Optimising associations of arbuscular mycorrhizal fungi with wheat

Thesis submitted for the degree of Doctor of Philosophy by

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ABSTRACT

Arbuscular mycorrhizal fungi (AMF) are ubiquitous symbionts of most vascular plants and essential contributors to soil health for which reason their application in agriculture has been investigated extensively. In wheat as one of the staple foods where large amounts of fertiliser and pesticides are used, the integration of mycorrhizal benefits such as increased nutrient uptake and plant health is desirable, but mutualistic outcomes of the symbiosis are determined by variety, agronomic management practices as well as nitrogen (N) and phosphorus (P) content of the soil. The present study investigated the impact of different fertiliser sources (biogas digestate, farmyard manure and mineral N) on AMF at five key development stages of two wheat varieties (Aszita and Skyfall) +/- crop protection over two cropping seasons 2017-18 and 2018-19 in a P-depleted soil. Additionally, the effect of a commercial AMF inoculum (INOQ Advantage) on plant performance, yield and grain quality was assessed. AMF-root colonisation (AMF-RC) was consistently higher in the modern variety Skyfall which also showed lower abundances of native AMF in response to AMF inoculation. Biogas digestate and farmyard manure application decreased AMF-RC in both years, whereas mineral N only reduced AMF-RC when soil N was high in the first season following grass-clover, but not in the second season following wheat (i.e. 2nd wheat crop). Amplicon sequencing of the ITS1-region revealed that mycorrhizal communities in roots were dominated by *Glomus* spp. and were not affected by agronomic management or variety. Differential abundance analyses based on sequences of the small subunit (SSU) however indicated increased diversity of fine root endopyhtes (FRE) in response to mineral N. Although the AMF inoculum was not detected in roots using strain-specific primers in digital droplet PCR, inoculation with AMF increased biomass production of wheat without fertiliser and decreased biomass production with mineral N treatment, but these changes did not affect grain yields. A pot experiment that tested a cellulose-based seed coating with the INOQ Advantage root powder showed negative effects on plant growth, but without root colonisation. The results of this study imply a key role of N that impacts AMF-RC, FRE and the effect of biostimulants. The use of such in wheat production requires further optimisation to guarantee economic benefit for farmers while excluding side-effects of exogenous strains on native AMF.

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DECLARATION

I declare that this thesis was written by myself and that the work within is my own unless explicitly stated otherwise. The work in this thesis has not been submitted for other degrees or qualifications.

CHAPTER 1. INTRODUCTION

1.1 CONSEQUENCES OF AGRICULTURAL DEVELOPMENT

Depending on the agroecological zone, the integration of modern varieties in wheat production after the 'Green Revolution' in the middle of the last century enabled a significant productivity increase leading to lower food prices and higher calorie intake (Evenson & Gollin, 2003). But this social benefit was only possible with extensive use of mineral fertilisers: The Special Report of the International Panel of Climate Change (IPCC) from 2019 depicts a 240% increase of cereal yields since 1961 which is accompanied by an 800% increase of nitrogen (N) application. Besides phosphorus (P) and potash (K), N is one of the essential plant nutrients and often the limiting factor in cereal production (Ladha *et al.*, 2016). The enormous N-depositions deriving from conventional[1] agricultural practices have altered the global N-cycle with drastic effects on the environment (Bodirsky *et al.*, 2014). Increasing emissions of the greenhouse gas (GHG) nitrous oxide (N_2O) and eutrophication of terrestrial and water ecosystems lead to biodiversity losses (Leff *et al.*, 2015; Zhang *et al.*, 2018) as well as pollution of ground- and surface waters through nitrate leaching (Di & Cameron, 2002). Also excessive application of inorganic P in conventional agriculture has been linked to eutrophication (Sharpley *et al.*, 2001). Additionally, studies have shown that up to 70 % of the anthropogenically used P is lost within food supply chains and cannot be recovered (Chowdhury *et al.*, 2017). A large proportion of this loss occurs already at the initial step when P is applied as fertiliser during early crop development. Only a small amount can be absorbed by the roots, whereas most of P is bound to clay particles and gets immobilized in the bulk soil (Holford, 1997). Hence, most conventionally managed soils are saturated with non-available P as a result of which farmers add more fertiliser. These fertilisers are produced from rock phosphate, a non-renewable resource of which 85% is derived from only three countries worldwide, none of them being where it is used the most (Elser & Bennett, 2011). In contrast to N, P is limited on a global scale as the resources are at risk of depletion in the near future (Gilbert, 2009). Announcing a potential P-crises, the Global Phosphorus Research Initiative (GPRI) was founded in 2008 and since then has urged the implementation of efficiency measures to guarantee future P-security (Cordell & White, 2013).

The fact that we must change our way how to produce food considerably has shaped the EU-elections in 2019 which showed strong vote increases for the Green party

[1] At this point is has to be acknowledged that agriculture as a form of land use is highly diverse and that there are various forms of farming systems which might not be considered in this thesis which focuses on central European agriculture.

(europeangreens.eu/2019results). One year later, the EU commission released the 'Farm-to-Fork' strategy as part of the European Green Deal for a more sustainable agriculture. On November 30th in 2020, the UK on its way out of the EU published an agricultural transition plan that describes the 'Path to sustainable Farming'. Overall, Europe is preparing for a substantial transformation in land use management to stop and compensate for the environmental impacts of modern agriculture. At the same time, research on agriculture as part of the solution to future challenges is gaining momentum.

1.2 BACK TO THE ROOTS: SOIL HEALTH A TOP PRIORITY

The need for a reformation of conventional food production and distribution has drawn attention to more holistic approaches such as sustainable intensification and agroecology which aim to minimize detrimental impacts of agriculture (Garnett *et al.*, 2013; Gliessman, 2016). The goal is to save resources while producing high quality food with less anthropogenic inputs and limit the negative environmental impact on the same or less currently available area of land (Wezel *et al.*, 2015). Soil degradation as consequence of intensive soil cultivation, erosion and chemical pollution threatens these objectives (Stavi & Lal, 2015). Therefore, agroecological practices prioritise soil conservation as a key element for the transformation to environmentally friendly food production (FAO, 2011). Since the 1970s, the term 'soil quality' has emerged and describes the ability of the soil to function depending on specific land use, i.e. relates to its potential to sustain animal and plant productivity (Lal, 2016). This term is distinguished from 'soil health' which presents soil as a dynamic living and finite resource with biotic activity and is directly linked to plant health (Lal, 2016). Functional and healthy soil is not only the foundation of sustainable farming but also the most important driver for crop health and other agroecosystem services (Gianinazzi *et al.*, 2010). For example, sustainable soil cultivation by direct drilling or the use of cover crops or green manure contribute to water retention, structural stability and nutrient transfer which ultimately can raise yields to similar levels as conventional agriculture (Tamburini *et al.*, 2020). Moreover, enhanced soil quality means increased carbon cycling capacities which increases soil fertility and carbon sequestration (Lal, 2016). Hence, agricultural practices that improve soil quality are essential to reduce GHG emissions. In fact, most recent scientific reports for policy makers (e.g. HLPE, 2019; IPCC, 2019) highlight the urge to support sustainable intensification methods through agricultural policies to prevent further loss of resources (Pe'er *et al.*, 2020). As the effects of global warming are becoming more tangible risks to agronomic productivity, soil health has also become of major concern for European farmers (Borrelli *et al.*, 2020). These concerns are calling out for enhanced research with focus on the versatile structures and processes within the pedosphere and

rhizosphere. Here, accumulating evidence has assigned an integral role in soil functioning to microbial activity (Bender *et al.*, 2016).

1.3 FROM FUNGI TO FORK

One group of microorganisms with particular contribution to soil health and hence important role in agroecology are arbuscular mycorrhizal fungi (AMF). As obligate biotrophs, AMF form mutualistic relationships with the majority of crop species during which they deliver nutrients (i.e. P, N and Zn) and water to their host in exchange for energy (i.e. C and lipids) (Smith *et al.*, 2008; Luginbuehl *et al.*, 2017; Savary *et al.*, 2020). AMF associations with plants are accompanied with a range of benefits which have also been recognised in sustainable farming practises (Gosling *et al.*, 2006; Rillig *et al.*, 2018; Bitterlich *et al.*, 2020). These benefits can provide potential yield increases (Hijri, 2015; Zhang *et al.*, 2019; Rocha *et al.*, 2020) with reduced fertilizer application and nutrient leaching (Kahiluoto *et al.*, 2009; Verzeaux *et al.*, 2017), increased tolerance to salinity (Evelin *et al.*, 2009), drought (Al-Karaki *et al.*, 2004; Garmendia *et al.*, 2017), pests (Imperiali *et al.*, 2017), diseases (Alaux *et al.*, 2018; Brito *et al.*, 2019) and can even improve the nutritional content of crops (Pellegrino & Bedini, 2014; Schubert *et al.*, 2020). More importantly in the context of conservation agriculture, AMF have been linked to improved soil structure by aggregation (Rillig & Mummey, 2006) and ultimately contribute to reduce GHG emissions (Bender *et al.*, 2015) and enhance carbon sequestration (Wilson *et al.*, 2009; Verbruggen *et al.*, 2021).

In connection with the sustainable intensification of agriculture, there is an increasing appeal for breeding of crops with improved rhizosphere-associated traits to enhance nutrient use efficiency and decrease fertiliser losses (Sawers *et al.*, 2008; Hohmann & Messmer, 2017). These traits are especially desired in important crops such as wheat which accounted for half of total European cereal production at 131.8 million tonnes per year in 2019 (Eurostat, 2020). However, not all symbiotic relationships of AMF and plants have shown beneficial outcomes (Johnson *et al.*, 1997; Klironomos, 2003). Particularly studies on the association of AMF and wheat reported varying results with yield increases up to 20 % (Pellegrino *et al.*, 2015), while other studies correlate growth depression to AMF colonisation (Ryan *et al.*, 2005) with great differences between varieties (Azcón & Ocampo, 1981; Hetrick *et al.*, 1992; Elliott *et al.*, 2019). The inconsistent results have led to intense discussions about the role of AMF in wheat production (Rillig *et al.*, 2018; Ryan & Graham, 2018). Nevertheless, decades of research point towards the pivotal importance of AMF in agroecosystems (Smith & Smith, 2011) for which reason these symbiotic fungi have been regarded with increasing commercial interest.

1.3.1 HOW MICROBES STIMULATE THE ECONOMY

Following the demand to lower environmental impacts of agriculture, plant microbiota have been investigated for their potential to substitute or supplement chemical inputs. This research has created an own terminology of so called 'biologicals' that depicts the respective targeted effect of microbial inoculants or natural compound formulations (Table 1.1). This categorisation is particularly difficult to fit to AMF as they provide a broad range of benefits to plant health. According to the definition by du Jardin (2015), AMF can be categorised as both 'biostimulant' or 'biofertilisers' whereas the latter represents a sub-category of the first.

Table 1.1 Definitions of biological alternatives to chemical compounds in plant production and examples (adapted from du Jardin, 2015)

Biological	**Definition**	**Example organism or compound**
Biocontrol agent	Living organism protecting plants against their enemies by e.g. competition, antibiosis, parasitism or Induced Systemic Resistance (ISR)	*Trichoderma* spp. *Bacillus* spp. *Pseudomonas* spp.
Biofertiliser	Microbial inoculant to increase the availability of nutrients and their utilization by plants, regardless of the nutrient content of the inoculant itself.	Biofertilisers may also be defined as microbial biostimulants improving plant nutrition efficiency, i.e. involve the same organisms
Biostimulant	Any substance or microorganism/group applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrients content.	AMF *Rhizobium* spp. *Azotobacter* spp. *Aszospirilium* spp. Seaweed extracts Chitosan

The integration of biostimulants in the new EU regulation (EU2019/1009) is the consequence of a rapidly growing market for microbial inocula, which has evolved from a few companies in Europe in the 1990s (M. Chen *et al.*, 2018) to a world-wide contributor to economic growth (Sessitsch *et al.*, 2018). According to the European Biostimulant Industry Council (EBIC), biostimulant application provide yield stabilisation and 5-10% increases in response to robust root development and improved water use efficiency (www.biostimulants.eu). In agriculture, certain practices can impede mycorrhizal symbiosis as their symbiotic contributions highly depend on environmental conditions and host identity (Johnson *et al.*, 2015). Furthermore, researchers have raised concerns about the risk of inoculation with non-local AMF strains as these can represent a threat to resident AMF populations (Rodriguez & Sanders, 2015;

Hart *et al.*, 2018). High prices of microbial products are another factor that constrain large-scale application in agricultural cropping systems (IJdo *et al.*, 2011; Bitterlich *et al.*, 2020). However, as soils are getting depleted of microbial activity as a result of conventional farming practices (Verbruggen *et al.*, 2010), reintroducing AMF by biostimulant application could represent a key element for the use of mycorrhizal ecosystem services in plant production (Bender *et al.*, 2016; Tamburini *et al.*, 2020). But with the uncertainties in symbiotic interactions of AMF and wheat, thorough research is required to optimize this important symbiosis in cereal production (Ryan *et al.*, 2019).

1.4 AIMS AND OBJECTIVES

This thesis aims to investigate the development of natural and exogenously applied AMF in wheat under the impact of different nutrient sources and crop protection programmes. This will be achieved by

- Monitoring the performance of two contrasting wheat varieties in relation to their mycorrhizal root colonisation over two growing seasons
- Analysing effects on yield parameters and grain quality as well as native AMF communities in response to AMF inoculation
- Molecular tracing of exogenously applied AMF strains in the field
- Formulation of AMF inoculum as seed coating for cost efficient biostimulant application in wheat

The core of the project is a field experiment that was repeated over two growing seasons which evaluated a range of agricultural input responses. Particular focus is set on the host-specific responses of AMF by using two wheat cultivars from different breeding backgrounds. The impact and interactions of nutrient source, genotype and AMF inoculum are analysed by a range of parameters from the macroscopic scale (i.e. yield) to the molecular level of fungal community structures. Here, the fate and behaviour of exogenously introduced AMF strains is examined after the design and application of strain-specific primers. While characterising the performance of AMF inoculum under field conditions, one target is to enhance the applicability of AMF inocula in wheat production which is approached by the development of a seed coating.

CHAPTER 2.LITERATURE REVIEW

2.1 ARBUSCULAR MYCORRHIZAL FUNGI

2.1.1 ORIGIN AND DISTRIBUTION

The term mycorrhiza derives from Greek and can be translated to “fungal roots” (*mýkēs*, "fungus", and ῥίζα *rhiza*, "root"). It was first used by Frank in 1885 (Frank, 2005) just one year after his description of the neutral coexistence of two dissimilar organisms as ‘symbiosis’(Smith & Read, 2008). Since then, mycorrhiza have been widely investigated under different scientific and economic aspects (Antoine *et al.*, 2021) and are considered as arguably the most common and most important symbiosis on earth (Tisserant *et al.*, 2013). Researchers distinguish between four different types of mycorrhizal symbiosis which are 1) arbuscular mycorrhiza, 2) ectomycorrhiza, 3) orchid mycorrhiza and 4) ericoid mycorrhiza (van der Heijden *et al.*, 2015). While ectomycorrhiza are only found in association with shrubs and trees, orchid and ericoid mycorrhiza are specialised on orchids and members of the Ericacea-family respectively. Fossilised spores and hyphae in plant roots from the Ordovician date back the association of fungi with land plants to 460 Million years ago and provide proof for the significance of mycorrhiza in the evolution of vascular plants (Simon *et al.*, 1993; Redecker *et al.*, 2000). More than 100 years of research on this ancient symbiosis have shown that around 80 % of all plant species form endomycorrhizal associations making arbuscular mycorrhizal fungi (AMF) the most common type of mycorrhizal symbioses (Smith & Read, 2008). They have been found on all continents and 34 % of the currently described AMF species occur on all of them except antarctica (Davison *et al.*, 2015). Despite the ancient character of the symbiosis, this cosmopolitan distribution and the lack of endemism has been linked to efficient recent dispersal rather than to paleogeographic events with a particular role of human activity and the spread of agriculture (Rosendahl *et al.*, 2009). These dispersal patterns are now additionally influenced by the intended spread of AMF species by the worldwide trading and application of AMF in form of commercial inocula (Schwartz *et al.*, 2006).

2.1.2 PHYLOGENY

Arbuscular mycorrhizal associations are formed by fungi that have long been classified as a separate monophyletic phylum called Glomeromycota (Schüßler *et al.*, 2001), which is a sister group of Ascomycota and Basidiomycota in the subkingdom Dikarya within the kingdom of fungi (Hibbett *et al.*, 2007). With publication of the first complete sequenced genome of *Rhizophagus irregularis* in 2013, Tisserant *et al.* (2013) suggested that this common representative of the Glomeromycota might be closer related to Mucoromycotina than to Dikarya. More recent phylogenetic analyses by Spatafora *et al.* (2016) have associated Glomeromycota to a sub-phylum (Glomeromycotina) within the phylum Mucoromycota (Fig. 2.1). However, the new order has not been officially accepted by the researcher community (http://www.amf-phylogeny.com/) suggesting that the relationship of Glomeromycotina with the other two sub-phyla Mucoromycotina and Mortierellomycotina has not been completely clarified (Bruns *et al.*, 2018; Walker *et al.*, 2018). Therefore, recent publications may refer to Glomeromycotina or new phylogenetic characterisations of soil fungi that do not consider the new sub-phyla (Tedersoo *et al.*, 2017). Further inconsistencies in phylogenetic assignment of AMF can be found up to genus level, where several changes of taxonomic ranks have caused conflicting opinions about systematic schemes (Redecker *et al.*, 2013). For example, the model species *Rhizophagus irregularis* has been renamed to *Rhizoglomus irregularis* due to confusions with a pathogenic fungi with the same genus name (Sieverding *et al.*, 2014). Nevertheless, there are current papers that do not adapt the new system (e.g. Jansa *et al.*, 2020) which will also be applied in this thesis.

Above all, analyses of nucleic genomes within single isolates confirmed earlier indications for sexual reproduction within AMF (Tisserant *et al.*, 2013; Ropars *et al.*, 2016) which have long been declared as asexual organisms (Redecker *et al.*, 2013). These findings open new perspectives not only regarding taxonomy and species concept definitions (Bruns *et al.*, 2018), but also to the biology and evolution of AMF (Yildirir *et al.*, 2020).

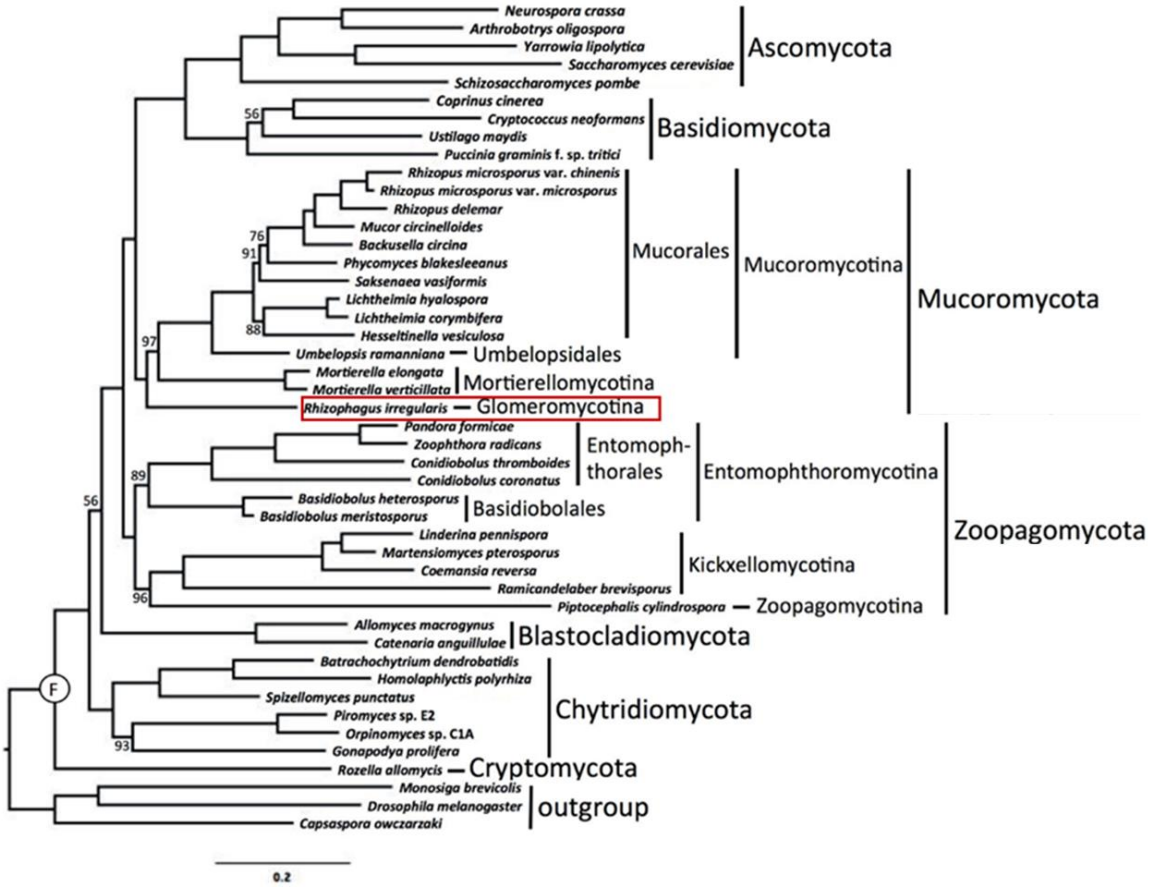


Fig. 2.1. Glomeromycotina (red box) within the phylogenetic tree of the Kingdom Fungi according to Spatafora *et al.* (2016).

Another ongoing discussion around mycorrhizal phylogeny is the choice of the most suitable marker region for metagenome studies (Lekberg *et al.*, 2018). With only 342 described species (http://www.amf-phylogeny.com, accessed 11/17/2021) organised in four to orders corresponding clades (Fig. 2.2), the number of known taxa within Glomeromycotina is much lower compared to the vast array of sequences that are produced by modern sequencing technology (Davison *et al.*, 2015). After ten years of AMF profiling by next generation sequencing (NGS), there is still no consensus on the ideal genetic barcode that can cover most of the phylogenetical variety of AMF across different sample types (Berruti *et al.*, 2017). Universal applicable approaches for reliable interpretation and comparison of metabarcoding studies on AMF have yet to be developed (Hart *et al.*, 2015). Nevertheless, high-throughput sequencing and other evolving molecular tools have initiated a new wave of mycorrhiza research that will also contribute to the development of new mycorrhizal technologies (Oviatt & Rillig, 2020). The most commonly used nuclear marker region in AMF amplicon sequencing is the small subunit of the nuclear ribosomal DNA (SSU or 18S DNA) (Öpik *et al.*, 2010), but concerns about the ability of this marker to recover full alpha diversities in microbial communities have been raised (Krüger *et al.*, 2009; Schlaeppi *et al.*, 2016). Consequently, the

use of more general primers targeting the internal transcribed spacer (ITS) of the ribosomal DNA has been proposed to assess AMF as part of the mycobiome in environmental samples (Lekberg *et al.*, 2018; Sommermann *et al.*, 2018). However, high variability and limited database representation of AMF for this marker region might hamper identification of unknown species in environmental samples (Stockinger *et al.*, 2010).

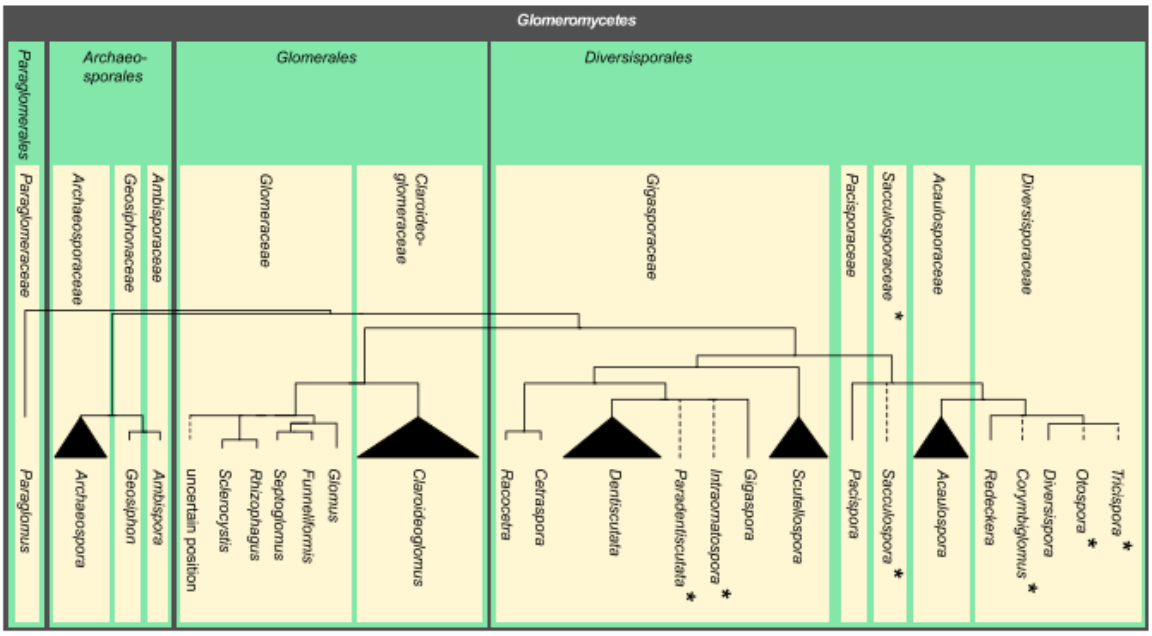


Fig. 2.2. Classification of Glomeromycota within the kingdom of fungi (Order, Family, Genus) according to Redecker *et al*. (2013). Dashed lines indicate genera of uncertain position, asterisk and triangles mark questionable genera with respect to data used for description and/or with respect to phylogenetic position (www.amf-phylogeny.com).

2.1.3 PHYSIOLOGY AND FUNCTIONING OF AMF SYMBIOSIS

Mycorrhizal fungi are obligate biotrophs that require a living plant host to develop and proliferate. A short asymbiotic phase occurs during spore germination either for non-host growth (without contact with the plant root) or in response to signal exchange and recognition of both symbiotic partners (Gianinazzi-Pearson, 1996). Compared to other fungal groups, spores of AMF are relatively large (30 µm - 1 mm), multinucleated and thick-walled with storages of carbohydrates and lipids (Gerdemann & Nicolson, 1963). These traits enable spores to endure long periods of dormancy (Tommerup, 1983; Douds *et al.*, 2011) or travel wide distances across the air (Chaudhary *et al.*, 2020). Hyphae that emerge from AMF spores are coenocytic and multinucleated which means that they are considered as a single cell containing thousands of independent nuclei (Kokkoris *et al.*, 2021). Different strains of the same AMF species can exchange genomic information as well as other cell compartments after fusion of these hyphae in a process called ‘anastomosis’ (Angelard *et al.*, 2010; de la Providencia *et al.*, 2013).

Attachment of an explorative hyphae to a root cell induces complex signalling processes in both symbiont cells and is followed by hyphopodium formation by the fungus to penetrate the root epidermis. In contrast to ectomycorrhiza which colonise the outer root layer and surrounding epidermal cells, AMF spread through intra- and inter-cellular hyphae into the inner root cortex (Bonfante & Genre, 2010). Inside the cells of this tissue, AMF form characteristic colonisation structures of finely branched hyphae which have been termed "arbuscules" (lat. *arbusculum* = bush or little tree) due to their tree-like structure. This phenotype of arbuscules is considered as *Arum*-type colonisation (Fig. 2.3 A), whereas during *Paris*-type (Fig. 2.3 B) colonisation hyphae spread via intracellular passages of cortical cells and form hyphal coils (Luginbuehl & Oldroyd, 2017). The occurrence of these two types of colonisation has been linked to host plant identity and functionality of their respiratory pathway (Mercy *et al.*, 2017; Cosme *et al.*, 2018). Arbuscule formation is a dynamic process of two to eight days involving the synthesis and degeneration of the periarbuscular membrane (Javot *et al.*, 2007). This thin layer around arbuscule branches represents the interface of AMF symbiosis and harbours a multitude of transporter proteins that facilitate nutrient exchange between the two symbiotic partners (Ivanov *et al.*, 2019; Savary *et al.*, 2020). Colonised plants have been shown to provide 4-20 % of their photosynthates to AMF (Johnson *et al.*, 1997). Carbohydrates in the form of hexoses have long been identified as the only currency that plants trade to their fungal symbionts (Helber *et al.*, 2011). Closer investigation of AMF interactions showed that these fungi lack certain genes that are required for the synthesis of fatty acids (Wewer *et al.*, 2014). Further analyses confirmed that besides sugars, AMF obtain lipids from their plant host (Bravo *et al.*, 2017; Luginbuehl *et al.*, 2017). In exchange, AMF provide essential minerals such as phosphorus (P) (Javot *et al.*, 2007; Elliott *et al.*, 2019) and nitrogen (N) (Mäder *et al.*, 2000; Govindarajulu *et al.*, 2005; Thirkell *et al*., 2019) as well as micronutrients and water to their plant host (Kothari *et al.*, 1991). The transfer of minerals and assimilates is regulated by both plant and fungus depending on the partners' ability to contribute to the symbiosis (Kiers *et al.*, 2011; Nouri *et al.*, 2014; Williams *et al.*, 2017). Mineral nutrients are mobilised and taken up through networks of fine hyphae (1-12 µm) which extend the plants root system beyond the rhizosphere. In this way, plants can gain access to nutrients and water resources that would not be available via the direct root pathway (Jacott *et al.*, 2017). Without AMF, plants have been shown to develop longer roots and higher root hair density to compensate for the lack of soil exploration and consequent nutrient acquisition (Kothari *et al.*, 1990). This underlines the important function of the extraradical mycelium which is often contrasted with the extent of intraradical structures (Sawers *et al.*, 2017). Until the beginning of this century, AMF have been described as "vesicular-arbuscular-mycorrhiza" (VAM) which refers to

another phenotypical characteristic of these fungi. Depending on the supply of fatty acids by the host, AMF form vesicles (Fig. 2.3 C) as storage organs on hyphal branches in the intercellular space of the root cortex (Keymer *et al.*, 2017). Similar to spores, vesicles are relatively robust, can endure non-active periods of symbiosis and can be reproductive stage for the germination of fungal hyphae (Mosse, 1973). For this reason, spores and vesicles are produced during later phases of the symbiosis (Ryan *et al.*, 1994) although their formation can be affected by environmental conditions (Oehl *et al.*, 2003; Orchard *et al.*, 2017) and are species-dependent too (Gerdemann & Nicolson, 1963; Oehl *et al.*, 2009). Morphological criteria of AMF root colonisation are not suitable to distinguish between different AMF species in most cases, but spore shape, size and colour can be used for species identification (Jansa *et al.*, 2002; Oehl *et al.*, 2010; Säle *et al.*, 2015). Although opposing opinions have been published (Wetzel *et al.*, 2014), such methods have been mostly replaced by molecular tools which are in particularly advantageous in mycorrhizal research since not all AMF form spores or can be cultured.

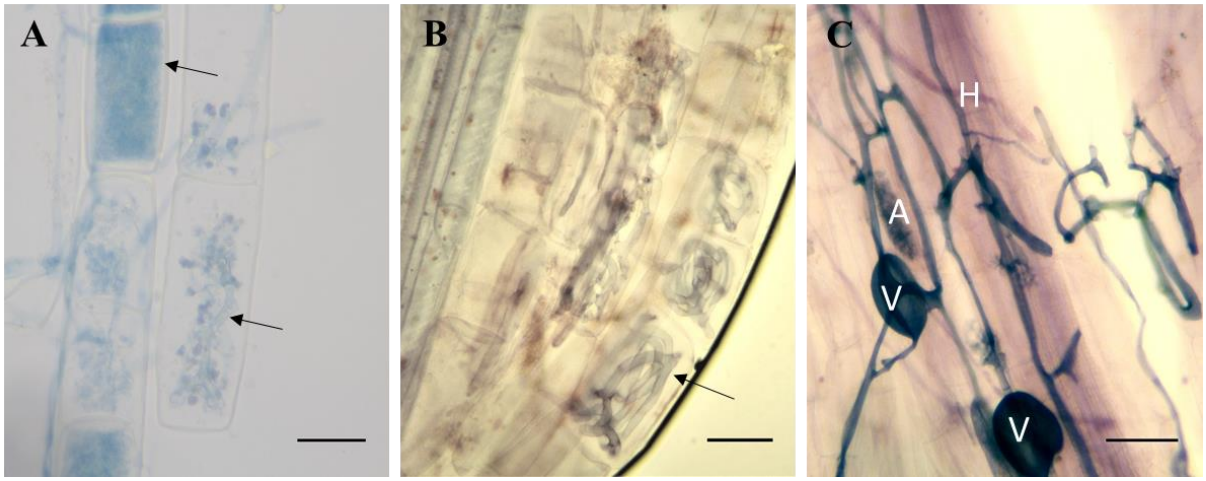


Fig. 2.3. Microscopy images of AMF colonisation structures (stained with China-ink) inside wheat roots at different growth stages **A**) *Arum*-type arbuscules at highest development stage filling the intracellular space (upper arrow) or starting/senescing branching structures (lower arrow). **B**) *Paris*-type arbuscules in form of intracellular hyphae coils **C**) Intercellular hyphae (H), arbuscules (A) and vesicles (V). Bar scale 50 µm.

2.1.4 FINE ROOT ENDOPHYTES

Besides AMF, there is another group of fungi that forms arbuscules and that has gained more attention in the past couple of years. The species complex of so called ‘fine root endophytes’ (FRE) was formerly collected within the species *Glomus tenue* (Hall, 1977), but recent molecular analyses showed that these fungi are phylogenetically different to ‘coarse’ AMF (Orchard *et al.*, 2017a). Consequently, *Glomus tenue* (basionym *Rhizophagus tenuis*) was renamed to *Planticonsortium tenue* and the new genus was assorted from Glomeromycotina to

the order of Endogonales within the subphylum Mucoromycotina, an early-diverging lineage of fungi (Desirò *et al.*, 2017; Walker *et al.*, 2018). However, the exact phylogenetic position of FRE is still uncertain and despite their discovery already more than 50 years ago (Greenall, 1963), research on the diversity of FRE is just at the beginning (Desirò *et al.*, 2017).

Spores of FRE are much smaller (≤ 20 µm) than AMF spores for which reason FRE have not been recorded during fungal community assessments using spore morphology (Sinanaj *et al.*, 2021). The characteristic fine hyphae (0.1-4 µm) and fan-shaped arbuscules (Fig. 2.4 A) facilitate differentiation of FRE from AMF by microscopy (Fig. 2.4 B and C). Instead of vesicles, FRE form terminal or intercalary hyphal swellings (5-15 µm) which are sometimes indistinguishable from immature spores (Hall, 1977; Field *et al.*, 2016; Orchard *et al.*, 2017b; Hoysted *et al.*, 2019). Detailed microscopy studies by Gianinazzi-Pearson *et al.* (1981) showed that FRE have thinner cell walls, but are overall similar to coarse AMF in both their ultrastructural features and colonisation behaviour. Probably due to this high similarity and their phylogenetic invisibility in the large group of *Glomus* spp., there are only few studies that distinguish between FRE and AMF during root colonisation assessments. Nevertheless, co-occurrence of both fungal symbionts have been frequently reported in different plant species and environments (Ryan *et al.*, 2005; Orchard *et al.*, 2017a,b; Hoysted *et al.*, 2019).

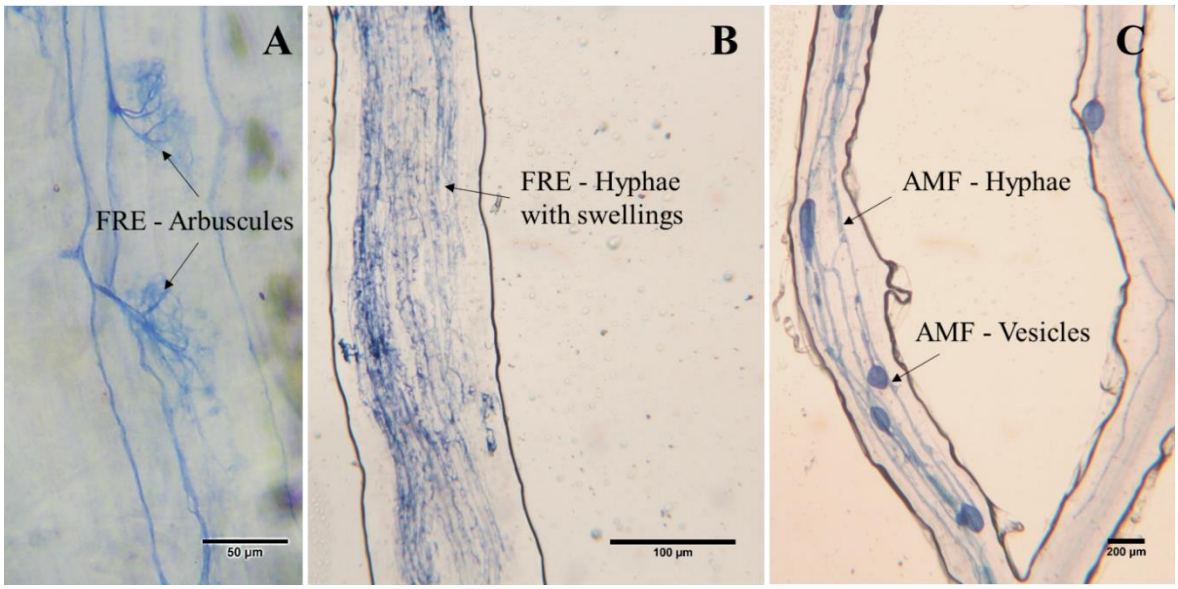


Fig. 2.4. Microscopy images of root colonisation structures of fine root endophytes (FRE) in **A)** wheat roots (GS64-Flowering) and **B**) in roots of *Plantago lancelota* in contrast to coarse arbuscular mycorrhizal fungi (AMF) in roots of **C**) *Plantago lancelota*. Fungal structures were stained with China-ink.

Like AMF, FRE are globally distributed and found in both natural and agricultural soils, but their functioning in these is far less understood than that of AMF (Orchard, *et al.*, 2017b).

Comparative studies of both fungal groups showed higher N-uptake by FRE than AMF while P was delivered in higher amounts by AMF than FRE suggesting a complementary role of both fungal endophytes regarding nutrient acquisition (Field *et al.*, 2016, 2019). This hypothesis is supported by the detection of FRE besides AMF in fossils indicating early cooperation of these two groups in plant-fungal associations (Strullu-Derrien *et al.*, 2014). Evidence was collected by the demonstration of nutritional mutualism of FRE in lycophytes and liverworts which represent groups of the earliest diverging lineages of land plants (Field *et al.*, 2015b; Field *et al.*, 2019; Hoysted *et al.*, 2019). Also saprotrophic characteristics that were demonstrated in these experiments and which could either be linked to FRE or associated microbes indicate that Mucoromycotina fungi might harbour the explanation for the obligately biotrophic lifestyle of Glomeromycotina (Field *et al.*, 2015a), but further research is required. Overall, these findings do not only give FRE a key position in the evolution of vascular plants but raise new research questions on the potential interaction of FRE in plant nutrition and ecosystem functioning (van der Heijden *et al.*, 2015; Desirò *et al.*, 2017; Sinanaj *et al.*, 2021).

2.2 USE AND EFFICACY OF ARBUSCULAR MYCORRHIZAL FUNGI IN CROP PRODUCTION

2.2.1 EFFECTS OF AMF ON PLANT PERFORMANCE AND AGROECOSYSTEM FUNCTIONING

Arbuscular mycorrhizal fungi can contribute significantly to plant nutrition due to their key function in phosphorus (P) uptake (Marschner, 2012). Depending on plant species and environmental conditions, plants can acquire up to 100 % of their P through the mycorrhizal pathway (Smith *et al.*, 2004). In particular P in form of inorganic phosphate is quickly depleted within the root zone, but AMF can overcome this process by solubilisation and transport of long-chained P-molecules through the hyphal network to the host (Marschner, 2012). For this reason, mycorrhizal fungi have been suggested as a strategy to recover immobilised P from agricultural soils which would otherwise not re-enter the P-cycle (Fig. 2.5) (Bovill *et al.*, 2013; Cordell & White, 2013). Vice versa, mycorrhizal research has set an early focus on the function of AMF in agriculture where AMF could improve the nutrient status of crops and reduce fertiliser inputs (Koide, 1991; Antoine *et al.*, 2021). Numerous studies have shown increased P contents in plants colonised by AMF compared to non-colonised plants (Manske, 1989; Jansa *et al.*, 2008; Gosling *et al.*, 2016).

While the processes that mediate P-uptake in mycorrhizal symbiosis have been widely investigated, the role of symbiotic N provided by AMF have just recently started to receive

more attention (Bücking & Kafle, 2015). The use of stable isotopes of N has helped to establish how AMF take up and transfer N between different host plants (Hodge *et al.*, 2001; Leigh *et al.*, 2009; Thirkell *et al.*, 2019). With high certainty, mycorrhizal N-uptake is driven by close interactions with other soil microorganisms such as nitrification bacteria that mobilise and mineralise N in the 'mycorrhizosphere' (Bukovská *et al.*, 2021). It is still under debate in which form AMF provide N to their host and if this might be regulated by agricultural management (Azcón *et al.*, 1992; Govindarajulu *et al.*, 2005; Thirkell *et al.*, 2019). In western agriculture, ammonium (NH_4^+) and nitrate (NO_3^-) are the main forms in which N is often excessively applied. In that context, AMF in agroecosystems have shown potential to reduce both N-leaching and N_2O emissions (Bender *et al.*, 2015).

Besides P and N, AMF also enhance uptake of other nutrients such as potassium, zinc, iron, copper, magnesium and manganese (Azcón *et al.*, 1992; Kahiluoto *et al.*, 2001; Mäder *et al.*, 2011; Ercoli *et al.*, 2017; Jansa *et al.*, 2020). In some cases, but not always (Smith *et al.*, 2004; Hodge *et al.*, 2010; Elliott *et al.*, 2019), increased nutrient assimilation due to AMF-RC results in enhanced biomass production and ultimately higher yields (Mohammad *et al.*, 2004; Hijri, 2015; Zhang *et al.*, 2019). The driving factor for increased growth is increased photosynthesis rate which is stimulated due to the additional C-sink in the roots (Kaschuk *et al.*, 2009; Lucini *et al.*, 2019). This increased metabolic capacity is supported by improved water supply for which reason mycorrhizal plants can be more resistant to drought and heat stress (Al-Karaki *et al.*, 2004; Lehnert *et al.*, 2018; Mathur *et al.*, 2019). Other biotic stressors like heavy metals and salinity have also been shown to be less damaging in plants associated with AMF (Evelin *et al.*, 2009; Bui & Franken, 2018). Additionally to enhanced metabolism and nutritional benefits, AMF can stimulate the plants immune system leading to increased capacity of the plant to resist pathogen, nematode and insect attacks (Liu *et al.*, 2007; Bedini *et al.*, 2018). This activation of defence mechanisms is also termed as 'priming' and has been found to be tightly linked to plant-microbe interactions (Conrath *et al.*, 2006). Mycorrhizal fungi themselves can induce this systemic response, but also affect microbial community composition in the rhizosphere that can contribute to the plants immune response (Jung *et al.*, 2012; Cameron *et al.*, 2013). Positive results on AMF-induced disease resistance have mostly been generated under controlled conditions (Gernns *et al.*, 2001; Mustafa *et al.*, 2016), but exist also for field experiments where mycorrhized cassava plants were more resistant to nematodes (Séry *et al.*, 2016) and in wheat where inoculation with AMF increased the survival of frit fly-infested seedlings (Imperiali *et al.*, 2017).

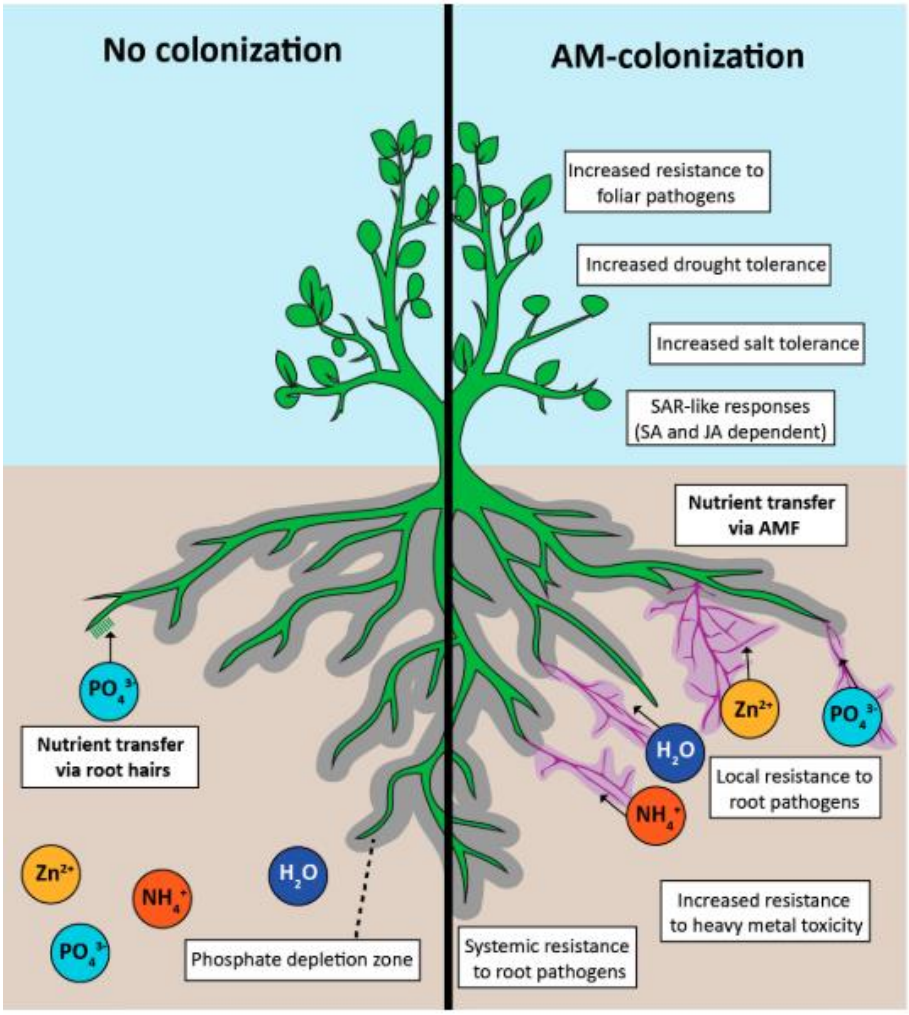


Fig. 2.5. Benefits conferred by arbuscular mycorrhizal fungi (AMF) vs. non-colonised roots (Jacott *et al.*, 2017). Fungal hyphae (purple) extend nutrient and water acquisition beyond the depletion zone (grey), but P-depletion zone will eventually also form around fungal hyphae. Root colonisation can induce system acquired resistance (SAR) mediated by salicylic acid (SA) and jasmonic acid (JA).

All of the outcomes of AMF symbiosis described here are strongly dependent on environmental and biotic factors, i.e. plant host and fungal identity (Hoeksema *et al.*, 2010; Mensah *et al.*, 2015). Not all associations with AMF result in mutualistic relationships, for which reason AMF symbiosis should be considered as a mutualism-parasitism continuum (Johnson *et al.*, 1997; Klironomos, 2003; Johnson & Graham, 2013). Depending on nutrient status of both symbiotic partners, mycorrhizal mediated nutrient uptake can cause a shut-down of direct nutrient uptake through the root, hence leading to nutrient deficiency and even reduced growth (Graham & Abbott, 2000; Smith *et al.*, 2004; Püschel *et al.*, 2016). Up to now, it remains a major challenge in mycorrhizal research to elucidate all the factors that determine mycorrhizal growth response (MGR) which would assist to make the benefits of this symbiosis more predictable and applicable (Sawers *et al.*, 2010; Lehnert *et al.*, 2018).

At this point it is also important to note that AMF in natural or agroecosystems are not only a single microbe-plant interaction, but network-forming associations of multiple fungi that colonise plant roots simultaneously and connect whole plant communities (Jansa *et al.*, 2008; Wagg *et al.*, 2011). Mycorrhizal community composition and even intraspecific diversity of AMF species have been shown to be relevant in ecosystem functioning and crop production (Dai *et al.*, 2014; Wagg *et al.*, 2014).

Another important aspect of AMF in agroecosystem is their influence on the soil. Nutrient leaching and emissions of greenhouse gasses are facilitated by improved soil aggregation due to glomalin excretion by AMF (Rillig & Mummey, 2006; Wilson *et al.*, 2009). Here, a pivotal role has been assigned to the extraradical mycelium not only for the translocation of C into the soil, but also for the transfer of nutrients, water and signalling molecules between plants (Bücking & Kafle, 2015). These hyphal networks can expand the bioactive zone of disease resistance molecules and even allelochemicals for which reason AMF have also been investigated for their potential to suppress weeds (Rinaudo *et al.*, 2010; Barto *et al.*, 2011).

2.2.2 IMPACT OF AGRICULTURAL PRACTICES ON AMF

The ecosystem services provided by AMF are not always pronounced, because mycorrhizal development is highly affected by environmental parameters such as soil type, pH, temperature, light and nutrient availability (Voříšková *et al.*, 2019). In agroecosystems, these factors are expanded by the impact of pesticides, soil treatment, fertiliser inputs and crop rotation (Gosling *et al.*, 2006). Especially conventionally managed field soils are usually impoverished in AMF-diversity compared to organic production systems (Oehl *et al.*, 2004). In particular, the detrimental effect of inorganic P on AMF development has been characterised as a general phenomenon (Smith & Read, 2008). High inorganic P contents due to extensive mineral fertilizer use impede mycorrhizal development in conventionally cultivated soils (Ryan *et al.*, 1994; Mäder *et al.*, 2000a; Kahiluoto *et al.*, 2009; Hildermann *et al.*, 2010; Bender *et al.*, 2019). At the same time, studies agree that the effect of P depends on the P-status of the soil (Mohammad *et al.*, 2004; Ceballos *et al.*, 2013; Köhl *et al.*, 2016; Johnson *et al.*, 2015), crop type or variety (Li *et al.*, 2005; Gosling *et al.*, 2013; Ercoli *et al.*, 2017; Martín-Robles *et al.*, 2018) and also on the form in which P is added to the cropping system (Smith & Smith, 2011; Zhou *et al.*, 2015; Caruso *et al.*, 2018).

Organic systems on the other hand are usually based on non-synthetic fertilisers such as cattle manure, more diverse crop rotations including legumes and the use of fertility building crops such as grass-clover leys. These practices promote microbial activity in the soil which the system ultimately depends on (Verbruggen *et al.*, 2010; Riedo *et al.*, 2021). Long-term field

experiments by Mäder *et al*. (2000a) confirmed that soils from such systems had a greater capacity to form AMF symbiosis. As a rather modern approach in fertilisation, the impact of biogas digestate on AMF-diversity and functionality has been scarcely studied (Wentzel & Joergensen, 2016; Caruso *et al.*, 2018). Multiple field trials investigating the interaction of different manure types and AMF have shown no detrimental effects on AMF-diversity (Oehl *et al.*, 2004; Hildermann *et al.*, 2010; Aguilar *et al.*, 2017). Nevertheless, long-term application of organic fertilisers can affect the composition of AMF-communities (Oehl *et al.*, 2004; Hassan *et al.*, 2013; Qin *et al.*, 2015). Similar results were found in the comparison of conventionally and no-till systems which showed different amount of spores of predominating AMF-species (Castillo *et al.*, 2006). Conventional tillage has been shown to disrupt hyphal networks of AMF and result in reduced AMF diversity compared to no-till farming systems (Jansa *et al.*, 2002; Verzeaux *et al.*, 2017). Detrimental impact on AMF can also occur in response to pesticide applications and especially to fungicides, even though the effect depends on the active compound in the chemical and the fungal community structure (Jin *et al.*, 2013). A recently published long-term study revealed negative correlation of AMF-RC and accumulating pesticide residues in agricultural soils (Riedo *et al.*, 2021). The authors concluded that organic agriculture is less likely to reduce microbial soil life and emphasise that pesticide-related studies should consider combinations of active compounds instead of single pesticides. Crop rotation can affect AMF and their availability to plants in agricultural soils. Although AMF form associations with most crop species, Brassicaceae and Chenopodiaceae that harbour important crops like sugar beet, rapeseed, quinoa and spinach are considered as non-mycorrhizal or rudimentary arbuscule mycorrhizal plants (Cosme *et al.*, 2018). Exclusion of such from crop rotations can avoid the decline of AMF potential (Lekberg & Koide, 2005; Gosling *et al.*, 2006), whereas strong mycorrhizal plants like maize have been shown to increase AMF diversity in the subsequent crop (Sommermann *et al.*, 2018). Fallow extracts the phototroph host from the obligative symbiotic fungi for which reason bare fields reduce the number of propagules in the soil (Douds *et al.*, 2011). Shortening of fallow periods is therefore recommended to increase mycorrhizal potential of soils (Lekberg & Koide, 2005).

2.3 INOCULATION WITH AMF FOR ENHANCED PLANT PERFORMANCE

2.3.1 COMMERCIAL INOCULUM PRODUCTION

Although AMF can be found in almost every soil around the world (Section 2.1.1), commercial AMF-based products are sold on a growing market as the benefits of AMF on plant performance

have attracted increasing interest from horticultural as well as agricultural growers (Sessitsch *et al.*, 2018; Bitterlich *et al.*, 2020). However, the obligative biotrophic character of the symbiosis represents a barrier to large-scale production of mycorrhizal inocula, as inoculum production always requires simultaneous plant cultivation. This obstacle was tried to be minimised by the development of *in-vitro* growing systems that use *Agrobacterium rhizogenes* (Ri)- transformed roots on specific nutrient media (Declerck *et al.*, 1996). Also referred to as root organ cultures ("ROC"), this system has been declared as more compact and hence transferable for production and application purposes (Rodriguez & Sanders, 2015). Despite its high yields of propagules, *in-vitro* AMF propagation is still cost intensive and not yet applicable for large-scale inoculum production (IJdo *et al.*, 2011; Ceballos *et al.*, 2013). Furthermore, not all AMF can be cultured for which reason only a limited selection of species are available for sterile production systems (Gianinazzi & Vosátka, 2004). But these hurdles might be removed in the future due to higher demands for biostimulants (M. Chen *et al.*, 2018) and advancements in research to overcome the dependency of AMF on phototrophic hosts (Rillig *et al.*, 2020).

The most widely adopted cultivation technique for AMF inoculum is the propagation of selected strains in inert substrates with strong mycorrhizal plant species in greenhouses (IJdo *et al.*, 2011). These strains can derive from *in-vitro* cultures or *in-planta* mono-specific trap cultures. Although cheaper than *in-vitro* systems, non-sterile production requires strict measurements to avoid contamination with undesired microbes. Unintended propagation of pathogens or invasive species in AMF production is only viable by regular control analyses to validate the absence of such strains in the inoculum.

After harvest, the AMF inoculum needs to be assessed for quality by infectivity assays. This step is required in both production systems as it has been shown that long term propagation of AMF can affect symbiotic functioning of the fungus (Kokkoris & Hart, 2019). Most probable number tests (MPN) can be used to assess the viability of the inoculum and to obtain information about the number of infective propagules (e.g. spores, hyphae or vesicles) (Wilson & Trinick, 1983).

The final step of the production process is the formulation to improve survival, applicability and effectiveness of the inoculum (Leggett *et al.*, 2011; Herrmann & Lesueur, 2013). This step can involve formulation with other beneficial microbes such as plant growth-promoting bacteria like *Rhizobium* spp. (Kaschuk *et al.*, 2009) or biocontrol agents like *Trichoderma* spp. (Colla *et al.*, 2015; Buysens *et al.*, 2016; Rocha, Ma, *et al.*, 2019) which have been shown to exert positive effects on plant performance in co-inoculation studies with AMF. Application methods of AMF inocula are less investigated but involve for example the formulation of the

product as alginate beads (De Jaeger *et al.*, 2011; Buysens *et al.*, 2017) or seed coating (Rocha *et al.*, 2018). Furthermore, the composition of species in mixed inocula can be decisive as some AMF species have shown to promote each other while others are more likely to become competitors (Thonar *et al.*, 2014).

2.3.2 APPLICATION OF AMF INOCULUM IN AGRICULTURAL SYSTEMS

Inoculation with AMF has been widely promoted as a tool in sustainable agriculture (Pellegrino *et al.*, 2011, 2015; Zhang *et al.*, 2019; Oviatt & Rillig, 2020). However, most results have been generated in greenhouse experiments where environmental factors like temperature, moisture and light can be controlled. Under field conditions, the outcome of inoculation with AMF is not only determined by environmental conditions, but also on the timing and application method (Verbruggen *et al.*, 2013). For example Buysens *et al.* (2016) reported increased potato yields after inoculation with AMF and *Trichoderma harzianum* of the preceding cover crop *Medicago sativa.* In contrast, microbial inoculation of potato directly had no effect on tuber yields or plant performance (Buysens *et al.*, 2017; Alaux *et al.*, 2018). Other studies report a lack of AMF inoculation effects even if the introduced AMF strains evidentially established in the field (Kokkoris *et al.*, 2019a) or after preliminary greenhouse trials had shown positive results (Rosa *et al.*, 2020). Furthermore, the applied formulation of AMF strains as well as host genotype determine the effect of AMF symbiosis on the plant or plant community (Hetrick *et al.*, 1992; Klironomos, 2003; Kiers *et al.*, 2011). Consideration needs to be given not only to the effect of the crop variety, but also genetic variation within the fungal partner. Greenhouse studies in rice showed that inoculation with closely related strains of *R. irregularis* had either growth reducing effects or up to five-fold higher growth responses depending on the fungal isolate (Angelard *et al.*, 2010). Similar results were generated under field conditions in cassava (Peña *et al.*, 2020) and indicate potential strategies for genetic manipulation of AMF to improve their application in crop production (Rodriguez & Sanders, 2015; Savary *et al.*, 2020).

Despite the mentioned obstacles of AMF inoculation in field experiments, Hijri (2015) documented profitability of this practice in potato after data analyses of 231 field studies which used the same strain of *R. irregularis* (DAOM 197198) in the form of a commercial inoculum. In contrast to many other field inoculation studies that describe effects of only a single growing season (Al-Karaki *et al.*, 2004; Ceballos *et al.*, 2013; Ercoli *et al.*, 2017), the dataset used by Hijri (2015) is unique in many aspects: the field trials were conducted by farmers during a fixed period of four years and in different areas across Europe and northeast America. This makes these results particularly valuable since most inoculation trials for research purposes often

ignore economic feasibility e.g. the use of large amounts of inoculum (Bender *et al.*, 2019) or unpractical application methods (Farmer *et al.*, 2007).

Besides positive effects in potato, inoculation of legumes have shown good responses to AMF inoculation i.e. in chickpea (Pellegrino & Bedini, 2014; Rocha *et al.*, 2019a), cowpea (Rocha *et al.*, 2020) and clover (Köhl *et al.*, 2016). Although cereals have been critically examined for their interaction with AMF (Section 2.4.3), a comprehensive meta-analyses by Zhang *et al.* (2019) showed overall positive effects of AMF inoculation on maize, rice and wheat yields. Interestingly, they found no effect of multi-species inocula or formulation with other soil microbes which was previously reported to have better effects than inoculation with single AMF species (Mäder *et al.*, 2011; Colla *et al.*, 2015; Taylor *et al.*, 2015). Another promising crop that has been investigated intensively in the context of AMF inoculation is cassava, an important staple food in Latin America (Rodriguez & Sanders, 2015). Several field trials gave significant yield increases and improved nematode resistance after inoculation with *R. irregularis* even under normal P-fertilisation (Ceballos *et al.*, 2013; Séry *et al.*, 2016).

With the growing market for microbial biostimulants, concerns about potential invasive character of commercialised strains have been raised among mycorrhizal researchers (Schwartz *et al.*, 2006; Hart *et al.*, 2018; Thomsen & Hart, 2018). Introduced fungi can replace native strains by competition or anastomosis and alter local microbiome compositions in the soil (Schlaeppi *et al.*, 2016; Bender *et al.*, 2019). On the other hand, native AMF-communities which are abundant in organically managed soils can outcompete exotic strains (Bender *et al.*, 2019). Douds *et al.* (2011) even speculated that indigenous AMF have to be reduced to less than one propagule cm^{-3} to enable exogenously applied AMF to establish. But natural AMF-abundance and composition should not only be considered because of potential risks associated with increasing biostimulant applications, but also for the exploitation of locally adapted microbes (Douds *et al.*, 2005). These have been shown to be superior regarding plant performance and yield increases in comparisons of commercial and locally sourced AMF inocula (Pellegrino & Bedini, 2014; Middleton *et al.*, 2015; Séry *et al.*, 2016). Berruti *et al.* (2016) encourage on-farm production of inoculum to make AMF application more feasible to farmers, because besides uncertainties of inoculation success, high costs for AMF inocula represent a hurdle to the implementation of AMF inoculation in agricultural systems (Ceballos *et al.*, 2013; Bitterlich *et al.*, 2020).

2.4 SYMBIOSIS OF AMF AND WHEAT

2.4.1 HISTORY AND PERSPECTIVES OF WHEAT BREEDING

Wheat is one of the oldest domesticated crops that has been tightly linked with the development of human civilization throughout history. With the onset of agriculture and settlement of men ~10,000 years ago, recurrent hybridization of closely related wild grass species (Triticae) resulted in the allohexaploid (AA,BB,DD) bread wheat (*Triticum aestivum*) of today (Bernhardt *et al.*, 2020). This large and complex genome makes wheat more adaptable to different environmental conditions (Voss-Fels *et al.*, 2019), but also a difficult crop for molecular research (Lukaszewski *et al.*, 2014). Targeted breeding for specific characteristics represents an essential tool in wheat production and was facilitated at the beginning of the 20th century with the switch from mass selection to hybridization (Borlaug, 1983; Harwood, 2012). The next and probably most prominent turning point in wheat production was the release of semi-dwarf varieties by the International Maize and Wheat Improvement Centre (CIMMYT) in 1961 (Harwood, 2012). These varieties were mostly bred for reduced height with enhanced nutrient uptake and grain filling which made them particularly responsive to mineral fertiliser (Dalrymple, 1985). This trait was efficient in that global grain yields doubled between 1950 and 1985 (Shiferaw *et al.*, 2013). But to provide continuously increasing yields, conventional wheat production has become progressively dependent on large amounts of N and P fertiliser as well as pesticides and herbicides (Torriani *et al.*, 2015; Ladha *et al.*, 2016; Rempelos *et al.*, 2018). Furthermore, the high-yielding varieties required constant water supply for which reason yield increases were most significant in industrialised countries in temperate climates (Shiferaw *et al.*, 2013). Today, 50 % of the worldwide cultivated common wheat varieties emanate from these CIMMYT-lines (Shiferaw *et al.*, 2013).

Wheat is the most widely grown crop and is of great importance for food security as it provides 20 % of the world's calorie supply (FAO, 2009). Highest yields of wheat are achieved in the temperate zone where under optimal growing conditions like in Western Europe, grain yields average around 7 t ha^{-1} whereas the world average at 3 t ha^{-1} is much lower (Röder *et al.*, 2014). Economic development and consequent changes towards a more affluent diet are predicted to increase the global demand for bread and feed wheat by 70 % in the near future (Gerbens-Leenes *et al.*, 2010; Alexandratos & Bruinsma, 2012). To meet this demand relies largely on the main wheat producers like the UK (Röder *et al.*, 2014), but especially industrialised countries are urged to reduce their environmental impact by employing more sustainable agricultural practices (IPCC, 2019). Furthermore, food security as well as wheat production is challenged by changing climate conditions and yield plateaus: after the continuous

increase in the second half of the 20th century, yields in industrialised agricultural systems slowed down in the 1990s and have recently stagnated in many countries (Shiferaw *et al.*, 2013). This phenomenon is most likely due to rising global temperatures that have much stronger detrimental effects on wheat yields than to other major staple crops e.g. maize or soy (Lobell *et al.*, 2011; Tian *et al.*, 2019). At the same time, land degradation and unsustainable farming practices have been associated with stagnation of crop yields (Stavi & Lal, 2015). Hence, to meet increasing food demands in a warming world will require new cultivation practices and potential expansion of the currently provided agricultural area (Shiferaw *et al.*, 2013; Röder *et al.*, 2014). The elite wheat breeding programmes have shown to be on the right track for further yield optimisation even in low-input production systems (Hildermann *et al.*, 2010; Voss-Fels *et al.*, 2019). Molecular tools that have enabled sequencing of the full wheat genome will aid to faster breeding successes in the future (Lukaszewski *et al.*, 2014), but more holistic transformation of agricultural systems are required to prevent further environmental degradation.

2.4.2 GENOTYPE-DEPENDENCY OF AMF IN WHEAT

To reduce chemical inputs, stabilise yield and grain quality and improve stress resistance at the same time, the association of AMF and wheat has been investigated for more than 50 years. Mutualistic relationship of AMF and wheat is characterised by increased grain yield which underlines also the potential financial gains from AMF-application in wheat production (Mohammad *et al.*, 2004; Mäder *et al.*, 2011; Pellegrino *et al.*, 2015; Zhang *et al.*, 2019). However, several studies report varying mycorrhizal responsiveness in different wheat varieties which makes efficiency predictions of AMF-application in wheat more difficult (Azcón & Ocampo, 1981; Hetrick *et al.*, 1984; Manske, 1989; Kapulnik & Kushnir, 1991).

Most grasses are less dependent on symbiosis with AMF compared to herbaceous plants because of their extensively branched root system (Li *et al.*, 2005). Wheat has been characterised as a facultative mycotroph (Hetrick *et al.*, 1992), but it has been shown that AMF-colonisation in some varieties can even lead to growth depression (Hetrick *et al.*, 1992, 1995; Ryan *et al.*, 2005). From these studies, evidence is accumulating that the difference in mycorrhizal dependency (MD = yield of plants with AMF/yield of plants without AMF) is a strong genetic trait in wheat (Hetrick *et al.*, 1992). Reciprocal crossing of a high yielding cultivar with low MD and a landrace with high MD indicated chromosomal heritability of MD (Manske, 1989), but attempts to correlate AMF- responsiveness of wheat varieties and wheat progenies with the ploidy level gave no clear answer and could not reveal the localisation of AMF-dependent genes in the wheat genome (Kapulnik & Kushnir, 1991; Hetrick *et al.*, 1995).

Genotype-dependent responses of crops to AMF have been found in several other crops (Tawaraya, 2003), such as citrus (Graham & Eissenstat, 1994), onion (Taylor *et al.*, 2015), potato (Alaux *et al.*, 2018), sorghum (Leiser *et al.*, 2016), maize (Sawers *et al.*, 2017), barley (Castillo *et al.*, 2012) and durum wheat (Singh *et al.*, 2012; Ercoli *et al.*, 2017). In common wheat, genotype-dependent mycorrhization was first reported by Azcón and Ocampo (1981) in spring wheat and was further studied by Hetrick *et al.* (1992, 1993, 1996) in winter wheat. In several experiments, the authors compared modern wheat varieties, ancestors and landraces from different countries after inoculation with AMF isolates in the greenhouse (Hetrick *et al.*, 1992). In these trials, mycorrhizal response was higher in older wheat varieties (released before 1950) and landraces in comparison to modern wheat lines. From these observations the authors concluded that breeding conducted in high nutrient environments for increased nutrient use efficiency has decreased the MD of modern wheat varieties (Hetrick *et al.*, 1996). This hypothesis was disproved in several greenhouse and field studies that found higher or similar AMF-RC levels in modern wheat and older varieties or landraces (Ryan *et al.*, 1994; Hildermann *et al.*, 2010; Ercoli *et al.*, 2017; Lehnert *et al.*, 2017). Moreover, re-analyses of the dataset of Hetrick *et al*. by Sawers *et al.* (2010) using linear regression models did not find the same correlation of variety and MD. A meta-analysis by Lehmann *et al.* (2012) manifested that breeding did not lower responsiveness of modern wheat varieties to mycorrhizal fungi hence rejecting the theory by Hetrick *et al.* (1993, 1996). However, screenings of different crop species have shown negative response of domesticated crops to AMF under addition of P-fertiliser in contrast to positive AMF-response in their wild progenies under the same conditions (Martín-Robles *et al.*, 2018). The authors of this study conclude that modern varieties might have lost the ability to recognise beneficial symbionts hence resulting in negative growth responses when the association with such is not benefitting the plant. These findings re-opened the discussion on varietal differences regarding AMF that may or may not be considered in breeding programmes.

2.4.3 ARE AMF RELEVANT FOR WHEAT PRODUCTION?

In contrast to the benefits provided by AMF to other crops (Section 2.2.1), the importance of AMF associations with wheat are still under debate among mycorrhizal scientists. The previously described variation in responsiveness of different wheat varieties to AMF is one factor that fuels this discussion. Another is the ambiguous results that have been reported in studies with AMF and wheat showing a strong dependency of agricultural management and environmental conditions (Ryan & Graham, 2018). Many greenhouse experiments with AMF inoculation of wheat show positive effects such as increased biomass production

(Azcón & Ocampo, 1981; Oliveira *et al.*, 2016), enhanced nutrient uptakes (Elliott *et al.*, 2019) and higher abiotic (Lehnert *et al.*, 2018; Mathur *et al.*, 2019) and biotic stress tolerance (Mustafa *et al.*, 2016). At the same time, there are studies that found negative growth responses in different combinations of AMF isolates and wheat genotypes with high and low P-supply (Graham & Abbott, 2000; Li *et al.*, 2005, 2006; Garmendia *et al.*, 2017). This potential detrimental outcome of AMF and wheat associations was confirmed in field studies (Ryan *et al.*, 2005; Dai *et al.*, 2014) while other studies could not find connection between AMF-RC and yield or nutrient uptake (Ryan *et al.*, 1994; Hildermann *et al.*, 2010; Mao *et al.*, 2014). But there are also positive reports from field trials where inoculation with AMF enhanced wheat growth and yield in a field with almost no indigenous mycorrhizal population (Mohammad *et al.*, 2004) and increased grain Zn and Fe-content of durum wheat (Ercoli *et al.*, 2017). Cabral *et al.* (2016) observed positive correlations of photosynthetic activity and AMF-RC under heat stress and inoculation of wheat with AMF could promote drought tolerance leading to increased yield in water stressed plants (Al-Karaki *et al.*, 2004). Such effects might be particularly beneficial in non-irrigated wheat producing regions where rising temperatures and unpredictable or missing precipitation events are likely to reduce yields (Shiferaw *et al.*, 2013). For example, a field trial in India showed increased mineral nutrient concentrations after inoculation with AMF and plant growth promoting bacteria (Mäder *et al.*, 2011).

These beneficial effects in wheat with up to 20 % higher N and P contents and yields, as well as 10 % higher Zn contents in response to AMF inoculation were summarised in a meta-analyses by Pellegrino *et al.* (2015) comprising results of 38 published field trials (Fig. 2.6 A). This study was often cited as positive example for AMF application in crop production (Bender *et al.*, 2016; Imperiali *et al.*, 2017; Verzeaux *et al.*, 2017), but was also criticised by mycorrhizal researchers who were of the opinion that farmers should not consider AMF in wheat production as they found opposing effects in their experiments (Fig. 2.6 B) (Ryan & Graham, 2018). In response, Rillig *et al.* (2018) argued that their statement was too focused on yields by listing the numerous other advantages of AMF on agroecosystem functioning. Thereupon, Ryan *et al.* (2019) emphasised the need of holistic field-based research that considers the complex interactions of crop genotype, environment and management (G × E × M) to draw rigorous conclusions on the role of AMF in agroecosystems (Ryan & Graham, 2018). The discussion was finalised by a new meta-analysis from the Rillig-group that included more field studies and variables and reported increased grain yields in response to AMF inoculation (Fig. 2.6 C) (Zhang *et al.*, 2019). Surprisingly, this effect seemed to be less pronounced in wheat varieties released after 1950 (Fig. 2.6 D), a factor that was

thought not to affect AMF symbiosis (Section 2.4.2). From these observations the authors conclude that AMF responsiveness should be considered in modern breeding programmes to enhance wheat yields and to enhance P-use efficiency in wheat as suggested by other mycorrhizal scientists (Sawers *et al.*, 2008;Jacott *et al.*, 2017; P. Campos *et al.*, 2018).

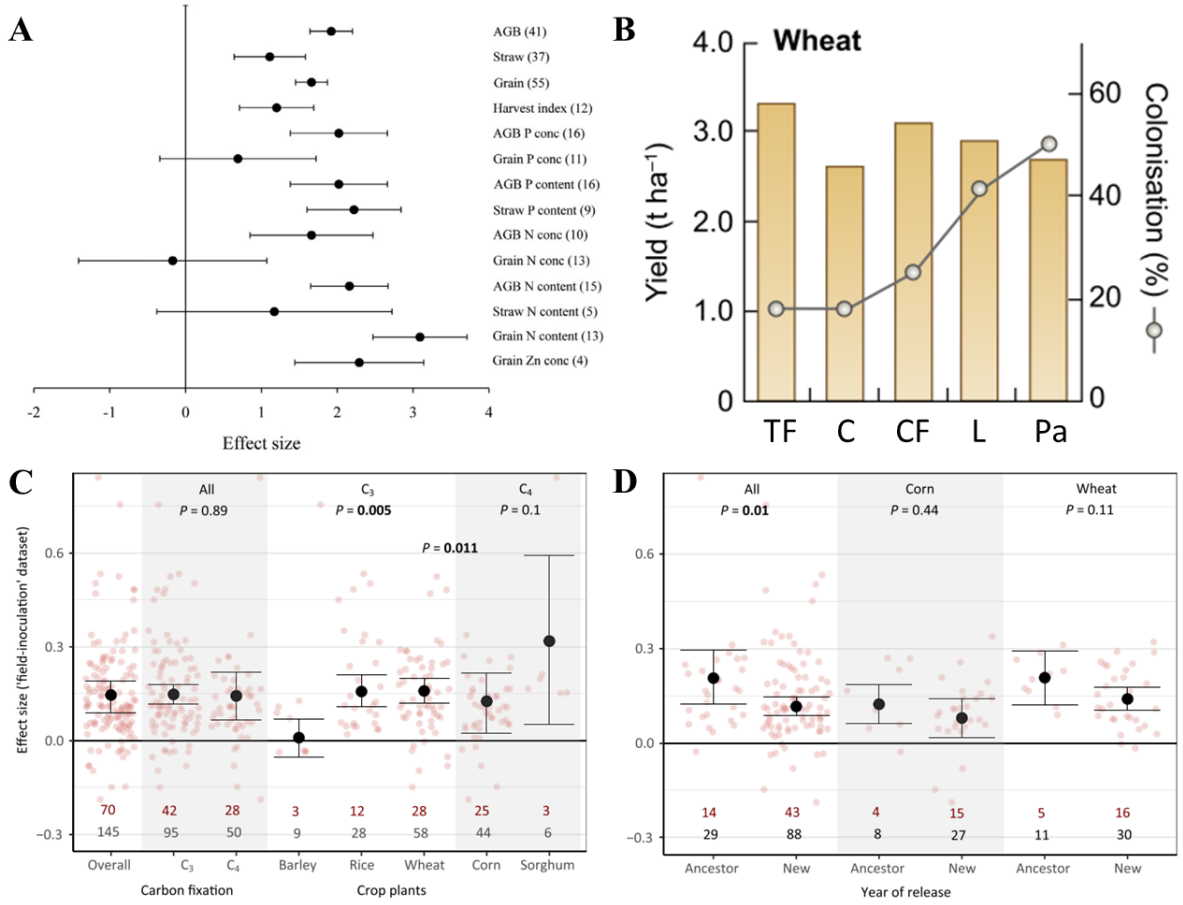


Fig. 2.6. Effects of AMF on yield and crop production parameters of different field-inoculation datasets with wheat **A**) Meta-analyses by Pellegrino *et al*. (2015) on different growth and yield parameters (AGB = above ground biomass, conc = concentration). A mean effect size (Hedge's g^{+}) is significantly different from zero when its 95% confidence interval does not overlap zero **B**) Relation of yield and AMF root colonisation in field trials by Ryan & Graham, (2018) with wheat (n = 4) grown after fallow maintained with tillage (TF), rape seed (C), fallow maintained with herbicides (CF), linola (L) and subterranean clover (Pa) **C**) Meta-analyses by Zhang *et al*. (2019) showing crop yields in response to AMF inoculation with effect of different crops and carbon fixation strategy. Numbers in red and black are study and trial numbers, respectively. Red data points represent original trail data **D**) Meta-analyses by Zhang *et al*. (2019) showing impact of year of release (new = after 1950, ancestor = before 1900) on effect of field inoculation with AMF.

2.5 SUMMARY

Decades of research have gathered results from greenhouse and field trials that show a pivotal role of arbuscular mycorrhizal fungi (AMF) in plant nutrition. This association was particularly beneficial for vascular plants to conquer land millions of years ago but is still evident in natural ecosystems. In agroecosystems, conventional management practices have shown to deplete arable soils of microbial activity for which reason commercially propagated AMF have been recommended to farmers. The application of such has not yet become standard practice as inoculation success varies strongly depending on environmental conditions, crop genotype and application method. The need of such implementations has raised conflicting opinions in the mycorrhizal researcher community which is currently confronted with striking discoveries in taxonomy, evolution and palaeobiology of mycorrhizal fungi. The new questions arising from these research areas will be met with rapidly advancing molecular tools, but to answer the agronomic research questions will require more holistic approaches. These should involve both phenological and molecular data of crops and their associated fungal microbiomes under the impact of different environmental factors. Wheat is one of the main staple foods and probably the most controversially discussed crop in mycorrhizal research where optimal conditions for beneficial outcome of AMF symbiosis remain to be elucidated. The role of specific host and fungal genotypes, but also the potential contribution of fine root endophytes in this association deserve further investigation.

CHAPTER 3. INTERACTIONS OF ARBUSCULAR MYCORRHIZAL FUNGI AND WHEAT GENOTYPES UNDER CONTRASTING AGRICULTURAL CONDITIONS

3.1 INTRODUCTION

The constant demand for yield increases in conventional wheat production is accompanied by the application of large amounts of mineral nitrogen (N) and phosphorus (P) fertilisers (Shiferaw *et al.*, 2013). In less intensive crop production systems, a significant proportion of P is acquired by arbuscular mycorrhizal fungi (AMF) (Smith & Smith, 2011; Smith *et al.*, 2011). Despite a growing body of evidence that AMF are able to mobilise and transfer N from inorganic and organic sources to their host (Hodge *et al.*, 2001; Govindarajulu *et al.*, 2005; Bender *et al.*, 2015; Thirkell *et al.*, 2019; Bukovská *et al.*, 2021), the significance of AMF for plant N-uptake is still under debate (Bücking & Kafle, 2015). Recent studies demonstrated that AMF contribute to N-acquisition in wheat under controlled conditions (Zhu *et al.*, 2016) and that mycorrhizal mediated N-uptake can differ significantly between wheat varieties (Elliott *et al.*, 2019; Thirkell *et al.*, 2020). These findings support the concept of breeding programmes for wheat varieties that benefit from mycorrhizal symbiosis, but there are conflicting opinions about this focus (Singh *et al.*, 2012; P. Campos *et al.*, 2018; Ryan & Graham, 2018). Besides the plant's nutritional status, mycorrhizal functioning is strongly affected by soil nutrient availability particularly N and P (Corkidi *et al.*, 2002). While the performance of AMF is limited in high-P environments (Smith & Read, 2008), raising the ratio of N:P has been suggested to enhance the efficiency of AMF-symbiosis (Johnson *et al.*, 2015; Püschel *et al.*, 2016; Verzeaux *et al.*, 2016). This effect in low-P soils can be anticipated by the addition of N in the form of mineral or organic fertilisers which have shown varying impact on AMF (Azcón *et al.*, 1992; Treseder, 2004; Ercoli *et al.*, 2017). Excessive application of N-fertilisers have detrimental impacts on soil microbiota such as AMF (Leff *et al.*, 2015; Ercoli *et al.*, 2017), while optimised N-management is important to promote mycorrhizal diversity in agricultural soils (Liu *et al.*, 2014). For the latter, increasing the mycorrhizal potential of a soil by the application of biostimulants that harbour multiple AMF species has been suggested to improve crop performance (Hoeksema *et al.*, 2010; Gosling *et al.*, 2016; Tamburini *et al.*, 2020), but this method was also reviewed as a potential risk for indigenous AMF species (Hart *et al.*, 2018; Thomsen & Hart, 2018). All of these discussion points leave open questions that are ideally answered by field-based research to suggest optimal agronomic conditions for AMF and wheat (Ryan *et al.*, 2019).

3.1.1 AIMS AND OBJECTIVES

The experiment described in this chapter was conducted to investigate the interaction of AMF and two contrasting winter wheat varieties under the impact of different fertiliser types and crop protection programmes in a low-P environment. The fertilisers (mineral nitrogen, biogas digestate and farmyard manure) provide inorganic and organic sources of N and will show how AMF are affected by the respective N-source. Furthermore, it was aimed to assess the effects of an AMF-based inoculum on plant growth and mycorrhizal root colonisation as well as yield and grain quality. The data from two consecutive growing seasons could then be analysed under the assumption that

I. Agricultural factors (variety, fertiliser, pesticides) have a significant impact on the symbiosis of AMF and wheat as host genotype, nutrient environment and plant health have shown to determine if the outcome of mycorrhizal associations is beneficial to negative in agroecosystems.
II. By identifying the optimal conditions, AMF can contribute to yield and plant health and decrease both fertiliser and pesticide use which has been demonstrated in several other crops and ultimately represents the sustainability aspect for applying biostimulants or promoting native mycorrhizal associations in agricultural systems.

In summary, these hypotheses will be tested by the following objectives:

1. Monitoring of colonisation of AMF in root systems of winter wheat at key development stages under the impact of inorganic and organic N-sources under conventional and organic crop protection management
2. Assessing the impact of a commercial AMF inoculum on plant health, performance, yield and grain quality
3. Comparing two wheat varieties from conventional and organic breeding backgrounds in their response to AMF colonisation under the influence of different agronomic management practices
4. Sample root material for molecular analysis of AMF communities and tracing of AMF inoculum

3.2 MATERIALS AND METHODS

3.2.1 FIELD SITE

The field trials were conducted at the research facilities of Newcastle University's Nafferton Experimental Farm in Northumberland (54°59'27.26"N, 1°54'26.96"W, Stocksfield, UK) in two consecutive years from autumn 2017-2018, and from autumn 2018-2019 (hereafter referred to as the 2018 and 2019 seasons).

3.2.2 CLIMATE CONDITIONS

The climate of the experimental area in Northern England is humid temperate with cool temperatures during winter and moderately warm summers with precipitation distributed fairly evenly throughout the year. Weather data at Nafferton Farm was recorded by an on-site automatic station located within 700 m of the field trials. The weather of the field site differed during the two years with dry weather conditions during the summer of 2018 (Appendix A, Fig. A.1) and high precipitation during the summer of 2019. Warm temperatures in summer 2018 passed to a mild winter season in 2018-2019 which was much warmer particularly in January/February compared to the previous winter of 2017-2018.

3.2.3 SOIL CONDITIONS

The soil type at the experimental field sites is a uniform sandy clay loam (Swain *et al.*, 2014) formed in slowly permeable glacial till deposits, *Cambic Stagnogley* (Avery, 1973) and had a pH of 6.25 in spring 2017 before the start of the experiment. At the same time point, available phosphorus, potassium and magnesium content were measured (Table 3.1). This analysis was repeated in spring 2019.

Table 3.1. Soil properties (topsoil) for the field trials in 2018 and 2019.

	pH	**P [mg L^{-1}]**	**K [mg L^{-1}]**	**Mg [mg L^{-1}]**
2018 (n=16)	6.4±0.13	6±0.44	66.8±7.74	145.8±7.83
2019 (n=4)	6.8±0.11	8.1±1.49	72±8.86	157±6.18

Available nitrogen (N) content in the form of ammonium and nitrate, was recorded during the spring of 2018 and 2019 for plots treated with farmyard manure (FYM) in the autumn and zero-input plots (n=64) each year at three different soil depths 0-30, 30-60 and 60-90 cm (Table 3.2). Soil sampling for available N was extended in the second season i.e. the spring of 2019 to include AMF-inoculation, crop protection and the effect of variety to try and identify any residual effects on N availability from the previous year's treatments. All soil analyses were conducted by NRM Laboratories (Berkshire, UK).

Table 3.2 Soil mineral nitrogen (means ± SE) at three depths for field trials in 2018 (n = 4) and 2019 (n = 16) in plots treated with farmyard manure (FYM, 170 kg N ha^{-1}) and plots without fertiliser and with conventional (conv. CP) or organic crop protection (org. CP).

NO_3 – N [mg kg^{-1}]	Depth [cm]	2018	2019
FYM - org. CP	0-30	4.9±1.34	1.8±0.30
	30-60	2.3±0.41	1.8±0.46
	60-90	1.6±0.26	1.6±0.40
FYM - conv. CP	0-30	3.6±0.75	2.3±0.28
	30-60	3.0±0.76	2.2±0.47
	60-90	1.6±0.53	1.7±0.30
Zero-input - org. CP	0-30	3.7±0.98	2±0.29
	30-60	3.6±0.8	1.7±0.27
	60-90	1.3±0.53	1.4±0.21
Zero-input - conv. CP	0-30	3.5±0.21	2.7±0.48
	30-60	3.0±0.46	3±0.48
	60-90	1.5±0.56	2.2±0.43
NH_4 – N [mg kg^{-1}]			
FYM - org. CP	0-30	2.8±0.51	2.2±0.27
	30-60	0.8±0.09	0.4±0.05
	60-90	0.6±0.09	0.4±0.09
FYM - conv. CP	0-30	3.7±0.38	3.0±0.25
	30-60	0.8±0.10	0.3±0.08
	60-90	0.7±0.05	0.2±0.05
Zero-input - org. CP	0-30	2.9±0.68	2.0±0.36
	30-60	0.9±0.12	0.4±0.05
	60-90	0.6±0.09	0.5±0.15
Zero-input - conv. CP	0-30	2.3±0.48	3.2±0.25
	30-60	0.7±0.13	0.3±0.07
	60-90	0.6±0.05	0.3±0.07
Total available N [kg ha^{-1}]			
FYM - org. CP	0-30	29.0±4.89	14.8±1.64
	30-60	11.6±1.35	8.4±1.67
	60-90	8.5±0.74	7.5±1.53
FYM - conv. CP	0-30	27.3±2.25	20.0±1.44
	30-60	14.3±2.71	9.5±1.84
	60-90	8.5±2.08	7.3±1.18
Zero-input - org. CP	0-30	24.7±1.83	15.0±1.68
	30-60	17.0±2.83	7.7±1.03
	60-90	6.9±1.92	7.0±1.03
Zero-input - conv. CP	0-30	21.8±1.58	22.0±2.39
	30-60	13.9±1.92	12.6±1.82
	60-90	7.7±2.24	9.1±1.63

3.2.4 EXPERIMENTAL SET-UP

The interactions of mycorrhizal inoculation, fertility management, variety and crop protection were assessed in a randomised split-split-split plot factorial design on an area of 0.3 ha (Fig. 3.1). Each of the four main blocks represented a replicate of the overall 32 tested conditions leading to a total of 128 plots. Within each replicate block, organic and conventional crop protection treatments were compared in two sub-blocks (24 m x 24 m). Each wheat variety was drilled into 24 m x 4 m strips, fertiliser sources were applied across the plots (6 m width) such that individual treatment plots were 6 m x 4 m. For each variety there was an inoculated treatment (+AMF) and a control without AMF with a 1 m gap between each inoculation treatment. Between the two different varieties, the conventional variety 'Skyfall' was drilled in both seasons to provide a buffer zone.

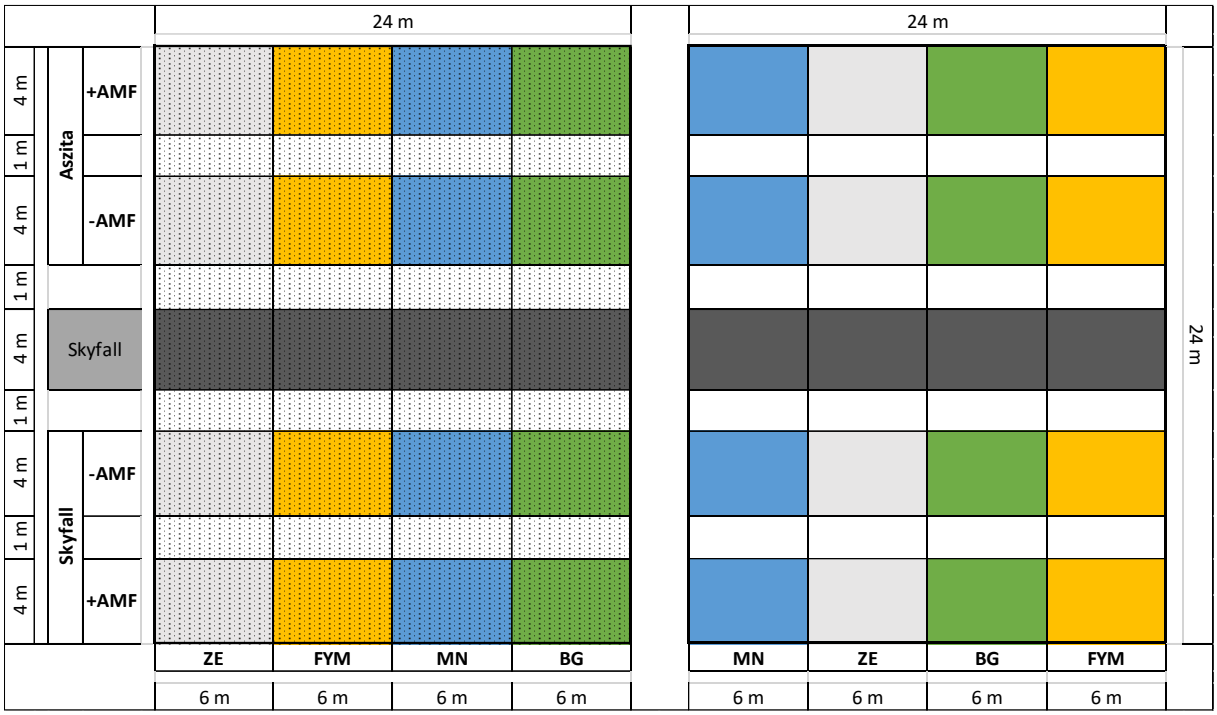


Fig. 3.1. Trial layout of one of the four replicate blocks of the 2018 and 2019 field trials showing crop protection (dots = conventional crop protection, plain = organic crop protection), and fertiliser source treatments (ZE= zero-input, FYM = farmyard manure, MN = mineral nitrogen, BG = biogas digestate) with + and - AMF inoculated seed of the two winter wheat varieties Aszita and Skyfall.

3.2.5 AGRONOMIC MANAGEMENT

All agronomic treatments and applications during the field trials from 2017-2019 are listed in Table 3.3. Before the set-up of the trials, the experimental fields had been covered with a grass-clover mixture in the two previous years. Winter wheat was drilled in early autumn after farmyard manure (FYM) had been applied to the treatment plots at a rate of 170 kg N ha^{-1}. Biogas digestate and mineral fertiliser (Ammonium nitrate, Nitram 34.5% N, CF fertilisers UK Ltd.) were applied in spring at the same N-rate. Samples of FYM and biogas digestate were analysed for nutrient composition at NRM Laboratories (Appendix A, Table A.2).

Table 3.3. Agronomic practices used during the 2018 and 2019 field trials at Nafferton Farm.

Application	2018	2019
Previous crop	Grass/clover	Winter wheat
Sowing date	12 October 2017	26 September 2018
Seed rate	400 seeds m^{-2} both varieties	Skyfall 400 seeds m^{-2} Aszita 550 seeds m^{-2}
Crop emergence	24 November 2017	15 November 2018
Herbicides		
Autumn 03/11/2017 01/10/2018	Liberator (400 g L^{-1} flufenacet and 100 g L^{-1} diflufenican) 0.6 L ha^{-1}	Liberator (400 g L^{-1} flufenacet and 100 g L^{-1} diflufenican) 0.6 L ha^{-1}
Spring-Summer 05/05/2018 10/05/2019	Axial (55 g L^{-1} pinoxaden) 0.3 L ha^{-1}, Adigor (47 % w/w methylated rapeseed oil) 1 L ha^{-1}, Fluroxypyr (200 g L^{-1}, 20.4% w/w) 1 L ha^{-1}	[1]Axial 0.3L ha^{-1} (55 g L^{-1} pinoxaden) Adigor (47 % w/w methylated rapeseed oil) 1 L ha^{-1}, Fluroxypyr (200 g L^{-1}, 20.4% w/w) 1 L ha^{-1}
Fungicides		
T1 04/05/2018 10/05/2019	Kestrel (160 g L^{-1} (16.2% w/w) prothioconazole, 80 g L^{-1} (8.1% w/w) tebuconazole and N,N,-Dimethyl decanamide) 1 L ha^{-1}, Bravo (500 g L^{-1} Chlorothalonil, 40.65% w/w) 1 L ha^{-1}	Turret (500 g L^{-1} (40.4% w/w) chlorothalonil) 2 L ha^{-1}, Enterprise (140 g L^{-1} boscalid and 50 g L^{-1} epoxiconazole) 2.5 L ha^{-1}
T2 30/05/2018 29/05/2019	Adexar (62.5 g L^{-1} epoxiconazole + 62.5 g L^{-1} fluxapyroxad) 1.5 L ha^{-1}, Bravo (500 g L^{-1} chlorothalonil, 40.65% w/w) 1 L ha^{-1}	Ceratavo Plus (100 g L^{-1} benzovindiflupyr) 0.75 L ha^{-1}, Daconil (500 g L^{-1} chlorothalonil) 2 L ha^{-1}, Epic (125 g L^{-1} epoxiconazole) 1 L ha^{-1}
T3 22/06/2018 21/06/2019	Kestrel (160 g L^{-1} (16.2% w/w) prothioconazole and 80 g L^{-1} (8.1% w/w) tebuconazole) 1 L ha^{-1}	Kestrel (160 g L^{-1} (16.2% w/w) prothioconazole & 80 g L^{-1} (8.1% w/w) tebuconazole) 1 L ha^{-1}
Plant growth regulators applied at T1	Chloromequat 1.25 L ha^{-1}	Chloromequat 1.25 L ha^{-1}
Fertilisers		
FYM (September)	170 kg N ha^{-1}	170 kg N ha^{-1}
Biogas digestate (April)	170 kg N ha^{-1}	170 kg N ha^{-1}
[2]**Ammonium nitrate** at 34.5 % (April)	170 kg N ha^{-1}	170 kg N ha^{-1}
Combine harvest	24/25 August 2018	29 August 2019

[1] This herbicide treatment was applied to all plots due to high weed pressure in 2019.
[2] Ammonium Nitrate was applied in two applications i.e. 70 kg ha^{-1} in mid-April and 100 kg two weeks later.

Wheat was drilled in early autumn using a twin hopper (Sprinter 4 ST, Horsch, Germany) with a working width of 4 m. This machine enabled drilling of wheat seeds at a soil depth of ~ 6 cm and the simultaneous placement of the inoculum 2 cm below the seed. In the first year, seed rates were similar for both varieties, but in the second year were unintentionally higher for Aszita due to the incorrect settings of the seed hopper (Table 3.3). Crop emergence was assessed by recording the average of counted plants in two randomly selected areas of 0.5 x 0.5 m for each plot.

Conventional crop protection and plant growth regulators (conv. CP) were applied throughout the cropping season at the required growth stages (Table 3.3). Pesticides were selected based on their AMF compatibility according to SmartRotations (Plantworks Ltd., UK).

3.2.6 WHEAT VARIETIES

Two winter wheat varieties were selected based on their contrasting characteristics (Table 3.4). Both varieties are considered as bread wheats, although classifications are different due to their release in different countries.

Table 3.4. Variety characteristics of the wheat varieties Skyfall and Aszita according to the breeder and www.wheatatlas.org

	Skyfall	**Aszita**
Release	2014, United Kingdom	2004, Switzerland
Breeder	RAGT Seeds Ltd.	Getreidezüchtung Peter Kunz
Growth habit	Winter	Winter
Breeding background	Conventional	Organic
Yield	High	Low
Growth	Short straw	Long straw
Protein content	11.8 %	13.1 %
Disease resistance:		
▪ Yellow Rust	medium	good
▪ *Septoria tritici* blotch	medium	excellent
▪ Fusarium head blight	high	very good
▪ Mildew	medium	good

Skyfall is a modern, high yielding, semi-dwarf variety, which is one of the most widely grown winter wheats on the UK market (RAGT, UK). Skyfall possesses a medium to high resistance to common wheat diseases such as Fusarium, *Septoria tritici* blotch and brown rust or eyespot. Aszita on the other hand is mostly used in organic and biodynamic wheat production (http://biosorten.de/wiki/Aszita). The variety is characterised by long straw growth of more

than 1 m and achieves low grain yields, but with high quality.

From the information provided by the breeders, Skyfall and Aszita also vary in their development time with Skyfall being an early flowering variety and Aszita a late flowering variety. This behaviour leads to slight shifts in the growth stages (~ 4 Zadoks growth stages difference), but due to practical reasons assessments and sampling were conducted for both varieties on the same day.

3.2.7 AMF INOCULUM

The inoculum (INOQ Advantage, INOQ GmbH, Germany) used in the field experiment was a powder of sheared roots of different host plants (Table 3.5) that were grown in beds filled with sterilized sand/vermiculite mixtures (36:65) under ambient light and temperature conditions in northern Germany (Loitze-Solkau, 52°54'18.0"N 10°50'01.5"E). The roots contained three different AMF species (*Rhizophagus irregularis*, *Funneliformis mosseae*, *Funneliformis caledonius*) according to the producer. The species composition in the inoculum as well as the number of propagules was different in the two cropping seasons (Table 3.5). This variation is due to climate conditions at the semi-controlled production facilities of INOQ GmbH that impact sporulation and mycorrhizal growth (Louis Mercy, pers. comm.).

Table 3.5. Composition of host plant and AMF species as well as number of propagules in mycorrhizal root powder 'INOQ Advantage' applied at the beginning of each field trial.

	2018	**2019**
Host plant species	*Plantago lanceolata, Zea mays, Ocimum basilicum, Euphorbia lathyrism, Allium schoenoprasum, Petroselinum crispum*	*Zea mays, Plantago lancelota*
AMF species:		
▪ *Rhizophagus irregularis*[1]	84 %	53 %
▪ *Funneliformis mosseae*	15 %	27 %
▪ *Funneliformis caledonius*	1 %	20 %
Number of propagules	11.08 Bio propagules kg^{-1}	180 Mio propagules kg^{-1}

[1] The proportion of *R. irregularis* in the inoculum of the first year contained a single strain (QS81) and two strains (QS81 and MA1) in the second year (Louis Mercy, pers. comm.).

The inoculum was applied at a rate of 100 g ha^{-1} using sterilised sand as a carrier substrate. Before drilling, 150 g of INOQ Advantage was mixed thoroughly into 15 kg of sterilised sand and filled into the hopper tank. After drilling and inoculation in the field, excess inoculum was collected from the hopper to calculate the actual applied amount. Sterilised sand without inoculum was applied to the plots in the control –AMF treatment.

3.2.8 SAMPLING FOR BIOMASS AND AMF ROOT COLONISATION

Sampling time points were selected based on physiological time points during wheat development that would be reflected in mycorrhizal root colonisation (Fig. 3.2). The first time point at crop emergence (GS12) was selected to show potential early colonisation by AMF, whereas the second time point at GS22 would be reached just after winter giving a base level of AMF root colonisation before the start of rapid spring growth. The next sampling was conducted at stem elongation (GS32) after the application of biogas digestate and mineral N fertilisers, as well as the first fungicide treatment of the crop protection programme (Table 3.3). Flowering at GS65 marks the shift in the development to grain set. The last sampling was done before harvest at GS90 to assess whether AMF were still colonising roots during maturity.

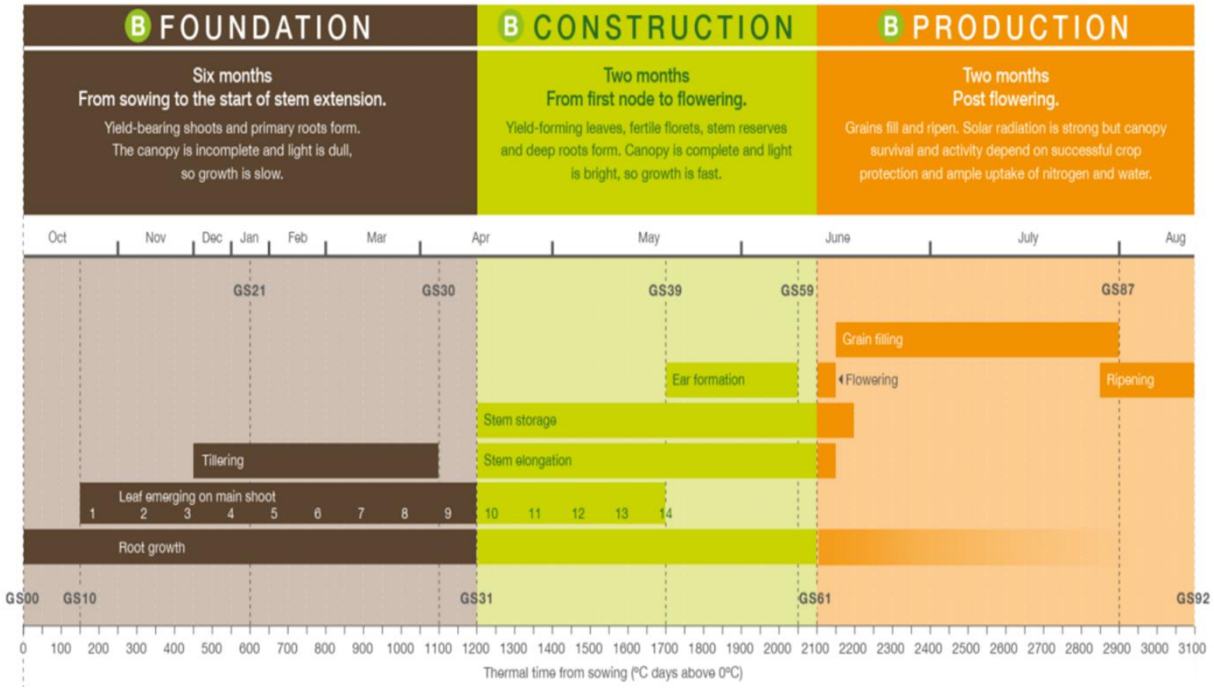


Fig. 3.2. Key development phases and growth stages of winter wheat in the UK (AHDB, 2018). For the present study, growth stages (GS) and benchmarks (B) might be shifted to slightly later calendar dates due to the location of the field trials in the north of the UK.

Shoot and root samples were collected at five key development stages of wheat according to Zadoks *et al.* (1974) for the monitoring of plant growth and AMF colonisation of root systems (Table 3.6). Due to their different physiology, Skyfall was usually ahead of Aszita in terms of development. Dates of sampling varied between the two seasons due to different weather conditions that affected plant growth and development.

Table 3.6. Sampling dates during the growing seasons 2017-2019

Sampling	Zadoks-growth stage	Description	Sampling dates during trials	
			2018	2019
1st	12-13	Seedling growth	06/12/2017	29/11/2018
2nd	22-23	Tillering	26/03/2018	05/03/2019
3rd	32-33	Stem elongation	22/05/2018	14/05/2019
4th	62-64	Flowering	19/06/2018	25/06/2019
5th	90-92	Maturity	01/08/2018	14/08/2019

For the first two growth stages, plants were sampled from an area of 0.25 m² in each plot to extract whole plants from the soil. From GS32 onwards roots and shoots were sampled separately by cutting off the shoots within the sampling area (0.25 m²) at the stem base, prior to digging the roots out. Tillers were counted and weighed before and after drying at 70°C. Dried shoot samples were milled (SK300, Retsch, Germany) and filled into tubes prior to processing for nutrient analysis.

Roots were extracted from the upper 15 cm of the topsoil layer around the stem base of 5-6 selected plants in the same sampling area. Roots were washed under running tap water to remove all soil residuals and were then transferred into 50 ml Falcon tubes with 50 % Ethanol. The samples were stored at 4°C in darkness prior to staining.

3.2.9 ROOT STAINING AND AMF-COLONISATION ASSESSMENT

Root samples were stained using the ink-vinegar method described by Vierheilig *et al.* (1998). First, roots were rinsed in tap water to remove ethanol residues after sampling. Then, samples were incubated in 10 % KOH solution at 80°C and subsequently in 8%-acetate-5%-China ink solution either over night or in the oven. Incubation times in the oven were between 1-2 h depending on the sampling time with younger roots using shorter incubation times than older roots from later growth stages. Roots were rinsed in tap water between each step.

For the colonisation assessment, 30 randomly chosen root pieces of approx. 1 cm length were cut from each sample and placed on a cover slide (24 x 50 mm; Carl Roth GmbH + Co. KG, Germany). This slide was then pressed on a microscope slide (Menzel Gläser; Thermo Fisher Scientific, USA) with 70 % Glycerol for sample preservation. Root colonisation [%] by AMF was assessed under the microscope (Primo Star, Zeiss, Germany). Microscopy data of the observed colonisation was entered in a Microsoft Excel spreadsheet (INOQ Calculator Advanced, Louis Mercy, 2017). This programme calculates mycorrhizal colonisation parameters as described by Trouvelot (1986) which are AMF frequency (F %), AMF intensity

(M%) and arbuscule intensities (A%) in the whole root system as well as mycorrhizal and arbuscule intensities in single mycorrhized root fragments (m% and a%). The INOQ Calculator Advanced extends this method for vesicle abundance (V%, v%) and intensities of intraradical hyphae (H%, h%). Vesicles are quantified by a scale of 0-3 according to the intensity of vesicles in each root fragment, a similar ranking as for arbuscule abundance. Hyphal intensities were obtained by subtraction of arbuscule and vesicle intensities from M% assuming that AMF are composed of three physiological parameters i.e. hyphae, arbuscules and vesicles (Mercy, 2017). These parameters are stated in relation to each other (Ar %, Hr%, Vr%) which add up to the overall M%. Additionally, the spreadsheet contains a column for the differentiation of *arum* and *paris*-type arbuscules, but this assessment was not possible for the field samples in this study. For the sake of clarity, results of AMF colonisation are summarised prioritising F%, M% and the three mycorrhizal components A%, H% and V%.

3.2.10 DISEASE ASSESSMENT

Disease severity, normalised difference vegetation index (NDVI) and leaf greenness (SPAD, Section 3.2.11) were assessed during the period between flag leaf emergence (GS39) and the end of flowering (GS69) at 10-14 day intervals. Assessments were conducted on 10 randomly selected tillers within each plot. Overall, four disease assessments on leaves and one assessment on wheat ears were conducted along with four to five SPAD and NDVI assessments in each year.

Disease severity was assessed for powdery mildew (*Blumeria graminis* f. sp. *tritici*), yellow rust (*Pucchinia striiformis* f. sp. *tritici*) and *Septoria tritici* blotch (*Zymoseptoria tritici*) on the first, second and third leaf by estimating the % of leaf area covered (Shaner, 1977). Diseases scored on the ears were Fusarium head blight (*Fusarium* spp.), yellow rust, powdery mildew as well as glume blotch (*Parastagonospora nodorum*). The area under the disease progress curve (AUDPC) was calculated for each disease and time point using the formula of Shaner and Finney (1977).

3.2.11 SPAD AND NDVI

Chlorophyll content was measured using a hand-held SPAD meter (SPAD 502 Plus; Konica Minolta, Japan). Ten readings on flag leaves from two areas within each plot were conducted and summarised to one average value.

The NDVI was measured using a Green Seeker® (Trimble, USA). Measurements were conducted by moving the sensor horizontally for around 10 seconds over the crop canopy at a height of 30 cm. Two duplicate readings were collected per plot to cover the whole plot area.

3.2.12 FINAL HARVEST AND GRAIN ANALYSES

Plant height as well as plant number were assessed on an area of 0.25 m² from each plot one week before harvest. Shoots were removed from the area and tillers were counted. Biomass of ears and shoots was determined separately before and after drying at 70 °C for 72 hrs. Dried subsamples of shoots (50 g) were stored for nutrient analyses and a subsample of ears (150 g) was threshed (LD350; Wintersteiger, Austria) for yield component analyses. Seeds were weighed and total seed number, as well as thousand grain weight (TGW) were determined using a C3-seed counter (Elmor, Switzerland).

Harvest (Dominator 38; Claas, Germany) was conducted under dry weather conditions after lodging had been mapped. Total fresh yield was recorded per plot during combining. A subsample of grain (200 g) was collected and dried at 70°C for three days for determination of grain moisture content. Grain yields throughout are presented at 15% moisture content. The grain was cleaned (Lainchbury HC1/7 W grain cleaner; Blair Engineering, UK) before TGW determination.

Grain samples of approx. 500 g were used for grain quality analyses which were conducted in the laboratory of Coastal Grains Ltd. (Belford, United Kingdom). Protein content, hectolitre weight and moisture content were measured by near-infrared transmittance technology in a grain analyser (Infratec ™ 1241, Foss, Denmark). For Hagberg falling number measurements (Hagberg, 1961), around 50 g of grain were milled (LM 3100, Perten, Sweden). Depending on the moisture content, a portion of flour was mixed with 25 ml of distilled, deionised water in a glass tube which was then placed in a Falling number system (FN 1310; Perten, Sweden).

3.2.13 PLANT NUTRIENT ANALYSIS

Nutrient and N-concentration in shoots and flour samples were analysed as described by Wang *et al.* (2020). In brief, contents of macro- and micro mineral nutrients were measured by Inductively Coupled Plasma Optical Spectrometry (ICP-OES). As preparation, samples were digested in nitric acid using a microwave reaction unit (CEM-Mars 6, USA). Nitrogen concentrations were assessed by the total combustion method using a vario MACRO cube C/N Analyzer (Elementar Ltd., Germany).

3.2.14 STATISTICAL ANALYSIS

The effects of season and agronomic management practices were analysed in linear mixed-effect models using the nlme-package (Pinheiro & Bates, 2000) in RStudio (vs. 4.0.2, R Core Team 2020). Mixed effect models are different to linear models and analyses of variances as they distinguish between fixed and random effects (Crawley, 2013). Fixed

effects describe factors which explain the mean of a variable of interest, whereas random effects explain potential variance of the variable. Given the experimental design of the field trial (Fig. 3.1), season, crop protection, variety, AMF inoculum and fertiliser were nested as fixed effects within blocks. Blocks containing the treatments up to inoculation-level were used as random effects to incorporate possible environmental heterogeneities at the field site (e.g. soil structure, slope) in the model. The last factor 'fertiliser' of the nested experimental design was not included in random effects as fitting these can saturate the model and is therefore not recommended (Pinheiro & Bates, 2000).

Biomass and AMF root colonisation data was analysed by growth stage to exclude the effect of plant development. Normal distribution was assessed by QQ-plotting of residuals as a pre-requisite for analyses of variance (ANOVA). The commonly applied arcsine-transformation of mycorrhizal colonisation data was refused as most AMF parameters showed normal distribution within the model (Warton & Hui, 2011). Multiple comparisons of fertiliser treatments were analysed with Tukey contrasts by general linear hypothesis testing (glht) using the multcomp-package (Hothorn *et al.*, 2008). Post-hoc analyses for significant two-way interactions ($p \leq 0.05$) were conducted using the same method. Figures were created using the ggplot2-package (Wickham, 2016).

3.3 RESULTS

There was one plot in the 2018 season with Skyfall, farmyard manure (FYM) and AMF inoculum application that showed a very low crop establishment and hence was not used for data collection.

3.3.1 GRAIN YIELD, TOTAL BIOMASS, PLANT HEIGHT AND HARVEST INDEX

Grain yield varied significantly between years ($p = 0.006$, Table 3.7) with 2018 showing higher yields than 2019. This difference was also visible in a lower harvest index in 2019. Although plants were significantly taller in 2019 compared to 2018 ($p = 0.002$), there was no seasonal variation in total biomass. Conventional crop protection (conv. CP) increased grain yield, total biomass and harvest index but organically managed wheat plants were taller ($p = 0.001$). Grain yield as well as harvest index were significantly higher in Skyfall compared to Aszita which was taller than the semi-dwarf variety. Inoculation with AMF had no effect on yield or biomass production, but fertiliser treatment had a significant impact on all yield parameters. Highest grain yield, plant height and biomass were in response to mineral nitrogen (N) and biogas digestate application. Harvest index was highest in plants with biogas digestate treatment and marginally lower in FYM and mineral N treated wheat. The lowest harvest index

was found in zero-input plots that also showed the lowest biomass production, plant height and grain yield in comparison to fertiliser treatments. Wheat with FYM showed intermediate grain yield, height and biomass production, but harvest index was not different to the mineral N-treatment.

Table 3.7. Effects of season, crop protection, variety, AMF inoculation and fertiliser on plant height, total biomass, grain yield and harvest index. ANOVA *p*-values in bold indicate significant main effects and interactions. Means within columns followed by the same letter are not significantly different at $p \leq 0.05$.

	Plant height [cm]	**Grain yield [t ha^{-1}]**	**Total biomass [t ha^{-1}]**	**Harvest index [%]**
Year (YR)				
2018 (n=127)	77.8±1.62b	6.6±0.18a	8.6±0.24	51±0.49a
2019 (n=128)	86.6±1.57a	4.1±0.16b	8.8±0.31	39±0.56b
Crop protection (CP)				
Conventional (n=128)	76.9±1.5b	6.3±0.2a	9.9±0.3a	47.2±0.72a
Organic (n=127)	87.5±1.64a	4.3±0.16b	7.5±0.21b	42.7±0.72b
Variety (VR)				
Aszita (n=128)	97.4±0.99a	4.9±0.16b	9±0.29	40.9±0.56b
Skyfall (n=127)	66.8±0.83b	5.7±0.22a	8.4±0.27	49±0.73a
Inoculation (AMF)				
-AMF (n=128)	81.7±1.63	5.2±0.19	8.7±0.28	45.1±0.72
+AMF (n=127)	82.7±1.65	5.4±0.2	8.7±0.27	44.8±0.77
Fertiliser (FT)				
Biogas digestate (n=64)	86±2.26a	6±0.27a	9.6±0.39a	46±1.12a
Farmyard manure (n=63)	79.8±2.41b	4.9±0.2b	8.5±0.35b	44.6±0.99ab
Mineral N (n=64)	87.1±2.19a	6.1±0.35a	10.2±0.39a	45.3±1.04ab
Zero-input (n=64)	75.9±2.16c	4.3±0.22c	6.5±0.28c	43.9±1.07b
ANOVA *p-values*				
Main effects				
YR	**0.002**	**0.006**	ns	**≤0.001**
CP	**≤0.001**	**≤0.001**	**≤0.001**	**≤0.001**
VR	**≤0.001**	**0.004**	ns	**≤0.001**
AMF	ns	ns	ns	ns
FT	**≤0.001**	**≤0.001**	**≤0.001**	**0.003**
Interactions				
YR:CP	**0.019**	ns	ns	ns
YR:VR	ns	ns	ns	ns
CP:VR	**0.002**	**0.019**	ns	ns
YR:AMF	ns	ns	ns	ns
CP:AMF	ns	ns	ns	ns
VR:AMF	ns	ns	ns	ns
YR:FT	**≤0.001**	**0.009**	**0.008**	ns
CP:FT	**0.004**	**≤0.001**	**0.008**	**0.041**
VR:FT	ns	ns	ns	**0.002**
AMF:FT	ns	ns	**0.028**	ns
YR:CP:VR	ns	ns	ns	ns
YR:CP:AMF	ns	ns	ns	ns

YR:VR:AMF	ns	ns	ns	ns
CP:VR:AMF	ns	ns	ns	ns
YR:CP:FT	ns	ns	ns	ns
YR:VR:FT	ns	ns	ns	ns
CP:VR:FT	ns	**0.044**	ns	ns
YR:AMF:FT	ns	ns	ns	ns
CP:AMF:FT	ns	ns	**0.022**	ns
VR:AMF:FT	ns	ns	ns	ns
YR:CP:VR:AMF	ns	ns	ns	ns
YR:CP:VR:FT	ns	ns	ns	ns
YR:CP:AMF:FT	ns	ns	ns	ns
YR:VR:AMF:FT	ns	ns	ns	ns
CP:VR:AMF:FT	ns	ns	**0.021**	ns
YR:CP:VR:AMF:FT	ns	ns	ns	ns

Crop protection affected plant height differently in the two seasons (p = 0.019, Table 3.7). Average plant height was lower in 2018 compared to 2019 independent of CP (Table 3.8). In both field trials plants were taller in plots with org. CP.

Table 3.8. Effect of season × crop protection (CP) on plant height.

Means ± SE (n=32)	**Season**	**Conv. CP**		**Org. CP**	
Plant height [cm]	2018	71.2±1.96	Bb	84.5±2.3	Ba
	2019	82.6±2.04	Ab	90.5±2.29	Aa

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

The interaction of variety × CP significantly affected plant height (p = 0.002) and grain yield (p = 0.019) in both seasons (Table 3.7). Aszita was taller than Skyfall independent of growth regulator application (Table 3.9). Grain yield of Skyfall was higher with the use of pesticides (conv. CP) than with org. CP. With the latter, grain yield levels of Skyfall were similar to those of Aszita which achieved lower yields than Skyfall also with conv. CP. The harvest index of Skyfall was higher than Aszita, even though it was lower when org. CP was applied.

Table 3.9. Effect of variety × crop protection (CP) on plant height, grain yield and harvest index.

Means ± SE (n=32)	**Variety**	**Conv. CP**		**Org. CP**	
Plant height [cm]	Aszita	90.7±1.21	Ab	104.2±1	Aa
	Skyfall	63.1±1.23	Bb	70.6±0.89	Ba
Grain yield [t ha^{-1}]	Aszita	5.5±0.21	Ba	4.3±0.22	Ab
	Skyfall	7±0.32	Ab	4.4±0.22	Aa
Harvest index [%]	Aszita	42.5±0.76	Ba	39.2±0.79	Bb
	Skyfall	51.8±0.91	Aa	46.2±1.05	Ab

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

The interaction of fertiliser × season influenced all grain yield parameters except harvest index (Table 3.7). In all tested nutrient regimes, plants were taller in 2019 than 2018 (Table 3.10). In 2018, plants were tallest in plots treated with mineral N, but the same level was reached with biogas digestate application in 2019. Wheat treated with FYM was taller than wheat without fertiliser application, but only in 2019. Total crop biomass at harvest was highest in plots with mineral N application and lowest in zero-input plots in 2018. Only in plots with biogas digestate did biomass production increase from 2018 to 2019 while other treatments showed no significant variation between seasons. With that increase, biogas digestate reached similarly high levels of biomass as in the mineral N-treatment which was slightly lower in 2019 than the biogas digestate treatment. Grain yield was highest in wheat which received biogas digestate or mineral N in both seasons. Compared to these two treatments, FYM application resulted in lower yields which were on a similar level with yields from zero-input plots in 2018, but higher in 2019. Under all nutrient regimes, grain yields were lower in 2019 than 2018.

Table 3.10. Effect of season × fertiliser on plant height, total biomass and grain yield.

Means ± SE (n=32)	**Fertiliser**	**2018**		**2019**	
Plant height [cm]	Biogas digestate	79±2.9	Bb	92.9±3.04	Aa
	Farmyard manure	76.1±3.64	Cb	83.4±3.08	Ba
	Mineral N	82.4±3.16	Ab	91.7±2.84	Aa
	Zero-input	73.7±3.17	Cb	78.2±2.93	Ca
Total biomass [t ha^{-1}]	Biogas digestate	8.9±0.44	Bb	10.3±0.63	Aa
	Farmyard manure	8.1±0.46	Ba	8.8±0.53	Ba
	Mineral N	10.5±0.42	Aa	9.9±0.66	ABa
	Zero-input	6.9±0.36	Ca	6.2±0.43	Ca
Grain yield [t ha^{-1}]	Biogas digestate	7.3±0.31	Aa	4.7±0.31	Ab
	Farmyard manure	5.8±0.23	Ba	4±0.24	Bb
	Mineral N	7.6±0.44	Aa	4.6±0.38	Ab
	Zero-input	5.5±0.22	Ba	3.1±0.23	Cb

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

The CP × fertiliser interaction had significant impact on all assessed yield parameters (Table 3.7). Wheat treated with conv. CP was shorter compared to wheat with org. CP (Table 3.11). Independent of CP application, plants were tallest with biogas digestate and mineral N followed by FYM and shortest in zero-input plots.

With or without fertiliser treatment, wheat with org. CP produced less biomass compared to wheat with conv. CP. With conv. CP, total biomass was highest with the mineral N and biogas digestate treatments and lowest in zero-input plots. With org. CP, biomass production in all

fertiliser treatments was not significantly different, but higher compared to zero-input plots. Grain yield was overall higher in wheat that received conv. CP compared to org. CP. In wheat with conv. CP, highest grain yield was achieved in response to mineral N application, followed by biogas digestate application. Lowest grain yield was harvested from FYM and the zero-input plots. In wheat with org. CP, highest grain yields were achieved with biogas digestate application followed by mineral N and FYM treatments and lowest yields in zero-input plots. Compared to plots with conv. CP, harvest index was lower with org. CP for all tested nutrient regimes. In wheat with conv. CP, harvest index was highest in wheat with biogas digestate and mineral N and lowest in wheat following FYM application and without fertiliser input. In wheat with org. CP, harvest index was highest with biogas digestate and significantly lower in zero-input plots than in wheat with fertiliser application.

Table 3.11. Effect of crop protection (CP) × fertiliser on plant height, total biomass, grain yield and harvest index.

Means ± SE (n=32)	**Fertiliser**	**Conv. CP**		**Org. CP**	
Plant height [cm]	Biogas digestate	81.8±2.89	Ab	90.1±3.36	Aa
	Farmyard manure	72.6±3.1	Bb	87.1±3.25	Ba
	Mineral N	83.1±2.86	Ab	91±3.2	Aa
	Zero-input	69.9±2.52	Cb	81.9±3.21	Ca
Total biomass [t ha^{-1}]	Biogas digestate	11.1±0.56	Aa	8±0.38	Ab
	Farmyard manure	9.4±0.53	Ba	7.5±0.39	Ab
	Mineral N	11.9±0.5	Aa	8.4±0.42	Ab
	Zero-input	7.2±0.43	Ca	5.9±0.33	Bb
Grain yield [t ha^{-1}]	Biogas digestate	6.8±0.36	Ba	5.2±0.36	Ab
	Farmyard manure	5.5±0.29	Ca	4.3±0.24	Bb
	Mineral N	7.9±0.41	Aa	4.2±0.32	Bb
	Zero-input	5±0.32	Ca	3.7±0.26	Cb
Harvest index [%]	Biogas digestate	48.1±1.46	Aa	43.8±1.64	Ab
	Farmyard manure	46.2±1.4	Ba	42.9±1.36	ABb
	Mineral N	48.4±1.4	Aa	42.2±1.35	ABb
	Zero-input	46±1.52	Ba	41.9±1.43	Bb

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

The harvest index varied in the two varieties depending on the applied fertiliser (p = 0.002, Table 3.7), but was higher in Skyfall than Aszita independent of fertiliser treatment (Table 3.12). No variation of harvest index in response to fertiliser treatments occurred in Aszita. In Skyfall, highest harvest index was achieved with biogas digestate and lowest without fertiliser input.

Table 3.12. Effect of variety × fertiliser on harvest index.

Means ± SE (n = 32)	**Fertiliser**	**Aszita**		**Skyfall**	
Harvest index [%]	Biogas digestate	40.7±1.23	Ab	51.2±1.35	Aa
	Farmyard manure	40.7±1.13	Ab	48.6±1.3	BCa
	Mineral N	41.3±1.04	Ab	49.3±1.51	Ba
	Zero-input	40.8±1.15	Ab	47±1.65	Ca

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

A significant interaction of AMF inoculation × variety was detected for total biomass (p = 0.028, Table 3.7). Without AMF inoculation, biomass production was highest with mineral N application and lowest without fertiliser application (Table 3.13). With AMF inoculation, total biomass of plots with biogas digestate, mineral N and FYM application was not significantly different. In zero-input plots, the application of AMF inoculum increased total biomass. In plots with mineral N-treatment, total biomass was decreased when AMF inoculum was applied.

Table 3.13. Effect of fertiliser × AMF inoculation on total biomass.

Means ± SE (n = 32)	**Fertiliser**	**-AMF**		**+AMF**	
Total biomass [t ha^{-1}]	Biogas digestate	9.7±0.47	Ba	9.5±0.63	Aa
	Farmyard manure	8.4±0.57	Ca	8.5±0.41	Aa
	Mineral N	10.7±0.53	Aa	9.6±0.57	Ab
	Zero-input	6±0.32	Db	7.1±0.45	Ba

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

3.3.2 YIELD COMPONENTS

Thousand grain weight (TGW) was lower in 2019 compared to 2018 (Table 3.14). Application of conv. CP increased TGW and ears per m². Variety differences were visible in all measured yield components with higher plants and ears per m² in Aszita, but lower grains per ear and TGW in comparison to Skyfall. In both varieties, fertiliser application affected ear number and TGW significantly (p < 0.001). Highest ear number was found in plants with mineral N

treatment and lowest without fertiliser input. Application of biogas digestate and FYM achieved similar ear numbers and these two treatments also showed highest TGW.

Table 3.14. Effects of season, crop protection, variety, AMF inoculation and fertiliser on yield components. ANOVA *p*-values in bold indicate significant main effects and interactions. Means within columns followed by the same letter are not significantly different at $p \leq 0.05$.

	Plants m^{-2}	**Ears m^{-2}**	**Grains per ear**	**TGW [g]**
Year (YR)				
2018 (n=127)	99±1.79	330.2±6.72	30.8±0.45	42.3±0.28a
2019 (n=128)	109.5±2.45	324.1±9.4	29.6±0.64	39.1±0.32b
Crop protection (CP)				
Conventional (n=128)	108.3±2.07	352±8.59a	30.4±0.55	42±0.33a
Organic (n=127)	100.2±2.27	302.1±7.08b	30±0.56	39.3±0.29b
Variety (VR)				
Aszita (n=128)	108.5±2.31a	341±8.4a	27.9±0.48b	39.1±0.21b
Skyfall (n=127)	100±2.01b	313.2±7.76b	32.4±0.55a	42.3±0.37a
Inoculation (AMF)				
-AMF (n=128)	106.4±2.08	326±8.41	30.4±0.52	40.5±0.33
+AMF (n=127)	102.1±2.3	328.3±7.95	30±0.59	40.9±0.34
Fertiliser (FT)				
Biogas digestate (n=64)	100.9±3.19	337.3±11.1b	30.9±0.78	41.7±0.45a
Farmyard manure (n=63)	107.7±2.82	318.2±9.52b	29.7±0.71	40.9±0.42a
Mineral N (n=64)	104±3.2	384.4±10.59a	31.1±0.76	40±0.54b
Zero-input (n=64)	104.5±3.19	268.6±10.15c	29±0.86	40.1±0.44b
ANOVA *p-values*				
Main effects				
YR	ns	ns	ns	**0.009**
CP	ns	**0.008**	ns	**0.002**
VR	**0.007**	**0.025**	**≤0.001**	**≤0.001**
AMF	ns	ns	ns	ns
FT	ns	**≤0.001**	ns	**≤0.001**
Interactions				
YR:CP	ns	ns	**0.035**	ns
YR:VR	**0.012**	ns	ns	ns
CP:VR	ns	ns	ns	**0.003**
YR:AMF	ns	ns	ns	ns
CP:AMF	ns	ns	ns	ns
VR:AMF	ns	ns	ns	ns
YR:FT	ns	ns	ns	ns
CP:FT	ns	ns	**0.021**	**≤0.001**
VR:FT	ns	ns	ns	**≤0.001**
AMF:FT	ns	**0.015**	ns	ns
YR:CP:VR	ns	ns	**0.017**	ns
YR:CP:AMF	ns	ns	ns	ns
YR:VR:AMF	ns	ns	ns	ns
CP:VR:AMF	ns	**0.033**	ns	ns
YR:CP:FT	**0.007**	**0.029**	**0.006**	**0.022**
YR:VR:FT	ns	ns	ns	ns

CP:VR:FT	ns	ns	ns	**≤0.001**
YR:AMF:FT	ns	ns	ns	ns
CP:AMF:FT	ns	**0.030**	ns	ns
VR:AMF:FT	ns	ns	ns	ns
YR:CP:VR:AMF	ns	ns	ns	ns
YR:CP:VR:FT	ns	ns	ns	ns
YR:CP:AMF:FT	ns	ns	ns	ns
YR:VR:AMF:FT	ns	ns	ns	ns
CP:VR:AMF:FT	ns	**0.009**	ns	ns
YR:CP:VR:AMF:FT	ns	ns	ns	ns

The season × crop protection interaction had significant impact on grains per ear (p = 0.035, Table 3.14). In 2018, ears with org. CP had lower grain numbers than with conv. CP (Table 3.15). In 2019, there was no difference in grain numbers per ear between crop protection programmes. From 2018 to 2019, grain numbers decreased in plots with conv. CP, but were not significantly different between seasons in plots with org. CP.

Table 3.15. Effect of season × crop protection on grains per ear.

Means ± SE (n=32)	**Season**	**Conv. CP**		**Org. CP**	
Grains per ear	2018	32±0.63	Aa	29.6±0.61	Ab
	2019	28.7±0.85	Ba	30.4±0.94	Aa

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

Season × variety showed a significant interaction on plants per m² (p = 0.012, Table 3.14). In 2018, plant numbers of Aszita and Skyfall were not significantly different; but were higher in Aszita in 2019 (Table 3.16).

Table 3.16. Effect of season × variety on plant number.

Means ± SE (n=32)	**Season**	**Aszita**		**Skyfall**	
Plants m^{-2}	2018	99.4±2.4	Ba	98.6±2.68	Aa
	2019	117.5±3.63	Aa	101.4±3	Ab

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

The varieties responded differently to CP which significantly affected TGW (p = 0.003, Table 3.14). Thousand grain weight (TGW) was overall higher in Skyfall than Aszita (Table 3.17). For this parameter, both varieties showed higher values with conv. CP compared with org. CP.

Table 3.17. Effect of variety × crop protection (CP) thousand grain weight (TGW).

Means ± SE (n = 32)	Variety	Conv. CP		Org. CP	
TGW [g]	Aszita	39.4±0.26	Ba	38.8±0.33	Bb
	Skyfall	44.7±0.39	Aa	39.8±0.47	Ab

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

Another interaction for CP was found in combination with fertiliser which affected grains per ear ($p = 0.021$, Table 3.14) and TGW ($p < 0.001$). Grains per ear were not significantly different between fertiliser treatments and zero-input plots with conv. CP. With org. CP, grains per ear were higher with fertiliser application than without fertiliser input (Table 3.18). Zero fertiliser treatment showed decreased grains per ear in organically managed plots compared to the pesticide treatment.

Thousand grain weights (TGW) were overall higher with conv. CP in comparison to org. CP. With conv. CP, highest TGW was found with biogas digestate and mineral N application whereas the latter showed lowest TGW when no pesticides were applied. With org. CP, FYM and biogas digestate showed highest TGW and zero-input plots yielded lower TGW. The latter were still higher than those from plants with the mineral N-treatment.

Table 3.18. Effect of fertiliser × crop protection (CP) on grains per ear and thousand grain weight (TGW).

Means ± SE (n = 32)	Fertiliser	Conv. CP		Org. CP	
Grains per ear	Biogas digestate	29.8±1	Aa	32.1±1.19	Aa
	Farmyard manure	29.5±0.91	Aa	29.8±1.1	Aa
	Mineral N	31.4±1.16	Aa	30.8±0.99	Aa
	Zero-input	30.8±1.28	Aa	27.2±1.08	Bb
TGW [g]	Biogas digestate	42.9±0.69	Aa	40.4±0.5	Ab
	Farmyard manure	41.5±0.65	Ba	40.3±0.51	Ab
	Mineral N	42.9±0.59	Aa	37.1±0.56	Cb
	Zero-input	40.9±0.67	Ba	39.4±0.54	Bb

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

The interaction of fertiliser × variety affected TGW significantly ($p = 0.001$, Table 3.14). Independent of fertiliser treatment, TGW was higher in Skyfall than in Aszita (Table 3.19). In both varieties, highest TGW were achieved with biogas digestate application, whereas in Skyfall similar levels were found also with FYM application. In Aszita, lowest TGW was found without fertiliser input. In Skyfall, TGW values were slightly higher without fertiliser than in the mineral N-treatment.

Table 3.19. Effect of fertiliser × variety on thousand grain weight (TGW).

Means ± SE (n = 32)	Fertiliser	Aszita		Skyfall	
TGW [g]	Biogas digestate	39.8±0.41	Ab	43.5±0.66	Aa
	Farmyard manure	39.1±0.38	Bb	42.8±0.59	ABa
	Mineral N	39.1±0.48	Bb	40.9±0.96	Ca
	Zero-input	38.4±0.39	Cb	41.9±0.65	BCa

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

A significant AMF × fertiliser interaction was found on ears per m² (p = 0.015, Table 3.14). While inoculation had no effect on this parameter when fertiliser was applied, ear number was higher in plots without nutrient input and AMF inoculum (Table 3.20).

Table 3.20. Effect of fertiliser × AMF inoculation on ears per m^2.

Means ± SE (n = 32)	Fertiliser	-AMF		+AMF	
Ears m^{-2}	Biogas digestate	337.6±13.15	Ba	337±18.12	Ba
	Farmyard manure	318.2±15.94	Ba	318.1±10.46	BCa
	Mineral N	400.5±13.71	Aa	368.4±15.86	Aa
	Zero-input	247.8±12.68	Cb	289.4±15.15	Ca

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

3.3.3 CROP GROWTH

3.3.3.1 CROP EMERGENCE

Plant emergence was higher in Aszita than Skyfall (p = 0.001, Table 3.21). Germination % varied significantly between seasons (p = 0.037) and was higher in 2018 compared to 2019.

Table 3.21. Effects of season, crop protection, variety, AMF inoculation and fertiliser on crop emergence. ANOVA p-values in bold indicate significant main effects and interactions. Means within columns followed by the same letter are not significantly different at $p \leq 0.05$.

	Plants m^{-2}	Germination [%]
Year (YR)		
2018 (n=127)	232.7±5.23	58.2±1.31a
2019 (n=128)	237.8±7.67	50.4±1.4b
Crop protection (CP)		
Conventional (n=128)	246.4±5.41	56.9±1.16
Organic (n=127)	224.1±7.29	51.7±1.61
Variety (VR)		
Aszita (n=128)	256.9±6.54a	55.2±1.53
Skyfall (n=127)	213.7±5.36b	53.4±1.34
Inoculation (AMF)		
+AMF (n=128)	234±5.84	54.1±1.29
-AMF (n=127)	236.6±7.22	54.5±1.58

Fertiliser (FT)		
Farmyard manure (n=63)	235.1±6.86	54.2±1.47
Zero-input (n=64)	235.5±6.27	54.4±1.41
ANOVA *p-values*		
Main effects		
YR	ns	**0.037**
CP	ns	ns
VR	**≤0.001**	ns
AMF	ns	ns
FT	ns	ns
Interactions		
YR:CP	ns	ns
YR:VR	ns	**0.011**
CP:VR	ns	ns
YR:AMF	ns	ns
CP:AMF	ns	ns
VR:AMF	ns	ns
YR:FT	ns	ns
CP:FT	ns	ns
VR:FT	ns	ns
AMF:FT	ns	ns
YR:CP:VR	ns	ns
YR:CP:AMF	ns	ns
YR:VR:AMF	ns	ns
CP:VR:AMF	ns	ns
YR:CP:FT	ns	ns
YR:VR:FT	ns	ns
CP:VR:FT	ns	ns
YR:AMF:FT	ns	ns
CP:AMF:FT	ns	ns
VR:AMF:FT	ns	ns
YR:CP:VR:AMF	ns	ns
YR:CP:VR:FT	ns	ns
YR:CP:AMF:FT	ns	ns
YR:VR:AMF:FT	ns	ns
CP:VR:AMF:FT	ns	ns
YR:CP:VR:AMF:FT	ns	ns

The only interaction that had an impact on crop emergence was season × variety (p = 0.011, Table 3.21). In 2018, germination % was lower in Skyfall than Aszita, whereas in 2019 germination % in Aszita was lower compared to the previous field trial season and not significantly different to Skyfall (Table 3.22).

Table 3.22. Interaction of season × variety on germination %.

Means ± SE (n = 32)	Season	Aszita		Skyfall	
Germination [%]	2018	62.1±1.3	Aa	54.2±2.07	Ab
	2019	48.2±2.17	Ba	52.6±1.73	Aa

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

3.3.3.2 FOUNDATION PHASE

Wheat seedlings (GS12) showed lower shoot biomass in 2018 than 2019 (Table 3.23). At the same growth stage, plant numbers and root biomass were higher in Aszita than Skyfall. Also, plots with AMF inoculum application showed higher plant number, but this effect did not result in increased shoot biomass. Wheat seedlings with conv. CP had higher root dry weight (DW) compared to wheat seedlings with org. CP. Both effects of AMF inoculum and conv. CP were not observed after the winter period at tillering (GS22). At this growth stage, Skyfall showed higher shoot and root biomass than Aszita ($p < 0.001$). At the same time point, wheat treated with FYM showed higher biomass production in both roots and shoots compared to wheat without fertiliser input ($p < 0.001$). Plant numbers and root DW were lower in 2019 at GS22 compared to 2018.

Table 3.23. Effects of season, crop protection, variety, AMF inoculation and fertiliser on plant numbers and shoot and root dry weight at early growth stages (GS12 and GS22). ANOVA *p*-values in bold indicate significant main effects and interactions. Means within columns followed by the same letter are not significantly different at $p \leq 0.05$.

	Seedling growth (GS12)			Tillering (GS22)		
	Plants m^{-2}	**Shoot DW [g m^{-2}]**	**Root DW [g m^{-2}]**	**Plants m^{-2}**	**Shoot DW [g m^{-2}]**	**Root DW [g m^{-2}]**
Year (YR)						
2018 (n=127)	277.6±9.24	6.4±0.23b	3.6±0.15	288.2±8.86a	23.9±1.28	5.7±0.33a
2019 (n=128)	250.6±10.96	9.2±0.4a	3.4±0.19	227.3±10.14b	29.7±1.8	3.2±0.25b
Crop protection (CP)						
Conventional (n=128)	274.7±10.84	8.1±0.4	3.8±0.16a	266.9±10.33	28±1.61	4.7±0.32
Organic (n=127)	253.1±9.51	7.5±0.34	3.2±0.16b	247.9±10.08	25.6±1.59	4.2±0.34
Variety (VR)						
Aszita (n=128)	286.3±10.88a	7.8±0.38	3.9±0.16a	274.3±9.41a	20.6±0.89b	3.7±0.26b
Skyfall (n=127)	241.3±8.76b	7.8±0.37	3.1±0.16b	240.4±10.68b	33.1±1.79a	5.2±0.37a
Inoculation (AMF)						
-AMF (n=128)	247.1±10.51b	7.4±0.35	3.3±0.15	248.6±10.85	26.2±1.51	4.3±0.32
+AMF (n=127)	281.2±9.59a	8.3±0.39	3.7±0.18	266.5±9.52	27.5±1.7	4.5±0.34
Fertiliser (FT)						
Farmyard manure (n=63)	269.5±10.12	8.1±0.35	3.8±0.16a	268.6±11.51	31±1.79a	5.5±0.36a
Zero-input (n=64)	258.6±10.41	7.5±0.39	3.3±0.18b	246.6±8.71	22.7±1.2b	3.4±0.24b
ANOVA *p-values*						
Main effects						
YR	ns	**0.027**	ns	**0.028**	ns	**0.026**
CP	ns	ns	**0.018**	ns	ns	ns
VR	**0.007**	ns	**0.002**	**0.017**	**≤0.001**	**≤0.001**
AMF	**0.023**	ns	ns	ns	ns	ns
FT	ns	ns	**0.028**	ns	**≤0.001**	**≤0.001**

Interactions						
YR:CP	ns	ns	ns	ns	ns	**0.045**
YR:VR	ns	ns	ns	ns	ns	ns
CP:VR	ns	ns	ns	ns	ns	ns
YR:AMF	ns	**0.042**	ns	ns	ns	ns
CP:AMF	ns	ns	ns	ns	ns	ns
VR:AMF	ns	ns	ns	ns	ns	ns
YR:FT	ns	ns	ns	ns	ns	ns
CP:FT	ns	ns	**0.013**	**0.015**	ns	ns
VR:FT	ns	ns	ns	ns	ns	ns
AMF:FT	ns	ns	ns	ns	ns	ns
YR:CP:VR	ns	ns	ns	ns	ns	ns
YR:CP:AMF	ns	ns	ns	ns	ns	ns
YR:VR:AMF	ns	ns	ns	ns	ns	ns
CP:VR:AMF	ns	ns	ns	ns	ns	ns
YR:CP:FT	ns	ns	ns	ns	ns	ns
YR:VR:FT	ns	ns	ns	ns	ns	ns
CP:VR:FT	ns	ns	ns	ns	ns	ns
YR:AMF:FT	ns	ns	ns	ns	ns	ns
CP:AMF:FT	ns	ns	ns	ns	ns	ns
VR:AMF:FT	ns	ns	ns	ns	ns	ns
YR:CP:VR:AMF	ns	ns	ns	ns	ns	ns
YR:CP:VR:FT	ns	ns	ns	ns	ns	ns
YR:CP:AMF:FT	ns	ns	ns	ns	ns	ns
YR:VR:AMF:FT	ns	ns	ns	ns	ns	ns
CP:VR:AMF:FT	ns	ns	ns	ns	ns	ns
YR:CP:VR:AMF:FT	ns	ns	ns	ns	ns	**0.014**

At the seedling stage, interaction of AMF inoculum × season had different effects on shoot DW (p = 0.042, Table 3.23). Shoot DW of seedlings was lower in 2018 than in 2019 (Table 3.24). Inoculation with AMF increased shoot biomass in 2019, but not in 2018.

Table 3.24. Effect of season × AMF inoculation on shoot dry weight at seedling stage (GS12).

Means ± SE (n = 32)	**Season**	**- AMF**		**+AMF**	
Shoot DW [g m^{-2}]	2018	6.4±0.33	Ba	6.4±0.33	Ba
	2019	8.3±0.58	Ab	10.1±0.51	Aa

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

Root biomass of seedlings varied depending on fertiliser and CP application (p = 0.013, Table 3.23). Root biomass with CP was significantly lower without fertiliser input than with FYM, but there was no difference between these two nutrient regimes in plots with org. CP (Table 3.25). With FYM, root DW was higher in org. CP plots than with conv. CP.

Table 3.25. Effect of fertiliser × crop protection (CP) on root dry weight at seedling stage (GS12).

Means ± SE (n = 32)	**Fertiliser**	**Conv. CP**		**Org. CP**	
Root DW [g m^{-2}]	Farmyard manure	3.8±0.24	Ab	3.7±0.2	Aa
	Zero-input	3.9±0.22	Ba	2.7±0.23	Aa

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

At tillering, plant numbers with conv. CP were lower in zero-input plots compared to plots with FYM (Table 3.26), but FYM application had no impact on plant number in plots with org. CP. Plots with conv. CP and FYM showed higher plant numbers than FYM plots with org. CP, but there was no difference between zero-input plots with and without conv. CP.

Table 3.26. Effect of fertiliser treatment × crop protection (CP) on plant number at tillering (GS22).

Means ± SE (n = 32)	**Fertiliser**	**Conv. CP**		**Org. CP**	
Plants m^{-2}	Farmyard manure	293.5±15.09	Aa	242.8±16.45	Ab
	Zero-input	240.4±12.69	Ba	252.9±12.04	Aa

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

Root biomass at tillering was higher in 2018 than in 2019 independently of CP application (Table 3.27). In 2019, wheat with conv. CP had higher root DW than wheat with org. CP, whereas pesticide application did not affect root DW in 2018.

Table 3.27. Effect of season × crop protection (CP) on root dry weight at tillering (GS22).

Means ± SE (n = 32)	**Season**	**Conv. CP**		**Org. CP**	
Root DW [g m^{-2}]	2018	5.5±0.5	Aa	5.8±0.44	Aa
	2019	3.8±0.33	Ba	2.6±0.34	Bb

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

3.3.3.3 CONSTRUCTION AND PRODUCTION PHASE

Plant growth at later growth stages was mostly affected by fertiliser ($p < 0.001$, Table 3.28), variety and season with higher shoot biomass in 2019 at GS32 and GS64 compared to 2018. Tiller numbers at GS32 were higher in plots with mineral N and FYM treatment and lowest in plots without fertiliser. This was still the case at flowering, but here biogas digestate and FYM showed the same tiller number per m² which was lower than tiller number in mineral N-plots. Shoot biomass of Skyfall was higher compared to Aszita at GS32 ($p < 0.001$), but the effect diminished with anthesis and reversed at maturity where Aszita showed higher biomass production than Skyfall. At this final assessment, wheat with conv. CP produced more biomass than wheat grown with org. CP. Wheat grown without fertiliser had lowest shoot weight and tiller number compared to other fertiliser treatments. At GS32 where mineral N and biogas digestate had just been applied, highest shoot DW was still found with FYM while tiller number was already increased in the MN treatment. This increase resulted in highest tiller number and shoot DW at GS64 while FYM and biogas digestate showed similar values in both parameters. Tiller numbers of biogas digestate and FYM were still not significantly different at GS90, however shoot DW of wheat with biogas digestate was now more similar to mineral N and lower in FYM.

Table 3.28. Effects of season, crop protection, variety, AMF inoculum and fertiliser on tiller and shoot dry weight after application of mineral N and biogas digestate in spring. ANOVA *p*-values in bold indicate significant main effects and interactions. Means within columns followed by the same letter are not significantly different at $p \leq 0.05$.

	Stem elongation (GS32)		**Flowering (GS64)**		**Maturity (GS90)**	
	Tillers m^{-2}	**Shoot DW [g m^{-2}]**	**Tillers m^{-2}**	**Shoot DW [g m^{-2}]**	**Tillers m^{-2}**	**Shoot DW [g m^{-2}]**
Year (YR)						
2018 (n=127)	508±9.51	214.1±6.54b	446.6±8.4	695.9±16.86b	339.9±7.64	312.5±9.99b
2019 (n=128)	555.8±14.33	270±10.34a	448±12.91	923.1±27.43a	344.9±9.37	412.5±14.53a
Crop protection (CP)						
Conventional (n=128)	550.6±13.13	248±9.43	459.2±10.19	838.3±24.72	369.9±9.1a	392.7±14.5a
Organic (n=127)	513.2±11.29	236.2±8.54	435.3±11.49	781.3±24.91	314.7±7.16b	332.4±11.23b
Variety (VR)						
Aszita (n=128)	520.7±12.72	207±7.86b	459.2±9.67	813.3±23.08	351.7±8.72	416±14.25a
Skyfall (n=127)	543.3±11.91	277.6±9.01a	435.3±11.94	806.5±26.7	333.1±8.31	308.9±10.11b
Inoculation (AMF)						
-AMF (n=128)	523.2±11.91	234.8±8.11	448.5±10.75	825.9±24.67	341±8.5	365.6±14.12
+AMF (n=127)	540.8±12.76	249.6±9.81	446.1±11.07	793.8±25.13	343.8±8.61	359.7±12.31
Fertiliser (FT)						
Biogas digestate (n=64)	525.1±18.94b	245.5±14.41b	441.7±13.54b	837.8±35.61b	346.8±11.09b	394.3±19.72ab
Farmyard manure (n=63)	560.3±15.99ab	278.5±12.9a	426.3±13.19b	792.4±35.45b	337.8±12.11b	362.9±18.65b
Mineral N (n=64)	590±16.6a	253.2±9.93ab	542.9±15.13a	979.8±29.93a	400.1±10.85a	417.4±18.19a
Zero-input (n=64)	452.9±13.25c	192±10.96c	377.9±11.9c	629.5±24.6c	285.1±9.88c	276.1±12.92c
ANOVA *p-values*						
Main effects						
YR	ns	**0.026**	ns	**0.043**	ns	ns
CP	ns	ns	ns	ns	**0.008**	**0.020**
VR	ns	**≤0.001**	ns	ns	ns	**≤0.001**
AMF	ns	ns	ns	ns	ns	ns
FT	**≤0.001**	**≤0.001**	**≤0.001**	**≤0.001**	**≤0.001**	**≤0.001**

Interactions						
YR:CP	ns	ns	ns	ns	ns	ns
YR:VR	ns	ns	ns	ns	ns	ns
CP:VR	ns	ns	ns	ns	ns	ns
YR:AMF	ns	ns	ns	ns	ns	ns
CP:AMF	ns	ns	ns	ns	ns	ns
VR:AMF	ns	ns	ns	ns	ns	ns
YR:FT	**≤0.001**	**≤0.001**	**0.006**	**0.001**	ns	**≤0.001**
CP:FT	ns	ns	ns	ns	ns	ns
VR:FT	ns	ns	**0.037**	ns	ns	ns
AMF:FT	ns	ns	**0.035**	**0.019**	ns	**0.027**
YR:CP:VR	ns	ns	ns	ns	ns	ns
YR:CP:AMF	ns	ns	ns	ns	ns	ns
YR:VR:AMF	ns	ns	ns	ns	ns	ns
CP:VR:AMF	ns	ns	ns	ns	ns	ns
YR:CP:FT	ns	ns	ns	ns	ns	ns
YR:VR:FT	ns	ns	ns	ns	ns	ns
CP:VR:FT	ns	ns	ns	ns	ns	ns
YR:AMF:FT	ns	ns	ns	ns	ns	ns
CP:AMF:FT	ns	ns	ns	ns	**0.031**	**0.003**
VR:AMF:FT	ns	ns	ns	ns	ns	ns
YR:CP:VR:AMF	ns	ns	ns	ns	ns	ns
YR:CP:VR:FT	ns	ns	ns	ns	ns	ns
YR:CP:AMF:FT	ns	ns	ns	ns	ns	ns
YR:VR:AMF:FT	ns	ns	ns	ns	ns	ns
CP:VR:AMF:FT	ns	ns	ns	**0.027**	**0.033**	**0.044**
YR:CP:VR:AMF:FT	ns	ns	ns	ns	ns	ns

The effect of fertiliser application on tiller number and shoot DW varied significantly between seasons ($p < 0.001$, Table 3.28) and affected plant growth at all growth stages. In 2018 at GS32, highest tiller number was found in plots with FYM application, but these were lower in comparison to 2019 (Table 3.29). In that year, all fertiliser treatments showed higher tiller numbers than zero-input plots which was different to 2018 where tiller numbers in zero-input and biogas digestate-treated plots were equally low. Tiller numbers in zero-input plots did not differ between the two field trials but were higher in plots with biogas digestate application in 2019 compared to 2018. At stem extension in 2018, shoot biomass was higher in wheat with FYM and mineral N than with biogas digestate and without fertiliser application. In 2019, mineral N-treated wheat and zero-input wheat showed the same level of shoot biomass, which was not significantly different to the previous year. Shoot biomass was higher with FYM and biogas digestate in 2019 than in 2018 and compared to mineral N and zero-input in 2019.

At flowering, wheat with mineral N-treatment had the highest tiller number in both seasons. Lowest were found in zero-input plots, and these were also lower in 2019 compared to 2018. Plots with FYM application showed similarly low tiller numbers in 2018 as zero-input plots, but were higher compared to zero-input plots in 2019. Shoot biomass at anthesis was highest in plots with mineral N, followed by FYM and biogas digestate. Wheat without fertiliser application had similarly low levels of shoot DW in both seasons, whereas all fertiliser treatments achieved higher biomass in 2019 than in 2018.

In contrast to previous growth stages, the interaction season × fertiliser only affected shoot DW and not tiller number at maturity. Here, shoot DW of all treatments was higher in 2019 compared to 2018. Biomass was lowest in zero-input plots and was higher in 2019 than 2018. This increase was most visible with biogas digestate application where similar levels as the mineral N application were reached in 2019.

Table 3.29. Effect of season × fertiliser treatment on both tiller number and shoot dry weight at stem elongation (GS32) and flowering (GS64), and on shoot dry weight at maturity (GS90).

Means ± SE (n = 32)	Fertiliser	2018		2019	
GS32					
Tillers m^{-2}	Biogas digestate	453.4±14.9	Cb	596.9±30.06	Aa
	Farmyard manure	515.1±20.98	Ab	604±21.65	Aa
	Mineral N	597.6±15.65	Ba	582.4±29.51	Aa
	Zero-input	466.1±12.87	Ca	439.8±23.17	Ba
Shoot DW [g m^{-2}]	Biogas digestate	179.8±10.07	Bb	311.2±21.55	Aa
	Farmyard manure	242.6±14.5	Ab	313.2±19.48	Aa
	Mineral N	257.8±11.61	Aa	248.6±16.26	Ba
	Zero-input	177±9.35	Ba	207±19.65	Ba
GS64					
Tillers m^{-2}	Biogas digestate	453.5±14.65	Ba	429.9±22.85	Ba
	Farmyard manure	410.7±12.42	Ca	441.4±22.92	Ba
	Mineral N	520.2±17.47	Aa	565.6±24.34	Aa
	Zero-input	400.8±13.74	Ca	355.1±18.81	Cb
Shoot DW [g m^{-2}]	Biogas digestate	681.7±26.22	Bb	993.8±53.8	Ba
	Farmyard manure	651.4±30.09	BCb	929±53.62	Ba
	Mineral N	855.7±36.19	Ab	1103.9±36.58	Aa
	Zero-input	593.3±22.53	Ca	665.7±43.21	Ca
GS90					
Shoot DW [g m^{-2}]	Biogas digestate	306.9±17.55	Bb	481.7±27.91	Aa
	Farmyard manure	304.8±21.87	Bb	419.1±26.71	Ba
	Mineral N	384.3±18.36	Ab	450.5±30.61	ABa
	Zero-input	253.7±15.34	Cb	298.5±20.26	Ca

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

A significant variety × fertiliser interaction affected tiller number at GS64 (p = 0.037, Table 3.28). Tiller number of both varieties was not significantly different in the respective fertiliser treatments (Table 3.30). In zero-input plots however, tiller numbers were higher in Aszita than in Skyfall. In comparison to fertiliser treatments, zero-input showed lowest tiller number in both varieties, whereas highest occurred in plots with mineral N application.

Table 3.30. Effect of fertiliser × variety on tiller number at anthesis (GS64).

Means ± SE (n = 32)	Fertiliser	Aszita		Skyfall	
Tillers m^{-2}	Biogas digestate	461.6±19.6	Ba	421.8±18.33	Ba
	Farmyard manure	441.8±17.56	BCa	410.3±19.63	Ba
	Mineral N	529.2±17.59	Aa	556.6±24.68	Aa
	Zero-input	404.1±16.25	Ca	351.8±16.36	Cb

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

Tiller number (p = 0.035, Table 3.28) and shoot DW (p = 0.019) at flowering varied significantly in plots with AMF inoculum treatment depending on the applied fertiliser. Tiller number was significantly lower in plots with AMF and mineral N application compared to the mineral N treatment without inoculation (Table 3.31). Highest shoot DW was achieved in non-inoculated plots with mineral N treatment but was decreased when AMF inoculum had been applied. Shoot weights of FYM and biogas digestate-treated plots were not affected by AMF inoculum application, and neither was the zero-input treatment which showed lowest shoot DW. The same interaction of fertiliser × AMF inoculation was observed at GS90 (p = 0.027, Table 3.28). Shoot DW was lower in plots with AMF and mineral N application compared to non-inoculated plots with mineral N. Shoot DW without fertiliser was higher with AMF inoculum application than without but were still lowest compared to fertiliser treatments.

Table 3.31. Effect of fertiliser treatment × AMF inoculation with effect on both tiller number and shoot dry weight at flowering (GS64) and on shoot dry weight at maturity (GS90).

Means ± SE (n = 32)	**Fertiliser**	**-AMF**		**+AMF**	
GS64					
Tillers m^{-2}	Biogas digestate	430.8±17.4	Ba	452.6±20.86	Ba
	Farmyard manure	422±15.05	Ba	430.7±22.09	BCa
	Mineral N	570.8±19.83	Aa	515.1±22.08	Ab
	Zero-input	370.5±16.08	Ca	385.4±17.72	Ca
Shoot DW [g m^{-2}]	Biogas digestate	833.4±48.13	Ba	842.1±53.25	Aa
	Farmyard manure	798.7±42.97	Ba	786±57.48	Aa
	Mineral N	1055.8±42.43	Aa	903.7±38.31	Ab
	Zero-input	615.8±29.28	Ca	643.2±39.87	Ca
GS90					
Shoot DW [g m^{-2}]	Biogas digestate	404.6±26.17	ABa	384±29.81	Aa
	Farmyard manure	360.9±30.06	Ba	364.8±22.29	Aa
	Mineral N	443.3±27.02	Aa	391.5±23.9	Ab
	Zero-input	253.5±17.27	Cb	298.7±18.63	Ba

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

3.3.3.4 SPAD

SPAD-readings were significantly higher in 2018 than 2019 ($p \leq 0.001$, Table 3.32). Application of conv. CP resulted in higher SPAD-readings at all assessed growth stages. SPAD was slightly higher in Skyfall than Aszita throughout the assessed growth period. Fertiliser application had strong impact on SPAD at all growth stages ($p < 0.001$) showing highest values in plots treated with mineral N at GS45. From GS58 to GS74, plots with biogas digestate application showed similar SPAD levels to mineral N, but with lower values at GS82. Farmyard manure application and zero-input plots showed lower SPAD compared to mineral N and biogas digestate. Mycorrhizal inoculation had no effect on SPAD at any measured time point.

Table 3.32. Effects of season, crop protection, variety, AMF inoculum and fertiliser on SPAD at different growth stages of wheat. ANOVA *p*-values in bold indicate significant main effects and interactions. Means within columns followed by the same letter are not significantly different at $p \leq 0.05$.

	SPAD GS45	**SPAD GS58**	**SPAD GS74**	**SPAD GS 82**
Year (YR)				
2018 (n=127)	46.6±0.41a	44.5±0.43a	38.9±0.54a	35.3±0.71a
2019 (n=128)	39.4±0.39b	32±0.6b	24.7±0.75b	12.1±0.53b
Crop protection (CP)				
Conventional (n=128)	43.8±0.52a	40.5±0.7a	35±0.89a	26.4±1.36a
Organic (n=127)	42.1±0.5b	36±0.77b	28.6±0.84b	21±0.97b
Variety (VR)				
Aszita (n=128)	42.4±0.52b	37.5±0.82b	29.9±0.99b	22±1.27b
Skyfall (n=127)	43.6±0.5a	39±0.69a	33.7±0.78a	25.4±1.11a
Inoculation (AMF)				
-AMF (n=128)	43±0.51	37.9±0.77	31.4±0.94	23.6±1.23
+AMF (n=127)	43±0.51	38.6±0.74	32.2±0.88	23.8±1.18
Fertiliser (FT)				
Biogas digestate (n=64)	45.2±0.7b	41.8±1.03a	35.3±1.31a	25.7±1.97b
Farmyard manure (n=63)	39.7±0.49b	35.3±0.79b	28.6±1.04b	20.3±1.51c
Mineral N (n=64)	47.9±0.51a	41.5±1.21a	36.2±1.35a	28.3±1.62a
Zero-input (n=64)	39.2±0.52c	34.4±0.86b	27.2±1.02b	20.5±1.46c
ANOVA *p-values*				
Main effects				
YR	**≤0.001**	**≤0.001**	**≤0.001**	**≤0.001**
CP	**0.002**	**≤0.001**	**≤0.001**	**0.002**
VR	**0.002**	**0.009**	**≤0.001**	**0.002**
AMF	ns	ns	ns	ns
FT	**≤0.001**	**≤0.001**	**≤0.001**	**≤0.001**
Interactions				
YR:CP	ns	**0.006**	ns	**0.009**
YR:VR	**0.004**	**≤0.001**	**≤0.001**	ns
CP:VR	ns	ns	ns	ns
YR:AMF	ns	ns	ns	ns
CP:AMF	ns	ns	ns	ns

VR:AMF	ns	ns	ns	ns
YR:FT	**0.002**	ns	**0.021**	**≤0.001**
CP:FT	**0.022**	**≤0.001**	**≤0.001**	**≤0.001**
VR:FT	ns	**0.016**	ns	ns
AMF:FT	ns	ns	ns	ns
YR:CP:VR	ns	ns	**0.004**	ns
YR:CP:AMF	ns	ns	ns	ns
YR:VR:AMF	ns	ns	ns	ns
CP:VR:AMF	ns	ns	ns	**0.044**
YR:CP:FT	ns	**≤0.001**	**0.001**	ns
YR:VR:FT	ns	**0.012**	ns	ns
CP:VR:FT	ns	ns	ns	ns
YR:AMF:FT	ns	ns	ns	ns
CP:AMF:FT	ns	ns	ns	ns
VR:AMF:FT	ns	ns	ns	ns
YR:CP:VR:AMF	ns	ns	ns	ns
YR:CP:VR:FT	ns	ns	ns	ns
YR:CP:AMF:FT	ns	ns	ns	ns
YR:VR:AMF:FT	ns	ns	ns	ns
CP:VR:AMF:FT	ns	ns	ns	ns
YR:CP:VR:AMF:FT	ns	ns	ns	ns

The interaction of season × CP significantly affected SPAD at GS58 (p = 0.006, Table 3.32) and GS82 (p = 0.009). SPAD-readings were higher with conv. CP compared to wheat with org. CP in both seasons (Table 3.33). SPAD-values were higher in 2018 than 2019 at both growth stages independent of CP.

Table 3.33. Effect of crop protection (CP) × season on SPAD at GS58 and GS82.

Means ± SE (n = 32)	**Season**	**Con. CP**		**Org. CP**	
GS58	2018	45.8±0.61	Aa	43.2±0.55	Ab
	2019	35.2±0.84	Ba	28.8±0.64	Bb
GS82	2018	39.9±0.84	Aa	30.7±0.78	Ab
	2019	12.8±0.95	Ba	11.4±0.45	Bb

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

Seasonal variations affected SPAD-readings in the two wheat varieties at the first three measured growth stages i.e. GS45, GS58 and GS74 (Table 3.32). SPAD of both Aszita and Skyfall was higher in 2018 than 2019 (Table 3.34). In 2018, there was no difference between Aszita and Skyfall at both GS45 and GS74, but Skyfall had lower SPAD-readings at GS58. In 2019, Skyfall showed higher SPAD than Aszita at all three growth stages.

Table 3.34. Effect of variety × season on SPAD at GS45, GS58 and GS74.

Means ± SE (n = 32)	Season	Aszita		Skyfall	
GS45	2018	46.5±0.54	Aa	46.7±0.62	Aa
	2019	38.2±0.51	Bb	40.5±0.57	Ba
GS58	2018	45.1±0.57	Aa	44±0.63	Ab
	2019	30±0.76	Bb	34±0.85	Ba
GS74	2018	38.8±0.68	Aa	39±0.84	Aa
	2019	21±0.99	Bb	28.4±0.92	Ba

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

The fertiliser × season interaction had a significant impact on SPAD at GS45 ($p = 0.002$, Table 3.32), GS74 ($p = 0.021$) and at GS82 ($p < 0.001$). At these growth stages, SPAD was higher in 2018 compared to 2019, but variations occurred in response to the applied fertilisers (Table 3.35). At GS45 in both seasons, highest SPAD was found in plots with mineral N followed by biogas digestate treatment. Lowest values were detected in the FYM and zero-input. The same pattern applied at GS74, but there was no significant difference between biogas digestate and mineral N-treatments anymore. At GS82 in 2018, mineral N and biogas digestate reached the same SPAD level which was higher than both the FYM and zero-input. In 2019, highest SPAD-readings were measured in mineral N-treated plots with lowest values in plots with FYM and without fertiliser. The latter however was not significantly different from plots with biogas digestate.

Table 3.35. Effect of fertiliser × season on SPAD at GS58, GS74 and GS82.

Means ± SE (n = 32)	Fertiliser	2018		2019	
GS45	Biogas digestate	49.7±0.62	Ba	40.6±0.55	Bb
	Farmyard manure	43±0.31	Ca	36.3±0.37	Cb
	Mineral N	51.2±0.32	Aa	44.6±0.51	Ab
	Zero-input	42.4±0.45	Ca	35.9±0.45	Cb
GS74	Biogas digestate	43.4±0.93	Aa	27.2±1.38	Ab
	Farmyard manure	35.8±0.51	Ba	21.4±0.87	Bb
	Mineral N	42.4±1.07	Aa	30±1.94	Ab
	Zero-input	34.2±0.62	Ba	20.2±0.84	Bb
GS82	Biogas digestate	40.2±1.1	Aa	11.2±0.98	Bb
	Farmyard manure	31.2±1.09	Ba	9.4±0.61	Cb
	Mineral N	38.6±1.58	Aa	17.9±1.17	Ab
	Zero-input	31.1±1.03	Ba	9.9±0.58	BCb

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

The interaction of fertiliser × CP was significant for all measured growth stages (Table 3.32). SPAD was consistently lower in org. CP plots compared to plots with conv. CP except for the FYM-treatment at GS45 (Table 3.36). Plots with conv. CP showed highest SPAD with mineral N application, followed by biogas digestate treatment and lowest values in the FYM and zero-input at all assessed growth stages. More variation occurred in org. CP plots, where biogas digestate application achieved higher SPAD-readings than mineral N-application at both GS58 and GS74, whereas levels were lower than the mineral N treatment at GS45 and not significantly different at GS82. SPAD-readings with FYM application were lower than biogas digestate and mineral N values at all time points, and were only at GS45 significantly higher than zero-input plots.

Table 3.36. Effect of crop protection (CP) × fertiliser on SPAD readings at GS45, GS58, GS74 and GS82.

Means ± SE (n = 32)	**Fertiliser**	**Con. CP**		**Org. CP**	
GS45	Biogas digestate	46.5±0.91	Ba	43.9±1.04	Bb
	Farmyard manure	39.8±0.72	Ca	39.5±0.67	Ca
	Mineral N	49±0.66	Aa	46.8±0.73	Ab
	Zero-input	40.1±0.76	Ca	38.2±0.67	Db
GS58	Biogas digestate	43.9±1.24	Ba	39.8±1.59	Ab
	Farmyard manure	35.9±1.03	Ca	34.7±1.2	BCb
	Mineral N	46.9±0.84	Aa	36.2±1.85	Bb
	Zero-input	35.4±1.22	Ca	33.3±1.22	Cb
GS74	Biogas digestate	39±1.53	Ba	31.5±1.94	Ab
	Farmyard manure	29.5±1.43	Ca	27.6±1.5	BCb
	Mineral N	43.2±1.08	Aa	29.2±1.75	Bb
	Zero-input	28.4±1.54	Ca	25.9±1.34	Cb
GS82	Biogas digestate	27.8±3.06	Ba	23.6±2.47	Ab
	Farmyard manure	21.6±2.45	Ca	19.1±1.77	Bb
	Mineral N	34.4±2.34	Aa	22.1±1.68	Ab
	Zero-input	21.7±2.41	Ca	19.3±1.67	Bb

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

The interaction of variety × fertiliser had significant impact on SPAD-readings at GS58 ($p = 0.016$, Table 3.32). At this growth stage, SPAD was higher in Skyfall with biogas digestate compared to Aszita (Table 3.37). In both varieties, SPAD-readings were highest with biogas digestate and mineral N fertiliser and lower with FYM and zero-input.

Table 3.37. Effect of fertiliser × variety on SPAD readings at GS58.

Means ± SE (n = 32)	Fertiliser	Aszita		Skyfall	
GS58	Biogas digestate	40.4±1.62	Ab	43.2±1.27	Aa
	Farmyard manure	35±1.29	Ba	35.6±0.92	Ba
	Mineral N	40.3±1.99	Aa	42.7±1.37	Aa
	Zero-input	34.4±1.31	Ba	34.4±1.15	Ba

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

3.3.3.5 CANOPY FORMATION

The normalised difference vegetation index (NDVI) was higher for the last three measured growth stages in 2018 compared to 2019 and was also higher in conv. CP than org. CP at the same time points (Table 3.38). The NDVI was significantly higher in Aszita compared to Skyfall at both GS45 and GS58, but not at later growth stages. Plots with AMF inoculation showed higher NDVI at GS45 ($p = 0.008$), but this effect was not observed at later growth stages. Fertiliser treatment affected NDVI significantly at all assessed growth stages ($p < 0.001$). Highest NDVI was measured in the mineral N treatment which was at a similar level to biogas digestate application at GS58 and GS74. Plots without fertiliser input showed lowest NDVI and similar values to the FYM treatment at GS74. The FYM treatment showed higher levels of NDVI at GS45 and GS58 but decreased to lower levels than zero-input at GS82.

Table 3.38. Effects of season, crop protection, variety, AMF inoculum and fertiliser on normalised difference vegetation index (NDVI) at different growth stages of wheat. ANOVA *p*-values in bold indicate significant main effects and interactions. Means within columns followed by the same letter are not significantly different at $p \leq 0.05$.

	NDVI GS45	NDVI GS58	NDVI GS74	NDVI GS82
Year (YR)				
2018 (n=127)	0.60±0.008	0.56±0.009a	0.49±0.006a	0.40±0.006a
2019 (n=128)	0.59±0.007	0.46±0.007b	0.39±0.007b	0.28±0.005b
Crop protection (CP)				
Conventional (n=128)	0.60±0.008	0.53±0.008a	0.47±0.008a	0.36±0.009a
Organic (n=127)	0.60±0.007	0.49±0.009b	0.41±0.007b	0.32±0.006b
Variety (VR)				
Aszita (n=128)	0.63±0.007a	0.53±0.009a	0.44±0.008	0.34±0.007
Skyfall (n=127)	0.56±0.007b	0.49±0.008b	0.44±0.008	0.34±0.008
Inoculation (AMF)				
-AMF (n=128)	0.59±0.008b	0.51±0.009	0.44±0.008	0.34±0.007
+AMF (n=127)	0.61±0.007a	0.51±0.009	0.44±0.008	0.34±0.008

Fertiliser (FT)				
Biogas digestate (n=64)	0.64±0.007b	0.56±0.011a	0.48±0.011a	0.36±0.012b
Farmyard manure (n=63)	0.55±0.008c	0.47±0.008b	0.40±0.007b	0.30±0.008d
Mineral N (n=64)	0.68±0.007a	0.58±0.012a	0.49±0.012a	0.38±0.011a
Zero-input (n=64)	0.52±0.008d	0.44±0.009c	0.39±0.008b	0.31±0.007c
ANOVA *p-values*				
Main effects				
YR	ns	**0.012**	**0.002**	**≤0.001**
CP	ns	**0.005**	**≤0.001**	**0.007**
VR	**≤0.001**	**≤0.001**	ns	ns
AMF	**0.008**	ns	ns	ns
FT	**≤0.001**	**≤0.001**	**≤0.001**	**≤0.001**
Interactions				
YR:CP	ns	**0.003**	**0.004**	ns
YR:VR	**0.035**	**≤0.001**	**0.009**	ns
CP:VR	ns	ns	ns	ns
YR:AMF	ns	ns	ns	ns
CP:AMF	ns	ns	ns	ns
VR:AMF	ns	ns	ns	ns
YR:FT	**≤0.001**	**≤0.001**	**≤0.001**	**≤0.001**
CP:FT	**0.014**	**≤0.001**	**≤0.001**	**≤0.001**
VR:FT	ns	**0.027**	**0.004**	**0.002**
AMF:FT	ns	ns	ns	ns
YR:CP:VR	ns	ns	ns	ns
YR:CP:AMF	ns	ns	ns	ns
YR:VR:AMF	ns	ns	ns	ns
CP:VR:AMF	ns	ns	ns	ns
YR:CP:FT	ns	**0.041**	ns	**0.007**
YR:VR:FT	**0.016**	ns	ns	ns
CP:VR:FT	ns	ns	ns	**0.037**
YR:AMF:FT	ns	ns	ns	ns
CP:AMF:FT	ns	ns	ns	ns
VR:AMF:FT	ns	ns	ns	ns
YR:CP:VR:AMF	ns	ns	ns	ns
YR:CP:VR:FT	ns	ns	ns	ns
YR:CP:AMF:FT	ns	ns	ns	ns
YR:VR:AMF:FT	ns	ns	ns	**0.047**
CP:VR:AMF:FT	**0.039**	ns	ns	ns
YR:CP:VR:AMF:FT	ns	ns	ns	ns

Significant interactions of season × CP were found at GS58 (p = 0.003, Table 3.38) and GS74 (p = 0.004). At both growth stages, NDVI readings were higher in 2018 than 2019 (Table 3.39). At GS58 in 2019, NDVI was higher in plots with conv. CP compared to org. CP. At GS74, NDVI with conv. CP was significantly higher compared to org. CP in both seasons.

Table 3.39. Effect of crop protection (CP) × season on NDVI at GS58 and GS74.

Means ± SE (n = 32)	Season	Conv. CP		Org. CP	
GS58	2018	0.56±0.013	Aa	0.56±0.012	Aa
	2019	0.5±0.01	Ba	0.42±0.007	Bb
GS74	2018	0.5±0.011	Aa	0.47±0.006	Ab
	2019	0.44±0.01	Ba	0.34±0.005	Bb

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

Differing weather conditions in the two seasons also affected canopy coverage in the two varieties at GS45 (p = 0.035, Table 3.38), GS58 (p = 0.001) and GS74 (p = 0.009). In both varieties, NDVI-readings were higher at these growth stages in 2018 than 2019 except for Skyfall at GS45 where NDVI measurements were not significantly different between seasons (Table 3.40). At GS45, GS58 and GS74 in 2018, Aszita showed higher NDVI than Skyfall. At GS58 in 2019, NDVI-values of Aszita and Skyfall were not significantly different and at GS74 in 2019, NDVI-readings were lower in Aszita than Skyfall.

Table 3.40. Effect of variety × season on NDVI at GS45, GS58 and GS74.

Means ± SE (n = 32)	Season	Aszita		Skyfall	
GS45	2018	0.65±0.011	Aa	0.56±0.01	Ab
	2019	0.62±0.008	Ba	0.57±0.01	Ab
GS58	2018	0.6±0.011	Aa	0.53±0.011	Ab
	2019	0.46±0.009	Ba	0.46±0.011	Ba
GS74	2018	0.5±0.008	Aa	0.48±0.01	Ab
	2019	0.38±0.009	Bb	0.41±0.011	Ba

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

The fertiliser × season interaction had a significant impact on NDVI at all measured growth stages (p < 0.001, Table 3.38). At GS45, highest NDVI-readings were found in plots with mineral N and these values were not significantly different in both seasons (Table 3.41). Plots with biogas digestate application showed second highest NDVI which reached higher levels in 2018 compared to 2019. Plots without fertiliser and those with FYM application showed similar NDVI in 2018, but levels were lower in zero-input plots and higher in plots treated with FYM

in 2019.

At GS58, NDVI was lower in 2019 than 2018 in all fertiliser treatments and zero-input plots. In both years, NDVI was highest in plots with mineral N and biogas digestate treatment. In 2018, NDVI levels in FYM and zero-input plots were not significantly different, but in 2019, NDVI-values were lower in zero-input plots than in wheat with FYM.

At GS74, NDVI levels were higher in 2018 than 2019 in all fertiliser treatments as well as zero-input plots. In 2018, NDVI-values in biogas digestate and mineral N were higher compared to FYM and zero-input plots. In 2019 at GS74, biogas digestate showed significantly lower NDVI levels than the mineral N treatment. The NDVI in wheat with FYM and zero-input was on similar levels which were lower than those of biogas digestate and mineral N in that year.

At GS82, NDVI in 2018 showed similar levels as in the previous growth stages with highest values in mineral N and biogas digestate plots and lowest in FYM and zero-input plots. In contrast to previous growth stages, plots without fertiliser showed higher NDVI than plots with FYM application at GS82 in 2019.

Table 3.41. Effect of fertiliser × season on NDVI at GS45, GS58, GS74 and GS82.

Means ± SE (n = 32)	**Fertiliser**	**2018**		**2019**	
GS45	Biogas digestate	0.66±0.012	Ba	0.62±0.007	Bb
	Farmyard manure	0.54±0.014	Cb	0.56±0.01	Ca
	Mineral N	0.68±0.011	Aa	0.67±0.009	Aa
	Zero-input	0.53±0.012	Ca	0.51±0.01	Db
GS58	Biogas digestate	0.63±0.012	Aa	0.5±0.009	Ab
	Farmyard manure	0.5±0.013	Ba	0.44±0.007	Bb
	Mineral N	0.64±0.009	Aa	0.52±0.017	Ab
	Zero-input	0.48±0.011	Ba	0.39±0.008	Cb
GS74	Biogas digestate	0.54±0.009	Aa	0.42±0.013	Bb
	Farmyard manure	0.43±0.007	Ba	0.36±0.008	Cb
	Mineral N	0.54±0.011	Aa	0.45±0.018	Ab
	Zero-input	0.43±0.007	Ba	0.35±0.008	Cb
GS82	Biogas digestate	0.44±0.01	Aa	0.28±0.009	Bb
	Farmyard manure	0.35±0.005	Ba	0.24±0.005	Cb
	Mineral N	0.44±0.011	Aa	0.32±0.011	Ab
	Zero-input	0.35±0.006	Ba	0.27±0.008	Bb

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

The interaction of fertiliser × CP affected NDVI at all measured growth stages (Table 3.38). In plots with conv. CP, highest NDVI was achieved with mineral N fertiliser at all growth stages, followed by biogas digestate application (Table 3.42). From GS58 onwards, NDVI with FYM

application and in zero-input plots were not significantly different and lower when compared to the mineral N and biogas digestate treatments. In plots with org. CP, there was little difference between mineral N and biogas digestate in NDVI but with a tendency for higher levels in biogas digestate treated plots at GS74 and GS82. Within fertiliser treatments, NDVI-readings in mineral N treatments were higher with conv. CP than with org. CP at all growth stages. The same applies to FYM, biogas digestate and zero-input, but only at GS74, whereas NDVI in these treatments was not affected by CP at other growth stages.

Table 3.42. Effect of fertiliser × crop protection (CP) on NDVI at GS45, GS58, GS74 and GS82.

Means ± SE (n = 32)	**Fertiliser**	**Conv. CP**		**Org. CP**	
GS45	Biogas digestate	0.65±0.009	Ba	0.63±0.01	Aa
	Farmyard manure	0.55±0.011	Ca	0.55±0.013	Ba
	Mineral N	0.69±0.008	Aa	0.65±0.011	Ab
	Zero-input	0.53±0.011	Da	0.52±0.011	Ca
GS58	Biogas digestate	0.58±0.013	Ba	0.55±0.018	Aa
	Farmyard manure	0.47±0.01	Ca	0.47±0.013	Ba
	Mineral N	0.62±0.008	Aa	0.53±0.02	Ab
	Zero-input	0.44±0.01	Ca	0.43±0.015	Ba
GS74	Biogas digestate	0.51±0.013	Ba	0.44±0.016	Ab
	Farmyard manure	0.41±0.007	Ca	0.38±0.011	BCb
	Mineral N	0.56±0.008	Aa	0.42±0.014	ABb
	Zero-input	0.41±0.009	Ca	0.38±0.012	Cb
GS82	Biogas digestate	0.38±0.02	Ba	0.34±0.013	Aa
	Farmyard manure	0.3±0.011	Ca	0.29±0.011	Ca
	Mineral N	0.42±0.015	Aa	0.33±0.011	ABb
	Zero-input	0.32±0.011	Ca	0.31±0.009	BCa

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

The interaction of variety × fertiliser affected NDVI at GS58 ($p = 0.027$, Table 3.38), GS74 ($p = 0.004$) and GS82 ($p = 0.002$). At GS58, both varieties showed highest NDVI with biogas digestate and mineral N, lower values in FYM and lowest with zero-input (Table 3.43). The NDVI of wheat with mineral N fertiliser was not significantly different between Skyfall and Aszita, whereas the other treatments had higher NDVI values in Aszita than Skyfall.

At GS74, NDVI was highest in plots with mineral N and biogas digestate and lower in FYM and zero-input plots. With mineral N, NDVI readings were higher in Skyfall than Aszita. Without fertiliser on the other hand, NDVI was higher in Aszita than Skyfall. At GS82, NDVI of Aszita was lowest in plots with FYM, followed by zero-input plots, biogas digestate application and highest in mineral N-treated plots. In Skyfall, biogas digestate and mineral N

reached similarly high NDVI levels, with lower levels in FYM and zero-input. NDVI of Skyfall was lower than Aszita in zero-input plots but higher following biogas digestate application.

Table 3.43. Effect of fertiliser × variety on NDVI at GS58, GS74 and GS82.

Means ± SE (n = 32)	Fertiliser	Aszita		Skyfall	
GS58	Biogas digestate	0.58±0.018	Aa	0.55±0.013	Ab
	Farmyard manure	0.49±0.012	Ba	0.44±0.009	Bb
	Mineral N	0.58±0.021	Aa	0.57±0.013	Aa
	Zero-input	0.46±0.013	Ca	0.41±0.011	Cb
GS74	Biogas digestate	0.47±0.017	Aa	0.48±0.015	Aa
	Farmyard manure	0.4±0.009	Ba	0.39±0.01	Ba
	Mineral N	0.48±0.018	Ab	0.5±0.016	Aa
	Zero-input	0.4±0.013	Ba	0.38±0.008	Bb
GS82	Biogas digestate	0.35±0.017	Bb	0.37±0.017	Aa
	Farmyard manure	0.3±0.011	Da	0.3±0.011	Ba
	Mineral N	0.37±0.013	Aa	0.39±0.018	Aa
	Zero-input	0.32±0.01	Ca	0.31±0.011	Bb

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

3.3.4 DISEASE

The observed diseases on wheat leaves during field trials at Nafferton Farm were *Septoria tritici* blotch (STB, *Zymoseptoria tritici*) and yellow rust (*Puccinia striiformis* f. sp. *tritici*). Diseases on ears were Fusarium head blight (FHB, *Fusarium* spp.), rust and glume blotch (*Parastagnospora nodorum*) (Table 3.44). There were only very low levels of powdery mildew (*Blumeria graminis* f. sp. *tritici*) in each trial season, for which reason this disease was not included in the statistical analyses.

Incidences of STB were very low in 2018 and no glume blotch and FHB were detected in that season. Yellow rust on the other hand dominated both years, but disease development was considerably reduced following the application of conv. CP ($p < 0.001$, Table 3.44). *Septoria tritici* blotch on the other hand occurred in wheat with conv. CP, but not at the same level as in plots with org. CP. Overall, the conv. CP programme reduced disease incidences of both foliar and ear diseases except for glume blotch. Varieties responded differently to foliar pathogens with Aszita showing higher susceptibility to STB and Skyfall showing higher susceptibility to yellow rust, but the latter was not significant on the flag leaf. Regarding ear diseases, higher FHB was observed in Skyfall than Aszita ($p = 0.002$). While AMF inoculation had no effect on disease levels, fertiliser affected the levels of STB on Leaf 2 and Leaf 3. Highest STB occurred in combination with mineral N and the lowest levels were observed in the biogas digestate.

Plots with FYM application showed intermediate STB symptom development. Disease levels of yellow rust ($p < 0.001$) as well as glume blotch ($p = 0.018$) and rust on ears ($p = 0.002$) were significantly higher in the mineral N treatment compared to the organic fertiliser and control treatments.

Table 3.44. Effects of season, crop protection, variety, AMF inoculum and fertiliser on disease AUDPC (Area under disease progress curve) of *Septoria tritici* blotch and yellow rust on leaves and Fusarium head blight (FHB), rust and Glume blotch (GB) on ears. ANOVA *p*-values in bold indicate significant main effects and interactions. Means within columns followed by the same letter are not significantly different at $p \leq 0.05$.

	***Septoria tritici* blotch**			**Yellow Rust**			**Ear diseases**		
	Leaf 1	**Leaf 2**	**Leaf 3**	**Leaf 1**	**Leaf 2**	**Leaf 3**	**FHB**	**Rust**	**GB**
Year (YR)									
2018 (n=127)	1.3±0.52b	17.8±2.24b	80.4±5.55b	96.8±12.09b	199.5±29.33	258.2±41.37a	0±0b	3.5±0.74	0±0
2019 (n=128)	23.8±1.94a	61.4±5.04a	132.7±7.33a	249.7±34.84a	231.2±38.76	80.6±18.1b	0.6±0.14a	4.1±0.75	2±0.61
Crop protection (CP)									
Conventional (n=128)	6.9±1.23b	15.3±2.15b	66.2±5.07b	0.5±0.21b	2.4±0.76b	16.2±3.85b	0.1±0.05b	0.1±0.08b	0±0.03
Organic (n=127)	18.2±2a	63.9±4.9a	146.9±6.62a	346±31.34a	454.4±40.33a	357.2±46.25a	0.5±0.13a	7.5±0.95a	1.9±0.61
Variety (VR)									
Aszita (n=128)	15±1.99a	45.6±5.01a	131.6±6.91a	193.2±33.52	138.3±26.99b	97.3±20.6b	0±0.01b	3.5±0.73	1.3±0.56
Skyfall (n=127)	10.1±1.4b	33.6±3.51b	81.4±6.13b	153.3±17.95	284.6±37.6a	246±41.4a	0.6±0.14a	4.1±0.76	0.7±0.25
Inoculation (AMF)									
-AMF (n=128)	13.4±1.82	40.6±4.71	106.1±6.82	173.6±25.83	196.5±28.73	183.9±36.4	0.3±0.1	3.5±0.66	0.8±0.32
+AMF (n=127)	11.7±1.64	38.6±3.97	107±6.99	172.9±28.01	232±38.23	166.6±32.16	0.3±0.11	4.1±0.82	1.2±0.53
Fertiliser (FT)									
Biogas digestate (n=64)	9.2±2.03	30.7±5.63b	88.2±8.35c	120±26.12b	97.8±18.45b	65.8±13.15b	0.1±0.04	2.1±0.5b	0.4±0.23b
Farmyard manure (n=63)	14.3±2.53	38.5±7.15ab	101.6±8.47bc	107.7±19.14b	132.3±22.68b	85.5±19.63b	0.3±0.15	2.6±0.8b	0.3±0.13b
Mineral N (n=64)	11.8±2.81	48.5±6.76a	124.5±12.78a	335.9±59.98a	450.8±78.72a	430.2±86.67a	0.5±0.21	7.3±1.59a	2.6±1.15a
Zero-input (n=64)	14.9±2.34	40.6±4.7ab	111.8±8.26ab	129.5±25.38b	192±38.04b	143.4±31.85b	0.2±0.12	3.2±0.9b	0.7±0.33b
ANOVA *p-values*									
Main effects									
YR	**0.004**	**0.003**	**0.005**	**0.021**	ns	**0.010**	ns	ns	ns
CP	**0.002**	**≤0.001**	**≤0.001**	**≤0.001**	**≤0.001**	**≤0.001**	**0.020**	**0.001**	ns
VR	**0.047**	**0.011**	**≤0.001**	ns	**0.005**	**≤0.001**	**0.002**	**0.632**	ns
AMF	ns	ns	ns	ns	ns	ns	ns	ns	ns
FT	**0.047**	**0.015**	**≤0.001**	**≤0.001**	**≤0.001**	**≤0.001**	**0.046**	**≤0.001**	**0.003**

Interactions									
YR:CP	**0.007**	**0.012**	ns	**0.004**	ns	**0.002**	**0.020**	ns	ns
YR:VR	ns	**≤0.001**	**≤0.001**	**0.019**	ns	**≤0.001**	**0.002**	**0.029**	ns
CP:VR	ns	ns	ns	ns	**0.006**	**≤0.001**	**0.011**	ns	ns
YR:AMF	ns	ns	ns	ns	ns	ns	ns	ns	ns
CP:AMF	ns	ns	ns	ns	ns	ns	ns	ns	ns
VR:AMF	ns	ns	ns	ns	ns	ns	ns	ns	ns
YR:FT	**0.012**	ns	**0.001**	**≤0.001**	ns	**≤0.001**	**0.046**	**0.008**	**0.003**
CP:FT	**≤0.001**	**≤0.001**	**≤0.001**	**≤0.001**	**≤0.001**	**≤0.001**	**0.018**	**≤0.001**	**0.002**
VR:FT	ns	ns	ns	**≤0.001**	ns	**≤0.001**	**0.024**	ns	ns
AMF:FT	ns	ns	ns	ns	ns	ns	**0.040**	ns	ns
YR:CP:VR	ns	**0.009**	**≤0.001**	**0.021**	ns	**0.002**	**0.011**	**0.024**	ns
YR:CP:AMF	ns	ns	ns	ns	ns	ns	ns	ns	ns
YR:VR:AMF	ns	ns	ns	ns	ns	ns	ns	ns	ns
CP:VR:AMF	ns	ns	ns	ns	ns	ns	ns	ns	ns
YR:CP:FT	**≤0.001**	ns	ns	**≤0.001**	ns	**≤0.001**	**0.018**	**0.015**	**0.002**
YR:VR:FT	ns	ns	ns	**≤0.001**	ns	**≤0.001**	**0.024**	**0.043**	ns
CP:VR:FT	ns	ns	ns	**≤0.001**	ns	**≤0.001**	**0.012**	**0.049**	ns
YR:AMF:FT	ns	ns	ns	ns	ns	ns	**0.040**	ns	ns
CP:AMF:FT	ns	ns	ns	ns	ns	ns	ns	ns	ns
VR:AMF:FT	ns	ns	ns	ns	ns	ns	**0.042**	ns	ns
YR:CP:VR:AMF	ns	ns	ns	ns	ns	ns	ns	ns	ns
YR:CP:VR:FT	ns	ns	ns	**≤0.001**	ns	**0.006**	**0.012**	ns	ns
YR:CP:AMF:FT	ns	ns	ns	ns	ns	ns	ns	ns	ns
YR:VR:AMF:FT	ns	ns	ns	ns	ns	ns	**0.042**	ns	ns
CP:VR:AMF:FT	ns	ns	ns	ns	ns	ns	ns	ns	ns
YR:CP:VR:AMF:FT	ns	ns	ns	ns	ns	ns	ns	ns	ns

Disease symptom development of STB on Leaf 1 and 2 was higher in 2019 than 2018 (Table 3.45). In both seasons, STB levels were higher in org. compared to the conv. CP treatment. In contrast to STB, yellow rust levels on flag leaves were the same in both trial years, but only in plots with conv. CP. Wheat with org. CP showed significantly higher yellow rust on flag leaves in 2019 compared to 2018. Leaf 3 on the other hand showed less disease in 2019 in both conv. and org. CP treatments. Fusarium head blight did not occur in 2018 but was found at higher levels on ears with org. CP than conv. CP in 2019.

Table 3.45. Effect of crop protection (CP) × season on AUDPC (area under disease progress curve) of *Septoria tritici* blotch (STB), leaf yellow rust and Fusarium head blight (FHB).

Means ± SE (n = 32)	**Season**	**Conv. CP**		**Org. CP**	
Foliar diseases					
STB Leaf 1	2018	0.2±0.21	Bb	2.4±1.01	Ba
	2019	13.5±2.17	Ab	34.1±2.66	Aa
STB Leaf 2	2018	2.2±0.53	Bb	33.3±3.52	Ba
	2019	28.4±3.6	Ab	94.4±7.41	Aa
Yellow rust Leaf 1	2018	0.8±0.32	Ab	192.9±17.22	Ba
	2019	0.3±0.27	Ab	499±54.03	Aa
Yellow rust Leaf 3	2018	30.1±7.18	Ab	486.4±72.09	Aa
	2019	2.3±1.47	Bb	138.7±29.72	Ba
Ear disease					
FHB	2018	0±0	Aa	0±0	Ba
	2019	0.1±0.11	Ab	1±0.25	Aa

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

Foliar disease levels as well as rust and FHB on ears were affected by the season × variety interaction (Table 3.44). In 2018, STB symptoms on Leaf 2 of Aszita were not significantly different to Skyfall, but were higher in 2019 (Table 3.46). Symptoms of STB on Leaf 3 of Skyfall did not vary between seasons, whereas Aszita showed higher STB disease levels in 2019 than 2018.

In both varieties, AUDPC-levels of yellow rust on flag leaves were higher in 2019 than 2018. Disease levels of yellow rust on Skyfall were higher than Aszita in 2018 and lower in 2019. On Leaf 3, yellow rust levels in Aszita were not affected by season, whereas Skyfall showed significantly lower disease in 2019 than 2018.

Yellow rust levels on ears of Skyfall were higher in 2018 and lower in 2019 than Aszita. Fusarium head blight was not detected in 2018 but was higher in Skyfall than Aszita in 2019.

Table 3.46. Interaction of variety × season on *Septoria tritici* blotch (STB) and leaf yellow rust as well as rust and Fusarium head blight (FHB) on ears.

Means ± SE (n = 32)	Season	Aszita		Skyfall	
Foliar diseases					
STB Leaf 2	2018	14±2.63	Ba	21.5±3.6	Ba
	2019	77.2±7.9	Aa	45.7±5.67	Ab
STB Leaf 3	2018	78.4±4.75	Ba	82.3±10.07	Aa
	2019	184.8±8.96	Aa	80.5±7.09	Ab
Yellow rust Leaf 1	2018	70.8±13.77	Bb	122.9±19.45	Ba
	2019	315.6±62.16	Aa	183.7±29.84	Ab
Yellow rust Leaf 3	2018	112±28.87	Ab	404.5±73.4	Aa
	2019	61.2±23.05	Aa	79.8±22.34	Ba
Ear diseases					
Rust	2018	1.5±0.39	Bb	5.4±1.38	Aa
	2019	5.4±1.36	Aa	2.8±0.62	Bb
FHB	2018	0±0	Aa	0±0	Ba
	2019	0±0.01	Ab	1.2±0.27	Aa

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

In both varieties, yellow rust levels were higher in org. CP compared to the conv. CP treatment (Table 3.47). Independent of CP, Aszita showed lower yellow rust than Skyfall on Leaf 2 and Leaf 3. With org. CP, Skyfall showed higher FHB than Aszita, but disease levels between the varieties were not significantly different with conv. CP.

Table 3.47. Effect of crop protection (CP) × variety on leaf yellow rust and Fusarium head blight (FHB).

Means ± SE (n = 32)	Variety	Conv. CP		Org. CP	
Foliar disease					
Yellow rust Leaf 2	Aszita	0.7±0.36	Bb	247.8±44.03	Ba
	Skyfall	4±1.44	Ab	547.4±55.49	Aa
Yellow rust Leaf 3	Aszita	2.6±1.47	Bb	170.6±34.04	Ba
	Skyfall	29.8±7.2	Ab	454.4±72.39	Aa
Ear disease					
FHB	Aszita	0±0	Aa	0±0.01	Ba
	Skyfall	0.1±0.11	Ab	1±0.25	Aa

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

Independent of fertiliser treatment, STB levels on flag leaf and Leaf 3 were significantly higher in 2019 than 2018 (Table 3.48). There was no response to fertiliser treatment of STB development on flag leaves in 2018, but biogas digestate showed lower disease levels than the other in 2019. On Leaf 3, STB levels were highest with mineral N application and lowest with biogas digestate and zero-input in 2018. In 2019, wheat without fertiliser showed similar STB levels as the mineral N treatment, whereas disease levels were lower in plots with FYM and biogas digestate treatment. Yellow rust on the flag leaf and Leaf 3 was highest following mineral N fertiliser application in both seasons. Glume blotch did not occur in 2018 but was found at higher levels with mineral N compared to other fertiliser treatments in 2019.

Table 3.48. Effect of fertiliser × season on *Septoria tritici* blotch (STB), leaf yellow rust as well as ear glume blotch.

Means ± SE (n = 32)	**Fertiliser**	**2018**		**2019**	
Foliar diseases					
STB Leaf 1	Biogas digestate	2±1.89	Ab	16.4±3.15	Ba
	Farmyard manure	0.7±0.37	Ab	27.8±3.74	Aa
	Mineral N	1.4±0.68	Ab	22.3±4.96	ABa
	Zero-input	1.2±0.55	Ab	28.6±3.14	Aa
STB Leaf 3	Biogas digestate	61.4±7	Cb	115.1±13.7	Ca
	Farmyard manure	79.7±10.69	Bb	123.4±12.11	BCa
	Mineral N	108.8±16.14	Ab	140.3±19.69	ABa
	Zero-input	71.7±6.36	BCb	152±11.52	Aa
Yellow rust Leaf 1	Biogas digestate	68.9±14.27	Bb	171.1±48.99	Ba
	Farmyard manure	56.6±12.59	Bb	158.8±34.09	Ba
	Mineral N	174.6±35.2	Ab	497.1±108.2	Aa
	Zero-input	87.3±22.79	Bb	171.6±44.53	Ba
Yellow rust Leaf 3	Biogas digestate	99.8±22.26	Ba	27.7±9.43	Bb
	Farmyard manure	125.9±33.27	Ba	39.7±16.03	Bb
	Mineral N	604.4±135.97	Aa	148.6±52.8	Ab
	Zero-input	203±51.15	Ba	66±27.9	Bb
Ear diseases					
Glume blotch	Biogas digestate	0±0	Aa	0.7±0.45	Ba
	Farmyard manure	0±0	Ab	0.6±0.25	Ba
	Mineral N	0±0	Ab	5.2±2.22	Aa
	Zero-input	0±0	Ab	1.4±0.64	Ba

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

The CP × fertiliser interaction affected disease levels on both leaves and ears (Table 3.44). In response to FYM and zero-input, conv. CP was effective for the reduction of yellow rust on leaves and rust on ears as well as STB in fertiliser treatments (Table 3.49). Zero fertiliser input showed highest STB symptom development with conv. CP compared to the fertiliser treatments, whereas wheat with org. CP showed highest STB levels following mineral N application on Leaf 2 and 3 and on flag leaves in response to FYM. When conv. CP was applied, plants with mineral N and biogas digestate showed lowest STB levels on Leaf 2 and 3.

Yellow rust on leaves was highest following mineral N application independent of CP. With conv. CP, wheat with biogas digestate application showed similar yellow rust symptom levels on flag leaves as mineral N-treated wheat, but with org. CP, biogas digestate similar yellow rust levels like FYM and zero-input which were lower compared to the mineral N-treatment.

Diseases on ears did not vary in response to fertiliser treatment with conv. CP. In the absence of pesticides, highest levels of FHB were recorded after FYM application. Highest disease levels of ear yellow rust were recorded in the biogas digestate and mineral N treatment while glume blotch was found mostly in plots with mineral N application.

Table 3.49. Effect of crop protection (CP) × fertiliser on *Septoria tritici* blotch (STB) leaf yellow rust as well as Fusarium head blight (FHB), rust and Glume blotch on ears.

Means ± SE (n = 32)	Fertiliser	Conv. CP		Org. CP	
Foliar diseases					
STB Leaf 1	Biogas digestate	4±1.76	Bb	14.4±3.46	Ca
	Farmyard manure	6.2±1.73	Bb	22.3±4.33	Aa
	Mineral N	2.1±0.89	Bb	21.6±5.02	ABa
	Zero-input	15.2±3.85	Aa	14.6±2.74	BCa
STB Leaf 2	Biogas digestate	10.6±3.01	BCb	50.8±9.68	Ba
	Farmyard manure	16±3.95	Bb	61.1±12.62	Ba
	Mineral N	6.8±2.51	Cb	90.2±8.18	Aa
	Zero-input	27.9±6.07	Aa	53.3±6.52	Ba
STB Leaf 3	Biogas digestate	56.8±9.4	Cb	119.7±11.45	Ba
	Farmyard manure	73.2±10.91	Bb	129.9±10.99	Ba
	Mineral N	43±6.87	Cb	206.1±13.71	Aa
	Zero-input	91.7±11.16	Ab	132±11.23	Ba
Yellow rust Leaf 1	Biogas digestate	0.8±0.48	ABb	239.2±43.08	Ba
	Farmyard manure	0±0.04	Bb	215.4±27.22	Ba
	Mineral N	1.3±0.67	Ab	670.4±86.04	Aa
	Zero-input	0±0	Bb	258.9±39.19	Ba
Yellow rust Leaf 2	Biogas digestate	1.1±0.6	Bb	185.4±27.12	Ca
	Farmyard manure	0.7±0.37	Bb	255.6±30.78	BCa
	Mineral N	6.7±2.78	Ab	796.3±105.62	Aa
	Zero-input	0.8±0.53	Bb	353.2±56.21	BCa
YR Leaf 3	Biogas digestate	8.5±3.2	Bb	119±21.46	Ba
	Farmyard manure	8.3±3.22	Bb	157.3±33.39	Ba
	Mineral N	34.5±13.13	Ab	718.4±130.04	Aa
	Zero-input	13.6±5.94	Bb	255.4±52.15	Ba
Ear diseases					
FHB	Biogas digestate	0±0	Ab	0.2±0.09	Ba
	Farmyard manure	0.2±0.21	Aa	0.5±0.22	ABa
	Mineral N	0±0	Ab	1±0.41	Aa
	Zero-input	0.1±0.06	Aa	0.4±0.24	Ba
Rust	Biogas digestate	0.1±0.04	Ab	4.2±0.86	ABa
	Farmyard manure	0.3±0.31	Ab	4.8±1.48	Ba
	Mineral N	0±0	Ab	14.5±2.62	Aa
	Zero-input	0±0	Ab	6.3±1.63	Ba
Glume blotch	Biogas digestate	0±0	Aa	0.7±0.45	Ba
	Farmyard manure	0.1±0.11	Aa	0.5±0.24	Ba
	Mineral N	0±0	Ab	5.2±2.22	Aa
	Zero-input	0±0	Ab	1.4±0.64	Ba

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

The two wheat varieties responded differently in terms of yellow rust on leaves and FHB on ears in response to fertiliser application (Table 3.50). Both varieties showed highest yellow rust following mineral N application. Flag leaves of Skyfall showed lower AUDPC-levels following biogas digestate application than in the mineral N and control treatments. Flag leaves of Aszita showed more yellow rust than Skyfall when mineral N was applied, whereas disease levels in response to the other fertiliser treatments were not significantly different between the two varieties. On Leaf 3, biogas digestate was the only fertiliser that showed similar levels of yellow rust in both varieties. Apart from that, symptoms were higher in Skyfall compared to Aszita. Symptoms of FHB on ears were higher in Skyfall than Aszita, but not significantly different when biogas digestate was applied. For Aszita, biogas digestate was the only fertiliser treatment where FHB symptoms occurred but at a very low level. In Skyfall, highest incidences of FHB were found following mineral N application with slightly lower levels with FYM and zero-input but again FHB incidence was very low.

Table 3.50. Effect of variety × fertiliser on disease levels of leaf yellow rust as well as Fusarium head blight (FHB).

Means ± SE (n = 32)	**Fertiliser**	**Aszita**		**Skyfall**	
Foliar diseases					
Yellow rust Leaf 1	Biogas digestate	142.1±46.36	Ba	97.9±24.33	Ca
	Farmyard manure	112.1±32.55	Ba	103.3±20.69	BCa
	Mineral N	413±107.17	Aa	258.7±52.47	Ab
	Zero-input	105.7±40.19	Ba	153.2±31.07	Ba
Yellow rust Leaf 3	Biogas digestate	47.8±14.85	Ba	79.7±20.77	Ba
	Farmyard manure	53.5±16.16	Bb	112.1±34.16	Ba
	Mineral N	202.4±65.58	Ab	550.5±135.54	Aa
	Zero-input	42.8±16.4	Bb	226.2±53.73	Ba
Ear disease					
FHB	Biogas digestate	0±0.02	Aa	0.2±0.08	Ba
	Farmyard manure	0±0	Bb	0.7±0.29	ABa
	Mineral N	0±0	Bb	1±0.41	Aa
	Zero-input	0±0	Bb	0.5±0.24	ABa

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

There was a significant fertiliser × AMF inoculum interaction on ear FHB (p = 0.04, Table 3.44). FHB levels showed no response to fertiliser without AMF-inoculation, but with the AMF inoculum, FHB levels varied depending on fertiliser treatment (Table 3.51). Here, biogas digestate and FYM showed lower FHB levels compared to the mineral N and control treatments.

Table 3.51. Effect of fertiliser × AMF inoculum on Fusarium head blight (FHB).

Means ± SE (n = 32)	**Fertiliser**	**- AMF**		**+ AMF**	
Ear disease					
FHB	Biogas digestate	0.1±0.07	Aa	0.1±0.06	Ba
	Farmyard manure	0.5±0.29	Aa	0.1±0.08	Ba
	Mineral N	0.3±0.21	Aa	0.7±0.37	Aa
	Zero-input	0.2±0.11	Aa	0.3±0.22	ABa

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

3.3.5 MYCORRHIZAL ROOT COLONISATION

The intensities of mycorrhizal colonisation structures such as arbuscules (A%), hyphae (H%) and vesicles (V%) in wheat roots followed the same pattern as mycorrhizal intensities (M%). For this reason, M% was selected to give an overview of mycorrhizal development in wheat over the two cropping seasons 2018 and 2019 (Fig. 3.3). Mycorrhizal colonisation intensities in root systems of wheat varied between seasons and were mostly affected by variety and fertiliser treatments. Peaks of M% were reached at flowering (GS64) in 2018 and at stem elongation (GS32) in 2019. Mycorrhizal colonisation intensity was not affected by CP in 2018 but was increased in response to conv. CP after tillering in 2019 (Fig. 3.3 A). Varying effects of fertiliser treatment on M% were observed (Fig. 3.3 B) with strong decreases in 2018 in response to mineral N and biogas digestate application and a constant lower M% with FYM. In 2019, the application of mineral N only affected M% at maturity (GS90) but had no impact on AMF root colonisation (AMF-RC) at stem elongation and flowering. Wheat without fertiliser input showed highest M% at all assessed time points. Skyfall showed overall higher M% than Aszita and this difference was more pronounced in 2019 than 2018 (Fig. 3.3 C). No main effects on M% were observed in response to inoculum application at all growth stages.

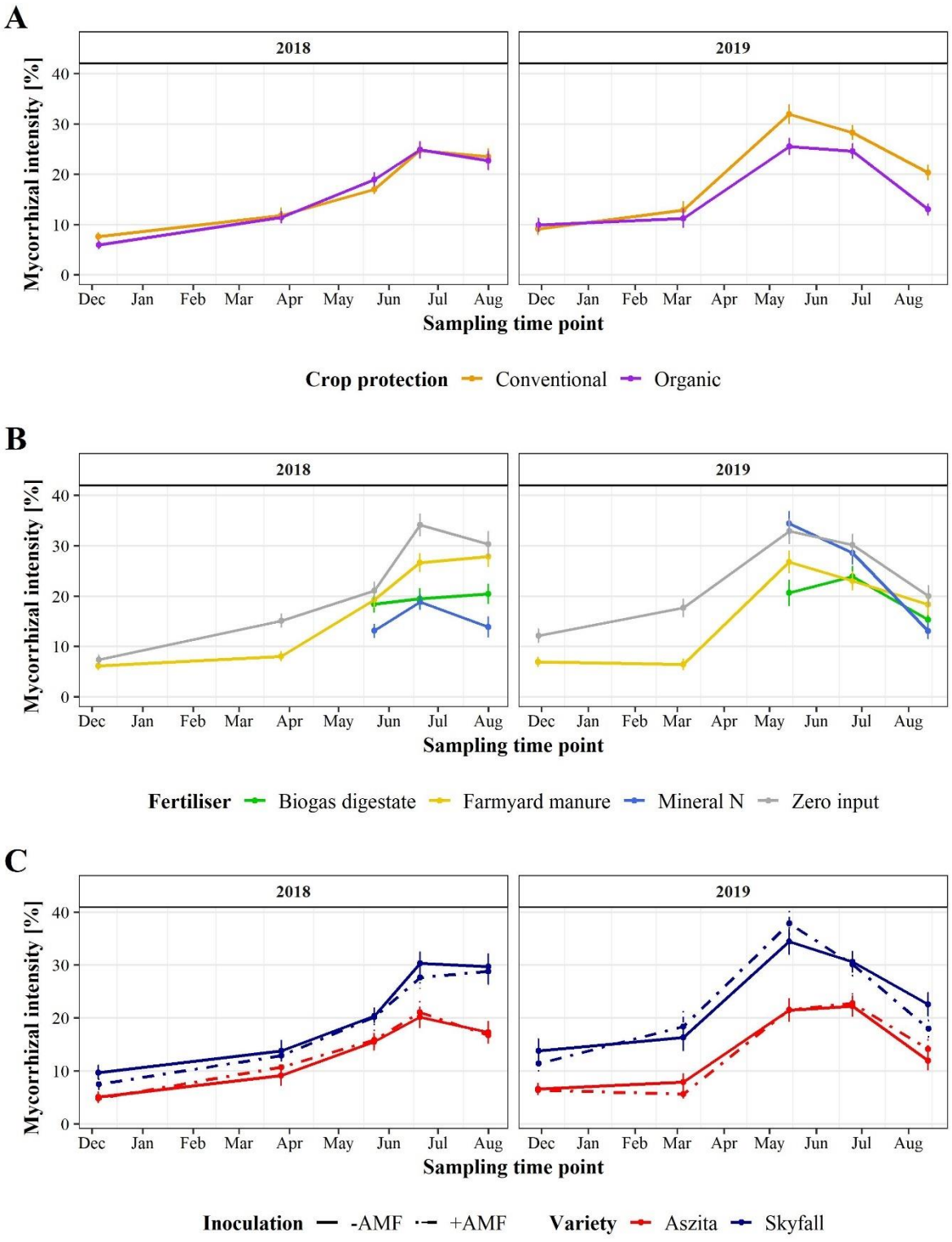


Fig. 3.3. Mycorrhizal intensity [%] in wheat roots over two cropping seasons (2018 and 2019) as affected by: **A**) organic and conventional crop protection (Dec. – Apr. n = 32, May – Aug. n = 64), **B**) fertiliser treatment (farmyard manure applied in autumn, mineral N and biogas digestate applied in spring) compared to zero-input (n = 32), **C**) variety Aszita and Skyfall with and without AMF inoculum application (Dec. – Apr. n = 16, May – Aug. n = 32). Data points show means ± SE.

3.3.5.1 SEEDLING GROWTH

Variety-specific colonisation patterns were already visible at seedling growth where Skyfall showed higher AMF frequencies (F%) and M% than Aszita ($p \leq 0.001$, Table 3.52). Arbuscule (A%) and hyphae (H%) abundances were higher in Skyfall too, but there was no significant difference in vesicle abundance (V%). Application of FYM reduced all AMF-RC parameters in wheat roots.

Table 3.52. Effects of season, crop protection, variety, AMF inoculum and fertiliser on mycorrhizal colonisation parameters (F = frequency, M = intensity, A, V and H = arbuscule, vesicle and hyphae abundances) at seedling stage (GS12). ANOVA *p*-values in bold indicate significant main effects and interactions. Means within columns followed by the same letter are not significantly different at $p \leq 0.05$.

	Seedling growth (GS12)				
	F%	**M%**	**A%**	**V%**	**H%**
Year (YR)					
2018 (n=127)	32.9±1.88	6.8±0.6	3.8±0.39	0±0.02	4.4±0.39
2019 (n=128)	38.3±2.05	9.5±0.89	5.7±0.58	0.2±0.08	5.9±0.54
Crop protection (CP)					
Conventional (n=128)	36.8±2.05	8.4±0.75	4.8±0.5	0.1±0.04	5.4±0.46
Organic (n=127)	34.4±1.94	7.9±0.81	4.6±0.52	0.2±0.07	5±0.5
Variety (VR)					
Aszita (n=128)	30.3±1.75b	5.7±0.5b	3.3±0.32b	0±0.01	3.7±0.32b
Skyfall (n=127)	41±2.01a	10.6±0.89a	6.2±0.6a	0.2±0.08	6.7±0.54a
Inoculation (AMF)					
-AMF (n=128)	36.5±2.03	8.8±0.86	5.3±0.58	0.2±0.07	5.4±0.52
+AMF (n=127)	34.7±1.97	7.5±0.68	4.1±0.42	0.1±0.04	5±0.44
Fertiliser (FT)					
Farmyard manure (n=63)	31.1±1.81b	6.5±0.62b	3.5±0.37b	0±0.01b	4.4±0.42b
Zero-input (n=64)	40.1±2.01a	9.8±0.87a	5.9±0.59a	0.2±0.08a	6±0.52a
ANOVA *p-values*					
Main effects					
YR	ns	ns	ns	ns	ns
CP	ns	ns	ns	ns	ns
VR	**≤0.001**	**≤0.001**	**≤0.001**	ns	**≤0.001**
AMF	ns	ns	ns	ns	ns
FT	**≤0.001**	**≤0.001**	**≤0.001**	**0.006**	**0.003**
Interactions					
YR:CP	ns	ns	ns	ns	ns
YR:VR	ns	ns	ns	ns	ns
CP:VR	ns	ns	ns	ns	ns
YR:AMF	ns	ns	ns	ns	ns
CP:AMF	ns	ns	ns	ns	ns
VR:AMF	ns	ns	ns	ns	ns
YR:FT	ns	**0.025**	**0.019**	ns	ns
CP:FT	ns	ns	ns	ns	ns
VR:FT	ns	ns	ns	**0.043**	ns
AMF:FT	ns	ns	ns	ns	ns

YR:CP:VR	ns	ns	ns	ns	ns
YR:CP:AMF	ns	ns	ns	ns	ns
YR:VR:AMF	ns	ns	ns	ns	ns
CP:VR:AMF	ns	ns	ns	ns	ns
YR:CP:FT	ns	ns	ns	ns	ns
YR:VR:FT	ns	ns	ns	ns	ns
CP:VR:FT	ns	**0.046**	ns	ns	**0.040**
YR:AMF:FT	ns	ns	ns	ns	ns
CP:AMF:FT	ns	ns	ns	ns	ns
VR:AMF:FT	**0.023**	ns	ns	ns	ns
YR:CP:VR:AMF	ns	ns	ns	ns	ns
YR:CP:VR:FT	ns	ns	ns	ns	ns
YR:CP:AMF:FT	ns	ns	ns	ns	ns
YR:VR:AMF:FT	ns	ns	ns	ns	ns
CP:VR:AMF:FT	ns	ns	ns	ns	ns
YR:CP:VR:AMF:FT	ns	ns	ns	ns	ns

In 2018, M% and A% were not significantly different in seedlings between the FYM and zero-input treatments, but in 2019, FYM application significantly decreased both M% and A% (Table 3.53). In plots with FYM application, there was no significant difference in A% and M% between the two seasons but in the zero-input treatment, M% and A% were higher in 2019 than 2018.

Table 3.53. Effect of season × fertiliser on mycorrhizal intensities (M%) and arbuscule abundances (A%) in wheat roots at seedling growth (GS12).

Means ± SE (n = 32)	**Season**	**Farmyard manure**		**Zero-input**	
M%	2018	6.1±0.83	Aa	7.4±0.87	Ba
	2019	6.9±0.93	Ab	12.1±1.39	Aa
A%	2018	3.3±0.54	Aa	4.2±0.57	Ba
	2019	3.8±0.5	Ab	7.5±0.95	Aa

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

Although vesicle numbers (V%) were low during early growth, V% in root systems of wheat seedlings were significantly affected by the variety × season interaction ($p = 0.043$, Table 3.52). In 2019, Skyfall showed higher V% compared to Aszita, whereas no difference occurred in 2018 (Table 3.54).

Table 3.54. Effect of season × variety on vesicle abundances (V%) in wheat roots at seedling growth (GS12).

Means ± SE (n = 32)	**Season**	**Aszita**		**Skyfall**	
V%	2018	0±0.02	Aa	0.1±0.04	Aa
	2019	0.1±0.02	Ab	0.3±0.15	Aa

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

3.3.5.2 TILLERING

Mycorrhizal colonisation after winter was higher in Skyfall than Aszita (Table 3.55). Besides variety, fertiliser application impacted all AMF-RC parameters ($p < 0.001$) except for V%. Plots without fertiliser showed almost twice the level of AMF-RC as wheat treated with FYM.

Table 3.55. Effects of season, crop protection, variety, AMF inoculum and fertiliser on mycorrhizal colonisation parameters (F = frequency, M = intensity, A, V and H = arbuscule, vesicle and hyphae abundances) at tillering (GS22). ANOVA *p*-values in bold indicate significant main effects and interactions. Means within columns followed by the same letter are not significantly different at $p \leq 0.05$.

	Tillering (GS22)				
	F%	**M%**	**A%**	**V%**	**H%**
Year (YR)					
2018 (n=127)	44.2±2.32	11.6±0.96	5.8±0.57	0.1±0.03	8±0.69
2019 (n=128)	38.2±2.62	12.1±1.28	7.3±0.86	0.3±0.09	7.4±0.76
Crop protection (CP)					
Conventional (n=128)	42±2.5	12.3±1.14	6.7±0.71	0.1±0.03	8.1±0.77
Organic (n=127)	40.4±2.5	11.3±1.13	6.4±0.77	0.3±0.08	7.3±0.67
Variety (VR)					
Aszita (n=128)	34.2±2.48b	8.3±0.83b	4.2±0.43b	0.1±0.02b	5.8±0.62b
Skyfall (n=127)	48.3±2.19a	15.4±1.23a	9±0.86a	0.3±0.09a	9.7±0.75a
Inoculation (AMF)					
-AMF (n=128)	40.8±2.48	11.8±1.1	6.7±0.71	0.2±0.04	7.5±0.73
+AMF (n=127)	41.6±2.53	11.9±1.17	6.4±0.77	0.3±0.08	7.9±0.72
Fertiliser (FT)					
Farmyard manure (n=63)	31.7±2.14b	7.2±0.75b	3.5±0.38b	0.2±0.08	5±0.55b
Zero-input (n=64)	50.6±2.26a	16.4±1.15a	9.5±0.81a	0.3±0.05	10.3±0.72a
ANOVA *p-values*					
Main effects					
YR	ns	ns	ns	ns	ns
CP	ns	ns	ns	ns	ns
VR	**≤0.001**	**≤0.001**	**≤0.001**	**0.031**	**0.003**
AMF	ns	ns	ns	ns	ns
FT	**≤0.001**	**≤0.001**	**≤0.001**	ns	**≤0.001**
Interactions					
YR:CP	ns	ns	ns	ns	ns
YR:VR	**0.008**	**0.032**	**0.016**	ns	ns
CP:VR	ns	ns	ns	ns	ns
YR:AMF	ns	ns	ns	ns	ns
CP:AMF	ns	ns	ns	ns	ns
VR:AMF	ns	ns	ns	ns	ns
YR:FT	ns	**0.038**	ns	ns	ns
CP:FT	ns	ns	ns	ns	ns
VR:FT	ns	**0.004**	**0.006**	ns	**0.020**
AMF:FT	**0.006**	ns	ns	ns	ns
YR:CP:VR	ns	ns	ns	ns	ns
YR:CP:AMF	ns	ns	ns	ns	ns
YR:VR:AMF	ns	ns	ns	ns	ns

CP:VR:AMF	ns	ns	ns	ns	ns
YR:CP:FT	**0.012**	**0.027**	ns	ns	**0.011**
YR:VR:FT	ns	ns	ns	ns	ns
CP:VR:FT	ns	ns	ns	ns	ns
YR:AMF:FT	ns	ns	ns	ns	ns
CP:AMF:FT	ns	ns	ns	ns	ns
VR:AMF:FT	ns	ns	ns	ns	ns
YR:CP:VR:AMF	ns	ns	ns	ns	ns
YR:CP:VR:FT	ns	ns	ns	ns	ns
YR:CP:AMF:FT	**0.036**	ns	ns	ns	**0.018**
YR:VR:AMF:FT	ns	ns	ns	ns	ns
CP:VR:AMF:FT	ns	ns	ns	ns	ns
YR:CP:VR:AMF:FT	ns	ns	ns	ns	ns

Mycorrhizal colonisation parameters at tillering were significantly affected by the season × variety interaction (Table 3.55). Aszita showed higher F% and M% in 2018 than 2019, but A% was not significantly different between the two seasons (Table 3.56). Mycorrhizal intensities (M%) and A% in Skyfall were lower in 2019 compared to the previous season, but F% was the same in both years. Mycorrhizal frequencies did not differ between the two varieties in 2018 but were higher in Skyfall than in Aszita in 2019. Mycorrhizal intensities and A% were higher in Skyfall than Aszita in both seasons.

Table 3.56. Effect of season × variety on mycorrhizal frequencies (F%), intensities (M%) and arbuscule (A%) abundances in wheat roots at tillering (GS22).

Means ± SE (n = 32)	**Season**	**Aszita**		**Skyfall**	
F %	2018	41.4±3.47	Aa	47.2±3.03	Aa
	2019	27±3.09	Bb	49.5±3.19	Aa
M%	2018	9.9±1.22	Ab	13.4±1.44	Ba
	2019	6.8±1.06	Bb	17.3±1.95	Aa
A%	2018	4.6±0.62	Ab	7.1±0.93	Ba
	2019	3.8±0.61	Ab	10.9±1.36	Aa

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

A significant interaction was found for fertiliser × season which had impact on M% (p = 0.038, Table 3.55) at tillering. However, Tukey-post hoc test revealed only lower M% in FYM-treated plots compared to zero-input plots with no differences between seasons (Table 3.57).

Table 3.57. Effect of fertiliser × season on mycorrhizal intensities (M%) in wheat roots at tillering (GS22)

Means ± SE (n = 32)	Fertiliser	2018		2019	
M%	Farmyard manure	8±0.97	Ba	6.4±1.14	Ba
	Zero-input	15.1±1.39	Aa	17.7±1.83	Aa

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for p ≤ 0.05 by Tukey's HSD test.

The effect of fertiliser on M%, A% and H% in wheat roots at tillering varied between the two varieties (Table 3.58). Root colonisation intensity (M%) as well as A% and H% were higher in Skyfall than in Aszita independent of fertiliser application. In both varieties, application of FYM decreased all the components of AMF-RC.

Table 3.58. Effect of fertiliser × variety on mycorrhizal intensity (M%), arbuscule (A%) and hyphae (H%) abundances in wheat roots at tillering (GS22).

Means ± SE (n = 32)	Fertiliser	Aszita		Skyfall	
M%	Farmyard manure	5.2±0.85	Bb	9.2±1.16	Ba
	Zero-input	11.4±1.2	Ab	21.3±1.54	Aa
A%	Farmyard manure	2.2±0.34	Bb	5±0.6	Ba
	Zero-input	6.2±0.63	Ab	12.9±1.25	Aa
H%	Farmyard manure	3.9±0.34	Bb	6.1±0.6	Ba
	Zero-input	7.6±0.63	Ab	13.1±1.25	Aa

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

There was a significant interaction of fertiliser × inoculation at tillering (p = 0.006, Table 3.55). Mycorrhizal frequencies (F%) were significantly lower in wheat with FYM and without inoculum compared to plots with AMF treatment (Table 3.59). Wheat without fertiliser input had overall higher F% and was not affected by inoculum application.

Table 3.59. Effect of fertiliser × AMF inoculum on mycorrhizal frequencies (F%) in wheat roots at tillering (GS22).

Means ± SE (n = 32)	Fertiliser	-AMF		+AMF	
F%	Farmyard manure	27.8±2.44	Bb	35.6±3.44	Ba
	Zero-input	53.8±2.85	Aa	47.4±3.45	Aa

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

3.3.5.3 STEM ELONGATION

Mycorrhizal root colonisation intensity (M%) as well as A%, V% and H% at stem elongation were lower in 2018 than 2019 (Table 3.60). Similar to previous growth stages, Skyfall showed higher AMF-RC than Aszita ($p < 0.001$). Application of biogas digestate and mineral N ~ 2-3 weeks prior to this sampling reduced F%, V% and H%. These two fertiliser types tended to have similar effects on AMF-RC, but on average the lowest M% and H% were found following biogas digestate application. Mycorrhizal frequencies and H% were highest in the FYM and zero-input. The latter treatment showed highest V% compared to plants that received fertiliser inputs. Arbuscule abundances (A%) at this growth stage were not significantly different in zero-input and mineral N-treated plots and in biogas digestate and FYM-treated plots respectively.

Table 3.60. Effects of season, crop protection, variety, AMF inoculum and fertiliser on mycorrhizal colonisation parameters (F = frequency, M = intensity, A, V and H = arbuscule, vesicle and hyphae abundances) at stem elongation (GS32). ANOVA *p*-values in bold indicate significant main effects and interactions. Means within columns followed by the same letter are not significantly different at $p \leq 0.05$.

	Stem elongation (GS32)				
	F%	**M%**	**A%**	**V%**	**H%**
Year (YR)					
2018 (n=127)	63.9±1.44	18±0.85b	9.8±0.49b	0.6±0.08b	11.8±0.6b
2019 (n=128)	55.5±1.75	28.7±1.31a	15.1±0.74a	2.1±0.29a	18.8±0.86a
Crop protection (CP)					
Conventional (n=128)	61.8±1.51	24.5±1.27	12.9±0.67	1.6±0.26	16.1±0.86
Organic (n=127)	57.5±1.75	22.2±1.12	12±0.66	1.1±0.16	14.5±0.75
Variety (VR)					
Aszita (n=128)	52.5±1.65b	18.6±1.02b	10±0.58b	0.5±0.13b	12.4±0.68b
Skyfall (n=127)	67±1.35a	28.2±1.22a	14.9±0.69a	2.3±0.26a	18.3±0.84a
Inoculation (AMF)					
-AMF (n=128)	59±1.59	22.9±1.14	12.4±0.67	1.3±0.2	14.9±0.76
+AMF (n=127)	60.4±1.69	23.8±1.26	12.4±0.67	1.4±0.24	15.7±0.85
Fertiliser (FT)					
Biogas digestate (n=64)	53.9±2.61b	19.5±1.54b	10.8±0.86b	0.7±0.16b	12.6±1.04c
Farmyard manure (n=63)	63.6±2.09a	23.1±1.46ab	11±0.7b	1.3±0.21b	16.1±1.09ab
Mineral N (n=64)	56.1±2.1b	23.8±1.93ab	13.6±1.09a	1.3±0.34b	14.9±1.26bc
Zero-input (n=64)	65.1±2.17a	27±1.71a	14.2±1.02a	2.1±0.44a	17.6±1.09a
ANOVA *p-values*					
Main effects					
YR	ns	**0.005**	**0.014**	**0.050**	**0.004**
CP	ns	ns	ns	ns	ns
VR	**≤0.001**	**≤0.001**	**≤0.001**	**≤0.001**	**≤0.001**
AMF	ns	ns	ns	ns	ns
FT	**≤0.001**	**≤0.001**	**≤0.001**	**≤0.001**	**≤0.001**

Interactions					
YR:CP	ns	**0.024**	**0.045**	ns	**0.020**
YR:VR	**0.023**	**0.002**	ns	**0.009**	**≤0.001**
CP:VR	ns	ns	ns	ns	ns
YR:AMF	ns	ns	ns	ns	ns
CP:AMF	ns	ns	ns	ns	ns
VR:AMF	ns	ns	ns	ns	ns
YR:FT	**≤0.001**	**≤0.001**	**≤0.001**	ns	**≤0.001**
CP:FT	ns	ns	ns	ns	ns
VR:FT	ns	ns	ns	ns	ns
AMF:FT	ns	ns	ns	ns	ns
YR:CP:VR	ns	ns	ns	ns	ns
YR:CP:AMF	ns	ns	ns	ns	ns
YR:VR:AMF	ns	ns	ns	ns	ns
CP:VR:AMF	ns	**0.029**	ns	ns	**0.022**
YR:CP:FT	ns	ns	ns	ns	ns
YR:VR:FT	**0.025**	ns	ns	ns	ns
CP:VR:FT	ns	ns	ns	ns	ns
YR:AMF:FT	**0.048**	ns	ns	ns	ns
CP:AMF:FT	ns	**0.031**	**0.029**	ns	**0.049**
VR:AMF:FT	ns	ns	ns	ns	ns
YR:CP:VR:AMF	ns	ns	ns	ns	ns
YR:CP:VR:FT	ns	ns	ns	**0.018**	ns
YR:CP:AMF:FT	ns	ns	ns	ns	ns
YR:VR:AMF:FT	ns	ns	ns	ns	ns
CP:VR:AMF:FT	ns	ns	ns	ns	ns
YR:CP:VR:AMF:FT	ns	ns	ns	ns	ns

There was a significant interaction of CP × season on AMF-RC (Table 3.61). Mycorrhizal intensity (M%) as well as A% and H% were higher in 2019 than 2018. Crop protection had no effect on these parameters in 2018, but increased AMF-RC rates compared to plots without conv. CP were scored in 2019.

Table 3.61. Effect of season × crop protection (CP) on mycorrhizal intensity (M%), arbuscule (A%) and hyphae (H%) abundances in wheat roots at stem elongation (GS32).

Means ± SE (n = 32)	**Season**	**Conv. CP**		**Org. CP**	
M%	2018	17±0.96	Ba	18.9±1.41	Ba
	2019	31.9±1.96	Aa	25.5±1.64	Ab
A%	2018	9.1±0.55	Ba	10.4±0.82	Ba
	2019	16.6±1.05	Aa	13.6±1.01	Ab
H%	2018	7.4±0.43	Ba	8.4±0.65	Ba
	2019	13.1±0.79	Aa	10.9±0.79	Ab

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

Seasonal effects of AMF-RC were also visible between the two varieties (Table 3.62). Mycorrhizal frequencies (F%) and M% as well as V% and H% of Aszita were significantly lower than Skyfall in both seasons. In 2019, F% in Aszita was lower than in 2018, whereas no difference between seasons was found in Skyfall. Mycorrhizal intensities and H% were lower in 2018 in both varieties. Vesicle numbers (V%) were lower in Skyfall in 2019, whereas Aszita showed the same V% in both years.

Table 3.62. Effect of season × variety on mycorrhizal frequency (F%), intensity (M%), vesicle (V%) and hyphae (H%) abundances in wheat roots at stem elongation (GS32).

Means ± SE (n = 32)	**Season**	**Aszita**		**Skyfall**	
F%	2018	59.3±2.09	Ab	68.5±1.81	Aa
	2019	45.7±2.27	Bb	65.5±1.99	Aa
M%	2018	15.7±1.25	Bb	20.3±1.09	Ba
	2019	21.5±1.53	Ab	36.1±1.68	Aa
V%	2018	0.2±0.05	Ab	1±0.14	Ba
	2019	0.7±0.26	Ab	3.6±0.45	Aa
H%	2018	10.8±0.88	Bb	12.8±0.8	Ba
	2019	14±1	Ab	23.8±1.11	Aa

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

The effect of fertiliser on AMF-RC parameters varied significantly between seasons ($p < 0.001$, Table 3.60). In 2018, F% was reduced in response to biogas digestate and mineral N application, but only by biogas digestate application in 2019 (Table 3.63). Mycorrhizal frequencies (F%) levels were not significantly different between years in the mineral N treatment, whereas all other fertiliser treatments showed lower F% in 2019 compared to 2018.

Mycorrhizal intensities were reduced by mineral N application in 2018, but not in 2019. In the same year, biogas digestate showed lowest M% rates, followed by FYM application. Pairwise comparison of this parameter between trial years showed that M% at GS32 was higher in 2019 in all fertiliser treatments except for biogas digestate where colonisation intensities were the same in both years. Also A% was not different between seasons with biogas digestate application, but increased from 2018 to 2019 in response to the other fertiliser sources. In 2018, A% was reduced by mineral N application, but not in 2019 where A% was decreased only in response to biogas digestate and FYM application.

Hyphae abundances (H%) were negatively affected by mineral N in 2018 and by biogas digestate in 2019. There was no effect of season on H% in wheat with biogas digestate whereas the same parameter increased in all other tested fertiliser regimes from 2018 to 2019.

Table 3.63. Effect of season × fertiliser on mycorrhizal frequency (F%), intensity (M%), arbuscule (A%) and hyphae (H%) abundances in wheat roots at stem elongation (GS32).

Means ± SE (n = 32)	Fertiliser	2018		2019	
F%	Biogas digestate	62.8±2.46	Ba	44.7±4.09	Bb
	Farmyard manure	69.4±2.23	ABa	58±3.25	Ab
	Mineral N	53.6±3.01	Ca	58.6±2.91	Aa
	Zero-input	69.9±2.85	Aa	60.3±3.09	Ab
M%	Biogas digestate	18.4±1.7	Aa	20.6±2.6	Ca
	Farmyard manure	19.3±1.65	Ab	26.8±2.22	Ba
	Mineral N	13.1±1.41	Bb	34.4±2.43	Aa
	Zero-input	21.1±1.77	Ab	32.9±2.56	Aa
A%	Biogas digestate	10.5±1.08	Aa	11.1±1.37	Ba
	Farmyard manure	9.8±0.84	ABb	12.2±1.08	Ba
	Mineral N	7.5±0.75	Bb	19.8±1.33	Aa
	Zero-input	11.3±1.12	Ab	17.2±1.54	Aa
H%	Biogas digestate	11.8±1.11	ABa	13.5±1.78	Ba
	Farmyard manure	13.1±1.28	Ab	19±1.6	Aa
	Mineral N	8.4±1.01	Bb	21.5±1.64	Aa
	Zero-input	13.9±1.19	Ab	21.2±1.59	Aa

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

3.3.5.4 FLOWERING

At flowering, all fertiliser input sources significantly reduced all assessed AMF-RC parameters ($p < 0.001$, Table 3.64) with higher levels in Skyfall than in Aszita observed ($p \leq 0.001$).

Table 3.64. Effects of season, crop protection, variety, AMF inoculum and fertiliser on mycorrhizal colonisation parameters (F = frequency, M = intensity, A, V and H = arbuscule, vesicle and hyphae abundances) at flowering (GS64). ANOVA *p*-values in bold indicate significant main effects and interactions. Means within columns followed by the same letter are not significantly different at $p \leq 0.05$.

	Flowering (GS64)				
	F%	**M%**	**A%**	**V%**	**H%**
Year (YR)					
2018 (n=127)	57.8±1.59	24.8±1.12	13.8±0.64	1.5±0.21	15.7±0.77
2019 (n=128)	58±1.61	26.4±1.06	13.7±0.54	1.5±0.21	17.7±0.75
Crop protection (CP)					
Conventional (n=128)	60.3±1.45	26.5±1.06	14.2±0.57	1.6±0.2	17.3±0.75
Organic (n=127)	55.5±1.71	24.7±1.12	13.2±0.6	1.4±0.22	16.1±0.77
Variety (VR)					
Aszita (n=128)	52.2±1.6b	21.5±1b	11.9±0.56b	0.5±0.08b	14.1±0.71b
Skyfall (n=127)	63.7±1.43a	29.7±1.06a	15.6±0.57a	2.5±0.26a	19.4±0.74a

Inoculation (AMF)					
-AMF (n=128)	57.8±1.62	25.8±1.1	13.9±0.62	1.5±0.2	16.9±0.76
+AMF (n=127)	58.1±1.58	25.4±1.08	13.6±0.56	1.5±0.22	16.6±0.77
Fertiliser (FT)					
Biogas digestate (n=64)	51.7±2.28b	21.7±1.47b	11.8±0.81b	1.2±0.23b	14.1±1.02b
Farmyard manure (n=63)	56.9±2.11b	24.8±1.34b	13.6±0.81b	1.5±0.24b	16.1±0.87b
Mineral N (n=64)	55.4±2.21b	23.7±1.52b	12.4±0.73b	1±0.26b	15.8±1.12b
Zero-input (n=64)	67.7±1.95a	32.1±1.54a	17.1±0.84a	2.3±0.41a	20.9±1.1a
ANOVA *p-values*					
Main effects					
YR	ns	ns	ns	ns	ns
CP	ns	ns	ns	ns	ns
VR	**≤0.001**	**≤0.001**	**≤0.001**	**≤0.001**	**≤0.001**
AMF	ns	ns	ns	ns	ns
FT	**≤0.001**	**≤0.001**	**≤0.001**	**≤0.001**	**≤0.001**
Interactions					
YR:CP	ns	ns	ns	ns	ns
YR:VR	ns	ns	ns	ns	ns
CP:VR	ns	ns	ns	ns	ns
YR:AMF	ns	ns	ns	ns	ns
CP:AMF	ns	ns	ns	ns	ns
VR:AMF	ns	ns	ns	ns	ns
YR:FT	**≤0.001**	**≤0.001**	**≤0.001**	**0.011**	**0.002**
CP:FT	**0.009**	**0.010**	**0.041**	**0.038**	**0.017**
VR:FT	ns	ns	ns	ns	ns
AMF:FT	ns	ns	ns	ns	ns
YR:CP:VR	ns	ns	ns	ns	ns
YR:CP:AMF	ns	**0.041**	ns	ns	ns
YR:VR:AMF	ns	ns	ns	ns	ns
CP:VR:AMF	ns	ns	ns	ns	ns
YR:CP:FT	ns	ns	ns	ns	ns
YR:VR:FT	ns	ns	ns	**0.041**	ns
CP:VR:FT	ns	ns	ns	ns	ns
YR:AMF:FT	ns	ns	ns	ns	ns
CP:AMF:FT	ns	ns	ns	ns	ns
VR:AMF:FT	ns	ns	ns	ns	ns
YR:CP:VR:AMF	ns	ns	ns	ns	ns
YR:CP:VR:FT	ns	ns	ns	ns	ns
YR:CP:AMF:FT	ns	ns	ns	ns	ns
YR:VR:AMF:FT	ns	ns	ns	ns	ns
CP:VR:AMF:FT	ns	ns	ns	ns	ns
YR:CP:VR:AMF:FT	ns	ns	ns	ns	ns

At flowering, all assessed AMF-RC parameters were affected by the season × fertiliser interaction (Table 3.64). The 2018 season showed a uniform pattern in all parameters where the highest AMF-RC levels were reached in the zero-input plots and the lowest levels occurred with application of mineral N and biogas digestate (Table 3.65). Samples from the FYM treatment showed intermediate colonisation rates except for V% which was not significantly different to the zero-input treatment.

In 2019, there was no statistical difference in V% between fertiliser treatments and zero-input plots. Other AMF-RC parameters such as F% and M% were lowest with biogas digestate and FYM whereas mineral N application showed almost the same colonisation level as zero-input plots. Application of biogas digestate resulted in similar levels of all AMF-RC parameters at flowering in both seasons. Zero-input plots showed decreased F% in 2019 but yielded overall the same AMF-RC rates in the two seasons. Roots from the FYM treatment showed lower F%, V% and A% in 2019 compared to 2018.

Table 3.65. Effect of season × fertiliser on mycorrhizal frequency (F%), intensity (M%), arbuscule (A%), vesicle (V%) and hyphae (H%) abundances in wheat roots at flowering (GS64).

Means ± SE (n = 32)	Fertiliser	2018		2019	
F%	Biogas digestate	48.4±3.27	Ca	54.9±3.12	Ba
	Farmyard manure	61.1±2.67	Ba	52.8±3.14	Bb
	Mineral N	50.9±2.59	Cb	59.8±3.45	ABa
	Zero-input	71±2.55	Aa	64.4±2.88	Ab
M%	Biogas digestate	19.5±2.06	Ca	23.9±2.05	BCa
	Farmyard manure	26.7±1.86	Ba	23±1.9	Ca
	Mineral N	18.8±1.6	Cb	28.6±2.32	ABa
	Zero-input	34.1±2.28	Aa	30.2±2.04	Aa
A%	Biogas digestate	11.1±1.2	Ca	12.4±1.08	Ba
	Farmyard manure	15.3±1.21	Ba	11.9±1.02	Bb
	Mineral N	10.3±0.88	Cb	14.6±1.04	ABa
	Zero-input	18.5±1.23	Aa	15.7±1.11	Ab
V%	Biogas digestate	1±0.24	BCa	1.3±0.4	Aa
	Farmyard manure	1.8±0.36	ABa	1.2±0.3	Ab
	Mineral N	0.3±0.14	Cb	1.7±0.47	Aa
	Zero-input	2.8±0.63	Aa	1.9±0.51	Aa
H%	Biogas digestate	12.2±1.43	Cb	16±1.41	BCa
	Farmyard manure	16.7±1.14	Ba	15.5±1.32	Ca
	Mineral N	12.4±1.18	Cb	19.3±1.72	ABa
	Zero-input	21.7±1.71	Aa	20.1±1.4	Aa

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

Also the fertiliser × CP interaction affected all assessed AMF-RC parameters at flowering (Table 3.64). With conv. CP, F% was lowest in wheat treated with FYM or biogas digestate whereas zero-input and mineral N treatment were not significantly different (Table 3.66). Without CP treatment however, mineral N showed lower F% compared to zero-input. Only biogas digestate reduced M% in wheat with conv. CP, whereas all fertilisers reduced M% compared to the zero-input treatment with org. CP. The application of conv. CP increased M% compared to org. CP for the mineral N treatment, but M% was not affected by CP in the other fertiliser sources. The same pattern of the interaction as described for M% was observed for A%. Vesicle intensities were not affected by fertiliser application when conv. CP was used. In wheat with org. CP however, fertiliser application reduced V% significantly compared to the zero-input plots. The mineral and FYM treatments showed lower V% with org. CP than with conv. CP. Treatment with biogas digestate decreased H% compared to other fertilisers and zero-input. With org. CP, all fertiliser treatments showed lower H% than zero-input. Within the mineral N treatment, H% was lower in roots with org. CP than conv. CP.

Table 3.66. Effects of fertiliser × crop protection (CP) on mycorrhizal frequency (F%), intensity (M%), (A%), vesicle (V%) and hyphae (H%) abundances in wheat roots at flowering (GS64).

Means ± SE (n = 32)	**Fertiliser**	**Conv. CP**		**Org. CP**	
F%	Biogas digestate	53±3	Ca	50.3±3.47	Ba
	Farmyard manure	58.6±2.8	BCa	55.1±3.19	Ba
	Mineral N	62.9±2.86	ABa	47.8±2.83	Bb
	Zero-input	66.8±2.44	Aa	68.6±3.07	Aa
M%	Biogas digestate	22.6±2.11	Ba	20.8±2.06	Ba
	Farmyard manure	25.6±1.97	ABa	24±1.83	Ba
	Mineral N	27.7±2.36	ABa	19.7±1.68	Bb
	Zero-input	30±1.91	Aa	34.3±2.37	Aa
A%	Biogas digestate	12.6±1.24	Ba	10.9±1.03	Ba
	Farmyard manure	13.9±1.19	ABa	13.2±1.11	Ba
	Mineral N	14.3±1.05	ABa	10.5±0.91	Bb
	Zero-input	16±1.07	Aa	18.2±1.29	Aa
V%	Biogas digestate	1.2±0.29	Aa	1.1±0.36	Ba
	Farmyard manure	1.9±0.4	Aa	1.1±0.23	Bb
	Mineral N	1.3±0.42	Aa	0.7±0.29	Bb
	Zero-input	1.8±0.46	Aa	2.9±0.66	Aa
H%	Biogas digestate	14.4±1.43	Ba	13.8±1.49	Ba
	Farmyard manure	16.6±1.25	ABa	15.6±1.23	Ba
	Mineral N	18.6±1.76	Aa	13.1±1.23	Bb
	Zero-input	19.6±1.39	Aa	22.2±1.69	Aa

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

3.3.5.5 MATURITY

Seasonal variations in mycorrhizal colonisation parameters were still visible at the last assessed time point just prior to harvest showing higher F% and H% in 2018 than 2019 (Table 3.67). At the same time point, wheat with conv. CP showed significantly higher F%, M% and A% than wheat with org. CP. Fertiliser and variety still significantly ($p < 0.001$) affected AMF colonisation patterns with the highest colonisation rates in zero-input plots and in Skyfall respectively.

Table 3.67. Effects of season, crop protection, variety, AMF inoculum and fertiliser on mycorrhizal colonisation parameters (F = frequency, M = intensity, A, V and H = arbuscule, vesicle and hyphae abundances) at maturity (GS90). ANOVA *p*-values in bold indicate significant main effects and interactions. Means within columns followed by the same letter are not significantly different at $p \leq 0.05$.

	Maturity (GS90)				
	F%	**M%**	**A%**	**V%**	**H%**
Year (YR)					
2018 (n=127)	50.3±1.84a	23.1±1.23a	9.5±0.64	1.2±0.16	17.1±0.88a
2019 (n=128)	39.2±1.93b	16.7±1.01b	8.2±0.51	1±0.16	11.4±0.72b
Crop protection (CP)					
Conventional (n=128)	48.1±1.95a	21.9±1.14a	9.9±0.57a	1.3±0.18	15.6±0.83
Organic (n=127)	41.3±1.91b	17.8±1.15b	7.8±0.57b	1±0.14	12.8±0.83
Variety (VR)					
Aszita (n=128)	37±1.82b	15±0.96b	6.8±0.48b	0.4±0.08b	10.8±0.71b
Skyfall (n=127)	52.5±1.84a	24.7±1.19a	11±0.61a	1.9±0.19a	17.6±0.86a
Inoculation (AMF)					
-AMF (n=128)	44.5±2.07	20.3±1.23	8.9±0.56	1.2±0.16	14.7±0.92
+AMF (n=127)	44.9±1.83	19.3±1.09	8.9±0.6	1.1±0.16	13.7±0.75
Fertiliser (FT)					
Biogas digestate (n=64)	42±2.67b	17.9±1.51b	7.6±0.73b	1±0.17b	13.1±1.13b
Farmyard manure (n=63)	48.9±2.57ab	23±1.6b	10.4±0.82a	1.5±0.26a	16.3±1.17b
Mineral N (n=64)	35.1±2.69b	13.5±1.29c	5.8±0.57b	0.6±0.15b	9.8±0.99c
Zero-input (n=64)	52.9±2.61a	25.1±1.74a	11.7±0.91a	1.4±0.29ab	17.7±1.23a
ANOVA *p-values*					
Main effects					
YR	**0.026**	ns	ns	ns	**0.022**
CP	**0.045**	**0.043**	**0.046**	ns	ns
VR	**≤0.001**	**≤0.001**	**≤0.001**	**≤0.001**	**≤0.001**
AMF	ns	ns	ns	ns	ns
FT	**≤0.001**	**≤0.001**	**≤0.001**	**0.006**	**≤0.001**
Interactions					
YR:CP	ns	ns	ns	ns	ns
YR:VR	ns	ns	ns	ns	ns
CP:VR	ns	ns	ns	ns	ns
YR:AMF	ns	ns	ns	ns	ns
CP:AMF	ns	ns	ns	ns	ns
VR:AMF	ns	ns	ns	ns	ns
YR:FT	ns	**0.032**	**0.028**	**0.037**	ns

CP:FT	**0.008**	ns	ns	ns	ns
VR:FT	ns	ns	ns	ns	ns
AMF:FT	ns	ns	**0.017**	ns	ns
YR:CP:VR	ns	ns	ns	ns	ns
YR:CP:AMF	ns	ns	ns	ns	ns
YR:VR:AMF	ns	ns	ns	ns	ns
CP:VR:AMF	ns	ns	ns	ns	ns
YR:CP:FT	ns	ns	ns	**0.025**	ns
YR:VR:FT	ns	ns	ns	ns	ns
CP:VR:FT	ns	ns	ns	ns	ns
YR:AMF:FT	ns	ns	ns	ns	ns
CP:AMF:FT	ns	ns	ns	ns	ns
VR:AMF:FT	ns	ns	ns	ns	ns
YR:CP:VR:AMF	ns	ns	ns	ns	ns
YR:CP:VR:FT	ns	ns	ns	ns	ns
YR:CP:AMF:FT	ns	ns	ns	ns	ns
YR:VR:AMF:FT	ns	ns	ns	ns	ns
CP:VR:AMF:FT	ns	ns	ns	ns	ns
YR:CP:VR:AMF:FT	ns	ns	ns	ns	ns

The previously described interaction of season × fertiliser application was still visible at GS90 where it affected M%, A% and V% (Table 3.68). In 2018, M% was highest in zero-input plots and with FYM application and lowest in the mineral N treatment. In 2019, mineral N and biogas digestate showed similar reduction of M% compared to higher levels in FYM and zero-input plots. Mycorrhizal intensities (M%) were overall lower in 2019 than in 2018 except for the mineral N treatment where similar levels were scored in both seasons.

Arbuscule abundances (A%) were lower in zero-input plots in 2019 when compared to 2018, whereas all fertiliser treatments achieved similar A% levels in both seasons at this growth stage. In 2018, highest A% was scored for zero-input and with FYM application while lowest levels were found with mineral N. In 2019, A% was decreased in response to biogas digestate and mineral N treatments, whereas A% levels in plots with FYM application were lower but not significantly different to zero-input plots. Vesicle abundances (V%) varied between fertiliser treatments in 2018 with higher levels in FYM and zero-input compared to the mineral N and biogas digestate treatments. In 2019 however, there was no significant difference of V% between the fertiliser treatments and zero-input plots.

Table 3.68. Effect of fertiliser × season on mycorrhizal intensity (M%), arbuscule (A%) and vesicle (V%) abundances in wheat roots at maturity (GS90)

Means ± SE (n = 32)	Fertiliser	2018		2019	
M%	Biogas digestate	20.4±2.03	Ba	15.3±2.19	ABb
	Farmyard manure	27.9±2.14	Aa	18.3±2.09	Ab
	Mineral N	13.9±2.09	Ca	13.1±1.55	Ba
	Zero-input	30.3±2.52	Aa	20±2.08	Ab
A%	Biogas digestate	7.9±1.06	Ba	7.3±1.03	Ba
	Farmyard manure	12±1.2	Aa	8.9±1.06	ABa
	Mineral N	5±0.81	Ca	6.6±0.8	Ba
	Zero-input	13.3±1.42	Aa	10.1±1.1	Ab
V%	Biogas digestate	0.8±0.16	Ba	1.3±0.29	Aa
	Farmyard manure	2±0.45	Aa	1.1±0.25	Aa
	Mineral N	0.5±0.15	Ba	0.7±0.26	Aa
	Zero-input	1.7±0.36	Aa	1.1±0.45	Aa

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

At GS90, fertiliser × crop protection showed a significant interaction that affected F% ($p = 0.008$, Table 3.67). With conv. CP, F% was highest without fertiliser application, whereas with org. CP similar levels were reached with the FYM treatment (Table 3.69). A significant difference was only found for mineral N application where root systems with org. CP showed lower F% than wheat with conv. CP.

Table 3.69. Effect of fertiliser × crop protection (CP) on mycorrhizal frequency (F%) in wheat roots at maturity (GS90)

Means ± SE (n = 32)	Fertiliser	Conv. CP		Org. CP	
F%	Biogas digestate	43.4±4.2	Ba	40.5±3.35	Ba
	Farmyard manure	48±3.68	Ba	49.9±3.65	Aa
	Mineral N	43.8±3.74	Ba	26.7±3.28	Cb
	Zero-input	57.1±3.59	Aa	48.6±3.68	ABa

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

The AMF inoculum × fertiliser interaction had a significant impact on A% at maturity ($p = 0.017$, Table 3.67). Inoculated plots with FYM showed higher A% than wheat without AMF application (Table 3.70). In wheat without AMF inoculum, lowest A% were found with mineral N fertilisation. With AMF application, mineral N and biogas digestate reduced A% compared to the FYM and zero-input treatment.

Table 3.70. Effect of fertiliser × AMF inoculation on arbuscule abundances (A%) in wheat roots at maturity (GS90)

Means ± SE (n = 32)	Fertiliser	-AMF		+AMF	
A%	Biogas digestate	8.6±1.24	Ba	6.5±0.76	Ba
	Farmyard manure	8.9±0.83	Bb	12.1±1.38	Aa
	Mineral N	5.8±0.87	Ca	5.9±0.76	Ba
	Zero-input	12.3±1.19	Aa	11.1±1.37	Aa

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

3.3.6 GRAIN QUALITY

Specific weight and Hagberg falling number were higher in 2018 than 2019 (Table 3.71). Grain from wheat with conv. CP showed lower protein content compared to org. CP ($p = 0.029$), but conv. CP increased both specific weight and Hagberg falling number. All measured grain quality parameters were higher in Aszita than Skyfall. Fertiliser treatment had a significant impact on all measured grain quality parameters ($p < 0.001$). Highest grain protein content and specific weight were observed following mineral N application, but falling number was highest with biogas digestate application. Lowest protein content and specific weights were measured in grain from the FYM and zero-input treatment.

Table 3.71. Effects of season, crop protection, variety, AMF inoculation and fertiliser on protein content, specific weight and Hagberg falling number (HFN) of grain. ANOVA *p*-values in bold indicate significant main effects and interactions. Means within columns followed by the same letter are not significantly different at $p \leq 0.05$.

	Protein content [%]	Specific weight [kg hl^{-1}]	HFN [s]
Year (YR)			
2018 (n=127)	10.9±0.15	75.6±0.26a	290.4±4.81a
2019 (n=128)	11.2±0.17	72.5±0.39b	191.8±7.29b
Crop protection (CP)			
Conventional (n=128)	10.8±0.16b	74.9±0.29a	254.6±6.97a
Organic (n=127)	11.2±0.16a	73.2±0.41b	226.8±7.99b
Variety (VR)			
Aszita (n=128)	12.2±0.13a	77.3±0.15a	298.2±4.81a
Skyfall (n=127)	9.9±0.12b	70.9±0.28b	182.2±6.25b
Inoculation (AMF)			
-AMF (n=128)	10.9±0.16	73.9±0.36	238.3±7.36
+AMF (n=127)	11.1±0.17	74.3±0.36	243.1±7.82
Fertiliser (FT)			
Biogas digestate (n=64)	11.2±0.21b	74.7±0.48b	255.4±11.09a
Farmyard manure (n=63)	10±0.16c	73.7±0.49c	236.5±10.67b
Mineral N (n=64)	12.8±0.2a	74.3±0.57a	233.2±11.3b
Zero-input (n=64)	10.1±0.16c	73.6±0.51c	237.4±9.81b

ANOVA *p-values*			
Main effects			
YR	ns	**0.002**	**≤0.001**
CP	**0.029**	**0.002**	**0.002**
VR	**≤0.001**	**≤0.001**	**≤0.001**
AMF	ns	ns	ns
FT	**≤0.001**	**≤0.001**	**≤0.001**
Interactions			
YR:CP	ns	**0.010**	**0.002**
YR:VR	ns	**0.024**	**≤0.001**
CP:VR	ns	ns	ns
YR:AMF	ns	ns	ns
CP:AMF	ns	ns	ns
VR:AMF	ns	ns	ns
YR:FT	**≤0.001**	ns	ns
CP:FT	ns	**≤0.001**	**0.037**
VR:FT	**0.038**	ns	ns
AMF:FT	ns	ns	ns
YR:CP:VR	ns	ns	ns
YR:CP:AMF	ns	ns	ns
YR:VR:AMF	ns	ns	ns
CP:VR:AMF	ns	ns	ns
YR:CP:FT	ns	ns	ns
YR:VR:FT	ns	ns	**0.005**
CP:VR:FT	ns	ns	ns
YR:AMF:FT	ns	ns	ns
CP:AMF:FT	ns	ns	ns
VR:AMF:FT	ns	ns	ns
YR:CP:VR:AMF	ns	ns	ns
YR:CP:VR:FT	ns	ns	ns
YR:CP:AMF:FT	ns	ns	ns
YR:VR:AMF:FT	ns	ns	ns
CP:VR:AMF:FT	ns	ns	ns
YR:CP:VR:AMF:FT	ns	ns	ns

The season × CP interaction influenced specific weight (p = 0.010, Table 3.71) and Hagberg falling number (p = 0.002). Specific weight was lower in the org. CP treatment in both seasons. In both CP treatments, specific weight was higher in 2018 compared to 2019 (Table 3.72). Falling numbers were higher in 2018 than 2019 independent of CP application. But in 2019, grain from wheat with conv. CP showed higher falling numbers than grain with org. CP.

Table 3.72. Effect of season × crop protection on specific weight and Hagberg falling number (HFN) of grain.

Means ± SE (n = 32)	Season	Conv. CP		Org. CP	
Specific weight [kg hl^{-1}]	2018	75.9±0.35	Aa	75.4±0.39	Ab
	2019	74±0.43	Ba	71.1±0.62	Bb
HFN [s]	2018	289.7±7.68	Aa	291±5.87	Aa
	2019	219.9±9.85	Ba	163.6±9.59	Bb

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

In both varieties, specific weight and falling number were lower in 2019 compared to 2018 (Table 3.73). Both quality traits were also lower in Skyfall than Aszita.

Table 3.73. Effect of season × variety on specific weight and Hagberg falling number (HFN) of grain.

Means ± SE (n = 32)	Season	Aszita		Skyfall	
Specific weight [kg hl^{-1}]	2018	78.4±0.1	Aa	72.8±0.14	Ab
	2019	76.2±0.19	Ba	69±0.42	Bb
HFN [s]	2018	333.9±4.25	Aa	245.5±3.5	Ab
	2019	262.6±5.91	Ba	121±4.5	Bb

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

Cropping season had different effects on grain protein content depending on the applied fertiliser (Table 3.74). In both years, highest protein contents were reached with mineral N, followed by biogas digestate application. Grain with FYM and zero-input treatments showed the lowest protein content. Wheat with biogas digestate application showed lower protein contents in 2019 than 2018, whereas grain protein was significantly higher in 2019 than 2018 in response to mineral N.

Table 3.74. Effect of season × fertiliser on grain protein content.

Means ± SE (n = 32)	Fertiliser	2018		2019	
Protein content [%]	Biogas digestate	11.4±0.3	Ba	11±0.3	Bb
	Farmyard manure	9.9±0.21	Cb	10.2±0.23	Ca
	Mineral N	12.4±0.23	Ab	13.2±0.31	Aa
	Zero-input	9.8±0.2	Cb	10.4±0.23	Ca

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

The fertiliser × crop protection interaction significantly affected grain specific weight (p = 0.001, Table 3.71) and Hagberg falling number (p = 0.037). Highest values for both quality traits were in grain with conv. CP in response to biogas digestate and mineral N application, whereas FYM and zero-input showed similar lower levels (Table 3.75). With org. CP, highest specific weights were found in response to the biogas digestate and FYM treatments, although the latter was not significantly different to lower values in mineral N and zero-input plots. Overall, grain specific weight was higher with conv. CP compared to org. CP. With conv. CP, falling number was highest with biogas digestate and mineral N and lower in grain following FYM application and with zero-input. With org. CP, falling number was lowest in wheat with mineral N application and highest with biogas digestate but the differences were relatively small and all values were <250 s. Overall, conv. CP increased falling number in all fertiliser treatments when compared to org. CP.

Table 3.75. Effect of fertiliser with crop protection (CP) on specific weight and Hagberg falling number (HFN) of grain.

Means ± SE (n = 32)	**Fertiliser**	**Conv. CP**		**Org. CP**	
Specific weight [kg hl^{-1}]	Biogas digestate	75.6±0.55	Aa	73.9±0.76	Ab
	Farmyard manure	74.3±0.59	Ba	73.2±0.79	ABb
	Mineral N	75.7±0.53	Aa	72.8±0.93	Bb
	Zero-input	74.2±0.61	Ba	73.1±0.81	Bb
HFN [s]	Biogas digestate	268.3±14.9	Aa	242.6±16.35	Ab
	Farmyard manure	248.9±13.65	Ba	223.7±16.39	Bb
	Mineral N	255.3±14.42	ABa	211.8±16.66	Cb
	Zero-input	245.8±13.02	Ba	229±14.73	Bb

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

The interaction of fertiliser × variety affected grain protein content significantly (p = 0.038, Table 3.71). Aszita showed an overall higher grain protein content than Skyfall, and in both varieties highest values were found in response to mineral N application (Table 3.76). Lowest values in both varieties were in the FYM and zero-input treatments.

Table 3.76. Effect of fertiliser × variety on grain protein content.

Means ± SE (n = 32)	**Fertiliser**	**Aszita**		**Skyfall**	
Protein content [%]	Biogas digestate	12.5±0.18	Ba	9.9±0.2	Bb
	Farmyard manure	11.1±0.1	Ca	9±0.13	Cb
	Mineral N	14±0.17	Aa	11.5±0.17	Ab
	Zero-input	11.1±0.1	Ca	9.1±0.13	Cb

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

3.4 DISCUSSION

The association of arbuscular mycorrhizal fungi (AMF) with wheat has revealed contradictory results with regards to growth response and economic benefit (Ryan & Graham, 2018). The field trials at Nafferton Farm could not assess the latter aspect as the applied mycorrhizal inoculum did not increase grain yield or quality. The two seasons showed differing responses of AMF to agronomic management practices for which reason a third season is required to corroborate the results of this study. The greatest difference was the responses of AMF root colonisation (AMF-RC, including mycorrhizal intensities and AMF-colonisation parameters such arbuscules and hyphae) to mineral N fertiliser where AMF-RC was drastically reduced in the first year, but had similar colonisation levels to the zero-fertiliser input treatment which showed highest AMF-RC levels in both years. Compared to zero-input plots, organic fertiliser in the form of farmyard manure (FYM) and biogas digestate reduced AMF-RC in both years. Consistent was also the effect of variety that showed higher AMF-RC in the modern variety Skyfall than Aszita. These two varieties were also contrasting regarding disease susceptibility, yield and grain quality. The application of conventional crop protection (conv. CP) prolonged AMF-RC by maintaining a higher SPAD later in the season likely as a result of extended leaf area duration. The organic crop protection (org. CP) treatment showed high disease levels following mineral N application which lowered AMF-RC for that treatment.

3.4.1 EFFECT OF SEASONAL VARIATIONS ON WHEAT PERFORMANCE

The results of the present study are based on two seasons of field trials with very contrasting weather conditions (Appendix A, Fig. A.1) such that almost all assessed parameters show significant season × treatment interactions. The first field trial (2018) was characterised by low precipitation rates especially during the production phase (April-June), whereas high precipitation in 2019 during the same phase increased disease and weed pressure (visual observation but no data collected). Also agronomic management was varied between the two trial seasons for example regarding seed rate which was with 550 seeds m^{-2} much higher in 2019 than the 400 seeds m^{-2} used in 2018 (Table 3.3). Even though plant numbers of Aszita were higher at the end of the season 2019 (Table 3.16), it is not expected that the altered seed rate of Aszita affected mycorrhizal parameters.

Vegetative growth largely depends on moisture which explains the increases in shoot height in 2019 where precipitation rates were higher than in 2018 especially during the stem elongation phase in May (Appendix A, Fig. A.1 A). Despite higher water supply in 2019, total biomass was the same in both field trial seasons whereas grain yields (Table 3.7) were much higher after

the dry summer of 2018. This contrast indicates a trade-off between grain production and shoot growth in 2019 which was confirmed by lower harvest indices. As the ratio of harvested grain to harvested total biomass (M. R. Smith *et al.*, 2018), the harvest index is most of all determined by variety and as expected was lower in the tall low-yielding variety Aszita compared to the semi-dwarf, high-yielding variety Skyfall. Both varieties achieved highest yields with mineral N or biogas digestate, followed by FYM and lowest yields without fertiliser. Despite slightly higher grain yields, parameters such as SPAD (Table 3.32) and grain protein content (Table 3.71) in plots with FYM application were similar to those measured in plots without fertiliser. This indicates that FYM and zero-input plots represented N-limited systems in the present study since N is often the limiting nutrient that determines yield and protein content in low-protein crops like wheat (Debaeke *et al.*, 2006; Büchi *et al.*, 2016). Nutrient concentrations of shoot parts (N & P) that are required to confirm this hypothesis were measured (Section 3.2.13) but could not be added to the results of this study due to time limitations. In contrast to FYM, the other organic fertiliser used in this study in the form of biogas digestate stood out with similar yield levels and higher harvest index than the mineral N treatment (Table 3.7). The same effect was found in other studies that compared digestates with mineral N-fertilisers (Andruschkewitsch *et al.*, 2013; Caruso *et al.*, 2018; Ren *et al.*, 2020), but only for applications of the liquid digestate fraction which can contain 45-80% of the NH_4^+-N (Möller & Müller, 2012). Although the application of biogas digestate has been linked with decreased soil microbial biomass (Andruschkewitsch *et al.*, 2013; Wentzel *et al.*, 2015), biogas digestate as a biproduct of bioenergy production represents an increasing opportunity for sustainable cropping systems (Caruso *et al.*, 2018; Magistrali *et al.*, 2020).

Higher precipitation in the spring and summer of 2019 enhanced disease development (Table 3.44) which is likely to have been a key factor contributing to the lower yields and grain quality (Table 3.71). Skyfall showed significantly higher infection with yellow rust than Aszita in both years (Table 3.44) with highest levels in plots treated with mineral N (Table 3.48). Although this effect requires confirmation from N-content analyses of shoots, increased disease symptoms with mineral N treatment could be explained by higher N-uptake of Skyfall in comparison to Aszita which was indicated in SPAD-readings (Table 3.32). Increased N-availability in leaves favours spread and development of the biotrophic pathogen *Puccinia striiformis* f. sp. *tritici* (Neumann *et al.*, 2004). Consequently, grain yield (Table 3.11) as well as thousand grain weight were decreased with mineral N application and in the absence of pesticides (Table 3.18) where yellow rust incidences were highest (Table 3.49). This marks a potential advantage of biogas digestate over mineral N which is that wheat with biogas digestate

showed lower disease levels than mineral N-treated plants while achieving the same yield levels (Magistrali *et al.,* 2020). Aszita on the other hand showed higher symptom development of *Septoria tritici* blotch than Skyfall in 2019. In contrast to 2018, the spread of *Septoria tritici* blotch in 2019 was likely facilitated by higher precipitation (Appendix A, Fig. A.1 A) that favoured splash-dispersal of the pathogen *Zymoseptoria tritici* (Torriani *et al.,* 2015). Another factor that could have aided to build up disease pressure of STB was that 2019 had wheat as the previous crop (Kirkegaard *et al.,* 2008), whereas the first season was drilled after a grass-clover ley. Since *Septoria tritici* blotch is a major threat in wheat production (Fones & Gurr, 2015), increasing focus on breeding for *Septoria tritici* blotch -resistance in modern varieties might explain the lower *Septoria tritici* blotch-disease in Skyfall compared to Aszita which was released ten years earlier than Skyfall (Table 3.4) and hence could have lost its resistance to *Septoria tritici* blotch (Brown *et al.*, 2015).

3.4.2 MYCORRHIZAL DEVELOPMENT

Most field-based studies on wheat report 20-40 % AMF-RC (Friedel *et al.,* 2008; Hildermann *et al.*, 2010; Castillo *et al.*, 2012; Bakhshandeh *et al.*, 2017; Ercoli *et al.,* 2017; Tian *et al.*, 2019) which is in line with the 20-30 % mycorrhizal intensities (M%) observed during the field trials at Nafferton Farm (Fig. 3.3). Mycorrhizal inoculum potential of the soil at Nafferton Farm must have been high considering the early colonisation of wheat seedlings six weeks after drilling and under low light and temperatures in both seasons. Due to these conditions, field trials with winter wheat in the US found no AMF-RC until late in the growing season (Hetrick & Bloom, 1983; Al-Karaki *et al.*, 2004). On the other hand, Ryan *et al.* (1994) report colonisation rates ~ 30 % already 4 weeks after drilling on an organic farm following pasture in Southern Australia. In their study, wheat sampled from an adjacent conventionally manged field was not colonised at this time point. Hence, the low soil P-content and also the preceding years of grass-clover lay and organic crop management most likely favoured the establishment of a rich mycorrhizal community in the soil at Nafferton Farm. In contrast to findings of other studies (Ryan & Angus, 2003; Sommermann *et al.*, 2018), there was no impact of preceding crop on AMF-RC at the seedling stage between the two seasons (Table 3.43).

Minor increases in AMF-RC were found between seedling growth (GS12) and tillering (GS22) in both years which corroborates results of Al-Karaki *et al.* (2004) and Hetrick *et al.* (1984) who found no AMF-development in wheat roots during winter months at similar temperatures. After tillering, peak colonisation was achieved earlier in 2019 than 2018 independent of agronomic management or variety (Fig. 3.3). This steep increase of AMF-RC in wheat roots

between GS22 and GS32 in 2019 may be linked to higher precipitation and soil temperatures after a mild winter (Appendix A, Fig. A.1). These conditions favoured plant and root growth which resulted in higher shoot biomass at stem elongation compared to lower values in the 2018 season (Table 3.60). More than half of total biomass of a wheat crop occurs during the construction phase i.e. GS32-G64 (AHDB, 2018) for which reason stem elongation marks the onset of enhanced nutrient and water demand. Assuming that AMF-association is actively regulated by both symbiotic partners (Kiers *et al.*, 2011), enhanced growth and nutrient demand increases AMF-RC mostly in the form of A% (Mandyam & Jumpponen 2008; Hazard *et al.*, 2014). In the present study, A% and H% were equally contributing to AMF-RC at all growth stages for which reason it cannot be deduced from A% values that nutrient transfer between fungus and wheat was increased at this growth stage. Monitoring of AMF-RC assessments throughout the growing season in *Lolium perenne* and *Trifolium repens* showed highest AMF-RC when plant growth rates increased (June) and the temporal dynamics were similar in both years (Hazard *et al.*, 2014). Also in wheat, field trials report highest colonisation at heading stage (Al-Karaki *et al.*, 2004; Smith *et al.*, 2015). The present findings indicate that peak AMF-RC in wheat is shaped by root growth in response to higher precipitation and warmer temperatures.

Overall, the assessment of AMF-RC at multiple growth stages of wheat seems crucial to understand AMF development in response to environmental conditions and agronomic management. Most field-based studies on the interaction of AMF and wheat assess AMF-RC once or twice around tillering and flowering (Hildermann *et al.*, 2010; Dai *et al.*, 2014; Verzeaux *et al.*, 2016; Bakhshandeh *et al.*, 2017), but the assessment of a minimum of three time points has been recommended (Ryan & Graham, 2018). With five assessed growth stages, this study belongs to the more extensive evaluations of AMF-RC in wheat in the field.

3.4.3 VARIETY-DEPENDENT MYCORRHIZAL ROOT COLONISATION

Host genotype-dependent variations in mycorrhizal colonisation of wheat have been widely reported in greenhouse experiments (Azcón & Ocampo, 1981; Hetrick *et al.*, 1992; Singh *et al.*, 2012; Lehnert *et al.*, 2017), but are not always pronounced under field conditions (Ryan *et al.*, 1994; Friedel *et al.*, 2008; Hildermann *et al.*, 2010; Mao *et al.*, 2014; Bakhshandeh *et al.*, 2017; Ercoli *et al.*, 2017). For this reason, it was fortunate to observe constantly higher AMF-RC in Skyfall compared to Aszita since the two varieties had not been screened previously with respect to AMF-RC. However, both varieties have been used in other studies: Skyfall acquired more P through AMF in comparison to two other modern wheat varieties and showed an intermediate root length colonisation of 34% in a study by

Elliott *et al.* (2019). Another study with the same compilation of varieties identified Skyfall as particularly dependent on mycorrhiza-mediated nutrient uptake (Thirkell *et al.*, 2020). Aszita on the other hand was revealed as one of the least colonised among 94 different wheat varieties in a greenhouse screening by Lehnert *et al.* (2018). While Elliott *et al.* (2019) concluded that breeding has weakened the response of modern wheat varieties to AMF (Hetrick *et al.*, 1992), Lehnert *et al.* (2018) could not link year of release of the screened varieties with AMF-RC. Their findings are in line with a meta-analysis by Lehmann *et al.* (2012) and support the detection of higher AMF-RC in the modern wheat variety in this study. The reason for higher AMF-RC in Skyfall however cannot be clarified solely based on AMF-RC data and would require more detailed comparison of Skyfall and Aszita regarding root architecture, root length and volume, exudates etc (Azcón & Ocampo, 1981; Hetrick *et al.*, 1992, 1993; Lucini *et al.*, 2019) as well as gene expression patterns (Sawers *et al.*, 2008) in different nutrient environments (Janos, 2007; Ryan & Graham, 2018). Nutrient use efficiency has been highlighted among the different drivers that determine AMF-associations with wheat (Yao *et al.*, 2001; P. Campos *et al.*, 2018), but there has not been a clear link to genotype-dependent AMF-associations. Koide (1991) hypothesised that modern wheat varieties might be more likely to associate with AMF as they require larger amounts of nutrients to produce high grain yields with appropriate protein contents. It was also suggested that shorter stems favour AMF-responsiveness as tall growing varieties allocate more C to shoot growth (Friedel *et al.*, 2008; Martín-Robles *et al.*, 2018). This would explain why Skyfall showed higher colonisation levels than Aszita, but it should be noted that AMF-RC does not necessarily correlate with effectiveness of mycorrhizal symbiosis (Corkidi *et al.*, 2002; Smith *et al.*, 2015; Leiser *et al.*, 2016; Hohmann & Messmer, 2017; Sawers *et al.*, 2017). In fact, empirical evaluation of mycorrhizal studies has shown little evidence for the use of AMF-RC as measure for AMF effectiveness (Treseder, 2013). More meaningful for the assessment of AMF efficiency of Skyfall and Aszita would be the mycorrhizal growth response (MGR, Hetrick *et al.*, 1992) or mycorrhizal responsiveness that describe the cost-benefit in plant performance as the ratio of colonised and non-colonised plants (Janos, 2007). Furthermore, the assessment of mycorrhizal mediated nutrient uptake have been proven as suitable tools for the evaluation of wheat varieties regarding mycorrhizal efficiency (Elliott *et al.*, 2019; Thirkell *et al.*, 2020). The implementation of both measures under field conditions is not straightforward as they require non-mycorrhizal controls and the potential addition of radio-labelled isotopes (Kahiluoto *et al.*, 2000; Ryan *et al.*, 2005; Smith *et al.*, 2015). While the first has been described as nearly impossible under field conditions (Smith & Smith, 2011), the latter can be of particular importance as nutrient concentrations in shoot parts do not always reflect

nutrients taken up through the mycorrhizal pathway (Smith *et al.*, 2015; Thirkell *et al.*, 2020). Combining the assessment of mycorrhizal mediated nutrient uptake with 'molecular phenotyping' for varietal comparison can be superior to the comparison with non-mycorrhizal plants since the performance of plants without AMF is based on different pathways than those harbouring AMF (Sawers *et al.*, 2008; Thirkell *et al.*, 2019; Lehnert *et al*., 2018). Finally, the extra-radical mycelium has been identified as another parameter that can vary between crop varieties and that can affect the efficiency of mycorrhizal-mediated uptake of nutrients (Sawers *et al.*, 2017). To measure the extent of hyphal networks in field trials requires hyphal traps, a method which was efficiently used in previous studies on wheat (Dai *et al.*, 2014) and which has been encouraged to complement AMF-RC assessments in mycorrhizal research (Ryan & Graham, 2018).

3.4.4 IMPACT OF FERTILISER SOURCE ON MYCORRHIZAL ROOT COLONISATION

Strong seasonal variations were detected for AMF-RC in response to fertiliser treatments (Fig. 3.3 B). The probably most intriguing finding in this regard is the ambivalent effect of mineral N in both field trials: in 2018, mineral N application prior to sampling at stem elongation (GS32) reduced AMF-RC to the lowest levels of all the fertiliser treatments (Table 3.62). In 2019, wheat without fertiliser and with mineral N fertiliser showed highest colonisation levels through to maturity (GS90) where AMF-RC was lower with mineral N compared to the control treatment (Table 3.68). Although there have been strong variations among studies, Treseder (2004) showed an overall negative effect of all N-fertilisers on AMF. Also Ercoli *et al.* (2017) found lowest AMF-RC in durum wheat, but only with maximum fertiliser rates of 40 kg N ha^{-1} while two or less applications showed no effect on AMF-RC. Their findings imply that AMF tolerate N up to a certain threshold which might be applicable to results of the present study. Here, this threshold was not defined by fertiliser application as the same rate was applied in both years (170 kg N ha^{-1}). Hence, the detrimental effect of mineral N on AMF in 2018 might be due to variations in soil-nitrogen supply between the two seasons. In the present study, N-availability in soils at the beginning of spring prior to mineral N application was higher in 2018 than in 2019 (Table 3.2). The first field trial was drilled after two years of grass-clover ley, whereas the second field trial was a 2nd wheat crop i.e. following winter wheat (Table 3.3). Pre-cropping with grass-clover enriches soil N (Mäder *et al.*, 2007) which can affect AMF in the following crop (Johnson *et al.*, 2015). On the other hand, wheat diverts a lot of its N to the grain which is removed from the field leaving a low N residue after harvest (Swain *et al.*, 2014). This was evident in a comparison of soil samples collected in early

spring 2019 that showed lower N-availability (especially of nitrate) in topsoil layers (0-30 cm) from plots with Skyfall compared to Aszita (Appendix A, Table A.1). Higher N-uptake in modern wheat varieties was also demonstrated in varietal screenings of wheat across different agronomic systems (Hildermann *et al.*, 2010; Büchi *et al.*, 2016). Lower N-concentrations in spring 2019 might have favoured AMF-RC whereas N-fertiliser additions in the spring of 2018 after grass-clover may have exceeded the N threshold of AMF. Further soil analysis of samples from spring 2018 would be required to prove this theory, but only samples from trials in 2019 were analysed with respect to treatment effects.

The presumed N-threshold that was created in mineral N-plots does not explain why biogas digestate and FYM decreased AMF-RC in both years since organic fertilisers were applied at similar N-rates as mineral N. Due to several reports of no or positive impact of organic fertilisers on AMF (Mäder *et al.*, 2011; Hazard *et al.*, 2014; Qin *et al.*, 2015; Aguilar *et al.*, 2017; Kokkoris *et al.*, 2019a), it was unexpected to observe reduced AMF-RC in response to FYM (Fig. 3.3 B). Main effects indicate a reduction of AMF-RC in response to FYM during early seedling growth (GS12), but the interaction of season × fertiliser showed lower M% and A% only in 2019 where FYM had been applied for two consecutive years (Table 3.53).

In contrast to studies on manure and AMF, there is a limited number of investigations on the effect of biogas digestate on AMF. Ren *et al.* (2020) found no impact of biogas digestate on AMF-RC in ryegrass roots and report even increases in hyphal length. More details can be derived from a field trial with AMF-inoculation of Triticale that differentiated between liquid and solid digestate fractions besides mineral NPK fertiliser (Caruso *et al.*, 2018). Although AMF-RC in that study was only found at late growth stages (hard dough), AMF development was the same in plots without fertiliser, with NPK and with the liquid biogas digestate fraction. With solid biogas digestate however, AMF intensities (M%) and frequencies (F%) were increased (Caruso *et al.*, 2018). The authors explain these AMF-responses with the low amount of available P in the solid biogas digestate fraction compared to the liquid fraction. Also Wentzel & Joergensen (2016) linked the reduction of AMF-RC in ryegrass to increases of plant available P after the application of biogas slurry. Although fractions of biogas digestate applied during field trials at Nafferton were not separated, the addition of P in both organic fertilisers may have been responsible for the reduction of AMF-RC in wheat roots. This effect was more pronounced with biogas digestate compared to FYM as the latter contained less available P (Appendix A, Table A.2). Mutualistic functioning of AMF-symbiosis and increased fungal biomass has been assumed for N-addition to low-P soils, whereas in soils with high P-content, N-addition can have the opposite effect (Johnson *et al.*, 2015; Azcón *et al.*, 1992;

Püschel *et al.,* 2016). While the addition of N in form of mineral N fertiliser increased the N:P ratio of the soil at Nafferton Farm, addition of P in form of organic fertilisers lowered the N:P ratio leading to less mutualistic AMF symbiosis for which reason AMF-RC decreased.

3.4.5 FINE ROOT ENDOPHYTES

There could be another explanation why N-fertiliser did not affect AMF-RC in 2019 which is that it was not AMF that dominated root colonisation, but another group of fungi that can play a role in N-acquisition. Many of the recorded root colonisation patterns observed by microscopy were similar to those of fine root endophytes (FRE, data not shown). The presence of these fungi in root samples would explain why vesicle abundances (V%) were low throughout the cropping seasons as FRE do not form vesicles, but hyphal swellings that were observed in addition to the characteristic fan-shaped arbuscules and finely branched hyphae (Gianinazzi-Pearson *et al.,* 1981; Orchard *et al.,* 2017b). After recognising these colonisation structures of FRE in root samples from the field season 2018, it was decided not to differentiate between AMF and FRE due to the phylogenetic assignment of *Glomus tenue* to Glomeromycotina at that time. Considering the new phylogenetic assignment of *Glomus tenue* to the subphylum of Mucoromycotina at the end of 2018 (Orchard *et al.*, 2017a; Walker *et al.*, 2018), reassessment of the root samples from both field seasons would be required to define the extent of FRE-colonisation compared to AMF. As a compromise, it was decided to validate the presence of FRE based on molecular evidence which will be further discussed in Chapter 5.

There are several reports on the association of FRE with wheat (Hetrick *et al.*, 1984; Ryan *et al.,* 2005; Smith *et al.*, 2015; Sinanaj *et al.,* 2020), but there is little information on the impact of agronomic management practices on these fungi. Recently, Albornoz *et al.* (2021) identified different environmental factors that characterise distinct ecological niches of FRE and AMF. They did not test the effect of N, but showed that like AMF, FRE are negatively affected by P which would confirm the reduction of fungal root colonisation in wheat treated with both biogas digestate and FYM (Section 3.4.4). Earlier studies by Sigüenza *et al.* (2006) had shown the resistance of FRE to N-accumulation in grass species whereby AMF-RC decreased. These findings could explain why fungal root colonisation was not decreased in response to mineral N in 2019, but why this was not the case in the previous season remains to be clarified. One explanation could be the higher precipitation rates in spring 2019 as FRE have been found to respond positively to waterlogging (Orchard *et al.,* 2016) and rainfall (Albornoz *et al.,* 2021). It cannot be excluded that precipitation events around the time of the application of mineral N in 2019 caused potential leaching of mineral N leading to less AMF-inhibition than in the previous season. But it is not likely that this was the case, since mineral

N was taken up by the crop effectively in both years as clearly confirmed by SPAD, crop performance and yield data. Overall, the finding of contrasting responses of AMF-RC to mineral N and a potential link to FRE is intriguing and requires further research in particular for this group of fungi which might have a role to play towards increasing the efficiency of nutrient acquisition in agricultural systems (Sinanaj *et al.*, 2020).

3.4.6 CROP PROTECTION EFFECTS ON MYCORRHIZAL ROOT COLONISATION

In contrast to reports about negative effects of pesticides on AMF in wheat production (Riedo *et al.*, 2021), F%, M% and A% were higher with conv. CP compared to non-sprayed wheat at the end of the growing season (Table 3.67). Depending on the mode of action, fungicides can have suppressive or stimulating effects on AMF-RC which was mostly demonstrated for seed-applied compounds (Jin *et al.*, 2013; Caruso *et al.*, 2018). But since foliar sprays were used for conv. CP in this study, direct impact of the applied chemicals on AMF are unlikely (Hage-Ahmed *et al.*, 2019). Hence, the maintenance of AMF symbiosis in form of higher AMF-RC in conv. CP plots compared to wheat with org. CP at maturity must be linked to improved crop health as a result of CP application (Diedhiou *et al.*, 2004) and is likely due to the prolonged green canopy of Skyfall. Although not always pronounced in different environments, increased leaf area duration post-anthesis can contribute to N-translocation during grain filling which results in increased protein content and grain yield (Bogard *et al.*, 2011; Simmonds *et al.*, 2014). The leaf area duration was not directly measured in this study, but an additional SPAD and NDVI assessment was conducted in 2018 in plots with conv. CP where flag leaves were still photosynthetically active in contrast to the senesced leaves in plots with org. CP. This assessment showed significantly higher SPAD and NDVI in Skyfall compared to Aszita (Appendix A, Table A.3) which would explain the significant difference of AMF-RC in the two varieties at GS90 in that year (Fig. 3.3 C). As AMF have been shown to stimulate N-uptake and photosynthesis in flag leaves of durum wheat and barley (Gernns *et al.*, 2001; Garmendia *et al.*, 2017), prolonged greeness in combination with prolonged AMF association could contribute to improved yield.

The differences in plant performance in response to org. and conv. CP were more pronounced in 2019 because disease and weed pressure were higher compared to the dry season in 2018 (Table 3.44). This higher stress level in plants with org. CP was clearly visible in an interaction of season × CP at stem elongation which resulted in decreased AMF-RC with org. CP in 2019 while no difference was found in 2018 (Table 3.61). At flowering however, the interaction of

fertiliser × CP affected all AMF-RC parameters (Table 3.65) suggesting that this effect occurred in both trial seasons. Post-hoc testing revealed that conv. CP only enhanced AMF-RC in combination with mineral N whereas all other nutrient regimes showed the same mycorrhization independent of chemical inputs. This interaction indicates that the significant increases of disease levels as a consequence of mineral N application reduced mycorrhizal development in wheat most likely because of reduced C-sink capacities due to less photosynthetic leaf area (Gernns *et al.*, 2001).

Arbuscular mycorrhizal fungi have been shown to enhance systemic defence reactions in the case of a pathogen or pest attack by inducing the inherent immune system of plants (Jung *et al.*, 2012). Besides root diseases where AMF can also have direct impact on pathogens (Pozo & Azcón-Aguilar, 2007), the interaction of biotrophic foliar pathogens and AMF have been investigated because both fungal groups rely on a living host and are affected by nutrient availability (Panstruga & Kuhn, 2019). A greenhouse experiment by Gernns *et al.* (2001) showed enhanced sporulation of the powdery mildew causing pathogen *Blumeria graminis* f. sp. *hordeii* in barley inoculated with AMF. But in contrast to diseased non-mycorrhizal controls, pathogen infection reduced neither yield nor grain quality in mycorrhizal plants (Gernns *et al.*, 2001). From their findings, the authors conclude that AMF in agricultural soils might be important to stabilise yields under pathogen attack. The results from the field trials at Nafferton suggest that under high disease pressure following high N-availability, the preservation of AMF symbiosis might be too costly for the plant for which reason AMF-RC is reduced. Nutrient availability as a driving factor for disease susceptibility was also found by Mustafa *et al.* (2016) who investigated the impact of AMF-inoculation on powdery mildew in wheat (*Blumeria graminis* f. sp. *tritici*). They showed that with reduced application of P-fertiliser, AMF-inoculation could significantly reduce haustoria formation of the pathogen, whereas higher P-supply reduced AMF-RC. Based on their findings, it cannot be concluded if the reduction of AMF-RC occurred in response to P-addition or increased disease infection.

3.4.7 EFFECTS OF AMF INOCULUM ON WHEAT PERFORMANCE

The application of AMF inoculum at the beginning of each field trial had no effect on the main agronomic parameters such as grain yield (Table 3.7) or grain quality (Table 3.71). Hence, the results of this study do not support the positive effects of AMF-inoculation on wheat yields as reviewed in the literature (Pellegrino *et al.*, 2015; Zhang *et al.*, 2019), but proof for the establishment of inoculation is required (Chapter 4). There were few direct effects of AMF-inoculum on plant performance. The first was observed in seedlings where plant number was higher with AMF-inoculum application (Table 3.23) that ultimately increased biomass

production (Table 3.24), but the latter difference was only significant in 2019. Although there have been reviews about the contribution of AMF to seedling establishment in natural ecosystems (van der Heijden & Horton, 2009), it is unlikely that AMF inoculum application was the reason for higher plant numbers six weeks after drilling since this effect was not abundant at tillering. Then again, crop canopy was marginally increased at GS45, but like early plant counts at establishment, this effect was not observed in NDVI measurements later in the season (Table 3.38).

Considering these random effects of the inoculum, it was surprising to measure increases in total biomass at harvest with AMF inoculation (Table 3.13). Furthermore, the inoculated zero-input plots showed significantly higher ear number (Table 3.20) while plant numbers were not increased. A possible explanation for this effect could be found in a pot experiment with barley where AMF inoculation induced late tillering, however without contribution to yield (Gernns *et al.*, 2001). This was also the case in the present field trials where the increased total biomass following AMF inoculum application in zero-input plots had no effect on grain yields. Then again, late tillering potentially leading to higher ear number would have been determined well before final harvest, but tiller numbers were not increased in zero-input plots with AMF-inoculation. The opposite to the previously described effect was found in plots with mineral N application and AMF-inoculation. At flowering, tiller numbers and shoot biomass were lower in plots with mineral N fertiliser and AMF inoculum application (Table 3.31). The same was found at maturity and at final harvest where less total biomass was harvested from plots with mineral N and AMF-inoculum application (Table 3.13). Unlike the effect of AMF inoculum in zero-input plots, decreased total biomass in plots with mineral N fertiliser and with AMF-inoculation was not reflected in lower ear numbers, but seed numbers were decreased (Appendix A, Table A.4, Table A.5). Nevertheless, grain yield in plots in this fertiliser treatment were not affected by AMF-inoculation (Table 3.7). Reduced biomass production in mycorrhizal plants has been linked to imbalances in symbiotic nutrient exchange which is mostly determined by the nutritional status of the plant, but also fungal identity (Corkidi *et al.*, 2002; Kiers *et al.*, 2011; Pellegrino *et al.*, 2011; Püschel *et al.*, 2016). As the soils at Nafferton Farm were mostly P-depleted, the addition of N should have favoured mycorrhizal benefits on plant growth, but the opposite was found with this reduction in total biomass. A possible explanation could be that the inoculated AMF strains replaced native AMF (Schlaeppi *et al.*, 2016; Bender *et al.*, 2019) which are likely better adapted to local soil conditions and ultimately provide greater benefits to the host plant (Pellegrino *et al.*, 2011; Johnson *et al.*, 2015; Middleton *et al.*, 2015). In association with AMF that might be less efficient in P-uptake, the depletion of P could be even more pronounced leading to reduced growth and reduced tillering

as described in multiple studies on wheat with low-P supply (Manske, 1989; Hetrick *et al.*, 1992; Graham & Abbott, 2000; Li *et al.*, 2005; Ryan *et al.*, 2005). This hypothesis implies why biomass was only decreased in plots with mineral N and AMF treatment and not when organic fertilisers were applied as these provided both N and P (Appendix A, Table A.2).

Overall, it is not guaranteed that the applied AMF inoculum ultimately affected wheat growth as the described effects were relatively small (Table 3.13), but evidence for inoculation success would be expected in terms of increased AMF-RC (Al-Karaki *et al.*, 2004; Farmer *et al.*, 2007; Buysens *et al.*, 2016; Ercoli *et al.*, 2017; Alaux *et al.*, 2018). Except for small reductions of arbuscule abundances (A%) at seedling growth with AMF-inoculation (Table 3.52), there were no main effects on AMF-RC. At tillering and at maturity, inoculated wheat with FYM application showed higher AMF-frequencies (F%, Table 3.59) and A% (Table 3.70) respectively. With arbuscules being the interface for symbiotic nutrient exchange, increased A% especially at early growth stages would be assumed to affect plant performance, but this was not observed. Besides the reports on increased AMF-RC in response to inoculation, there are a considerable number of inoculation trials which have shown no effect on AMF-RC (Pellegrino *et al.*, 2012; Middleton *et al.*, 2015; Smith *et al.*, 2015; Schlaeppi *et al.*, 2016; Buysens *et al.*, 2017). These controversial findings indicate that AMF-RC may not be a suitable parameter to assess inoculum establishment where a high level of native AMF are present in the soil (Hart *et al.*, 2018). Instead, molecular markers have been suggested for the tracing of inoculated AMF strains (Kokkoris *et al.*, 2019a; Rosa *et al.*, 2020). That native AMF were abundant at Nafferton Farm was observed already six weeks after drilling with wheat seedlings being colonised by AMF in both inoculated and non-inoculated plots (Table 3.52). Hence, competition with indigenous AMF might have hindered inoculum establishment (Verbruggen *et al.*, 2013; Buysens *et al.*, 2017). This scenario becomes more likely considering the extremely low dosage of 100 g ha^{-1} that was applied to the field. However, the low quantity could have been compensated by the high concentration of propagules in the inoculum (Table 3.5). The amount of applied propagules was higher in the first year and lower in the second year compared to rates described by Ercoli *et al.* (2017) who used 9.3 g m^{-2} i.e. 8,333 spores m^{-2} (= 83.3 Mio propagules ha^{-1}). They reported strong increases in AMF-RC in a field trial with durum wheat as well as higher grain N-concentrations in unfertilised plots with AMF-inoculation, but no effects on grain yield. In their case, the increase in AMF-RC was only found in an old variety of durum wheat. The results of the current field trials 2018 and 2019 show no differences between Aszita and Skyfall in response to AMF inoculation as no variety × AMF-inoculation interaction was observed. These could be expected considering

studies that showed different root colonisation and nutrient use efficiency of wheat varieties in association with *Rhizophagus irregularis* under controlled conditions (Elliott *et al.*, 2019; Thirkell *et al.*, 2020). Since the inoculum used during field trials at Nafferton Farm harboured three AMF species, effects of AMF-inoculation on wheat might be more pronounced, or vice versa, less effective due to competition among inoculated strains (Jansa *et al.*, 2008; Thonar *et al.*, 2014). The applied rate of AMF inoculum was probably even lower as it was observed that particles of the root powder disintegrated during mixing with sand and stuck to plastic surfaces. For this reason, it was decided to develop a more direct approach for the application of AMF powder in wheat production i.e. seed coating (Chapter 6). Buysens *et al.* (2017) trapped propagules of *R. irregularis* in alginate beads and applied these at a rate of 1,560 propagules per potato plant in a field trial after calculating the required amount of inoculum to colonise 90 % of the plant. Despite this precise application method and preceding pot experiments, no effects on crop yield or AMF-RC were observed in their study as the inoculum hardly established (Buysens *et al.*, 2017). The outcome of their study might be similar to the results of the present field trials, for which reason molecular evidence will be presented in the following chapter to validate the effects of the AMF-inoculum observed in both seasons (Chapter 4).

3.5 CONCLUSION

The colonisation of wheat roots by AMF in both varieties was mostly shaped by environmental conditions and closely linked to wheat growth stage. Consistently lower AMF root colonisation (AMF-RC) in Aszita compared to Skyfall do not support the notion that breeding programmes have reduced AMF-responsiveness in modern wheat varieties although this is based on limited evidence i.e. two varieties. Further information about nutrient uptake and concentrations (particularly P and N) in the two varieties are needed since AMF-RC alone cannot be used to draw conclusions about mycorrhizal efficiency in varietal comparisons. The same applies to the assessments of different growth stages of wheat where it was assumed that late AMF-RC in senescing wheat could have negative impacts on yield and grain quality. Leaf senescence (measured by SPAD) was delayed following the application of conventional crop protection in Skyfall for which reason beneficial (or negative) impacts of prolonged AMF symbiosis are expected in particular under P limitations in modern varieties with high mycorrhizal responsiveness. If mycorrhiza-compatible fungicides should be considered in breeding programmes for mycorrhizal-responsive wheat varieties, inputs of mineral fertilisers should be adjusted accordingly as the application of mineral N decreased AMF-RC when no fungicides were applied. This was not the case in non-fertilised plots and with application of organic

fertilisers where biogas digestate seems to be a promising alternative to mineral N regarding grain yields and disease susceptibility, but negative impacts on AMF-RC were found in both seasons. This effect may have been due to P that was added by the application of both farmyard manure and biogas digestate. In contrast, the addition of N in the form of mineral N to a low-P soil can have both positive and negative impacts on AMF-RC and most likely depends on the N-status of the soil. The role of fine root endophytes in this variable response to N-fertiliser poses an intriguing research question, but presence of these fungi at Nafferton Farm requires validation at the molecular level. The same applies to the AMF strains that were used in the inoculum in low dosages and faced strong competition from the local and potentially more adapted AMF population. Marginal, but significant increases in total biomass at harvest with AMF-inoculation in the absence of applied fertiliser were contrasted by reduced biomass following AMF-inoculation in plots with mineral N application which indicate a potential growth promoting effect of the inoculum under low nutrient supply. But like all the described effects, further field trials as well as more detailed investigations of mycorrhizal efficiency are required to validate conclusions about the role of AMF in wheat. These might still not be applicable to different locations or environmental conditions which have been shown to have strong impact on AMF-symbiosis in the present study

CHAPTER 4. MOLECULAR TRACING OF AMF INOCULUM USING STRAIN-SPECIFC PRIMERS

4.1 INTRODUCTION

Inoculation successes with AMF under field conditions are usually reported with regard to biomass or other plant performance parameters, but proof of inoculum establishment is often missing (Hart *et al.*, 2018). The problem here is the discrimination of the abundances of natural and introduced AMF communities for which reason molecular tools are required to clarify the fate and success of fungal inoculants (Kokkoris *et al.*, 2019a). High genetic variability in AMF form an obstacle to molecular tracing of specific strains as even single isolates can harbour multiple haplotypes (Koch *et al.*, 2004; Ropars *et al.*, 2016). Therefore, the choice of a suitable molecular marker is essential to obtain sufficient phylogenetic resolution while avoiding highly variable regions as found in mycorrhizal nucleic DNA (Börstler *et al.*, 2008; Croll *et al.*, 2008). The ribosomal large subunit of the mitochondrial ribosomal DNA (mtLSU) as a more conserved region of the genome has been recognised for distinguishing variability between AMF isolates (Raab *et al.*, 2005; Börstler *et al.*, 2008, 2010).

Common methods for the tracing of inoculated AMF strains are quantitative PCR (Krak *et al.*, 2012; Pellegrino *et al.*, 2012; Buysens *et al.*, 2017; Alaux *et al.*, 2018) and nested PCR (Farmer *et al.*, 2007; Williams *et al.*, 2017; Thilagar, 2018). More recently, digital droplet PCR (ddPCR) has been introduced to mycorrhizal research with the potential to replace hitherto applied methods (Kokkoris *et al.*, 2019a, 2020a; Barceló *et al.*, 2020; Rosa *et al.*, 2020). High precision and sensitivity of ddPCR enables the detection and quantification of rare molecules (Hindson *et al.*, 2011) which are characteristics that have been extremely valuable in clinical studies and have more recently also been applied in ecological studies (Kokkoris, *et al.*, 2021b). The technology behind ddPCR is based on an assumption of a Poisson-distribution of the target molecule in up to 20,000 droplets which are generated in a specific oil emulsion of the PCR master mix with the template DNA sample (Basu, 2017). Each droplet represents a single endpoint PCR amplification that produces a fluorescent signal if the target DNA sequence was present in that particular reaction. The original concentration of the target molecule in the sample DNA extract dictates the probability of an individual droplet giving a positive signal and thus can be calculated from the number of positive count droplets in relation to negative count droplets without the need for standard curves (Pinheiro *et al.*, 2012).

4.1.1 AIMS AND OBJECTIVES

This work aimed to develop oligonucleotide DNA target sequences that are specific for an isolate of *Rhizophagus irregularis* (R.i.) which was the main component of the AMF inoculum (INOQ Advantage) that was applied in the field experiments reported earlier (Chapter 3, Table 3.5). These target sequences could then be used in ddPCR to trace this fungal strain in soil and root samples and ultimately investigate if, and to what extent, the strain colonised roots of the two tested wheat varieties (Chapter 3, Table 3.4). Furthermore, high specificity of the target sequence will enable the discrimination of the inoculum strain from native R.i. strains. Abundances and responses of native R.i. strains to inoculation, crop protection and wheat variety will be analysed using less specific primers that amplify most R.i. haplotypes.
In summary, the aims will be achieved by:

1. Identifying polymorphisms within the mtLSU region of selected R.i. strains
2. Designing highly specific molecular targets based on these sequences
3. Comparing target specificity with other R.i.-isolates
4. Running strain-specific markers in optimized ddPCR assays with DNA extracted from wheat roots recovered from field trials and environmental samples
5. Demonstrating absence of the introduced R.i.-genotype in the field prior to inoculation
6. Investigating impacts of inoculation and agronomic factors on native R.i. strains

4.2 MATERIALS AND METHODS

Preparation work for the molecular tracing of the inoculum strains were conducted at the laboratory of Dr Jan Jansa at the Institute of Microbiology in Prague (MBU, Czech Academy of Science). Extractions of DNA from field samples and digital droplet PCRs (ddPCR) were conducted at Newcastle University in the lab of Prof. Neil D. Gray and Prof. Ian Head.
In brief, the steps for primer design at MBU included DNA extraction from root organ cultures (ROC) which were then used for amplification and sequencing of the mtLSU region by PCR (Fig. 4.1 A). Edited sequences were aligned with each other and a gene bank library as a template for the design of strain-specific primers.
For molecular tracing of inoculum strains by ddPCR, DNA was extracted from root and soil samples (Fig. 4.1 B). These samples were used and tested with the newly designed primers in endpoint PCRs and then run in ddPCR assays. The results were statistically analysed after threshold setting.

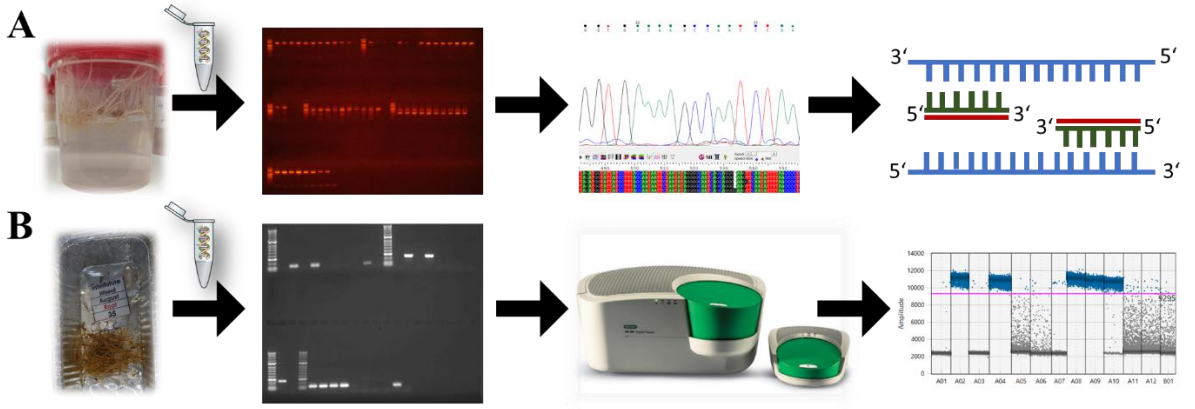


Fig. 4.1. Steps during the procedure of **A)** primer design for the inoculum strain starting with DNA extraction from root organ cultures, followed by amplification with RNL-primers that yielded sequences of the mtLSU region which were then used as base for the design of specific oligo nucleotides. **B)** molecular tracing of the inoculum strain in the field starting with DNA extraction from root samples, amplification of mtLSU with new oligo targets first by normal PCR and then by ddPCR. The process was finalised by threshold setting of ddPCR results.

4.2.1 DNA EXTRACTION FROM RHIZOPHAGUS IRREGULARIS ISOLATES

To identify DNA sequence variations between the AMF inoculum strain QS81 from INOQ GmbH compared to other R.i. strains, DNA was extracted from a selection of 13 different ROC of R.i. with different origins (Table 4.1). All isolates were grown for two months in a specific ROC system developed by Martin Rozmos (MBU Prague) using transformed chicory roots on modified Strullu-Romand medium (Declerck *et al.*, 1996). All steps with the ROC were conducted under sterile conditions.

Table 4.1. Sample list of *Rhizophagus irregularis* isolates from INOQ and MBU used during primer design

Isolate	Place of origin	Environment	Lab	Reference/Project
MA1	Mayotte, Coconi	Garden soil	INOQ	"Afforestation of bad-lands using endo-mycorhizas and local tree species in Mayotte"
MA2	Mayotte, Kahani	Forest soil (Padza)	INOQ	
MA3	Mayotte, Kahani	Grassland (Padza)	INOQ	
MA4	Mayotte; Kahani	Bare soil (Padza)	INOQ	
QS69	Czechia	Unknown	INOQ	Savary *et al.* (2018), here: ESQLS69-1
QS73	Subculture of QS69	-	INOQ	
QS81	Subculture of QS69	-	INOQ	
STS1	Germany, Loitze	Field soil	INOQ	EIPAgri
L1	Czechia, Litoměřice	Grassland	MBU	Řezáčová *et al.* (2016)
L2	Czechia, Litoměřice	Grassland	MBU	Řezáčová *et al.* (2016)
L4	Czechia, Litoměřice	Grassland	MBU	Řezáčová *et al.* (2016)
L21	Czechia, Litoměřice	Grassland	MBU	Řezáčová *et al.* (2016)
L23	Czechia, Litoměřice	Grassland	MBU	Řezáčová *et al.* (2016)

For DNA extraction from ROC, roots were removed from the surface of the culture medium. The material was subsequently ground using a pestle and mortar with the addition of 300-500 µl lysis buffer from the DNeasy PowerSoil kit (Qiagen, Germany). The liquid was pipetted into PowerBead tubes filled with lysis buffer from the same kit. Samples were stored at 4 °C until further processing.

For DNA extraction from pure fungal material, culture medium containing mycelium was extracted with a sterile spoon and placed in glass bottles filled with 500 ml of 10 mM citric buffer (pH 6.0, Doner & Bécard, 1991). Bottles were agitated on a shaker until all residues were dissolved and hyphae visibly conglomerated in the liquid. The bottle content was then passed through sterilised 5 µm Teflon filters using a vacuum pump. Fungal material was collected from the filter membrane and homogenised with the addition of lysis buffer using a pestle and mortar. The liquid was pipetted into lysis buffer filled PowerBead Tubes for storage until further processing. As a control treatment, both procedures for DNA extraction from roots and hyphae were conducted for an empty ROC without AMF inoculation.
As the first step of DNA extraction, all samples were incubated at 70°C for 10 min in the heating block. Then, cells were lysed in the PowerBead Tubes using a vortex mixer for 10 min at max. speed. Subsequent steps were conducted using the DNeasy PowerSoil kit following the manufacturers protocol. Samples were eluted in two steps using 50 µl of elution buffer from the kit.

4.2.2 AMPLIFICATION AND SEQUENCING OF THE MTLSU REGION OF RHIZOPHAGUS IRREGULARIS

Prior to sequencing, DNA from the selected isolates was amplified by polymerase chain reaction (PCR) using RNL-primers by Raab *et al.* (2005) and Börstler *et al.* (2008) as well as newly designed primers by Jansa (2019) (Table 4.2). Each reaction was set up in 25 µl containing 1 µl of DNA template, 12.5 µl of Combi PPP Master Mix (TopBio, Czechia), 0.5 µl forward primer (25 µM) and 0.5 µl reverse primer (25 µM) as well as 10.5 µl of nuclease free water. PCRs were run with initial denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 15 sec, 50°C for 30 sec, 72°C for 1 min and finalized with a 5 min extension step at 72°C. Results were checked by gel electrophoresis (1 % agarose, 10 % ethidium bromide). Amplicons were purified using the QiaQuick PCR purification kit (Qiagen, Germany) following the manufacturers protocol.

Table 4.2. Primers (forward = fwd, reverse = rev) used for amplification and sequencing of mtLSU region of *Rhizophagus irregularis* isolates

Primer	Sequence 5'-3'	Orientation	Used for:		Reference
			PCR	Sequencing	
RNL1	AGACCCGAARCCWRGTGATCT	fwd	x	x	Raab *et al*. 2005
RNL5	GAGCTTCCTTTGCCATCCTA	rev	x	x	Raab *et al*. 2005
RNL7	CTTCTGCTTTCGGCGAAGAG	rev		x	Raab *et al*. 2005
RNL7c	AATACCAACATACTCATTAC	rev		x	Jansa 2019
RNL12	CATTATATGCTCCGGCGTAG	fwd		x	Raab *et al*. 2005
RNL14	AGGATAGGCCTGGAAACCAAGC	rev	x	x	Raab *et al*. 2005
RNL15	CTGAGCTGTTACGCTATC	rev	x	x	Raab *et al*. 2005
RNL26	CTTGTGCAAGTAGGCCTTCT	rev		x	Börstler *et al.* 2008
RNL28	CCATGGCCAAGTGCTATTTA	fwd	x	x	Börstler *et al.* 2008
RNL29	TAATAAGACTGAACGGGTGT	fwd	x		Börstler *et al.* 2008
RNL30	TAGCATCGGGCAGGTATCAG	rev	x		Börstler *et al*. 2008
RNL63rev.	CTACGCCGGAGCATATAATG	rev		x	Börstler *et al*. 2008
mt5rev	TAATAAGACTGAACGGGTGT	rev		x	Jansa 2019

Amplicons of DNA from fungal mycelium were submitted for Sanger sequencing at the Centre for DNA sequencing at MBU. Two sequencing runs with different primer sets were required to obtain the full mtLSU sequence of the selected strains (Table 4.2). The sequences were edited in Chromas (vs. 2.6.6, Technelysium Pty Ltd, Australia) and contiguous sequences (contigs) were produced by merging the two shorter but partially overlapping sequences manually in MS Word (Microsoft Corporation, 2018).

4.2.3 SEQUENCE PROCESSING AND PRIMER DESIGN

To identify polymorphisms within the aligned sequences, but also in comparison to known R.i. haplotypes that could possibly be abundant in the field soil at Nafferton Farm, the sequences were aligned with the mtLSU region sequences of a selection of R.i. haplotypes extracted from GenBank (http://www.ncbi.nlm.nih.gov).

Table 4.3. Reference library of *Rhizophagus irregularis* isolates from previous studies used during strain-specific primer design based on mitochondrial ribosomal DNA sequences.

Isolate	Mt genome accession no.	Origin	Reference
C2	KU127234.1	Tänikon, Switzerland	Ropars *et al*. 2016
A4	KU162859.1	Tänikon, Switzerland	Ropars *et al*. 2016
B3	KU127233.1	Tänikon, Switzerland	Ropars *et al*. 2016
A5	KU127232.1	Tänikon, Switzerland	Ropars *et al*. 2016
A1	KU127231.1	Tänikon, Switzerland	Ropars *et al*. 2016
FACE#494	FJ648425.1	Eschikon, Switzerland	Lee & Young, 2009
MUCL46241	JQ514225.2	Quebec, Canada	Formery *et al*. 2012
DAOM234179	KC164354.1	Quebec, Canada	Beaudet *et al.* 2013
MUCL46239	JQ514223.2	Quebec, Canada	Formery *et al*. 2012
DAOM234328	JX993114.1	Finland	de la Providencia *et al.* 2013
DAOM197198	NC_014489.1	Quebec, Canada	de la Providencia *et al.* 2013
DAOM240415	JX993113.1	Manitoba, Canada	de la Providencia *et al.* 2013
MUCL41833	KY347874	Canary Islands, Spain	Buysens *et al*. 2017
L8	FN377590	Landskron, France	Börstler *et al*. 2010

All sequences were aligned using the MUSCLE tool in the Molecular Evolutionary Genetics Analysis software (MEGA X, Kumar *et al.*, 2018). Phylogenetic relationships were inferred by applying the Neighbour-Joining method in the same software. Evolutionary distances were computed using the Maximum Composite Likelihood algorithm with a bootstrap test for 1000 replicates. Target specific sequences suitable for use as primers for the R.i. isolate QS81 were designed by Jan Jansa using the software AlleleID (version 6, PREMIER Biosoft, USA).

4.2.4 CROSS-REFERENCE TESTING

Primer sequences were tested *in silico* with the GenBank tool Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Based on these results, two sets of oligos composed of forward and reverse primers, as well as FAM1 and BHQ1 dual labelled HPLC-purified TaqMan probes were synthesised (Eurofins, Luxembourg). The primer pairs were first tested without probes by normal (end-point) PCR using 12.5 µl of MegaMix-blue (Microzone Ltd, UK), 1.25 µl of each primer (10 µM), 5 µl ddH_2O and 3 µl of hyphal DNA extract templates from R.i. isolates (Table 4.1). PCR conditions were 5 min at 95°C for initial denaturation, followed by 30 s at 95 °C, 30 s at 55°C and 1 min at 72°C in 35 cycles, with termination for 5 min at 72°C. DNA from four field samples from inoculated and non-inoculated plots were included in each PCR run to see if inoculum detection was possible even without probes.

4.2.5 GENOMIC MATERIAL FOR ASSAYS WITH STRAIN SPECIFIC MARKERS

Abundances of the AMF inoculum strains was mainly assessed for the 2019 field trial at growth stage (GS) 32 (stem elongation, Chapter 3, Table 3.6) where the highest AMF colonisation had been observed during colonisation assessments (Chapter 3, Fig. 3.3). Additionally, samples from other time points (GS90 2018, GS12 2018, GS22 2019) were selected, but quality assessments of DNA extractions showed that most of these samples had been degraded for which reason only a small number of samples could be analysed. All root samples (~15 mg) had been collected from plots with crop protection, +/- AMF inoculation and zero fertiliser application treatments (Chapter 3, Section 3.2.4).

To prove delivery of the inoculum to the field soil, DNA was extracted from the remaining sand-inoculum mixture (250 mg) that had been sampled from the hopper after drilling (Chapter 3, Section 3.2.5). DNA from soil samples (250 mg) of inoculated and uninoculated plots sampled at 0-30 cm depth in spring 2019 (during the tillering phase and about one month before GS32) were used as positive and negative environmental controls respectively. To evaluate the presence of the targeted mtLSU haplotypes in the field prior to inoculation, DNA was extracted from five randomly selected soil samples which had been collected from the same site before the start of the field trials (March 2017). All samples were extracted using the DNeasy PowerSoilPro Kit (Qiagen, Germany) following the manufacturers protocol. Prior to DNA extraction, samples were homogenised using a FastPrep-24™ (MP Biomedicals, USA) at 6 m s^{-1} with 2 x 40 sec and an incubation step for 5 min at 4°C between sessions. Each DNA extraction was accompanied by positive (AMF inoculum) and negative controls (ddH_2O). DNA concentrations were measured by fluorometric quantification (Qubit™ Fluorometer 3.0) using the Qubit™ dsDNA high sensitivity assay kit (Invitrogen, USA) following the manufacturers protocol.

4.2.6 DIGITAL DROPLET PCR

Each ddPCR assay was set up in 22 µl reactions with a standard master mix of 17µl containing 10 µl Supermix for probes (no dUTP, Bio-Rad Laboratories, USA), 2 µl forward and reverse primer (0.9 µM), 0.5 µl probe (0.25 µM), 2.5 µl ddH_2O and 5 µl of template DNA. The master mix was distributed in specific ddPCR 96-well plates (Bio-Rad Laboratories). After adding a single sample DNA template to a single well, the plates were sealed with a Pierceable Foil Heat Seal (PX1 PCR Plate sealer, BioRad Laboratories), vortexed and spun down (PCR plate spinner, VWR, USA) to facilitate an even distribution of the DNA template in the master mix. Droplets were then generated automatically in the AutoDG (Bio-Rad Laboratories) using the droplet generation oil for probes (Bio-Rad Laboratories). The resultant 96-well plate with the

droplets was sealed and placed in a thermo cycler (C1000 Touch, Bio-Rad Laboratories). After endpoint amplification by PCR, droplets were analysed in the Droplet Reader (QX200, Bio-Rad Laboratories). Droplet counts and signal amplitudes (λ) were collected using the software QuantaSoft™ (vs. 1.7.4.0917, Bio-Rad Laboratories). Raw data was exported into QXManager Software (Standard Edition vs. 1.1, Bio-Rad Laboratories) for manual threshold adjustment and visualisation. Runs with less than 10,000 generated droplets were excluded from the analyses since data collection from 12,000-16,000 droplets have been recommended by Bio-Rad.

4.2.7 OPTIMIZATION OF DIGITAL DROPLET PCR ASSAYS

Each primer set was run with a temperature gradient using a positive control as DNA template to identify optimal annealing temperatures for probes and primers. PCR conditions for the temperature gradient were 10 min at 95°C, followed by 39 cycles of a two-step thermal profile of 30 s at 94 °C for denaturation, annealing from 50 to 58°C for 1 min each, and termination at 98°C for 10 min and 12 °C for 10 min. This programme was adjusted for assays with field samples according to Witte *et al.* (2016) to more cycles (44), lower ramp rate (1°C s^{-1} instead of 2.5 °C s^{-1}) and doubled annealing times.

Troubleshooting in environmental samples was conducted by construction of a dilution series (up to 100-fold) and the spiking of samples with positive controls. Field samples were run undiluted and in 5-fold dilutions as it was recognised that some undiluted samples were accompanied by a lot of noise. All assays were run with three negative template controls (NTC) per plate using ddH_2O instead of DNA to exclude potential contamination during the assay setup.

4.2.8 STATISTICAL ANALYSES

If contamination was detected in NTCs, the acquired concentration of target sequences μl^{-1} was subtracted from the sample signals. Copy numbers per gram of sample (Cpgs) were calculated from these values using a formular adopted from Kokkoris *et al*. (2019a):

$$Cpgs = \left(Quantalife\ value * \left(\frac{RV}{TV}\right) * DF * EQ\right) / QS$$

The Quantalife value was generated in the QuantaSoft™ software during droplet reading and was multiplied by the ratio of reaction volume (RV, 22 µl) and template volume used in the assay (TV, 5µl). Diluted samples were then multiplied with the dilution factor (DF) and with the initial elution volume of the DNA sample (EQ, 50 µl). The subsequent value was divided by the quantity of material that was used for DNA extraction from the respective sample (QS)

which was known for soil (250 mg) and root samples (15 mg), but not for pure culture samples. Hence, copies per ROC are stated per sample and not per gram of fungal material.

The effects of crop protection, variety and inoculation on copy numbers were analysed using the same model and test procedure as used for field data (linear mixed effect model, Section 3.2.14) without fertiliser as fixed effect since only samples from zero-input plots were used for ddPCR assays. Copy numbers were cube root transformed to achieve normality which was assessed by QQ-plotting of residuals. Data sets from runs with strain-specific and haplotype-specific primers were analysed separately in RStudio (R-Core Team, 2019). Spearman's correlation analyses of copy numbers and microscopy data were plotted using the ggplot2-package (Wickham, 2016).

4.3 RESULTS

4.3.1 MTDNA MARKERS FOR R.I. ISOLATES

Sequencing of selected isolates of *Rhizophagus irregularis* (R.i.) indicated significant length polymorphisms within the mitochondrial large subunit (mtLSU) of strains from INOQ (~ 2300 bp) and MBU (~1550 bp). A provisional alignment after the first sequencing showed that INOQ-strains harbor a long insertion of around 800 bp towards the end of the mtLSU region (Fig. 4.2, RNL5 downstream).

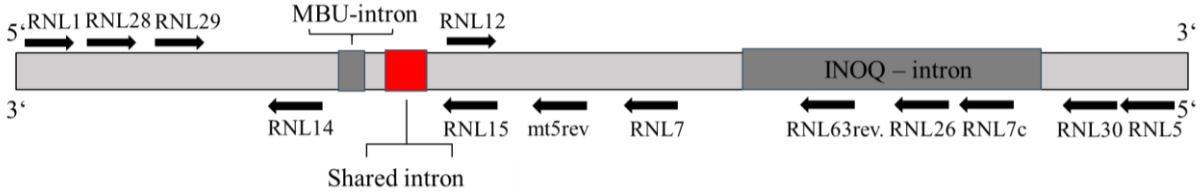


Fig. 4.2. Map of RNL-primers (adapted from Raab *et al*. 2005) used for amplification and sequencing of mtLSU region of *Rhizophagus irregularis* isolates from INOQ and MBU. Grey boxes mark introns specific for strains from the respective lab. Red box marks the variable region on a shared intron where primers for inoculum strains were located.

Alignment of the mtLSU sequences revealed three distinct haplotypes which grouped those R.i. isolates with almost identical sequences (Fig. 4.3). The main differentiation were two introns which characterised strains from the respective labs (Fig. 4.2, grey boxes). A further grouping criterion was a variable region between these two introns (Fig. 4.2, red box) that occurred in L-lines (L1, L2, L4, L21, L23) from Czechia and the group of haplotypes similar to QS81 (QS73, QS69, MA3, MA4). In comparison to the L-lines, INOQ-strains with this intron showed a C-insertion which was only found in one isolate of the library strains (MUCL41833, Table 4.3). The whole intron was missing in strains similar to MA1 (STS1, MA2) which had

been part of the AMF inoculum used during the 2019 season. With the lack of this intron or any other characteristic region of this strain, it was only possible to design target sequences for strains like QS81 (haplotype-specific primers, HSP), but not for MA1.

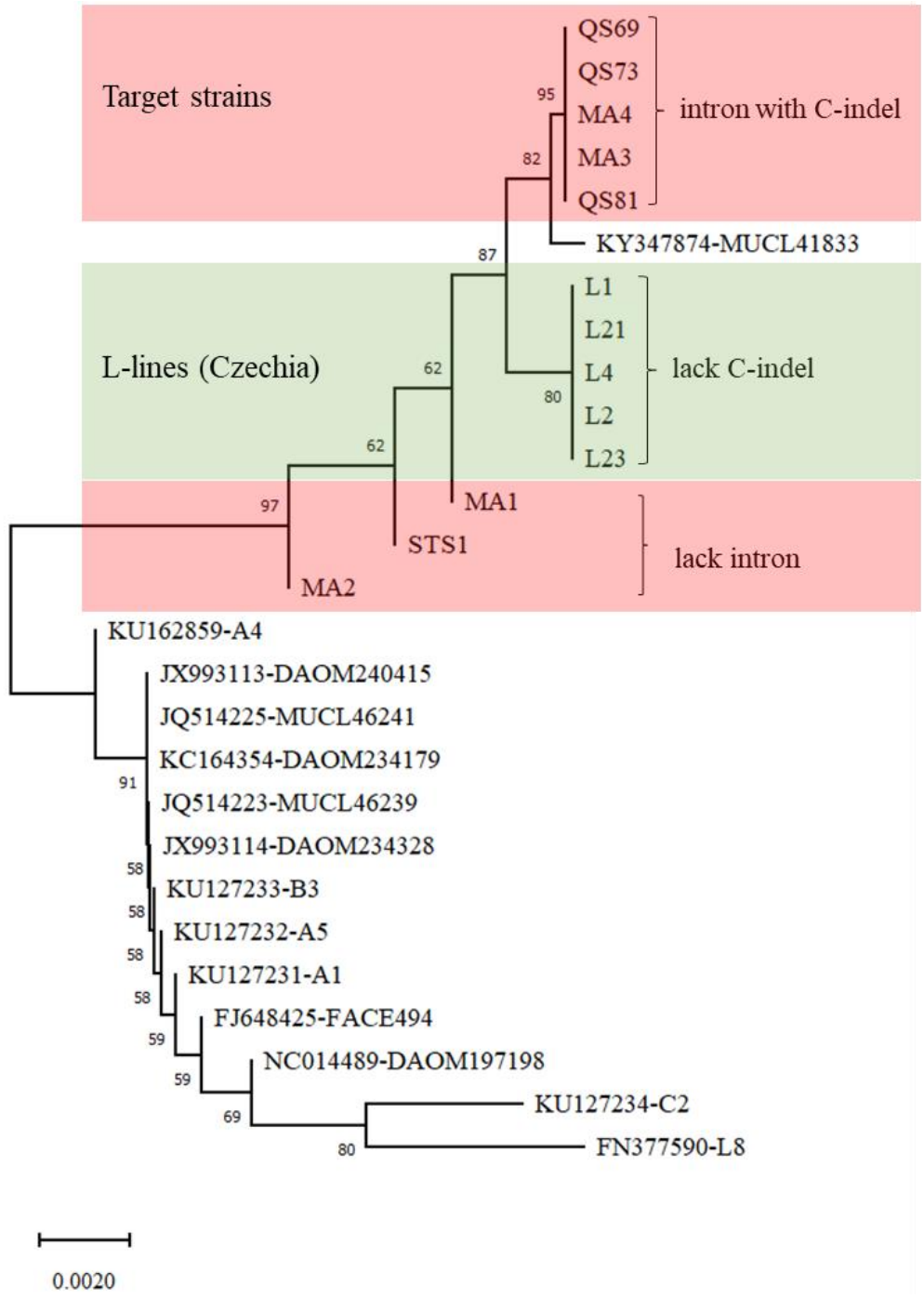


Fig. 4.3. Phylogeny of selected strains of *Rhizophagus irregularis* (R.i) used for the development of oligonucleotide primers and probes targeting the mitochondrial ribosomal RNA large subunit gene. Strains within coloured rectangles represent R.i. isolates from Czechia (green) and INOQ GmbH (red). Target strains for primer design were those similar to QS81. Brackets describe groups of haplotypes based on characteristics in their sequence alignment with library strains (not coloured). Branch numbers show percentages of trees in which the associated taxa cluster together in the bootstrap test (1000 replicates). Branch lengths show evolutionary distances according to Maximum Composite Likelihood method.

Additionally, more general primers (species-specific primers, SSP) targeting most mtLSU-haplotypes of R.i. were designed to assess if native strains of R.i. could be detected in the field samples despite the presence or absence of the inoculant (Table 4.4). Application of these primers was made to clarify at what ratio inoculated and native R.i. strains occurred in the field and if the inoculated strains replaced native R.i. strains to any extent.

Table 4.4. Oligo sets designed by Jansa (2019) with different specificity grades for the detection of *Rhizophagus irregularis* strains in field samples

Specificity	Oligo	Orientation	Sequence 5'- 3'	T_m	Product
Haplotype-specific	Primer	sense	TACCTATGCCGCTACG	48.8	100 bp
	Primer	antisense	GCTTCCACAATATTATATCATG	49.2	
	TaqMan	antisense	TTTTCAACCATGTTTAGACCA	51.8	
Species-specific	Primer	sense	GGCAAGGGGTGAAAAGC	53.1	127bp
	Primer	antisense	AGCTGCGTGGCTTCC	52.7	
	TaqMan	sense	AATCTAACCTGGAGATA	67.0	

4.3.2 PRIMER SPECIFICITY TESTS

Cross-reference testing with the new primers by PCR confirmed *in-silico* results of primer BLAST (data not shown) as only haplotypes like QS81 were amplified with HSP (Fig. 4.4 A) whereas all strains were amplified with SSP (Fig. 4.4 B). No amplification was achieved in field samples using HSP, but faint bands of the correct amplicon fragment size (in addition to the bands of smaller fragment size resulting from primer dimer) were visible in reactions with SSP in samples with and without inoculation.

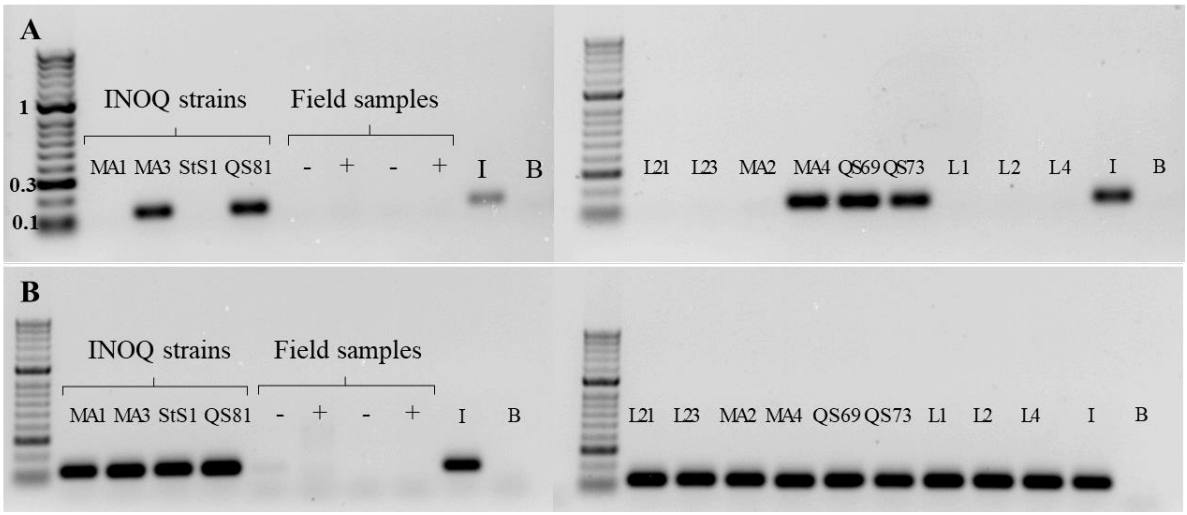


Fig. 4.4 Validation of primer specificity and amplicon size (c.f. 50 bp ladder) by PCR using two sets of oligos with, **A**) haplotype-specificity for QS81 (INOQ strains) and **B**) species-specificity for all other *Rhizophagus irregularis* haplotypes. Positive control was DNA from AMF-inoculum (I), water as blank (B). Field samples represent samples from inoculated (+) and uninoculated (-) plots.

Cross reference tests by ddPCR showed similar results to end-point PCR with positive signals predominantly in QS81-like haplotypes using the HSP assay (Fig. 4.5 A) and complete response in all selected R.i. strains with SSP assay (Fig. 4.5 B). In assays with HSP, positive droplets were detected also in strains from Czechia (L-lines). These few stronger signals derived from a population of weaker signals deriving from a cloud of unspecified amplification products ('dust'). In reactions with SSP on the other hand, enhanced noise derived from positive signals was observed ('rain').

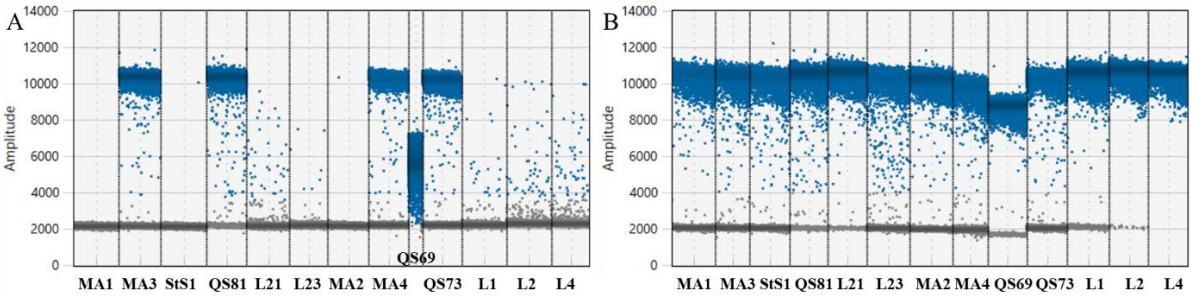


Fig. 4.5. Amplitude plots for amplification of mtLSU of selected culture isolates of *Rhizophagus irregularis* (R.i.) using, **A**) haplotype-specific primers for QS81-like haplotypes and **B**) species-specific primers for R.i.-mtLSU. Positive (blue) signals are distinguished from negative signals (grey) by a threshold around $\lambda = 4000$.

4.3.3 TRACING OF INOCULUM STRAINS IN FIELD SAMPLES

DNA from the sand-inoculum mix used in both 2017 and 2018 showed positive signals using the HSP assay with 2,911.5 copy number per gram sample (cp no. g^{-1}) and 47,842 cp no. g^{-1} respectively. This result implies that the inoculum was delivered to the soil successfully during drilling in both years. Nevertheless, soil samples from the field trial did not show amplification when they were run with the same assay (Fig. 4.6). This finding was also the case for soil samples collected from the field site before inoculum application which indicated that the haplotype of the inoculated strain of R.i. was not present in the field prior to inoculation, but was also absent after inoculation at the assessed time point.

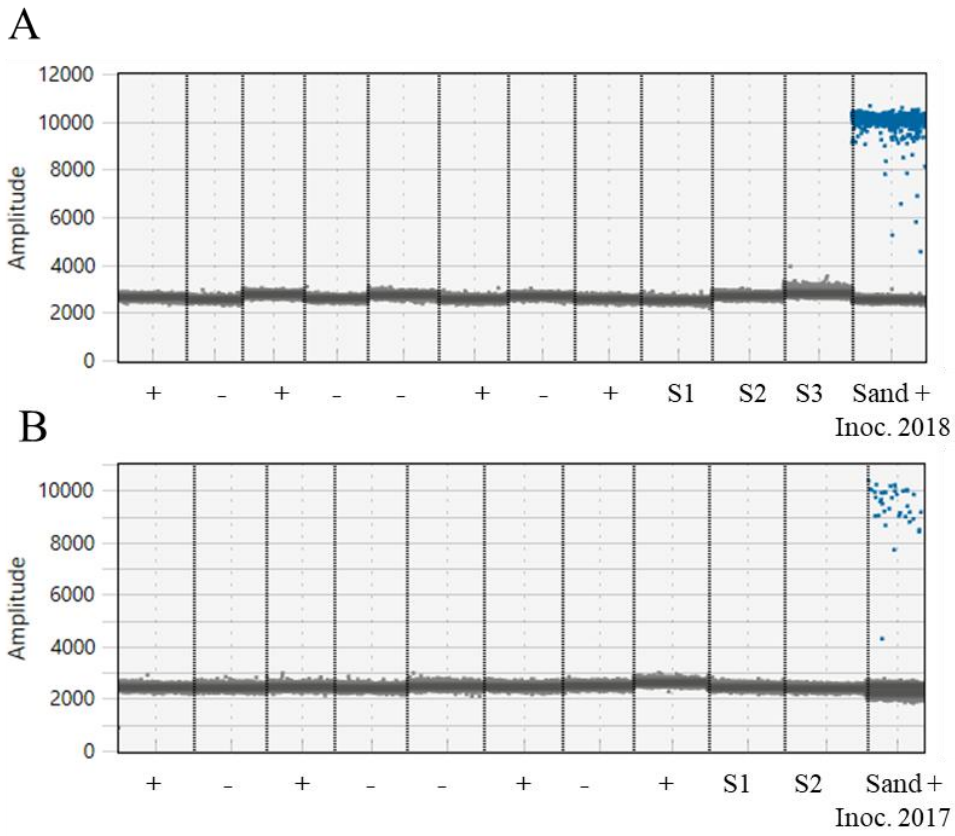


Fig. 4.6. Amplitude plots with haplotype-specific primers targeting QS81 in soil samples with (+) and without (-) inoculation, as well as soil from the field site before the start of the experiment (S1-S3). **A**) 100-fold dilution of DNA samples positive control was sand-inoculum mix from drilling in autumn 2018 **B**) 50-fold dilution of DNA samples, positive control was sand-inoculum mix from drilling in autumn 2017. Positive (blue) signals are distinguished from negative signals (grey) by a threshold at $\lambda = 4000$.

Statistical analyses could not be applied to data from the HSP assays as positive signals were scarce even when samples were run at different dilutions (Fig. 4.7 A). In root samples, positive signals were achieved only after the spiking of diluted samples with DNA from the AMF inoculum (Fig. 4.7 B). These runs yielded strong signals and indicate that the lack of amplification with HSP in root samples is unlikely to be linked to PCR inhibitors.

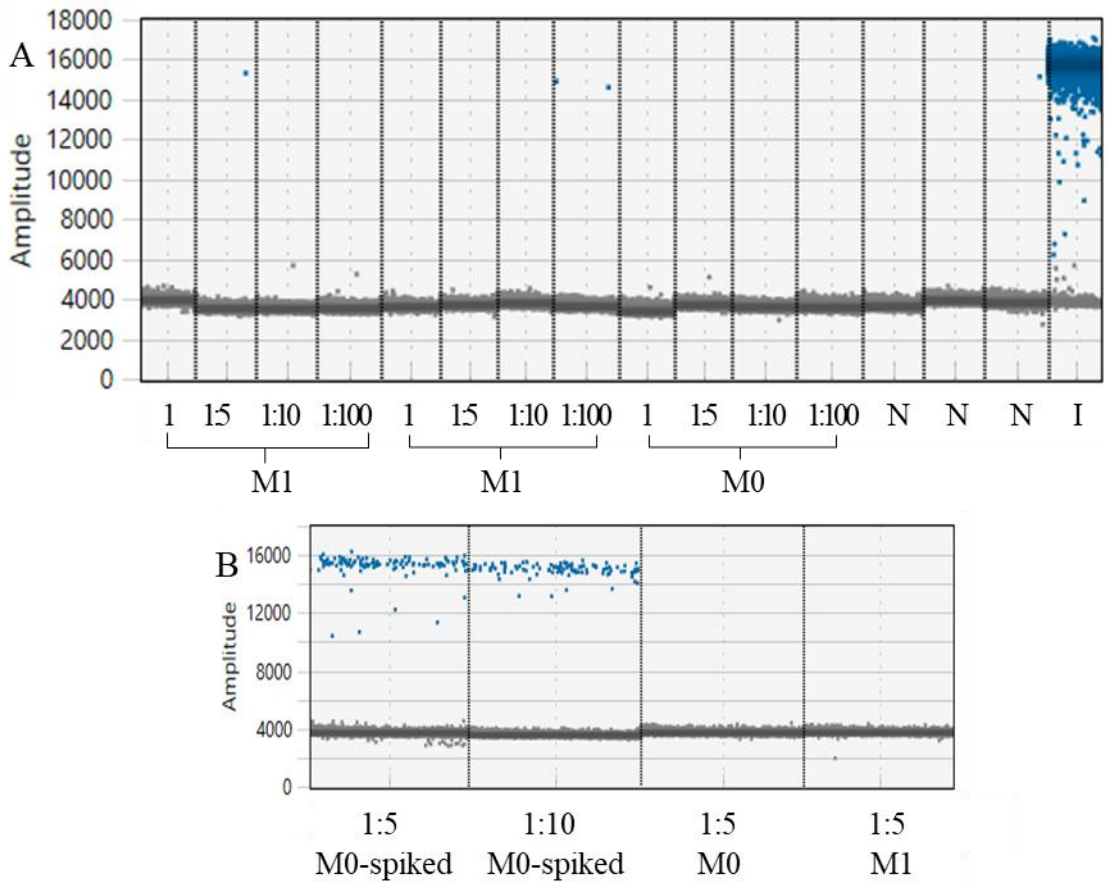


Fig. 4.7. Dilution series of field samples taken from plots which were inoculated (M1) and from plots which were not inoculated (M0) using haplotype-specific primers for QS81. **A**) Dilution series with root samples (taken at GS32) and non-template control (N), the positive control was DNA extracted from the AMF inoculum (I). **B**) Assessment of inhibition in PCR reactions by spiking (M0-spiked, 0.1 µl of AMF inoculum DNA) root samples (taken at GS22). Positive (blue) signals are distinguished from negative signals (grey) by a threshold at $\lambda = 6000$.

The few samples from earlier time points that were analysed showed no or a very scarce numbers of signals that would indicate potential detection of the QS81-haplotype. One positive droplet occurred in a sample from GS90 that was harvested at the end of the first growing season in 2018, but this observation was accompanied by a positive signal in an uninoculated sample (Fig. 4.8 A). A selection of samples at the seedling stage (GS12) of the second growing season (autumn 2018) showed no signs of amplification in inoculated samples (Fig. 4.8 B). Instead, positive signals were detected in the root sample that was adjacent to the positive control (AMF inoculum) on the plate, indicating possible cross-contamination of samples during ddPCR assays. The only albeit weak indication for the presence of QS81 was found in one sample from GS22 (Fig. 4.8 C) which had also shown positive signals at GS90 (plot 131).

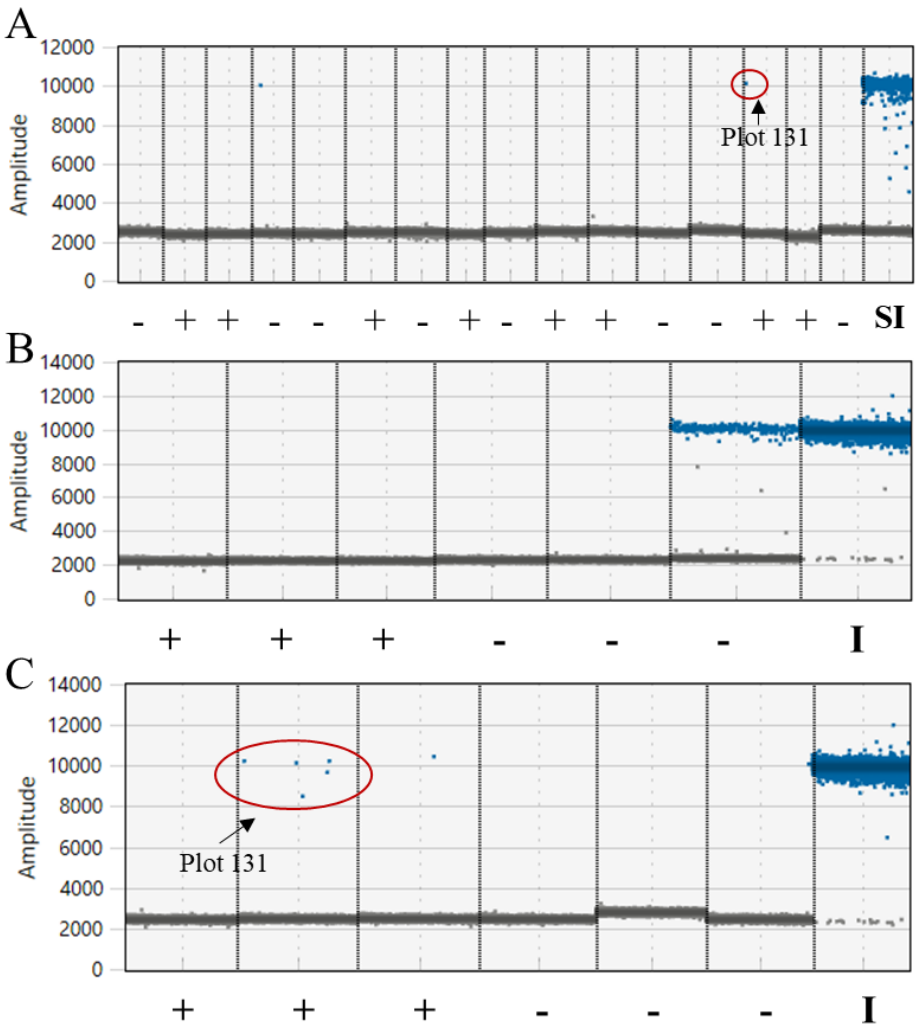


Fig. 4.8. Amplitude plots of ddPCR assays with haplotype-specific primers targeting inoculum strain QS81 in inoculated (+) and non-inoculated (-) root samples from: **A**) GS90 of the first growing season (2018) with sand + inoculum mix (SI) as positive control, **B**) GS12 of the second growing season (2019) and **C**) GS22 of the second growing season (2019) and pure inoculum DNA (I) as positive control. Positive (blue) signals are distinguished from negative signals (grey) by a threshold at $\lambda = 4000$.

Positive signals in samples harvested at GS32 occurred in both inoculated samples and samples without AMF inoculation (Fig. 4.9 A, blue circles). Ten-fold dilution did not reproduce these patterns but showed scarce signals of which one appeared in the previously described sample from plot 131 (Fig. 4.9 B). Overall, assays with HSP yielded positive signals in only 20 % out of all the reactions with inoculated samples (n = 66), whereas 14 % were positive for samples which were not inoculated (n = 59).

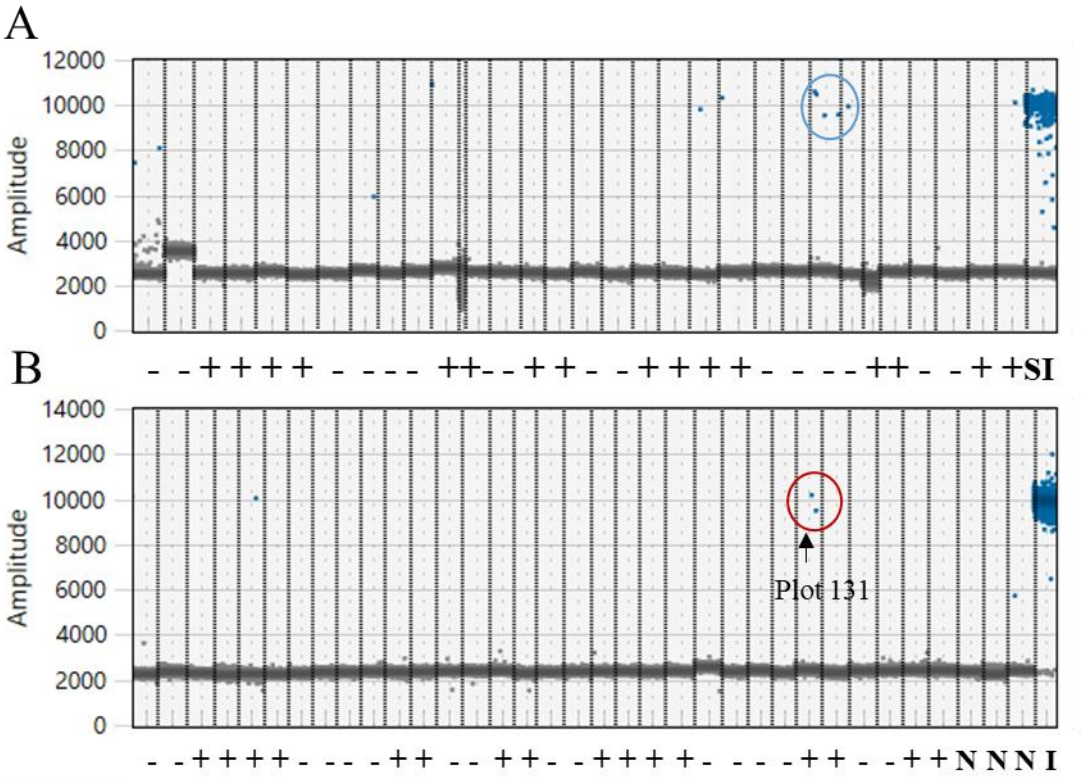


Fig. 4.9. Amplitude plots of root samples (GS32, May 2019) from inoculated (+) and uninoculated plots (-) with haplotype-specific primers targeting inoculum strain QS81. **A**) Undiluted samples with sand-inoculum mix (SI) as positive control, negative controls are not shown and **B**) 10-fold diluted samples, non-template controls (N) and AMF inoculum (I) as positive control. Red circle indicates positive signals at inoculated target sites, blue circle marks signals in samples from non-inoculated plots. Positive (blue) signals are distinguished from negative signals (grey) by a threshold at $\lambda = 4000$.

4.3.4 DETECTION OF NATIVE R.I. STRAINS IN ROOT SAMPLES

Contrary to assays with HSP, 75% (n = 110) of the samples assayed with primers targeting most haplotypes of R.i. (SSP, Table 4.4) yielded positive amplification signals. However, these signals were accompanied by a lot of noise (Fig. 4.10) which hampered clear separation of positive and negative signal clouds by threshold setting and did not vanish after dilution of samples (Appendix B, Fig. B.2).

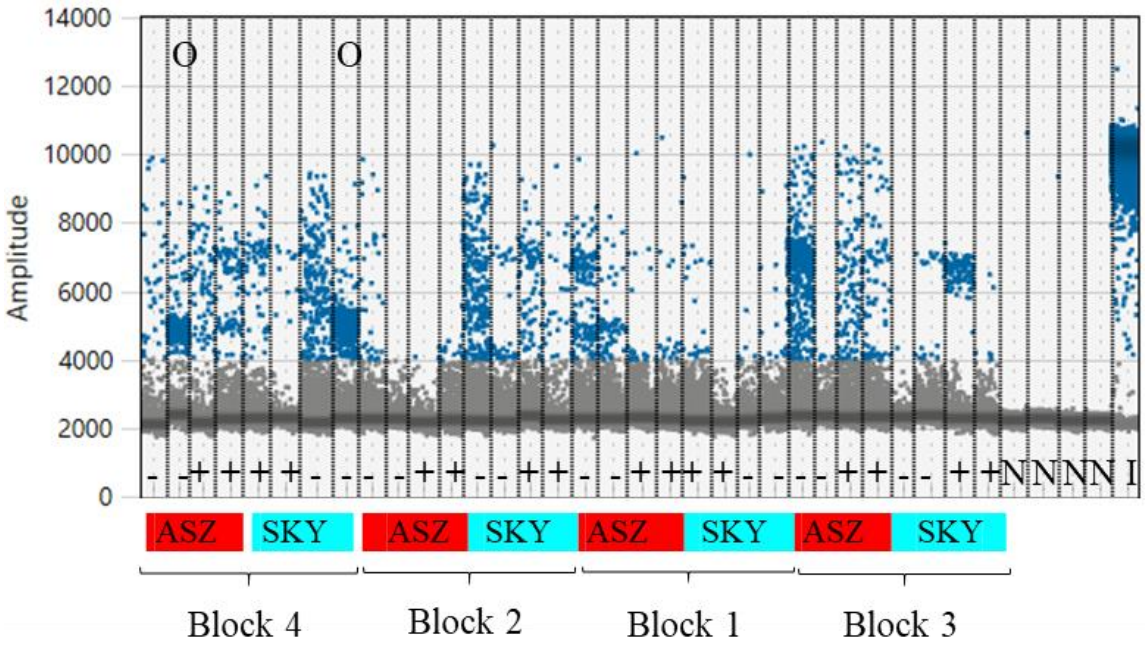


Fig. 4.10. Amplitude plots of ddPCR assays with species-specific primers for *Rhizophagus irregularis* in root samples of 'Aszita' (ASZ) and 'Skyfall' (SKY) at GS32 (May 2019) harvested from plots with (+) and without (-) AMF inoculation. No template controls (N) were run to exclude contamination, DNA from the AMF inoculum (I) served as positive controls. Outliers (O) were identified after plotting of statistical analyses results. Positive (blue) signals are distinguished from negative signals (grey) by a threshold at $\lambda = 4000$.

R.i-abundances varied greatly between samples which resulted in large standard errors in statistical analyses (Table 4.5). Despite these deviations, copy numbers of R.i. were significantly higher in the roots of Skyfall compared to Aszita ($p = 0.012$, Table 4.5).

Inoculum application appeared to lower R.i.-copy numbers, but this effect was only visible in the conventional crop protection treatment ($p = 0.036$)

Table 4.5. Effects of crop protection, variety and AMF inoculation on copy numbers of *Rhizophagus irregularis* (detected by the use of species-specific primers) in root samples from zero-input (without fertiliser and pesticides) plots at GS32 in May 2019. Numbers presented are means ± SE of the mean. ANOVA *p*-values in bold indicate significant main effects and interactions. Means within columns followed by the same letter are not significantly different at $p \leq 0.05$.

	Copy no. per gram sample
Crop protection (CP)	
Conventional (n = 16)	$8.2 \times 10^5 \pm 2.98 \times 10^5$
Organic (n = 16)	$2.4 \times 10^5 \pm 6.70 \times 10^4$
Variety (VR)	
Aszita (n = 16)	$3.0 \times 10^5 \pm 1.71 \times 10^5$ b
Skyfall (n = 16)	$7.5 \times 10^5 \pm 2.62 \times 10^5$ a
Inoculation (AMF)	
-AMF (n = 16)	$8.2 \times 10^5 \pm 3.00 \times 10^5$
+AMF(n = 16)	$2.3 \times 10^5 \pm 5.48 \times 10^4$
ANOVA *p-values*	
Main effects	
CP	ns
VR	**0.012**
AMF	*0.053*
Interactions	
CP:VR	ns
CP:AMF	**0.036**
VR:AMF	ns
CP:VR:AMF	ns

Non-inoculated plots with conventional crop protection showed significantly higher R.i.-abundances than samples from plots without crop protection (Table 4.6). Inoculation with AMF reduced R.i.-mtLSU copy numbers in sprayed plots but had no effect in plots without crop protection.

Table 4.6. Interaction means ± SE (n = 8) of AMF inoculation with impact of crop protection (CP) on copy numbers of *Rhizophagus irregularis* per gram root.

	- AMF inoculation	**+ AMF inoculation**
Conventional CP	$1.4 \times 10^6 \pm 5.27 \times 10^5$ Aa	$2.3 \times 10^5 \pm 6.97 \times 10^5$ Ab
Organic CP	$2.4 \times 10^5 \pm 1.06 \times 10^5$ Ba	$2.4 \times 10^5 \pm 8.95 \times 10^4$ Aa

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

Plotting of the interaction of crop protection and AMF inoculation revealed two samples with particularly high copy numbers from the same block (Fig. 4.11 A, circled). These two samples had already stood out in amplification plots with dense clouds of positive signals (Fig. 4.9, O-markings) and indicate location-specific variation in R.i abundances at the field site. Interestingly, these variations only occurred in non-inoculated plots and in both varieties (Fig. 4.11 B, circled), but not in the adjacent inoculated plots of the same variety.

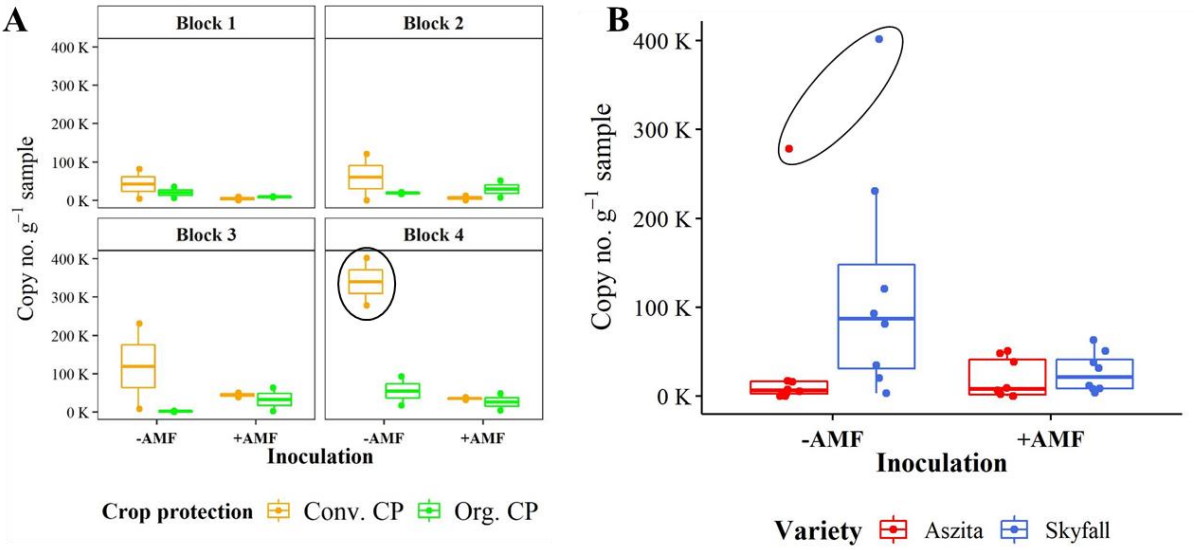


Fig. 4.11. Boxplots showing the interaction of: **A**) crop protection (conventional vs. organic) and AMF inoculation in four replicate blocks (n = 2) and **B**) variety and AMF-inoculation (n = 8) with impact on copy numbers of *Rhizophagus irregularis* in wheat roots. Circled data points mark outliers within the data set.

Exclusion of outliers did not reduce standard deviations but removed site-specific variations in R.i.-abundances in the statistical analyses as well as marginal main effects of AMF inoculum application (Table 4.7). ANOVA of the dataset without outliers showed the variety-dependent abundance of R.i. in root samples (p = 0.007). The same analyses showed an interaction between AMF inoculation and variety (p = 0.046) which was not significant when outliers were included (Table 4.6).

Table 4.7. Effects of crop protection, variety and AMF inoculation on copy numbers of *Rhizophagus irregularis* (detected by the use of species-specific primers) in root samples from zero-input plots at GS32 in May 2019 after removal of outliers. Numbers presented are means ± SE of the mean. ANOVA *p*-values in bold indicate significant main effects and interactions. Means within columns followed by the same letter are not significantly different at $p \leq 0.05$.

	Copy no. per gram sample
Crop protection (CP)	
Conventional (n = 14)	$4.5 \times 10^5 \pm 1.71 \times 10^5$
Organic (n = 16)	$2.4 \times 10^5 \pm 6.70 \times 10^4$
Variety (VR)	
Aszita (n = 15)	$1.4 \times 10^5 \pm 4.56 \times 10^4$ b
Skyfall (n = 15)	$5.3 \times 10^5 \pm 1.56 \times 10^5$ a
Inoculation (AMF)	
-AMF (n = 14)	$4.5 \times 10^5 \pm 1.76 \times 10^5$
+AMF(n = 16)	$2.3 \times 10^5 \pm 5.48 \times 10^4$
ANOVA *p-values*	
Main effects	
CP	ns
VR	**0.007**
AMF	ns
Interactions	
CP:VR	ns
CP:AMF	ns
VR:AMF	**0.047**
CP:VR:AMF	ns

Without inoculation, roots of Skyfall showed higher abundances of R.i. compared to Aszita whereas this effect vanished in plots with AMF application (Table 4.8). Copy numbers were significantly reduced in Skyfall treated with AMF inoculum, but no effect was observed in Aszita. This tendency was already indicated when outliers were still included (Fig. 4.11 B).

Table 4.8. Interaction means ± SE (n = 8) of two wheat varieties with impact of AMF inoculation on copy numbers of *Rhizophagus irregularis* per gram root sample.

Variety	**- AMF inoculation**	**+ AMF inoculation**
Skyfall	$8.3 \times 10^5 \pm 2.92 \times 10^5$ Aa	$2.7 \times 10^5 \pm 7.88 \times 10^4$ Ab
Aszita	$7.3 \times 10^4 \pm 2.67 \times 10^4$ Ba	$2.0 \times 10^5 \pm 7.91 \times 10^4$ Aa

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

4.3.5 CORRELATION OF ROOT COLONISATION AND RHIZOPHAGUS IRREGULARIS COPY NUMBERS IN WHEAT ROOTS

As higher AMF colonisation rates in Skyfall had been observed through microscopy assessments (Chapter 3, Fig. 3.3 C), it was questioned whether there was a connection of this observation and the mtLSU-copy numbers of R.i. A weak but significant (R = 0.39, $p = 0.027$) correlation of copy numbers with root colonisation assessments (Fig. 4.12 A) indicates a significant contribution of R.i. to mycorrhizal intensities in wheat roots at the assessed growth stage (Fig. 4.12 A). In more detail, copy numbers were correlated with vesicle (Fig. 4.12 B) and hyphae (Fig. 4.12 C) abundances, but not with arbuscule abundances (R = 0.32, p = ns). Correlation analyses of mtLSU-copy numbers with AMF colonisation parameters for Aszita and Skyfall separately were not significant (data not shown).

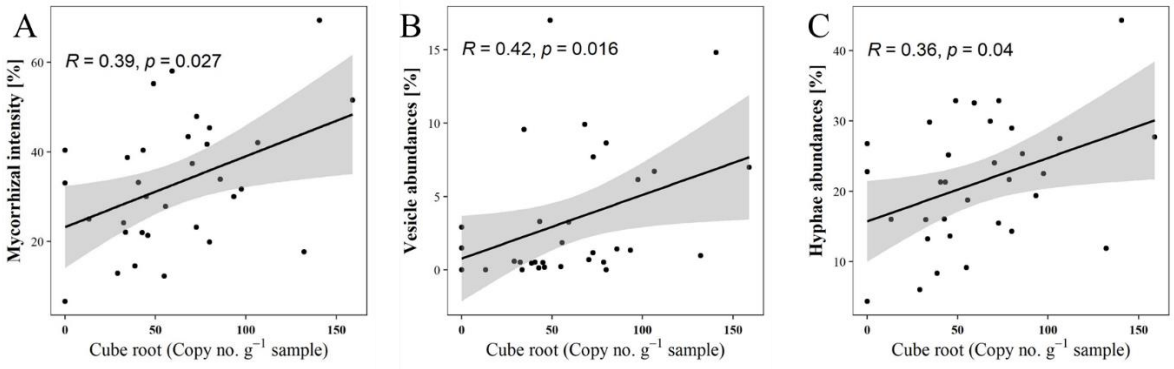


Fig. 4.12. Correlation analysis of **A**) mycorrhizal intensity (M%), **B**) vesicle (V%) and **C**) hyphae abundances (H%) with copy numbers of mtLSU per gram root sample of wheat at GS32 (May 2019) after ddPCR with species-specific primers targeting most haplotypes of *Rhizophagus irregularis*. Spearman's correlation coefficient *R* describes the relationship of AMF root colonisation parameters and cube-root transformed mtLSU copy numbers. This relationship is fitted as a linear model (black line) with 95% confidence intervals (grey shading) and is significant for $p \leq 0.05$.

4.3.6 ABUNDANCE OF NATIVE RHIZOPHAGUS IRREGULARIS STRAINS IN FIELD SOIL

Soil samples from before and during field trials assessed with the SSP assay showed positive signals which indicates that R.i. was present at the field site (Fig. 4.13). There was no clear pattern of signals from inoculated and uninoculated plots as positive signal yield was generally low. An interesting observation was found in one sample (block 4, OCP, -AMF) which showed a double band when run with the SSP assay. In contrast to other soil samples, this sample had not been diluted during the ddPCR assay as DNA yield was lower compared to the extremely high yields from other soil samples (>1000 ng μl^{-1}). Reducing the dilution factor from 100 to 50-fold yielded more positive signals in the same soil samples and reproduced the double band for this samples but did not result in similar patterns in other soil samples (Fig. 4.13 B).

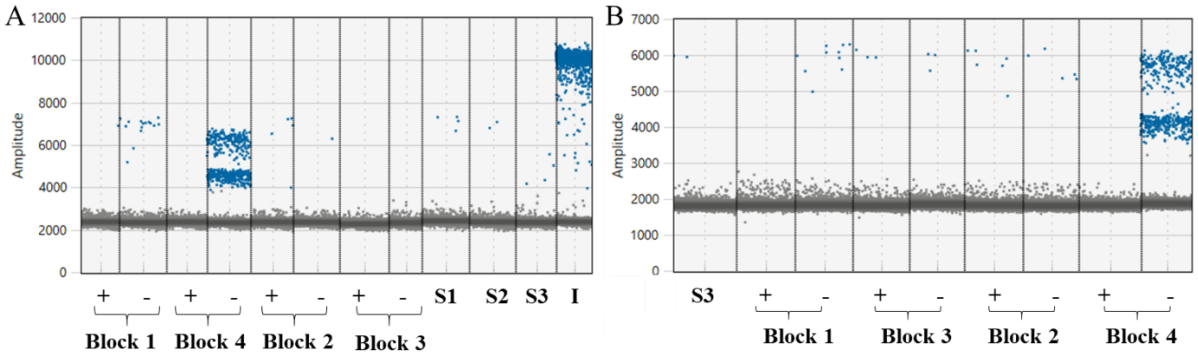


Fig. 4.13. Amplitude plots of ddPCR assay with species-specific primers for *Rhizophagus irregularis* in soil samples from the experimental field site before inoculation (S1-S3) and sampled from inoculated (+) and uninoculated (-) plots during field trials. **A**) 100-fold dilution except block 4, -AMF. DNA from AMF inoculum (I) was used as positive control. Threshold was set at λ = 4000 and **B**) 50-fold dilution except block 4, -AMF. Threshold was set at λ = 3000. Positive signals are marked as blue droplets, negatives in grey.

4.4 DISCUSSION

The increases in market share of microbial products/biostimulants (Sessitsch *et al.*, 2018) requires the need to clarify the fate and side effects of the applied microbes on the environment (Schwartz *et al.*, 2006; Rodriguez & Sanders, 2015; Hart *et al.*, 2018). This clarification was the objective of the present study that aimed to trace specific strains based on the mitochondrial DNA (mtDNA) polymorphisms of an isolate of *Rhizophagus irregularis* (R.i.) which had been introduced during field trials to observe potential effects on plant growth and performance. The application of newly designed haplotype-specific primers (HSP) showed only a few amplification events that would indicate successful establishment of the AMF inoculum strain in the field. More responses were gained from application of species-specific primers (SSP)

that amplified most R.i-strains in the field. These results revealed negative effects of AMF inoculation on native R.i. populations inside wheat roots of the variety ‘Skyfall’ which had shown overall higher abundance of this fungal species. Further research is required to validate these observations since they were based on analyses of only one time point and were potentially biased by signal interferences during digital droplet PCR (ddPCR) assays.

4.4.1 NOVEL MARKERS FOR QS81-LIKE HAPLOTYPES

When it comes to the tracing of commercially produced AMF inocula in field environments, it is often necessary to distinguish to the isolate level as these products usually contain cosmopolitan species which might already be present in the soil (Antoine *et al.*, 2021). Therefore, the target haplotype needs to be compared to a broad range of other haplotypes to exclude amplification of native strains of the same species (Kokkoris *et al.*, 2019a). Ideally, the local haplotype is known e.g. from root organ cultures (ROC) and can be excluded directly via strain-specific primer design, but this step is impeded by obstacles of AMF *in-vitro* cultivation (Declerck *et al.*, 2005) and the great diversity among haplotypes that has been described for R.i. (Koch *et al.*, 2004; Börstler *et al.*, 2008, 2010). These variations were found in samples from all over the world, therefore it was surprising to observe almost identical sequences among the selected cultures of isolates with different geographical origin that were used in this study (Table 4.1). Although identical isolates of the same strain have been found in fields of 30 km distance (Formey *et al.*, 2012), there is a low chance to extract the same haplotype of the mitochondrial large subunit (mtLSU) from soils in Mayotte and Germany. Moreover, it is dubious that the selection of R.i.-isolates turned out to be highly similar or identical within the respective labs (Fig. 4.3). Cross-contamination of strains might be an explanation for this observation and is not unusual during propagation and sub-culturing processes (Walker & Vestberg, 1994; Vosátka *et al.*, 2012). As shown in many dispersal studies, AMF are highly mobile and spread easily through the air (Chaudhary *et al.*, 2020). Therefore, to prevent cross-contamination of strains in open-production facilities for AMF inoculum requires strict control measures (von Alten *et al.*, 2002; Declerck *et al.*, 2005). With accumulating proof of diverging functionality among mycorrhizal strains (Farmer *et al.*, 2007; Peña *et al.*, 2020), regular quality controls of AMF inoculum should involve sequencing approaches to confirm the propagation of the target strain. The oligo targets identified and used in this study and their specificity demonstrated by PCR (Fig. 4.4 A) and ddPCR (Fig. 4.5) represent suitable tools for this purpose with QS81-like isolates. In fact, the primers could probably be used even without Taqman-probes which would reduce costs on the one hand, but might lower assay specificity at the same time.

Alignment with several library strains aimed to compensate for the lack of genetic variation that was found between the selected isolate cultures. At the same time, this step of the analysis showed that strains like MUCL41833 harbour the targeted C-indel and would hence be amplified if present in the experimental environment. Even though this strain originated from the Canary Islands (Table 4.3) and the haplotype was proven to be absent from the field site before inoculation (Fig. 4.6), the new markers target a non-unique polymorphism which represents a limitation for their applicability. On the other hand, the primers could also be applied in trials with MUCL41833 which has shown yield increases in potato following cover crop inoculation (Buysens *et al.*, 2016). The authors of the study established qPCR assays with new primers for this haplotype (Buysens *et al.*, 2017; Alaux *et al.*, 2018), but mapping of their markers in the alignment of the present analysis detected their selected target sequence in the L-lines from Czechia (data not shown). Therefore, the oligo targets described in this study might be more suitable for the tracing of MUCL41833 as their specificity is based on a single nucleotide polymorphism. This observation emphasises that strain-specificity of molecular markers is not only limited by the presence of native AMF strains, but also depends on the reference sequences that are aligned with the target sequence during primer design. These issues are considerations that need to be tackled by thorough *in-silico* and *in-situ* testing when it comes to the design of universally applicable primers for the tracing of promising AMF strains that would save costs and time in future inoculation studies (Corradi & Bonen, 2012). The new molecular markers for the QS81-haplotype provide promising traits for this purpose, but ddPCR runs for strain specificity showed that non-target amplification can occur with these primers too (Fig. 4.5 A). Therefore, all steps described in this study would need repetition and comparison with more mtLSU-haplotypes and other AMF species to guarantee sole amplification of the targeted R.i.-isolate.

4.4.2 AMF INOCULUM PERSISTANCE UNDER FIELD CONDITIONS

The application of HSP in ddPCR assays did not show clear responses that would indicate the presence of the targeted QS81-haplotype in wheat roots from plots which had been inoculated with the same R.i. strain at the beginning of each field trial. Here, the previously discussed indications for cross-contamination of strains raise the question if the applied root powder actually contained the target strain QS81. This was confirmed by using DNA extracts from the same AMF inoculum as positive control in normal PCR and during ddPCR assays with HSP (e.g. Fig. 4.7 A and Fig. 4.8 B). The R.i. strain QS81 was also detected in the inoculum-sand mix that had passed through the hopper during drilling which proved that the inoculum was delivered to the soil, but there was little to no indication for the presence of the inoculum strain

in environmental samples.

At the mainly assessed time point of GS32 in May 2019, positive signals were scarce in root samples and could not be clearly assigned as true amplification of the QS81-haplotype because signals were also derived from non-inoculated plots. Although it has been recommended to ignore counts of one to four droplets in ddPCR results (Witte *et al.*, 2016; Kokkoris *et al.*, 2021a), these signals could be signs for non-target amplification or indicate that inoculated fungi spread to non-inoculated plots (Janoušková *et al.*, 2017; Kokkoris *et al.*, 2019a). This spreading is not excluded in the experimental design where plots with and without inoculation were only 1 m apart in the field and AMF hyphae can grow 3.2 m yr^{-1} (Powell, 1979). However, extensively spreading extra-radical mycelium would indicate competitiveness of the introduced AMF strain (Janoušková *et al.*, 2017) and since signals were overall rare in root samples from inoculated plots it is more likely that the QS81-haplotype was not present in wheat roots from this growth stage. Moreover, extensive mycelium of the inoculated strain should have been detectable in soil samples from the same time point, but these showed no amplification (Fig. 4.6). Then again, only a few soil samples were analysed and amplification of mycorrhizal DNA in soil samples can be hampered due to excess DNA of other soil organisms or the presence of PCR inhibitors (Barceló *et al.*, 2020). Hyphal traps for the collection of pure fungal material in the root zone could have been used to circumvent these obstacles, but the method was not used in this project.

Interestingly, the few observed positive droplets tended to appear in samples from the same location (Fig. 4.8 and Fig. 4.9). In one of the samples from an earlier growth stage (GS22, tillering), a population of clear positives was observed, whereas no signals occurred in non-inoculated samples (Fig. 4.8 B). The signals were reproducible (data not shown) and derived from the same plot that showed positive signals also at GS32 (plot 131, Fig. 4.9 B). These findings suggest site-specific establishment of the AMF inoculum (Farmer *et al.*, 2007; Kokkoris *et al.*, 2019a), however on a very low level. Amplification success with HSP in one sample is not sufficient to speculate that the AMF inoculum colonised wheat roots earlier in the season and ultimately did not persist until later growth stages. To prove this hypothesis would require analyses involving more samples from earlier time points, especially from seedlings. The few samples that were available from this growth stage had shown very low DNA concentrations which could explain why no amplification occurred (Fig. 4.8 B). Nevertheless, high sensitivity of ddPCR would most likely detect traces of the target R.i.-haplotype if the AMF inoculum had established after six weeks or was still present from the first growing season.

According to INOQ, the AMF inoculum contained the R.i.-strain MA1 besides *Funneliformis mosseae*, *Funneliformis caledonium* and R.i.-strain QS81 (Chapter 3, Table 3.5). The ratios of the respective R.i.-strain however were not stated in the product description. Sequences of MA1 could not be used for primer design due to the lack of characteristic polymorphisms in the mtLSU of this strain (Fig. 4.3). Hence, sequencing of other mtDNA genes such as the cox3-rnl intergenic region (Kokkoris *et al.*, 2019a) would be required to identify suitable markers for this isolate. For the present study this means that the establishment of MA1 in the field could not be validated, but since QS81 did not persist in wheat roots and was not present in the soil it is questionable if MA1 was able to do so.

Although the limited performance of the AMF inoculum is not convincing from a practical point of view, this is not an uncommon observation (Buysens *et al.*, 2017; Alaux *et al.*, 2018) and represents an obstacle for large-scale application of biostimulants in agriculture (du Jardin, 2015; Hart *et al.*, 2018). Besides agronomic factors like high nutrient content (Mäder *et al.*, 2000a), the use of pesticides (Rivera-Becerril *et al.*, 2017) or tillage practices (Jansa *et al.*, 2002) that hamper AMF symbiosis, there are numerous reasons that explain why the AMF inoculum did not establish in the field which will be elaborated in the General Discussion (Chapter 7).

4.4.3 IMPACT OF INOCULATION AND HOST GENOTYPE ON NATIVE AMF

It cannot be completely excluded that the viability of the applied inoculum was reduced during transportation or storage, but it is more likely that competition with natural AMF that occupy the same ecological niche hampered establishment of the introduced strains (Krak *et al.*, 2012; Janoušková *et al.*, 2017; Niwa *et al.*, 2018). This hypothesis gained more support with the results from ddPCR assays using species-specific primers (SSP) that amplified also the native R.i strains. With site-specific variations, the analyses conducted revealed high abundances of this fungal group in both wheat roots (Fig. 4.10) and field soil (Fig. 4.13) and showed that it contributed significantly to AMF root colonisation at the assessed time point (Fig. 4.12). Here, higher abundances in the modern wheat variety Skyfall compared to Aszita indicate host genotype-specific root colonisation behaviour of R.i. in wheat which has been demonstrated in many other studies (e.g. Hetrick *et al.*, 1992; Lehnert *et al.*, 2017; Elliott *et al.*, 2019). More intriguingly was the impact of AMF inoculation on R.i. in the two contrasting wheat varieties. Although it was not possible to detect AMF inoculum strains in soil or root samples (Section 4.4.2), inoculation with AMF affected abundances of local R.i. strains. This effect was first indicated in two samples from the same block where both wheat varieties showed lower copy numbers in inoculated plots compared to extremely high mtLSU copy numbers of R.i. in

plots without AMF inoculation (Fig. 4.11 A). These site-specific variations of R.i. abundances in the field were also visible in ddPCR assays with soil samples and SSP (Fig. 4.13) but could not be linked to the field history. Removal of the two outlier samples validated the effect of AMF inoculation leading to decreased mtLSU copy numbers of local R.i. strains in Skyfall (Table 4.8). As there were indications for the presence of the AMF inoculum strain QS81 at earlier growth stages (Fig. 4.8 C), the observed decreases of native R.i. strains could indicate that the R.i. inoculum strain occupied the ecological niche at earlier growth stages of R.i. but then withdrew from wheat roots as part of seasonal species dynamics as described by Gao *et al.* (2019). In their study, R.i. predominated among fungal communities identified in sorghum roots and the rhizosphere until six weeks after seeding, but then declined in favour of other AMF species. Their results from field trials are in line with *in-vitro* experiments on the colonisation behaviour of R.i. by Hart *et al.* (2001) which classified Glomeraceae as fast root colonisers. These reports suggest that decreased copy numbers of R.i. were attributable to the inability of the native R.i. strains to replace the inoculum strain inside wheat roots of Skyfall after colonisation by the inoculum strain QS81 declined. But this assumption is opposed to the correlation of mtLSU copy numbers of R.i. with microscopy data that confirmed significant contribution of R.i. to the AMF root colonisation intensities at the assessed time point (Fig. 4.12). Moreover, proof for co-colonisation of both native and inoculated R.i. strains would require proof of inoculum establishment in the first place, but this was not confirmed in the present analyses.

It is more likely that the declines in mtLSU copy numbers of native R.i. were due to intra-specific competition of AMF in the rhizosphere that has been shown to result in reduced root colonisation (Wilson, 1984; Koch *et al.*, 2011; Symanczik *et al.*, 2015; Niwa *et al.*, 2018; Rocha *et al.,* 2019a). Krak *et al.* (2012) showed that co-inoculation of two R.i. strains significantly reduced mtLSU copy numbers in plant roots compared to single inoculation treatments. Janoušková *et al.* (2013) observed the same phenomenon as a consequence of AMF inoculation into an established fungal community which did not only have detrimental effects on AMF root colonisation, but also on growth of *Medicago sativa*. They related these effects to oversaturation of propagules which might have happened also in the present field trial where a potent AMF inoculum with high amounts of propagules was added to an agroecosystem with rich soil life. The results of this experiment demonstrate that the competitive potential of an inoculum can vary between host plant genotypes since the inoculum had no effect on native R.i. strains in roots of Aszita (Table 4.8).

A study by Elliott *et al.* (2019) found only positive effects among three wheat varieties

including Skyfall in response to inoculation with a commercial R.i.-strain grown in a non-sterilised field soil under greenhouse conditions. Their reports are contrary to the observed interactions in the present study of native and exogenous AMF which underlines the differences among soil ecosystems and consequently varying effectiveness of commercial AMF inocula (Schwartz *et al.*, 2006; Hart *et al.*, 2018; Thomsen & Hart, 2018). A recommendation to test biostimulant products under field conditions seems obvious, but the present findings reveal once more the complexity of agronomic and environmental conditions that affect inoculation success with AMF (Verbruggen *et al.*, 2013). At the same time, the analyses show how ddPCR can be used to depict these complex interactions which will be useful for future inoculation studies that have access to this tool.

4.4.4 DDPCR FOR ANALYSES OF AMF COMMUNITIES IN ENVIRONMENTAL SAMPLES

After the proven robustness of ddPCR in medicinal studies (Sanders *et al.*, 2011), the results of assays with strain-specific primers (SSP) showed that protocols with environmental samples still require optimisation. This was attempted by adjusting e.g. sample dilution and cycling conditions, but unspecific signals from negative droplet clouds still occurred (Fig. 4.10). Therefore, it has to be taken into account that the previously discussed effects of inoculum and host genotype on mtLSU copy numbers of native R.i. could be biased since it was not possible to separate clear negative and positive signals. If not set properly, too high or too low thresholds might include non-specific amplification or exclude real positives which ultimately affects copy number counts and the deduced treatment effects (Witte *et al.*, 2016; Kokkoris *et al.*, 2021b). The most common signal interferences described for ddPCR assays are descending droplets from positive signal clouds which have also been termed as ‘rain’ (Witte *et al.*, 2016). Rain was also found in the present study but only in assays with SSP and pure fungal DNA samples (Fig. 4.5 B). The predominant form of noise observed in the current study however might represent the opposite to rain and will be further referred to as ‘dust’. These clouds of droplets rising from negative signal clouds occurred in most root samples run with SSP and have not previously been described in the literature. In fact, even the patent holder and producer of ddPCR was not able to give advice in this matter (Bio-Rad, pers. communication). Ten-fold dilution of samples resolved the issue partially but did not facilitate ideal droplet separation (Appendix B, Fig. B.2). The reproducibility of noise signals indicates actual amplification in these droplets that is likely derived from non-target sequences such as other microbial or plant DNA (Dreo *et al.*, 2014). Lievens *et al.* (2016) point out that specific primer design is crucial to exclude non-target amplification which might explain why dust in environmental samples

only appeared with SSP but not with HSP. This pattern was reversed looked different in culture samples where dust appeared only with HSP and here mostly in the L-lines (Fig. 4.5 A) which harboured the same variable region as the QS81-haplotype but without the C-indel (Fig. 4.3). The absence of both amplification and noise in ddPCR assays with environmental samples and HSP hence indicates that neither QS81-like haplotypes nor R.i.-haplotypes similar to the L-lines were present at the field site. PCR inhibition due to chemical composition of samples can be excluded since spiking with DNA from root powder clearly detected the targeted haplotype as compound of the AMF inoculum (Fig. 4.7 B). Thus, interference in form of dust in ddPCR might occur when the target as well as highly similar sequences are abundant in high concentrations.

Increasing annealing temperatures has been suggested to enhance primer specificity (Lievens *et al.*, 2016; Witte *et al.*, 2016) while Demeke and Dobnik (2018) recommend lowering annealing temperatures to reduce noise. In the present study, all ddPCR assays were run with the same annealing temperature (52°C). However, temperature gradient assays had shown multiple optimal annealing temperatures (Appendix B, Fig. B.1. A, B), so potential adjustments might change the results from assays with root samples. Another explanation for the occurrence of intermediate droplet signals could be found in the fact that our primers targeted circular mtDNA which delayed fluorescence signals in digital PCR studies when no restriction enzymes were applied in advance (Sanders *et al.*, 2011). Pre-digestion of plasmids is recommended by Bio-Rad and was found to reduce intermediate fluorescent signals in ddPCR (Demeke & Dobnik, 2018). To prevent this issue, we used an extended PCR programme with 44 cycles which should provide sufficient time for denaturalisation and amplification of the target DNA (Kokkoris, *et al.*, 2021b). Increasing cycle number has also been suggested to reduce noise in ddPCR (Lievens *et al.*, 2016), but this was not the case in the present study. Programmes with less cycles and without pre-treatment of mtDNA worked for the ddPCR assays of Kokkoris *et al.* (2019a) and other studies using qPCR for molecular tracing of AMF inoculum under field conditions (Buysens *et al.*, 2017; Alaux *et al.*, 2018). This suggests that the reason for dust is not related to the structure of mtDNA, but to the specificity of the applied primers.

In contrast to root samples, ddPCR with soil samples and SSP showed no signs of noise which is contrary to protocols from Barcelo *et al* (2020) who describe several cleaning steps prior to successful amplification of mycorrhizal DNA in soil samples. The role of PCR inhibitors in ddPCR has been assessed for clinical samples (Dingle *et al.*, 2013), but not for environmental samples. Therefore, it cannot be excluded that root samples contained compounds that inhibited

PCR and ultimately delayed fluorescent signals, hence underestimating actual presence of R.i. in this sample type. Then again, the only undiluted soil sample yielded the highest numbers of positive signals in the form of a double band (Fig. 4.13) which was also indicated in some root samples (Fig. 4.10). Double bands have been described in previous studies as off-target amplification due to sequence variations that create different fragment lengths and ultimately intermediate fluorescence (Lievens *et al.*, 2016; Jacchia *et al.*, 2018). These studies also showed that double bands can be eliminated by adjustment of annealing temperatures, but this was not tested in the present study.

In summary, repetition of ddPCR assays with altered annealing temperatures might be the first step to optimize the application of the here designed SSP targeting R.i. in environmental samples. If this does not reduce signal interference, it is most likely that dust and double bands were caused by amplification of other R.i.-haplotypes that were present at the field site (Börstler *et al.*, 2010). Hence, the application of more specific primers for native R.i.-haplotypes probably represents the best option to reduce ddPCR interferences in this case. To gain insight into local R.i.-mtLSU haplotypes would be facilitated by sequencing of amplicons from non-inoculated plots which had shown faint bands during primer specificity tests after PCR with SSP (Fig. 4.4 B). Based on these sequences it would be possible to design haplotype-specific primers for native R.i. strains. This approach would also represent a more targeted approach to distinguish between local and exotic strains than using general R.i.-primers, but it is not guaranteed that all native R.i.-haplotypes can be revealed.

4.5 CONCLUSIONS

The main objective of this chapter was to assess if the AMF inoculum strain QS81 colonised wheat roots during field experiments in the second year. Application of newly designed molecular markers targeting the mitochondrial large subunit (mtLSU) with high specificity for this haplotype of *Rhizophagus irregularis* (R.i.) showed that the strain was neither present in the field before inoculation, nor after inoculation in the soil or in roots when highest mycorrhizal colonisation occurred. Here, native R.i. strains contributed significantly to the fungal community inside wheat roots in which Skyfall showed overall higher R.i abundances than Aszita. Genotype-specific effects were also visible in response to AMF inoculation. Although only one sample from an earlier growth stage indicated presence of the inoculum strain, mtLSU copy numbers of native R.i. declined when Skyfall had been inoculated with AMF harbouring root powder. Intra-specific competition due to excess propagule numbers after inoculation in an established fungal community could be the reason why the inoculum did not establish in the field and hampered native R.i. from colonising wheat roots. These detailed observations of host-

genotype dependent microbial interactions were only possible by total quantification of molecular signatures of R.i. using digital droplet PCR (ddPCR), but further optimisation steps as well as analyses of more samples from different time points are required to validate the results from the present analyses.

Overall, the new molecular markers that result from this study will be useful in future experiments that involve the same R.i.-haplotype as used for field inoculation in this project. Their application will aid to elucidate the fate of AMF inocula in the environment and ultimately assist to improve the effectiveness of biostimulants and their application in sustainable agriculture. The results described in the current study however do not support the use of such products in wheat, especially if native AMF are already present in the field.

CHAPTER 5. ENDOPHYTIC FUNGAL COMMUNITIES IN WHEAT ROOTS UNDER THE IMPACT OF DIFFERENT AGRICULTURAL MANAGEMENT PRACTICES

5.1 INTRODUCTION

Barcode sequencing of nuclear ribosomal DNA (nrDNA) is a common tool for the analysis of microbiomes and has revealed how AMF are strongly affected by agronomic management practices in agricultural soils. Besides tillage (Jansa *et al.*, 2002) and crop rotation (Sommermann *et al.*, 2018), particularly nitrogen (N) (Egerton-Warburton *et al.*, 2007; Williams *et al.*, 2017) as well as phosphorus (P) inputs (Hijri *et al.*, 2006; Gosling *et al.*, 2013) are considered to shape mycorrhizal community structure. In contrast, there are only few studies such as in onion (Gosling *et al.*, 2016) and wheat (Mao *et al.*, 2014) that report host genotype-specific AMF associations. But there are several studies that used next generation sequencing (NGS) to demonstrate that some AMF species are more resistant to specific conditions than others while positive and negative growth responses of crops have been associated with the abundance of certain species (Dai *et al.*, 2014; Bender *et al.*, 2019). Hence, molecular analyses of the fungal microbiome should supplement phenotypic data in field experiments as it can harbour crucial information about the functionality of agroecosystems (Rodriguez & Sanders, 2015). With the increasing interest for mycorrhizal biotechnology in crop production, NGS has been suggested as a tool for the assessment of quality and purity of commercial AMF inocula (Vosátka *et al.*, 2012). However, the outcome of NGS studies of AMF highly depends on the molecular marker used (Hart *et al.*, 2015). A popular marker for mycobiomes, but due to its low species resolution often criticised in the context of AMF is the internal transcribed spacer region (ITS) (Schoch *et al.*, 2012; Lekberg *et al.*, 2018). The discussion around this marker derives from its hypervariability especially in the ITS1 region which is shorter than the more conservative ITS2 region (Thiéry *et al.*, 2012). Therefore, most phylogenetic classifications of AMF have been based on sequence variations of the small subunit (SSU) nrDNA (Öpik *et al.*, 2010). This marker was used for the novel classification of fine root endophytes (FRE) within the sub-phylum Mucoromycotina (Orchard *et al.*, 2017a; Walker *et al.*, 2018). This group of fungi has recently gained new attention in terms of plant evolution (Field *et al.*, 2015b), but also regarding symbiotic functioning with potential application in crop production (Sinanaj *et al.*, 2020). Colonisation structures of FRE in root samples of the present study were observed during microscopy assessments which suggests that Mucoromycotina were abundant at the field site, but their presence remains yet to be confirmed by molecular analyses. At the same time, these studies represent the first approach under field

conditions to investigate host genotype-specificity of FRE in wheat with the impact of fertiliser application. The latter is likely to affect Mucoromycotina according to greenhouse studies which showed similarities between AMF and FRE in response to nutrient inputs (Albornoz *et al.*, 2021). In the present study, great variations of mycorrhizal colonisation following mineral nitrogen (N) fertiliser application raised the hypothesis that Mucoromycotina FRE might be less affected by mineral N than Glomeromycotina (Chapter 3, Section 3.4.5). Overall, the presence of FRE besides AMF in wheat opens new perspectives to answer crucial ecological characteristics of FRE for which reason these fungi cannot be ignored during mycobiome analyses.

5.1.1 AIMS AND OBJECTIVES

This chapter describes three amplicon sequencing approaches targeting different marker regions on the nuclear ribosomal DNA (nrDNA) using Illumina MiSeq technology. The first dataset was produced from *Rhizophagus irregularis* (R.i.) isolates which had been used for strain-specific primer design (Chapter 4, Table 4.1). These samples were analysed to compare phylogenetic segregation based on mitochondrial large subunit (mtLSU) analysis to nrDNA. Additionally, two samples from the AMF inoculum (INOQ Advantage, INOQ GmbH) were sequenced to validate the identity of the three AMF species that had been applied during field trials (Chapter 3, Table 3.5). The same sequencing run included DNA from a selection of root samples to gain preliminary information on the presence of AMF phylotypes in the field trials reported previously without assessing the impact of agricultural management.

The second approach targeted the AMF community in roots where the highest root colonisation was detected (at stem elongation, GS32) during the second field season 2019. These samples were analysed to investigate the impact of conventional and organic crop protection, fertiliser type and wheat variety on composition of the mycobiome. For the latter factor, it was hypothesised that Aszita and Skyfall from organic and conventional breeding backgrounds respectively show distinct patterns in their mycorrhizal community composition.

From the same growth stage (stem elongation, GS32, 2019), a subset of samples was extracted from roots for the third sequencing approach that focused on the relative abundance of FRE in comparison to AMF. This dataset was further examined to evaluate how these two groups were influenced by mineral nitrogen (N)-fertiliser application and wheat variety. In summary, the objectives of this chapter were:

1. Investigate genetic segregation of R.i. isolates used for primer design based on nrDNA
2. Validate AMF species identity in the root powder used for inoculation in the field

3. Gain insights into AMF communities present inside wheat roots
4. Characterise the impact of fertiliser (organic vs. mineral), variety and crop protection on mycorrhizal communities
5. Acquire information on the FRE community present at the field site and investigate the impact of N-application and wheat variety.

5.2 MATERIALS AND METHODS

The three sequencing approaches targeted three different markers associated with fungal nuclear ribosomal DNA (nrDNA). For objective 1-3 and in cooperation with Dr Jan Jansa at the Institute of Microbiology in Prague (MBU, Czech Academy of Science), a nested PCR was conducted to amplify the ITS2-region of AMF using primer mixes according to Krüger *et al*. (2009) in the first run and ITS4 and AM 5.8S in the second run (Fig. 5.1, green). For objective 4 and in cooperation with the Geomicrobiology group of Prof. Neil D. Gray and Prof. Ian Head at Newcastle University, more general primers were applied in a single endpoint PCR that targeted the ITS1 region of most fungal groups (Fig. 5.1, blue). For objective 6 and in cooperation with Prof. Gary D. Bending and Dr Sally Hilton at Warwick University, selected root samples were run with primers targeting the small subunit (SSU/18S nrDNA gene) of AMF that would also include FRE (Fig. 5.1, red).

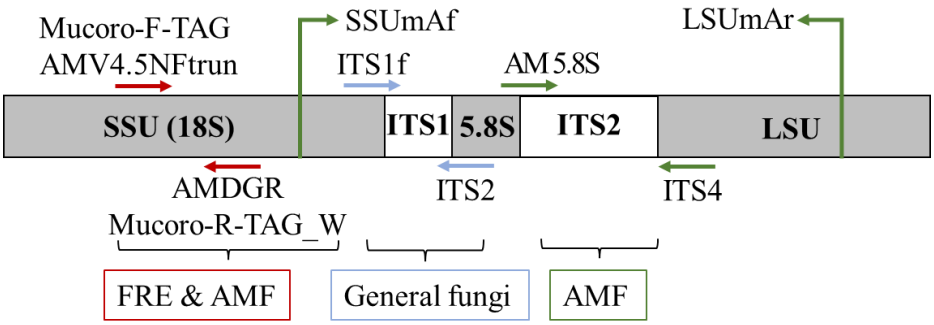


Fig. 5.1. Position and orientation of primers (arrows) and Krüger-primer mixes (bent arrows) for the amplification of the small subunit (SSU/18S gene, red) of fine root endophytes (FRE) and arbuscular mycorrhizal fungi (AMF), internal transcribed spacer region 1 (ITS1, blue) and mycorrhizal specific ITS2 region (green) were used for tagged amplicon sequencing of the nuclear ribosomal DNA.

5.2.1 ITS2-SEQUENCING OF AMF COMMUNITIES, RHIZOPHAGUS IRREGULARIS ISOLATES AND ROOT POWDER SAMPLES

To gain the preliminary insights into AMF communities at the field site, DNA was extracted from a selection of root samples from both wheat varieties (Aszita and Skyfall) without fertiliser or conventional crop protection and from three different sampling time points: the last sampling

of the first growing season (grain ripening, GS90, 2018) and the subsequent first two sampling points of the second growing season (seedling growth, GS12 and tillering, GS22, 2019) (Chapter 3, Table 3.6). Roots were ground with liquid nitrogen using a sterilised mortar and pestle. Samples were lysed in heated SLS buffer (65°C, 45 min) from the innuPREP Plant DNA extraction kit (Analytik Jena, Germany). Subsequent DNA extraction steps were followed according to the manufacturer's instructions. DNA was eluted from the filter tube in two centrifugation steps with the addition of 50 µl of elution buffer onto the membrane. DNA yields and quality were determined by spectrophotometry (Synergy HT, BioTek, USA) and by gel electrophoresis using ethidium bromide stained 1 %- agarose gels.

To assess strain segregation of the *Rhizophagus irregularis* (R.i.) isolates that were used during primer design (Chapter 4, Table 4.1), DNA was extracted from root organ cultures (hyphae and colonised roots) using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) as described in Section 4.2.1. To validate species identity in the AMF inoculum used for field inoculation, one gram of root powder from the production of *Funneliformis mosseae* and *Funneliformis caledonium* inoculum (INOQ GmbH, Germany) was ground using a ball mill (MM200, Retsch, Germany). DNA was extracted using the same kit and protocol as for R.i. isolates described in Section 4.2.1.

All samples were amplified using a nested PCR approach targeting the ITS region of Glomeromycota (Krüger *et al.*, 2009). For the first reaction, 2.5 µl of each primer mix (SSUmAf and LSUmAr, Table 5.1) were added at 1 µM equimolar concentration to a master mix containing 12.5 µl of 2xPPP Combi (TopBio, Czechia) with 4% proof-reading Pfu Polymerase (ThermoFisher Scientific, USA), 5.5 µl ddH_2O and 2 µl template DNA. After initial denaturation for 5 min at 95°C, 38 cycles were run starting with 95 °C for 45 s for polymerase activation, followed by annealing for 1.5 min at 58°C and elongation at 72°C for 3 min. The reaction was terminated with final extension at 72°C for 10 min. Based on the band intensities during gel electrophoresis (1% agarose, 10 % ethidium bromide), the purified amplicons (QiaQuick PCR purification kit, Qiagen, Germany) were used either diluted (1:10 or 1:100) or undiluted in the subsequent PCR step.

The second PCR was run with 1.5 µl of the tagged primer AM 5.8S and ITS4 (Table 5.1) at 0.5 µM concentration in a master mix containing 12.5 µl of 2x PPP Combi, 8 µl ddH_2O and 2 µl of DNA template. The PCR conditions were set to initial denaturation at 95 °C for 5 min, followed by 20 cycles starting with 30 s at 95°C for polymerase activation, followed by annealing for 10 s at 58°C and elongation for 1 min. The reaction was terminated with a final extension for 10 min at 72°C. The PCR products were checked on agarose gels with the

expectation of a band between 350 and 400 bp. Amplicons were purified as described previously and then fused with flagged primers by applying 1.25 µl (10 µM) of each primer to a master mix containing 12.5 µl 2xPPP Combi, 8 µl ddH_2O and 2 µl of DNA template in a PCR with the following programme: 5 min of initial denaturation, 10 cycles of 30 s at 95°C, 10 s at 58°C and 30 s at 72 °C. The reaction was terminated with final extension for 10 min at 72°C. The resulting amplicons were purified and concentration was measured by PicoGreen fluorescence (Quant-iT, Invitrogen, USA) before samples were sent for sequencing on a 2x300 bp platform (Illumina MiSeq).

Table 5.1. Primer mixes and primers used for the amplification of the ITS2 region of mycorrhizal nrDNA in a nested PCR approach.

Primer	Sequence 5'- 3'	Product	Reference
	1st PCR reaction		
SSUmAf	TGGGTAATCTTTTGAAACTTYA TGGGTAATCTTRTGAAACTTCA	~ 1800 bp	Krüger *et al.*, 2009
LSUmAr	GCTCACACTCAAATCTATCAAA GCTCTAACTCAATTCTATCGAT TGCTCTTACTCAAATCTATCAAA GCTCTTACTCAAACCTATCGA		
	2nd PCR reaction		
AM 5.8S	TCGCATCGATGAAG AACG	~ 350-400 bp	Řezáčová *et al.*, 2019
ITS4	TCCTCCGCTTATTGATATGC		White *et al.*, 1990

5.2.2 ITS1-SEQUENCING OF THE FUNGAL MICROBIOME IN ROOT SAMPLES

For the analyses of AMF communities in wheat as influenced by agricultural management practices, DNA was extracted from the root samples of the 2019 growing season (GS32, stem elongation, Chapter 3). Cell lysis and DNA extractions were conducted using the DNeasy PowerSoilPro Kit (Qiagen, Germany) as described in Section 4.2.5. In consequence of the lack of positive signals from inoculated roots during ddPCR assays (Chapter 4, Section 4.3.3) it was decided to exclude inoculation as a factor from the microbiome assessment. Therefore, only samples from the three fertiliser treatments (biogas digestate, mineral N and farmyard manure) together with the control plots as well as both varieties and with organic and conventional crop protection were further processed for sequencing. Extraction yields were measured by fluorometric quantification (Qubit™ Fluorometer 3.0) using the Qubit™ dsDNA high sensitivity assay kit (Invitrogen, USA). DNA quality was assessed by spectrophotometry (NanoDrop Lite, Thermo Fisher, USA). To guarantee amplifiability, samples were run in a provisional PCR with the ITS-primers for fungi targeting the ITS region (ITS4 and ITS3,

White *et al.*, 1990). When all quality criteria where met, samples were transferred in 20fold dilutions to NU-OMICS, the sequencing facility at Northumbria University (Newcastle upon Tyne, UK). Samples were processed following the protocol of the Earth microbiome project (D. P. Smith *et al.*, 2018) using specific primers (Table 5.2) for the amplification of the fungal ITS1 region in Illumina MiSeq V3 chemistry.

Table 5.2. Primers used for amplification of the fungal ITS1 region on nuclear ribosomal DNA.

Primer	Sequence 5'- 3'	Product	Reference
ITS1f	CTTGGTCATTTAGAGGAAGTAA	~ 230 bp	Smith *et al.*, 2018
ITS2	GCTGCGTTCTTCATCGATGC		White *et al.*, 1990

5.2.3 SSU-SEQUENCING OF FINE ROOT ENDOPYHTES IN CONTRAST TO COARSE ARBUSCULAR MYCORRHIZAL FUNGI

From the same growth stage as selected for ITS1-sequencing (stem elongation, GS32, 2019), 12 root samples were sent to Prof. Gary D. Bending and Dr Sally Hilton at Warwick University. This subset was composed of samples from both wheat varieties without AMF inoculation and with crop protection as these had shown higher AMF-root colonisation than non-sprayed samples (Chapter 3, Fig. 3.3 A). Half of the samples were roots from plots with the mineral nitrogen treatment whereas the other half were from the control plots with zero-fertiliser input. In the lab at Warwick University, DNA was extracted by Sally Hilton using the same protocol as described in Section 4.2.5. Amplicons for the analyses of FRE and AMF were produced using two different primer sets targeting the small subunit (SSU) of the nrDNA of FRE and AMF (Table 5.3). The first primer set with adapted primers by Sato *et al.* (2005) targeted both Glomeromycotina and Mucoromycotina, whereas Mucoro-primers targeted only FRE.

Table 5.3. Primers used for the amplification of SSU of fine root endophytes (FRE) and arbuscular mycorrhizal fungi (AMF).

Primer	Sequence 5'- 3'	Product	Reference
AMV4.5NFtrun	AAGCTCGTAGTTGAATTT	~ 300 bp	adapted from
AMDGR	CCCAACTATCCCTATTAATCAT		Sato *et al*. 2005
Mucoro-F-TAG	GTTGAATTTTAGCCYTGGC	~440 bp	Sally Hilton,
Mucoro-R-TAG_W	CCCAAAAACTTTGATTTCTCW		2020

For the amplification with adapted SSU-primers AMV4.5NFtrun and AMDGR (AM-primers), 15 ng of DNA were used in a master mix containing 12.5 µl 2x Q5® High Fidelity Hot Start Master Mix (New England BioLabs® Inc., USA), 1.25 µl of each primer (10 µM) and 5 µl ddH_2O. The PCR programme started with denaturation at 98°C for 30 s, followed by 35 cycles starting with polymerase activation at 98°C for 10 s, annealing at 60°C for 15 s and elongation

at 72°C for 20 s. Final extension was conducted at 72°C for 5 min.

PCR reactions with Mucoromycota-specific primers (FRE-primers) were performed in a reaction volume of 25 µl containing Q5® Hot Start High-Fidelity 2X Master Mix and 1.25 µl (0.5 µM) of each primer. Thermocycling consisted of an initial denaturation at 98°C for 30 s followed by 35 cycles of polymerase activation at 98°C for 10 s, annealing at 55°C for 15 s and elongation at 72°C for 20 s. The reaction was terminated with final extension for 5 min at 72°C. Amplicons were checked by gel electrophoresis expecting a band of ~440 bp (Table 5.3). The PCR products were purified using paramagnetic SPRI (Solid Phase Reversible Immobilisation) beads (Rohland & Reich, 2012).

5.2.4 PIPELINE BASED PROCESSING OF AMPLICON SEQUENCING DATA

Each dataset was analysed using a pipeline in R (R Core Team, 2019) with code provided by Dr Peter J. Leary (Bioinformatician, University of Zürich, Switzerland). The main packages of the pipeline are described in Table 5.4. Not all steps were conducted for each dataset since the procedure also depended on the results of the preceding step and if the analysis would be meaningful in the context of the objectives of the study (Section 5.1.1).

Table 5.4. Most relevant steps in the pipeline for processing and analysis of three sequencing datasets (ITS2, ITS1, SSU) using different packages in R and MEGAX for phylogenetic analyses. Code and workflow are described by Callahan *et al.* (2016).

R-Package/Software	**Procedure**	**ITS2**	**ITS1**	**SSU**
DADA2	‣ Import of demultiplexed FASTQ files ‣ Quality inspection ‣ Trimming and filtering of reads ‣ Error rate estimation ‣ Sequence inference (ASVs) ‣ Chimera checking and removal ‣ Assigning taxonomy	x	x	x[1]
decontam	‣ Identification and removal of contaminant ASVs	x[2]	x	
Phyloseq	‣ Diversity indices (Evenness, observed richness, Shannon's alpha-diversity) ‣ Beta-diversity (NMDS) ‣ Visualisation of taxonomy composition	x	x	x
Phylogenetic tree in MEGAX	‣ BLAST of ASVs in Genbank ‣ Multiple sequence alignment ‣ Maximum-likelihood modelling	x		x
DESeq2	‣ Stabilising variance transformation ‣ Differential abundance analysis ‣ PCoA			x

[1] DADA2-pipeline and decontamination assessment were conducted by Sally Hilton, Warwick University
[2] No negative controls were run during Illumina MiSeq run for ITS2 (Jan Jansa, pers. comm.), therefore decontam was used with DNA from a non-colonised root organ cultures as control sample

5.2.4.1 DADA2

Sequences of all runs were provided already demultiplexed and with primer sequences removed. As the first step in the pipeline, the quality of forward and reverse reads was inspected by plotting. Sequences were trimmed at both ends to exclude reads with quality scores < 30. A maximum of two expected errors (maxEE) per read were accepted. After removing low quality sequences from the data set, the remaining reads were fed into the subsequent step which contains the characteristic feature of DADA2 ('Divisive Amplicon Denoising Algorithm'). In contrast to most traditional sequencing pipelines for microbial community profiling, DADA2 does not cluster sequence reads randomly to operational taxonomic units (OTU) based on a set similarity threshold. Instead, DADA2 forms amplicon sequencing variants (ASVs) during sequence inference before errors can be integrated (Callahan *et al.*, 2017). The DADA2 algorithm circumvents this scenario by creating and applying a self-trained error model to the sequencing data. This error model was acquired from the data set and checked by plotting. Then, paired ends were merged under the exclusion of error-prone sequences and chimera were removed accordingly. The UNITE database (Kõljalg *et al.*, 2013) was used for subsequent taxonomy assignment of ITS-sequencing runs and SILVA132 vs. 2019 (Quast *et al.*, 2013) was used for analysis of the SSU dataset. These sequences were provided by Dr Sally Hilton already denoised and with assigned taxonomy using DADA2 in Qiime2™ (Bolyen *et al.*, 2019).

5.2.4.2 DECONTAMINATION AND FILTERING

Taxonomy tables, meta data and ASV tables from the DADA2-output were imported into phyloseq (McMurdie & Holmes, 2013). Before data evaluation and visualisation with the same package, sequences were assessed for contamination using the R-package decontam (Davis *et al.*, 2018). This was only conducted for ITS-datasets as contaminants had already been removed from the SSU-dataset (Sally Hilton, pers. comm.). For this step, the negative control that was run with the samples during sequencing was analysed for potential ASVs and their probability to occur also in true samples. If that was the case, these ASVs were removed from the dataset. Further filtering was conducted based on the prevalence of ASVs at phylum level. Phyla containing rare ASVs that occurred in less than five samples were blasted in GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and removed if clear taxonomy assignment to genus-level (100 % query cover) was not possible.

5.2.4.3 PHYLOSEQ

The R-package phyloseq was used for visualisation of relative abundances as well as calculation and plotting of ecological metrics such as species evenness, observed richness and Shannon's diversity index (combination of richness and evenness). Relative abundances were calculated as the quotient of the ASV counts divided by the total library size of the respective sample. For the investigation of treatment effects (i.e. fertiliser, variety, crop protection) on mycobiome composition, beta diversity was visualised by non-metric multidimensional scaling (NMDS) of Bray-Curtis dissimilarities. All plots were edited with ggplot2 (Wickham, 2016).

5.2.4.4 PHYLOGENETIC ANALYSIS BY TREE CONSTRUCTION

All tables (ASV, taxonomy and metadata) were also exported from R to Microsoft Excel (Microsoft Corporation, 2019) for visualisation of relative abundances as pie charts. In Excel, the ASVs of interest were filtered from the tables and each representative sequence was run using the online BLAST-tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify closely related reference sequences in GenBank. The accession numbers, FASTA-sequence and isolate information of the organism with highest query-cover were gathered in a Microsoft-Word document together with the respective ASVs and a selection of more distantly related sequences (outgroup). All sequences were uploaded into MEGA X (Kumar *et al.*, 2018) for multiple alignment using MUSCLE following the steps described by Hall (2013). The resulting alignment formed the basis for inference of a maximum-likelihood phylogenetic tree after identification of the most optimal substitution model in the same software which was in most cases the Tamura 3-parameter model (Tamura, 1992). This model was applied together with a bootstrap test for phylogeny (1000 iterations) and partial deletion of missing data. Initial tree(s) for the heuristic search were obtained by applying the Neighbour-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach.

5.2.4.5 DESEQ2

Differential abundance analyses were conducted with the R-package DESeq2 (Love *et al.*, 2014) which can be used in microbiome studies to extract ASVs that are more or less frequent in specific groups under consideration of the experimental design. This analysis was done for the present data when treatment effects were detected based on NMDS or hierarchical clustering and confirmed by statistical testing (Section 5.2.5). DESeq2 uses a negative-binomial generalised linear model that gives log2 fold changes on all counted ASVs. The model accounts for overdispersion of the data (variance > mean) for which reason DESeq2 has been discussed to be more reliable than other normalisation methods such as rarefaction

(McMurdie & Holmes, 2014). Treatment groups are compared with the *DESeq*-function that uses a Wald-test to test the H_0-hypothesis (i.e. there are no differences between treatment groups). Additional to the *p*-values of the Wald-test, DESeq2 produces Benjamini-Hochberg adjusted *p*-values which lowers the false discovery rate (Weiss *et al.*, 2017). The results of the differential abundance analysis were plotted as heatmap using the pheatmap-package in R (Kolde, 2019). Additionally, principial coordinates analysis (PCoA) was used to visualise similarities and dissimilarities of the ASVs identified by DESeq2.

5.2.5 STATISTICAL ANALYSES

Alpha diversity measures were compared by non-parametric testing using Kruskal-Wallis and Wilcoxon rank-sum tests for statistical comparison of multiple groups and two groups respectively in RStudio (R Core Team, 2019). To incorporate random effects ('block') in the analyses of mycobiome composition, the datasets were investigated using a mixed-effect model as described in Section 3.2.14 (Chapter 3) assessing the main effects and interactions of crop protection, variety and fertiliser treatment on fungal communities. If the data was not normally distributed according to QQ-plotting of residuals, non-parametric testing (Kruskal-Wallis) was applied. Beta-diversity ordination as NMDS was statistically documented to test the hypothesis if mycobiome communities could be grouped in response to different treatments. Samples were compared with permutational multivariate analysis of variance (PERMANOVA) using the *adonis*-function of the vegan-package in R (Oksanen *et al.*, 2020). Prior to the analyses, ASV-counts were normalised with DESeq2 (Love *et al.*, 2014). Based on the produced Euclidean distance matrix, the dispersion of homogeneity within treatment groups was assessed using the *betadisper*-function from the vegan-package. Groups showing a sufficient homogeneity of variances with $p \geq 0.05$ were run in PERMANOVA. If significant treatment effects on mycobiome composition were detected for $p \leq 0.05$, pairwise comparison of the respective groups was conducted using the *pairwise.adonis2*-function of the pairwiseAdonis-package (Martinez Arbizu, 2017). Treatment groups showing significant differences for $p \leq 0.05$ were analysed in DESeq2 for differential abundance of ASVs (Section 5.2.4.5).

5.3 RESULTS

5.3.1 ANALYSIS OF RHIZOPHAGUS IRREGULARIS ISOLATES AND AMF-INOCULUM BASED ON ITS2-REGION

Sequencing of the ITS2-region of nrDNA from root organ cultures (ROC) which had been used for strain-specific primer design (Chapter 4, Table 4.1) resulted in 134,858 sequences. These were separated into a total of 18 ASVs which were all identified as *Rhizophagus irregularis* (R.i., Fig. 5.2). Root powder samples from the AMF inoculum production process were also assigned to R.i and did not contain the expected species *Funneliformis mosseae* and *F. caledonium*. According to prevalence assessment, no contamination by other fungal taxa occurred (Appendix C, Fig.C.1). However, plotting of relative abundances showed that the negative control (chicory roots from empty ROC) contained ASVs assigned to R.i. This was not detected by the prevalence method because this ASV had the most abundant sequences in the whole data set. Non-targeted DNA amplification in this sample was already visible during nested PCR with Krüger-primers (Appendix C, Fig.C.2).

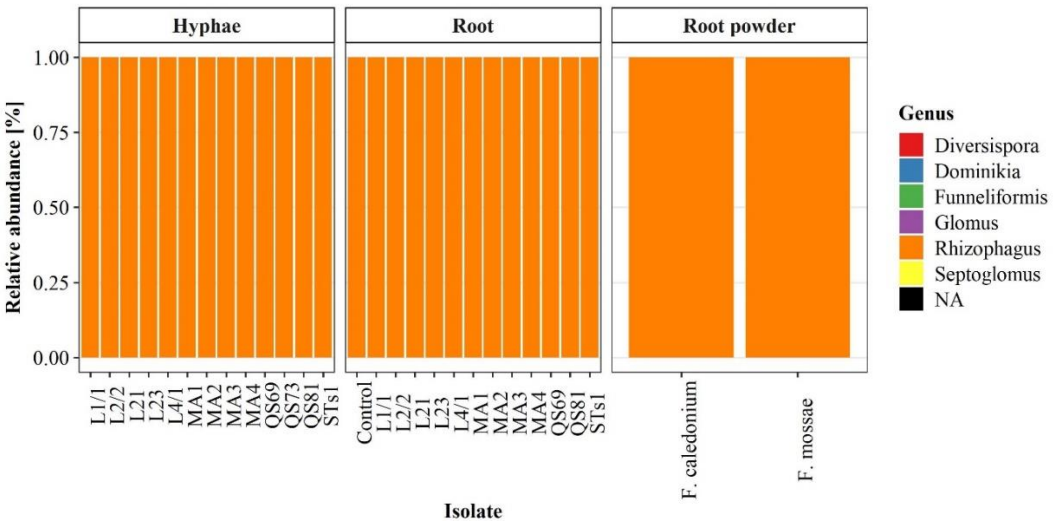


Fig. 5.2. Relative abundances [%] of fungal genera assorted to ASVs in root organ cultures of *Rhizophagus irregularis* isolates used for inoculum strain-specific primer design.

Each of the R.i.-isolates contained multiple populations of ASVs which revealed three groups according to non-metrical multidimensional scaling (NMDS, Fig. 5.3): the L-lines from Czechia were identical to each other irrespective of whether the DNA had been isolated from root or hyphae. The contaminated control sample was more closely related to this group than to other isolates which indicates that the ASV found in this sample originated from the L-lines. The QS-lines from the INOQ-inoculum formed a group with MA3-ASVs separate of the L-lines while STs1, MA1 and MA2-ASVs from the same lab formed another group with similar distances to the other two groups.

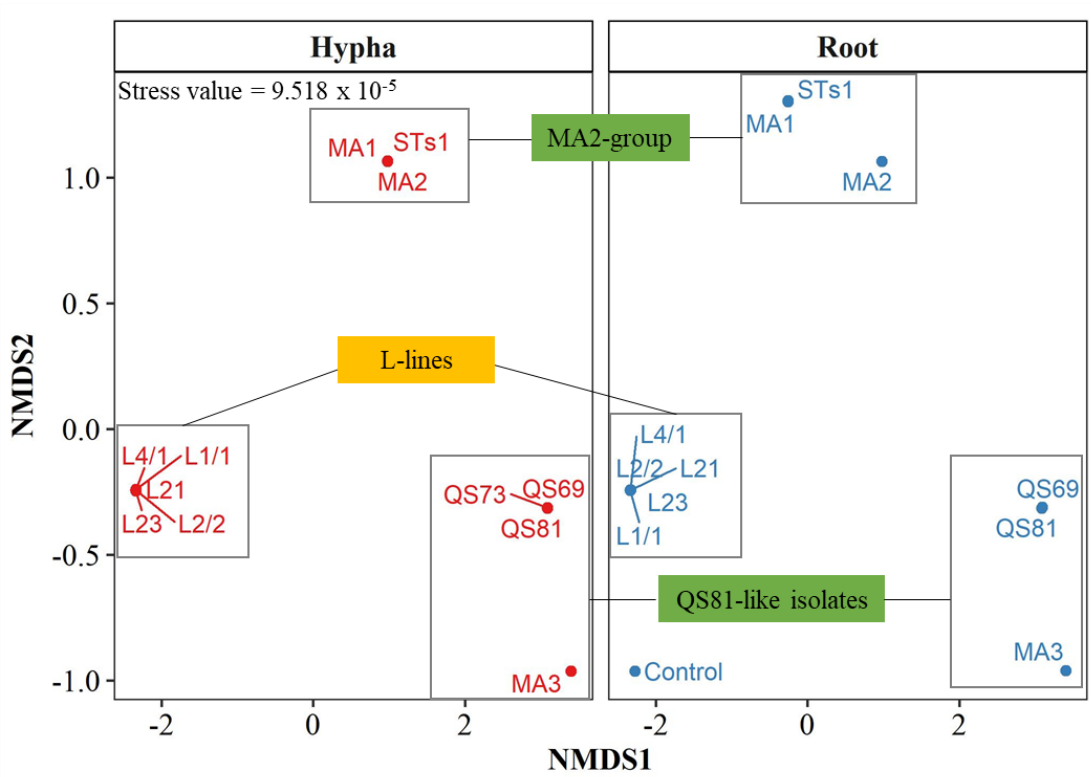


Fig. 5.3. Non-metric multidimensional scaling (NMDS) of Bray-Curtis distances of different isolates of *Rhizophagus irregularis* with DNA extracted from roots and hyphae. Boxes mark groups of isolates from the lab of INOQ GmbH (green label) and MBU Prague (yellow label).

5.3.2 FIRST INSIGHTS INTO AMF COMMUNITIES IN WHEAT SAMPLES OF THE FIELD TRIALS

Fungal community analyses based on ITS2- sequencing of the root samples from different growth stages of wheat from control plots (two wheat varieties, no fertiliser or AMF inoculation, organic crop protection) of the field experiment yielded 140,479 sequences which were separated into 117 ASVs. After removal of features that could not be assigned to the phylum level, it was found that the main phyla of this sequencing run were Glomeromycota, followed by Mortierellomycota and Basidiomycota (Fig. 5.4 A). Most abundant genus within Glomeromycota was *Glomus* spp. (28 %) followed by *Rhizophagus* spp. (19 %), *Funneliformis* spp. and *Diversispora* spp. (Fig. 5.4 B). Sequences of the genus *Dominikia* spp. (0.11%) were only found in one sample and *Septoglomus* spp. only contributed 0.04 % to the ASVs within Glomeromycota. A large proportion (40.72 %) of the sequences within this phylum could not be assigned at the genus level.

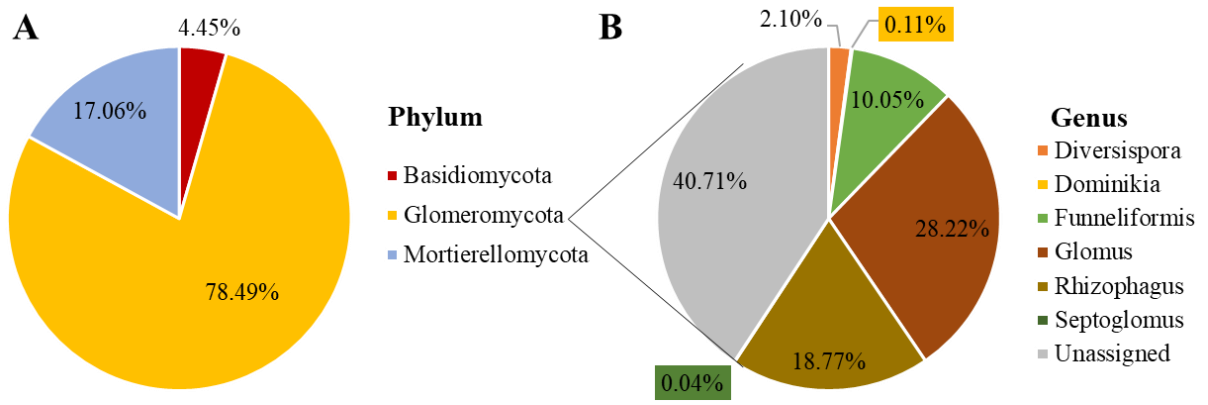


Fig. 5.4. Composition of **A**) fungal phyla and **B**) Glomeromycota genera from ITS2-sequencing of root samples of wheat.

Several root samples from the field experiment could not be sequenced for ITS2 as it was not possible to recover amplicons during nested PCR (Appendix C, Fig.C.2 B), probably due to DNA degradation (data not shown). Also, library sizes for Glomeromycota-sequences based on ITS2 varied with some samples showing only Mortierellomycota (data not shown) and no AMF-sequences (Fig. 5.5). Wheat roots at maturity (GS90) of the first trial season (2018) were dominated by R.i. whereas AMF communities at seedling growth (GS12) and tillering (GS22) in the second season (2018-19) were more diverse according to richness calculations (Appendix C, Fig.C.3). This predominance of *Rhizophagus* spp. at GS90 contrasted with absence of the same genus at GS22 where *Glomus* spp. and *Funneliformis* spp. dominated AMF communities.

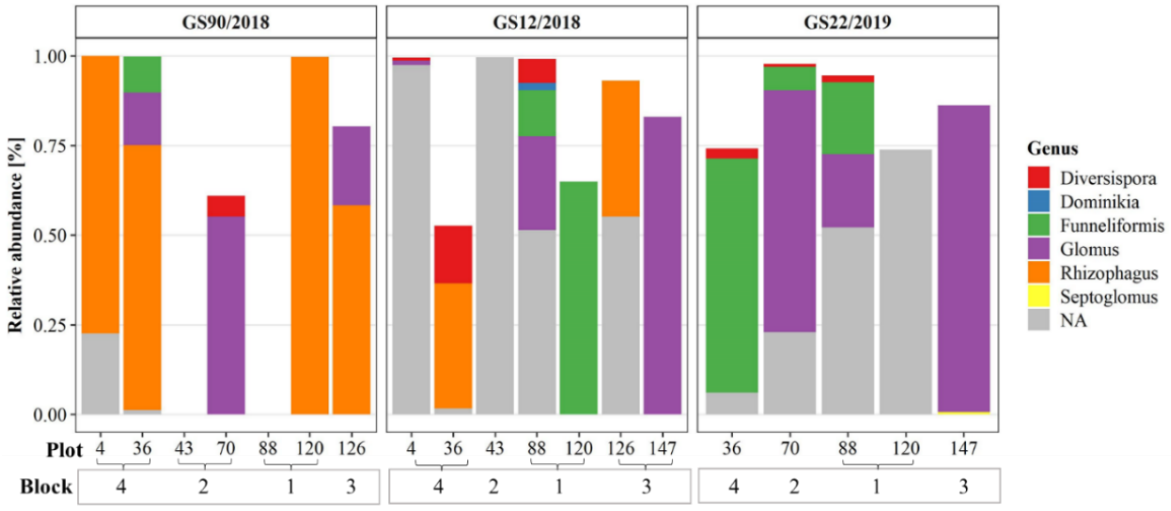


Fig. 5.5 Relative abundances [%] of Glomeromycota genera in wheat roots (zero fertiliser, org. crop protection) at three sampling time points. First panel shows final harvest of first field trial (2018) at maturity (GS90). Second panel shows first sampling (2018) at seedling growth (GS12, in the second season (2019). Third panel shows sampling at tillering (GS22) in the 2019 season. X-axis shows numbers of plots of two varieties (not shown) within blocks at the field site.

5.3.3 CLOSER INVESTIGATION OF THE ITS2-SEQUENCE POPULATION ASSIGNED TO THE GENUS RHIZOPHAGUS SPP.

Phylogenetic analysis confirmed that *Rhizophagus* spp. was not present in the selected samples from GS22 of 2019 (Fig. 5.6). The same analyses showed that some of the most abundant ASVs (with ASV1 having the highest counts within the whole data set) which had been extracted from the R.i.- ROCs were also present in the field: ASV3 was found in the R.i.-strain QS81 and in high abundance in plot 36 (Skyfall) but only at GS90 (Fig. 5.6, blue box). Sequencing variants from the same plot at early growth stages (GS12 and GS22) in the second season (2019) showed different clustering. Another overlap of R.i.-haplotypes in field samples and ROCs occurred for ASV2 which was found in the plots 126 (Skyfall) and 120 (Aszita) of the field trial. This haplotype was identical with ASVs in the isolates MA1, Sts1 and MA2 which were often located in phylogenetic proximity to the root powder samples (Fig. 5.6, red boxes) indicating the origin of the R.i. strain that contaminated the inoculum of *Funneliformis* spp. None of the ASVs found within L-lines from Czechia were detected in root samples from the field at Nafferton Farm. Sequencing variants from L-lines and non-colonised chicory roots clustered together confirming the previously suggested cross-contamination of the control sample with this haplotype (Fig. 5.6, green box). Also, unique sequences which did not show 100%-overlap with the GenBank strains such as ASV7 or ASV12 were identified and showed different arrangement of R.i.-strain groups than previously observed in NMDS ordination (Fig. 5.3). These two ASVs were only found in QS-lines and MA-lines respectively which could relate the original background of these R.i.-isolates being from different countries (Chapter 4, Table 4.1).

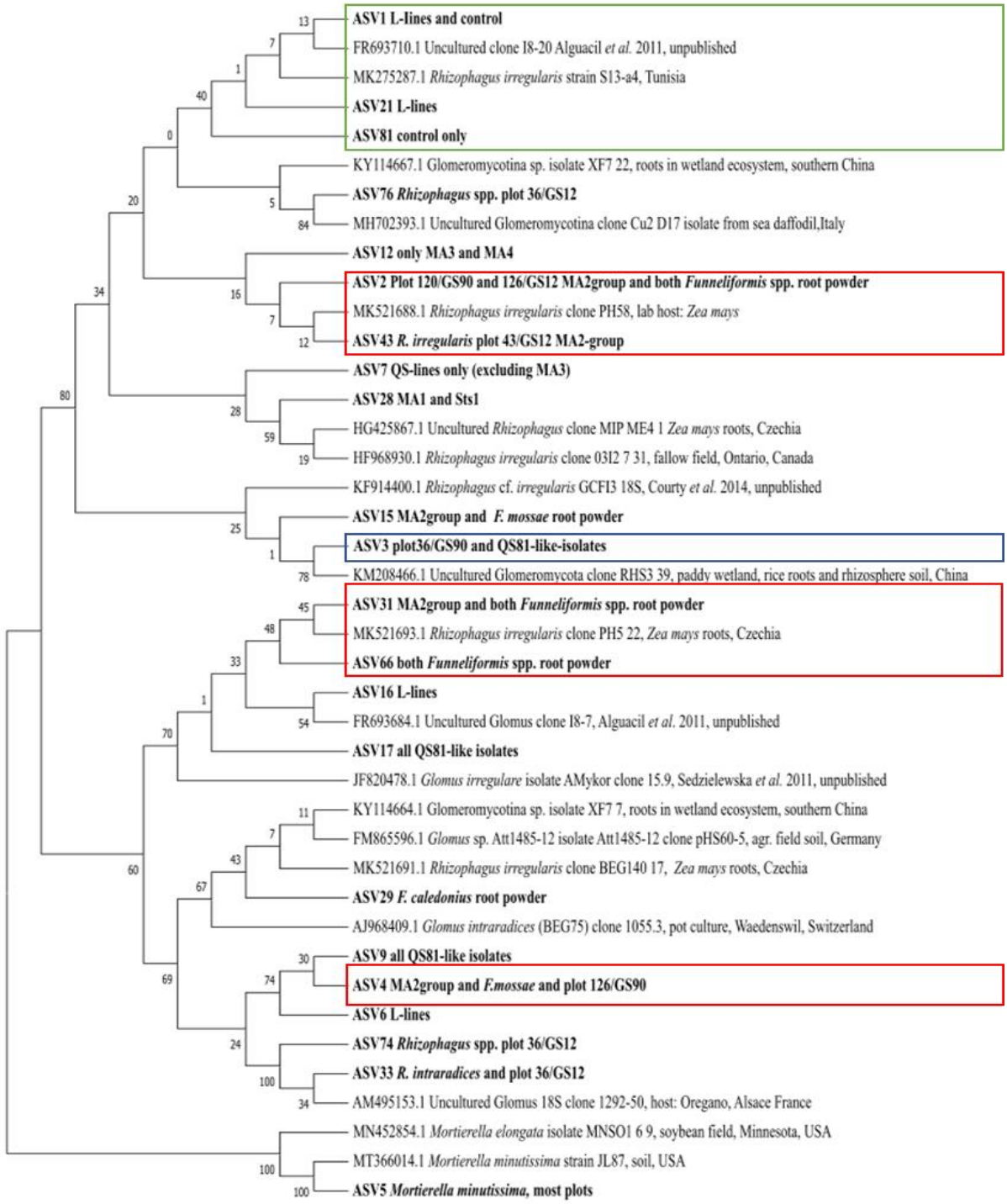


Fig. 5.6. Phylogenetic tree of *Rhizophagus* spp. amplicon sequencing variants (ASVs, bold) based on the ITS2-region of cultured *R. irregularis* isolates and DNA extracted from wheat roots at three growth stages (GS). The green box marks the cross-contamination event of the control sample and L-lines. The red boxes mark the cross-contamination event of *Funneliformis* spp. inoculum with R.i. strains. The blue box marks presence of QS81- inoculum nrDNA haplotype in the field. Reference strains and strain information (if provided) were extracted from GenBank. Node labels show bootstrap values (1000 iterations) of maximum-likelihood test (log likelihood -734.92). *Mortierella* spp. was included as an outgroup.

5.3.4 IMPACT OF AGRICULTURAL PRACTICES ON MYCORRHIZAL COMMUNITY COMPOSITION

From the MiSeq-run with general fungal primers targeting the ITS1 region, 3,207,784 sequences and 3,121 ASVs were recovered after filtering. No contaminants were discovered (Appendix C, Fig.C.4). Most ASVs were assigned to the phyla Ascomycota (68.62 %, Fig. 5.7 A) and Basidiomycota (15.87 %). While Glomeromycota (11.78 %) represented the third most abundant phylum, the Mucoromycota was the phylum with the lowest relative ASV representation (0.002 %) after Mortierellomycota (2.54 %) and Olpidiomycota (1.19 %).

From the 377,797 reads that were assigned to the phylum Glomeromycota, the largest proportion was assigned to the family Glomeraceae (88.32 %, Fig. 5.7 B). A 6.95 %-fraction was assigned to Archaesporaceae as the second most abundant family. Only 2.44 % were assigned to ASVs of the Paraglomeraceae, followed by 1.29 % of Diversisporaceae. Sequences of the families Ambisporaceae (0.99 %) and Claroideoglomeraceae (0.01 %) represented the AMF with the lowest abundances.

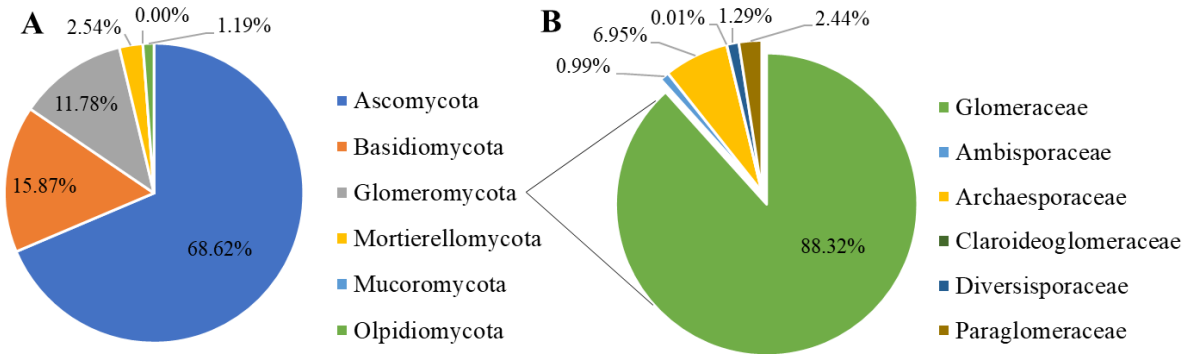


Fig. 5.7. Composition of **A**) fungal phyla and **B**) Glomeromycota families in the form of relative abundances [%].

There was no visible effect of wheat variety, crop protection or fertiliser source on relative abundances of the detected AMF genera (Fig. 5.8). *Glomus* spp. dominated samples from each treatment. According to taxonomy interferences with the UNITE-database, *Glomus*-sequences were all identified as *Glomus invermaium*. The other most abundant genera such as *Rhizophagus* and *Paraglomus* were also found more or less evenly distributed across treatments. *Funneliformis* occurred in low abundances and was present in all treatment groups. A fraction (6 %) of the sequences could not be assigned to the genus level and were mostly composed of unassigned Glomeraceae, Achaesporaceae, Diversisporaceae and Ambisporaceae decreasing in abundance in this order (data not shown).

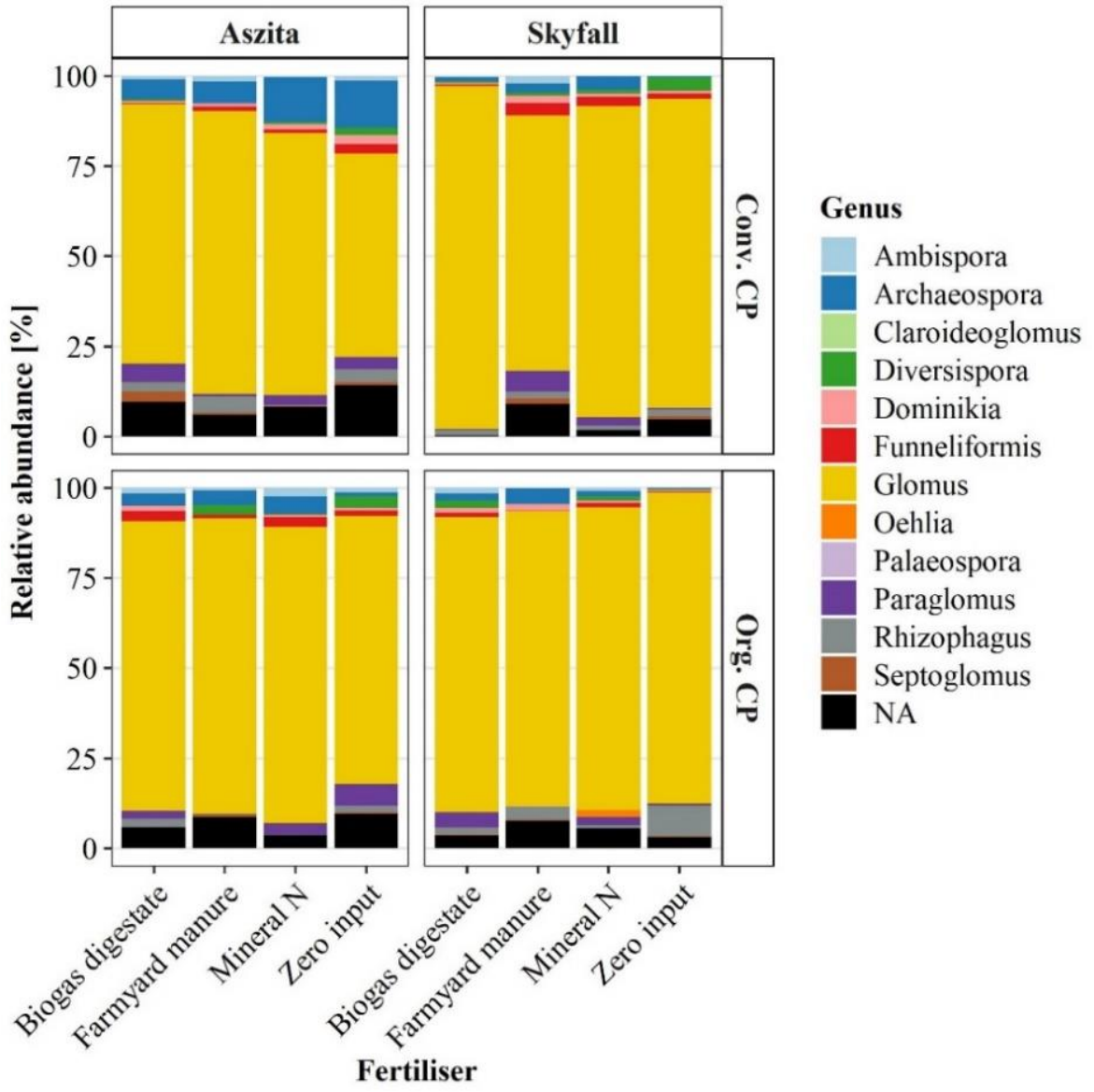


Fig. 5.8. Relative abundances [%] of genera within Glomeromycota in response to organic and conventional crop protection (CP), fertiliser source and variety (n = 4).

Statistical comparison of Glomeromycota genera (Table 5.5.A,B) did not show significant effects of agricultural practices or variety on mean relative abundances of AMF. Only variety seemed to affect abundance of the genus *Dominikia* according to ANOVA, but post-hoc analyses showed that there was no significant difference between the two varieties.

Table 5.5.A. Effects of crop protection, variety and fertiliser source on relative abundances of AMF genera in wheat roots (GS32) in the 2019 season. Numbers presented are means ± standard error of the mean. ANOVA *p*-values in bold indicate significant main effects and interactions. Means within columns followed by the same letter are not significantly different at $p<0.05$.

	Ambispora	**Archaespora**	**Diversispora**	**Dominikia**	**Funneliformis**	**Glomus**	**Paraglomus**
Crop protection (CP)							
Conventional (n=32)	0.01±0.003	0.06±0.019	0.01±0.004	0.01±0.003	0.02±0.005	0.77±0.041	0.03±0.008
Organic (n=32)	0.01±0.004	0.03±0.008	0.01±0.005	0.01±0.002	0.01±0.003	0.82±0.029	0.02±0.008
Variety (VR)							
Aszita (n=32)	0.01±0.003	0.06±0.019	0.01±0.005	0.01±0.002a	0.02±0.004	0.75±0.041	0.03±0.008
Skyfall (n=32)	0.01±0.003	0.02±0.007	0.01±0.005	0.01±0.003a	0.01±0.005	0.84±0.027	0.02±0.007
Fertiliser (FT)							
Biogas digestate (n=16)	0.01±0.005	0.03±0.011	0.01±0.005	0.01±0.003	0.01±0.004	0.82±0.044	0.03±0.012
Farmyard manure (n=16)	0.01±0.005	0.04±0.019	0.01±0.004	0.01±0.006	0.01±0.008	0.78±0.055	0.02±0.011
Mineral N (n=16)	0.01±0.005	0.06±0.021	0.01±0.003	0.01±0.003	0.02±0.006	0.81±0.041	0.03±0.007
Zero-input (n=16)	0.01±0.004	0.04±0.03	0.02±0.011	0.01±0.004	0.01±0.006	0.76±0.062	0.03±0.012
ANOVA *p-values*							
Main effects							
CP	ns	ns	ns	ns	ns	ns	ns
VR	ns	ns	ns	**0.030**	ns	ns	ns
FT	ns	ns	ns	ns	ns	ns	ns
Interactions							
CP:VR	ns	ns	ns	ns	ns	ns	ns
CP:FT	ns	ns	ns	ns	ns	ns	ns
VR:FT	ns	ns	ns	ns	ns	ns	ns
CP:VR:FT	ns	ns	ns	ns	ns	ns	ns

Table 5.5.B. The effect of crop protection, variety and fertiliser source on relative abundances of AMF genera in wheat roots (GS32) in the 2019 season. Numbers presented are means ± standard error of the mean. ANOVA *p*-values in bold indicate significant main effects and interactions. Means within columns followed by the same letter are not significantly different at p<0.05.

	Rhizophagus	**Septoglomus**	**Unassigned**	**Claroideoglomus[1]**	**Oehlia[1]**	**Paleospora[1]**
Crop protection (CP)						
Conventional (n=32)	0.02±0.008	0.01±0.004	0.07±0.015	0±0.0002	0±0	0±0
Organic (n=32)	0.02±0.011	0±0.001	0.06±0.016	0±0	0.002±0.0023	0±0.002
Variety (VR)						
Aszita (n=32)	0.02±0.008	0.01±0.004	0.08±0.017	0±0.0002	0±0	0±0
Skyfall (n=32)	0.03±0.011	0±0.002	0.04±0.012	0±0.0001	0.002±0.0023	0±0.002
Fertiliser (FT)						
Biogas digestate (n=16)	0.02±0.008	0.01±0.008	0.05±0.016	0±0.0001	0±0	0±0
Farmyard manure (n=16)	0.02±0.012	0.01±0.005	0.08±0.027	0±0.0003	0±0	0±0
Mineral N (n=16)	0.01±0.003	0±0.001	0.05±0.015	0±0	0.005±0.0046	0±0.005
Zero-input (n=16)	0.04±0.021	0.01±0.003	0.08±0.025	0±0.0001	0±0	0±0
ANOVA *p-values*						
Main effects						
CP	ns	ns	ns	ns	ns	ns
VR	ns	ns	ns	ns	ns	ns
FT	ns	ns	ns	ns	ns	ns
Interactions						
CP:VR	ns	ns	ns	ns	ns	ns
CP:FT	ns	ns	ns	ns	ns	ns
VR:FT	ns	ns	ns	ns	ns	ns
CP:VR:FT	ns	ns	ns	ns	ns	ns

[1] Residuals of these genera were not normally distributed, hence *p*-values show Kruskal-Wallis test results instead of ANOVA.

There was no effect of agronomic practices or variety on the alpha-diversity of Glomeromycota in wheat roots using liner mixed-effect models (Table 5.6). The same outcome was found after non-parametric testing (Appendix C, Fig.C.5).

Table 5.6. The effect of crop protection, variety and fertiliser source on alpha-diversity indices of arbuscular mycorrhizal communities in wheat roots (GS32) in the 2019 season. ANOVA *p*-values in bold indicate significant main effects and interactions Means within columns followed by the same letter are not significantly different at p<0.05

	Observed richness	**Evenness**	**Shannon**
Crop protection (CP)			
Conventional (n=32)	15.5±1.82	0.65±0.03	1.63±0.116
Organic (n=32)	14.6±1.33	0.63±0.025	1.59±0.09
Variety (VR)			
Aszita (n=32)	15.3±1.8	0.66±0.033	1.63±0.123
Skyfall (n=32)	14.8±1.36	0.62±0.021	1.58±0.082
Fertiliser (FT)			
Biogas digestate (n=16)	15.1±2.16	0.62±0.041	1.53±0.142
Farmyard manure (n=16)	14.7±2.33	0.65±0.041	1.7±0.181
Mineral N (n=16)	15.9±1.8	0.61±0.036	1.63±0.134
Zero-input (n=16)	14.5±2.75	0.69±0.037	1.58±0.136
ANOVA *p-values*			
Main effects			
CP	ns	ns	ns
VR	ns	ns	ns
FT	ns	ns	ns
Interactions			
CP:VR	ns	ns	ns
CP:FT	ns	ns	ns
VR:FT	ns	ns	ns
CP:VR:FT	ns	ns	ns

Ordination of Bray-Curtis distances did not show any distinct groupings of samples which indicates no consistent pattern of beta-diversity in AMF community composition in response to fertiliser treatment (Fig. 5.9 A). For both wheat varieties with and without crop protection, there were clusters of samples with similar genus compositions around a Bray-Curtis dissimilarity of 0 indicating high similarity. Ordination including block in NMDS showed that these clusters most likely indicate similarity of AMF communities at the respective sampling location (Fig. 5.9 B).

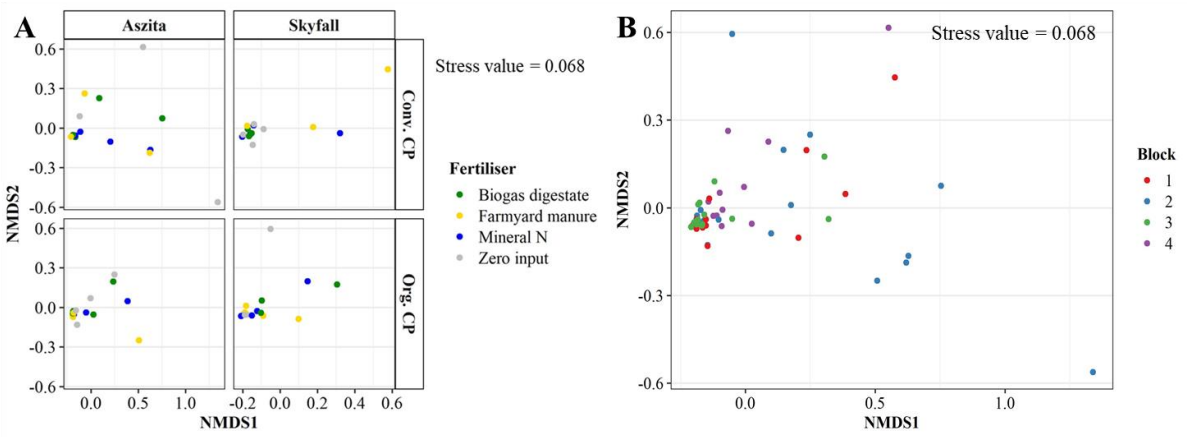


Fig. 5.9. Non-metric multidimensional scaling (NMDS) of Bray-Curtis distances of mycorrhizal community composition with effect of **A**) Crop protection, variety and fertiliser. **B**) Effect of sampling location (Block) on AMF community diversity at trial site.

Permutational ANOVA of a normalized distance matrix of the Glomeromycota sequencing counts did not show main effects of treatments, but there was a significant interaction of crop protection × variety ($p < 0.016$, Table 5.7).

Table 5.7. Permutational Analysis of Variance (PERMANOVA) of the effects and interactions of crop protection, variety and fertiliser on Glomeromycota community composition associated with wheat roots at GS32 of field trial season 2018-19. P_r-values in bold indicate significant differences between treatment groups for $p \leq 0.05$.

Main effects	**Df**	**Sums of Squares**	**Mean Squares**	**F- Model**	**R^2**	**P_r(>F)**
Crop protection (CP)	1	1290	1290.5	1.00394	0.02	ns
Variety (VR)	1	1289	1289.4	1.00310	0.02	ns
Fertiliser (FT)	3	3828	1276.0	0.99265	0.05	ns
Interactions						
CP:VR	1	1330	1330.0	1.03473	0.02	**0.016**
CP:FT	3	3859	1286.3	1.00070	0.05	ns
VR:FT	3	3862	1287.3	1.00145	0.05	ns
CP:VR:FT	3	3816	1272.0	0.98956	0.05	ns
Residuals	48	61699	1285		0.76197	
Total	63	80973			1	

Pairwise comparison revealed that AMF-communities varied in Aszita and Skyfall with conv. CP ($p = 0.027$, Table 5.8). However, differential abundance analyses with DESeq2 did not confirm this contrast of treatments and showed no significant differences in any of the treatment groups (data not shown).

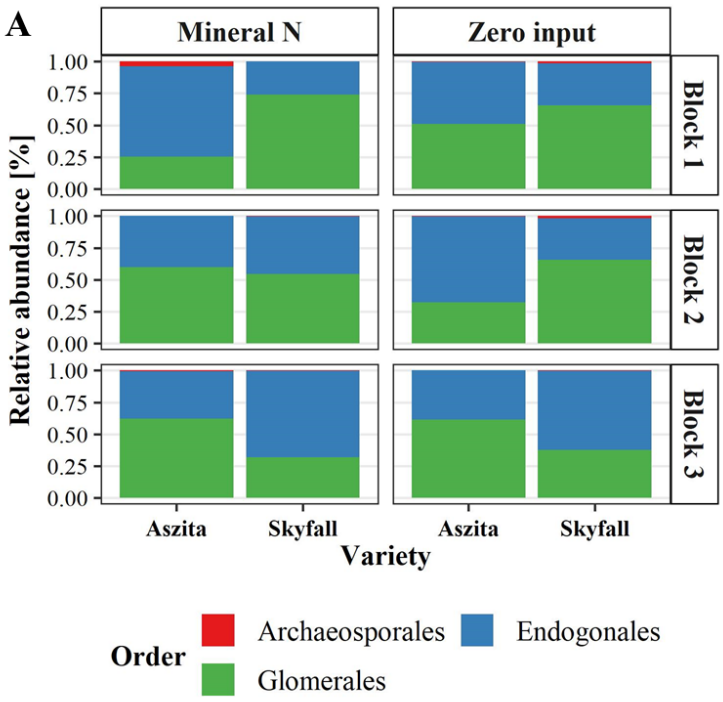
Table 5.8. Pairwise comparison of the interaction crop protection (OCP = organic crop protection, CCP = conventional crop protection) and variety (ASZ = Aszita, SKY = Skyfall). P_r-values in bold indicate significant differences between treatment groups for $p \leq 0.05$.

Pairwise comparison	Df	Sum of Squares	R_2	F-Model	P_r(>F)
OCP×ASZ vs CCP×ASZ	1	1241	0.03299	1.0235	ns
OCP×ASZ vs OCP×SKY	1	1446	0.03251	1.008	ns
OCP×ASZ vs CCP×SKY	1	1256	0.03256	1.0097	ns
CCP×ASZ vs OCP×SKY	1	1324	0.03223	0.9992	ns
CCP×ASZ vs CCP×SKY	1	1174	0.03333	1.0345	**0.027**
OCP×SKY vs CCP×SKY	1	1380	0.03279	1.0172	ns

5.3.5 ANALYSES OF FINE ROOT ENDOPHYTES USING GENERAL AND SPECIFIC SSU-PRIMERS

Sequencing of the nuclear ribosomal small subunit (SSU) with primers targeting both fine root endophytes (FRE) and AMF (adapted Sato-primers, further referred to as AM-primers) yielded 241,089 sequences. These were assignable to 252 ASVs after removing saprophytes (Mortierellomycotina) and contaminating sequences from the dataset. More than half of the ASVs (145) were assigned as Mucoromycotina which accounted for 44 % of all reads (Fig. 5.10 A). The remaining reads were composed of Glomeromycotina (107 ASVs) which accounted for 47.5 % of all reads. Glomeromycotina-ASVs were predominantly genus *Rhizophagus* spp. followed by a much smaller fraction of *Ambispora* spp. (Fig. 5.10 B). Other common Glomeromycota-genera (e.g. *Funneliformis* spp., *Glomus* spp.) were represented in the dataset by fewer reads (Fig. 5.10 B). Most of the Mucoromycotina reads were assigned as uncultured Endogonaceae and only 0.4 % of these were assigned to the genus *Endogone*.

Relative abundances of the main fungal orders within Glomeromycotina and Mucoromycotina showed small variations among treatments and across blocks (Fig. 5.10 A). Statistical comparison of AMF:FRE ratios did not reveal any effects of variety or fertiliser treatment on the two subphyla in wheat roots at this growth stage (Appendix C, Table.C.2).



B

Genus	Counts	%-reads
Unassigned	123,874	51.38
Rhizophagus	111,781	46.37
Ambispora	1,980	0.82
Endogone	996	0.41
Funneliformis	892	0.37
Glomus	628	0.26
Septoglomus	565	0.23
Archaeospora	210	0.09
Acaulospora	135	0.06
Diversispora	18	0.01
Claroideoglomus	13	0.01
Sum	241,089	

Fig. 5.10. Composition of Mucoromycotina and Glomeromycotina reads based on tagged amplicon sequencing of SSU: **A**) Relative abundances [%] of ASVs assorted to orders within Mucoromycota in root samples of two wheat varieties (Aszita and Skyfall) treated with mineral nitrogen or without fertiliser input replicated in three blocks (n = 2); **B**) List of all detected genera as well as unassigned Mucoromycotina and Glomeromycotina in root samples with respective count numbers of amplicon sequencing variants (ASVs) based on SSU-sequencing.

Neither Mucoromycotina nor Glomeromycotina diversity was affected by mineral N application or variety when ASVs generated with AM-primers were compared (Appendix C, Fig.C.7). Statistical analyses using linear mixed-effect models showed that there was no effect of variety and/or mineral N on relative abundance of Mucoromycotina or Glomeromycotina (Table 5.9). There was no impact on orders within these subphyla either, but the already very low relative abundances of a group of unassigned Glomeromycetes were significantly reduced in response to mineral N application.

Table 5.9. Effect of wheat variety and mineral N fertiliser on relative abundances of Mucoromycota subphyla and orders in wheat roots (GS32) in the 2019 season. ANOVA *p*-values in bold indicate significant main effects and interactions. Means within columns followed by the same letter are not significantly different at $p < 0.05$ according to Tukey-HSD test.

	Subphylum		**Order**				
	Glomeromycotina	**Mucoromycotina**	**Archaesporales**	**Diversisporales**	**Endogonales**	**Glomerales**	**Unclassified Glomeromycetes**
Variety (VR)							
Aszita (n=6)	0.43±0.062	0.47±0.038	0.01±0.006	0±0	0.47±0.038	0.41±0.067	0.01±0.002
Skyfall (n=6)	0.46±0.069	0.43±0.051	0.01±0.004	0±0.001	0.43±0.051	0.45±0.068	0±0.002
Fertiliser (FT)							
Mineral N (n=6)	0.45±0.075	0.45±0.043	0.01±0.006	0±0	0.45±0.043	0.44±0.08	0±0.001b
Zero-input (n=6)	0.45±0.055	0.45±0.048	0.01±0.004	0±0.001	0.45±0.048	0.43±0.053	0.01±0.002a
ANOVA *p*-values							
Main effects							
VR	ns	ns	ns	ns	ns	ns	ns
FT	ns	ns	ns	ns	ns	ns	**0.021**
Interaction							
VR:FT	ns	ns	ns	ns	ns	ns	ns

Primers for the specific amplification of FRE produced 86,922 reads of the phylum Mucoromycota and were grouped into 121 ASVs solely assigned to the order Endogonaceae within the order Endogonales. Only 5 % of these sequence types could be assigned to the genus *Endogone*, the remaining 95 % were described as unassigned Endogonaceae. Despite this homogenity of taxonomic assignment, differences in ASVs were detected and showed increased alpha-diversity of the Endogonales in response to mineral N-appliction, but evenness and richness were not affected by fertiliser application (Fig. 5.11 A). There was no difference in diversity measures when the two wheat varieties were compared regarding Endogonales-ASVs (Fig. 5.11 B).

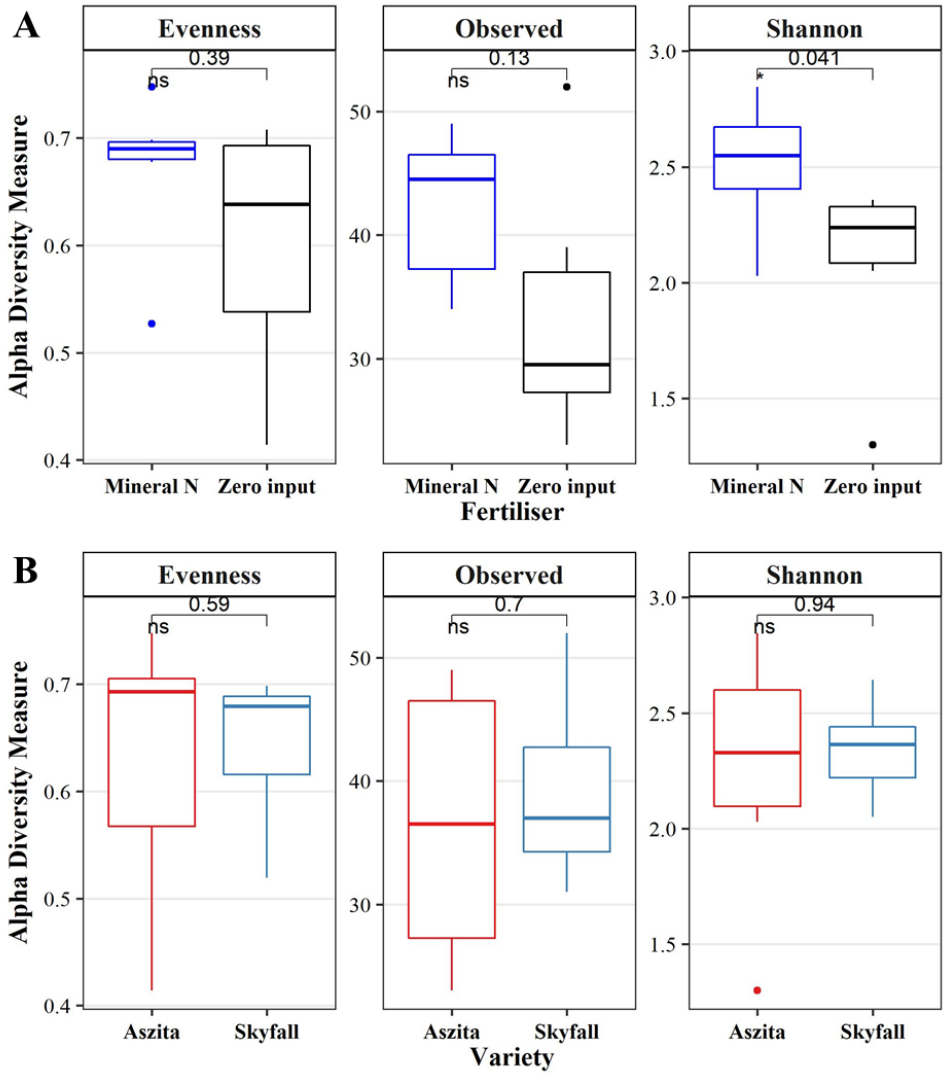


Fig. 5.11. Box plot showing alpha diversity measures of Endogonales in samples analysed by amplicon sequencing of small subunit (SSU) assessing the impact of **A**) mineral N application vs. zero-input and **B**) wheat variety. Numbers indicate *p*-values for pairwise comparison by Wilcoxon-rank test (ns = not significant, * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001).

Closer investigation of the beta-diversity of Endogonales showed clustering of all samples (except plot 37) which had been treated with mineral N (Fig. 5.12 A). This effect was confirmed by PERMANOVA of the zero-input and mineral N-treated samples (p = 0.02). In contrast, Endogonales communities produced by the AM-primers showed no clustering of samples in repsonse to mineral N application (Fig. 5.12 B) and were not significantly different in response to fertiliser treatment (p = 0.21). Neither NMDS nor PERMANOVA showed effects of wheat variety on beta-diversity of Endogonales in both SSU-data sets (Fig. 5.12, p_{FRE} = 0.88, p_{AM} = 0.22).

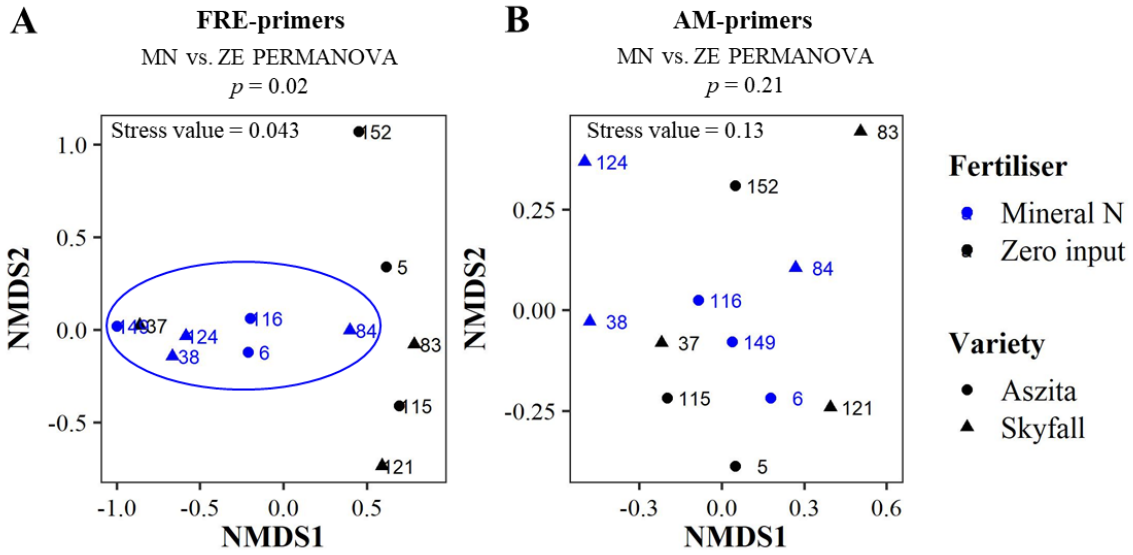


Fig. 5.12. Beta-diversity of Endogonales in wheat roots (GS32) of two wheat varieties +/- mineral N application in the 2019 season assessed using non-metric multidimensional scaling (NMDS) and PERMANOVA. A p-value ≤ 0.05 indicates significant differences of community composition. **A**) Analyses based on amplification of the small subunit (SSU) using specific FRE-primers. **B**) Analyses based on SSU-amplification of Endogonales using more general AM-primers. Numbers show plot-labels.

Differential abundance analyses of the FRE-primer sequences extracted a list of 23 ASVs that contributed to variations in Endogonales communities in response to mineral N application (Appendix C, Table.C.3). The key driver here was ASV3 which occurred in all samples but showed the highest abundances in experimental plots with mineral N (Fig. 5.13). Overall, there were more ASVs that occurred in higher abundances in mineral N-treated plots than in control plots. There were a few ASVs like ASV29 or ASV57 that showed higher abundance in control plots than in samples with mineral N-application. In contrast to ASV3, ASV1 was less abundant in the mineral N-treated plots, but reached very high abundances in one sample from a control plot without fertiliser (plot 152). Variety as a factor did not contribute to variation in this sequencing data set (Appendix C, Fig.C.8).

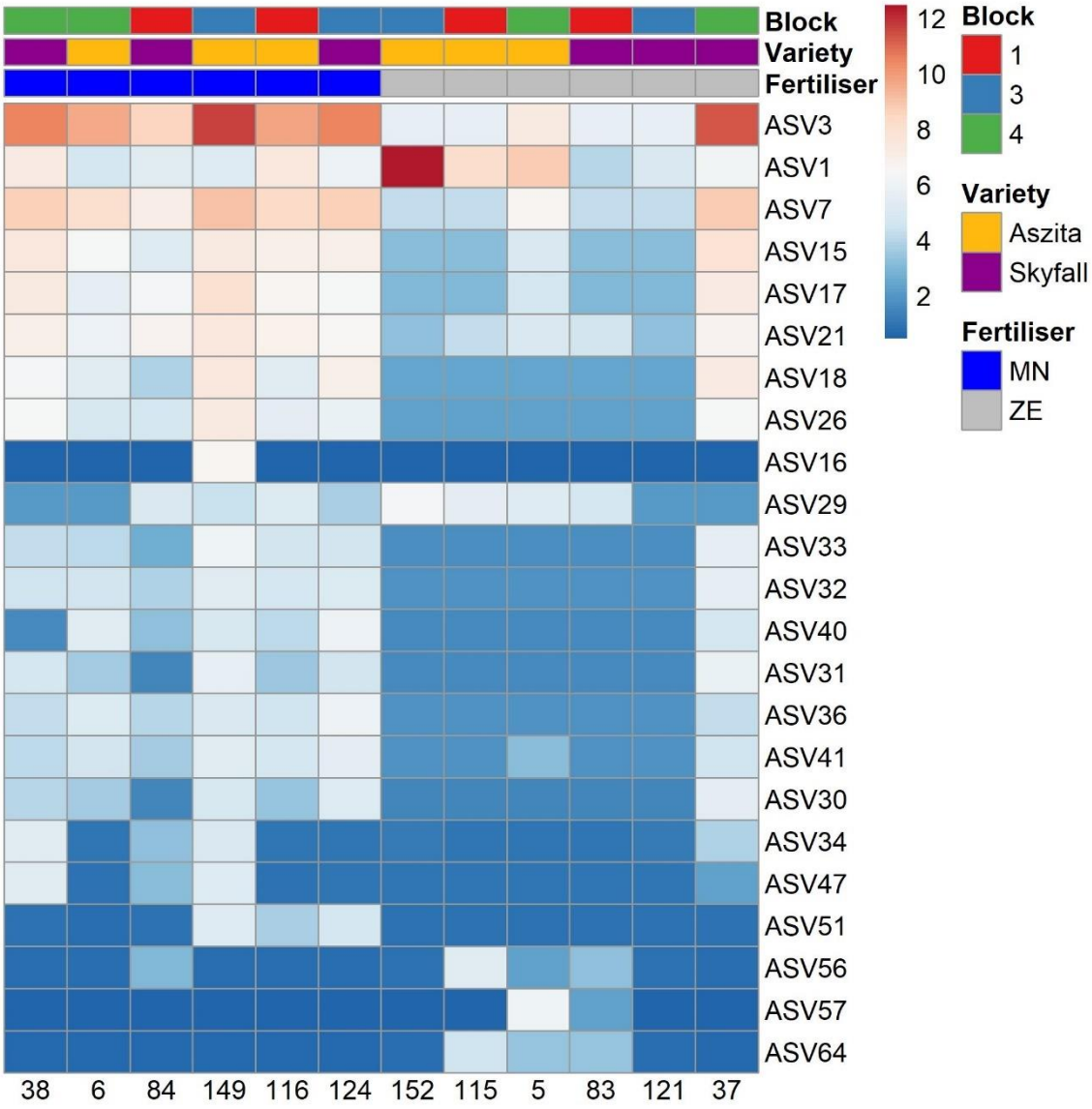


Fig. 5.13. Heatmap of signature ASVs of Endogonales identified by differential abundance analyses. Colour code shows regularised log-transformed variances of normalised ASV-counts in relation to mineral N (MN) application and zero-input (ZE) as well as two wheat varieties grown in three different blocks (replicates).

The Endogonales ASVs that were identified during differential abundance analyses were BLAST-searched to create a phylogenetic tree with a selection of reference strains. Most sequences were similar to BMVT-lines isolated from different liverwort species by Rimington *et al*. (submitted 2019, unpublished) where the highest sequence homology was found between ASV47 and isolate BMVT_30 (Fig. 5.14). Another match between an ASV and a reference strain with reliable bootstrap support was found between OTU4 by Orchard *et al*. (2016) and ASV1. There were also more distinct sequences like ASV30, ASV32 and ASV18 that formed their own clade without association to reference strains from GenBank. ASV3 was further separated from these ASVs and from the rest of the reference library and formed a clade with other ASVs that were more abundant in mineral N-plots, however with low bootstrap support.

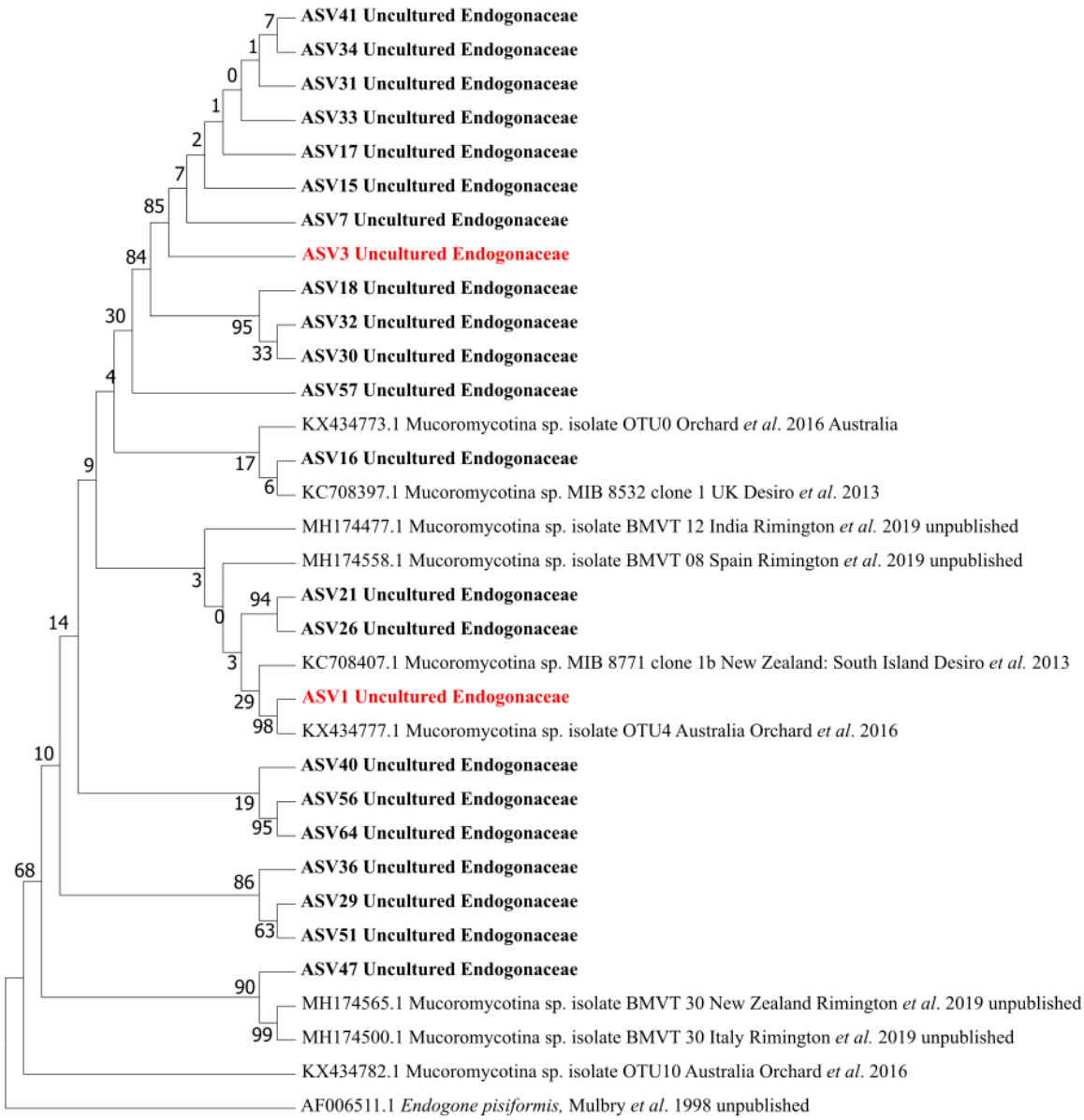


Fig. 5.14. Phylogenetic tree of Endogonales based on amplicon sequencing variants (ASVs, bold) from SSU-sequencing of fungal communities with FRE-specific primers. Phylogenetic distances were inferred using the Maximum Likelihood method and Tamura 3-parameter model. Node labels show bootstrap values (1000 iterations) of maximum-likelihood test (log likelihood = -1200.54). *Endogone pisiformis* was included as outgroup.

5.4 DISCUSSION

In contrast to most reports about the sensitivity of arbuscular mycorrhizal fungi (AMF) towards anthropogenic influences (e.g. Verzeaux *et al.*, 2016; Riedo *et al.*, 2021), there were no effects of agricultural practices on fungal community composition (Section 5.3.4) which might be explained by low taxonomic resolution of ITS1 (Maeda *et al.*, 2018). The less variable ITS2 region has been criticised for the same reason (Berruti *et al.*, 2017), but in the present study it was possible to identify the same groups of *Rhizophagus irregularis* (R.i.) strains based on ITS2 as found after Sanger-sequencing of the mitochondrial large subunit (mtLSU, Fig. 5.3). Using the same approach showed that the AMF inoculum which had been applied to the field trials contained only R.i. instead of the supposed *Funneliformis* spp. (Fig. 5.2). The consequences for the interpretation of the field trial results in this study will be further discussed in Chapter 7. Intriguingly, ITS2-sequencing detected R.i.-strains in the inoculum powder and cultured isolates that were identical to those at Nafferton Farm which might give indications about the fate of the R.i.-inoculum in the field. Another interesting observation was made during analyses of the fine root endophyte (FRE) communities based on variations in the small subunit (SSU): Endogonales diversity was increased with the application of mineral nitrogen (Section 5.3.5), but this effect was only detected using specific FRE-primers and not with more general primers. Overall, all three sequencing approaches touched on some of the obstacles which have caused frequent discussion in molecular research of AMF (Hart *et al.*, 2015; Stefani *et al.*, 2020).

5.4.1 STRAIN POLYMORPHISM ANALYSES BASED ON THE ITS2-REGION OF RHIZOPHAGUS IRREGULARIS

One major challenge in molecular studies of AMF is the huge number of heterogenous nuclei and the genetic variation within these fungi throughout their life cycle (Kokkoris *et al.*, 2020a). In the present study, this feature of AMF was examined in the phylogenetic analyses of selected R.i. isolates and amplicon sequencing variants (ASVs) of *Rhizophagus* spp. from the experimental field trial site (Fig. 5.6). In some cases, ASVs from the same R.i.-isolate were more closely related to reference strains or those from the field than to other ASVs from the same mycelium. This lack of phylogenetic clustering based on nucleic ribosomal DNA (nrDNA) was also reported by Thiéry *et al.* (2012) who investigated intra-sporal sequence variation of ITS in *Diversispora* spp. They concluded that this phenomenon confirms the hypothesis of random partitioning of heterogeneous nucleotypes that are distributed across the coenocytic mycelium (Kuhn *et al.*, 2001). The same probably occurred in the present study where DNA had been extracted from whole cultures instead of single spores as it was accomplished in other studies (Börstler *et al.*, 2008; E. C. H. Chen *et al.*, 2018;

Kokkoris *et al.*, 2021a). Although not all strains show heterokaryosis (Ropars *et al.*, 2016), intra-isolate variation based on nrDNA sequences has been described many times (e.g. Hijri & Sanders, 2005; Stockinger *et al.*, 2009) and has led to critical reviews on the use of ITS as a marker region for AMF (Bruns *et al.*, 2018; Maeda *et al.*, 2018). For this reason, phylogenetic analyses of AMF based on ITS should be interpreted with care (George *et al.*, 2019) and might also explain the low bootstrap-support found in the ITS-based phylogenetic tree in the present study (Fig. 5.6).

To avoid the problems associated with the use of nrDNA, mitochondrial DNA (mtDNA) has frequently been suggested to provide more reliable strain-level resolution which has particularly been demonstrated for R.i. (Raab *et al.*, 2005; Börstler *et al.*, 2008, 2010; Croll *et al.*, 2008). Some of these studies opposed their own findings from ITS-sequencing approaches of the same isolate to prove the superiority of mtDNA (Börstler *et al.*, 2008; Formey *et al.*, 2012). Therefore, in the current study it was unexpected to observe the same strain-clusters of R.i.-cultures from INOQ GmbH and from Czechia based on ITS2-sequencing (Fig. 5.3) as found by analyses of the mtLSU of the same isolates (Chapter 4, Fig. 4.3). It should be noted that these clusters were not observed in phylogenetic analyses after alignment of individual ASVs (Fig. 5.6) which implies that only the combination of these different sequences made up the characteristic genotype of the respective strains. This demonstrates the importance of considering ASVs as single features instead of clustered sequences in form of operational taxonomic units (OTUs) when it comes to strain-level detection (Hart *et al.*, 2015; Callahan *et al.*, 2017; Stefani *et al.*, 2020). For AMF, the ITS-region has been declared as not suitable for species-assignment for which reason many studies have used long fragments of the nrDNA region (Fig. 5.1) for strain-level identification (Stockinger *et al.*, 2010; Krüger *et al.*, 2012; Schlaeppi *et al.*, 2016). To my knowledge, the differentiation of AMF strains based on ITS2-sequence variations has not been previously reported.

The detection of the same ITS2-sequences in R.i. isolates from INOQ root powder samples intended to include only *Funneliformis* spp. demonstrate that the prevention of cross-contamination in AMF inoculum production under non-sterile conditions is not that simple (von Alten *et al.*, 2002; IJdo *et al.*, 2011). In the phylogenetic analyses of this study, an attempt was made to track where the contamination of both the control sample (uncolonized chicory roots) and the root powder samples originated from (Fig. 5.6). In the case of the control sample from ROCs it is obvious that the contamination must have occurred during nested PCR which was confirmed by clustering of the ASVs from this sample with L-lines from the MBU-lab in both NMDS and phylogenetic analyses (Fig. 5.3 and Fig. 5.6). Furthermore, it is noticeable

how the root powder samples of *Funneliformis* spp. clustered only with MA2-like haplotypes which could indicate that the contaminating R.i.-strain in these inoculum batches was derived from one of the MA2-like isolates. As there were no traces of *Funneliformis* spp. sequences in both root powder samples (Fig. 5.2), the contamination of this material probably occurred at an early stage of the production process. Hence, regular sequencing of *in-vitro* cultures and inoculum samples as a quality control measure is needed to ensure propagation of the target species or strain (Vosátka *et al.*, 2012).

5.4.2 SIMILARITY OF R.I. BETWEEN FIELD SAMPLES AND IN THE INOCULUM

Another intriguing detail of the ITS2-dataset analyses was that the R.i.-isolate QS81 and the R.i.-haplotypes found in the *Funneliformis* spp.-root powder samples shared two of the most abundant ASVs (ASV2 and ASV3) with the R.i.-population at the field site (Fig. 5.6, blue and red boxes). In contrast, ASVs from Czechia (L-lines) were not detected in root samples from the field. Both QS81- and root powder samples were the main components of the same AMF inoculum which was used during inoculation of the field trial in each growing season (Chapter 3, Table 3.5). Even though assessed root samples were obtained from non-inoculated plots, these findings could indicate potential mixing of exogenous and native R.i.-strains. Fusion of hyphal networks of two AMF isolates leading to exchange of nuclei via anastomosis has been described in AMF tissue cultures but has been rarely documented in field experiments (de la Providencia *et al.*, 2004). A comparable phenomenon was reported by Schlaeppi *et al*. (2016) who observed the colonisation of an inoculated R.i. strain in a wheat field which almost replaced the native R.i. population. Also Pellegrino *et al*. (2012) successfully applied an nrDNA-based marker to trace an exogenous *Funneliformis mosseae*-strain up to two years after inoculation. In contrast to the present approach, both studies used a much larger nrDNA fragment spanning SSU, the whole ITS region and parts of the LSU region (Krüger *et al.*, 2009, 2012; Pellegrino *et al.*, 2012; Berruti *et al.*, 2017). Sequencing of this up to 2500 bp-long fragment has been suggested as the most optimal method to achieve reliable AMF taxa resolution (Redecker *et al.*, 2013; Kolaříková *et al.*, 2021). Then again, Gao *et al.* (2019) reported immigration and extinction of mycorrhizal OTUs in sorghum over a whole growing season based on ITS2. Something similar was observed in the present study where R.i. dominated relative abundances around grain maturity (GS90) when it was mostly represented by those highly abundant ASVs (ASV3 and ASV2) which were also found in the inoculum strains QS81 and in MA2-like strains (Fig. 5.6). The high similarity of INOQ-strains and the R.i.-ASVs found in the field at GS90 would represent a desirable signal for the detection of

successful AMF-inoculation which has been frequently requested by the scientific community (Gianinazzi & Vosátka, 2004; Schwartz *et al.*, 2006; Schlaeppi *et al.*, 2016). Thomsen & Hart (2018) pointed out that in particular generalists like R.i. possess characteristics that assist this species to rapidly invade new environments. Applied to the present study, this assumption would support the hypothesis that the R.i. strains from the AMF inoculum spread outside of the inoculated plots and were able to outcompete native R.i. populations during the field trials. If this could be done within the time frame of one cropping season however remains to be elucidated.

If the first years (2018) AMF inoculum was as potent as assumed, it would be expected to be able to detect the same ASVs of R.i. in the subsequent field season, but different ASVs were found at GS12 (Fig. 5.6) and there was no R.i. in any of the analysed samples at GS22 of the second field trial in 2019 (Fig. 5.5). This indicates that different nucleotypes dominated root colonisation in seedlings at the beginning of the second field season which could be explained by selective pressure due to differing environmental conditions or management practice (Jansa *et al.*, 2002; Sommermann *et al.*, 2018). Plasticity of AMF genomes in response to environmental conditions and host identity has been demonstrated *in-vitro* (Kokkoris *et al.*, 2021a), but if the same can be detected in the field using such a highly variable marker region like ITS2 requires further investigation with a much greater and more consistent set of samples. Alternative to the above discussion about apparent colonisation and considerable spatial dispersal of an inoculum is the notion simply that these strains are present as indigenous R.i. Mycorrhizal fungi are ubiquitously distributed and identical AMF strains have been identified across continents (Börstler *et al.*, 2008; Davison *et al.*, 2015). This was also possible in the current study where strains of fine root endophytes (FRE) from the field at Nafferton Farm showed high similarity to those described in soils in Southern Australia as it will be discussed in Section 5.4.5.

5.4.3 ARTEFACTS OF MOLECULAR RESEARCH ON AMF

A common problem in molecular profiling of AMF communities in environmental samples is that taxa composition can be biased depending on the applied molecular marker and the database used for taxonomic assignment (George *et al*., 2019; Stefani *et al*., 2020). Both issues were encountered during analyses of the three sequencing runs of the present study but could be optimised in future analyses. One of the most obvious aspects that was also found in the present study is that ITS1-primers amplify mostly Ascomycota which leads to the dilution of Glomeromycota reads (Fig. 5.7 A). Therefore, the use of general fungal primers has not been recommended for research questions that focus on AMF (Kohout *et al*., 2014;

Tedersoo *et al*., 2015; Řezáčová *et al*., 2016). The use of AMF-specific primer mixes prior to sequencing has been proposed for higher taxonomic resolution (Krüger *et al*., 2009) which was demonstrated in the ITS2-sequencing run of the present study (Fig. 5.4). With this approach, wheat roots at GS22 seemed to be colonised by a more diverse AMF community (Fig. 5.5), whereas the mycorrhizal microbiome was almost solely occupied by the genus *Glomus* according to ITS1-sequencing of roots sampled two months later at GS32 (Fig. 5.8). *Glomus* spp. are very common in arable soils (Hijri *et al*., 2006; Gosling *et al*., 2013; Leiser *et al*., 2016; Sommermann *et al*., 2018) and have developed life-strategies to persist under non-optimal environmental conditions (Verbruggen & Kiers, 2010). However, only SSU and ITS1-sequencing approaches are directly comparable as these sequences were generated from the same samples. When these results are compared, it is noticeable that according to ITS1 *Glomus* predominated in the wheat roots at GS32 (Fig. 5.8) while SSU-sequencing revealed *Rhizophagus* as the prevalent genus (Fig. 5.10 B). These differences of SSU and ITS-taxonomic assignment based on the databases SILVA and UNITE has been discussed in mycorrhizal research (Kohout *et al*., 2014; Berruti *et al*., 2017; George *et al*., 2019) which should be prevented by frequent updates of the more AMF-specific database 'MaarjAM' (Öpik *et al*., 2010; Stefani *et al*., 2020). The same obstacle was found in the ITS-sequencing approaches of this study which assigned all *Glomus*-ASVs to the species *Glomus invermaium*. This taxon was renamed to *Rhizophagus invermaius* in 2016 according to the official AMF-phylogeny (Walker 2016, unpublished, www.amf-phylogeny.com), but was not updated in the UNITE database. The adaptation of the new name in UNITE would clearly change the picture of the current analyses as then both SSU and ITS metabarcoding studies would show *Rhizophagus* as the most abundant AMF genus. But it should be noticed that taxonomy assignment of Glomeromycota based on ITS has been declared as not reliable for higher ranks than family-level (Tedersoo *et al*., 2015; Thiéry *et al*., 2016). Then again, the presence of *Glomus invermaium* (also referred to as *Rhizoglomus invermaium*) in temperate agricultural soils has been reported before (Säle *et al*., 2015; Oehl & Koch, 2018), but it is questionable if all *Glomus*-ASVs in the present study can be assigned to this single taxon. That the *Glomus*-group deserves further investigation in the context of wheat production was demonstrated in a field study by Dai *et al*. (2014) that showed negative correlations of *Glomus* abundances with nutrient concentrations, nutrient use efficiency and biomass production of organically managed wheat. Graham & Abbott (2000) even grouped *Glomus* sp. and *Glomus invermaium* as 'aggressive colonisers' in wheat that can induce growth depression depending on P-supply and growth stage. Since the molecular data of this study was not correlated with biomass data it remains open if this impact would have been observed under the experimental conditions of the current field study.

5.4.4 EFFECTS OF AGRICULTURAL MANAGEMENT ON THE MYCOBIOME ACCORDING TO ITS1-SEQUENCING

Probably the most surprising outcome of the ITS1-sequencing study was the lack of treatment effects on AMF community composition and diversity: No differences between mycorrhizal communities with fertiliser treatment (organic vs inorganic) were found when compared to samples without fertiliser nitrogen (N)-input in both varieties with or without crop protection (Fig. 5.8, Table 5.5). These findings conflict with the literature that showed lower AMF diversity in response to N (Leff *et al.*, 2015; Qin *et al.*, 2015; Bakhshandeh *et al.*, 2017; Verzeaux *et al.*, 2017) or phosphorus (P)-fertilisers (Gosling *et al.*, 2013). According to these studies, we would expect similar results at least in samples with mineral N application, especially since the second year of the field trial was assessed where the same amount of mineral N fertiliser had already been applied in the previous year. Adaptation to the addition of N could have favoured root colonisation by fungal species that are more resistant to high nutrient concentrations (Egerton-Warburton *et al.*, 2007). Consequently, the observed predominance of generalists like *Glomus* spp. which are less affected by agricultural management could be interpreted as an effect of N-fertilisation itself (Egerton-Warburton *et al.*, 2007; Porras-Alfaro *et al.*, 2007; Dai *et al.*, 2014; Liu *et al.*, 2014; Qin *et al.*, 2015). On the other hand, it is questionable whether such selection processes would be detectable one year following the first fertiliser application. Egerton-Warburton *et al.* (2007) suggest seasonal differences are important in the response of AMF to N-fertilisation, but they also report community composition homogenisation after three years. Considering time frames, it should be noted that in the current study the assessed time point was only 2-3 weeks after the application of both mineral N and biogas digestate which could be too soon to observe potential effects of these two treatments on AMF. Furthermore, amplicon sequencing was conducted for only one growth stage which can provide limited idea of the actual effects of the agricultural management practices under the assessed conditions. Hence, analyses from the previous field season as well as later sampling time points are required to validate this hypothesis for the present study. Time is also an important factor that should be considered in the context of pesticide applications. Fungicides can have significant impact on AMF community composition and AMF diversity in soils which can even affect plant growth (Jin *et al.*, 2013; Hage-Ahmed *et al.*, 2019), but such effects were not observed in the ITS1-dataset. However, no soil samples were analysed that could indicate if the applied pesticides did not initially hamper AMF spore germination (Dodd & Jeffries, 1989). Such detrimental effects of chemical compounds could have selected for AMF species that could pass this bottleneck which again would highlight the competitiveness of *Glomus* spp.

The few investigations on the effect of biogas digestate on AMF specifically do not report AMF diversity (Caruso *et al.*, 2018; Ren *et al.*, 2020), but would be intriguing considering that the application of this fertiliser type adds high concentrations of plant available nutrients to the soil, but also a whole microbial cocktail from the anaerobic digestion process that could potentially interact with AMF (Wentzel & Joergensen, 2016). While some studies suggest that organic fertilisers contribute to AMF community diversity (Gosling *et al.*, 2006), Hazard *et al.* (2014) found no effects on AMF communities in response to application of up to 5 Mg DM ha^{-1} of biosolids which could also be applied to the present study where biogas digestate or FYM did not affect mycorrhiza diversity (Table 5.6). The lack of treatment effects could also be explained by the soil chemical properties that are essential drivers for AMF community dynamics (Egerton-Warburton *et al.*, 2007). With 170 kg N applied per ha this study investigated the effect of a reduced N-application and not the recommended 220 kg N ha^{-1} in conventional agricultural practice (Verzeaux *et al.*, 2017). Optimum N-management was shown to be essential for the protection of AMF diversity (Liu *et al.*, 2014). But even if this threshold was met in the tested system, we would still expect a difference between plots with fertiliser treatments compared to control plots as their nutrient status was likely to be N-limited in the 2019 season (Chapter 3, Table 3.2). This indicates that the observed homogeneity of the fungal mycobiome must be explained by conditions that date back to conditions before the experiment. A long-term field study by Sommermann *et al.* (2018) showed the impact of the previous crop on *Glomus* spp. which dominated wheat roots in both of their separate ITS1 and ITS2 amplicon sequencing approaches. They found effects of extensive and intensive fertiliser on a few less abundant AMF genera while *Glomus* spp. was only marginally affected by tillage, but not by other farming practices. Neither pre-crop nor tillage were subject of this study but represent interesting research questions, for example to identify potential changes in AMF composition in the first cropping season after a grass-clover ley in comparison to being grown as a second wheat crop.

It was presumed that the two contrasting wheat varieties Aszita and Skyfall may show differences in their mycobiome composition, but this was not the case or at least was not detected with the applied bioinformatic and statistical approaches (Section 5.4.6). It is not clear if the interaction of crop protection × variety (Table 5.7) that revealed different AMF-community composition in Aszita and Skyfall with conv. CP (Table 5.8) was actually significant as it was not possible to confirm differences between groups using DESeq2 (Section 5.4.4). There is not much information about variety-specific AMF community composition in crops whereas the species-specific differences of mycobiomes are well

documented (Gosling *et al.*, 2013; Řezáčová *et al.*, 2016). In fact, a simultaneous experiment in adjacent fields at Nafferton Farm with potato revealed quite a different AMF community composition (e.g. *Paraglomus* as the most abundant genus) than the present study using the same molecular tools (Salisa Suchitwarasan, pers. comm.). In wheat, Mao *et al.* (2014) provided the rare evidence that AMF community assembly can operate at a variety level, whereby 21 wheat varieties showed significantly different AMF mycobiomes under field conditions, but their composition was also correlated with drought stress tolerance. However, these variations had no effects on wheat performance (Mao *et al.*, 2014). Although opposing opinions have been published (Leiser *et al.*, 2016; Ryan & Graham, 2018), such findings support the idea of microbe-orientated plant breeding which has been raised by mycorrhizal researchers (Taylor *et al.*, 2015; Hohmann & Messmer, 2017; P. Campos *et al.*, 2018; Bitterlich *et al.*, 2020). Recent reviews on factors that drive AMF communities in their association with plants point out that host identity might be less important than, for example, environmental conditions (Lekberg & Waller, 2016; Kokkoris *et al.*, 2020b). Considering the prevalence of *Glomus* independent of agricultural management practices used, it is questionable if a specific wheat variety could elicit a more effective fungal symbiosis under given environmental conditions (Kiers *et al.*, 2011). Hence, it might be more meaningful to create favourable conditions for AMF in agricultural systems rather than selecting for strong genetic traits in host plants.

5.4.5 ABUNDANCE OF FINE ROOT ENDOPHYTES ARE AFFECTED BY NITROGEN FERTILISER

There were noticeable differences in the two datasets of Endogonales sequences also referred to as fine root endopyhtes (FRE) (Table 5.3): with the more general primers targeting both Mucoromycotina and Glomeromycotina (AM-primers) it was possible to assess the ratio of AMF and FRE, but no effects of agricultural management practices were detected (Appendix C, Table.C.2). In contrast, with the more specific FRE-primers, abundances of certain Endogonales-assigned ASVs were altered in response to mineral N use (Fig. 5.12). Sequence analyses with AM-primers showed that the number of reads of Mucoromycotina and Glomeromycotina were almost the same (Section 5.3.5) which indicates that FRE occurred in high abundance in wheat roots probably in equal proportion to AMF. Co-colonisation or ‘dual’ symbiosis by the two fungal subphyla is a common observation in studies that differentiate between AMF and FRE (Ryan *et al.*, 2005; Orchard *et al.*, 2016; Orchard, 2017b; Field *et al.*, 2019; Hoysted *et al.*, 2019). To distinguish between AMF and FRE during microscopy has been encouraged (Ryan & Graham, 2018), but was only repeated for the

12 samples used for SSU-amplicon sequencing (Chapter 7, Section 7.3.4). For now, evidence is provided that FRE were part of the mycobiome in wheat roots during the field trials which confirms observations of characteristic Endogonales colonisation structures by microscopy. Further, it was demonstrated that the provided primers can be used to differentiate between Glomeromycotina and Mucoromycotina, but with different resolution for community shifts. Therefore, it would be interesting to compare both primer pairs regarding efficiency with those that have been used for the same purpose in other studies (Desirò *et al.*, 2017; Hoysted *et al.*, 2019; Sinanaj *et al.*, 2020).

The only detected impact of agricultural management on mycorrhizal community composition in root samples was found in the sequencing approach with specific primers for Endogonales, whereby the application of mineral N increased alpha-diversity (Fig. 5.11 A) and resulted in significantly higher abundances of certain ASVs compared to samples without fertiliser input (Fig. 5.13). These findings are surprising considering the evidence for detrimental impact of N-fertilisers on soil microbiomes (Leff *et al.*, 2015). However, diversity indices do not represent real biological numbers and might not be relevant in the studies of a single family (Endogonaceae) especially considering the high intra-specific variation of nrDNA as found in the closely related Glomeromycotina (Section 5.4.1). Furthermore, this effect of mineral N on FRE was only detected with FRE-primers, but not with AM-primers (Fig. 5.12). This difference might be explained by potential primer-bias (George *et al.*, 2019) and can only be circumvented by using longer fragments for DNA barcoding (Stockinger *et al.*, 2010; Redecker *et al.*, 2013) or selective amplification approaches prior to sequencing (Krüger *et al.*, 2009; Stefani *et al.*, 2020). A longer fragment is also required to identify reliable relationships of ASVs (Orchard *et al.*, 2017a) which explains the low bootstrap support for most branches of the SSU-based phylogenetic tree (Fig. 5.14). Then again, matches with strong support (99 %) were found in ASV47 with BMVT_30 by Rimington *et al*. (2019) and in ASV1 with OTU4 from the study by Orchard *et al*. (2016) whereby the latter is identical to *Planticonsortium tenuis* that was described by Walker *et al*. (2018) (Gary D. Bending, pers. comm.). With this observation, we can confirm that this genus was present in the field soil at Nafferton Farm. The same ASV1 also stood out in analyses regarding treatment effects where it occurred in zero N-treatment and at a higher level than when mineral N was applied (Fig. 5.13). These results would indicate preference of this taxon for low-N environments, but this hypothesis requires further investigation since this ASV occurred in high abundances in only one sample. More interestingly would be the comparison of ASV3 and ASV1 which occurred in contrasting patterns regarding mineral N application (Fig. 5.13). These two highly abundant sequences

could potentially mark distinct strains with different ability to cope with the presence of inorganic N-sources. Their functionality regarding N-transfer as well as the identity and phylogenetic relation of ASV3 to AMF would assist to add crucial information to the interactions of FRE and nutrient conditions in agricultural systems. Field *et al.* (2019) demonstrated that in contrast to Glomeromycotina, Mucoromycotina fungi contribute significantly to N-uptake of liverworts. Whether their model is also applicable to inorganic N-sources and to vascular plants requires further investigation.

5.4.6 LIMITATIONS OF THE PRESENT STUDY

In the present study, DNA extracts of six soil samples were included for ITS1-sequencing but these samples were from a different field at Nafferton Farm (Somerville) and had been collected in 2020, one year after the second field season (Leonidas Rempelos, pers. comm.). This makes these samples less comparable to the results presented here, but their analyses confirmed the previously discussed dilution effect of the ITS1-marker region for AMF by other fungal phyla (Section 5.4.3) as the proportion of Glomeromycota was much lower in soil samples than in root samples (Appendix C, Fig.C.6 A). Furthermore, *Glomus* was no longer the most abundant genus which is in line with results of other studies which compared AMF communities in roots with bulk soil and rhizosphere samples (Liu *et al.*, 2014; Berruti *et al.*, 2018; Barceló *et al.*, 2020; Stefani *et al.*, 2020). The same studies emphasize the importance of analysing both root and soil fractions which are important for nascent AMF symbiotic interactions (Kokkoris *et al.*, 2020b). Therefore, the outcome of this study cannot be used to extrapolate the observed effects of agronomic management practises from the AMF community inside wheat roots to the overall mycobiome at the field trial site.

The most recommended tool in combination with the DADA2-pipeline is DESeq2 which was encouraged for use in mycorrhizal research (Hart *et al.*, 2015; Callahan *et al.*, 2016; Savary *et al.*, 2020). At the same time, DESeq2 has been declared as not ideal for microbial abundance data since it was originally developed for 'RNA-Seq' gene expression studies (Love *et al.*, 2014; Lin & Peddada, 2020). For the analyses of the mycobiomes in the present study, the *DESeq*-algorithm applied to FRE-community composition could identify differentially abundant ASVs (Fig. 5.13), but this was not possible for the ITS1-sequences where PERMANOVA and pairwise comparison revealed a significant difference of Aszita and Skyfall with conv. CP (Table 5.8). There is conflicting information about the ability of DESeq2 to adjust for random effects which could explain the different results of PERMANOVA compared to *DESeq*-analyses. The need to incorporate 'block' as a random factor in mixed-

effect models and in microbiome analyses of the present analyses becomes clear considering the shift in AMF community composition between the different blocks that was indicated in the ITS1-dataset (Fig. 5.9 B). Other R-packages that can include random effects such as Maaslin2 (Mallick *et al.*, 2021) were inspected, but there are also other options that include different programmes besides R (C. Campos *et al.*, 2018).

Considering the low taxonomic resolution and high variability of the ITS1-region in Glomeromycota as previously discussed (Section 5.4.3), it is questionable if further bioinformatic analyses could reveal differences in microbiome composition based on this marker. Perhaps the SSU would have been more useful to elucidate potential shifts in response to agronomic management or wheat variety as it was found with specific FRE-primers (Fig. 5.12). To achieve higher taxonomic resolution for the SSU, a nested PCR with specific Glomeromycota primers and a reduced number of cycles has been suggested (Stefani *et al.*, 2020). As previously mentioned, the most optimal approach would be the use of a long nrDNA fragment as recently suggested by Kolaříková *et al.* (2021). Such long amplicons can be sequenced for example by Single Molecule Real Time (SMRT) with PacBio platforms which might become more feasible in the future, but could not be used in the present study.

5.5 CONCLUSIONS

The results of this study supplemented the microscopy data from root colonisation assessment with molecular evidence for the presence of arbuscular mycorrhizal fungi (AMF) and fine root endophytes (FRE). Additional to this main objective, it was shown that the root powder used for field inoculation only contained *Rhizophagus irregularis* (R.i.) instead of three AMF species (as stated by the manufacturer) and these strains were also detected in three field samples that were not actually inoculated. This observation could either indicate potential invasive behaviour of the R.i.-inoculum during the first field season or the presence already of these strains in the soil, but further sample analyses are required to discriminate these hypotheses. Although it was possible to separate R.i.-strains based on ITS2-sequence variations, the results of both ITS-sequencing runs should be interpreted with care as it has been suggested by mycorrhizal scientists especially for ITS1. Therefore, it is not clear if the observed lack of treatment effects are due to primer-bias or can be explained by high resilience of Glomeraceae towards the agronomic management practices used in this study. These fungi dominated AMF communities in wheat roots at the studied growth stages independently of fertiliser, variety or crop protection. Further investigation using more samples is required to explain the conflicting findings of prevalent AMF genera based on ITS and SSU markers. The latter seem to be more trustworthy since they identified that besides AMF also FRE contributed to approximately half

of the arbuscule forming fungal community in wheat roots at the assessed time point. The increased diversity of Endogonales in response to mineral nitrogen (N) has not been reported in the literature before but might be due to the functional differentiation of these fungi regarding N-uptake.

In summary, with the present study the efficiency of two novel primer pairs was examined for their efficiency to generate Endogonales-sequences which will assist future studies of this yet understudied group of endophytes. At the same time, the use of short nrDNA markers might become obsolete in the future as more advanced molecular tools are developed for the investigation of AMF in environmental samples. The approaches applied here gave a preliminary insight into the fungal endophytic community in wheat in the tested system, but more sampling, e.g. of soil, and the use of highly resolving DNA markers is required to further validate final conclusions. Overall, the presented studies highlight critical points which still need to be optimised in molecular research on AMF.

CHAPTER 6. DEVELOPMENT OF A SEED COATING FOR MYCORRHIZAL FUNGI APPLICATION IN WHEAT

6.1 INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) based biostimulant products can be found as pure inocula or formulated with other beneficial microorganisms (Colla *et al.*, 2015). Although the market for biostimulant products has grown significantly during recent years (Sessitsch *et al.*, 2018), large scale application of AMF has not yet been established due to the economic feasibility of mycorrhizal technology in low value crops such as cereals (Vosátka *et al.*, 2012).

One of the difficult hurdles that prevent the successful implementation of biostimulant products in agriculture is the application method. Pedrini *et al.* (2017) reviewed seed coating as a cost efficient method for the application of agrochemicals which can reduce the overall amount of required compounds, and the same principle can be used for biostimulants (Rocha *et al.*, 2019b). Seed coating with AMF inocula has been suggested as an attractive method for large-scale application for example in wheat production (Rillig *et al.*, 2018). Furthermore, since sufficient phosphorus supply is important during early seedling growth, colonisation of seedlings by beneficial microbes is desirable (Castillo *et al.*, 2012). Seed coating with AMF could provide an early inoculation raising the chances of applied strains to colonise the roots (Wilson, 1984). Hence, it is important to select compatible coatings which do not inhibit seed germination or AMF viability, but which are also biodegradable as well as non-hazardous for humans and the environment (Malusá *et al.*, 2012; Vassilev *et al.*, 2015). At the same time, active ingredients such as protectants, soil adjuvants or nutrients can be added to the seed coat which could induce and promote both plant and fungal growth (Pedrini *et al.*, 2017). Sugars have been suggested to stimulate mycorrhization which can ultimately contribute to plant growth (Bedini *et al.*, 2018). Additives in seed coatings such as sugars can be formulated together with binder and filler substances to a product that can be used by seed companies or farmers for the application of AMF and other microorganisms. Moreover, high costs of AMF-inoculum production need to be overcome by using a high-quality inoculum formulated with low-cost binder/filler yielding positive effects under field conditions. A small number of studies is available on the seed coating with AMF. These either used silicon dioxide (Oliveira *et al*., 2016, Rocha *et al*., 2018), natural gums (Rocha *et al*., 2019a) or did not describe the formulation process (Colla *et al*. 2015) while cellulose-based seed coatings with AMF have not been investigated.

6.1.1 AIMS AND OBJECTIVES

The aim of this study is to develop a formulation that can be used to apply a dried root powder-based inoculum harbouring a consortium of different AMF strains to wheat seeds. This treatment should be practical, non-hazardous for humans and the environment and of low cost for farmers. In summary, the main objectives of this part of the study are:

1. Evaluate a selection of compounds with the potential to be used as seed coatings for the inoculation of wheat with AMF
2. Find the optimal composition of binder, filler and active substances that can form a stable seed coat
3. Assess the effectiveness of seed coating on AMF root colonisation, wheat growth and nutrient uptake

6.2 MATERIALS AND METHODS

The assessment of the applicability of the AMF containing root powder was conducted in three different steps (Fig. 6.1). The first phase comprised collection of information about suitable options for seed coating and therefore involved qualitative research. The second phase was based on observations while screening and handling of different substances and substance combinations. Quantitative data was collected from experiments conducted in phase 3 in which the performance of coated seeds was assessed under controlled conditions. The final formulation and its impact on plant development was tested in a greenhouse experiment.

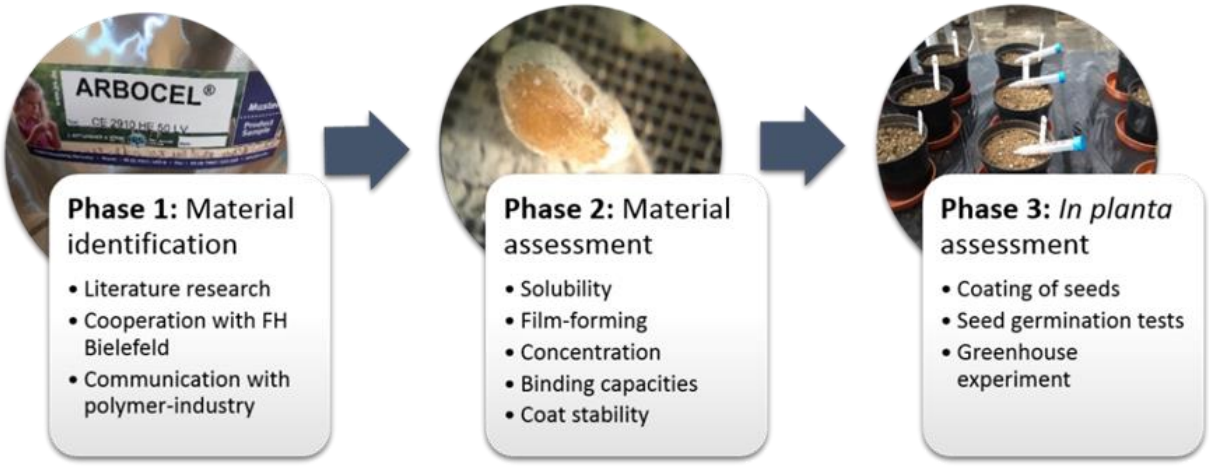


Fig. 6.1. Phases during the development and testing of a seed coating approach for the application of AMF-inoculum in form of root powder.

6.2.1 MATERIAL IDENTIFICATION

In the initial phase of the development of a seed coating with AMF, a screening of various substances was conducted. Most important criteria for the selection of a substance were the compatibility with AMF or other microorganisms, biodegradability, non-toxicity for humans and low cost.

During the initial phase, a co-operation between INOQ GmbH and the University for Applied Science of Bielefeld (FH Bielefeld, Working Group Patel, Désirée Jakobs-Schönwandt) was established to exchange information and experience about the formulation of biologicals. From this cooperation, contact with companies from the polymer producing industry was obtained. Representatives from the companies JRS (J. Rettenmaier & Söhne GmbH + Co KG, Germany) and Ashland™ (Wilmington, USA) were first consulted to provide information for common products used for seed coating purposes, and later provided samples of their recommended substances.

6.2.2 MATERIAL ASSESSMENTS

After a literature research and correspondence with partner institutions and experts, a selection of compounds was tested for their physical characteristics (Fig. 6.1, Phase 2) which are essential for the application in seed coatings (Fig. 6.2). The first screening was conducted using multiple substances which were considered to have binding as well as film-forming capacities to adhere the inoculum powder evenly around the seed (Binders, Appendix D). Since the AMF-inoculum is provided in the form of a fine powder (< 200 µm), it was decided to add a filler substance (Appendix D) to increase the volume of the product to make the seed coating process more applicable. Another advantage of using a filler is that it can be formulated with small dosages of active ingredients (Additives, Appendix D) with potentially beneficial effects on seed germination and the nutrition of symbiotic organisms.

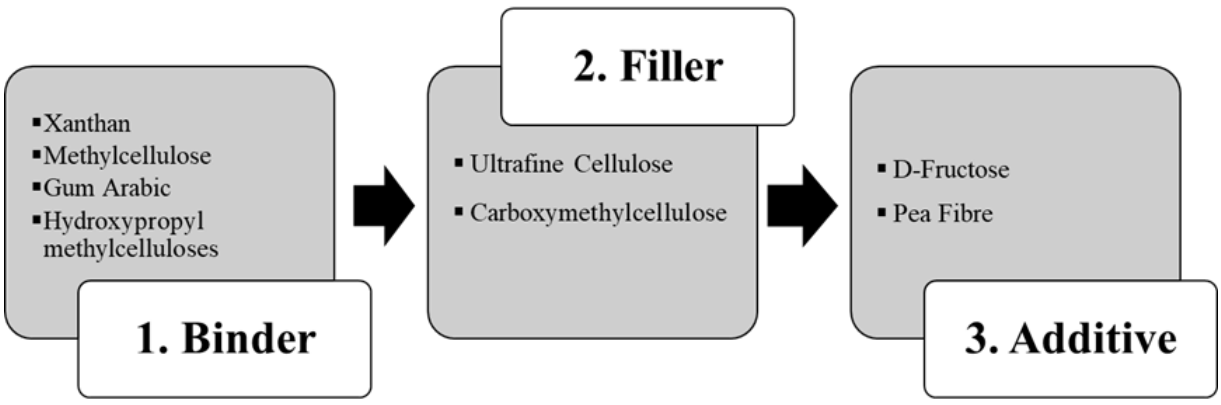


Fig. 6.2. Screening of materials used for coating wheat seeds with AMF-harbouring inoculum.

6.2.3 GERMINATION TEST WITH CELLULOSE-BASED COMPOUNDS

For germination tests with cellulose-based seed treatments, 25 wheat seeds ('Skyfall') per treatment (n = 3) were filled in 50 ml Falcon tubes. Solutions with Blanose 7ULC (3%), Blanose 7M (3 %), Culminal (2%) and a combination of the JRS-compounds with Arbocel HPMC (3%), Arbocel UFC100 (4%) and pea fibre (3%) in the ratio 7:1:2 were prepared by mixing the respective amount of the compounds in 100 ml sterilised tap water (Appendix D). Seeds were agitated in the tube until the surface of all seeds was covered with liquid. Sterilised water was used as a control for the untreated seeds. After coating, the seeds were placed on a metal mesh to dry in a laminar flow cabinet. The dried seeds were distributed in glass petri dishes with three layers of moistened kitchen paper. Glass dishes were incubated in the climate chamber (25°C, 16 h light, 4000 lux). After five days, the percentage seed germination was determined.

6.2.4 SCREENING OF SEED COATING FORMULATION UNDER GREENHOUSE CONDITIONS

A selection of compounds was screened for their potential to deliver AMF-inoculum as a seed coating. The experiment was set up in May 2019 in the greenhouse facilities at Cockle Park Farm. Prior to set up, field soil from unfertilised, non-inoculated plots from the field experiment (Chapter 3) was sampled and sterilised in the oven at 120 °C for two consecutive days with a break of 8 h between sessions. After oven sterilisation, soil nutrient content was determined with phosphorus = 14.6 mg L^{-1}, potassium = 77 mg L^{-1} and magnesium = 116 mg L^{-1} (NRM laboratories, UK). The sterilised soil was sieved and filled into one-litre pots.

6.2.5 FORMULATION

Ten different treatments were tested in the greenhouse experiment (Table 6.1). For each treatment, 100 wheat seeds were coated with different formulations of cellulose-based coating agents and additives in form of fructose and pea fibre. Arbocel®HPMC by JRS and Culminal™ by Ashland™ are both hydroxypropyl methylcelluloses (HPMC) that were used as binders (Appendix D). Filler substance was an ultra-fine cellulose (Arbocel® UFC100, JRS) and additives were D-Fructose and pea fibre (Vitacel®, JRS, Germany). Fructose was selected assuming stimulating effects on plant metabolism that would also affect mycorrhization (Lucic & Mercy, 2014). Pea fibre was suggested by JRS as a potential compound to support seedling vigour with an additional source of nitrogen to promote early seedling growth (Martin Kleinert, JRS pers. comm.).

The inoculum used in this experiment was a fine root powder (RP) with particle sizes of <200 µm (INOQ Advantage, INOQ GmbH, Germany). The inoculum contained overall 6.6 million propagules kg^{-1} of three different AMF species (*Rhizophagus irregularis* (50%), *Funneliformis mosseae* (25%) and *Funneliformis caledonium* (25%)). The treatment dose was set at 6.6 propagules per seed. For treatment 10 (T10), 1 g of the same inoculum was mixed into the medium for each pot leading to a concentration of 6600 propagules L^{-1}.

Table 6.1. Formulation of the coating treatments of wheat seeds (Skyfall) with and without AMF inoculum and active ingredients (binder, filler and additives). Treatment 9 with uncoated seeds was used as a seed coating control while Treatment 10 represents a control to test the activity of the inoculum from soil application. Each treatment was replicated six times.

Treatment	Binder	Filler	Additive	AMF Inoculum
1	Culminal™	Arbocel® UFC	Pea Fibre, Fructose	✓
2	Culminal™	Arbocel® UFC	Pea Fibre, Fructose	-
3	Culminal™	Arbocel® UFC	Pea Fibre	✓
4	Culminal™	Arbocel® UFC	Pea Fibre	-
5	Arbocel® HPMC	Arbocel® UFC	Pea Fibre	-
6	Arbocel® HPMC	Arbocel® UFC	Pea Fibre	✓
7	Arbocel® HPMC	Arbocel® UFC	Pea Fibre, Fructose	-
8	Arbocel® HPMC	Arbocel® UFC	Pea Fibre, Fructose	✓
9	-	-	-	-
10	-	-	-	Soil inoculation

6.2.6 SEED COATING PROCEDURE AND EXPERIMENTAL SETUP

For preparation of the seed coating binders 1g of Culminal and 1.5g of Arbocel HPMC were dissolved in 50 ml deionised water. For the complete coating powder used in treatment one (T1) and six (T6), Arbocel UFC (used as filler), pea fibre and Fructose (used as additives) and AMF inoculum were mixed in a ratio of 7:1:1:1 (Table 6.1). For the other treatments, the coating powders were mixed without the AMF inoculum but maintaining the same ratio i.e. 7:1:1.

The seeds for each treatment were placed in a glass beaker and were coated under constant agitation. First, 1 ml of the respective binder substance was gradually added with a pipette. When the glue was distributed evenly around the seed surface, 0.5 g of the coating powder was added slowly to the beaker until a first coating layer was established. This step was repeated by adding another 0.5 ml of glue and another 0.5 g of coating powder. The beaker was agitated until all seeds were covered. The coated seeds were placed on a wire mesh to dry at 30°C in the

oven. At the same time, a batch of untreated wheat seeds was placed in the oven and was later used for the control treatments T9 and T10 (Table 6.1). After drying, the seeds were placed in 15 ml Falcon tubes for transport to the greenhouse. Here, six treated seeds were placed on the surface of each pot and were subsequently covered with a thin layer of soil. All pots were completely randomised within the greenhouse.

Plants were watered regularly and grew under ambient light and temperature conditions (23 ± 2°C /16 ± 2°C day/night). Germination was recorded at 7 and 14 days after sowing (DAS). At 14 DAS, all treatments were thinned to four plants per pot.

6.2.7 PLANT GROWTH PARAMETERS AND NUTRIENT ANALYSIS

After six weeks, the plants were harvested at GS32 (Zadoks *et al.*, 1974). Shoots were cut at the stem base and were weighed before and after drying at 70°C for 72 hrs. The dried shoots were milled (Cyclone MILL TWISTER, Retsch, Germany) with 100,000 rpm and a sieve size of 250 µm. One gram of milled shoot was digested with concentrated HNO_3 and HCl in an open-vessel microwave reaction system using a temperature-controlled digestion block (CEM-Mars 6, USA). The concentrations of phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca), sulphur (S), manganese (Mn), zinc (Zn), copper (Cu), iron (Fe) and boron (B) were analysed by inductively coupled plasma-optical emission spectrometry (ICP-OES) by NRM Laboratories (Berkshire, UK). Nitrogen (N) concentration was determined by DUMAS combustion using a LECO TruSpec Automated C/N Analyzer (LECO Corporation, USA) according to the manufacturer's instructions.

6.2.8 ROOT COLONISATION

Roots were washed thoroughly under running tap water. A fraction of each root sample was weighed before and after oven drying with the shoot parts. The remaining sample was stored in 50% ethanol in the fridge before further processing. Staining and colonisation assessment was conducted as described in section 3.2.9 (Chapter 3).

6.2.9 STATISTICAL ANALYSIS

All data was collected and processed in MS Excel (vs. 2013, Microsoft Corporation, 2019). Tables were saved and imported into RStudio (vs. 3.6.1, R-Core Team, 2019). Data distribution was tested using Shapiro-Wilk test assuming normal distribution at $p > 0.05$. For data which did not show normal distribution, natural logarithm, square root, cube root or reciprocal transformations were applied. Analysis of variance (ANOVA) was carried out with subsequent Tukey- contrasts for multiple pairwise-comparison of means using the multcomp-package

in R (Hothorn *et al.*, 2008). Data sets from coating and control treatments (no inoculum, soil inoculation) were analysed separately. Data that could not be fitted to normal distribution of residuals (i.e. germination data) was analysed by non-parametrical tests. Plots were generated in R using the ggplot2 package (Wickham, 2016).

6.3 RESULTS

6.3.1 GERMINATION UNDER CONTROLLED CONDITIONS

Seed treatment with celluloses and pea fibre did not affect germination compared to seeds treated with water (Fig. 6.3 A). Examination of coating quality under the stereo microscope showed high porosity of Blanoses (Fig. 6.3 B) for which reason both tested Blanoses were excluded from further screenings.

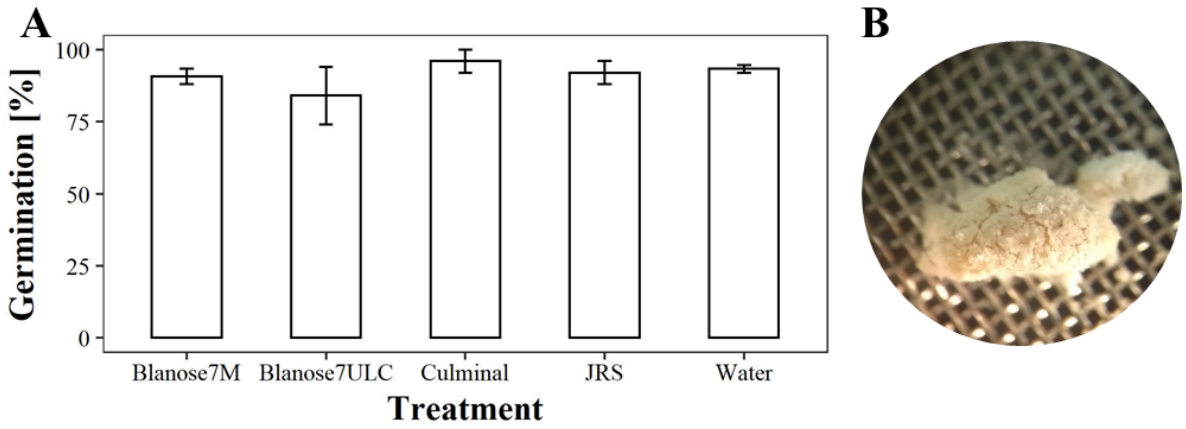


Fig. 6.3. Screening tests for seed coat with cellulose **A)** Wheat seed coated with Blanose after drying **B)** Average germination [%] of wheat seeds (n = 3) coated with celluose provided by JRS (blend of ultrafine cellulose, pea fibre and HPMC in the ratio of 1:1:2) and Ashland (Culminal (2%), Blanose 7M (2 %), Blanose 7ULC (2%)). Water was used as a control treatment. Error bars show means ± SE.

6.3.2 GERMINATION UNDER GREENHOUSE CONDITIONS

Coating of wheat seeds in sterilised soil affected germination % significantly in the first (p = 0.006, Fig. 6.4 A) and in the second week (p < 0.001) after seeding. Control treatments germinated the fastest, whereas 100% germination of seeds in pots with soil inoculation was reached only in the second week. No significant differences were found between binders (Arbocel and Culminal) or in response to fructose application. AMF inoculum application in the soil (p = 0.034) as well in form of seed coating (p = 0.013) reduced germination % (Fig. 6.4 B).

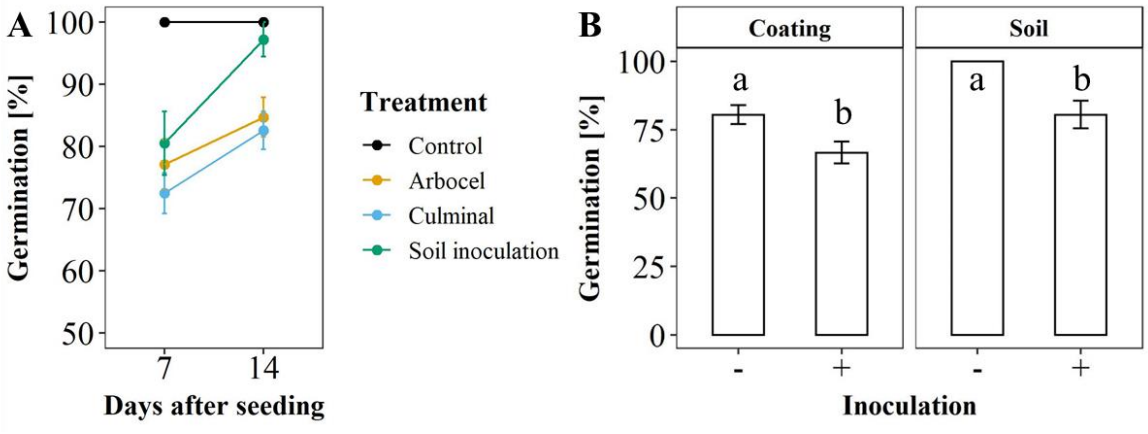


Fig. 6.4. Germination [%] of coated and uncoated wheat seeds. **A)** Germination of seeds with AMF inoculum applied as seed coating (Arbocel and Culminal, n = 24) or soil inoculation (n = 6) compared to untreated seeds (Control, n = 6) at two time points. Data points represent means ± SE. **B)** Germination of coated (Coating, n = 24) and uncoated seeds (Soil, n = 6) at 7 days after planting. Bars represent means ± SE. Letters indicate significant differences between treatments for $p \leq 0.05$.

6.3.3 CROP GROWTH

AMF inoculation significantly decreased shoot biomass in both, coated (p = 0.046) and non-coated seeds (p = 0.041, Table 6.2). Root biomass was reduced in plants grown from untreated seeds in soil containing AMF inoculum (p =0.027), but not in plants grown from seeds coated with AMF inoculum. No main effects were observed in response to binder or fructose application.

Table 6.2. Effects of binder substance, inoculation and additive on root and shoot dry weight [g] of wheat during greenhouse experiment. Control plants represent untreated seeds +/- AMF inoculum soil application. Numbers presented are means ± SE of the mean. ANOVA *p*-values in bold indicate significant main effects and interactions. Means within columns followed by the same letter are not significantly different at $p \leq 0.05$.

	Shoot dry weight [g]	**Root dry weight [g]**
Binder (B)		
Arbocel (n=24)	3.2±0.1	1.2±0.07
Culminal (n=24)	3.4±0.12	1.4±0.13
Inoculation (I)		
+AMF (n=24)	3.1±0.12 b	1.3±0.12
-AMF (n=24)	3.4±0.1 a	1.2±0.09
Additive (A)		
+Fructose (n=24)	3.2±0.13	1.3±0.12
-Fructose (n=24)	3.4±0.09	1.3±0.09
Control		
+AMF (n= 6)	3±0.25 b	1±0.2 b
-AMF (n=6)	3.7±0.19 a	1.7±0.17 a
ANOVA *p-values*		
Main effects		
B	ns	ns
I	**0.046**	ns
A	ns	ns
Interactions		
B x I	ns	ns
B x A	ns	ns
I x A	ns	**0.028**
B x I x A	ns	ns
Control +/- AMF	**0.041**	**0.027**

Plants grown from seeds with fructose and coating without AMF inoculum showed lower root biomass compared to plants without fructose coating (Table 6.3). This effect was not observed when the seed coat contained AMF inoculum. Within fructose treatments, AMF inoculum application had no effect on root biomass.

Table 6.3. Effect of fructose × AMF inoculum on wheat root biomass.

Means ± SE (n = 12)	**Additive**	**-AMF**		**+AMF**	
Root biomass [g]	- Fructose	1.5±0.09	Aa	1.2±0.15	Aa
	+ Fructose	1±0.14	Ba	1.4±0.2	Aa

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test.

6.3.4 SHOOT NUTRIENT CONTENTS

Seed coating with fructose increased concentrations of N ($p = 0.024$, Table 6.4), P ($p = 0.046$), Cu ($p = 0.021$) and B ($p = 0.01$), but decreased Ca concentration in shoots ($p = 0.002$). The AMF inoculum had no effect on nutrient concentrations seed coating treatments, but shoots from soil-inoculated pots showed elevated K ($p = 0.012$) and Cu ($p = 0.033$) concentrations as well as decreased Mg ($p = 0.028$) concentrations. Plants treated with Culminal showed significantly higher S concentrations in shoots compared to seeds coated with Arbocel.

Table 6.4. Effects of binder, +/- AMF inoculation and additive on concentrations [mg kg^{-1}] of nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca), sulphur (S), manganese (Mn), copper (Cu), zinc (Zn), iron (Fe) and boron (B) in dried wheat shoots. Control plants represent seeds without seed coating +/- AMF inoculum application to the soil. Values show means ± SE for coated seeds (n=48) and control groups (n=12). ANOVA *p*-values in bold indicate significant main effects and interactions. Means within columns followed by the same letter are not significantly different at $p \leq 0.05$.

Main Effects	**N**	**P**	**K**	**Ca**	**Mg**	**S**	**Mn**	**Cu**	**Zn**	**Fe**	**B**
Binder (B)											
Arbocel (n=24)	2.8±0.08	0.2±0.01	2.7±0.08	0.3±0.01	0.1±0	1942.2±70.97b	234.6±10.41	7.6±0.23	38.6±1.22	93.4±8.55	7.3±0.21
Culminal (n=24)	2.7±0.07	0.3±0.01	2.6±0.1	0.3±0.01	0.1±0	2171.1±104.82a	240.1±13.8	7.4±0.27	38.9±1.55	88.7±2.46	7.3±0.17
Inoculation (I)											
+AMF (n=24)	2.9±0.07	0.3±0.01	2.7±0.11	0.3±0.01	0.1±0	2149.2±111.2	247.7±13.31	7.8±0.3	38.5±1.26	95.5±8.51	7.6±0.19
-AMF (n=24)	2.7±0.08	0.3±0.01	2.7±0.07	0.3±0.01	0.1±0	2031.3±81.89	226.8±12.48	7.4±0.19	39.8±1.56	86.2±2.49	7.2±0.2
Additive (A)											
+Fructose (n=24)	2.9±0.09a	0.3±0.01a	2.7±0.1	0.3±0.01a	0.1±0	2150.6±111.18a	252.6±13.79	7.8±0.28a	39.2±1.44	96.2±7.5	7.6±0.22a
-Fructose (n=24)	2.7±0.05b	0.2±0.01b	2.6±0.07	0.3±0b	0.1±0	1967.8±60.05b	219±8.86	7.2±0.19b	38.2±1.35	84±2.55	7±0.12b
Control											
+AMF (n= 6)	3±0.18	0.3±0.01	2.9±0.11a	0.4±0.03	0.1±0	2075.3±117.96	255.2±9.92	8.2±0.43a	36.1±1.18	102.2±8.62	7.9±0.48
-AMF (n=6)	2.7±0.11	0.3±0.01	2.4±0.14b	0.3±0.02	0.1±0	1902.3±89.98	241±11.09	6.8±0.37b	35.7±2.51	90.4±3.75	6.8±0.06
ANOVA p-values											
Main effects											
B	ns	ns	ns	ns	ns	**0.027**	ns	ns	ns	ns	ns
I	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
A	**0.024**	**0.046**	ns	**0.002**	ns	**0.095**	ns	**0.021**	ns	ns	**0.010**
Interactions											
B x I	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
B x A	ns	ns	ns	ns	ns	**0.046**	ns	ns	ns	**0.0002**	ns
I x A	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
B x I x A	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Control +/- I	ns	ns	**0.012**	ns	**0.028**	ns	ns	**0.033**	ns	ns	ns

The adhesives in combination with fructose had significant impact on S ($p = 0.046$, Table 6.4) and Fe-concentrations ($p < 0.001$). In comparison of both adhesives, shoots grown from seeds coated with Culminal had higher S concentrations when fructose was applied compared to seeds without fructose (Table 6.5). In shoots from Arbocel treatments, this effect was not observed for S, but for Fe where concentrations increased in response to sugar application to the same level as Culminal-treated seeds which were not affected by fructose.

Table 6.5. Effect of fructose × adhesives Arbocel and Culminal on Sulphur and Iron concentration of shoots.

Means ± SE (n = 12)	**Additive**	**Arbocel®**		**Culminal™**	
Sulphur [mg kg^{-1}]	- Fructose	1954±88.67	Aa	1981.6±88.52	Ba
	+ Fructose	1930.3±114.79	Ab	2495±179.34	Aa
Iron [mg kg^{-1}]	- Fructose	76.2±1.64	Bb	91.9±3.81	Aa
	+ Fructose	94±3.28	Aa	84.8±3.43	Aa

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different $p \leq 0.05$.

6.4 DISCUSSION

Arbuscular mycorrhizal fungi (AMF) are considered as a promising tool in sustainable agriculture, but the production of AMF-based biostimulants for large-scale field use is cost intensive (IJdo *et al.*, 2011). This study describes the approach to formulate a biostimulant product in the form of a root powder (RP) for seed treatment. Screenings of binder substances and additives revealed suitable compounds which did not affect biomass production, but altered nutrient concentrations under greenhouse conditions. The results of this experiment could not demonstrate if the tested formulation affected AMF as no root colonisation was observed in any of the treatments. The absence of AMF root colonisation (AMF-RC) was most likely due to inactivity of the inoculum since no AMF colonisation occurred when AMF were directly applied to the soil either. These results underline the importance of extensive formulation screening in combination with viable inoculum for seed-coating of AMF application to be a feasible method in wheat production.

6.4.1 SEED COATING MATERIALS

Prior to the *in-vivo* experiments with celluloses, different compounds such as gum arabic, xanthan gum and polyvinylpyrrolidone (PVP) that are used for the inoculation of rhizobacteria for legumes (Deaker *et al.*, 2004) were included in the material screenings (Section 6.2.2), but

were not further tested due to different factors. For example, the complete biodegradability of PVP is not guaranteed under certain environmental conditions (Vanharova *et al.*, 2017), but biodegradability is an important criteria for the application of biostimulants in crop production (Malusá *et al.*, 2012). Gum arabic on the other hand is a natural product that was successfully used as a film coating with biochar and AMF to increase yields in chickpea (Rocha *et al.*, 2018). To reduce costs, seed coating with gum arabic requires a drum coater where the binder can be applied as a spray at low dosages (Pedrini *et al.*, 2017). This equipment was not available during material screenings for which reason gum arabic was later excluded from further screenings. The same applies to xanthan gum which represents a low priced alternative to gum arabic as it can be produced on a large-scale in bioreactors and has shown effective film-forming characteristics at lower dose (García-Ochoa *et al.*, 2000).

Experiments were conducted with cellulose-based adhesives such as methylcellulose which showed no negative effects on germination (Fig. 6.3 A). Methylcelluloses are synthetically produced, hence cheaper than natural gums and are commonly used binders in seed coatings (Pedrini *et al.*, 2017). Hydroxypropylmethyl celluloses (HPMC) show increased solubility compared to pure methylcelluloses and are commonly applied in drug and food production, thus should be compatible with living organisms such as AMF in crop production. In contrast to HPMCs, carboxymethyl celluloses (CMC) like the two tested Blanoses (7M and 7 ULC) are not film-forming which explains the brittle coat that was observed under the stereo microscope (Fig. 6.3 B). Blanoses could have been more suitable as filler substances to improve breathability of the coat which would have been an advantageous trait considering the observed inhibition of seed germination during the greenhouse experiment (Fig. 6.4 A). CMCs were not involved in further screenings as the instability of the coat would not pass the Heubach-test for the assessment of free floating dust of treated seeds (Heitbrink, 1990). The Heubach-test would probably not be passed either by seeds which were used during the greenhouse experiment, but coating quality was sufficient for the present experimental approach since almost no substance disintegration from the seed surface after drying and shaking was observed (data not shown). For larger-scale or even commercial production however, seeds would be coated using a pan coater or similar machine which could ultimately change the coating characteristics and possibly also results of the experiment.

6.4.2 PLANT GROWTH AND NUTRIENT CONTENT WITHOUT AMF COLONISATION

Delayed or reduced seed germination is not uncommon after seed treatment, but should be compensated at later growth stages (Scott, 1975). This was aimed by the application of AMF inoculum which can increase seedling vigour and growth as shown e.g. in durum wheat and chickpea (Colla *et al.*, 2015; Rocha *et al.*, 2018). The opposite was observed in the current seed coating experiment where the presence of AMF inoculum in the seed coat or applied directly to the soil reduced seed germination (Fig. 6.4). This is likely due to a permeability barrier which impedes water uptake, aeration and hence germination (Scott, 1975). In this case, germination tests involving the full formulation of binder, filler and additives prior to the greenhouse trial could have highlighted these constrains. Furthermore, potential negative effects of seed coating compounds should be excluded by assessment of multiple germination parameters besides the germination % (Hotta *et al.*, 2016). However, these tests cannot simulate actual conditions in the soil and do not explain why soil inoculation also delayed germination. A methodical explanation for this observation could be found in differences of seeding depth during setup of the experiment as pots were set up by different persons. This would explain why germination of this treatment was delayed, but not overall reduced (Fig. 6.4 A). Nevertheless, delayed germination was visible in terms of reduced shoot biomass of all treatments involving AMF-application (Table 6.2). Growth depression in wheat has been associated with AMF inoculation (e.g. Graham & Abbott, 2000; Ryan *et al.*, 2005), but no AMF-RC was observed in this experiment. Assuming the AMF inoculum was densely colonised and contained numerous fungal propagules, it remains unclear why the inoculum was not active during the experiment. Kloepper & Schrot (1981) report lower survival rates of applied microorganisms in seed coatings with methylcellulose compared to xanthan, for which reason it could be assumed that mycorrhizal germination might have been impeded due to cellulose application. But since no AMF-RC was detected when the inoculum was applied directly to the soil, the inoculum was obviously not active. Damage or loss of activity could have been occurred during shipping/storage where the conditions are unclear but once received at Newcastle University the inoculum was stored at 4°C, in the dark. Viability of AMF in the inoculum and after coating could have been tested by MTT staining (An & Hendrix, 1988), but it was assumed that the coating process would not damage AMF propagules.

If the viability of the inoculum was not affected during transport or by the application method, the inactivity of AMF propagules can only be explained by the environmental conditions in the greenhouse or the substrate. The first can be excluded as AMF development from natural inoculation was observed in other experiments running at the same time in the same greenhouse

(data not shown). The use of field soil in this study was important to simulate *in-situ* environmental conditions, but its chemical properties might have been changed during the sterilisation processes in the oven. High temperatures as applied during autoclaving or microwave incubation have been shown to alter pH, aggregation and soil chemistry significantly which can ultimately affect plant growth and associated microorganisms (Salonius *et al.*, 1967; Lees *et al.*, 2018). Further chemical analyses are required to evaluate if the applied method for soil pasteurization in this experiment created non-favourable growth conditions for AMF.

Beneficial effects on plant growth in treatments with inactive AMF could still have been expected (Jansa *et al.*, 2020), but shoots showed lower biomasses from treatments with AMF inoculum application (Table 6.2). A chemical profile of the RP revealed high copper (Cu) concentration (213 ppm, Louis Mercy INOQ pers. com.) which could explain the increases of Cu in the shoots and reduced biomass as Cu can have phytotoxic properties (Luo & Rimmer, 1995). Cu concentrations were elevated in shoots grown from seeds treated with RP, but not significantly as the inoculum had been applied in much lower quantities compared to soil inoculation. This might also explain why root biomass was reduced in soil inoculated pots, but did not affect plants grown from seed treatments with RP (Table 6.3).
Similar to the RP, pea fibre was formulated in the seed coat with the purpose to stimulate plant growth by providing nitrogen (N) to the germinating seeds. However, there were no measurable increases of N concentrations after seed coating and plants grown from uncoated seeds showed similar N concentrations to coated seeds (Table 6.4). The wheat plants were thus not able to use pea fibre as N-source, likely because pea fibre would contain organic N which needs to be broken down over time before it can be taken up as inorganic N and sterilised soil would contain low microbial activity.

6.4.3 EFFECT OF FRUCTOSE ON NUTRIENT CONTENTS

Fructose in seed coatings increased N, P, Cu, B and S concentration in shoots (Table 6.4), while it decreased root biomass if no inoculum was applied (Table 6.3). Application of even small quantities of ‘trigger molecules’ such as fructose has been shown to stimulate mineralisation by soil microbes resulting in an increase of plant available nutrients (De Nobili *et al.*, 2001; Hamer & Marschner, 2005). This effect is also known as ‘priming’ which describes the constant state of alert of cells under increased turnover rates of energy. Priming of soil microbes has also shown to be sustained in the presence of cellulose (De Nobili *et al.*, 2001) which could explain the increased concentrations of nutrients following fructose use in seed coatings in the present

study. Although the experiment was conducted in sterilised soil and no AMF colonisation was found to support this hypothesis, microbes could still derive from the seed surface or the RP. DNA-profiling of the microbiome of substrate and root samples of the wheat plants could have revealed the association of microbial communities with different treatments (Jansa *et al.*, 2020), but was not part of this project.

In the whole analyses, the only significant differences between Arbocel and Culminal were increased sulphur concentrations in the shoots of seeds coated with Culminal which was more pronounced with the addition of fructose (Table 6.5). The opposite was observed for iron concentrations which were significantly higher in treatments with Arbocel HPMC and fructose. The reason for these alterations in mineral profiles of plants might lay in the molecular structure of the respective adhesives or in differences during synthesis, but further information about these processes is not available (Norbert Nüchter Ashland, pers. com.).

However, the assumption that fructose application stimulated microbial priming in the soil does not explain decreases in root dry weights of seeds coated without AMF and sugar (Table 6.3). This is particularly surprising since increased concentrations of essential macronutrients such as N and P were found (Table 6.4). Perhaps fructose did not only attract beneficial, decomposing microbes, but also fungal pathogens as levels of powdery mildew were greater at later growth stages (data not shown).

6.4.4 ECONOMIC FEASIBILITY OF AMF SEED COATING IN WHEAT PRODUCTION

Seed coating with AMF has been suggested as a viable method in cereal production which facilitates targeted application of small inoculum doses to enhance AMF presence and colonisation in the field (Rillig *et al.*, 2018). This has been demonstrated in one study where the combination of AMF and *Trichoderma atroviride* increased grain yield and nutrient concentrations in wheat shoots (Colla *et al.*, 2015). However, seeds with only AMF were not tested, while *Trichoderma* spp. is a well-known biocontrol agent that also mobilizes P and stimulates plant growth (Srivastava *et al.*, 2010; Buysens *et al.*, 2016). Due to the lack of a comparable control treatment without *T. atroviride* and AMF only, this study does not prove the applicability of AMF through seed coating under field conditions.

A greenhouse trial with AMF and wheat showed that the application of AMF by seed coating could reduce the amount of required fertiliser (Oliveira *et al.*, 2016). Following these results, the authors present seed coating with AMF as a promising eco-technological approach for sustainable production of wheat, whereas few years earlier they had stated that AMF inoculation

is economically not realistic for cereal production (Vosátka *et al.*, 2012). Interestingly, more recent publications from the same research group focus on seed coating with AMF in other crops such as maize (Rocha *et al.*, 2018), chickpea (Rocha *et al.*, 2019a) and cowpea (Rocha *et al.*, 2020). In their field trials, legume inoculation with AMF and plant growth promoting rhizobacteria (PGPR) consortia showed yield increases up to 50 % and ameliorated crop quality. At the same time, these studies represent one of the few examples that consider application costs for AMF inocula and revealed in this way that the investment for the farmers is profitable for the respective crops. If we do the same for wheat with a seeding rate of 400 seeds per m^2 and calculate the costs for the inoculation of 200 kg of wheat seeds with 6.6 propagules per seed as used in the present study, farmers would pay €700 just for the inoculum (€2400 kg^{-1}, February 2020) to drill one hectare (ha) of wheat. The recommended rate by INOQ GmbH of 50 g inoculum per ha (February 2020), the inoculation would still cost €240 ha^{-1} for 200 kg of wheat seeds which would then receive 0.08 propagules per seed if 1 g of inoculum contains 6600 propagules. This amount of inoculum might be too low as suggested by Rocha *et al.* (2019, 2020) who applied 20 propagules per seed to achieve profitable yield increases. Adopting their inoculation rate for wheat, seed treatment of 200 kg wheat seeds for one hectare would cost €2100 with the tested inoculum. For example, winter wheat yields in Germany are around 7.5 t ha^{-1} and farmers are currently getting 176.25 € t^{-1} (boerse-online.de, accessed: 28 June 2020,), hence €1321.24 for one ha of wheat. To compensate the costs for AMF-inoculation would require a yield increase of at least 2 t ha^{-1} which does not include the required coating materials and actually coating of the seeds. In the end it is obvious that AMF application on this price scale is not feasible for large scale application in a low-value crop like wheat. This is particularly true as long as clear benefits of AMF for wheat production are not proven (Vosátka *et al.*, 2012; Ryan & Graham, 2018).

6.5 CONCLUSION

Inoculation of low commodity crops like wheat with arbuscular mycorrhizal fungi (AMF) to increase plant health and growth in agricultural requires large quantities of root powder (RP) inoculum. Seed coating is likely to be a more efficient and cheaper method than application directly to the soil, especially when dealing with small amounts/concentrations of biostimulant product. This approach was tested for wheat in a greenhouse experiment after screening different binder substances. It is likely that the AMF inoculum was not active as no colonisation was observed following the use of AMF in the seed coating treatment or applied directly to the soil. The use of cellulose-based seed coating reduced germination % and plant biomass during early growth. Some nutrient concentrations in shoots were partially increased following

fructose application which can be due to microbial priming. The effect of fructose in providing an energy source for AMF could not be evaluated as the inoculum in this study was inactive.

Positive reports on the effects of seed coating with AMF can be found in the literature but are mostly available for high value crops. The results of the present study show that it is important and difficult to optimise conditions for biostimulant activity. The current high cost of AMF inoculum means that potential for use on high volume crops like wheat is limited. Seed coating formulations with other plant growth promoting microbes have shown promising results under field conditions and therefore warrant further investigation with an active AMF inoculum.

CHAPTER 7.GENERAL DISCUSSION

The association of plants and arbuscular mycorrhizal fungi (AMF) has been investigated extensively with the aim to evaluate and optimise the symbiotic benefits in crop production (Pellegrino & Bedini, 2014; Thirkell *et al.*, 2017; Bitterlich *et al.*, 2020). The two main approaches to achieve this objective are either the inoculation with AMF strains that have been selected based on their efficiency to take up nutrients, or adapted agricultural management practices to support naturally abundant mycorrhiza (Douds *et al.*, 2005). Both aspects were covered by the present study which investigated the effect of an AMF-harbouring inoculum on the performance of wheat and the impact of agronomic management practices and variety on native AMF. A clear advantage of AMF association with wheat resulting from biostimulant application or natural AMF could not be demonstrated by the employed methods of this study. Nevertheless, the results offer several implications for future studies on the optimisation of exogenous and native AMF in agricultural systems.

7.1 EFFECTS OF AMF INOCULATION

7.1.1 PLANT GROWTH

Efficiency measures of the inoculated AMF strains were implemented by targeted application as a seed coating in a greenhouse experiment (Chapter 6) and by application to a field trial (Chapter 3). Both approaches did not show that the application of this biostimulant product provides benefits under the tested conditions except for increased biomass production in the absence of fertiliser inputs that did not contribute to improvements in grain yield or quality. Digital droplet PCR (ddPCR) with strain-specific molecular primers showed that the inoculated strains did not establish in the second field trial in 2019 (Chapter 4). Despite this result and the lack of root colonisation during seed coating experiments, application of the AMF-harbouring root powder affected wheat growth under environmental and controlled conditions. From these observations the question arises if the root powder stimulated plant growth by providing access to an additional source of nutrients. This effect has been demonstrated in greenhouse experiments using inactivated AMF inoculum (Jansa *et al.*, 2020) for which reason the establishment of appropriate control treatments in mycorrhizal experiments requires reconsideration (Gryndler *et al.*, 2018). It is unlikely that such effects would be measurable under field conditions in response to the extremely low quantities at which the root powder was applied. Compared to field inoculation rates (100 g ha^{-1}), more pronounced effects would be expected in the seed coating experiment where the concentration of the root powder in the

substrate was much higher i.e. 1 g per pot. But in contrast to enhanced wheat biomass in plots without fertiliser, the effect of the AMF inoculum in the seed coating experiment reduced wheat biomass which was also found with a combination of mineral N and AMF inoculation in the field trials. Although these experiments are not directly comparable as no N-fertiliser was used in the seed coating experiment, N-fertilisation can have a great impact on plant response to AMF-inoculation (Hoeksema *et al.*, 2010). Ercoli *et al.*, (2017) report 50 % increased P-uptake with AMF inoculation but only when no N-fertiliser was added. To elucidate this potential effect for the present study requires nutrient analyses of the harvested shoots which had not been completed by the end of this project. Furthermore, Ercoli *et al.* (2017) did not trace the AMF inoculum as they observed significant changes in mycorrhizal root colonisation (AMF-RC) in response to AMF inoculation. Such effects were not found in the present study for which reason a strain-specific primer assay was developed, but only root samples from zero-input plots were used for these analyses. Then again, it is unlikely that the introduced AMF strains were active in mineral N plots as zero-input plots represented nutrient-limited systems where AMF-inoculation is evidently most successful (Hoeksema *et al.*, 2010; Mäder *et al.*, 2011; Ercoli *et al.*, 2017). These contrasting findings from greenhouse and field studies demonstrate that the outcome of biostimulant application is unpredictable and difficult to replicate across different environments and conditions (Faye *et al.*, 2013; Hart *et al.*, 2018). Although hardly comparable, both field and greenhouse trials indicated that the impact of the AMF inoculum on plant growth is not necessarily due to active mycorrhizal root colonisation for which reason further investigations are required to exclude negative effects of the product in future applications.

7.1.2 NATIVE AMF

Although no indications for the establishment of the inoculated strain of *Rhizophagus irregularis* (R.i.) QS81 were found during ddPCR, the same approach revealed that inoculation of Skyfall decreased mitochondrial DNA (mtDNA) markers for the native R.i. strains. At the same time, ITS2-amplicon sequencing of the same R.i. strain and those in root samples from the field trials revealed that similar nucleotypes of R.i. were present in non-inoculated wheat roots and in the applied root powder (Chapter 5). Taken together, these findings could indicate that the applied R.i. strain even spread to uninoculated plots in 2018, but did not establish in the consecutive field season 2019. This hypothesis can only be tested by the probe-based ddPCR assay with root samples from 2018, but these were only collected for microscopy.

Besides its effects on plant growth (Section 7.1.1), the decrease of mtLSU markers provides another indication for an indirect effect of the inoculum. Similar findings based on mtLSU

sequences are not reported in the literature, but deserve further investigation to exclude potential negative effects of the QS81 strain on native R.i. (Thomsen & Hart, 2018). There have been suggestions that nuclear ribosomal DNA (nrDNA) markers are more efficient than mtDNA for the quantification of fungal biomass in roots (Köhl *et al.*, 2016; Voříšková *et al.*, 2017). However, the indication of a correlation of mtLSU counts and AMF-RC in this study contradict this notion. Furthermore, the mtLSU instead of nrDNA as a molecular marker for tracing of the applied R.i. strain was probably the better choice considering the identical nrDNA-nucleotypes in the targeted R.i. strain QS81 and the R.i. strains from uninoculated plots at Nafferton Farm according to ITS2- sequencing.

The impact of the AMF inoculum on mtLSU copy numbers in roots of Skyfall was detected after DNA samples had been sent for ITS1-amplicon sequencing. In another sequencing approach with higher taxonomic resolution than ITS1, AMF inoculation would be included as a factor in the community profiling by amplicon sequencing. In contrast to the impact of fertiliser and its impact on mycorrhizal diversity (Oehl *et al.*, 2004; Gosling *et al.*, 2013), there are less studies on the effects of commercial biostimulants on native AMF communities. Ercoli *et al.* (2017) report reduced abundance of *Funneliformis* spp. after inoculation with R.i. in durum wheat which supports other studies where the same species modified the composition of native AMF communities (Schlaeppi *et al.*, 2016; Elliott *et al.*, 2019). The results of the present study support the demand for further research on the effects of commercial inocula on native AMF communities (Schwartz *et al.*, 2006; Rodriguez & Sanders, 2015; Hart *et al.*, 2018). The use of high-resolution molecular tools such as ddPCR and novel sequencing methods in mycorrhizal research are strongly encouraged to generate such information.

7.2 OPTIMISING AMF APPLICATION IN AGRICULTURE

7.2.1 COMPETITION

Competition with native AMF can antagonise establishment of introduced strains (Farmer *et al.*, 2007; Martignoni *et al.*, 2020) and was discussed as a major aspect that affected the AMF inoculum used during field trials at Nafferton Farm. Although it is nearly impossible to avoid competition with native AMF due to their ubiquitous distribution (Tedersoo *et al.*, 2014; Davison *et al.*, 2015), assessment of the natural mycorrhizal inoculum potential of the soil at Nafferton Farm prior to the start of the field trial would have given an idea about the abundance of local AMF. This assessment can be done by a most probable number test (MPN-test) which is more commonly conducted to calculate the number of infective propagules of an inoculum (Farmer *et al.*, 2007; Srivastava *et al.*, 2010; Niwa *et al.*, 2018), but can also be used

to determine the mycorrhizal potential of a soil (Ramos-Zapata *et al.*, 2011; Alaux *et al.*, 2018). The MPN-method in the context of AMF has been criticised due to the strong dependence of the assay results on environmental conditions (Wilson & Trinick, 1982). For the same reason, the two MPN-tests that were established for each field trial season are not reported in this thesis due to low seed germination and poor plant growth (*Plantago lancelota*). MPN tests conducted prior to field inoculation would have shown that the soil at Nafferton Farm (with low P status and the lack of use of pesticides and mineral fertilisers during recent years) already harboured native AMF and fine root endophytes (FRE) as later demonstrated by amplicon sequencing, and therefore does not require inoculation with exogenous AMF (Schwartz *et al.*, 2006). When the natural AMF-inoculum potential of a soil is low, significant yield increases can be expected even in low-mycorrhizal responsive crops such as wheat (Mohammad *et al.*, 2004). Furthermore, positive results have been generated in phytoremediation of contaminated soils (Lenoir *et al.*, 2016) or grassland restoration (Koziol & Bever, 2017) which provide examples where AMF inocula can be used besides crop production.

7.2.2 DOSAGE AND FREQUENCY

The dosage of AMF inoculum for field inoculation needs to offset mycorrhizal symbiosis efficiency with financial expense as too little will result in limited root colonisation while excess amounts create unnecessarily high costs for farmers (Verbruggen *et al.*, 2013; Hart *et al.*, 2018). At the same time, inoculum dosage depends on the presence of native AMF (Section 7.2.1) as inoculation success is determined by the balance of introduced and native mycorrhizal propagules (Wilson, 1984). Mohammad *et al.* (2004) showed yield increases following the application of AMF in wheat grown after 20 years of fallow in an extremely low-P soil. They used a monospecific inoculum of *Rhizophagus intraradices* (former *Glomus intraradices*) in the form of sheared roots at a rate of 0.5 g per 1000 seeds containing 1.75×10^5 AMF propagules g^{-1} which is comparable to the inoculum used in the 2019 season of the present study (Chapter 3, Table 3.5). Applied to the seed rate of the present study, this dosage would mean the application of 2 kg ha^{-1} of AMF inoculum. Although the dosage of 100 g ha^{-1} of AMF inoculum that was recommended by the producer (INOQ GmbH) and therefore used in the field trials of this study was possibly too low, such rates as described by Mohammad *et al.* (2004) are not feasible for small scale or medium sized farms (Oviatt & Rillig, 2020) considering the high costs (Chapter 6, Section 6.4.4).

Repeated inoculation with AMF propagules is supposed to increase the mycorrhizal potential of a substrate or soil (Kokkoris *et al.*, 2019a), but the results of the present study do not confirm

this hypothesis. The ddPCR assays with strain-specific primers showed that the inoculated R.i. strain did not establish in spring 2019 although the AMF inoculum had been applied twice to the same area of soil by this time point. Similar results were published by Kokkoris *et al.* (2019a) who found no build-up of mycorrhizal inoculum potential after re-inoculation with a commercial biostimulant at different farm sites. So far, there is little discussion on inoculation frequencies with AMF, most likely because there are only a small number of publications that have used realistic dosages and found corresponding meaningful increases in crop production under field conditions (Hart *et al.*, 2018).

7.2.3 APPLICATION METHOD AND TIMING

Primary root infection or the so called 'priority effect' represents a competitive advantage for AMF and hence determines inoculation success (Wilson, 1984; Verbruggen *et al.*, 2013; Thonar *et al.*, 2014). For this reason, seed coating appeared to be the ideal method for AMF inoculum application, but no inoculum activity was observed using this method in the present study. Nevertheless, several positive results of seed coatings with AMF even under field conditions have been reported (Colla *et al.*, 2015; Oliveira *et al.*, 2016; Rocha *et al.*, 2019b, 2020) for which reason this method should be further developed if cost-benefit balances are guaranteed (Section 7.2.2).

In general, inoculation methods should be straight forward and feasible for farmers by using standard machinery (Malusá *et al.*, 2012). For the field trials, this was aimed by use of the twin hopper which was originally designed for the simultaneous application of fertiliser and seeds during drilling. The concurrent placement of inoculum and wheat seeds should facilitate early colonisation of seedlings, but it is not clear if the low temperatures and light conditions in autumn hampered germination of the inoculated fungi (Hetrick *et al.*, 1984). Although there has been little research on inoculation timing, higher light intensities and warmer temperatures would help activate applied AMF propagules leading to early symbiotic establishment (Wilson, 1984; Konvalinková & Jansa, 2016). Therefore, inoculation of spring wheat rather than winter wheat or other spring crops might represent a more compatible option for inoculation with AMF (Verbruggen *et al.*, 2013; P. Campos *et al.*, 2018).

7.2.4 FUNGAL IDENTITY

Multiple studies as well as modelling of inoculation experiments have shown that the application of multiple AMF instead of a single species improves inoculation success (Verbruggen *et al.*, 2013; Gosling *et al.*, 2016; Martignoni *et al.*, 2020). For the same reason, the AMF inoculum in this study contained *Funneliformis mosseae* and *Funneliformis*

caledonium and R.i. with distinct colonisation behaviour (Louis Mercy, pers. comm.). However, amplicon sequencing of the inoculum compounds showed that instead of three AMF species, only R.i. was present in the AMF inoculum. It is not unusual that commercial fungal inoculants do not contain the species or strains which are stated by the producer for which reason standardised quality assessments of these products are needed (Faye *et al.*, 2013; Hart *et al.*, 2018). Inoculation with solely R.i. means that potential benefits by the other two fungal species could not be exploited. In particular *F. mosseae* has been described as a fast coloniser that facilitates efficient nutrient transfer (Jansa *et al.*, 2008) and that has the ability to enhance wheat production under field conditions (Al-Karaki *et al.*, 2004; Colla *et al.*, 2015). Also R.i. has been reviewed as a competitive AMF (Thomsen & Hart, 2018), but amplicon sequencing of the small subunit (SSU) of a selection of root samples from the field trials showed that R.i. belonged to the most abundant species in the soil at Nafferton Farm. Although AMF strains of the same species can be considerably different in their colonisation behaviour and also in their impact on plants (Mensah *et al.*, 2015; Rodriguez & Sanders, 2015), enhanced competition is expected if inoculated AMF target the same ecological niche as resident fungi (Farmer *et al.*, 2007; Verbruggen *et al.*, 2013; Martignoni *et al.*, 2020). Therefore, application of a single AMF species instead of three might have lowered both the competitiveness and effectivity of the inoculum during field trials.

7.3 MANAGING MYCORRHIZAL SYMBIOSIS IN WHEAT PRODUCTION

7.3.1 CROP PROTECTION

The results from mycorrhizal root colonisation assessments and amplicon sequencing showed no detrimental effects of conventional crop protection on AMF-RC or fungal community composition which confirms that the selected chemicals were compatible with AMF (SmartRotations, 2017). However, it cannot be excluded that negative effects of pesticides would be detected longer-term or that e.g. sporulation or mycorrhizal efficiency were not affected (Dodd & Jeffries, 1989). The selection of mycorrhizal compatible pesticides might be particularly important as AMF alone cannot confer plant protection comparable to fungicides (Alaux *et al.*, 2018). Proof of mycorrhiza-induced disease resistance based on field trials are scarce (Pozo & Azcón-Aguilar, 2007), but nevertheless scientists emphasize that this potential outcome of mycorrhizal symbiosis should be incorporated in plant breeding strategies (Hohmann & Messmer, 2017). From the data of the present study, it cannot be stated how this mycorrhizal use efficiency is characterised for Aszita and Skyfall since no comparison to

disease levels in non-mycorrhizal plants was conducted. If mycorrhizal responsiveness should be considered in wheat breeding programmes for organic agriculture, future studies should investigate if AMF symbiosis can contribute to yield and grain quality by extension of leaf area duration and photosynthetic activity without the use of fungicides. In low-input agricultural systems where pesticides are not banned, field studies are required that use gradients of chemicals and fertilisers to investigate if reduced inputs can exploit benefits of AMF-symbiosis. The pesticides/fertilisers applied during such studies should be screened for mycorrhizal compatibility in advance, ideally over several cropping seasons and in relation to multiple parameters that characterise AMF-plant interactions besides AMF-RC.

7.3.2 VARIETY

The seed coating experiment included non-mycorrhizal treatments and involved nutrient content analyses for which reason it represented a possibility to gain more information about the interaction of AMF on Skyfall. This variety was not only stronger colonised by AMF than Aszita in the field trials, but also showed altered mtLSU counts of native R.i. strains in roots in response to AMF inoculum. Therefore, it was presumed that Skyfall is more responsive to AMF than Aszita for which reason it was selected for the seed coating approach. But since the AMF inoculum was not active in the greenhouse experiment, this study does not provide information about the characteristics of mycorrhizal responsive wheat varieties. Besides suitable genotypes, cereal breeders require genetic factors that can be manipulated to develop wheat varieties which profit from the association with AMF (Sawers *et al.*, 2008). The comparison of marker genes that characterise mycorrhizal responsiveness in Skyfall and Aszita would have been interesting, but localising mycorrhizal dependency in the complex and highly variable wheat genome is challenging and was not part of this thesis. Two genome wide association studies (GWAS) by Lehnert *et al*. (2017, 2018) of 94 wheat varieties identified potential quantitative trait loci (QTLs) for genes that might be involved in AMF-symbiosis. They estimated heritability of mycorrhizal colonisation as moderate which is advantageous for targeted breeding for improved AMF-RC (Lehnert *et al.*, 2017). If these markers will be applied in wheat breeding is questionable as modern wheat varieties have been shown to outcompete the performance of older varieties even in low-input systems (Hildermann *et al.*, 2010; Voss-Fels *et al.*, 2019), but stronger responses of older wheat varieties (released before 1950) to AMF inoculation have been reported (Zhang *et al.*, 2019). Moreover, it is not guaranteed that selecting for mycorrhizal compatibility will also improve other important traits such as nutrient uptake (Singh *et al.*, 2012; Leiser *et al.*, 2016) or disease resistance (Jacott *et al.*, 2017), although these are characteristics

of mutualistic AMF symbiosis, however which are not always pronounced (Johnson *et al.*, 1997).

7.3.3 FERTILISER INPUT

According to AMF-RC data from the field trials, it was concluded that addition of P in the form of farmyard manure (FYM) and biogas digestate had detrimental impact on AMF. In contrast, there was no effect of organic fertilisers on AMF diversity or community composition indicating that the applied P-rates were too low to affect AMF communities (Hijri *et al.*, 2006; Gosling *et al.*, 2013). Based on these contrasting findings from microscopy and sequencing data that has also been reported in other studies (Jumpponen *et al.*, 2005; Gosling *et al.*, 2013; Mao *et al.*, 2014), it is difficult to draw conclusions about mycorrhiza-compatible fertiliser treatments. Here, most promising might be the application of biogas digestate which achieved high yields comparable to mineral N while showing lower disease levels. Hence, biogas digestate offers the possibility to reduce pesticides, but the consistent decrease of AMF-RC in both field trials contradicts this sustainability aspect and requires further investigation.

The addition of N altered the effect of AMF-inoculation on wheat (Section 7.1.1), but also indicated that AMF are only decreased by mineral N if soil N-supply is high and that similar to biogas digestate and FYM this fertiliser does not affect AMF community composition. It remains open if the latter effect would also be identified for the first trial season where mineral N significantly decreased AMF-RC, or if molecular markers with higher resolution had been employed. These findings are contrary to those of Egerton-Warburton *et al.* (2007) who showed positive effects of N-fertilisation on AMF in P-limited soils and suggest a key role of soil N-content in the interaction of AMF and wheat. If AMF are to be considered in wheat production where grain yields are mostly limited by N-supply (Debaeke *et al.*, 2006), this N-threshold for AMF should be looked at and characterised in future studies.

7.3.4 FINE ROOT ENDOPHYTES

After the confirmation of fine root endophytes (FRE) in wheat roots by molecular analyses, the sub-set of samples used for amplicon sequencing (n =12) was re-examined by microscopy. As compared with AMF, the FRE root colonisation data indicates that these fungi accounted for the majority of what had previously been scored as AMF (Fig. 7.1). On the one hand, this rough estimation demonstrates that mycorrhizal root colonisation by microscopy is a subjective method that should be complemented by molecular evidence (Kokkoris *et al.*, 2019b). On the other hand, the reassessment implies that abundance of Glomeromycotina-AMF was most likely over-estimated by ignoring FRE during root colonisation assessments. Besides of

microscopy, future studies should employ molecular tools to differentiate between FRE and AMF, for example by using specific primers in ddPCR that could provide detailed quantification of both fungal groups in environmental samples.

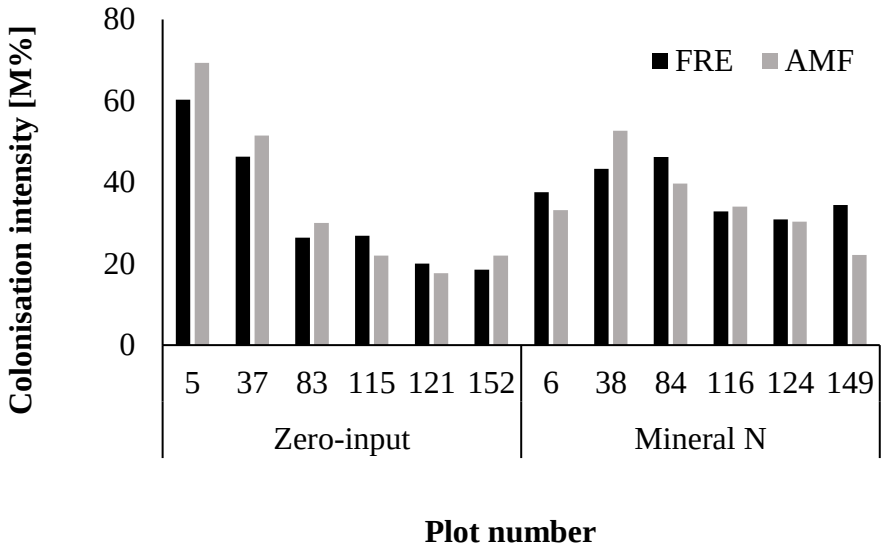


Fig. 7.1. Colonisation intensities (M%) of arbuscular mycorrhizal fungi (AMF) and fine root endophytes (FRE) in selected root samples from wheat treated with mineral N or without fertiliser input at the stem elongation phase (GS32) in 2019. Numbers on the x-axis show plot numbers.

Colonisation assessment of FRE in all samples of the field trials could have revealed important information about the response of FRE to agronomic management practices. The diversity promoting effects of mineral N on selected FRE-taxa that were found during molecular analyses implied that FRE might have a higher tolerance towards N-fertilisers than AMF (Sigüenza *et al.*, 2006) which suggests that these fungi might be quite robust regarding destructive environmental factors as found in agricultural production systems (Hetrick *et al.*,1984). As mutualistic symbionts with the potential to increase nutrient uptake and plant performance (Powell, 1979; Hoysted *et al.*, 2019), FRE might soon be recognised as potential biostimulants which is supported by recent advances in development of the methodology for the extraction and propagation of these fungi (Sinanaj *et al.*, 2021).

7.4 CONCLUSION AND FUTURE WORK

In his definition about biostimulants, du Jardin (2015) uses the allegory of the human gut microbiome for the management of AMF in plant production: "[…] adding inoculants (i.e. 'probiotics') is one thing, but feeding beneficial bacteria with prebiotics seems even more important". Applied to the context of agriculture, this means that before the use of biostimulants becomes common practice, it is most important to create favourable environmental conditions

to exploit the agroecosystem services provided by AMF. As shown by the presence of natural AMF in the field trials of this study, these conditions are developed by using low nutrient inputs, minimal tillage, compatible or no pesticides and cover crops. Intriguingly, the same agronomic management practices have been discussed in the context of sustainable intensification of agricultural systems (Section 1.2) which means that sustainable transformation of crop production systems goes hand in hand with mycorrhizal management. To accelerate this process in European agriculture, research results on the benefits of healthy soil microbiomes should be communicated to farmers and political decision-makers. The latter should provide financial support to research and practitioners for the transition process. The use of biostimulants still needs to be optimised with regards to economic benefits for farmers. But before this aspect becomes relevant, further research is required to exclude potential side-effects of exogenously introduced strains on plant performance of native AMF as found in the present study. The enhanced incorporation of molecular tools in mycorrhizal research in combination with standardised quality assessments in the biostimulant industry will ultimately assist in providing farmers with effective products. If these need to be used in an agricultural system should be analysed prior to inoculation in co-operation of farmers, scientists and industry. The same applies to breeding programmes that aim to exploit mycorrhizal associations in crop development. Here, but also in food production in general, the consideration of mycorrhizal agroecosystem services instead of extensive inputs presumes a shift from a focus on quantity towards quality and sustainability. With regards to the predominating notion in Europe that yields need to be constantly increased, it remains questionable if such a rethink will happen in the near future. Instead, mycorrhizal technology should be adapted to areas e.g. where extreme environmental conditions or conventional farming practices have degraded soils the most. Here, biostimulants should be used as a bridging structure with the aim to re-introduce mycorrhizal benefits to an environment without creating dependencies of farmers on product applications. The role of fine root endophytes besides AMF in all these processes harbours intriguing research questions that still need to be elucidated.

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Appendix A CHAPTER 3

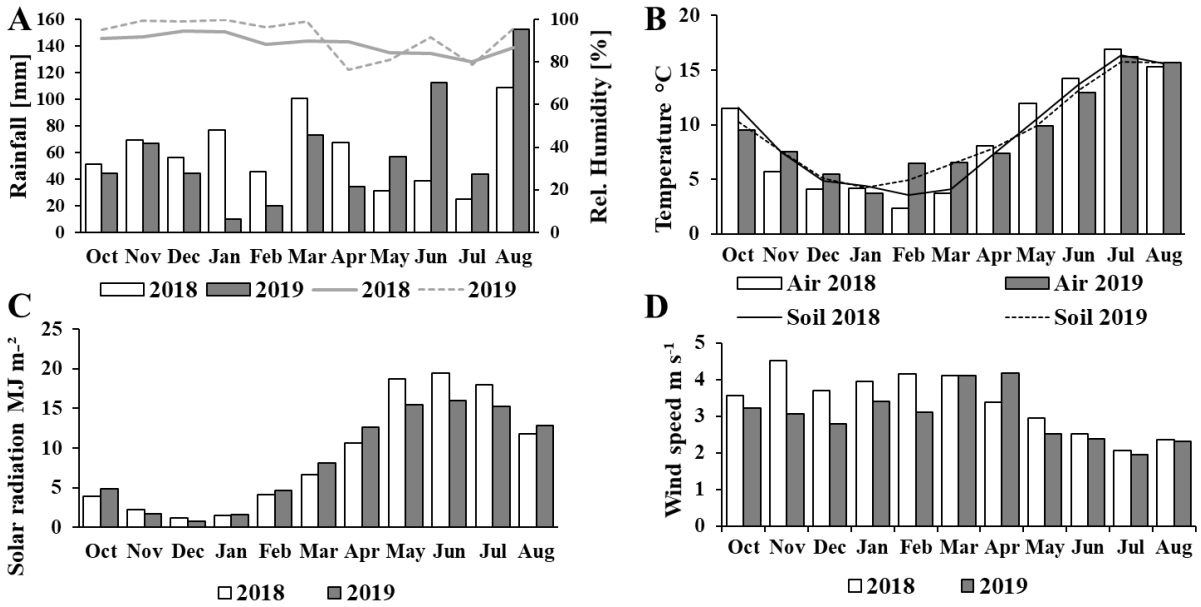

Fig. A.1. Climate parameters measured at Nafferton Farm during two growing seasons 2017-2019. **A**) Average monthly rainfall and relative humidity **B**) Average monthly temperature of air and soil **C**) Average solar radiation **D**) Average wind speed.

Table A.1. Effects (and interactions) of crop protection, variety, AMF inoculation and fertiliser application on soil mineral nitrogen in the second field trial (2019) at three depths [cm]. ANOVA *p*-values in bold indicate significant main effects and interactions. Means within columns followed by the same letter are not significantly different at $p \leq 0.05$.

	Nitrate			Ammonium			Total available N		
Depth [cm]	**0-30**	**30-60**	**60-90**	**0-30**	**30-60**	**60-90**	**0-30**	**30-60**	**60-90**
Crop protection (CP)									
Conventional (n = 32)	2.5±0.27	2.6±0.34	1.9±0.26	3.1±0.17a	0.3±0.05	0.2±0.04	21±1.38a	11±1.3	8.2±1
Organic (n = 32)	1.9±0.21	1.7±0.26	1.5±0.22	2.1±0.22b	0.4±0.03	0.4±0.09	14.9±1.16b	8±0.97	7.3±0.91
Variety (VR)									
Aszita (n = 32)	2.8±0.28a	2.6±0.3	2.1±0.27	2.6±0.24	0.4±0.04	0.4±0.09	20.2±1.62a	11.3±1.11	9.3±1.06a
Skyfall (n = 32)	1.6±0.13b	1.7±0.3	1.4±0.2	2.6±0.21	0.4±0.05	0.3±0.04	15.7±0.94b	7.8±1.16	6.2±0.74b
Inoculation (AMF)									
-AMF (n = 32)	2.1±0.26	1.9±0.25	1.6±0.22	2.8±0.22	0.4±0.04	0.4±0.09	18.4±1.5	8.4±0.98	7.5±0.92
+AMF (n = 32)	2.3±0.23	2.5±0.35	1.8±0.26	2.4±0.22	0.4±0.05	0.3±0.05	17.5±1.25	10.7±1.31	8±0.99
Fertility (FT)									
Farmyard manure (n = 32)	2±0.21	2±0.32	1.7±0.24	2.6±0.2	0.4±0.05	0.3±0.05	17.4±1.17	9±1.23	7.4±0.95
Zero-input (n = 32)	2.3±0.28	2.3±0.3	1.8±0.24	2.6±0.24	0.3±0.04	0.4±0.08	18.5±1.57	10.1±1.12	8.1±0.96
ANOVA *p-values*									
CP	ns	ns	ns	**0.038**	ns	ns	**0.039**	ns	ns
VR	**0.019**	ns	ns	ns	ns	ns	**0.040**	ns	**0.050**
AMF	ns	ns	ns	ns	ns	ns	ns	ns	ns
FT	ns	ns	ns	ns	ns	ns	ns	ns	ns
Interactions									
CP:VR	ns	ns	ns	ns	ns	ns	ns	ns	ns
CP:FT	ns	ns	ns	ns	ns	ns	ns	ns	ns
VR:FT	ns	ns	ns	ns	ns	ns	ns	ns	ns
CP:AMF	ns	ns	ns	ns	ns	ns	ns	ns	ns
VR:AMF	ns	ns	ns	ns	ns	ns	ns	ns	ns
FT:AMF	ns	ns	ns	**0.046**	ns	ns	ns	ns	ns
CP:VR:FT	ns	ns	**0.026**	ns	ns	ns	ns	ns	ns
CP:VR:AMF	ns	ns	ns	**0.02**	ns	ns	ns	ns	ns

CP:FT:AMF	ns	ns	ns	ns	ns	ns	ns	ns	ns
VR:FT:AMF	ns	ns	ns	ns	ns	ns	ns	ns	ns
CP:VR:FT:AMF	ns	ns	ns	ns	ns	ns	ns	ns	ns

Table A.2. Nutrient content of organic fertilisers applied during the 2018 and 2019 field seasons based on dry matter analyses.

Biogas digestate				
	2018		**2019**	
Nutrient	**Content [% w/w]**	**Rate applied per 170 kg N ha^{-1}**	**Content [% w/w]**	**Rate applied per 170 kg N ha^{-1}**
Total P	0.44	83.97	0.45	42.74
Total K	0.095	226.49	0.049	264.13
Total Mg	0.488	32.78	0.583	11.48
Total N	0.051	170	0.018	170
Farmyard manure				
	2018		**2019**	
Nutrient	**Content [% w/w]**	**Rate applied per 170 kg N ha^{-1}**	**Content [% w/w]**	**Rate applied per 170 kg N ha^{-1}**
Total P	3.51	73.09	3.26	72.96
Total K	0.659	181.91	0.611	173.34
Total Mg	3.13	57.32	2.77	36.18
Total N	0.713	170	0.418	170

Table A.3. Effect of variety, AMF inoculum and fertiliser on SPAD during grain filing (GS86) wheat. ANOVA *p*-values in bold indicate significant main effects and interactions. Means within columns followed by the same letter are not significantly different at $p \leq 0.05$.

	GS86, 2018	
	SPAD	**NDVI**
Variety (VR)		
Aszita (n=32)	13.9±0.96b	0.3±0.009b
Skyfall (n=31)	31.3±1.73a	0.35±0.01a
Inoculation (AMF)		
-AMF (n=32)	24±2.12	0.33±0.01
+AMF (n=31)	21.2±2.03	0.32±0.011
Fertiliser (FT)		
Biogas digestate (n=16)	25.6±2.77a	0.36±0.018a
Farmyard manure (n=15)	19.6±2.5b	0.29±0.007b
Mineral N (n=16)	27±3.37a	0.35±0.015a
Zero-input (n=16)	18.1±2.67b	0.3±0.008b
ANOVA *p*-values		
Main effects		
VR	**0.002**	**0.040**
AMF	ns	ns
FT	**≤0.001**	**≤0.001**
Interactions		
VR:AMF	ns	ns
VR:FT	ns	ns
AMF:FT	ns	ns
VR:AMF:FT	ns	ns

Table A.4. Effects of season, crop protection, variety, AMF inoculation and fertiliser on total seed number. ANOVA *p*-values in bold indicate significant main effects and interactions. Means within columns followed by the same letter are not significantly different at $p \leq 0.05$.

	Seeds m^{-2}
Year (YR)	
2018 (n=127)	10359.3±276.59
2019 (n=128)	10485.1±389.71
Crop protection (CP)	
Conventional (n=128)	11718.6±355.65a
Organic (n=127)	9116.1±274.54b
Variety (VR)	
Aszita (n=128)	10037.7±317.81
Skyfall (n=127)	10810.2±354.77
Inoculation (AMF)	
-AMF (n=128)	10553.6±349.36
+AMF (n=127)	10290.2±326.47
Fertiliser (FT)	
Biogas digestate (n=64)	11264.9±475.24b
Farmyard manure (n=63)	9803.5±401.59c
Mineral N (n=64)	12580.2±481.79a
Zero-input (n=64)	8031.4±346.5d
ANOVA *p-values*	
Main effects	
YR	ns
CP	**0.005**
VR	ns
AMF	ns
FT	**≤0.001**
Interactions	
YR:CP	ns
YR:VR	ns
CP:VR	ns
YR:AMF	ns
CP:AMF	ns
VR:AMF	ns
YR:FT	ns
CP:FT	**0.021**
VR:FT	ns
AMF:FT	**0.006**
YR:CP:VR	ns
YR:CP:AMF	ns
YR:VR:AMF	ns
CP:VR:AMF	**0.023**
YR:CP:FT	ns
YR:VR:FT	ns
CP:VR:FT	ns
YR:AMF:FT	ns
CP:AMF:FT	ns
VR:AMF:FT	ns
YR:CP:VR:AMF	ns

YR:CP:VR:FT	ns
YR:CP:AMF:FT	ns
YR:VR:AMF:FT	ns
CP:VR:AMF:FT	**0.014**
YR:CP:VR:AMF:FT	ns

Table A.5. Effect of fertiliser × AMF inoculation on seed number.

Means ± SE (n = 32)	**Fertiliser**	**-AMF**		**+AMF**	
Seeds m^{-2}	Biogas digestate	11190.2±615.03	Ba	11339.6±734.42	Aa
	Farmyard manure	9778.2±651.67	Ca	9829.6±473.64	Ba
	Mineral N	13676.5±625.48	Aa	11483.9±689	Ab
	Zero-input	7569.5±414.25	Da	8493.4±550.11	Da

Means followed by the same upper-case letter within a column and the same lower-case letter within a row are not significantly different for $p \leq 0.05$ by Tukey's HSD test

Appendix B CHAPTER 4

Optimisation of ddPCR assays

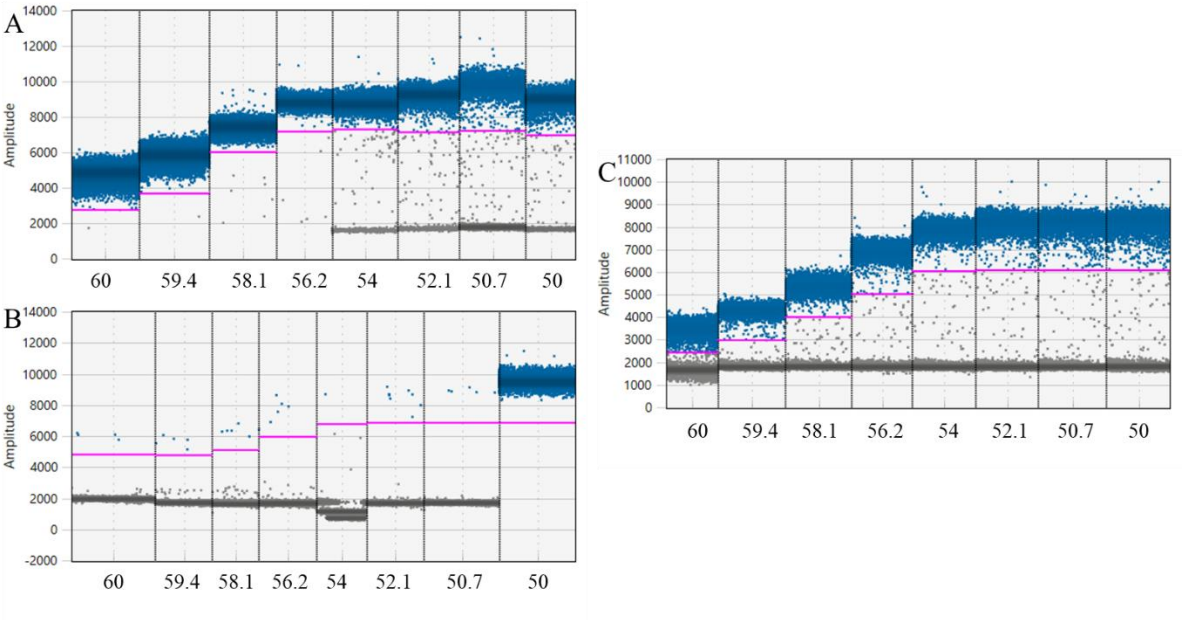

Fig. B.1. Amplitude plots of temperature gradients using: **A**) and **B**) species-specific primers (SSP) for mtLSU region of *Rhizophagus irregularis* and **C)** haplotype-specific primers (HSP) for AMF inoculum strain QS81. Annealing temperatures [°C] were tested for mycorrhizal inoculum (A,C) and in wheat roots harvested from an inoculated plot at GS22 in 2019 (B).

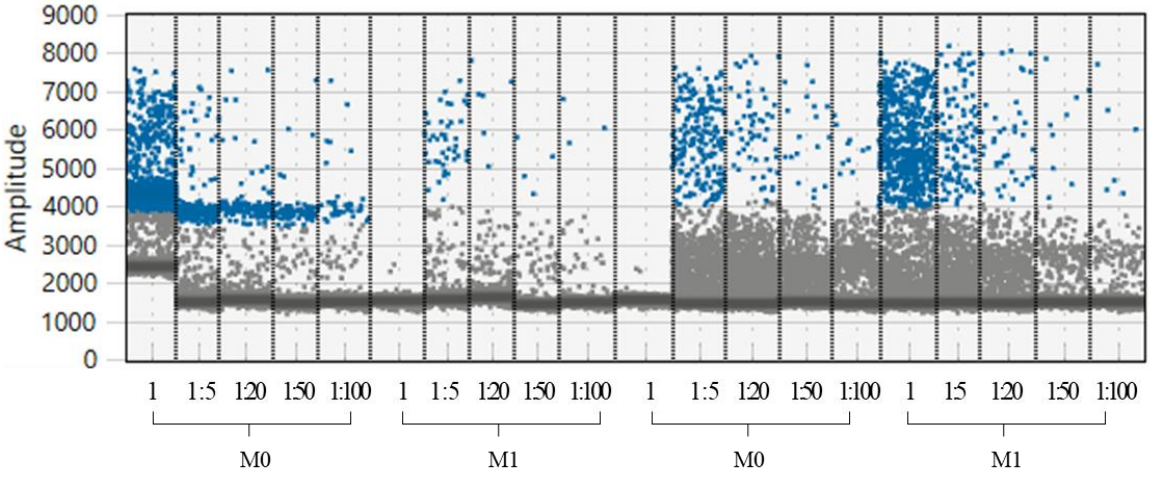

Fig. B.2. Amplitude plots of dilution series with selected root samples from inoculated and non-treated plots run with species-specific primers for mtLSU region of *Rhizophagus irregularis.*

Appendix C CHAPTER 5

ITS2-sequencing

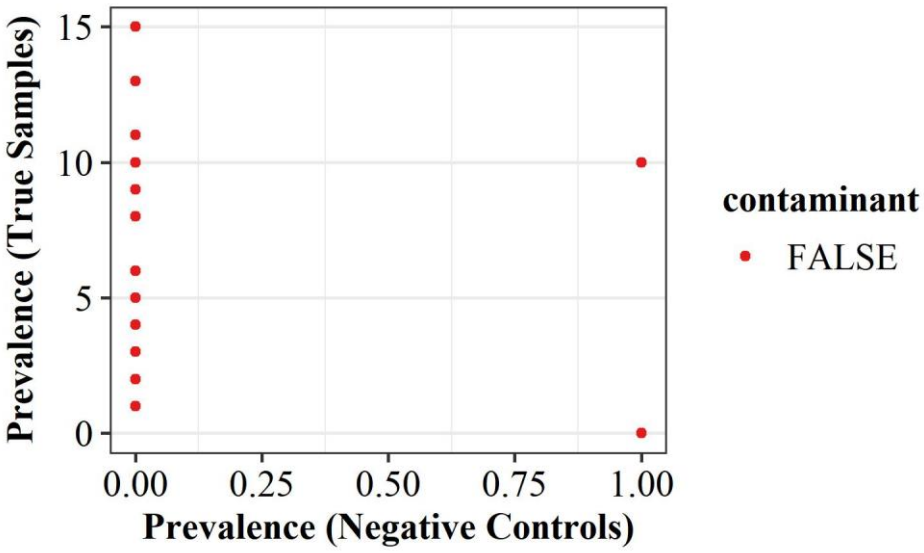

Fig.C.1. Prevalence of contaminating sequences in ITS2-library.

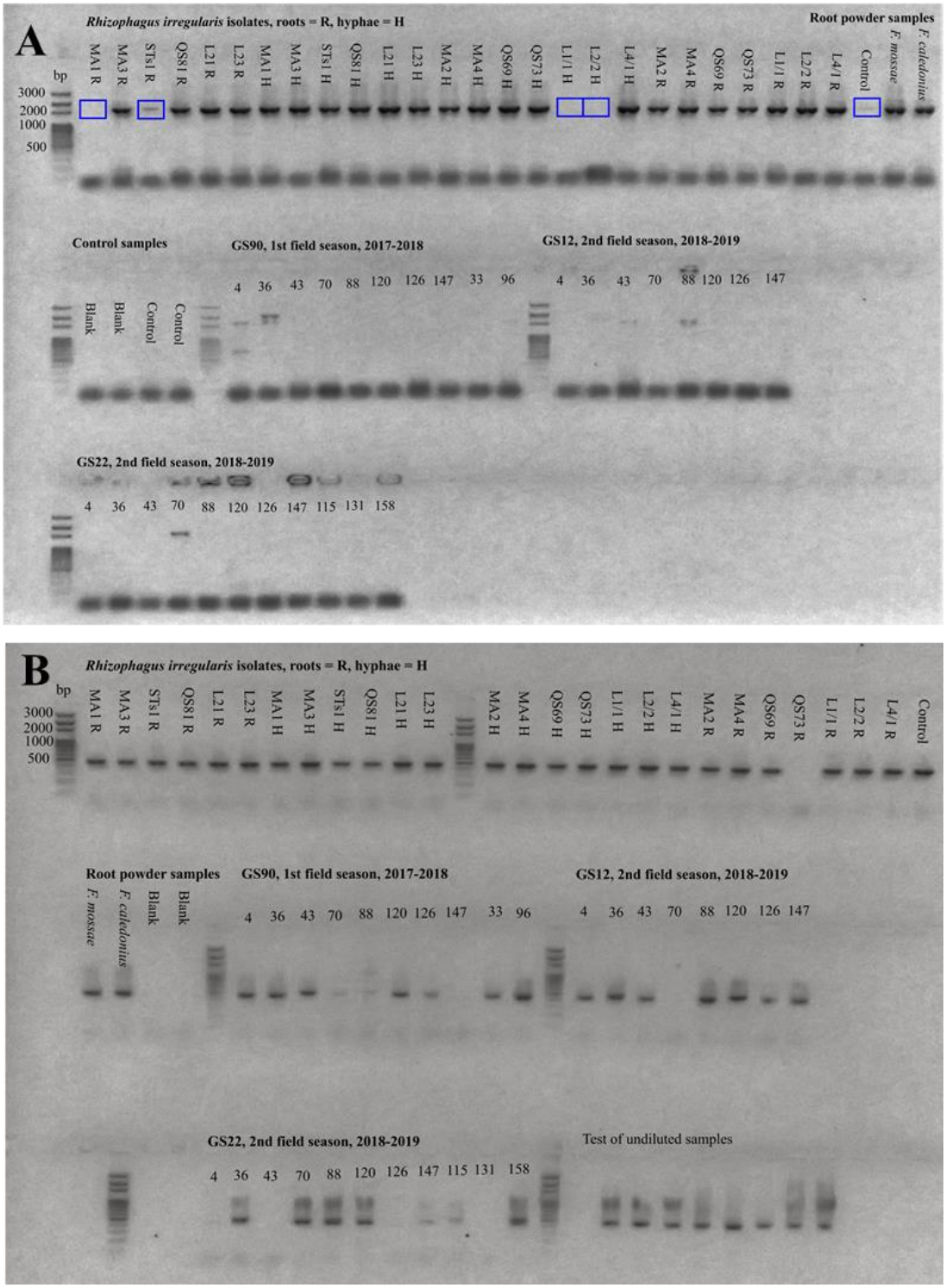

Fig.C.2. Results of nested PCR targeting the ITS2 region of *Rhizophagus irregularis* in hyphae (H) and roots (R) from root organ cultures (1st row respectively) and in root samples from field trials **A**) First PCR using primer mixes SSUmAf and LSUmAr by Krüger *et al*. (2009), amplicon size ~ 1800 bp. Blue boxes mark reactions which were repeated in a separate PCR (not shown) where control samples did not show amplification in contrast to isolate samples. **B**) Second PCR using the primers AM 5.8S and ITS4, amplicon size ~300-400 bp. Undiluted samples were run to assess effect of dilution on amplification results. Control samples were DNA from non-colonised chicory roots, blanks were reactions with ddH_2O instead of template DNA.

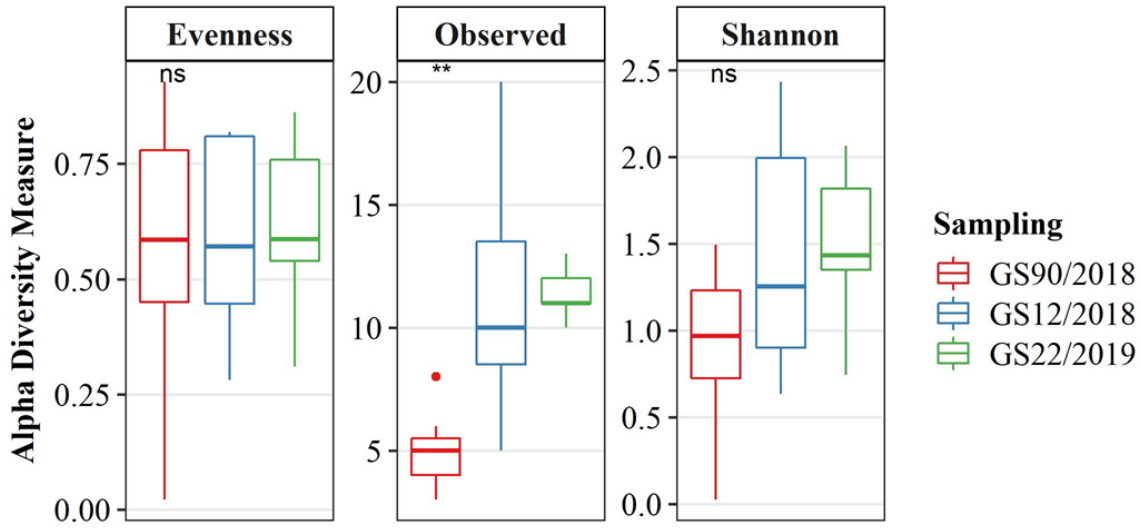

Fig.C.3. Box plots of alpha-diversity measures (Evenness, observed richness, Shannon-index) of fungal community composition based on ITS2-sequencing at three different growth stages (GS) of wheat: maturity (GS90), seedling growth (GS12) and tillering (GS22). Kruskal-Wallis test of multiple groups (C, ns = not significant, * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001).

ITS1-sequencing

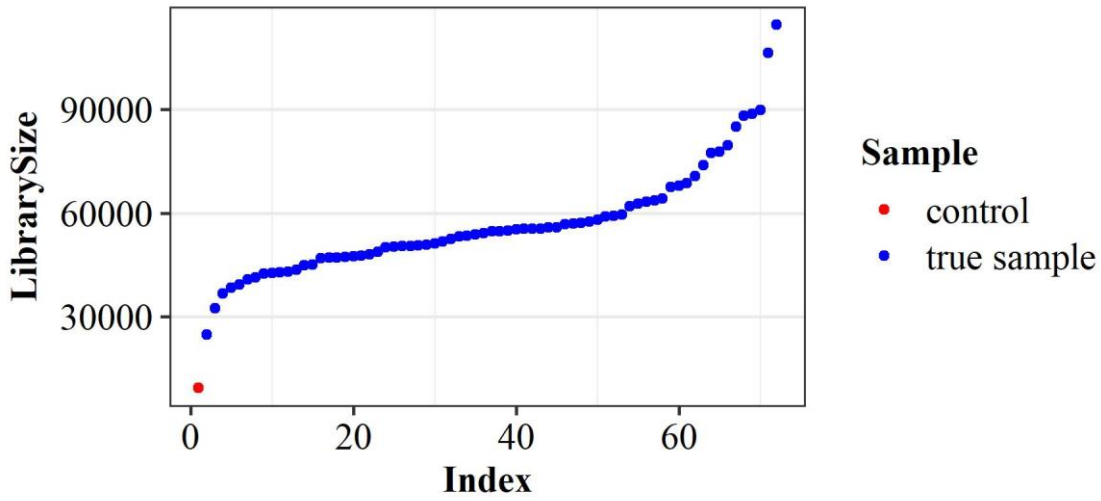

Fig.C.4. Identification of decontaminants in ITS1-library based on frequency of ASVs detected in the negative control (red) compared to other samples (blue).

Table.C.1. Effects of crop protection, wheat variety and fertiliser with on fungal community composition based on ITS1-amplicon sequencing. Results are significant for $P_R \leq 0.05$ according to permutational ANOVA analyses (PERMANOVA).

Main effects	**Df**	**Sums of Squares**	**Mean Square**	**F-Model**	**R^2**	**P_r(>F)**
Crop protection	1	16605	16605	0.9778	0.015	0.571
Variety (VR)	1	17869	17869	1.05225	0.016	0.183
Fertiliser (FT)	3	50764	16921	0.99642	0.047	0.446
Interactions						
CP:VR	1	15601	15601	0.91867	0.014	0.864
CP:FT	3	48824	16275	0.95835	0.045	0.806
VR:FT	3	49929	16643	0.98003	0.047	0.606
CP:VR:FT	3	47435	15812	0.93108	0.044	0.956
Residuals	48	815139	16982	0.76743		
Total	63	1062166	1			

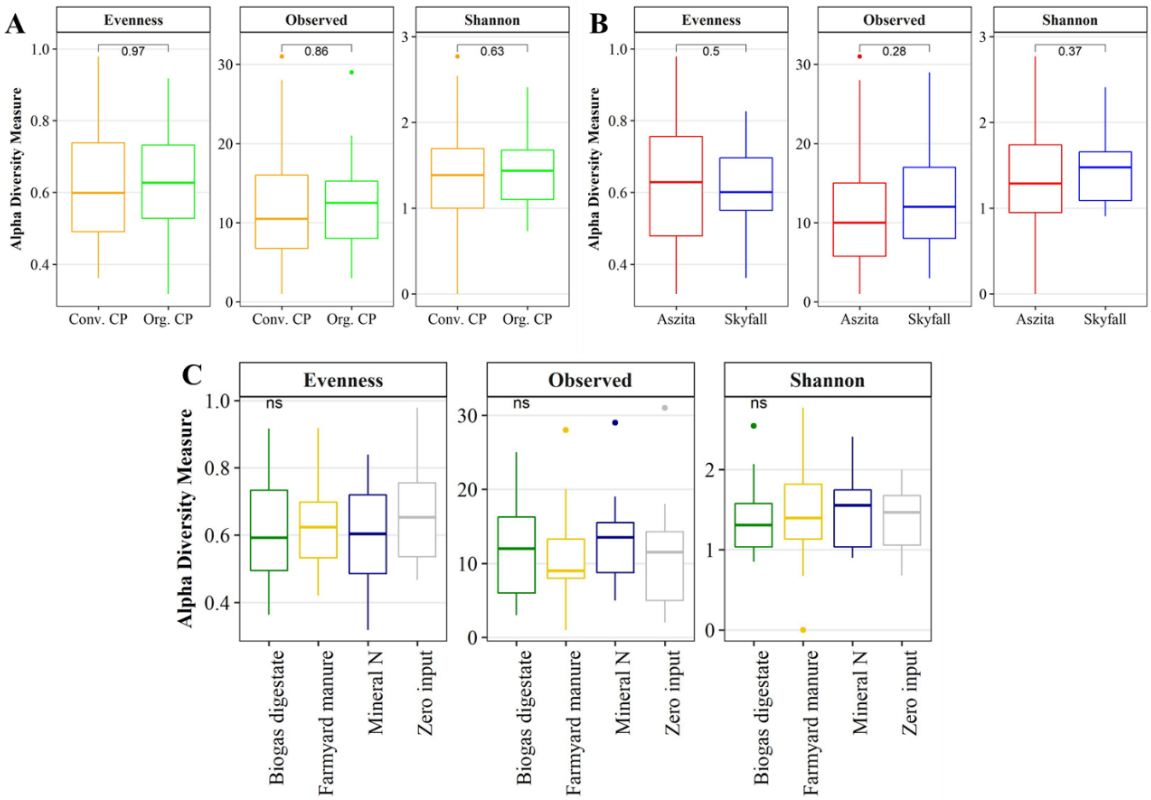

Fig.C.5. Box plots showing alpha diversity measures (Evenness, observed richness, Shannon-index) of arbuscular mycorrhizal community composition in samples analysed by amplicon sequencing of ITS1-region assessing the impact of **A**) conventional and organic crop protection, **B**) variety and **C**) N fertiliser source treatments. Numbers indicate p-values for pairwise comparison by Wilcoxon-rank test with $p \leq 0.05$ (A,B) and Kruskal-Wallis test of multiple groups (C, ns = not significant, * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001).

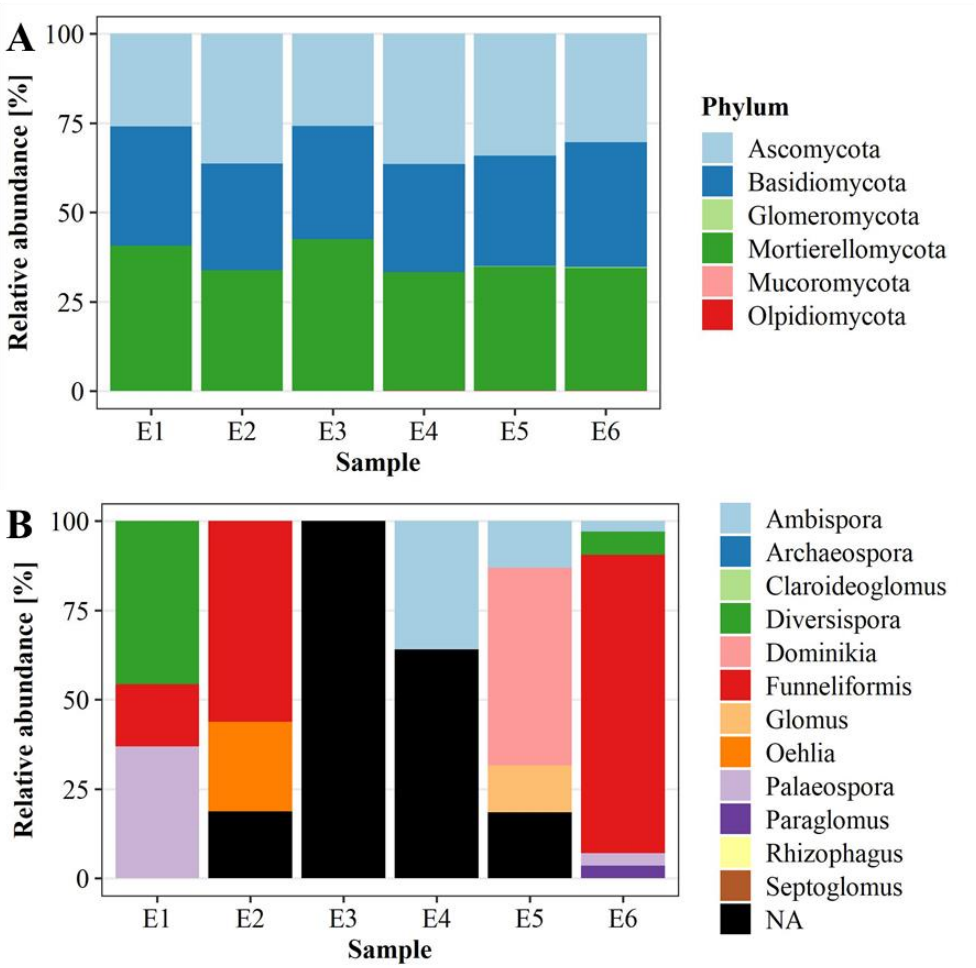

Fig.C.6. Relative abundances [%] of **A**) fungal phyla and **B**) Glomeromycota ASVs based on ITS1-sequencing of six soil samples from Nafferton Farm.

SSU-sequencing

Table.C.2. Effect of variety and fertiliser on ratio of Glomeromycotina:Mucoromycotina sequences of SSU-nrDNA in wheat roots at stem elongation (GS32). Ratios were generated using different normalisation methods. Rarefaction was conducted to minimum sampling depth of each sample.

	Untransformed	**DESeq2**	**Rarefied**
Variety (VR)			
Aszita (n=6)	1±0.192	0.71±0.019	1.01±0.194
Skyfall (n=6)	1.25±0.301	0.69±0.019	1.26±0.312
Fertiliser (FT)			
Mineral N (n=6)	1.14±0.291	0.7±0.016	1.17±0.304
Zero-input (n=6)	1.11±0.221	0.71±0.023	1.1±0.218
ANOVA *p-values*			
Main effects			
VR	0.659	0.582	0.672
FT	0.919	0.768	0.830
Interactions			
VR:FT	0.927	0.683	0.926

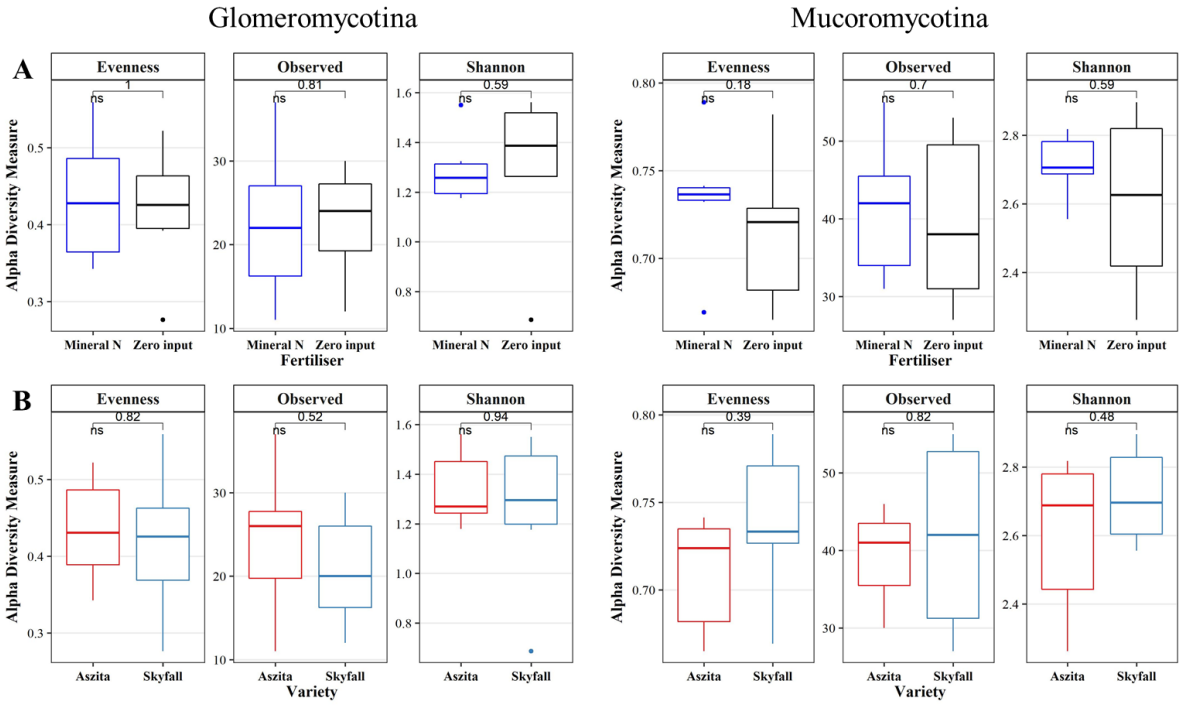

Fig.C.7. Alpha diversity measures (Evenness, observed richness, Shannon-index) of Glomeromycotina and Mucoromycotina community composition in samples analysed by amplicon sequencing of small subunit (SSU) assessing the impact of **A**) mineral nitrogen application vs. zero-input and **B**) variety. Numbers indicate *p*-values for pairwise comparison by Wilcoxon-rank test (ns = not significant, * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001).

Table.C.3. Results of differential abundance analyses of FRE-communities in roots with impact of fertiliser treatment and wheat variety. P-values and adjusted *p*-values according to Benjamini-Hochberg are shown.

ASV	**baseMean**	**log2FoldChange**	**Lfc SE**	**stat**	***p*-value**	***p*-adj**
ASV3	1607.36	10.57	1.79	5.90	0.00000	0.00000
ASV7	339.29	8.16	1.62	5.03	0.00000	0.00001
ASV15	132.26	7.14	1.59	4.50	0.00001	0.00009
ASV17	121.44	7.23	1.59	4.56	0.00001	0.00009
ASV26	69.42	7.29	1.64	4.44	0.00001	0.00010
ASV18	93.84	7.21	1.68	4.30	0.00002	0.00016
ASV33	28.34	6.05	1.59	3.80	0.00015	0.00116
ASV21	98.28	4.96	1.38	3.61	0.00031	0.00211
ASV36	25.79	5.36	1.50	3.58	0.00035	0.00211
ASV16	50.83	5.62	1.60	3.51	0.00045	0.00226
ASV32	26.23	5.60	1.59	3.53	0.00041	0.00226
ASV31	26.12	5.38	1.61	3.34	0.00082	0.00378
ASV41	23.33	4.60	1.41	3.27	0.00108	0.00456
ASV40	26.13	5.34	1.68	3.17	0.00151	0.00593
ASV30	20.75	4.71	1.62	2.90	0.00375	0.01374
ASV51	14.09	4.06	1.50	2.71	0.00665	0.02285
ASV1	738.23	-4.11	1.53	-2.68	0.00735	0.02378
ASV64	7.57	-3.81	1.46	-2.61	0.00910	0.02781
ASV57	10.64	-4.14	1.69	-2.45	0.01441	0.04170
ASV56	10.83	-3.46	1.43	-2.42	0.01533	0.04217
ASV34	15.85	4.19	1.78	2.35	0.01868	0.04671
ASV47	15.73	4.13	1.75	2.35	0.01865	0.04671
ASV29	34.08	-3.27	1.42	-2.31	0.02090	0.04998

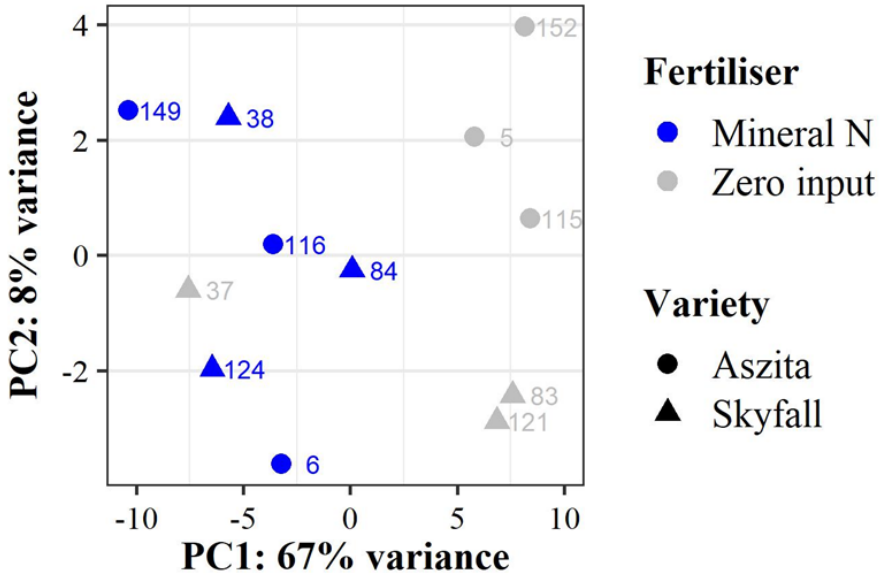

Fig.C.8. Principal component plot of FRE-communities in root samples with impact of fertiliser treatment and wheat variety. Axes show factors that explain %-variation of FRE-community composition. Counts were regularized log-transformed prior to ordination.

Appendix D CHAPTER 6

Binders

At first, solubility of all selected compounds was tested by dissolving 3 g of each binder substance in 100 ml of deionised water on a heated magnetic stirrer (IKAMAG® RCT CombiMAG, Janke & Kunkel, Germany). The hydroxypropyl methylcelluloses (HPMC) Arbocel®HPMC by JRS and Culminal™ by Ashland™ were added to cold water which was then heated briefly to 70-80 °C and then cooled under constant stirring. Texture, film-forming and drying behaviour of the binder liquids was tested by dipping wheat seeds into the liquid and then leaving them to dry at room temperature. In a second approach, seeds were added to a glass beaker together with a blend of the binder liquid and inoculum powder. When the seeds were fully covered, they were placed in glass dishes to dry at room temperature. All seeds were checked on consecutive days for drying and stickability by agitating the seeds in the glass dishes. Depending on the amount of disassociated inoculum, the concentrations of the binder substances were adjusted for a repetition of the same screening set-up.

Fillers

Two different types of cellulose were screened as potential filler substances. In contrast to the methylated celluloses used during the binder screenings, these polymers are insoluble and not film-forming. The first filler substance tested was a carboxylmethylcellulose (CMC, Blanose™, Ashland™), the second filler was an ultra-fine cellulose (Arbocel® UFC100, JRS). Both polymers were applied to the seed in combination with the binder liquids after the initial screening. For this approach, wheat seeds were first dipped into the binder liquid using forceps to coat with the cellulose powder. Another approach was to add binder and fillers with root powder to a glass vial and then cover the seeds by agitating. Diverse combination of binders and fillers were tested while the interaction of the substances was observed. After drying at room temperature, porosity of the seed coat was assessed under a dissecting microscope. Several coated seeds were agitated in a container to observe if the coat sticks to the seed surface or disintegrates during shaking (adapted Heubach-test, Heitbrink, 1990).

Additives

Additives which had been considered as active ingredients in seed coat formulations were selected to assess for positive effects on the symbiotic organisms. For this reason, additives were tested *in-vivo* formulated with binder and filler substances