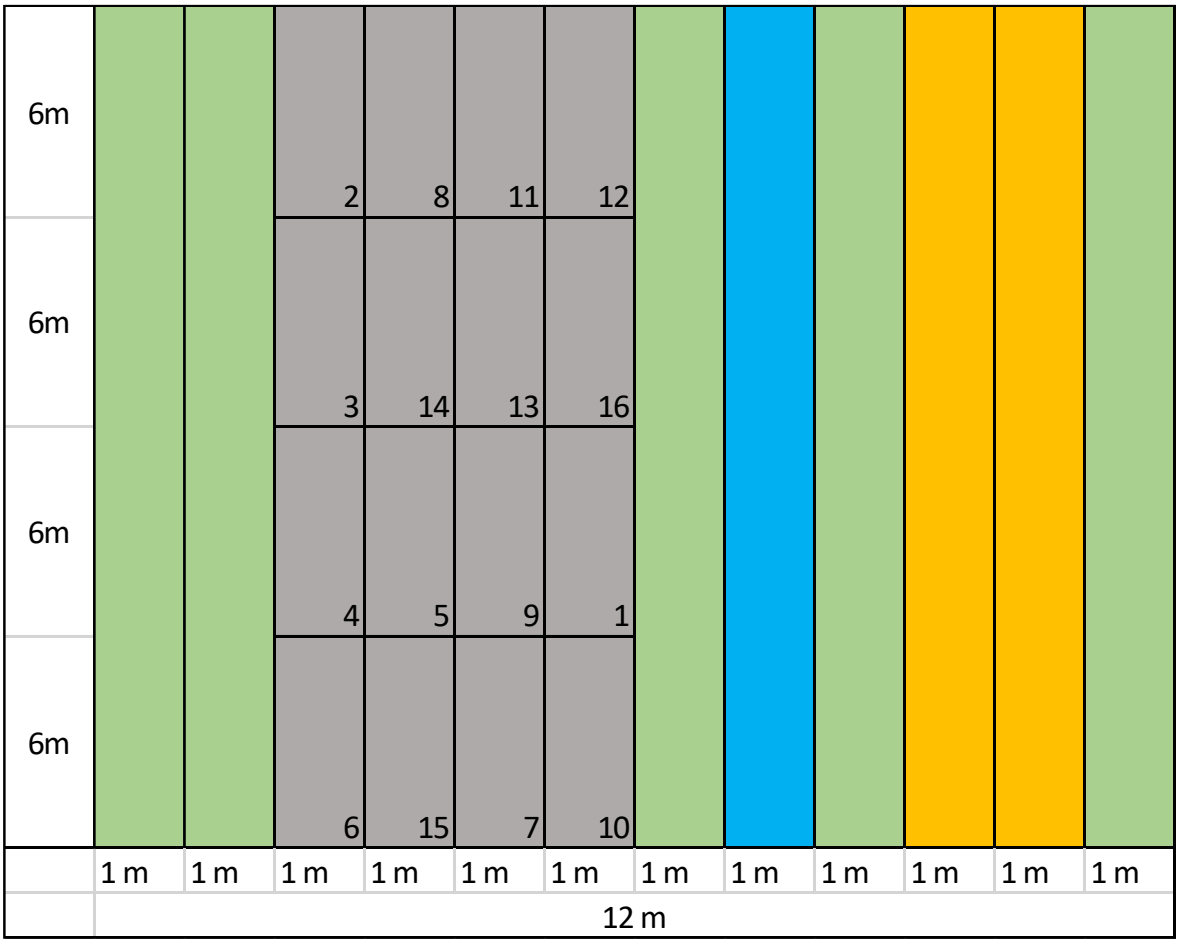



**Figure 4. 1** Diagram illustrating potato planting in a single plot of the Quarry field in 2017 and 2018. Numbers indicate the random assignment of 16 potato varieties to the sub-plots. Coloured strips represent potato in-fill with green-Sarpo Mira, blue-Cara, and yellow-Desiree.

### *4.3.2 Experimental planning for analysis*

Techniques used for potato root analysis are illustrated in Figure 4. 2. Potato roots from the fields were collected and analysed two achieve two objectives: assessment of AMF abundance and AMF diversity. Traditional root staining and qPCR were used to quantify the abundance of AMF in the roots, while Illumina MiSeq NGS was used to assess species diversity. Meanwhile, for agronomic analysis, potato plants and tubers were collected to study tissue P concentration, total P uptake, tuber yield, and P use efficiency at harvest.

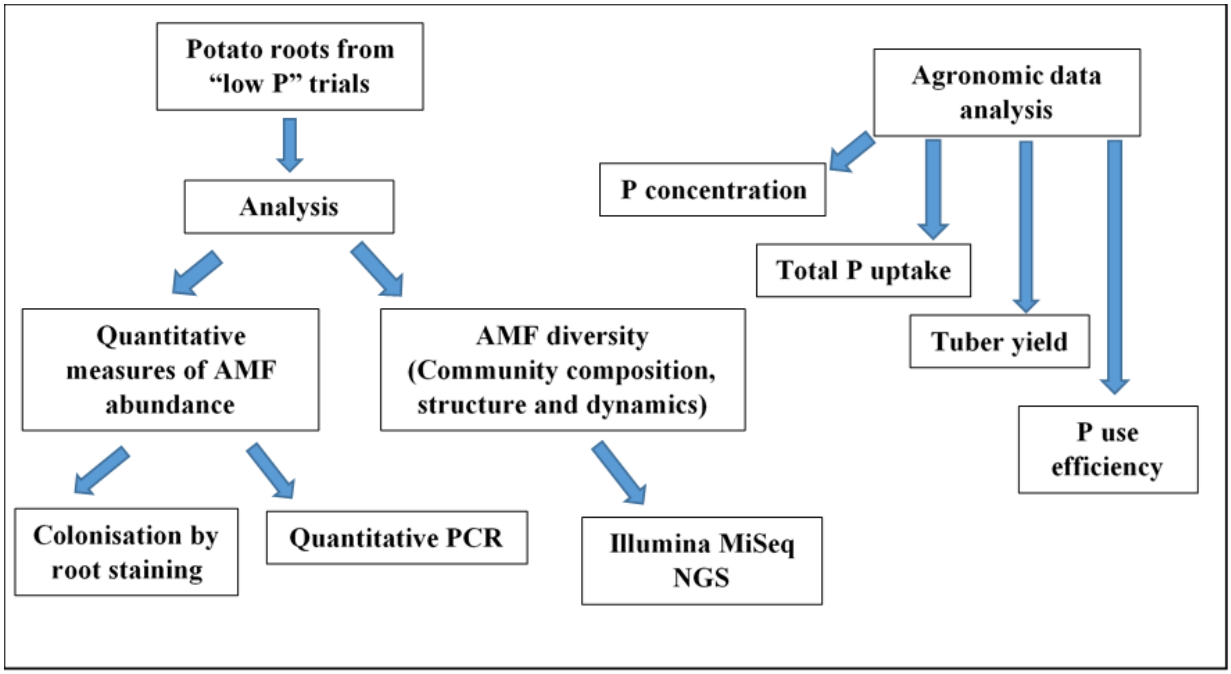



**Figure 4. 2** Techniques used in the low P potato trials.

### *4.3.3 Baseline soil sampling in each year*

Soil samples were collected from all six plots before potato planting each year (28/03/2017 and 25/04/2018). The aim was to assess the baseline native AMF community in the soils before experimental treatments were applied. Soils were taken using a soil core sampler from a depth of 10-20 cm with 10-12 samples per plot. The soil samples were then mixed and stored at -20$^{o}$C until required.

### *4.3.4 P concentration, total P uptake, tuber yield analysis, and P use efficiency*

**Potato plant and tuber sampling for P concentration, total P uptake, and P use efficiency analysis**

Potato plants were collected on the 15$^{th}$ of August in 2017 and 10$^{th}$ of August in 2018 (Table 4. 2) at the tuber filling stage (vegetative growth; 90-115 days after planting) (Obidiegwu *et al.*, 2015). The above-ground canopy of two plants selected randomly was sampled from each sub-plot (single variety), and fresh weight and dry weight were recorded for analysis. All collected samples were put in the oven at 70$^{o}$C for 4-5 days until dry.

Final tuber yields were obtained after plant desiccation by harvesting a full row of potatoes, and fresh weight (t ha$^{-1}$) was recorded as tuber yield. For calculating tuber P uptake, 5-6 tubers were randomly selected per sub-plot (single variety), washed and air-dried (Figure 4. 3A) and cut into small pieces using a potato chipper (Figure 4. 3B), then put in the oven at 70$^{o}$C for 4-5 days (Figure 4. 3C) until dry (Figure 4. 3D).

All dry plant and tuber samples were milled using a Retsch model SK 300 (Haan, Germany) milling machine to a size of 1 mm. Then, sub-samples (5 g) were sent to an external laboratory (SAC Commercial Ltd) for P analysis by nitric acid microwave digestion, with P determination by ICP spectrometry (Esslemont *et al.*, 2000).

**Calculations**

Where P was presented as the concentration (mg g$^{-1}$) of dry matter, calculations were carried out as follows:

$$P\ (mg\ g^{-1}) = P\ (\%) \times 10$$

Where P was presented as the P uptake (kg ha$^{-1}$) of dry matter, calculations were carried out as follows:

$$P\ uptake\ (kg\ ha^{-1}) = P\ (\%) \times 10 \times DM\ (kg\ ha^{-1})$$

Total P uptake (kg ha$^{-1}$) was calculated as the combined P uptake in the plant and tuber.

Where P was presented as P use efficiency. P use efficiency was calculated in terms of P efficiency ratio (Baligar *et al.*, 2001), calculations were carried out as follows:

$$P\ use\ efficiency = Tuber\ yield\ (kg)/P\ concentration\ in\ tuber\ (kg)$$


**Figure 4. 3** Tuber preparation for dry matter determination and P analysis.

### *4.3.5 Potato root sampling for root colonisation and molecular study*

Potato root samples were collected randomly from two plants in each sub-plot on the 27$^{th}$ of July in 2017 and 18$^{th}$ of July in 2018 (tuber filling stage, 90-115 days after planting) (Table 4. 2). Roots were cut and separated from the above-ground plant parts and were rinsed with tap water. Then, root samples from two plants in each sub-plot were cut into small pieces (2-3 inches) and mixed together. Root samples were then separated into two parts for a) root colonisation study and b) molecular analysis. Both sub-samples were frozen immediately in liquid nitrogen and stored at -20$^{o}$C until required (Padamsee *et al.*, 2016).

### *4.3.6 AMF colonisation by root staining (Ink and vinegar method)*

Root staining was carried out as detailed in section 3.3.4.

### *4.3.7 DNA extraction for all root samples and soil*

DNA was extracted from all freeze-dried and milled potato root samples (section 4.3.5) and soils (section 4.3.3) using the method described in section 3.3.5 (Method 3 FastDNA SPIN kit for soil, MP Biomedicals, 101 Inc., La Jolla, CA, USA) following the manufacturer's protocol.

### *4.3.8 Nucleic acid quantification and purity checked*

The quality of the DNA was checked as described in 3.3.5.

### *4.3.9 Quantification of AMF abundances using qPCR*

A subset of DNA samples from the 2017 low-P potato trial with low and high levels of AMF colonisation identified by root staining were chosen for analysis. In this chapter, four potato varieties; Fortyfold, Lady Balfour, Shelagh, and Sheriff (high colonisation) and four potato varieties; Alpha, Anya, Darwina, and Stamina (low colonisation) from both fertility treatments (with and without P) were selected for qPCR. qPCR was done as detailed in section 3.3.7. Briefly, DNA extracts from samples were diluted (1:10) prior to PCR amplification. SYBR Green-based qPCR assays with plasmid standards were used for AMF SSU gene fragment quantification. The primer pair AMG1F and AM1 (Salvioli et al., 2008; Hewins et al., 2015) were used for amplification. Plasmid standard dilutions were prepared to give standards containing $10^2$, $10^3$, $10^4$, and $10^5$ copies of the SSU rDNA gene target per µL. Negative controls containing water were used to check for potential contamination in the master mix. All samples, including standards and negative controls, were amplified in three replicate qPCR reactions using a Bio-RAD C1000 TouchTM Thermal Cycler. At the end of the runs, AMF SSU concentrations in the templates were carefully recorded and used for the final calculation.

### *4.3.10 AMF community and diversity analysis using Illumina MiSeq NGS*

AMF community and diversity analysis by NGS was conducted as detailed in section 3.3.10.

### *4.3.11 Statistical analysis in this chapter*

**General description of statistical analysis**

The data of P concentration, total P uptake, tuber yield, P use efficiency, and the percentages of AMF colonisation from all samples were subjected to ANOVA with "Year," "Two fertility treatments," and "Yield potential group of potato variety" as the main factors. The data were checked for normal distribution of the residuals using the qqnorm command; if the residuals were not normally distributed, then the data was transformed; log base 10 data transformation was employed. A linear mixed-effects (lme) model was used to produce ANOVA P-values for

main effects and all interactions with the nested structure of the split-plot design reflected in the random error term:block/plot/sub-plot (Pinheiro and Bates, 2000) using the nlme package in the R programming language (RCore, 2016). When ANOVAs indicated a significant effect ($p<0.05$), differences between individual means were analysed using Tukey's honestly significant difference (HSD) post hoc test in the multcomp package in R. A 95% significance level ($p<0.05$) was applied to discriminate the means. Where the interaction between factors were significant, Tukey's HSD was applied to compare the interaction means.

**Root colonisation data**

The percentages of AMF colonisation from all samples in each year (2017 and 2018) were also analysed with two factors, "Two fertility treatments" and "Potato variety." Then, subjected to ANOVA and following the general description of statistical analysis as described above.

**AMF abundance by qPCR**

The abundance of AMF (gene copy number) from all root samples were subjected to two-way ANOVA with "Two fertility treatments" and "Potato variety" as factors. Then, subjected to ANOVA and following the general description of statistical analysis as described above.

**Comparison of AMF abundance measured by root staining and qPCR using AMF-specific primers**

To compare the AMF abundance data determined with microscopic colonisation assessment by root staining or qPCR, a Pearson's product-moment correlation was performed. This analysis was carried out using the devtools package in the R programming language.

**AMF community and diversity analysis by NGS**

From the ASVs table, fungal taxonomic data were normalised as a proportion of the total number of sequences in the library prior to analysis (McGee *et al.*, 2017). The fractional abundance of individual taxa (phylum, family, and genus) within each community was calculated by dividing the number of sequences in different taxonomic groups (phylum, family, and genus) by the number of total sequences in the library obtained for that sample.

The composition of general fungi and AMF communities were analysed as described in the data analysis section 3.3.10 with "Year," "Two fertility treatments," and "Yield potential

group of potato variety" as factors. Diversity analysis and taxonomic composition analysis were also analysed, as described in the data analysis section 3.3.10.

## 4.4 Results

### *4.4.1 AMF colonisation by root staining*

**AMF colonisation in each year of the trial for all potato varieties**

Treatments had a significant effect on the percentage of AMF colonisation in both years of the trials (Table 4. 4). AMF colonisation in the without P treatment was higher than with P treatment in both years of the trials. In contrast to treatment, potato variety had no significant effect on AMF colonisation. There was no significant interaction between treatment and potato variety for AMF colonisation in both years of the trials.

From the 2017 low-P potato trial, four potato varieties; Fortyfold, Lady Balfour, Shelagh, and Sheriff (high colonisation group) and four potato varieties; Alpha, Anya, Darwina, and Stamina (low colonisation group) from both fertility treatments (with and without P) were selected for qPCR.

**AMF colonisation in each year of the trial for yield potential group of potato variety**

According to Table 4. 5, treatments had a significant effect on the percentage of AMF colonisation in both years of the trials. AMF colonisation in the without P treatment was higher than with P treatment in both years of the trials. In both years trials, the high yield potential group of potato variety had numerically higher AMF colonisation than the low yield potential group of potato variety; however, this effect was not statistically significant. There was no significant interaction between treatment and yield potential group of potato variety for AMF colonisation in both years of the trials.

**Table 4. 4** Effect of each treatment and potato variety on the percentage of AMF colonisation in each year trial by the root staining method (Main effect means±SE, ANOVA p-values, n=96).

| Factor | AMF colonisation (%) | |
|---|---|---|
| | **2017** | **2018** |
| **Treatment (TR)** | | |
| With P | 14.73±2.15 | 7.17+1.04 |
| Without P | 66.69±1.85 | 73.31+2.18 |
| **Potato variety (VA)** | | |
| Alpha | 39.67±11.70 | 35.50±14.30 |
| Anya | 35.83±12.52 | 35.17±13.12 |
| Cramond | 45.17±11.66 | 45.50±17.39 |
| Darwina | 33.00±13.76 | 37.67±15.47 |
| Estima | 37.67±12.33 | 38.17±14.52 |
| Fortyfold | 42.50±16.25 | 46.83±18.59 |
| Golden Wonder | 38.83±13.23 | 31.50±11.61 |
| Lady Balfour | 43.50±13.48 | 41.83±12.47 |
| Libertas | 41.00±13.10 | 39.50±13.00 |
| Markies | 38.67±10.21 | 45.67±18.70 |
| Pentland Crown | 36.67±13.39 | 41.67±14.47 |
| Romano | 40.83±13.30 | 32.83±13.12 |
| Sassy | 43.83±11.76 | 47.33±17.52 |
| Shelagh | 46.50±14.59 | 45.83±19.85 |
| Sheriff | 47.00±13.38 | 38.00±16.66 |
| Stamina | 40.37±12.84 | 40.83±14.49 |
| **ANOVA p-values** | | |
| TR | **< 0.0001** | **0.0051** |
| VA | 0.8010 | 0.1681 |
| TR x VA | 0.5691 | 0.0652 |

**Table 4. 5** Effect of each treatment and yield potential group of potato variety on the percentage of AMF colonisation in each year trial by the root staining method (Main effect means±SE, ANOVA p-values, n=96).

| Factor | AMF colonisation (%) | |
|---|---|---|
| | **2017** | **2018** |
| **Treatment (TR)** | | |
| With P | 14.73±2.15 | 7.17+1.04 |
| Without P | 66.69±1.85 | 73.31+2.18 |
| **Yield potential group of potato variety (YP)** | | |
| High yield group | 42.46±4.45 | 42.33±5.32 |
| Low yield group | 38.96±4.10 | 38.15±4.89 |
| **ANOVA p-values** | | |
| TR | **0.0248** | **0.0051** |
| YP | 0.1313 | 0.0618 |
| TR x YP | 0.4814 | 0.1759 |

As the results in Table 4. 4 showed that the potato variety had no significant effect on AMF colonisation; however, there was a pattern of high yielding varieties being different from the low yielding ones (Table 4. 5). Then, the "yield potential group" was selected as an experimental factor rather than variety for the root colonisation and agronomic data analysis.

**AMF colonisation by "yield potential group" of potato variety**

**Effects on AMF colonisation**

As highlighted in Table 4. 6, AMF colonisation was significantly affected by both treatment and yield potential group of potato variety, but not the experimental year. The AMF colonisation in the treatment without P was significantly higher than the treatment with P, and the high yield potential group of potato variety had significantly higher AMF colonisation than the low yield potential group of potato variety. AMF colonisation in both years was equal at around 40% of colonisation. There was a significant year by treatment interaction for AMF colonisation.

**Identification of the dominant AMF structure in the roots assessed by the root staining method**

Treatment had a significant effect on the colonisation percentages of all three AMF structures observed in potato roots (Table 4. 6). Treatments without P showed significantly higher hyphae, arbuscule, and vesicle levels than the treatment with P.

In the year 2018, there were significantly more arbuscules than in the year 2017. The high yield potential group of potato variety had significantly higher colonisation percentages for both hyphae and arbuscules than the low yield potential group of potato variety. There was a significant interaction between year and treatment for the colonisation percentages of both hyphae and arbuscules (Table 4. 6).

The year by treatment interaction was significant for the percentage of colonisation, hyphae, and arbuscule (Figure 4. 4). For all structures, the without P treatments were significantly higher than the with P treatments in both years. For the with P treatments, colonisation and hyphae were significantly higher in 2017 compared to 2018, while for the without P treatments, colonisation and hyphae were higher in 2018 compared to 2017. For the without P treatment, there were significantly more arbuscules in 2018 compared to 2017, while for the with P treatments, there were no significant differences in numbers of arbuscules between the years.

**Table 4. 6** Effect of each year, treatment, and yield potential group of potato variety on the percentage of AMF colonisation and each AMF structure observed in the roots by the root staining method (Main effect means±SE, ANOVA p-values, n=192).

| Factor | AMF colonisation (%) | AMF structure (%) | | |
|---|---|---|---|---|
| | | Hyphae | Arbuscule | Vesicle |
| **Year (YR)** | | | | |
| 2017 | 40.71±3.02 | 40.71±3.02 | 29.33±2.42 | 3.95±0.53 |
| 2018 | 40.24±3.60 | 40.24±3.60 | 34.86±3.17 | 3.98±0.75 |
| **Treatment (TR)** | | | | |
| With P | 10.95±1.25 | 10.95±1.25 | 7.08±0.73 | 1.03±0.19 |
| Without P | 70.00±1.46 | 70.00±1.46 | 57.11±1.53 | 6.90±0.79 |
| **Yield potential group of potato variety (YP)** | | | | |
| High yield group | 42.40±3.45 | 42.40±3.45 | 34.42±3.00 | 4.36±0.69 |
| Low yield group | 38.55±3.17 | 38.55±3.17 | 29.78±2.63 | 3.56±0.59 |
| **ANOVA p-values** | | | | |
| **Main effects** | | | | |
| YR | 0.7986 | 0.7986 | **0.0004** | 0.9696 |
| TR | **0.0020** | **0.0020** | **0.0009** | **0.0189** |
| YP | **0.0386** | **0.0386** | **0.0029** | 0.3297 |
| **Interaction** | | | | |
| YR x TR | **0.0002** | **0.0002** | **<0.0001** | 0.3686 |
| YR x YP | 0.8515 | 0.8515 | 0.6268 | 0.8095 |
| TR x YP | 0.7040 | 0.7040 | 0.0719 | 0.6208 |
| YR x TR x YP | 0.2079 | 0.2079 | 0.6962 | 0.8292 |

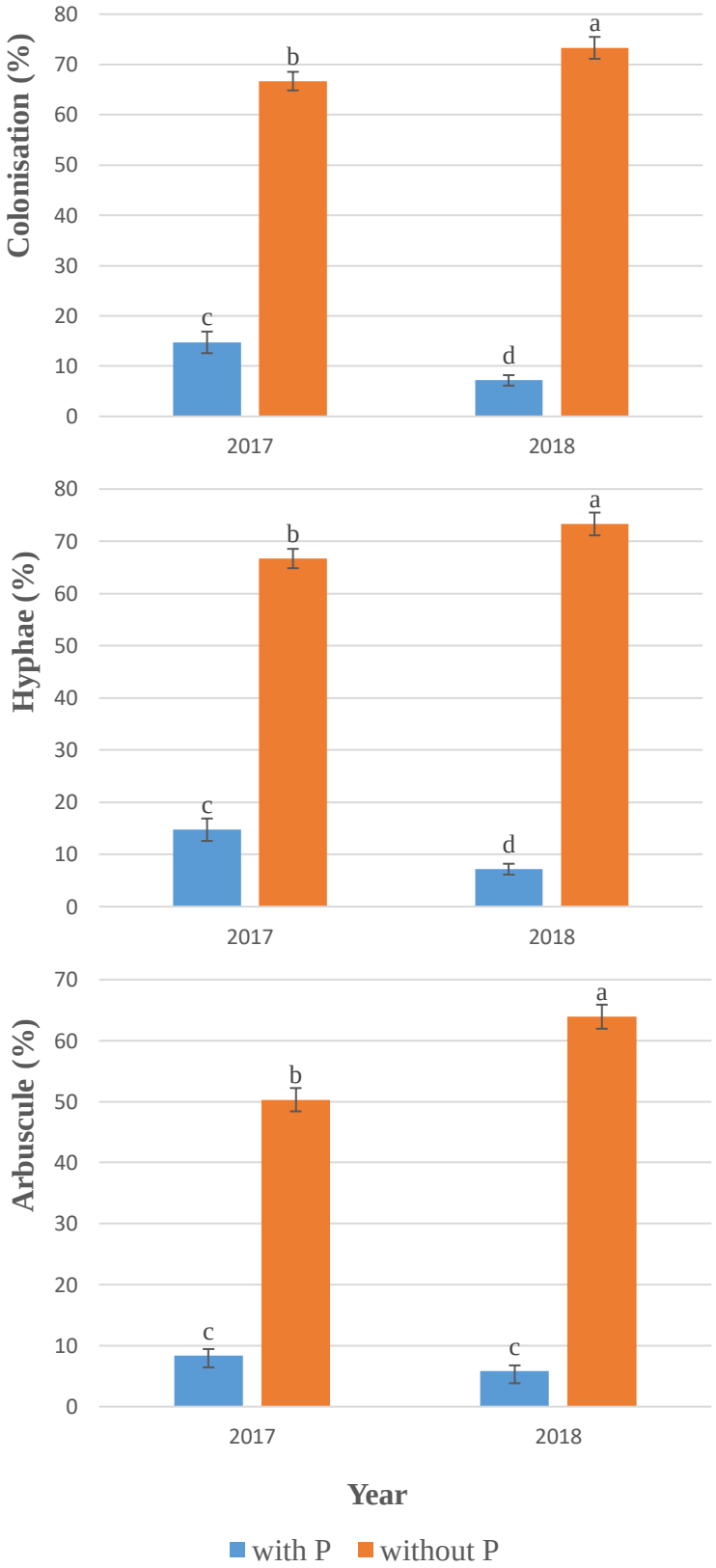



**Figure 4. 4** Effect of treatment (with P and without P) in 2017 and 2018 on the percentage of AMF colonisation, hyphae, and arbuscule. Bars labelled with the same letter are not significantly different (Tukey's honestly significant difference test, $p<0.05$). Standard errors of the mean (error bars) were calculated from all three field plot replicates.

### *4.4.2 Quantification of AMF in roots using qPCR*

**Standard curve and amplification curve generation**

A qPCR technique was used successfully for the assessment of AMF quantity in all DNA samples using AMF-specific primers AMG1F and AM1. According to Figure 4. 5, the graph showed a linear relationship between Cq values (Quantification cycle) and the log of the starting quantity for standards ($R^2$= 0.991). The unknowns that are plotted on the graph shows individual samples. The DNA concentrations of all samples generally plotted between $10^2$ to $10^5$ gene copies per µL on the standard curve.

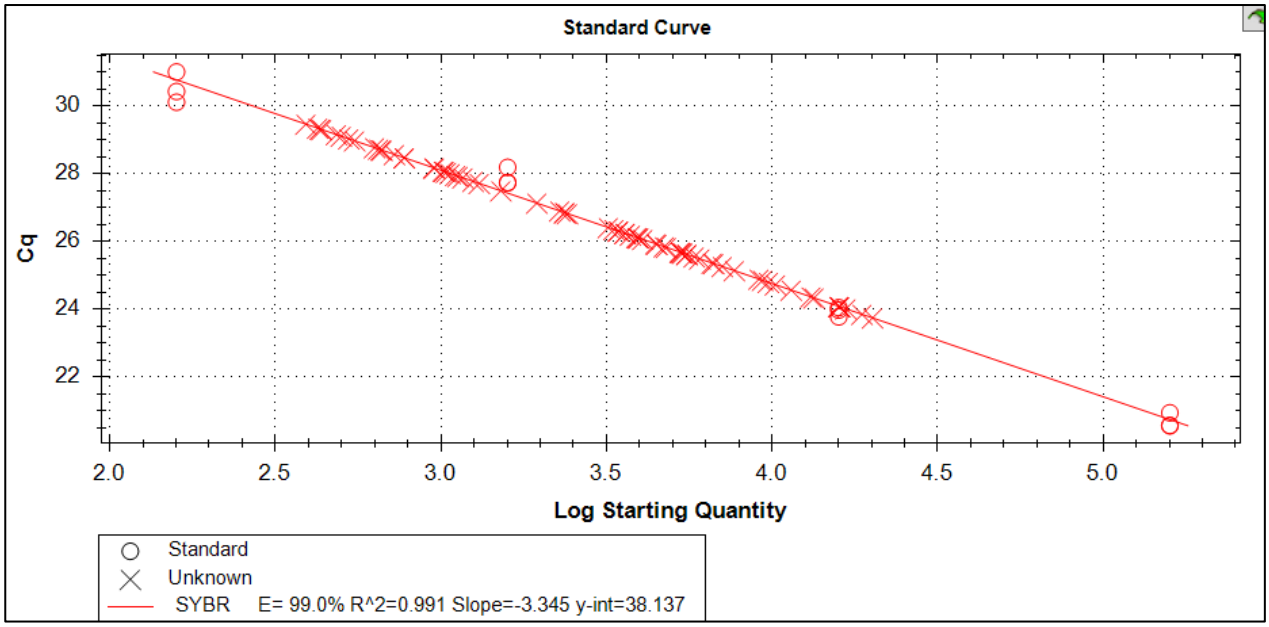
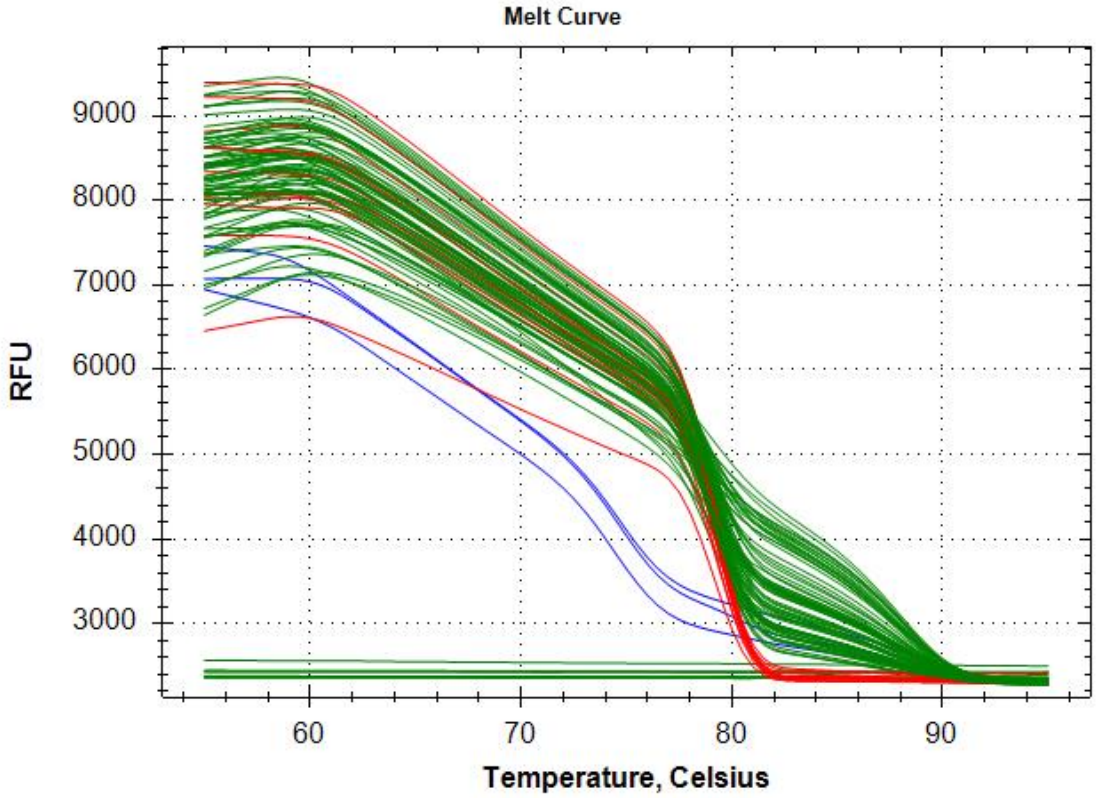



**Figure 4. 5** Standard curve obtained by plotting Cq value (Quantification cycle) against log starting quantity (genomic DNA concentration). Individual samples included in the study are indicated as "unknown" in the figure legend. E=amplification efficiency.

According to Figure 4. 6, all samples were successfully amplified. The melting curve and the amplification curve for the known standards, negative control, and samples are shown in Figure 4. 6A and Figure 4. 6B.

**A**




**B**

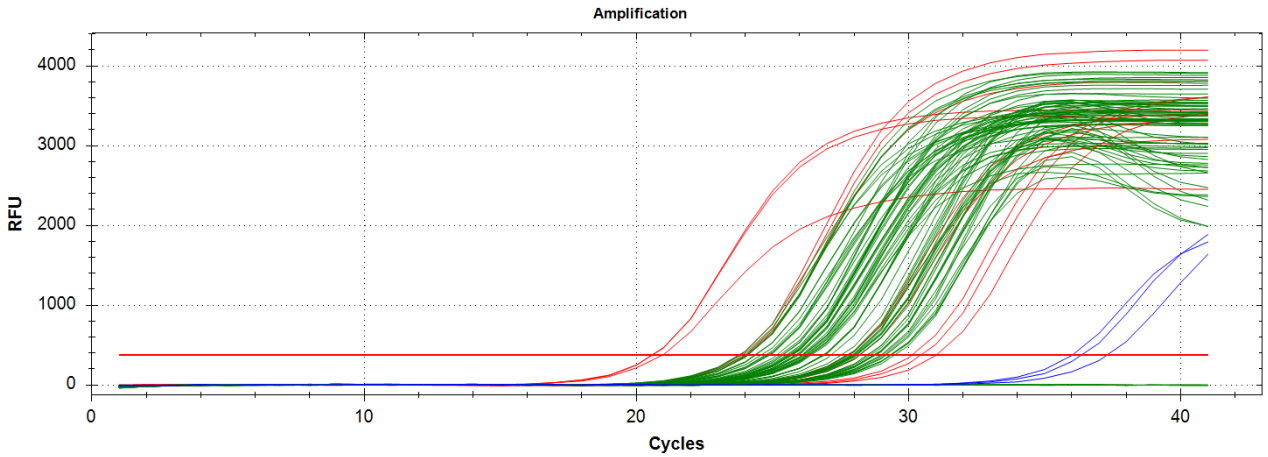



**Figure 4. 6** qPCR melting curve (A) and amplification curve (B) obtained by plotting temperature ($^{o}$C) and cycles against relative fluorescence units (RFU). The red lines show standards, green lines show samples, and blue lines show the negative control.

**AMF gene abundance**

AMF gene abundance results are presented in terms of gene copy number per unit mass of roots in each sample. Analysis of variance, as highlighted in Table 4. 7 shows that the main effects of treatment and potato variety were not significantly different for copy number of AMF and that there was no interaction between treatment and potato variety for gene copy number. However, Figure 4. 7 shows that gene copy numbers in the treatment without P were consistently numerically higher than the with P treatment for all eight potato varieties, with the highest gene copy number in both treatments for Alpha and the lowest gene copy numbers for Anya.

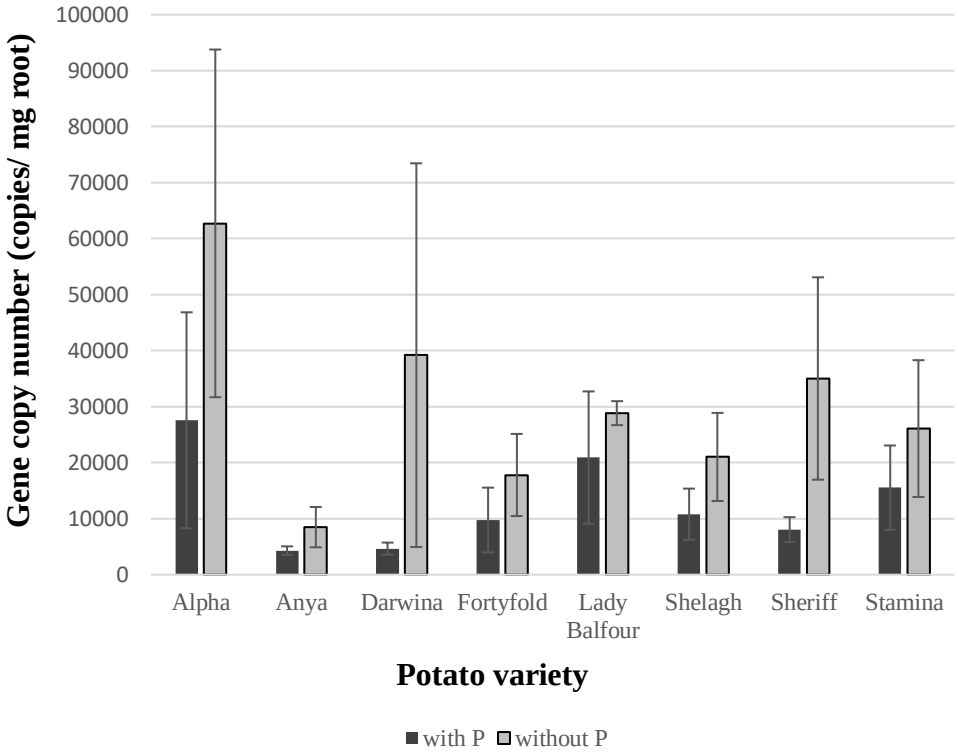



**Figure 4. 7** AMF gene copy number (gene copies per mg of the freeze-dried root) in two treatments (with P and without P) for eight potato varieties in 2017 trial. Values represent the interaction means, n=48. Error bars±SE.

**Table 4. 7** Effect of each treatment and potato variety on AMF gene copy number (gene copies per mg of the freeze-dried root) in 2017 trial (Main effect means±SE, ANOVA p-values, n=48).

| Factor | Gene copy number (copies/mg root) |
|---|---|
| **Treatment (TR)** | |
| With P | 12699.22±3060.64 |
| Without P | 29899.84±6334.36 |
| **Potato variety (VA)** | |
| Alpha | 45151.13±18134.78 |
| Anya | 6391.97±1894.49 |
| Darwina | 21923.69±17166.59 |
| Fortyfold | 13783.55±4546.79 |
| Lady Balfour | 24880.28±5652.14 |
| Shelagh | 15910.62±4665.40 |
| Sheriff | 21539.50±10134.28 |
| Stamina | 20815.49±6834.99 |
| **ANOVA p-values** | |
| TR | 0.1636 |
| VA | 0.1795 |
| TR:VA | 0.8161 |

### *4.4.3 Comparison of root colonisation by root staining with qPCR*

The correlation between the AMF colonisation and gene copy number is presented in Figure 4. 8. There was no significant correlation between AMF colonisation and gene copy number (r=0.17; p=0.25).

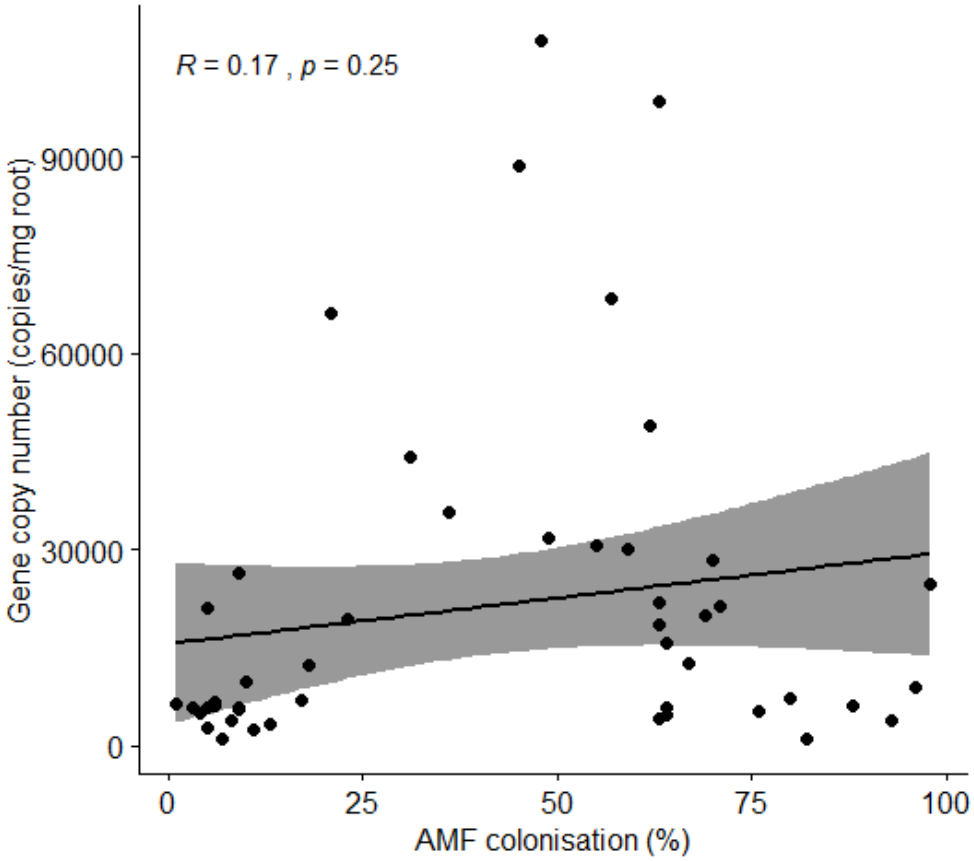



**Figure 4. 8** The percentage of AMF colonisation plotted against the AMF gene copy number for each root sample (a subset of 2017 samples). (Pearson's product-moment correlation, r=0.17; p=0.25, n=48).

### *4.4.4 AMF community analysis in the baseline soil before the experiment started*

According to fractional abundance results in Figure 4. 9, *Paraglomus* has the highest fractional abundance in both years compared with other AMF genera.

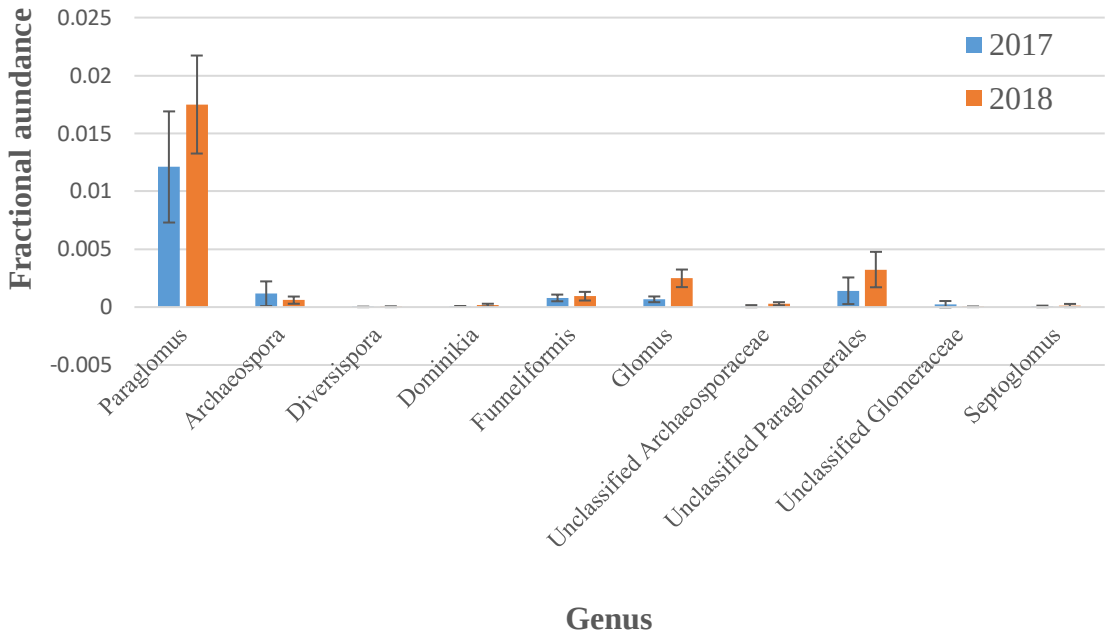



**Figure 4. 9** The fractional abundance of each genus in the Phylum Glomeromycota in each year for all baseline soil samples (expressed as a fraction of the total sequences within the Kingdom Fungi). Values represent the means of all samples, n=12. Error bars±SE.

Taxa bar plots of the relative abundances of AMF genera-related sequences identified in amplicon libraries for phylum Glomeromycota obtained from the baseline soil samples in each P plot in the year 2017 and 2018 are shown in Figure 4. 10.

In 2017, the taxonomic diversity analysis shows that the main AMF genus present in most plots is *Paraglomus* (blue taxa bars). Only one plot (No. 28) has a different main genus: *Archaeospora* (yellow taxa bars) in a treatment without P (noting that P treatments had not been applied at the time of baseline soil sampling). In addition to the *Paraglomus* and *Archaeospora*, various other genera in this phylum (*Funneliformis*, *Glomus*, and *Septoglomus*) were found in different proportions in each plot.

In 2018, the taxonomic diversity results show that the main genus is *Paraglomus*. Furthermore, *Archaeospora*, *Funneliformis*, *Glomus*, and *Septoglomus* were also found in each plot.

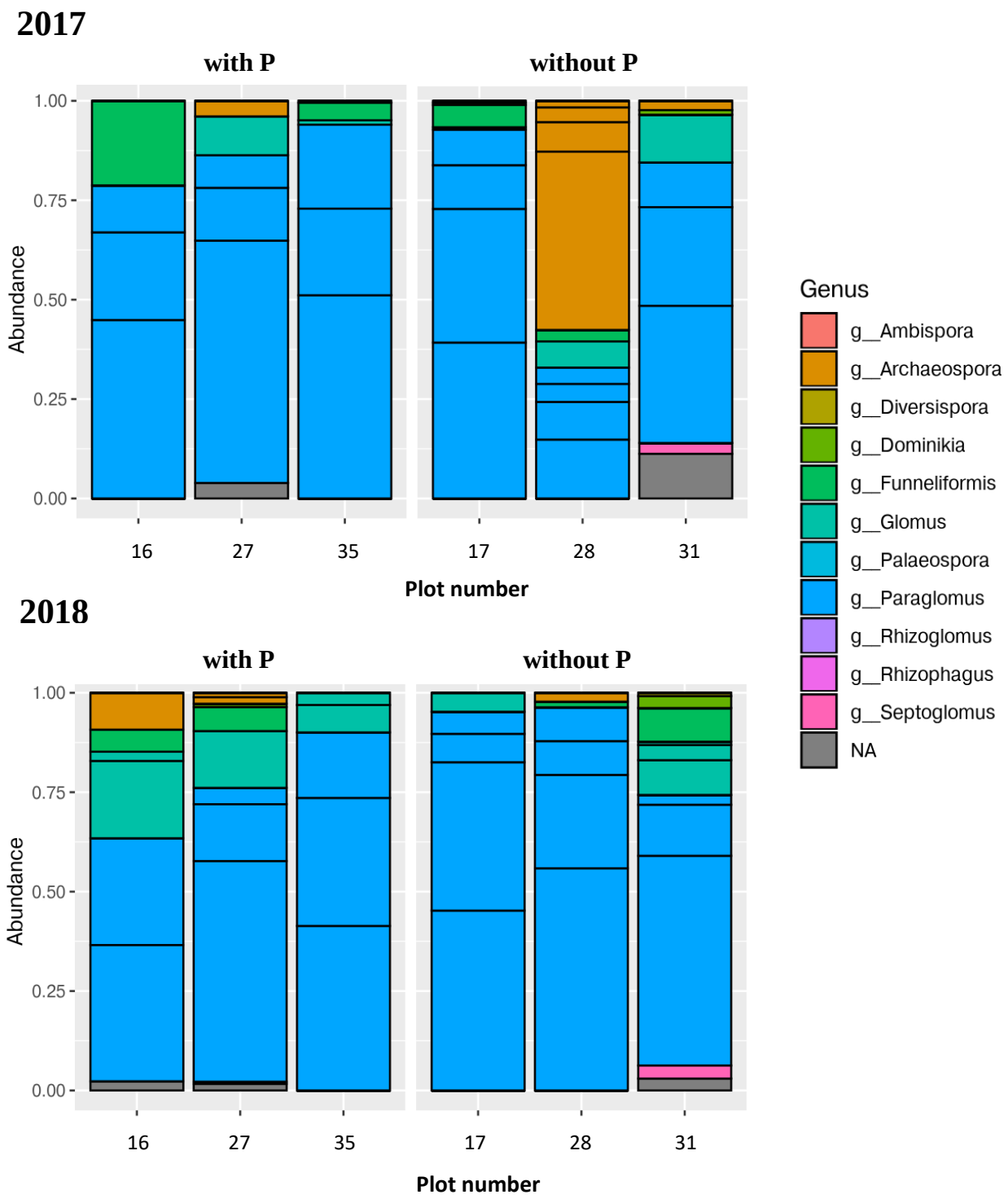



**Figure 4. 10** Taxa bar plots of the relative abundances of AMF genera-related sequences identified in amplicon libraries obtained from baseline soil samples before the experiment started in each plot of the two-year low P trials. The plots are separated in two treatments with P and without P, although samples were taken before P application. Soil samples in each plot plotted against the relative abundance.

### *4.4.5 AMF diversity and community analysis in potato roots*

General fungal sequences, including AMF, were found in all samples from both treatments in the 2017 low-P potato trial (Table 4. 8). General fungal sequences were also found in all samples from the without P treatment in the 2018 low-P potato trial, whereas seven samples from the with P treatment did not contain AMF sequences, leaving 41 samples from 48 samples with AMF sequences detected.

**Table 4. 8** The number of samples in each treatment of the two-year low P trials for NGS analysis.

| Year | Treatment | Number of samples sending to NGS analysis | Number of samples found general fungal sequences | Number of samples found AMF sequences |
|---|---|---|---|---|
| 2017 | With P | 48 | 48 | 48 |
| | Without P | 48 | 48 | 48 |
| 2018 | With P | 48 | 48 | 41 |
| | Without P | 48 | 48 | 48 |

**General composition of the fungal community**

In the year 2017, a total of 1,783,055 sequences and 3,391 ASVs were obtained using ITS primers (Table 4. 9). These were comprised of eight phyla (Ascomycota, Basidiomycota, Glomeromycota, Mortierellomycota, Rozellomycota, Olpidiomycota, Chytridiomycota, and Mucoromycota) and 1 group of unclassified fungi. Phylum Ascomycota was the main fungi present with 1,507 ASVs and 1,035,076 sequences, whereas there were 680 ASVs of phylum Glomeromycota (AMF) with 125,806 sequences. The percentage of ASVs in each phylum is presented in Figure 4. 11. The highest percentage of ASVs was in the phylum Ascomycota at 44.44%, while the phylum Glomeromycota (AMF) had 20.05%.

In the year 2018, a total of 939,940 sequences and 1,985 ASVs were obtained (Table 4. 9). These were comprised of 7 phyla (Ascomycota, Basidiomycota, Glomeromycota, Mortierellomycota, Rozellomycota, Olpidiomycota, and Mucoromycota) and 1 group of unclassified fungi. Phylum Ascomycota is the main fungi with 959 ASVs and 495,060 sequences, whereas there were 256 ASVs of phylum Glomeromycota (AMF) with 11,276

sequences. The highest percentage of ASVs was in the phylum Ascomycota at 48.31%, while the phylum Glomeromycota (AMF) represented 12.9% (Figure 4. 11).

**Table 4. 9** The number of ASVs and sequence library abundance in each phylum of Kingdom Fungi of the two-year low P trials.

| | **Number of ASVs** | | **Sequence library abundance** | |
|---|---|---|---|---|
| **Phylum** | **2017** | **2018** | **2017** | **2018** |
| 1. Ascomycota | 1507 | 959 | 1035076 | 495060 |
| 2. Basidiomycota | 838 | 520 | 183073 | 119270 |
| 3. Glomeromycota | 680 | 256 | 125806 | 11276 |
| 4. Unclassified fungi | 193 | 99 | 7131 | 1685 |
| 5. Mortierellomycota | 85 | 75 | 401578 | 307608 |
| 6. Rozellomycota | 42 | 32 | 3122 | 309 |
| 7. Olpidiomycota | 43 | 43 | 27246 | 4688 |
| 8. Chytridiomycota | 1 | 0 | 2 | 0 |
| 9. Mucoromycota | 2 | 1 | 21 | 44 |
| **Total** | **3391** | **1985** | **1783055** | **939940** |

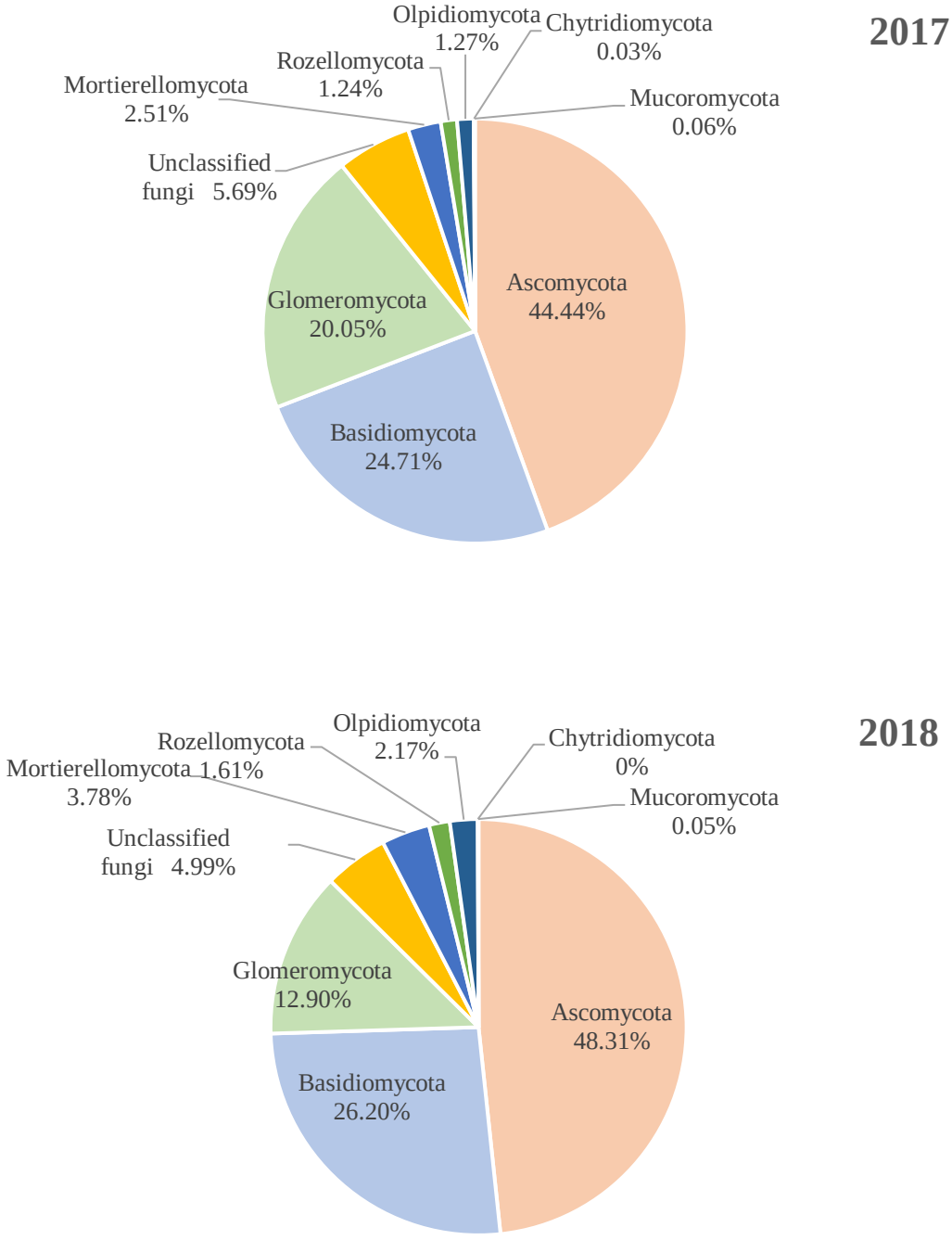



**Figure 4. 11** The percentage of ASVs in each phylum of Kingdom Fungi found in potato roots from two treatments (with P and without P) in this study.

The fractional abundance of the two-year low P potato trials (Figure 4. 12) showed that the highest fractional abundance was in the phylum Ascomycota, whereas the fractional abundance of phylum Glomeromycota was less than 0.1.

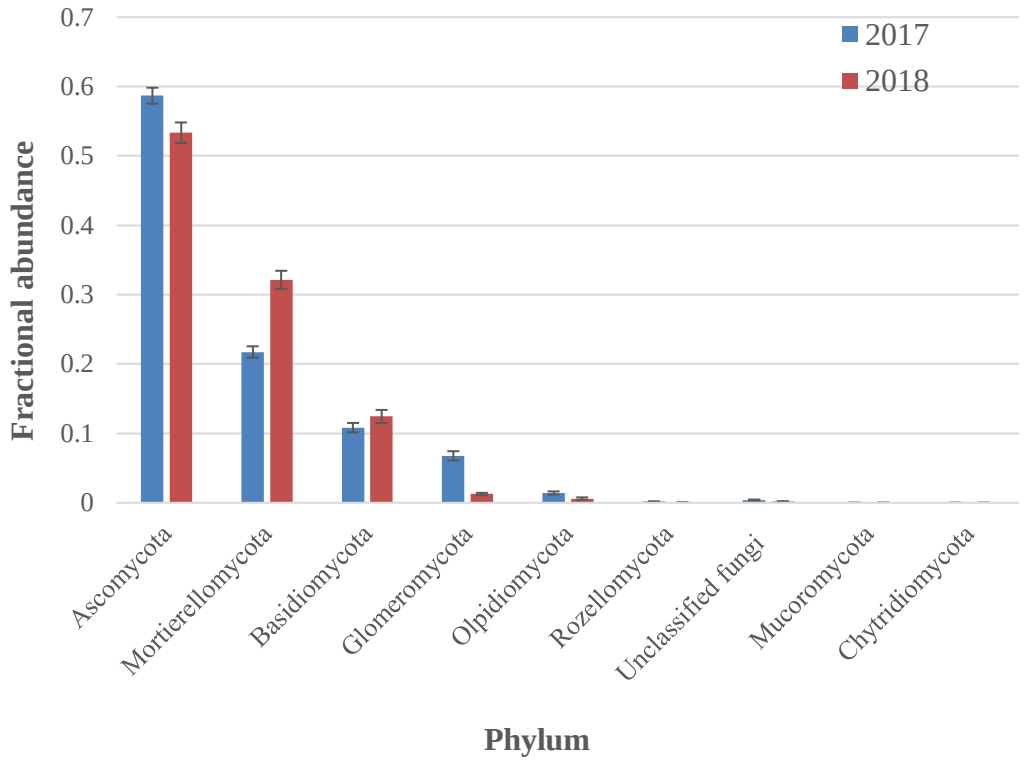



**Figure 4. 12** The composition of the fungal community in each year of the experiment (expressed as a fraction of the total sequences within the Kingdom Fungi) in potato roots. Values represent the means of all samples n=192. Error bars±SE.

**General composition of the AMF community**

The AMF community (phylum Glomeromycota) in the potato roots was comprised of 5 families (Paraglomeraceae, Glomeraceae, Archaeosporaceae, Ambisporaceae, and Diversisporaceae) and nine genera (*Paraglomus*, *Glomus*, *Rhizophagus*, *Funneliformis*, *Septoglomus*, *Dominikia*, *Archaeospora*, *Ambispora*, and *Diversispora*), as well as unclassified Glomeraceae, unclassified Archaeosporaceae, unclassified Paraglomerales and unclassified Glomeromycota (Table 4. 10). In the year 2017, the highest number of ASVs was in the genus *Archaeospora* (188 ASVs), while *Paraglomus* had the highest number of ASVs in 2018 with 88 ASVs.

The highest sequence library abundance in the year 2017 was Glomeraceae (47,893 sequences), whereas Paraglomeraceae had the highest sequence library abundance in the year 2018 with 7,889 sequences.

**Table 4. 10** The number of ASVs and sequence library abundance in each family and genus of phylum Glomeromycota in the two-year low P potato trials.

| Family | Genus | Number of ASVs | | Total number of ASVs | | Sequence library abundance | |
|---|---|---|---|---|---|---|---|
| | | **2017** | **2018** | **2017** | **2018** | **2017** | **2018** |
| Paraglomeraceae | *Paraglomus* | 170 | 88 | 170 | 88 | 43282 | 7889 |
| Glomeraceae | *Glomus* | 85 | 30 | 220 | 103 | 47893 | 1980 |
| | *Rhizophagus* | 30 | 26 | | | | |
| | *Funneliformis* | 50 | 32 | | | | |
| | *Septoglomus* | 7 | 3 | | | | |
| | *Dominikia* | 4 | 5 | | | | |
| | Unclassified Glomeraceae | 44 | 7 | | | | |
| Archaeosporaceae | *Archaeospora* | 188 | 28 | 218 | 34 | 30099 | 346 |
| | Unclassified Archaeosporaceae | 30 | 6 | | | | |
| Ambisporaceae | *Ambispora* | 15 | 3 | 15 | 3 | 236 | 9 |
| Diversisporaceae | *Diversispora* | 18 | 11 | 18 | 11 | 347 | 98 |
| Unclassified Paraglomerales | | 25 | 17 | 25 | 17 | 3393 | 954 |
| Unclassified Glomeromycota | | 14 | 0 | 14 | 0 | 556 | 0 |
| **Total** | | **680** | **256** | **680** | **256** | **125806** | **11276** |

According to fractional abundance values shown in Figure 4. 13, *Paraglomus* had the highest fractional abundance in both years of the low P trial compared with other AMF genera.

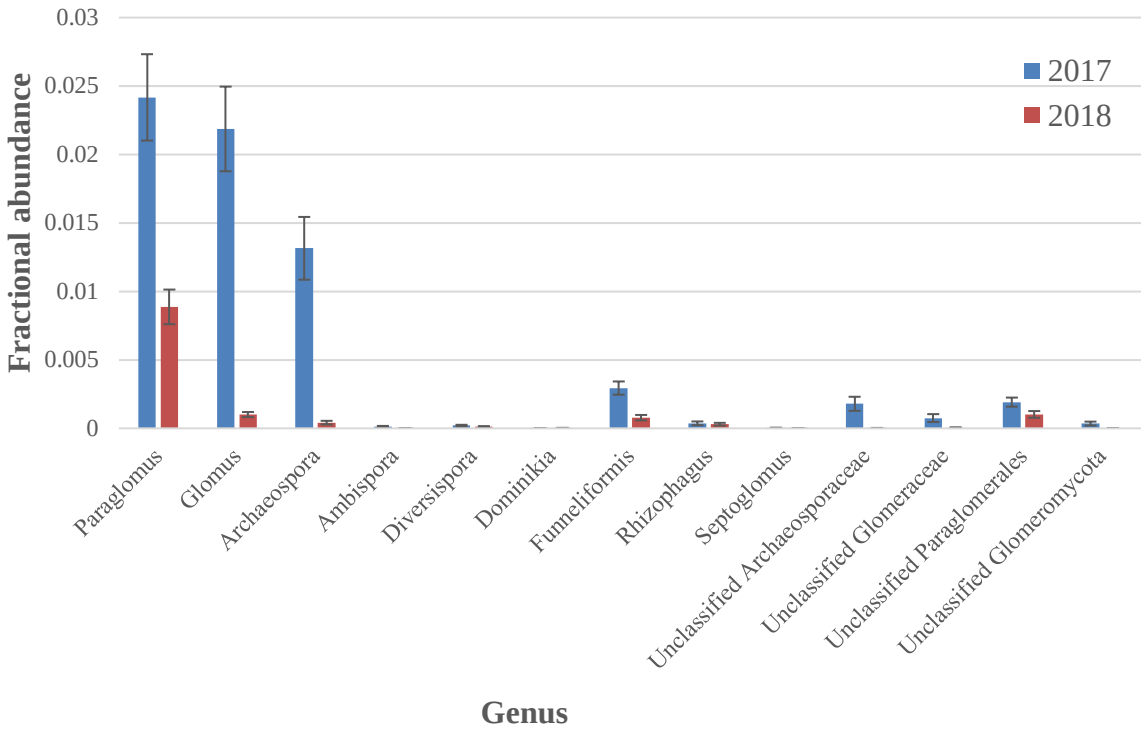



**Figure 4. 13** The distribution of genera in the Phylum Glomeromycota in each year for all potato root samples (expressed as a fraction of the total sequences within the Kingdom Fungi). Values represent the means of all samples, n=192. Error bars±SE.

**Effects of experimental factors on the AMF community**

The fractional abundance of the AMF genera *Paraglomus*, *Glomus*, *Archaeospora*, *Ambispora*, *Funneliformis*, and the unclassified group were significantly higher in 2017 compared to 2018 (Table 4. 11). In general, the fractional abundance of AMF genera in the year 2017 was higher than the year 2018, except for the genus *Dominikia*. *Paraglomus* had the highest fractional abundance in both years.

Treatments without P had significantly higher fractional abundances than those with P for two AMF genera: *Paraglomus* and the unclassified group. The fractional abundance in the without P treatment was numerically higher than the with P treatment in all AMF genera, with the exception of the genus *Ambispora*.

The yield potential group of potato variety significantly affected the fractional abundance in only genus *Funneliformis*. The fractional abundance of *Funneliformis* in the low yield group of potato variety was significantly higher than the high yield group.

The year by treatment interaction was significant for the fractional abundance of *Archaeospora*, *Ambispora,* and the unclassified group (Table 4. 11; Figure 4. 14). For the fractional abundance of *Archaeospora* and the unclassified group, the without P treatments were significantly higher than the with P treatments in the year 2017. In contrast, there were no significant differences between treatments in the year 2018. The fractional abundance of *Ambispora* in the with P treatment was significantly higher than the without P treatment in the year 2017, while there were no significant differences between treatments in the year 2018.

The fractional abundance of *Ambispora* in the with P treatment was significantly higher in 2017 compared to 2018, while there were no significant differences in the without P treatment between the two years. The fractional abundance of the unclassified group in the without P treatment was significantly higher in 2017 compared to 2018, while there were no significant differences in the with P treatment between the two years.

**Table 4. 11** Effect of each year, treatment, and yield potential group of potato variety on the fractional abundance of each AMF genus (expressed as a fraction of the total sequences within the Kingdom Fungi) (Main effect means$x10^{-4}$±SE$x10^{-4}$, ANOVA p-values, n=192).

| Factor | Fractional abundance | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ***Paraglomus*** | ***Glomus*** | ***Archaeospora*** | ***Ambispora*** | ***Diversispora*** | ***Dominikia*** | ***Funneliformis*** | ***Rhizophagus*** | ***Septoglomus*** | **Unclassified Group** |
| **Year (YR)** | | | | | | | | | | |
| 2017 | 242±32 | 219±31 | 131±23 | 1.33±0.44 | 2±0.6 | 0.09±0.05 | 29±5 | 4±1 | 0.34±0.14 | 48±8 |
| 2018 | 89±13 | 10±2 | 4±1 | 0.08±0.05 | 1±0.4 | 0.24±0.13 | 8±2 | 3±1 | 0.10±0.06 | 11±2 |
| **Treatment (TR)** | | | | | | | | | | |
| With P | 79±10 | 78±20 | 32±7 | 1.01±0.40 | 1±0.6 | 0.13±0.08 | 18±4 | 0.6±0.2 | 0.11±0.06 | 10±3 |
| Without P | 251±31 | 146±27 | 101±23 | 0.37±0.17 | 2±0.4 | 0.21±0.11 | 19±4 | 6.1±1.6 | 0.33±0.14 | 48±8 |
| **Yield potential group of potato variety (YP)** | | | | | | | | | | |
| High yield group | 164±26 | 104±20 | 83±22 | 0.44±0.15 | 0.8±0.3 | 0.06±0.04 | 11±2 | 3±1 | 0.27±0.10 | 30±7 |
| Low yield group | 163±24 | 120±27 | 50±11 | 0.93±0.41 | 2.3±0.7 | 0.28±0.13 | 25±5 | 4±1 | 0.17±0.11 | 28±5 |
| **ANOVA p-values** | | | | | | | | | | |
| YR | **<0.0001** | **<0.0001** | **<0.0001** | **0.0001** | 0.2151 | 0.5199 | **<0.0001** | 0.9559 | 0.1132 | **<0.0001** |
| TR | **0.0370** | 0.1107 | 0.2165 | 0.1722 | 0.4136 | 0.7189 | 0.7061 | 0.1738 | 0.4299 | **0.0457** |
| YP | 0.7950 | 0.8721 | 0.0775 | 0.4182 | 0.1176 | 0.1186 | **0.0234** | 0.4540 | 0.2328 | 0.4689 |
| YR x TR | 0.3321 | 0.0581 | **0.0009** | **0.0153** | 0.8655 | 0.2858 | 0.6819 | 0.9430 | 0.7974 | **0.0316** |
| YR x YP | 0.7444 | 0.4586 | 0.0827 | 0.5860 | 0.9275 | 0.6890 | 0.6778 | 0.2233 | 0.4996 | 0.2987 |
| TR x YP | 0.7391 | 0.2342 | 0.1541 | 0.2683 | 0.1522 | 0.9070 | 0.8696 | 0.6186 | 0.6583 | 0.4792 |
| YR x TR x YP | 0.6667 | 0.1944 | 0.1023 | 0.4214 | 0.6061 | 0.9686 | 0.4307 | 0.3730 | 0.9230 | 0.1167 |

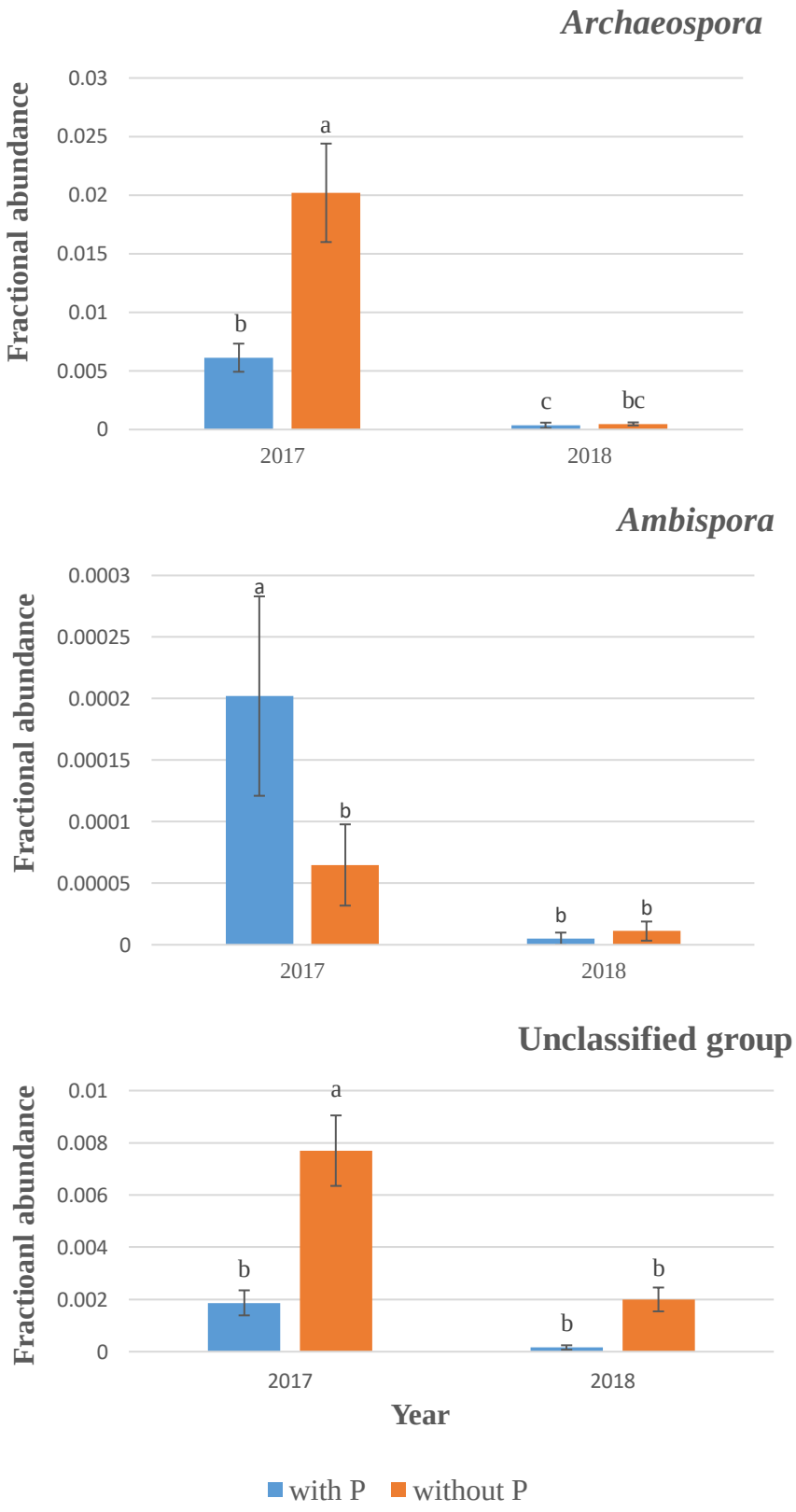



**Figure 4. 14** Effect of treatment (with P and without P) in 2017 and 2018 trials on the fractional abundance of *Archaeospora*, *Ambispora*, and an unclassified group (expressed as a fraction of the total sequences within the Kingdom Fungi). Bars labelled with the same letter are not significantly different (Tukey's honestly significant difference test, $p<0.05$). Standard errors of the mean (error bars) were calculated from all three field plot replicates.

Year significantly affected the fractional abundance of the total AM fungal sequences in the AMF genera *Paraglomus*, *Glomus*, *Archaeospora*, *Ambispora*, and *Rhizophagus* (as a fraction of sequences in the Phylum Glomeromycota; Table 4. 12). The fractional abundance of AMF genera in the year 2018 was higher than the year 2017, except for three genera *Glomus*, *Archaeospora*, and *Ambispora*. *Paraglomus* had the highest fractional abundance in both years.

The year by treatment interaction was significant for the fractional abundance of *Ambispora* (Figure 4. 15). For the fractional abundance, the with P treatments were higher than the without P treatments in both years, and the fractional abundance from both treatments was higher in 2017 compared to 2018.

**Table 4. 12** Effect of each year, treatment, and yield potential group of potato variety on the fractional abundance of each AMF genus (expressed as a fraction of the total sequences within the Phylum Glomeromycota) (Main effect means±SE, ANOVA p-values, n=192).

| Factor | Fractional abundance | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ***Paraglomus*** | ***Glomus*** | ***Archaeospora*** | ***Ambispora*** | ***Diversispora*** | ***Dominikia*** | ***Funneliformis*** | ***Rhizophagus*** | ***Septoglomus*** | **Unclassified Group** |
| **Year (YR)** | | | | | | | | | | |
| 2017 | 0.419±0.027 | 0.286±0.023 | 0.169±0.016 | 0.003±0.001 | 0.002±0.001 | 0.000±0.000 | 0.057±0.009 | 0.004±0.001 | 0.001±0.000 | 0.042±0.006 |
| 2018 | 0.670±0.034 | 0.071±0.012 | 0.038±0.013 | 0.001±0.001 | 0.011±0.005 | 0.009±0.008 | 0.079±0.019 | 0.031±0.010 | 0.001±0.000 | 0.089±0.022 |
| **Treatment (TR)** | | | | | | | | | | |
| With P | 0.505±0.036 | 0.203±0.025 | 0.109±0.019 | 0.003±0.001 | 0.005±0.003 | 0.009±0.008 | 0.099±0.020 | 0.014±0.007 | 0.000±0.000 | 0.047±0.015 |
| Without P | 0.576±0.030 | 0.160±0.018 | 0.101±0.013 | 0.000±0.000 | 0.008±0.004 | 0.001±0.000 | 0.039±0.006 | 0.021±0.008 | 0.001±0.000 | 0.082±0.016 |
| **Yield potential group of potato variety (YP)** | | | | | | | | | | |
| High yield group | 0.540±0.034 | 0.180±0.020 | 0.121±0.017 | 0.002±0.001 | 0.006±0.004 | 0.001±0.000 | 0.062±0.016 | 0.017±0.008 | 0.001±0.000 | 0.063±0.018 |
| Low yield group | 0.545±0.032 | 0.182±0.023 | 0.089±0.015 | 0.002±0.001 | 0.007±0.003 | 0.008±0.008 | 0.073±0.014 | 0.018±0.006 | 0.001±0.000 | 0.067±0.014 |
| **ANOVA p-values** | | | | | | | | | | |
| YR | **<0.0001** | **<0.0001** | **<0.0001** | **0.0033** | 0.1881 | 0.3024 | 0.5437 | **0.0070** | 0.4786 | 0.9929 |
| TR | 0.2979 | 0.3687 | 0.5923 | 0.2551 | 0.5787 | 0.4545 | 0.1976 | 0.2476 | 0.4216 | 0.0808 |
| YP | 0.6959 | 0.9172 | 0.0718 | 0.7852 | 0.3181 | 0.2651 | 0.3711 | 0.4203 | 0.8062 | 0.2529 |
| YR x TR | 0.5313 | 0.6829 | 0.0519 | **0.0239** | 0.6513 | 0.7538 | 0.9958 | 0.7260 | 0.4031 | 0.3750 |
| YR x YP | 0.0789 | 0.8632 | 0.0633 | 0.3505 | 0.7803 | 0.3722 | 0.7416 | 0.2400 | 0.7145 | 0.6752 |
| TR x YP | 0.3886 | 0.6430 | 0.1861 | 0.4582 | 0.0806 | 0.3404 | 0.4747 | 0.5749 | 0.9810 | 0.3300 |
| YR x TR x YP | 0.8105 | 0.3388 | 0.4514 | 0.6425 | 0.3393 | 0.3503 | 0.9352 | 0.8322 | 0.8733 | 0.7189 |

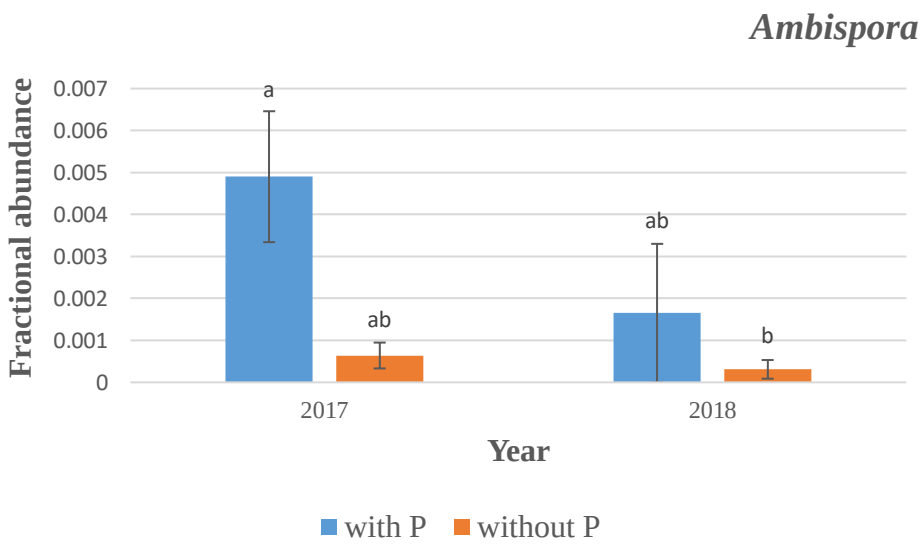



**Figure 4. 15** Effect of treatment (with P and without P) in the year 2017 and 2018 trial on the fractional abundance of *Ambispora* (expressed as a fraction of the total sequences within the Phylum Glomeromycota). Bars labelled with the same letter are not significantly different (Tukey's honestly significant difference test, $p<0.05$). Standard errors of the mean (error bars) were calculated from all three field plot replicates.

**AMF diversity**

In both years, the total number of ASVs (observed) and Shannon diversity index (H') in the without P treatment were higher than the with P treatment (Figure 4. 16). Species evenness was not significantly affected by treatment in 2017, while in 2018, species evenness in the without P treatment was significantly higher than the treatment with P.

There were no significant effects of potato variety on species evenness, the total number of ASVs, or Shannon's diversity index (H') in either experimental year (Figure 4. 17).

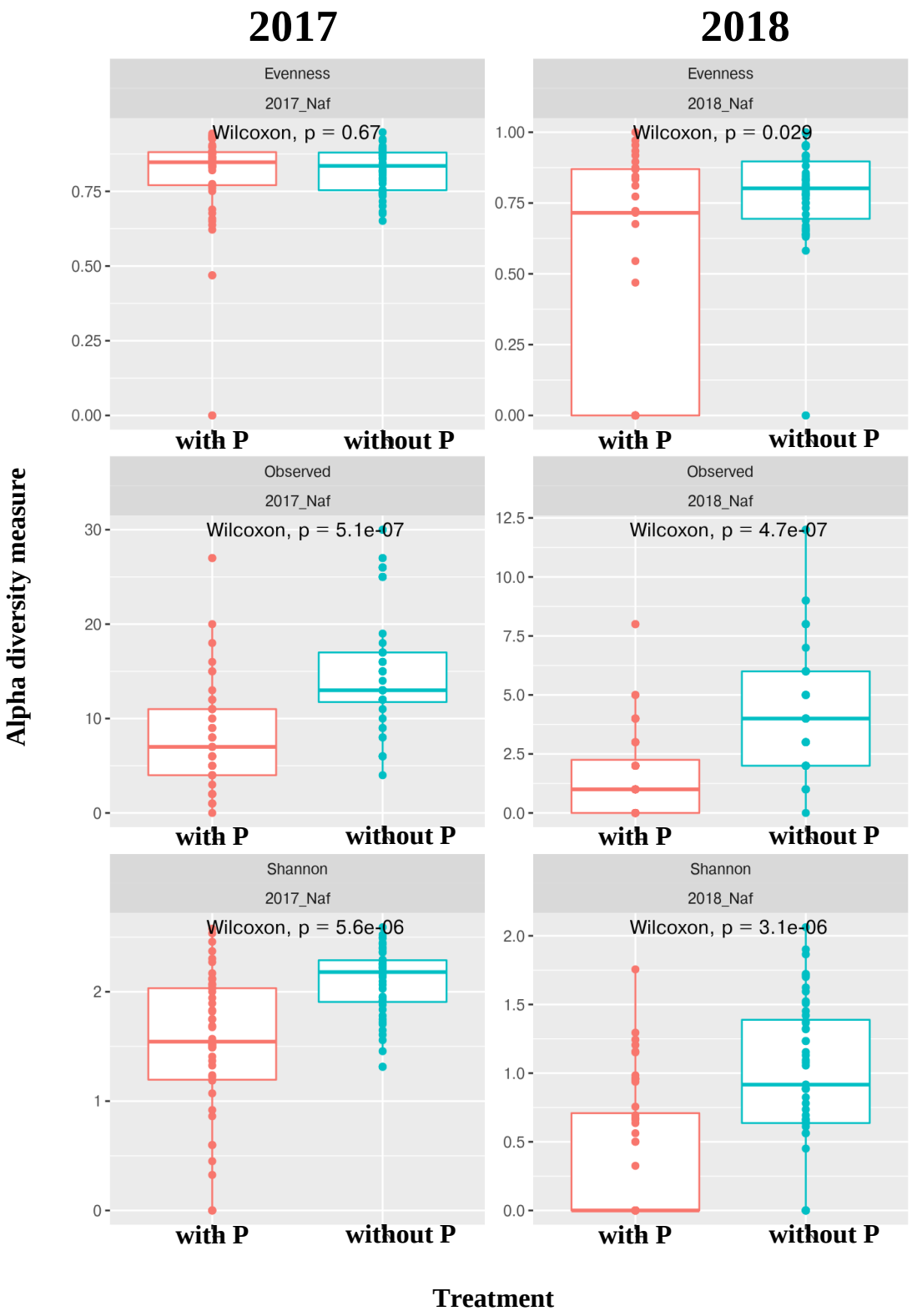
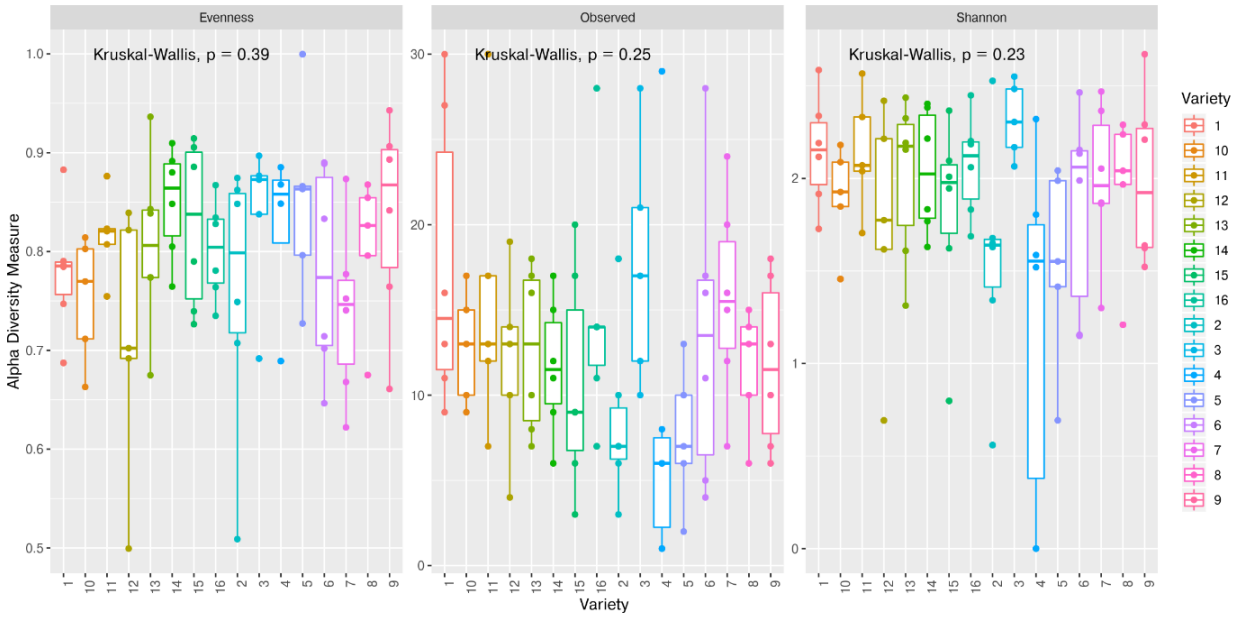



**Figure 4. 16** Boxplots showing evenness, number of ASVs (observed), and Shannon's diversity index (H') of AMF in potato roots in the year 2017 and 2018 trials. Treatment plotted against the alpha diversity measure.

# 2017




# 2018

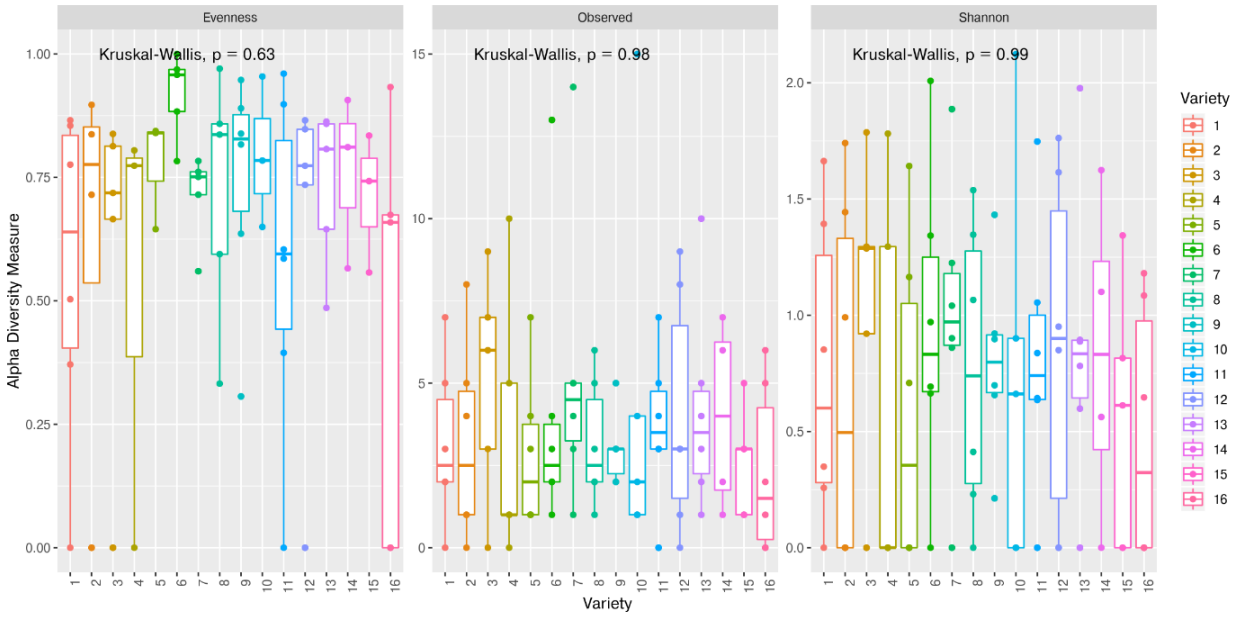



**Figure 4. 17** Boxplots showing evenness, number of ASVs (observed), and Shannon's diversity index (H') of AMF in potato roots in the year 2017 and 2018 trials. Potato variety plotted against the alpha diversity measure (Potato variety; 1=Alpha, 2=Anya, 3=Cramond, 4=Darwina, 5=Estima, 6=Fortyfold, 7=Golden Wonder, 8=Lady Balfour, 9=Libertas, 10=Markies, 11=Pentland Crown, 12=Romano, 13=Sassy, 14=Shelagh, 15=Sheriff, 16=Stamina).

**AMF taxonomic composition**

Taxa bar plots of relative abundances of AMF genera-related sequences identified in amplicon libraries for phylum Glomeromycota obtained for both treatments in each year are shown in Figure 4. 18. In both years, the taxonomic diversity results show that the main AMF genus present in all samples is *Paraglomus* (blue taxa bars). In addition to the *Paraglomus,* various other genera in this phylum (*Ambispora*, *Archaeospora*, *Diversispora*, *Dominikia*, *Funneliformis*, *Glomus*, *Rhizophagus,* and *Septoglomus*) were found in different proportions in each treatment. In 2017, no AM fungal genera were detected at all in seven samples from the treatment with P.

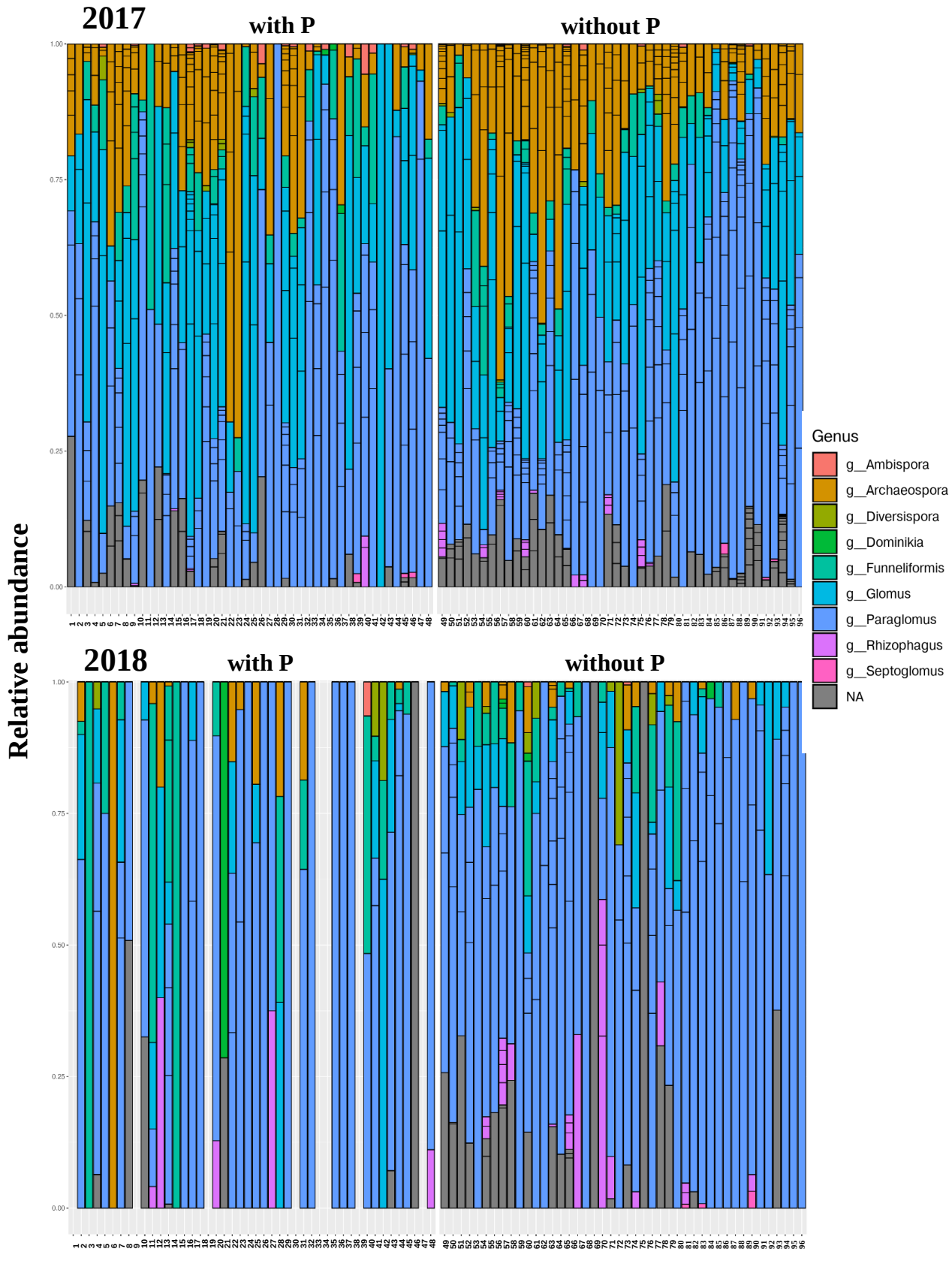



**Figure 4. 18** Taxa bar plots of the relative abundances of AMF genera-related sequences identified in amplicon libraries obtained from two treatments with P and without P in the two-year low P trials. Potato root samples numbers plotted against the relative abundance.

### *4.4.6 Effect of year, treatment, and yield potential group of potato variety on P concentration, total P uptake, tuber yield, and P use efficiency*

Total P uptake and tuber yield in 2018 were significantly higher than in the 2017 (Table 4. 13); however, the year had no significant effect on P concentration and P use efficiency. Treatment had no significant effect on P concentration, total P uptake, tuber yield, and P use efficiency. P concentration, total P uptake and tuber yield values were numerically higher in the treatments that received P compared to the treatment without P, whereas P use efficiency in the treatment without P was numerically higher than the treatment with P (Table 4. 13).

Total P uptake, tuber yield, and P use efficiency in the high yield potential group of potatoes were significantly higher than for the low yield potential group. Whereas yield potential group of potato variety had no significant effect on P concentration.

The year by treatment interaction was significant for P concentration (Figure 4. 19). For the with P treatment, P concentration was significantly higher in 2017 compared to 2018, while for the without P treatment, P concentration was higher in 2018 compared to 2017. In 2017 P concentration in the treatment with P was significantly higher than the treatment without P, while in 2018 addition of P had no effect on P concentration.

**Table 4. 13** Effect of each year, treatment, and yield potential group of potato variety on P concentration, total P uptake, tuber yield and P use efficiency (Main effect means±SE, ANOVA p-values, n=192).

| Factor | P concentration (mg $g^{-1}$ DW) | Total P uptake (kg $ha^{-1}$) | Tuber yield (t $ha^{-1}$) | P use efficiency (Efficiency ratio*) |
|---|---|---|---|---|
| **Year (YR)** | | | | |
| 2017 | 3.56±0.06 | 5.14±0.26 | 15.91±0.80 | 3152.03±63.91 |
| 2018 | 3.61±0.05 | 7.48±0.33 | 22.78±1.05 | 3038.66±48.39 |
| **Treatment (TR)** | | | | |
| With P | 3.70±0.05 | 7.17±0.34 | 21.23±1.09 | 2941.60±53.55 |
| Without P | 3.47±0.05 | 5.45±0.27 | 17.46±0.85 | 3249.09±55.94 |
| **Yield potential group of potato variety (YP)** | | | | |
| High yield group | 3.52±0.06 | 7.23±0.33 | 23.26±1.01 | 3298.47±59.12 |
| Low yield group | 3.65±0.05 | 5.39±0.27 | 15.43±0.80 | 2892.21±46.15 |
| **ANOVA p-values** | | | | |
| **Main effects** | | | | |
| YR | 0.4610 | **<0.0001** | **<0.0001** | 0.0980 |
| TR | 0.1608 | 0.0615 | 0.0803 | 0.1300 |
| YP | 0.0721 | **<0.0001** | **<0.0001** | **<0.0001** |
| **Interaction** | | | | |
| YR x TR | **0.0006** | 0.0712 | 0.3039 | **0.0007** |
| YR x YP | 0.3742 | 0.7780 | 0.8449 | 0.0987 |
| TR x YP | 0.8678 | **0.0163** | **0.0269** | 0.4298 |
| YR x TR x YP | 0.7423 | 0.2586 | 0.3304 | 0.8026 |

* Efficiency ratio= tuber yield (kg)/P concentration in tuber (kg)

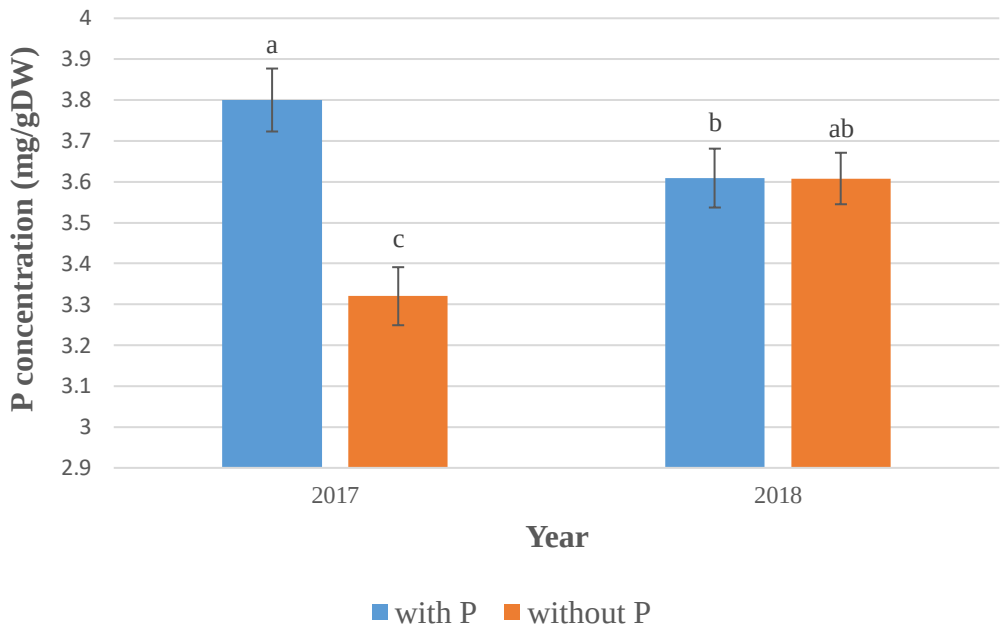



**Figure 4. 19** Effect of treatment (with P and without P) in the year 2017 and 2018 trials on P concentration. Bars labelled with the same letter are not significantly different (Tukey's honestly significant difference test, $p<0.05$). Standard errors of the mean (error bars) were calculated from all three field plot replicates.

The yield potential group of potato variety by treatment interaction was significant for total P uptake (Figure 4. 20). For the high yield group, total P uptake was significantly higher in the with P treatment compared to the low yield group, while for the without P treatment, P concentration did not differ between yield potential groups. In with P treatment, total P uptake in the high yield group was significantly higher than the treatment without P, while in low yield group addition of P had no effect on total P uptake.

The yield potential group of potato variety by treatment interaction was significant for tuber yield (Figure 4. 21). For the high yield group, tuber yield was significantly higher for both treatments compared to the low yield group. In with P treatment, tuber yield in the high yield group was significantly higher than the treatment without P, while in low yield group addition of P had no effect on tuber yield.

The year by treatment interaction was significant for P use efficiency (Figure 4. 22). In 2017, P use efficiency was significantly higher in the without P treatment compared to 2018, while for the with P treatment, P use efficiency did not differ between two years. In 2017, P use

efficiency in the without P treatment was significantly higher than the treatment with P, while in 2018 addition of P had no effect on P use efficiency.

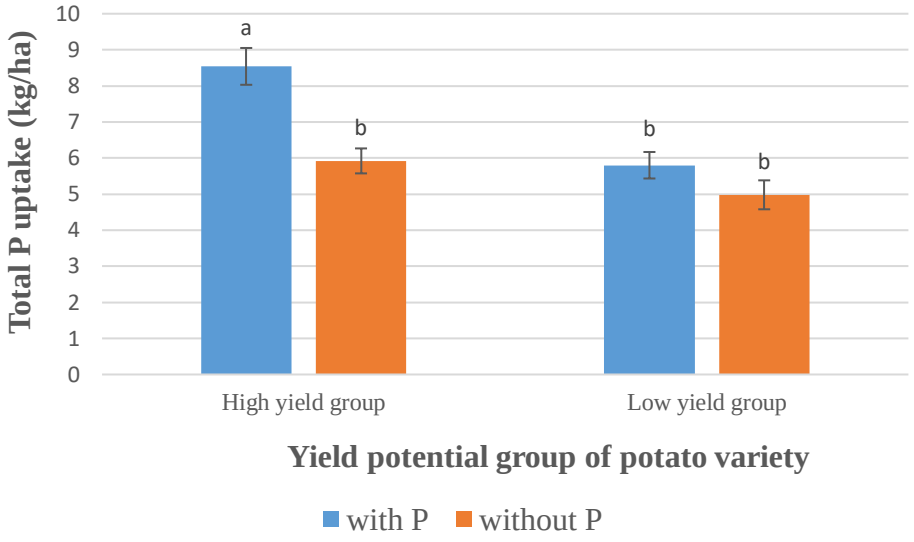



**Figure 4. 20** Effect of treatment (with P and without P) in the yield potential group of potato variety on total P uptake. Bars labelled with the same letter are not significantly different (Tukey's honestly significant difference test, $p<0.05$). Standard errors of the mean (error bars) were calculated from all three field plot replicates.

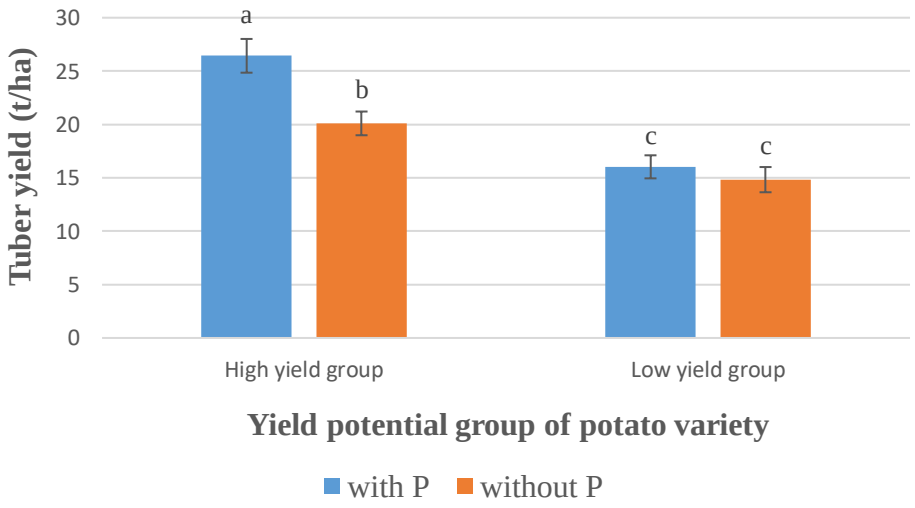



**Figure 4. 21** Effect of treatment (with P and without P) in the yield potential group of potato variety on tuber yield of potato. Bars labelled with the same letter are not significantly different (Tukey's honestly significant difference test, $p<0.05$). Standard errors of the mean (error bars) were calculated from all three field plot replicates.

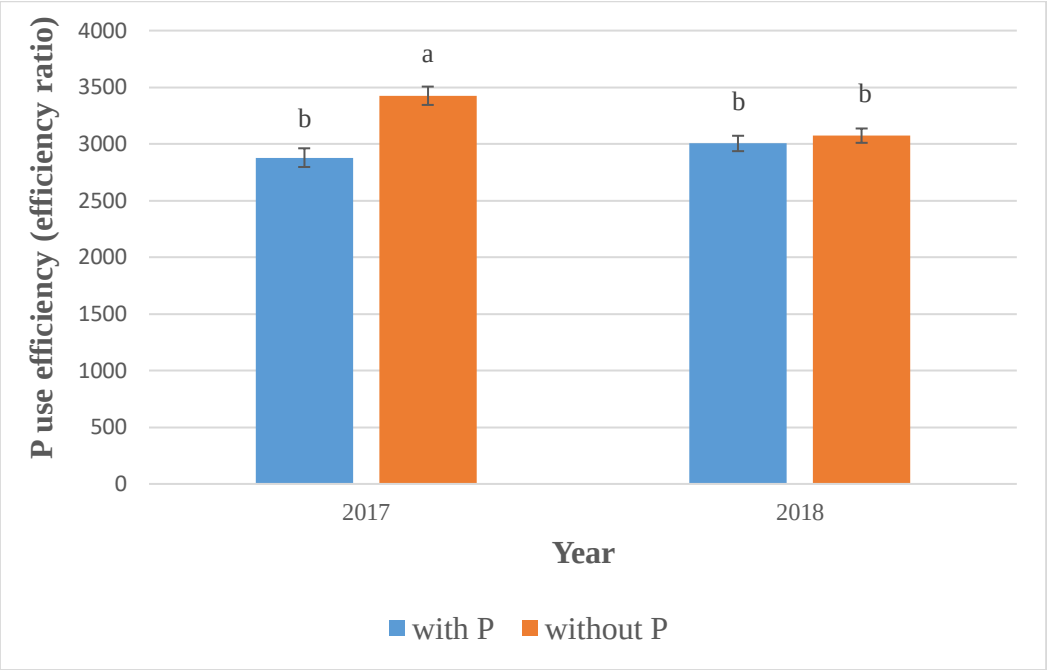



**Figure 4. 22** Effect of treatment (with P and without P) in the year 2017 and 2018 trials on P use efficiency. Bars labelled with the same letter are not significantly different (Tukey's honestly significant difference test, $p<0.05$). Standard errors of the mean (error bars) were calculated from all three field plot replicates.

### *4.4.7 Tuber yield of the 2017, 2018 low P trials*

According to Figure 4. 23, most of the potato varieties in 2018 had higher tuber yield than in 2017, with the exception of only two potato varieties, "Pentland Crown" and "Alpha." Two high-yielding potato varieties, "Sheriff" and "Shelagh," were selected for the glasshouse experiment (see Chapter 5) based on the results of the tuber yield.

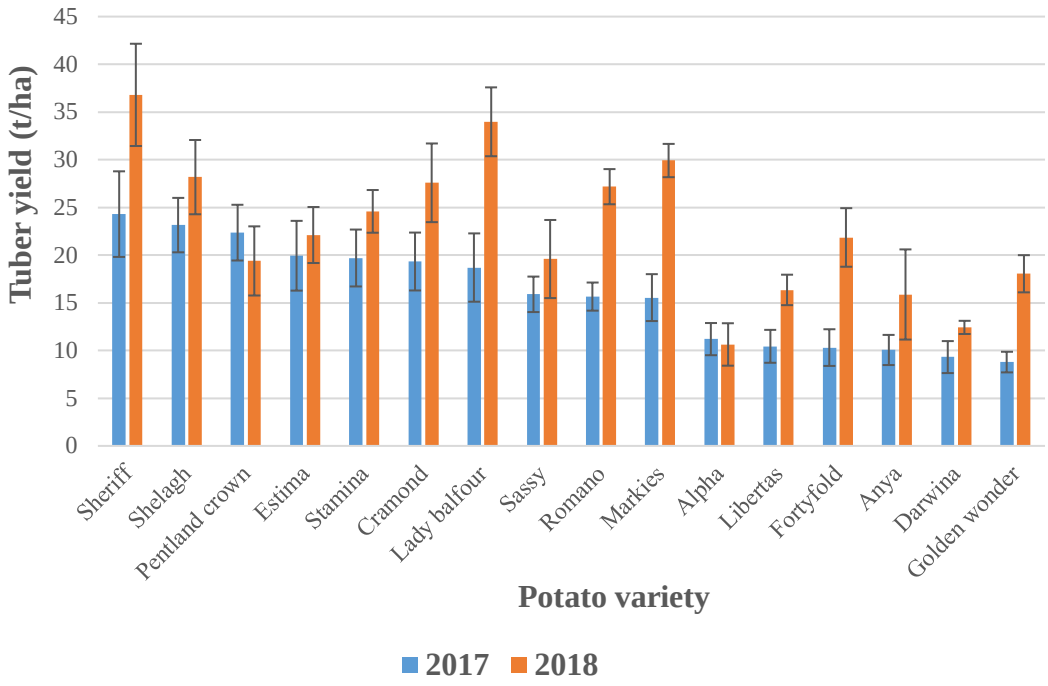



**Figure 4. 23** Tuber yield of each potato variety in the 2017 and 2018 low P trials. Standard errors of the mean (error bars) were calculated from all three field plot replicates.

### *4.4.8 Correlation between AMF colonisation in each sample and P concentration, total P uptake, tuber yield, P use efficiency*

AMF colonisation was significantly negatively correlated with P concentration in the year 2017 (r= -0.38; p=0.00016) and was not correlated with P concentration in the year 2018 (r= 0.0061; p=0.99) (Figure 4. 24). AMF colonisation was significantly negatively correlated with total P uptake in 2017 (r= -0.35; p=0.00043) and not correlated with P uptake in 2018 (r= -0.12; p=0.24). AMF colonisation was significantly negatively correlated with tuber yield in 2017 (r= -0.23; p=0.024), but there was no correlation between AMF colonisation and yield in 2018 (r= -0.069; p=0.5). AMF colonisation was significantly correlated with P use efficiency in 2017 (r= 0.38; p=0.00017) and not correlated with P use efficiency in 2018 (r=0.12; p=0.26).

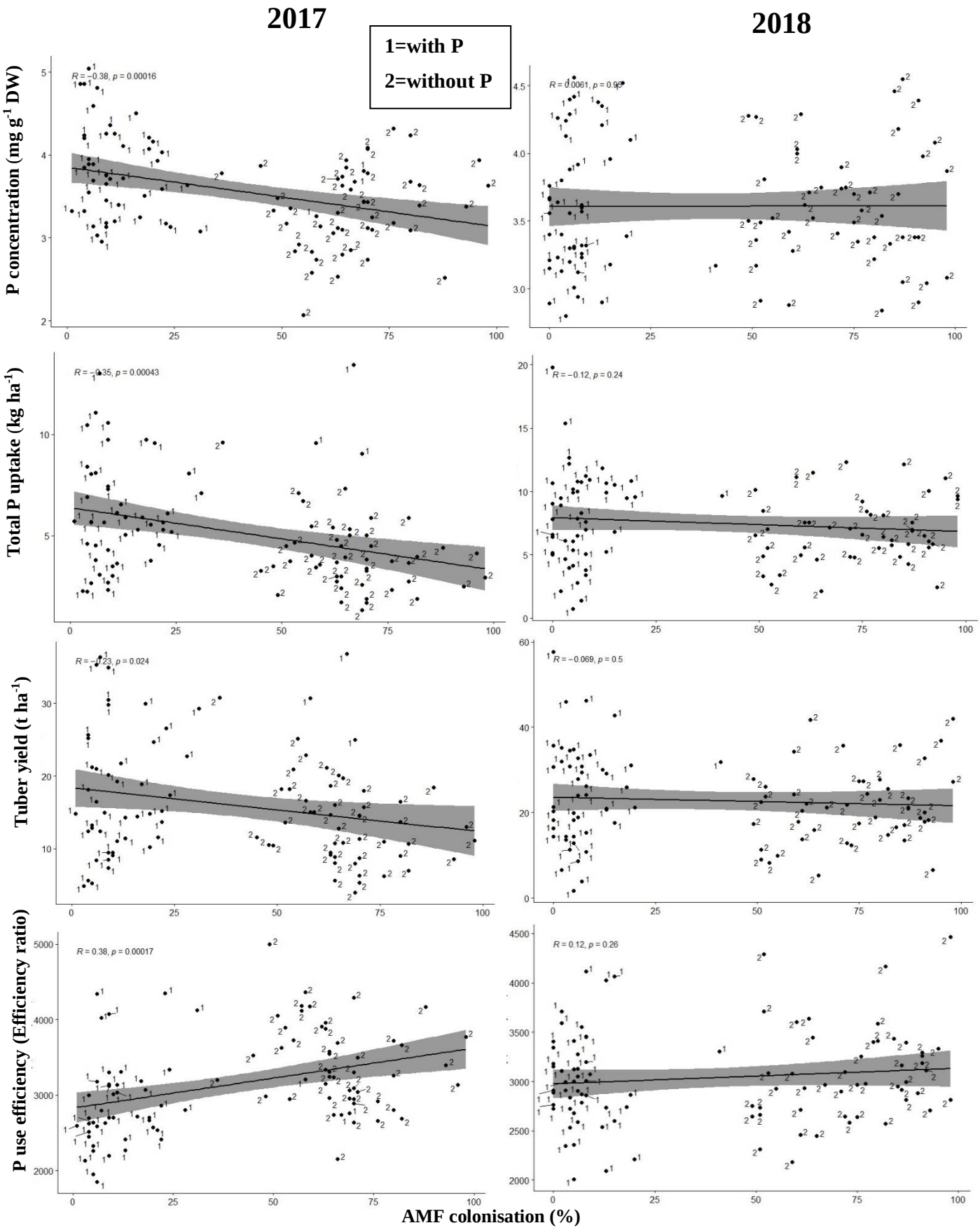



**Figure 4. 24** The percentage of AMF colonisation in each sample plotted against P concentration, total P uptake, tuber yield and P use efficiency in 2017 and 2018 low-P potato trials (Pearson's product-moment correlation, n=96 in each year).

### 4.5 Discussion

Table 4. 14 summarises the findings from this study, indicating that added P fertiliser negatively affected AMF abundance, colonisation, and diversity in both years of the experiment. These findings reflect the results from many previous studies showing that increases in P availability decreased AMF colonisation in many host plants, including barley (Khaliq and Sanders, 2000), sweet potato (Minemba *et al.*, 2019), and potatoes (Loit *et al.*, 2018; Gao *et al.*, 2020).

**Table 4. 14** Effect of P fertiliser on the abundance and diversity of AMF from each year in this study.

| | | **AMF abundance** | | **AMF diversity (Illumina MiSeq NGS)** | | |
|---|---|---|---|---|---|---|
| **Factor** | **Year** | **AMF colonisation** | **Gene abundance (qPCR)** | **Evenness** | **Richness** | **Shannon's diversity index** |
| **Addition of P** | **2017** | ↓ | ns | ns | ↓ | ↓ |
| | **2018** | ↓ | - | ↓ | ↓ | ↓ |

ns=not significant, indicated by p-value

arrows represent the level variation; increased expression (upward position) and reduced expression (downward position)

Many studies have reported that qPCR was not correlated with microscopy. Voříšková *et al.* (2017) reported that information about the relationship between the copy number of DNA by qPCR and other quantification approaches for AMF colonisation is still limited. Gamper *et al.* (2008) suggested that qPCR assesses different biological units than the microscopic approach; thus, these approaches are not comparable. Labonova *et al.* (2018) reported that microscopic studies can evaluate the colonisation level from all AMF structures, including senescent structures; in contrast, qPCR only quantifies the active fungal structures containing DNA.

According to the classification of soil P index from AHDB (2020), the soil P availability is classified using an index between 0 and 9 (zero to more than 280 mg $L^{-1}$ of Olsen-P in soil). In this study, the P index from the initial soil test results (Table 4. 1) indicated that the P index from all three without P treatment plots in this study were low; ranging from 0 to 1 (8.40-9.60

mg $L^{-1}$). This study confirmed that without P treatment plots had higher AMF abundance and diversity than with P treatment plots in both years. Deng *et al.* (2014) showed that root colonisation and AMF-specific Pi transporter genes are significantly up-regulated when the soil P is below 10 mg $kg^{-1}$. Liu *et al.* (2016) also reported that root colonisation is significantly up-regulated when the soil Olsen-P is below a critical level (10 mg $kg^{-1}$). Furthermore, the field study of Gosling *et al.* (2013) presented that AMF diversity was decreased when the soil P index was more than 2 (more than 25 mg $L^{-1}$). This thesis study provides further evidence to support all findings; low soil P concentration is frequently presented to increase AMF abundance and diversity. The critical P level for increasing AMF abundance and diversity in this study is 9.60 mg $L^{-1}$.

The total P uptake and tuber yield were highest in the high yield potential group of potato variety when P fertiliser was added; this group also showed the highest AMF root colonisation. It appears that the high yield potential group of potato variety were able to form associations with AMF that may have resulted in improved P uptake and crop growth. As the results of AMF colonisation did indicate differences between with P and without P treatments, this suggests that under low P levels (below 9.60 mg $L^{-1}$), the presence of the AMF allowed the plants to compensate for the low P, so that similar agronomic results were achieved. In the treatment without P fertiliser, potato plants may uptake P from the soil by a direct pathway of their roots together with the indirect pathway of an AMF-specific Pi transporter. (Smith *et al.*, 2011; Bücking *et al.*, 2012). This can be confirmed from the results of the AMF genera abundance derived from NGS results. The genera abundance of AMF in the year 2018 trial was higher than the 2017 trial. The higher abundance of AMF in 2018 may have contributed to the higher P uptake in that year.

P use efficiency in this study was measured by physiological P use efficiency of plant for yield production using the P absorbed in tubers. The treatment without P in this study had numerically higher P use efficiency than the treatment with P in both years. Interestingly, the treatment without P had higher AMF colonisation than the with P treatment in both years as shown in Figure 4. 24. It is a definite confirmation that AMF colonisation in the treatment without P significantly increased P use efficiency, AMF can improve P use efficiency by accessing P from soil. Increasing P use efficiency can be also achieved by increasing P uptake capacity (Neto *et al.*, 2016). P use efficiency has been shown to be closely associated with total P uptake for the yield potential group of potato variety results. As shown in this study,

total P uptake was highest in the high yield potential group of potato variety which resulted in highest P use efficiency. This high yield potential group of potato variety which had the highest AMF root colonisation is definite suitable for AMF symbiosis to increase total P uptake and P use efficiency. Thus, AM colonisation may supply soil P to potato under no P fertilisers condition.

A possible reason why *Paraglomus* was the dominant genus in most root samples was that it was also dominant in the bulk (baseline) soils before potato planting in both years. As AMF has a low host specificity, *Paraglomus* was able to effectively form associations with the potatoes grown in this study.

The results from Table 4. 13 showed that treatment had no significant effect on total P uptake and tuber yield at the $P<0.05$ level, however, there was significance at the $P<0.1$ level. The treatments that received P had numerically higher total P uptake and tuber yield than the treatment without P. It is possible that the low replication and high variance may have lead to a non-significant result at the $P<0.05$ level (Gibson, 2021).

### 4.6 Conclusion

This study demonstrated that increased P availability through additions of P fertiliser negatively affected AMF abundance and diversity in both years. The results of this study showed higher AMF abundances and diversity in the treatment without P. At the same time, there were no significant differences in tuber P concentration, total P uptake, and tuber yield when compared to the with P treatment. This suggests that the presence of AMF compensated for low P supply in the without P treatments by supplying P through their mycelium. It is concluded that AMF positively influences total P uptake, especially in potatoes with a high yield potential when grown under conditions of low P availability.

# CHAPTER 5. The potential for AMF inoculant to promote the growth of potato

## 5.1 Introduction

Beneficial soil microorganisms, including AMF, have been produced as bio-inoculants for nutrient management, the control of plant pathogens, and plant growth-promoting benefits (Vassilev *et al.*, 2005; Owen *et al.*, 2015; Mohan and Rajendran, 2019). The use of bio-inoculants as bio-fertilisers is considered a good alternative to reduce chemical fertiliser use without negative environmental impact (Cely *et al.*, 2016; Selvakumar *et al.*, 2018). AMF inoculants have been applied to many agricultural crops such as potato, wheat, rice, corn, pulses, tomato, onion, and soybean (Hijri, 2016; Igiehon and Babalola, 2017; Hart *et al.*, 2018; Elliott *et al.*, 2019). AMF are classified as phosphate mobilising bio-inoculants (Mohan and Rajendran, 2019). An application of AMF inoculants to the host plants acts as a bio-stimulant to promote the effective use of existing soil nutrients, especially phosphorus (Owen *et al.*, 2015; Elliott *et al.*, 2019). It is theorised that using AMF inoculants can reduce the crop's requirement for P fertiliser addition while maintaining plant growth and yield, and reducing reliance on non-renewable sources of P (Elbon and Whalen, 2015).

AMF inoculation technology is limited because these fungi cannot be cultured on synthetic growth media without hosts (Siddiqui and Kataoka, 2011). AMF inoculants can be produced using various techniques in two main systems; soil-based and soil-less systems (Siddiqui and Kataoka, 2011). The soil-based system inoculant production starts by using AMF spores, and AMF colonised root segments amended to the substrate used for plant seedling production; AMF spread in the substrate and colonise the root seedlings. Both colonised substrates and roots then serve as an AMF inoculant (Brundrett, 2004). However, to produce the AMF inoculant on a large-scale requires control and optimisation of host growth and AM fungal development (Siddiqui and Kataoka, 2011). Also, the soil-based system generally takes time and risks contamination by other microorganisms (Mohammad *et al.*, 2000). Soil-less techniques can also be used to produce AMF inoculant. These techniques include the nutrient film technique, aeroponic cultures, axenic cultures, root organ cultures, and production from a polymer-based inoculum (Mohammad *et al.*, 2000; Siddiqui and Kataoka, 2011; Lone *et al.*, 2017). These soil-less techniques can produce a high quality of AMF inoculant greater than that of the soil-based technique and, furthermore, the selected AMF can be produced in pure

culture free from contamination by other microorganisms (Setiadi, 2002; De Santana *et al.*, 2014).

Recently, the production of AMF inoculants has focused on finding AMF strains with a high potential to enhance crop yield (Adeyemi *et al.*, 2020). Such single AMF species or a mixture of AMF species have been produced in the form of commercial inoculants by many companies (Gianinazzi and Vosátka, 2004). The formulation of AMF inoculant usually consists of AMF fragments (fungal hyphae and spores) mixed into the carrier (peat, perlite, zeolite, inorganic clay, vermiculite, sand, etc.) (Gianinazzi and Vosátka, 2004). The essential criteria for AMF inoculant quality control checking are the physical and chemical properties, the density of AM fungal propagules, the effectiveness of inoculant, and absence of contaminants (Gianinazzi and Vosátka, 2004). The effectiveness of AMF inoculants can be evaluated by increased root colonisation and nutrient capture on their application (Elliott *et al.*, 2019).

Several researchers have studied the potential of AMF inoculants to promote the growth of potatoes under field and glasshouse conditions and showed that AMF inoculants benefit potato production (Hijri, 2016). Black and Tinker (1977) conducted an early study on the use of *Glomus macrocarpus* as an inoculant in a potato field and showed that AMF inoculant enhanced potato growth. After that, many studies using different potato cultivars and different AMF inoculants had been reported (Hijri, 2016). For example, yield improvements occurred after inoculation with *Rhizophagus intraradices* (formerly *Glomus intraradices*) of a potato variety "Golden Wonder" in a greenhouse experiment (Duffy and Cassells, 2000); *Gigaspora* sp. of a potato variety "Cipira" in a field experiment in Cameroon (Ngakou *et al.*, 2006); *Rhizophagus intraradices* and *Funneliformis mosseae* (formerly *Glomus mosseae*) of potato varieties "Jyoti" and "TPS" (Lone *et al.*, 2015) and; *Rhizophagus intraradices* of a potato variety "Yungay" in a field experiment in Peru (Davies Jr *et al.*, 2005). Mechanistically, in the study of Duffy and Cassells (2000), the two commercial AMF inoculants (a mixture of isolates) applied to the potato variety "Golden Wonder" in a greenhouse experiment were found to promote potato flowering. In addition, some studies investigated the mycorrhizal association between AMF inoculant and potato, and found that various AMF inoculants can colonise various potato varieties; for example, the potato variety "King Edward," inoculated with *Funneliformis mosseae* in Sweden (Bharadwaj *et al.*, 2007) and an Italian potato field inoculated with *Rhizophagus irregularis* (formerly *Glomus irregulare*) (Cesaro *et al.*, 2008).

In terms of potato growth stage and AMF associations, Hannula *et al.* (2012) and Hannula *et al.* (2010) found that the diversity and function of the AM fungal communities in the rhizosphere soil of six potato varieties (Modena, Karnico, Aveka, Aventra, Désirée and Premiere) were affected by three potato growth stages (seedling/young, flowering and senescence), the change in quality and quantity of root exudates in the different potato growth stages effects AM fungal community. Senés-Guerrero *et al.* (2014), in a field study in Peru, showed that AMF diversity was the highest in potato roots (varieties Yungay and Unica) and in the associated rhizosphere soils at the emergence stage; the main AMF species was found to be *Funneliformis mosseae* (*Glomeraceae*).

*Rhizophagus irregularisis* (*Glomeraceae*) is one of the AMF species widely used in commercial inoculant products. This species is easy to cultivate in vitro and in vivo for mass production (Buysens *et al.*, 2016; Buysens *et al.*, 2017; Alaux *et al.*, 2018). In addition, this species can colonise several varieties of plants, including potato (Alaux *et al.*, 2018).

In the current study, a commercial AMF inoculant from PlantWorks Ltd. purported to be composed of a mixture of AMF species and an inoculant of *Rhizophagus irregularis* MUCL41833-colonised maize roots were compared. The objective was to study their effects on potato varieties in a pot experiment conducted under glasshouse-controlled conditions following growth through three potato growth stages. Two high yielding potato varieties, "Sheriff" and "Shelagh," were selected based on the results of the experiment carried out in 2017 (see Chapter 4). To my knowledge, no publication regarding these two potato varieties and their association with AMF has been published. AMF abundances determined by the root staining method and AMF diversity assessment by Illumina MiSeq NGS were used to evaluate the colonisation success of these inoculants. Above-ground plant biomass and tuber yield when harvested, P concentration, and total P uptake from potato plants and tubers were also analysed as indicators of the agronomic impacts of AMF inoculation.

## 5.2 Aim and Objectives

1. To study the effect of two different kinds of AMF inoculant on potato growth and nutrient status from agronomic data analysis such as above-ground plant biomass, tuber yield, P concentration, and total P uptake.

2. To evaluate the potential for colonisation of the added AMF inoculant by studying the abundance and the diversity of AMF in potato roots after inoculation.

3. To investigate the effect of potato variety and potato growth stage on abundance and the diversity of AMF in potato roots after inoculation.

## 5.3 Materials and Methods

### *5.3.1 Experimental set up and planning*

The experiment was established, and potatoes were planted on 14$^{th}$ June 2018. Two potato varieties (Sheriff and Shelagh) were selected for plant growth experiments under glasshouse conditions. The growing medium for cultivation was John Innes No. 1, which has the lowest fertility of the John Innes suite of growing media (Bazzaz and Harper, 1976). The ingredients in John Innes No. 1 typically include a mixture of seven parts loam, three parts sphagnum peat moss, and two parts grit sand. For each cubic metre of the mixture, nutrients are supplied by additions of ground horticultural-grade limestone (0.6 kg), hoof and horn meal (1.2 kg), superphosphate (1.2 kg), and potassium sulfate (0.6 kg) (https://www.gardeningdata.co.uk/soil/john_innes/john_innes.php).

The pot volume used was 3L, with a top diameter of 19 cm. Two commercial AMF inoculants and one negative control inoculant were used in this study. The three inoculants (shown in Figure 5. 1) were as follows:

Control (no AMF treatment): A granular inoculant carrier without AMF inoculant added, supplied by PlantWorks Ltd.

Inoculant 1: A granular AMF inoculant, supplied by PlantWorks Ltd. The granular inoculant contained propagules of spores and hyphae of AMF.

Inoculant 2: The AMF strain *Rhizophagus irregularis* MUCL41833 provided by the Declerck lab, Université Catholique de Louvain, Belgium, and supplied as colonised maize roots.


**Figure 5. 1** AMF inoculants used in this study (Control/no AMF treatment, Inoculant 1: AMF inoculant from PlantWorks Ltd. and Inoculant 2: *Rhizophagus irregularis* MUCL41833).

Control and inoculant 1 were mixed with the growing medium before planting (10 g per pot), whereas inoculant 2 was placed 2-3 cm below the tuber in each pot (1g per plot). Then, the potato tuber was put into the growing medium, one tuber per pot. Potato seeds from the 2017 experiment (see Chapter 4), which had been stored in the fridge at 4 °C since harvesting, were used in this pot trial. The glasshouse was maintained at a temperature of 18 °C and pots were watered until the water drained from the bottom of the pot every two days; no fertilisers were added.

There were three sampling dates used to assess the different potato growth stages. The three sampling dates were classified according to the three stages of potato growth as described previously (Obidiegwu *et al.*, 2015);

1. Stolon initiation stage (30 days after planting); this first sampling date was, therefore, on 13th July 2018.

2. Tuber initiation stage (60 days after planting), the second sampling date was, therefore, on 13h August 2018.

3. Tuber filling stage (90 days after planting), the third sampling date (harvesting time) was on 13th September 2018.

The total number of potato pots in this study reflected the experimental design, namely the evaluation of 2 potato varieties (x2), both tested with the three inoculants (x3) with replicated (x5) treatment pots sacrificially sampled on the three different sampling dates (x3) = 90 pots. The design was a randomised complete block design. Figure 5. 2 shows the pot experiment in the glasshouse.

**Figure 5. 2** Pot experiment in the glasshouse at Cockle Park Farm, Newcastle University.

### *5.3.2 Experimental analysis conducted in the context of experimental sampling*

Potato roots for all three sampling dates for the three different growth stages of potato were analysed to assess AMF colonisation by root staining and AMF diversity by Illumina MiSeq NGS (Figure 5. 3). Furthermore, above-ground plant biomass, P concentration, total P uptake, and tuber yield at harvest were used for agronomic data analysis.

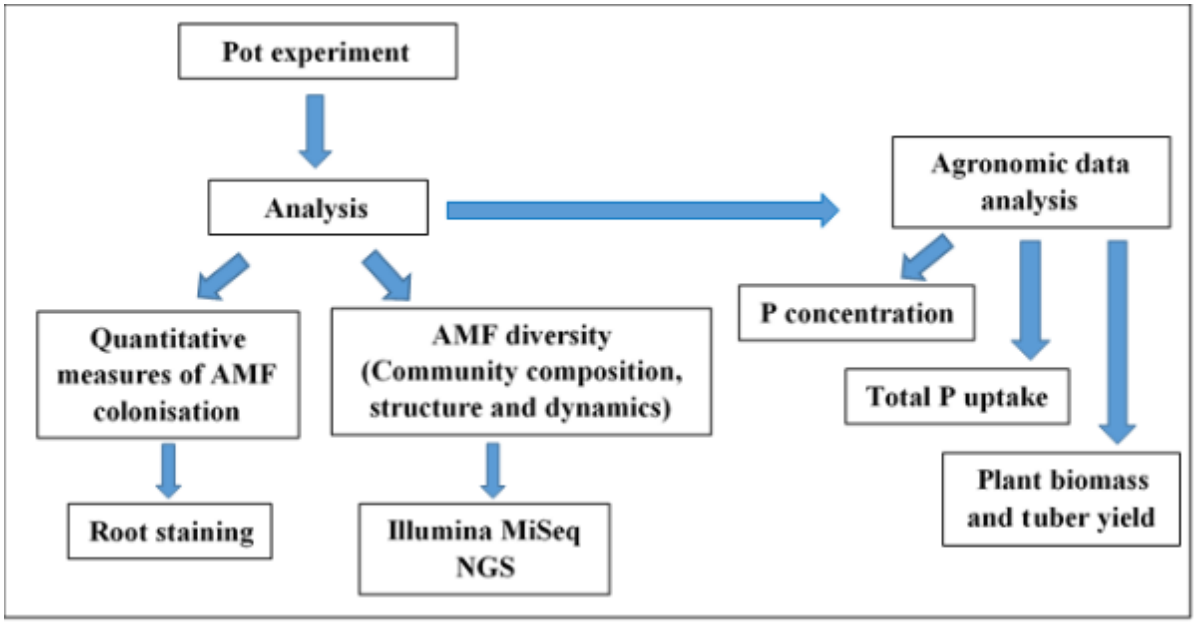



**Figure 5. 3** Techniques used for assessment of AMF effects on potatoes in the study.

### *5.3.3 Growing medium and inoculant analysis*

The aim of this step was to check the purity of the growing medium and to check the diversity of all AMF inoculants, including the control. Samples (1g) were collected from the growing medium, and all AMF inoculants before starting the experiment and stored at -20$^{o}$C prior to DNA extraction.

### *5.3.4 Above-ground plant biomass, plant P concentration, and plant P uptake analysis*

#### Above-ground plant sampling for P concentration and P uptake analysis

The above-ground plant materials were harvested on all three sampling dates. The cut material was placed in an oven (70$^{o}$C) for 4-5 days until dried. Plant biomass fresh weight and dry weight (g per plant) were then recorded.

All dried samples were milled to 1 mm fragments (Retsch milling machine model ZM 200, Haan, Germany), and sub-samples (5 g) were sent to SAC Commercial Ltd. for P analysis by nitric acid microwave digestion coupled to an analysis by ICP spectrometry (Esslemont *et al.*, 2000).

Plant P concentration was calculated by using the following equation.

$$P\ (mg\ g^{-1}) = P\ (\%) \times 10$$

Where P was presented as the concentration (mg g$^{-1}$) of dry matter.

Plant P uptake was calculated by using the following equation.

$$P\ uptake\ (g\ plant^{-1}) = P\ (\%) \times 10 \times plant\ biomass\ DW\ (g)/1000$$

Where P was presented as the P uptake (g plant$^{-1}$) of dry matter.

#### Statistical analysis for above-ground plant biomass, plant P concentration, and plant P uptake

Plant biomass fresh weight and dry weight, plant P concentration, and plant P uptake were subjected to ANOVA with three factors “AMF inoculant (treatment),” “Potato variety,” and “potato growth stage”. The data were checked for normal distribution of the residuals using the qqnorm command; if the residuals are not normally distributed, then the data was

transformed using a square root data transformation. A linear mixed-effects (lme) model was used to produce ANOVA P-values for main effects and all interactions with the nested structure of the split-plot design reflected in the random error term:block/subblock (Pinheiro and Bates, 2000) using the nlme package in the R programming language (RCore, 2016). When ANOVAs indicated a significant treatment effect ($p<0.05$), differences between individual means were analysed using Tukey's honestly significant difference (HSD) post hoc test in the multcomp package in R. A 95% significance level ($p<0.05$) was applied to discriminate the means. Where the interaction between factors was significant, the data was subset by factor and a separate one-way ANOVA conducted at each level of the factors.

### *5.3.5 Tuber yield, tuber P concentration, and tuber P uptake analysis*

#### Tuber sampling for P concentration and P uptake analysis

Potato tubers were collected at the harvest time point (the 3$^{rd}$ sampling date, 90 days after planting), as shown in Figure 5. 4C. All potato tubers were separated from roots, washed, and air-dried, cut into small pieces (around 1cm x 3cm). All subsequent treatment, analysis, and statistical comparison were as described above for the analysis of the above-ground plant biomass; however, ANOVA was performed with "AMF inoculant" and "Potato variety" as factors.

### *5.3.6 Potato root sampling for root colonisation and molecular study*

Destructive sampling for potato roots was conducted at the same time as the above-ground plant sampling at the three different potato growth stages (stolon initiation stage; 30 days after planting, tuber initiation stage; 60 days after planting and tuber filling stage; 90 days after planting). Figure 5. 4A, B, and C show the roots and potato tubers on the three different sampling dates. Briefly, all root samples were cut and separated from the above-ground plant matter and rinsed with tap water. Root samples were divided into two parts for analysis, namely, for the root colonisation and the molecular studies. All these root samples were then frozen by immersion in liquid nitrogen and stored at -20$^{o}$C until required.

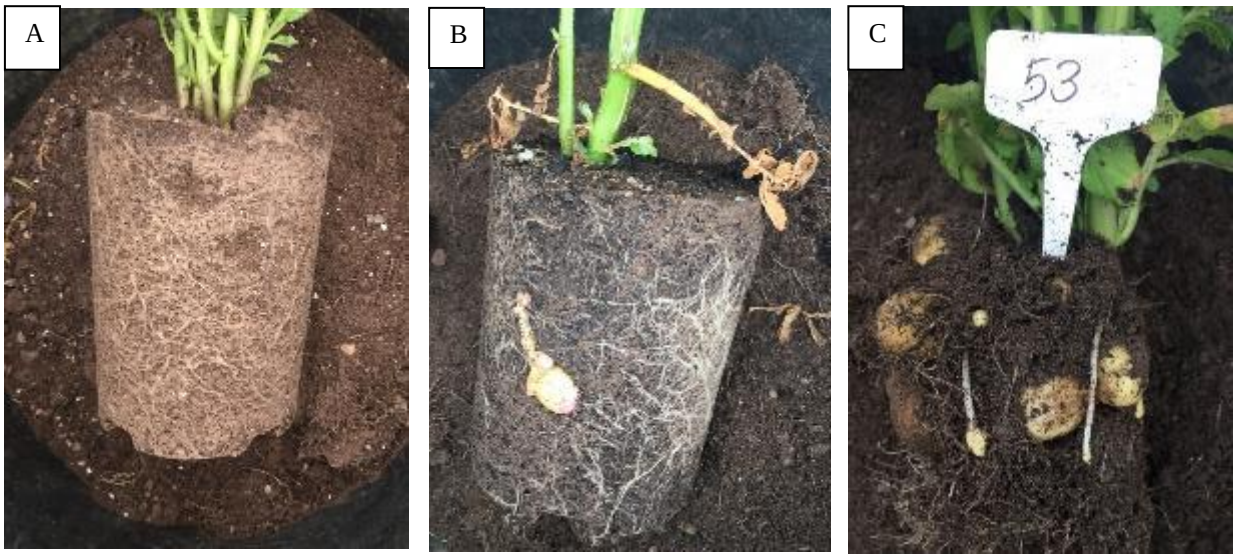



**Figure 5. 4** Roots and potato tubers at the different growth stages; A=Stolon initiation stage (30 days after planting), B=Tuber initiation stage (60 days after planting), and C=Tuber filling stage (90 days after planting).

### *5.3.7 AMF colonisation by root staining (Ink and vinegar method)*

Root staining was carried out as detailed in section 3.3.4.

In terms of statistical analysis, the percentages of AMF colonisation from all samples were subjected to ANOVA, as described above for the analysis of agronomic data in section 5.3.4 with "AMF inoculant" "Potato variety" and "Potato growth stage" as factors.

### *5.3.8 DNA extraction*

DNA was extracted from all potato root samples at the three potato growth stages, as well as the growing medium and AMF inoculants, including the AMF free carrier control, using the method detailed in section 3.3.5 (Method 3), FastDNA SPIN kit for soil (MP Biomedicals, 101 Inc., La Jolla, CA, USA) following the manufacturer's protocol using freeze-dried and milled root samples before DNA extraction.

### *5.3.9 Nucleic acid quantification and purity check*

DNA quality checks were carried out using a Nanodrop 8000 spectrophotometer, as detailed in section 3.3.5.

### *5.3.10 AMF community and diversity analysis using Illumina MiSeq NGS*

AMF community and diversity analysis by NGS of all root samples, growing medium, and all AMF inoculants were conducted as detailed in section 3.3.10. Briefly, from the ASVs table, fungal taxonomic data were normalised as a proportion of the total number of sequences in the library prior to analysis (McGee *et al.*, 2017). The fractional abundance of individual taxa (phylum, family, and genus) within each community was calculated by dividing the number of sequences in different taxonomic groups (phylum, family, and genus) by the number of total sequences in the library obtained for that sample.

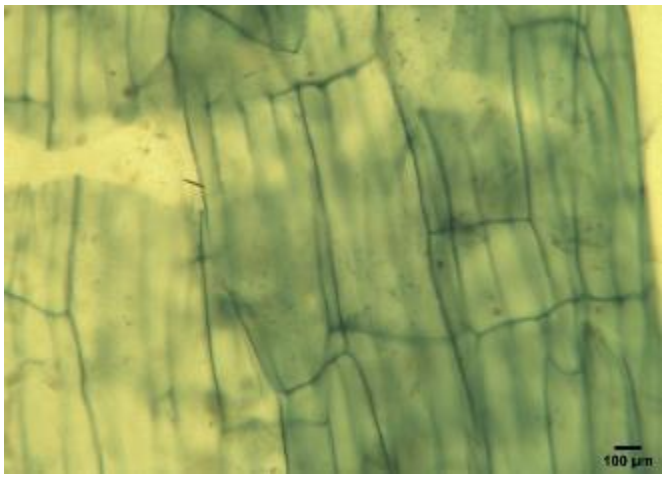
The general composition of general fungi and AMF communities was analysed as described in the data analysis section 3.3.10 with "AMF inoculant," "Potato variety," and "Potato growth stage" as factors. Diversity analysis and taxonomic composition analysis were also analysed, as described in the data analysis section 3.3.10.

## 5.4 Results

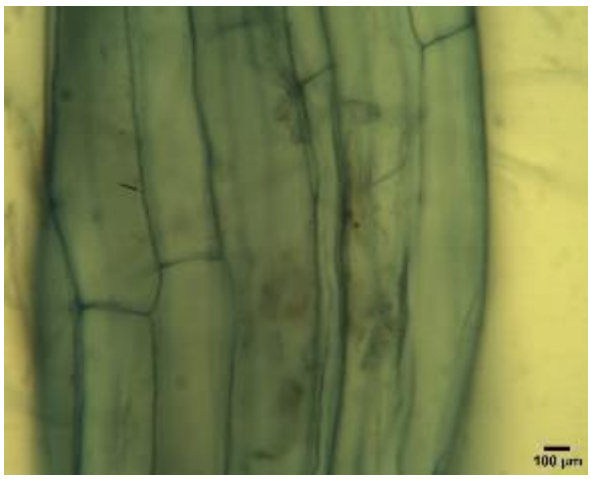
### *5.4.1 AMF colonisation by root staining*

The results of root colonisation assessments showed no AMF infection in any root samples of potato plants that were or were not (i.e., the control) inoculated with AMF. Figure 5. 5 shows the roots with no AMF colonisation of some samples from the three potato growth stages. These images can be compared with others with clear evidence of infection (see Chapter 3, Figure 3. 5)

**Stolon initiation stage**
(30 days after planting)




**Tuber initiation stage**
(60 days after planting)




**Tuber filling stage**
(90 days after planting)

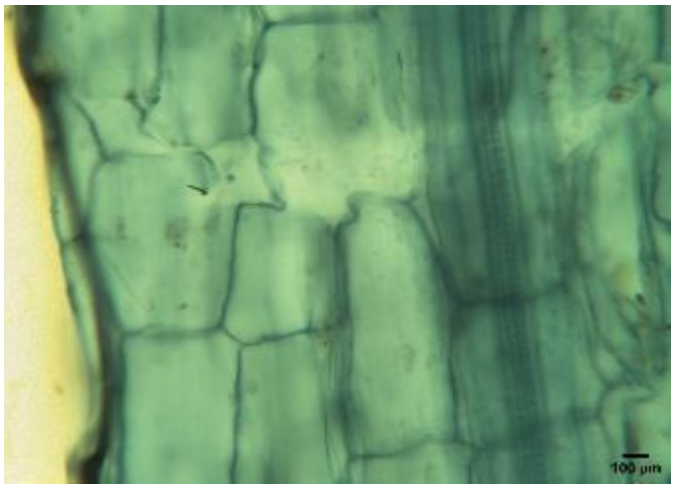



**Figure 5. 5** Samples of roots stained with an ink-vinegar solution from three potato growth stages in this study, showing the absence of AMF colonisation.

### *5.4.2 AMF diversity and community analysis in the growing medium (John innes No. 1), AMF inoculant and control*

#### General composition of the fungal community

A total of 62,935 fungal sequences and 129 ASVs were obtained. The number of ASVs and sequence library abundance in each phylum of the Kingdom Fungi are shown in Table 5. 1. There were eight phyla (Ascomycota, Basidiomycota, Glomeromycota, Chytridiomycota, Mortierellomycota, Mucoromycota, Olpidiomycota, and Rozellomycota) and 1 group of

unclassified fungi. Phylum Ascomycota was the main fungi identified in this study with 63 ASVs and 40,948 sequences, whereas there were 22 ASVs of phylum Glomeromycota (AMF) with 7,870 sequences.

**Table 5. 1** The number of ASVs and sequence library abundance in each phylum of Kingdom Fungi.

| Phylum | Number of ASVs | Sequence library abundance |
|---|---|---|
| 1. Ascomycota | 63 | 40948 |
| 2. Basidiomycota | 22 | 12472 |
| 3. Glomeromycota | 22 | 7870 |
| 4. Chytridiomycota | 1 | 14 |
| 5. Mortierellomycota | 12 | 631 |
| 6. Mucoromycota | 1 | 4 |
| 7. Olpidiomycota | 2 | 127 |
| 8. Rozellomycota | 2 | 67 |
| 9. Unclassified fungi | 4 | 802 |
| **Total** | **129** | **62935** |

According to fractional abundances shown in Figure 5. 6, phylum Ascomycota and Glomeromycota were the main groups of fungi identified in the AMF inoculant from PlantWorks Ltd. Whereas, phylum Ascomycota was the main group in the AMF inoculant *Rhizophagus irregularis* MUCL41833 provided by the Declerck laboratory and the fractional abundance of Glomeromycota was less than 0.1.

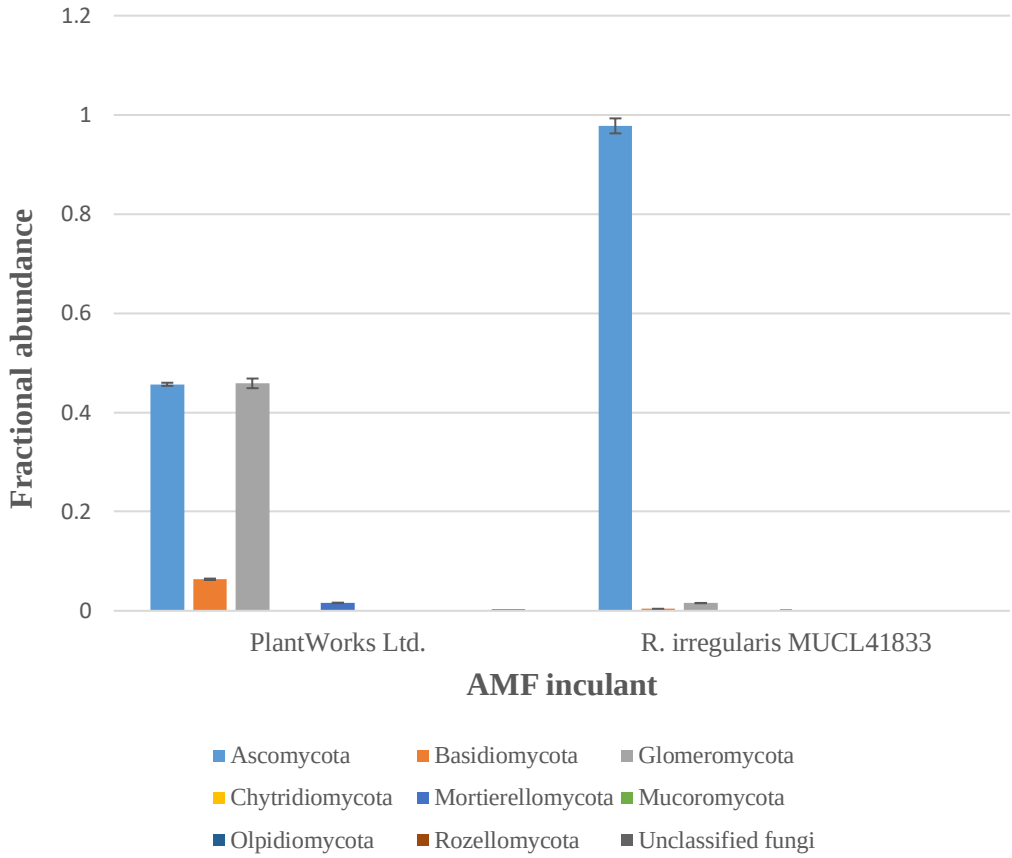



**Figure 5. 6** The distribution of sequences among each of the fungal phylum in AMF inoculants PlantWorks Ltd. and *R. irregularis* MUCL41833 (expressed as a fraction of the total sequences in the Kingdom fungi). Values represent the means. Error bars±SE.

**General composition of the AMF community**

According to Table 5. 2, from a total of 22 ASVs and 7,870 sequences of phylum Glomeromycota, there were only two AMF species (*Rhizophagus irregularis* and unclassified *Rhizophagus* species) in the family Glomeraceae. The highest number of ASVs and sequences of both AMF species were present in the AMF inoculant from PlantWorks Ltd. Unclassified *Rhizophagus* species was present in only the AMF inoculant from PlantWorks Ltd. No AMF was detected in the growing medium (John Innes No. 1).

According to the fractional abundance data shown in Figure 5. 7, *Rhizophagus irregularis* and unclassified *Rhizophagus* species were highest in AMF inoculant from PlantWorks Ltd. No AMF were present in the John Innes No. 1 growing medium.

**Table 5. 2** The number of ASVs and sequence library abundance of each inoculant and growing medium.

| Species | | Control | AMF (PlantWorks Ltd.) | *R. irregularis* MUCL41833 | John Innes No. 1 |
|---|---|---|---|---|---|
| *Rhizophagus irregularis* | No. of ASVs | 1 | 8 | 6 | 0 |
| | Sequence library abundance | 54 | 3938 | 508 | 0 |
| unclassified *Rhizophagus* species | No. of ASVs | 0 | 7 | 0 | 0 |
| | Sequence library abundance | 0 | 3370 | 0 | 0 |

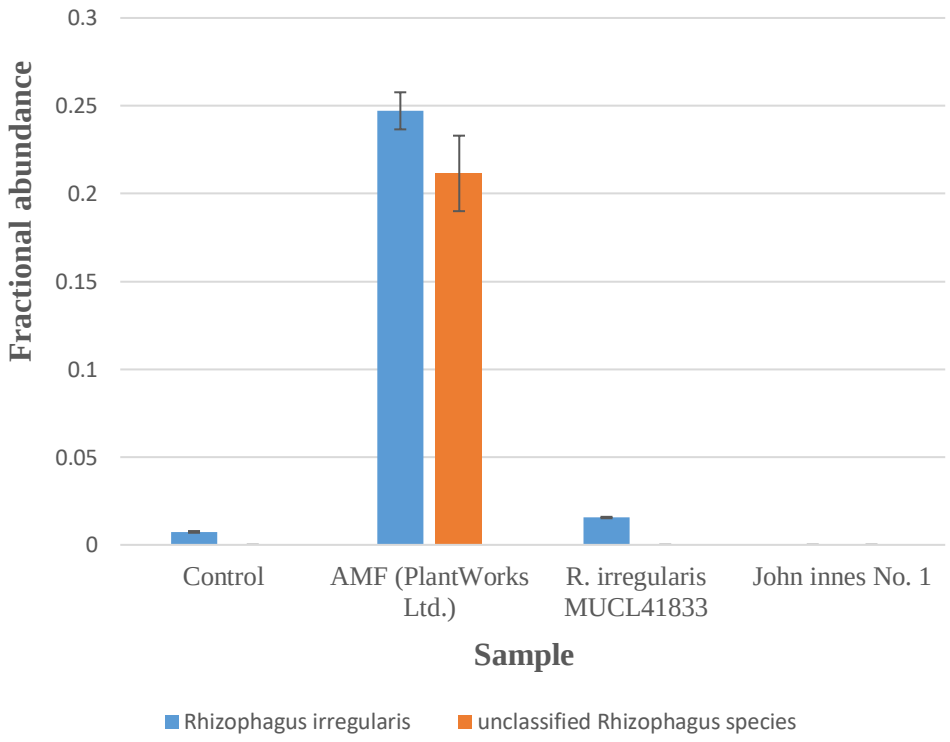



**Figure 5. 7** The species composition of the AMF community in the growing medium (John Innes No. 1), AMF inoculants, and control (expressed as a fraction of the total sequences in each species of *Rhizophagus* within the Kingdom Fungi). Values represent the means. Error bars±SE.

### *5.4.3 AMF diversity and community analysis in potato roots*

**General composition of the fungal community**

A total of 721,434 sequences and 877 ASVs were obtained. The number of ASVs and sequence library abundance in each phylum of the Kingdom Fungi are shown in Table 5. 3. There are eight phyla (Ascomycota, Basidiomycota, Glomeromycota, Mortierellomycota, Rozellomycota, Olpidiomycota, Monoblepharomycota, and Mucoromycota) and 1 group of unclassified fungi. Phylum Ascomycota is the main fungi in this study with 463 ASVs and 483,995 sequences, whereas there are 13 ASVs of phylum Glomeromycota (AMF) with 208 sequences. The percentage of ASVs in each phylum is presented in Figure 5. 8. The highest percentage of ASVs is for the phylum Ascomycota at 52.79%, while the phylum Glomeromycota (AMF) represents just 1.48% of the ASVs identified.

**Table 5. 3** The number of ASVs and sequence library abundance in each phylum of Kingdom Fungi.

| Phylum | Number of ASVs | Sequence library abundance |
|---|---|---|
| 1. Ascomycota | 463 | 483995 |
| 2. Basidiomycota | 288 | 219803 |
| 3. Unclassified fungi | 71 | 14588 |
| 4. Glomeromycota | 13 | 208 |
| 5. Mortierellomycota | 27 | 1920 |
| 6. Rozellomycota | 3 | 104 |
| 7. Olpidiomycota | 6 | 617 |
| 8. Monoblepharomycota | 1 | 3 |
| 9. Mucoromycota | 5 | 196 |
| **Total** | **877** | **721434** |

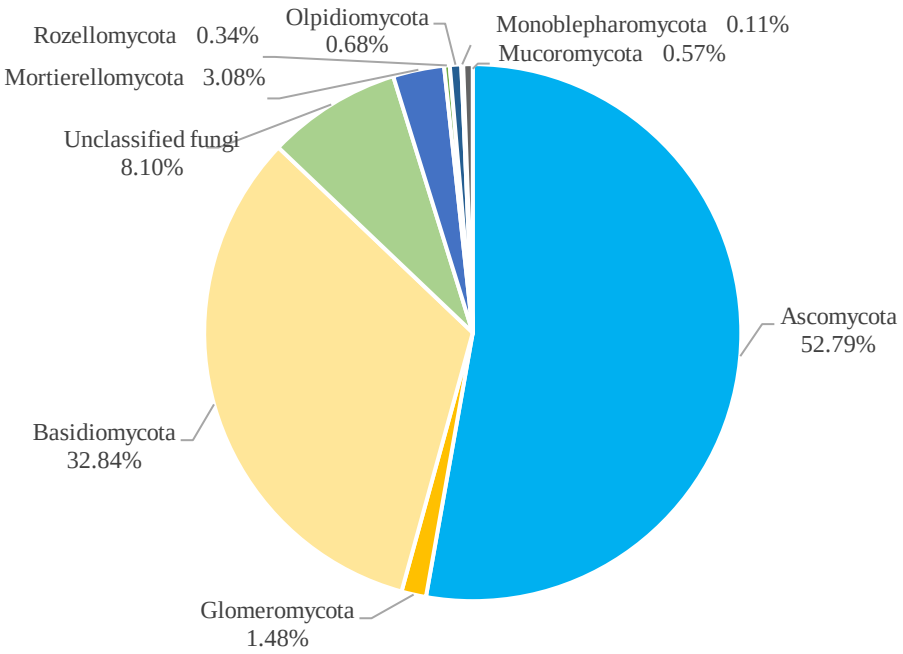



**Figure 5. 8** The percentage of ASVs in each phylum of Kingdom Fungi found in potato roots from a glasshouse trial with and without AMF inoculant.

The highest fractional abundance in each phylum of the Kingdom Fungi was for Ascomycota (Figure 5. 9), whereas the fractional abundance of phylum Glomeromycota was less than 0.1.

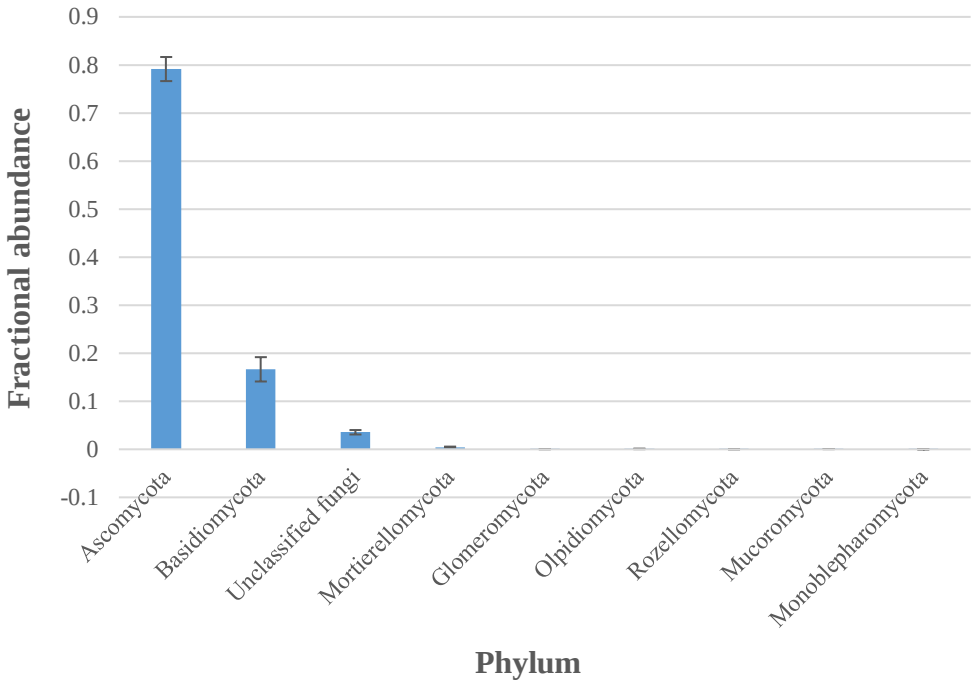



**Figure 5. 9** The composition of the fungal community (expressed as a fraction of the total sequences within the Kingdom Fungi) in potato roots. Values represent the means of all samples. Error bars±SE.

**General composition of the AMF community**

According to Table 5. 4, from a total of 13 ASVs and 208 sequences of phylum Glomeromycota, there was only one family Glomeraceae represented with two species, *Rhizophagus irregularis,* and unclassified *Rhizophagus* species in this study. The number of ASVs and sequence library abundance of *Rhizophagus irregularis* was higher than unclassified *Rhizophagus* species

The fractional abundance of the two species (Figure 5. 10) shows that slightly more ASVs were classified as unclassified *Rhizophagus* species compared to *Rhizophagus irregularis.*

**Table 5. 4** The number of ASVs and sequence library abundance in each species of the family Glomeraceae.

| Family | Species | Number of ASVs | Sequence library abundance |
|---|---|---|---|
| Glomeraceae | *Rhizophagus irregularis* | 9 | 114 |
| | unclassified *Rhizophagus* species | 4 | 94 |
| | **Total** | **13** | **208** |

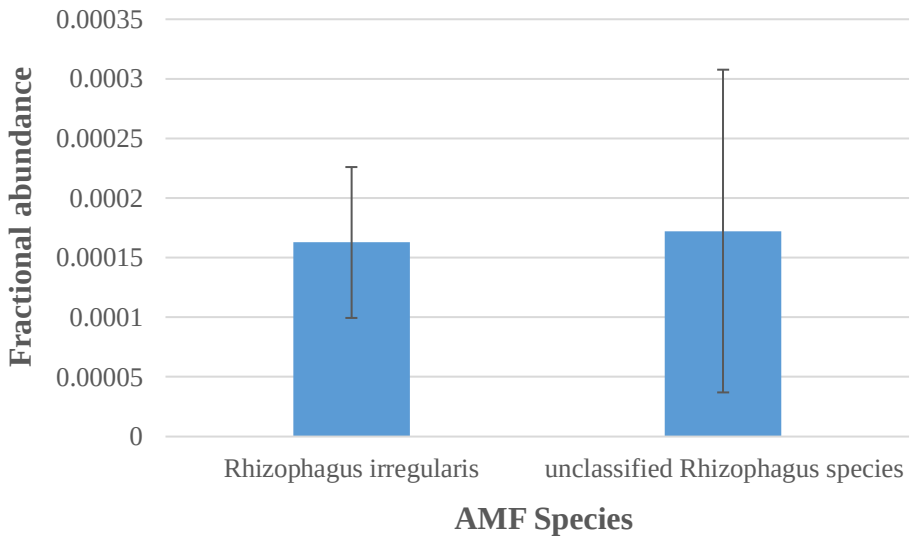



**Figure 5. 10** The species composition of the AMF community (expressed as a fraction of the total sequences in each species of *Rhizophagus* within the Kingdom Fungi). Values represent the means. Error bars±SE.

**Fungal taxonomic composition**

The taxa bar plots of fractional abundance of all fungal phyla in all potato root samples for the two potato varieties Sheriff and Shelagh (Figure 5. 11 and Figure 5. 12) showed that the dominant fungi in most samples were in the phylum Ascomycota. However, the dominant fungi of some samples in potato variety Shelagh was from the phylum Basidiomycota. Seven root samples were detected the AMF (Glomeromycota) colonisation in only the treatment receiving the inoculant from PlantWorks Ltd., namely: three potato plants of the variety Sheriff (Figure 5. 11) and four potato plants of the variety Shelagh (Figure 5. 12). These seven root samples that sampled during the tuber initiation stage and tuber filling stage (60 and 90 days after planting, respectively). The AMF (Glomeromycota) showed relatively small quantities in both potato varieties when compared to the phylum Ascomycota.

**AMF taxonomic composition**

According to the taxa bar plots showing the relative abundances of AMF genera within the phylum Glomeromycota (Figure 5. 13), only one genus (*Rhizophagus*) was detected in seven root samples.

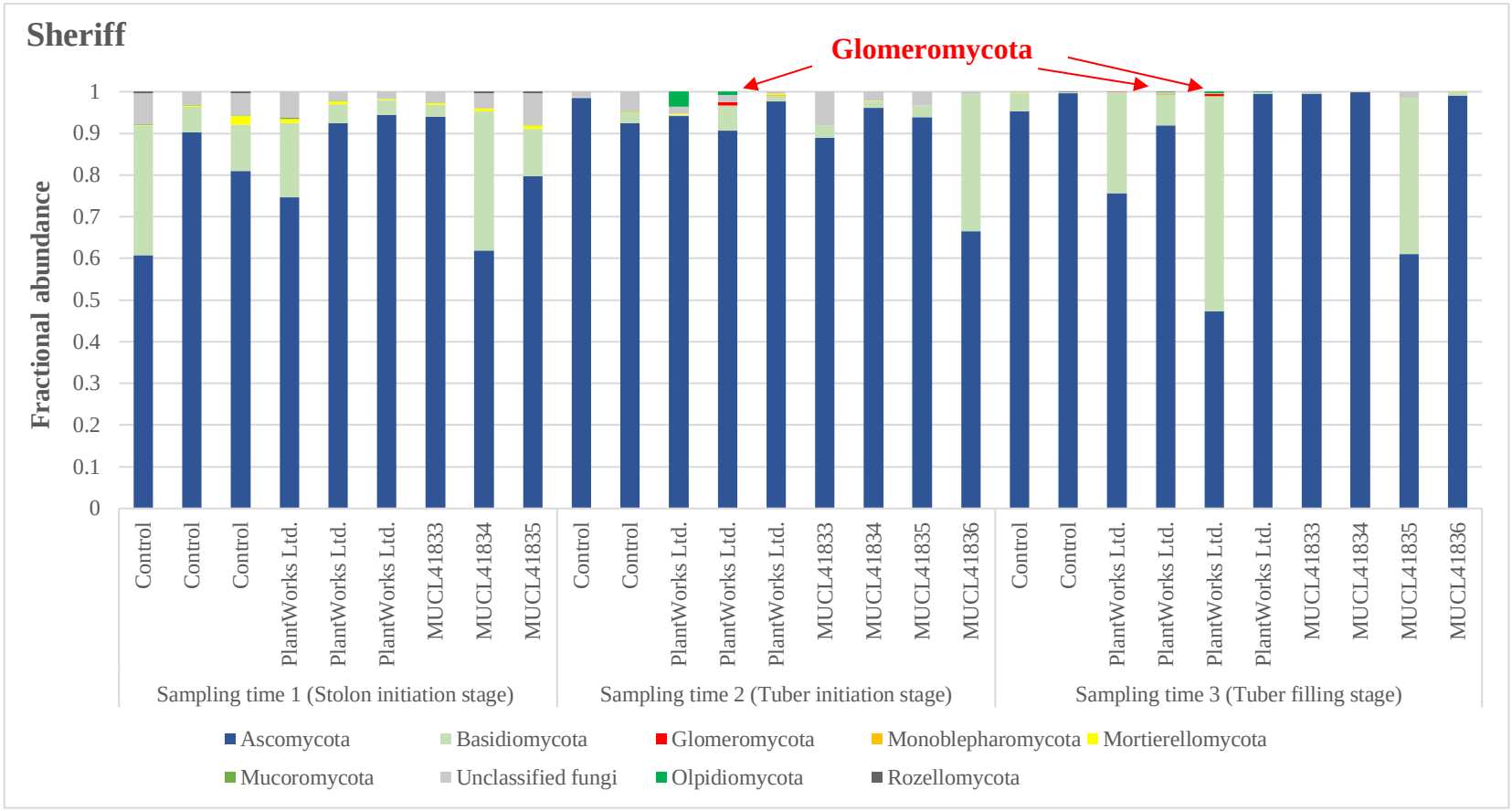



**Figure 5. 11** Taxa bar plots of the relative abundances in each phylum of Kingdom Fungi related sequences identified in amplicon libraries obtained from the potato varieties Sheriff. Potato root samples plotted against the relative abundance.

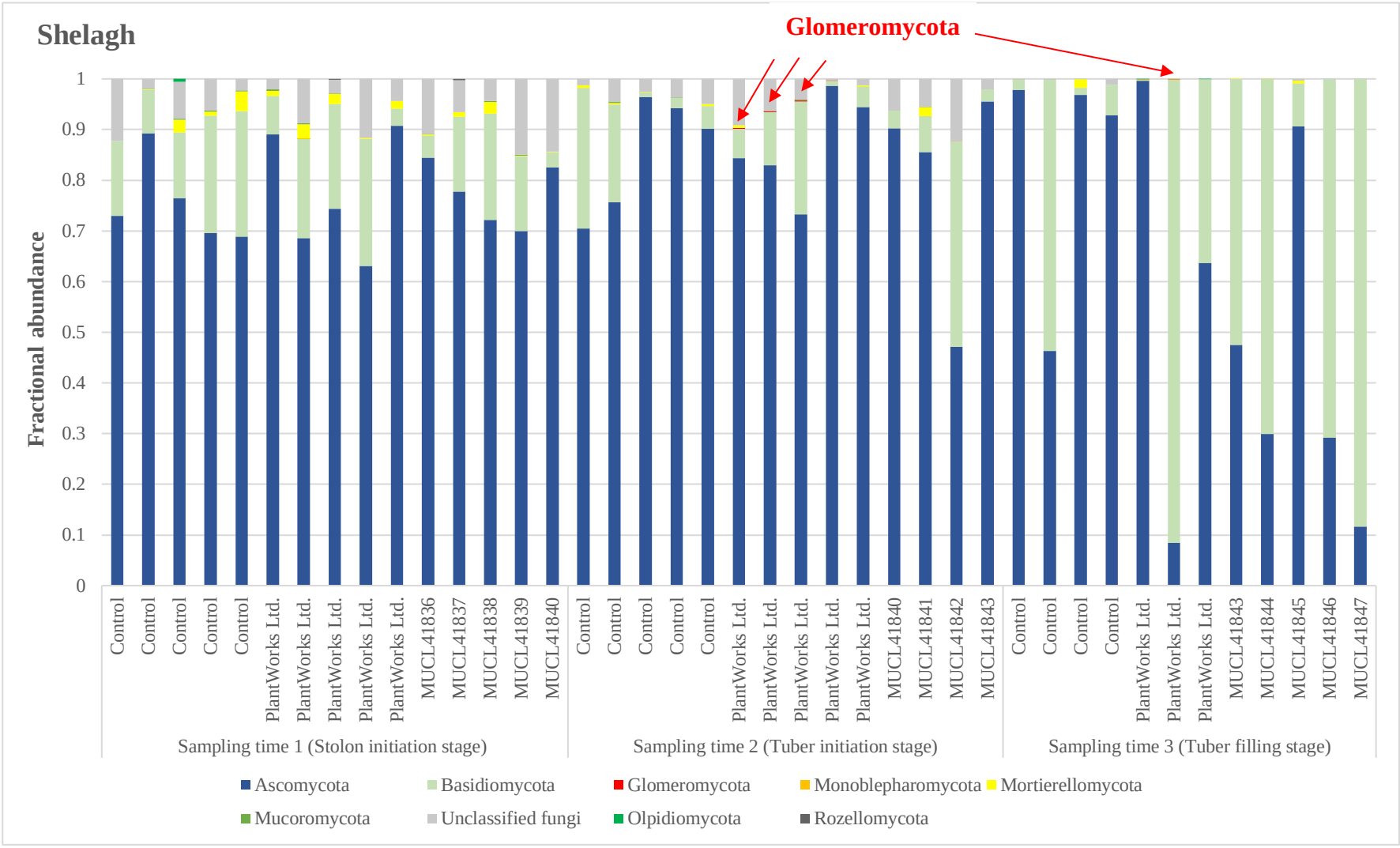



**Figure 5. 12** Taxa bar plots of the relative abundances in each phylum of Kingdom Fungi related sequences identified in amplicon libraries obtained from the potato varieties Shelagh. Potato root samples plotted against the relative abundance.

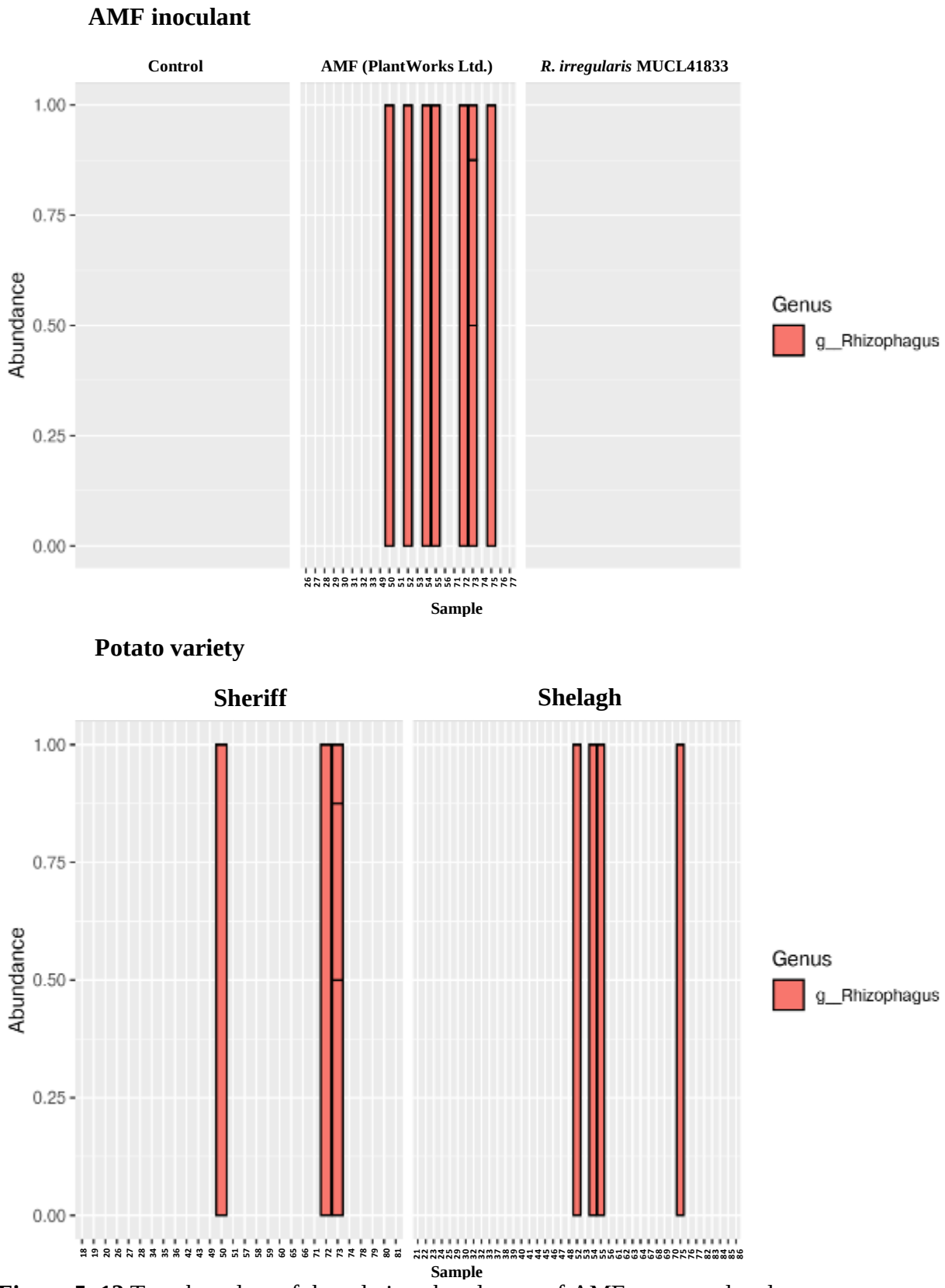



**Figure 5. 13** Taxa bar plots of the relative abundances of AMF genera-related sequences identified in amplicon libraries presented for each inoculant treatment (top figure) and each potato variety (bottom figure). Potato root samples plotted against the relative abundance; empty columns indicate that no AMF genera were detected in those samples.

### *5.4.4 Effect of AMF inoculant, potato variety, and potato growth stage on above-ground plant biomass, plant P concentration, and plant P uptake*

There were no significant effects of AMF inoculant treatment on above-ground plant biomass (fresh weight and dry weight), plant P concentration, or plant P uptake (Table 5. 5). Furthermore, potato variety had no significant effect on plant biomass (fresh weight and dry weight) and plant P uptake. However, potato variety was found to have a significant impact on plant P concentration. The potato variety "Shelagh" had a significantly higher P concentration than "Sheriff."

In contrast, the potato growth stage was shown to have a significant effect on all the agronomic parameters, including above-ground plant biomass (fresh weight and dry weight), plant P concentration, and plant P uptake. The tuber initiation stage (60 days after potato planting) showed the highest above-ground plant biomass (fresh weight and dry weight) and plant P uptake whereas, the stolon initiation stage (30 days after potato planting) showed the highest plant P concentration.

A significant interaction was observed between potato variety and potato growth stage for above-ground plant biomass (fresh weight) (Figure 5. 14). For both potato varieties, "Sheriff" and "Shelagh" above-ground plant biomass (fresh weight) was highest at the tuber initiation stage compared to the other two potato growth stages (stolon initiation stage and tuber filling stage). At the tuber initiation stage, above-ground plant biomass (fresh weight) for the "Sheriff" variety was significantly higher than "Shelagh." In contrast, at the tuber filling stage, "Shelagh" had above-ground plant biomass (fresh weight) significantly higher than "Sheriff." There were no significant differences in the above-ground plant biomass (fresh weight) at the stolon initiation stage for both potato varieties.

**Table 5. 5** Effect of each AMF inoculant, potato variety, and potato growth stage on above-ground plant biomass (fresh weight and dry weight), plant P concentration, and plant P uptake in potato (Main effect means±SE, ANOVA p-values, n=90). Means followed by the same letter within the same column and experimental factor are not significantly different at the level of $p<0.05$.

| Factor | Plant biomass (g/plant FW) | Plant biomass (g/plant DW) | Plant P concentration (mg $g^{-1}$ DW) | Plant P uptake (g/plant) |
|---|---|---|---|---|
| **AMF inoculant (AMF)** | | | | |
| Control with no AMF | 84.27±7.32 | 8.65±0.72 | 3.36±0.29 | 0.028±0.003 |
| PlantWorks Ltd. | 90.60±6.45 | 9.90±0.54 | 3.25±0.29 | 0.031±0.003 |
| *Rhizophagus irregularis* MUCL41833 | 87.68±7.55 | 9.77±0.62 | 3.01±0.34 | 0.028±0.003 |
| **Potato variety (VA)** | | | | |
| Sheriff | 87.14±8.37 | 10.17±0.67 | 2.84±0.25 | 0.028±0.003 |
| Shelagh | 87.94±3.93 | 8.99±0.39 | 3.44±0.24 | 0.030±0.002 |
| **Potato growth stage (PGS)** | | | | |
| Stolon initiation stage | 81.25±5.94 b | 7.78±0.65 b | 4.71±0.16 a | 0.035±0.003 a |
| Tuber initiation stage | 114.73±6.12 a | 11.65±0.55 a | 3.31±0.11 b | 0.038±0.002 a |
| Tuber filling stage | 66.21±5.07 b | 9.04±0.33 b | 1.42±0.09 c | 0.013±0.001 b |
| **ANOVA p-values** | | | | |
| AMF | 0.8506 | 0.3582 | 0.1543 | 0.6285 |
| VA | 0.9913 | 0.1380 | **0.0008** | 0.4890 |
| PGS | **<0.0001** | **<0.0001** | **<0.0001** | **<0.0001** |
| AMF x VA | 0.9645 | 0.7850 | 0.4002 | 0.6290 |
| AMF x PGS | 0.5472 | 0.7881 | 0.6263 | 0.1976 |
| VA x PGS | **0.0068** | 0.8317 | 0.1445 | 0.7401 |
| AMF x VA x PGS | 0.0867 | 0.3639 | 0.9112 | 0.4145 |

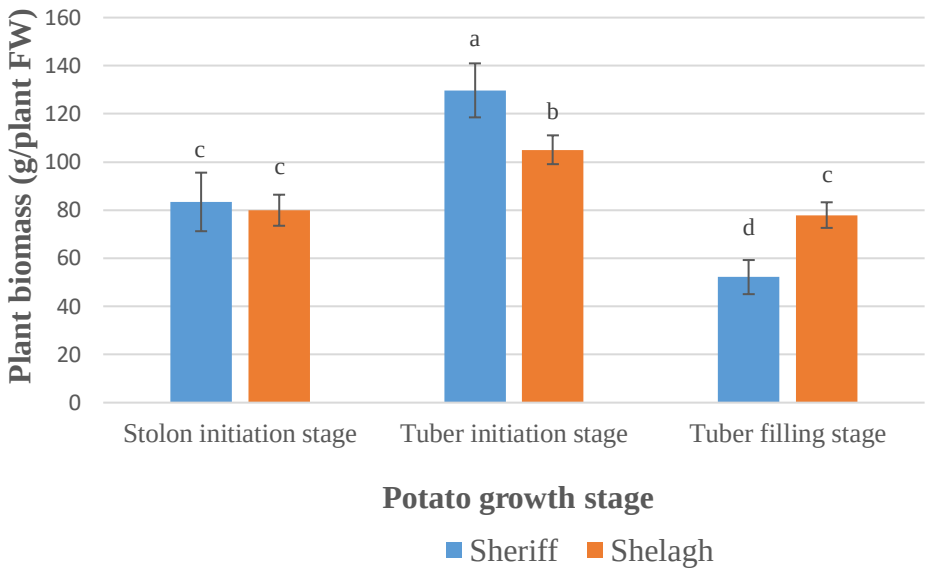



**Figure 5. 14** Effect of potato variety (Sheriff and Shelagh) and potato growth stage on above-ground plant biomass (fresh weight). Bars labelled with the same letter are not significantly different (Tukey's honestly significant difference test, $p<0.05$). Standard errors of the mean (error bars) were calculated from all five-pot replicates.

### *5.4.5 Tuber yield, tuber P concentration, and tuber P uptake*

As shown in Table 5. 6, AMF inoculant and potato variety did not affect tuber yield, tuber P concentration, or tuber P uptake. There were also no significant interactions between the AMF inoculant and potato variety for tuber yield, tuber P concentration, and tuber P uptake.

**Table 5. 6** Effect of each AMF inoculant and potato variety on tuber yield, tuber P concentration, and tuber P uptake in tuber filling stage (90 days after planting) (Main effect means±SE, ANOVA p-values, n=30).

| Factor | Tuber yield (g/plant) | Tuber P concentration (mg $g^{-1}$ DW) | Tuber P uptake (g/plant) |
|---|---|---|---|
| **AMF inoculant (AMF)** | | | |
| Control with no AMF | 56.16±16.27 | 2.61±0.73 | 0.03±0.01 |
| PlantWorks Ltd. | 67.42±15.45 | 3.23±0.74 | 0.05±0.01 |
| *Rhizophagus irregularis* MUCL41833 | 81.16±12.23 | 3.54±0.43 | 0.06±0.01 |
| **Potato variety (VA)** | | | |
| Sheriff | 63.23±12.97 | 2.82±0.57 | 0.04±0.01 |
| Shelagh | 73.27±11.08 | 3.44±0.48 | 0.05±0.01 |
| **ANOVA p-values** | | | |
| AMF | 0.5040 | 0.5820 | 0.2474 |
| VA | 0.5652 | 0.4094 | 0.5114 |
| AMF x VA | 0.3658 | 0.2137 | 0.3946 |

## 5.5 Discussion

### *5.5.1 The potential and function of AMF inoculant in potato*

The primary purpose of this study was to examine the effects of two AMF inoculants on the growth of the potato. The AMF genus *Rhizophagus* was detected in both inoculants identified by NGS. However, after inoculation with these two AMF inoculants in potato, *Rhizophagus* colonisation was only detected in the treatment receiving the inoculant from PlantWorks Ltd. in both potato varieties; the MUCL41833 inoculant did not colonise any root samples. It seems that only PlantWorks Ltd. inoculant could successfully colonise the roots of either of the potato varieties.

An additional factor that could have caused colonisation differences between the two treatments had they both contained similar populations of AMF, was the inoculation technique, which differed between the two inoculants. The PlantWorks Ltd inoculant was mixed with the growing medium, whereas the *Rhizophagus irregularis* MUCL41833 was placed below the tuber. Placing the inoculant underneath the tuber could be not suitable for inoculation; it may have been difficult for the developing roots of the potato to come in contact with the colonised maize roots of *Rhizophagus irregularis* MUCL41833. In addition, the propagules of spores and hyphae of AMF inoculant can be easily leached from the pot with watering (Loján *et al.*, 2017).

Another possible reason for the different efficiency for two inoculants is maybe from the very different fractional abundances of the AMF sequences in both inoculants. AMF species in the PlantWorks Ltd inoculant were higher than the AMF species in MUCL41833 inoculant by more than ten times (Figure 5. 7).

Several studies indicate that indigenous AMF species are more effective than commercial inoculant species. This could be because the conditions provided in the pot culture did not relate to the natural environment, for example, the substrate type (Faye *et al.*, 2013; Selvakumar *et al.*, 2018). The John Innes No. 1 was used as the substrate in this study. Peat moss is one of the components in this substrate. There are studies showing both positive and negative impacts of peat moss on AMF (Ma *et al.*, 2007). It is also possible that the AMF genus *Rhizophagus* in both commercial inoculants was not compatible with the two host plants of potato varieties, Sheriff and Shelagh. Whereas the indigenous AMF *Paraglomus* was the dominant genus colonised in both potato varieties in the field experiment.

Although one genus of *Rhizophagus* (formerly *Glomus*) was detected by NGS in the PlantWorks treatment, the root staining technique did not indicate any AM fungal structures in the roots. Morton and Redecker (2001) explain that several AMF may not be stained at all within the roots using the standard dyes. It seems that some *Glomus* species (*G. occultum*, *G. brasilianum*, *G. clarum*, *G. diaphanum*, and *G. intraradices*) are difficult to detect in roots by standard staining (Dodd *et al.*, 2000; Millner *et al.*, 2001).

Although I detected some AMF DNA in the PlantWorks Ltd. granular inoculant, there was no evidence of root colonisation or promotion of potato growth. Apparently, the AMF failed to form the mycorrhizal associations in almost all root samples since there were only seven root

samples in which *Rhizophagus* sp. were detectable by sequencing. Since no AMF structures were present in the roots, the association detected by NGS was unlikely to have resulted in any benefits to crop growth.

### *5.5.2 The effect of potato variety on abundance and diversity of AMF inoculant*

In this study, potato variety did not affect AMF (*Rhizophagus*) colonisation. Potato seeds of the two potato varieties were from the 2017 field experiment reported in Chapter 4; in this earlier study, it was found that the main AMF genus which formed associations in both potato varieties in the field experiment was *Paraglomus*. This genus was also found to dominate in the bulk soil from this field (see Fig. 4.9 and Fig. 4.10). It is possible that the potato varieties in this study may have a specific symbiosis with the native AMF genus *Paraglomus* (Cesaro *et al.*, 2008). The host plant can affect AMF by managing carbon allocation to roots and secondary metabolite production. Also, the host preferences and the varying symbiotic efficiency of AMF may be from the AMF secreted proteins, which act as a fungal effector to control symbiotic efficiency (Zeng *et al.*, 2018). The host preference of AMF has been presented in many reports (Helgason *et al.*, 2002; Vandenkoornhuyse *et al.*, 2003; Torrecillas *et al.*, 2012; Van Der Heijden *et al.*, 2015; Van Geel *et al.*, 2016b). However, there is no publication available in the literature which documents a host preference between *Paraglomus* and the potato varieties Sheriff and Shelagh. Only one report presented that *Paraglomus* is the main genus found in the potato variety "Kennebec" in Argentina (Stephan *et al.*, 2019).

However, potatoes have quite a broad range of potential AMF partners, as described by Senés-Guerrero and Schüßler (2016), and an individual plant root system is usually colonised by several AMF species. Potato colonised with several AMF genera including *Rhizophagus irregularis* (formerly *Glomus intraradices*) was also reported (McArthur and Knowles, 1992; Davies Jr *et al.*, 2005; Senés-Guerrero and Schüßler, 2016). Hence, it is possible that no colonisation by AMF inoculants was detected in this experiment was because the inoculants included a limited number of AMF strains. Inoculants with only a few very closely related strains are much less likely to produce a match between the plant and AMF.

Because of the low host specificity of AMF reported in the literature (Clapp *et al.*, 1995; Santos *et al.*, 2006; Lee *et al.*, 2013; Muneer *et al.*, 2019), competition between the AMF and other fungal groups within inoculant may be the most important factor affecting colonisation of the potato roots by AMF species in the inoculants. The NGS results clearly demonstrated

that the dominant fungi in the MUCL41833 were in the phylum Ascomycota (Figure 5. 6), and after inoculation, Ascomycota was also the main fungal group colonising in potato roots (Figure 5. 11 and Figure 5. 12). For the PlantWorks Ltd. inoculant, although the proportions of AMF species (Glomeromycota) and Ascomycota were equal based on the fractional abundance results (Figure 5. 6), the Ascomycota represented a more diverse range of genera than Glomeromycota, which only had one genus (data not shown). Increasing the diversity of Glomeromycota species in the inoculant should be an option to increase the probability of colonisation.

### *5.5.3 The effect of potato growth stage on abundance and diversity of AMF inoculant*

DNA from *Rhizophagus* was detected at two stages of potato growth: tuber initiation stage and tuber filling stage (60 and 90 days after planting, respectively) for both potato varieties, but not at the stolon initiation stage. This could be related to fungal development. The development of *Rhizophagus irregularis* colonisation was studied by Chaudhary *et al.* (2019), who reported that it is comprised of three stages: initiation (20 days; pre-infection stage of hyphae), progression (40 and 60 days; arbuscule and vesicle development), and maturity stage (40, 60 and 80 days; extraradical spores production). In this study, the root sampling for the first time occurred at 30 days, which may have been too early to detect any AMF at all, as evidenced by the lack of fungal structures visualised in the roots.

## 5.6 Conclusion

Both AMF inoculants had no beneficial impact on potato growth; the inoculants apparently failed to colonise, indicating that they may not be compatible with their potential host plants, i.e., potatoes (Elliott *et al.*, 2019). The lack of viable AMF species in two of the inoculants may also have been a factor explaining the lack of colonisation for these treatments. Using local soil with a diversity of AMF species may increase plant growth more productively than the commercial products (Emam, 2016). Another point is the lack of AMF diversity in the inoculant; both inoculants included only one genus of AMF (Figure 5. 7). Several reports have shown that the association of AMF and plants may not always be species-specific (Santos *et al.*, 2006; Öpik *et al.*, 2009; Torrecillas *et al.*, 2012). Thus, in this study, the inoculant probably needs to have several AMF taxa in the different genera to increase the probability of a match.

# CHAPTER 6. General discussion

## 6.1 General introduction

AMF are known as beneficial soil fungi that improve plant nutrient uptake, especially P, whereas potato is a P-demanding plant. Several reports have shown the positive influences of AMF on potatoes in various varieties and different areas. Recently, there has been increasing interest in the effect of AMF on many plants, including potato. Many researchers have proposed the use of AMF in cropping systems. AMF can be managed by two main approaches, the first is using inoculum of selected AMF strains, and the second is adopting agricultural practices that promote indigenous fungi (Roy-Bolduc and Hijri, 2011). The management of agricultural practices to support indigenous AMF symbiosis are important in cropping system to maintain the AM fungal community (Plenchette *et al.*, 2005; Van Geel *et al.*, 2016a). Management practices in agricultural soils could promote indigenous AMF abundance and AMF diversity. Because of the importance of AMF for promoting plant nutrient uptake and plant productivity, many studies have focused on the response of AMF abundance and their community to crop management.

In this thesis, the main aim was to understand the influence of crop management practice and potato variety on species abundance and the diversity of AMF. This study also assessed the impact of AMF on the agronomic performance of potatoes in the field and under glasshouse conditions. Two AMF inoculants for growth enhancement in potatoes under controlled conditions in the glasshouse were also investigated. The abundance and diversity of AMF were assessed using the traditional root staining method and molecular methods (qPCR, T-RFLP, and Illumina MiSeq NGS). A total of twenty-two potato varieties were selected to use in this study. AMF function in field and glasshouse was evaluated from the agronomic data, i.e., P concentration, total P uptake, plant biomass, and tuber yield (Daniell *et al.*, 2001; van de Wiel *et al.*, 2016).

## 6.2 Crop management practice

The first hypothesis that crop management practice would affect the abundance and diversity of AMF was confirmed under field conditions in this study. Fertility, organic crop protection, and conventional crop protection were employed in the initial study (Chapter 3) to investigate

the effect of mineral fertilisers (NPK) and conventional pesticides on AMF abundance and AMF diversity. The findings clearly showed that organic crop management practices significantly enhance AMF colonisation and AMF diversity in potato, whereas mineral fertiliser NPK significantly decrease AMF abundance measured by root staining and AMF diversity by assessed by NGS. The pesticides increased AMF abundance indicated by root staining but did not impact on AMF diversity.

As expected, several previous studies have reported the negative effects of mineral fertilisers on AMF as found in this thesis, including P and N. In addition, the development and diversity of AMF are often decreased by fertiliser combinations, such as P, N, NP, and NPK (Wang *et al.*, 2011). The colonisation and species diversity are often reported to be higher in the organic systems compared to conventional systems (Mäder *et al.*, 2000; Oehl *et al.*, 2004). Decreasing AMF abundance when fertilisers are applied may be due to mineral fertilisers reducing AMF development to form the symbiosis with the host plants under field conditions. Wang *et al.* (2011) proposed that the negative effects of fertilisation on AMF may be caused by changes in soil properties, which may influence the formation of AMF symbiosis. This is because mineral fertilisers inhibit the length and density of AMF hyphae in soils, which most likely resulted in reducing the colonisation of the host plant roots (Gryndler *et al.*, 2006; Sheng *et al.*, 2013). However, this study did not assess the length and density of AMF hyphae in soils.

An increased level of soil available P in soil generally decreases the abundance and diversity of AMF, as illustrated in Chapter 4. High soil P concentration is often reported in the literature to reduce root colonisation, as shown by the results in Table 4. 4. The negative effect of soil P availability on AMF abundance and AMF diversity is also indicated by the P index of soils before potato planting. The P index of soils before potato planting in Chapter 3 was higher than Chapter 4 (P index of 3 in Chapter 3 and 0 or 1 in Chapter 4). When comparing the results of the fractional abundance of all AMF genera to the total fungal sequences between the control treatment (no fertilisers) in Chapter 3 (Fractional abundance=$578.70 \times 10^{-5}$, Table 3. 16) and the without P treatment in Chapter 4 (Fractional abundance=$574.01 \times 10^{-4}$, Table 4. 11), the fractional abundance of AMF in the control treatment when the P index was 3, was ten times lower than the without P treatment in Chapter 4 when the P index was much lower.

In field conditions, the fungicides are sprayed on the above-ground plant parts to control potato blight. The AMF colonisation in the root cells was not affected by these fungicides.

Several reports have shown the negative or positive influences of pesticides on the abundance and diversity of AMF. The impact of pesticides on AMF associations is complex and not as clearly predictable (Kurle and Pfleger, 1994; Schreiner and Bethlenfalvay, 1997). Several pesticides can cause significant reductions in AMF colonisation and sporulation, which depends on the fungal species. However, some pesticides have a positive effect on the AMF associations.

### 6.3 Potato variety

To study the effect of potato variety and their interaction with crop management practice on the abundance and diversity of AMF was the second hypothesis. Interestingly, the results from Chapter 3 and 4 showed that there was no significant interaction between crop management practice (mineral NPK fertilisers, pesticides, and P fertiliser applied) and potato variety (22 varieties) for the abundance and diversity of AMF. Also, there were no significant differences among the potato varieties for either the abundance or the diversity of AMF. This is explained by several studies which indicate that AMF has no or low host specificity in their symbiosis with plants (Clapp *et al.*, 1995; Santos *et al.*, 2006; Lee *et al.*, 2013; Muneer *et al.*, 2019). The results from Chapter 3 and Chapter 4 clearly demonstrated that the main factor affecting AMF and potato associations is the crop management practice, not potato variety. The main genus in the field, *Paraglomus,* is the dominant genus in potato roots. Potato variety has no impact on AMF abundance and diversity. This is as discussed in section 3.5.4; the main factor that affects the AMF community is an abiotic factor. AMF have very low partner specificity in plant species and plant variety.

### 6.4 Indigenous AMF and AMF inoculants on the growth of potato

In this study, the agronomic data were analysed to clarify the third hypothesis for assessing the effects of indigenous AMF and AMF inoculants on the growth of potato in the field and glasshouse, respectively. The results in Chapter 4 clearly demonstrated that the presence of AMF can compensate for low P supply, which suggests that their hyphae enhanced P supply in the without P treatments. Interestingly, in the 2017 experiment, the potato variety “Sheriff” showed the highest AMF colonisation compared to other potato varieties (Table 4. 4) and also had the highest tuber yield (Figure 4. 23). It seems that AMF can support the P uptake, which increases the tuber yield. This is presented in several studies; P uptake and P use efficiency

play an important role in enhancing potato yield (Rosen and Bierman, 2008; Choudhary *et al.*, 2019; Cui *et al.*, 2020; Stark *et al.*, 2020). In the 2018 experiment, the potato variety "Sheriff" also had the highest tuber yield compared to other potato varieties (Figure 4. 23); however, in this experimental year, this variety did not have the highest root colonisation by AMF.

In terms of P use efficiency, indigenous AMF colonisation can increase P use efficiency under low P condition (no P fertilisers) by accessing P from soil especially in high yield potential group of potato variety. This enhancing P use efficiency can reduce P fertiliser input while maintaining high tuber yield.

It is cleared that *Paraglomus* is the main indigenous AMF in Nafferton farm's soil. *Paraglomus* is the dominant AMF genus found in all potato roots of all three field experiments (the year 2011, 2017, and 2018). According to the biogeography database of AMF by Stürmer *et al.* (2018), they proposed that *Paraglomus* has been demonstrated to have a cosmopolitan distribution (found in at least four of seven continents); *Paraglomus* was found in 6 continents except for only the Oceanian. Mello *et al.* (2013) also indicated that *Paraglomus* has a worldwide distribution.

The two AMF inoculants did not successfully colonise the potato roots. There was no evidence of colonisation of roots based on root staining methods, although some DNA from AMF was detected in roots using NGS. Focusing on the total fungal community of the two AMF inoculants before inoculating in potato, the main fungal group in the AMF inoculants from PlantWorks Ltd. was from the phylum *Ascomycota* and *Glomeromycota*, which presented an equal fractional abundance (Figure 5. 6). After inoculation, the results showed that the main fungal group in potato roots was from the phylum *Ascomycota* (Figure 5. 9). It can be assumed that the competition between AMF (*Glomeromycota*) and the *Ascomycota* was intense. Several studies have shown that many fungal genera in *Ascomycota* are endophytic fungi which can live in the tissues of the host plants such as roots, leaves, stems, fruits, and flower. Several fungal genera in the phylum *Ascomycota* had been reported as endophytic fungi in potato, both beneficial and plant pathogen (Posada and Vega, 2005; Posada *et al.*, 2007; Zimudzi *et al.*, 2018).

Because the AMF inoculant *Rhizophagus irregularis* MUCL41833 was supposed to be a pure culture colonised in maize roots, this inoculant may have been contaminated with *Ascomycota* when the inoculant was prepared. Berruti *et al.* (2017) explained that undesired contamination

with other fungi and bacteria is the most important factor to consider when preparing AMF inoculants.

### 6.5 Molecular techniques

Since the advancement to improve the primers in specific amplification of the AMF gene, several publications on AMF molecular studies have been presented. A variety of molecular techniques as well as different DNA marker regions have been applied to study the AMF and plant association. The molecular techniques including qPCR, T-RFLP and NGS had been used in this study. In fact, each molecular approach has its own strengths and weaknesses when applied to characterise the abundance and community structure of AMF. Namely, DNA-based techniques can vary based on sample type, DNA marker genes, and sequencing approach (Öpik and Davison, 2016). From the three methods used in this study, the different target DNA regions, i.e., SSU, LSU, and ITS, with varying primer sets were applied. All three DNA marker genes are widely used in the molecular study of AMF. A weakness of this study is that the specific primers that amplified the SSU and LSU regions cannot amplify the main genus *Paraglomus* which was the dominant species identified by NGS. This is due to both DNA markers have poor species resolution for some AM fungal groups, including Paraglomeraceae. In comparison, the generally accepted DNA marker for AMF is the ITS region (Yang *et al.*, 2012; Kolaříková *et al.*, 2021).

Nowadays, several primers targeting the different regions of rDNA have been used for DNA amplification. However, some primers are poor DNA amplification in some species of AMF, as shown in this study. All specific primers cannot amplify *Paraglomus.* A qPCR method using AMF-specific primers AMG1F and AM1 for SSU gene fragment quantification was cleared that this primer pair did not cover *Paraglomus* (Bodenhausen *et al.*, 2021). T-RFLP method using AMF-specific primers FLR3 and FLR4 for LSU gene fragment quantification also did not amplify the *Paraglomus.* On the other hand, the use of general ITS primers for NGS were advantageous as they can show AMF community structure. The SSU and LSU regions had limited species-level resolution compared with the ITS region. In terms of the primers, the determination of primer pairs would be a main important practice to clarify the community composition of AMF. This will overcome the limitation of molecular technique in AMF study.

Consequently, from the results of this study, the molecular approach NGS which used the universal fungal primers ITS1f and ITS2 for the ITS rDNA amplification, is suitable to amplify the main genus *Paraglomus*. The other molecular techniques, qPCR and T-RFLP, were also successful in rDNA region amplification; however, it should use the ITS primers for amplification so that *Paraglomus* were amplified. This study seems that the most important to select the molecular approach is based on the primer set. At present, the NGS technique with ITS primer set has been used to study AMF community compositions with success in several studies (Taylor *et al.*, 2016; Kryukov *et al.*, 2020; Suzuki *et al.*, 2020).

## 6.6 Conclusion

### *6.6.1 General conclusions*

According to the results presented in this thesis, the following conclusions can be drawn:

: Organic crop protection management (no mineral fertilisers and no conventional pesticides) significantly enhances AMF colonisation and the genetic diversity of the AMF community.

: The use of mineral fertiliser NPK, especially P significantly decreased the abundance and diversity of AMF. Whereas the use of conventional pesticides significantly increased the AMF abundance but had no impact on AMF diversity. Soil available P being the dominant factor affecting colonisation levels in the field.

: Potato variety and the interaction between potato variety and crop management practice did not impact the abundance and diversity of AMF.

: The highest AMF species abundance in soils and inoculants influence AMF colonisation in potato roots. The highest AMF species abundance are the dominant AMF species that colonise in potato roots.

: The indigenous AMF in the field can compensate for P uptake under low P conditions, although this study did not illustrate a strong relationship between AMF colonisation and P uptake or potato tuber yield.

: The indigenous AMF in the field enhance P use efficiency for potato under low P conditions. P use efficiency in this study presents to produce more yield in high yield potential group of potato variety.

: AMF inoculant probably needs to have several AMF taxa in the different genera to increase the probability of a match. The competition between the AMF and other fungal groups within inoculant may be the most important factor affecting colonisation of the potato roots by AMF species in the inoculants.

### *6.6.2 Recommendations and future work*

: In this study, it seems that the dominant AMF species of both indigenous AMF under field conditions and of AMF inoculants under glasshouse conditions are the main AMF that colonised the potato roots. It would improve our understanding of host preferences for species of AMF if we also checked the species composition of the AMF in the rhizosphere soils and growing medium by NGS technique at the same time as when roots were collected for assessment. To identify AMF actively associated with potatoes at the same time point maybe necessary.

: It seems that the potato varieties “Cara” and “Sarpo Mira” form strong associations with AMF. Further study on the mechanisms favouring AMF associations in “Cara” and “Sarpo Mira” may need to be studied under glasshouse conditions.

: As the results show that AMF can compensate for P uptake under low P conditions, the next steps for further research should focus on the study of P uptake by AMF under field conditions. Several researches reported the mechanisms how AMF improved P uptake from soil by various reasons, for example, the release of acid phosphatase enzyme in the hyphal tips, AMF inducing activity of phosphate solubilising bacteria/fungi and AMF symbiosis increases the root area to uptake more nutrients by their hyphae in the soils. These mechanisms need to be explored to confirm the role of AMF in P uptake.

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