FUNGAL DIVERSITY AND MICROBIAL COMMUNITY STRUCTURE IN THE PERFORMANCE OF PULSE AND WHEAT-BASED ROTATION SYSTEMS

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ABSTRACT

Crop rotation is a key strategy of sustainable agriculture in the Canadian Prairie. Improving crop productivity and yield stability in pulses-based cropping systems with better soil biology is the ultimate goal of this research. Firstly, my studies provide information on the effect of pulses on the biodiversity of soil fungi: arbuscular mycorrhizal fungi (AM) and non-AM fungi, associated with the main pulse species grown in the Canadian Prairie (field pea, lentil, and chickpea), and their influence on wheat-based cropping systems. Secondly, the optimum 4-yr crop rotation for wheat production was determined, based on the relationship among fungal communities associated to the different crops and the yield and quality of these crops.

My research included two experiments. First, in a field experiment replicated in time and site, the effect of previous pulse crops on wheat root-associated microbial communities and crop performance was assessed in four 2-yr rotation systems. Second, a 4-yr field experiment evaluated the relative influence of eight different crop rotations on root-associated microbial communities and on wheat productivity in the last year of the rotations. A greenhouse assay was conducted to evaluate, under controlled conditions, the influence of the microbial communities selected by these previous field crop rotations on wheat performance, using soil from the field as inoculant. The response of root-associated microbial communities was characterized using next generation sequencing technologies, phospholipid fatty acid markers, microscopic observation of roots and soil dehydrogenase assay. Plant response was evaluated based on crop density, biomass, yield and tissue nutrient content.

My studies showed that community composition of AM and non-AM fungal communities in the roots of wheat were largely influenced by host plant identity and environmental conditions. The structure of the overall fungal community in wheat roots was not affected by the previous crops. The soil microbial legacies of previous crops were different from the fungal communities found in the roots of the following wheat, suggesting that wheat, as a host plant, selects and associates with a specific fungal community. Seasonal variations in soil moisture, temperature, pH, and nutrient cycling between sampling times have a great influence on soil microbes and could also be influencing these effects.

The 2-yr crop rotation experiment revealed that wheat after a pulse crop had higher plant density and produced more seed biomass and total yield. The 4-yr crop rotation studies revealed that, in the field, diversified rotations including pea or lentil in alternate years, largely contributed to wheat performance. However, rotations including chickpea contributed little to the rotation benefits, suggesting that a careful selection of plant species is essential to improve the performance of the agroecosystem. Contrary to the field results' findings, under greenhouse conditions, rotations that included chickpea before wheat contributed the best to wheat productivity, suggesting that in the field, factors other than the microbial community selected by chickpea were responsible for the poor performance of chickpea-wheat rotations in the field.

Soil bacterial and fungal biomasses were positively correlated with wheat yield in the field experiments, which suggests that an abundant and diversified microbial community positively influences wheat productivity. Also, possible antagonistic and synergistic

interactions between different AM species and root pathogens could be inferred. These results suggest that many AM fungi can potentially contribute to combat pathogens and enhance plant performance, whereas other might produce detrimental effects on the plants.

Overall my studies revealed that host plant identity and environmental conditions influence the fungal community structure and dynamics. The frequency and sequence of crops in the rotations strongly influences productivity in wheat based agroecosystems. Lentil and pea alternating with wheat largely contribute to wheat performance. Thus, the productivity of wheat can be improved by selecting and including the plant species most beneficial to the rotation in order to increase soil available water and N, while promoting beneficial microbial associations and reducing disease incidence.

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LIST OF ABBREVIATIONS / NOMENCLATURE

AAFC: Agriculture and Agri-Food Canada

Al: Aluminum

AM: Arbuscular mycorrhizal

ANOVA: Analysis of variance

C: Carbon

$CaCO_3$: Calcium carbonate

CHP: Chickpea

dd H_2O: Double distilled water

DNA: Deoxyribonucleic acid

DSE: Dark septate endophytes

ISR: Induced systemic resistance

ITS: Internal transcribed spacer

K: Potassium

KOH: Potassium hydroxide

LEN: Lentil

LSD: Least Significance Difference

MAP: Monoammonium phosphate ($NH_4H_2PO_4$)

MID: Multiplex identifiers

MRPP: Multiresponse permutation procedures

NaOCl: Sodium hypochlorite

NMS: Non-metric multidimensional scaling

OTU: Operational taxonomical unit

PCR: Polymerase chain reaction

PLFA: Phospholipid fatty acid

N: Nitrogen

NLFA: Neutral lipid fatty acid

P: Phosphorus

PerMANOVA: Permutational Multivariate Analysis of Variance

PGPB: Plant growth promoting bacteria

Ra_P-II: Roots at anthesis in phase II

Ra_P-IV: Roots at anthesis in phase IV

RDA: Redundancy analysis

rDNA: Ribosomal DNA

Sf_P-I: Soil in the fall of phase I

Sf_P-III: Soil in the fall of phase III

SPARC: Semiarid Prairie Agricultural Research Centre

TPF: Triphenyl formazan

TTC: 2,3,5-triphenyltetrazolium chloride

WHT: Wheat

1.0 General Introduction

Crop rotation is a traditional strategy to maintain microbial diversity in the agroecosystem, reduce the incidence of disease and weeds, decrease herbicides requirements, and increase yields (Fiers 2012). Farmers include broadleaf crops such as field pea, lentil, chickpea, canola and mustard in cereal-based rotations in the Canadian Prairie, in order to replace summer fallow and reduce fertilizer requirements. Pulses play an important role in cropping systems. Through their symbiosis with rhizobia, they fix atmospheric N and increase the availability of soil N for the following crops, reducing the dependence of cropping systems on fertilizer (Alvey 2003; Marenco and Santos 1999).

Growing pulses has the advantage of contributing to grain yield and protein content of economically important crops such as durum wheat that follow in rotation. Most pulses leave residual N from N_2 fixation in the soil and good levels of plant available soil water as compared to continuous cereal systems (Gan et al. 2003). Pea, chickpea and lentil are generally well-adapted to the semi-arid conditions of the Canadian Prairie and show better drought tolerance than other crops (Angadi et al. 2008; Cutforth 2009) such as canola and mustard which have a better production on summer fallow (Angadi et al. 2008). Faba bean, alfalfa, field pea and lentil improve soil N economy, especially over the long term, but chickpea and common bean contribute little N to cropping systems based on published estimates of grain and N_2 fixation in the Great Plains (Walley 2007). Including pulses in rotations also facilitates the decomposition of crop residues by microbes because the C: N ratio of these crops is usually low. The additional N provided by legumes is used by the soil

microorganisms to break down the C-rich residues of crops such as corn or wheat following in the rotation, reducing the immobilization of soil N.

Evidence shows that diversified crop rotations provide more benefits to the agroecosystem than monoculture (Campbell et al. 2007; Gan et al. 2003; Gaudin et al. 2015; Walley 2007). Several studies have focused on the agronomic performance, water relationships and nutrient cycling benefits brought by crop rotation and how the inclusion of pulses in these systems have considerably reduced the need for N fertilizers, the frequency of summer fallow, and disease incidence (Fernandez et al. 2008; Gan et al. 2003). The inclusion of broad leaf legumes in the Canadian Prairie rotations not only modifies the soil environment, but also those who live and depend upon it: e.g., soil microorganisms.

Little is known about the influence of the crop rotation systems of the Canadian Prairie on fungal community composition and dynamics. Changes in the soil physicochemical properties due to agricultural practices can drive shifts in the microbial communities (Doi and Ranamukhaarachchi 2009; Marais et al. 2012). Previous studies have used culture-dependent methods to evaluate selected soil physicochemical and microbial parameters under different management systems in wheat-based rotations (Marais et al. 2012). The fact that only a small fraction of the soil microorganisms are culturable represents a limitation of culture-dependent studies (Kent and Triplett 2002). In contrast, culture-independent methods based on the analysis of soil DNA, help to explore fungal communities with the ability of discriminating among microbial types and subtypes (Buee et al. 2009).

Pyrosequencing of amplicons offers the same accuracy as conventional DNA sequencing for short reads, but it is more flexible and a large number of samples can be processed in

parallel (Ronaghi and Elahi 2002). Pyrosequencing of barcoded amplicons has been used effectively to determine the composition of fungal and bacterial communities in different ecosystems (Buee et al. 2009). This method uses amplicon libraries to produce high quality sequences with less cost than using other molecular techniques, which makes pyrosequencing a powerful tool to assess soil microbial diversity (Buee et al. 2009; Ronaghi and Elahi 2002).

Previous studies by Dunfield and Germida (2003) showed that soil microbial community structure and diversity is significantly influenced by plant species. Thus, it would be expected that including different legume crops in wheat-based rotation systems will diversify the microbial community composition in response to the selective influence of different plant species in comparison with monoculture. It has been reported that highly diversified rotations harbor healthier and more diverse microbial communities compared with crop monoculture (Ellouze et al. 2014; Lehman et al. 2012; Nayyar et al. 2009). Introducing pulse breaks in wheat-based systems can also reduce disease incidence by disrupting pathogens' cycles while improving crop yields (Gan et al. 2003).

Factors such as fungal biodiversity, distribution, and dominant species associated with pulse crops may play important roles in boosting the grain yields and quality of subsequent cereal crops. The size and structure of the soil microbial communities can influence yield and crop performance in wheat-based rotation systems (Mandal et al. 2007). The selection of crop rotation systems that promote the proliferation of beneficial fungal communities while preventing or reducing the incidence of fungal pathogens in the root endosphere could help reduce farmer expenses of fertilizers and pesticides. The influence of host crop species and crop sequence and frequency on microbial communities demands a greater understanding.

The relationships between the microbial communities associated with different rotation systems and crop performance should be understood.

Thus, the following hypotheses were tested:

- Chickpea, pea, lentil and wheat select specific soil microbial communities, in particular, fungal communities (e.g., AM and non-AM fungi).
- Chickpea, pea and lentil influence the fungal community composition and the interactions within the rhizosphere and endosphere of the following wheat crop.
- The fungal community structure and patterns of community dynamics related with different crop rotation systems correlates with the yield and quality of the final wheat crop in 4-year rotation system.

The specific research objectives were:

- To provide information on the diversity of all soil fungi associated with chickpea, pea, lentil and wheat, and the subsequent cereal crop, in support for the development of cropping systems that improve soil biology.
- To define the relative productivity of eight crop rotation systems involving pulses and wheat and the contribution of plant-associated microbial communities to system productivity.

2.0 LITERATURE REVIEW

2.1 PrefaceThis chapter includes three main sections related to plant-microbe interactions and the management of soil resources through plant species selection and rotation. The section 2.4 Management of Soil Microbial Resources through Crop Rotation in the Canadian Prairie has been reprinted from the manuscript by Ellouze, W., Esmaeili Taheri, A., Bainard, L. D., Yang, C., Bazghaleh, N., Navarro-Borrell, A., Hanson, K. and Hamel, C. 2014. Soil fungal resources in annual cropping systems and their potential for management. BioMed Res Int 2014:15.

2.2 Plant-Microbe Interactions

Plants and microorganisms have co-evolved through millennia since life on Earth began. The rhizosphere, defined as the soil in direct contact with the root surface, is a diverse ecosystem containing communities of bacteria, fungi, protozoa and nematodes in dynamic interaction with each other and with the plant roots (Mocali and Benedetti 2010). Understanding the complexity of plant-microbe interactions can support the development of strategies to increase the sustainability of agricultural systems.

Depending on the cost-benefit balance of an interaction, the relationship between plants and microbes can be classified as beneficial, detrimental or neutral (Fig. 2.1). In many circumstances seemingly neutral plant-microbe relationships have an indirect effect on plant performance. Microbes may use plant-derived compounds as source of energy, facilitating nutrient cycling, ultimately modifying the rhizosphere environment, and influencing plant growth (Schenk et al. 2012)

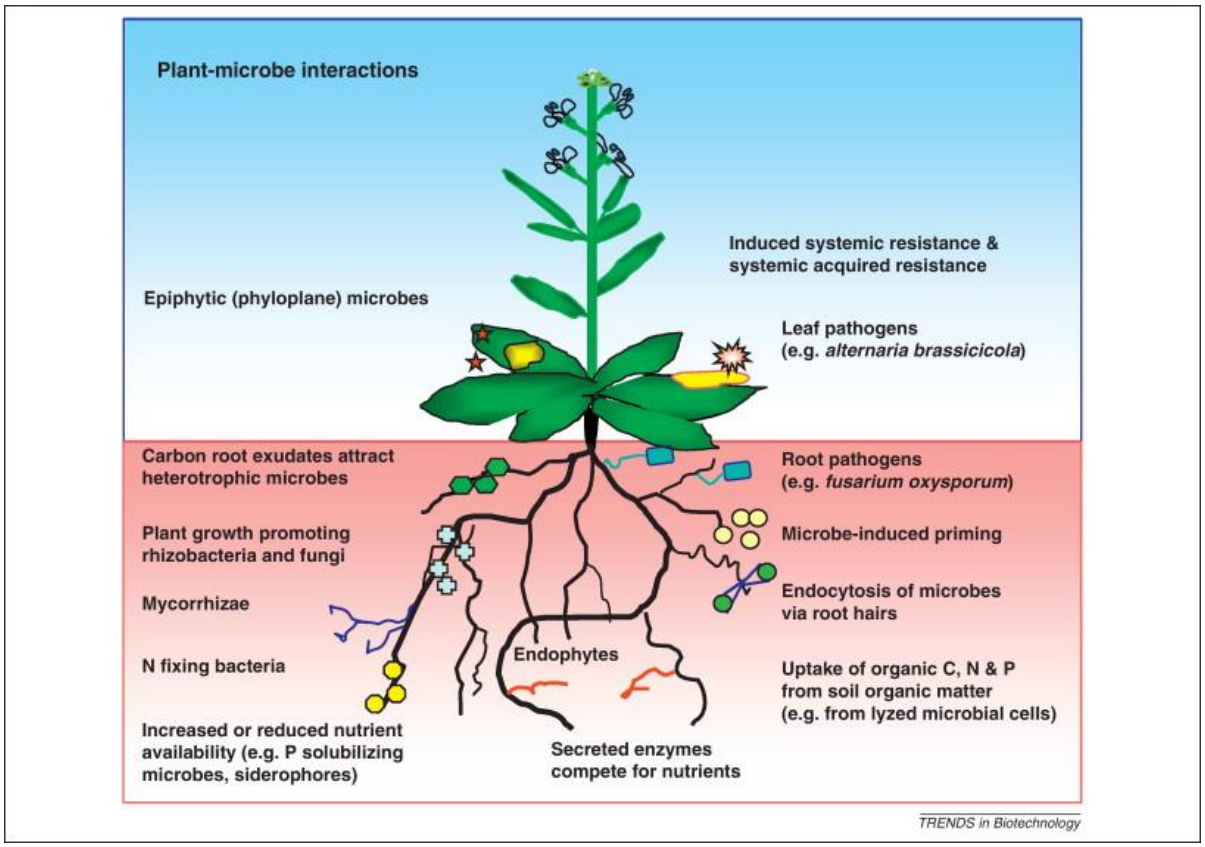

Figure. 2.1 General overview of plant-microbe interactions. Reprinted from Trends in Biotechnology, March 2012, Vol. 30, No.3, Peer M. Schenk, Lilia C. Carvalhais and Kemal Kazan, Unraveling plant–microbe interactions: can multi-species trancriptomics help?, p 177-184, Copyright 2014. Permission to use granted by ELSEVIER in October 14th 2014.

Agricultural research has previously focused on plant interactions with pathogens and the use of chemical inputs such as pesticides to combat them. More recently, studies on the diversity of beneficial symbiotic associations have gained importance. Beneficial symbionts, such as AM fungi and plant growth promoting bacteria (PGPB) improve nutrient and/or water acquisition, boost crop yields and enhance plant protection against pathogens by producing antagonistic compounds (e.g. siderophores, antibiotics) (Jousset et al. 2010; Schenk et al. 2012) or by priming (Conrath et al. 2002).

Priming is the process by which plant resistance to pathogens is increased (Conrath et al. 2002). It involves systemically inducing the physiological pathways of plant defense. This mechanism is also known as induced systemic resistance (ISR) when mediated by non-pathogenic rhizobacteria (Schenk et al. 2012; Shoresh et al. 2010).

On the other hand, pathogenic microbes can cause disease, yield loss, necrosis in different organs of the plant, and ultimately may cause plant death. Plant pathogens can be classified as biotrophic, necrotrophic and hemibiotrophic depending on their location and the damage they cause to the host. Biotrophic pathogens live within the plant tissues and extract nutrients from plant cells using a structure called haustoria. This structure arises from intercellular hyphae, appressoria, or external hyphae and penetrates the host cellular wall causing an invagination on the plasma membrane and the cytoplasm. The host wall is highly modified in the invaginated zone causing thinning and the creation of an interfacial matrix were nutrient extraction occurs (Jennings and Lysek 1996). Necrotrophic pathogens infect and kill host tissue and extract nutrients from the dead host cells. Hemibiotrophs initially form associations with living cells of the host, much like a biotrophic pathogen and in the later stages of infection it becomes necrotrophic, killing the host cells. In general plant pathogens are responsible for huge economic losses and they represent a threat to global food security (Koeck et al. 2011).

Host plants are able to promote and regulate their interactions with the microbial community by releasing root exudates containing signal molecules that trigger specific pathways within beneficial microbes. Carbon compounds released as root exudates account for more than 25 % of the total carbon fixed by the plant (Pond et al. 2011). Therefore, the

plant-microorganism interaction has a great energetic cost for the plant, although it can obtain much benefit from the association. The physiological mechanisms used by the plant to interact and regulate the relationship with soil microorganisms in the soil environment are not totally understood.

Microbial communities play a key role in soil dynamics impacting plant cover in both natural and agricultural ecosystems. They are involved in organic matter decomposition and in the cycling of C, N, P and S. These nutrients are particularly important for plant life. Microbial diversity has a tremendous impact on soil ecosystem stability and resilience to disturbance (Garbeva et al. 2004). Microorganisms mediate biochemical processes through different interactions with plant cover and amongst themselves. Plant species and genotypes, and soil management through rotation are major factors affecting soil biology.

Plant-microbe interactions are affected by abiotic factors. Geography, climate, soil type and anthropogenic activity can cause changes in microbial community dynamics. Plants are able to adjust and regulate their interaction with the belowground communities which are constantly influenced by the abiotic soil environment (Erb and Lu 2013). For example the interactions with AM fungi are favored by limited soil P level (Jasper and Davy 1993). Furthermore, plants may recruit specific groups of beneficial microbes to deal with a stressful soil environment (Yi et al. 2011).

2.3 Plant Influence on Soil Biology

Plant cover is a major factor influencing soil microbial communities since plants are the main providers of carbon and energy to microorganisms (Garbeva et al. 2004). The rhizosphere is a dynamic ecosystem where plant roots and microbes interact. The interaction is complex and regulated by chemical signals (Bais et al. 2006). Plant roots release a wide variety of bioproducts targeting different microbial groups to regulate their interaction with the plant. With the increased sensitivity of modern techniques and equipment, a wide range of root exudates is being discovered (Uren 2007), however how they regulate community structure remains largely unknown. Among the known root exudates, free oxygen and water, sugars, organic acids, amino-acids, ethylene, vitamins, polysaccharides and enzymes (Bais et al. 2006; Bertin et al. 2003) modify the conditions of the rhizosphere environment with impact on the root-associated microorganisms.

The active processes of root exudation can be sub-classified as root excretion or secretion. The first implies the transport of waste material following a concentration gradient. On the other hand, secretion includes the exudation of compounds with specific functions, either for defense or lubrication (Uren 2007). Root exudates are divided into two types of compounds: low molecular weight (i.e., secondary metabolites, amino acids, sugars and phenolics) and high molecular weight compounds (i.e., polysaccharides and proteins) (Uren 2007). Many of the carbohydrates exudated by the roots support heterotrophic rhizobacterial communities in the rhizoplane and rhizosphere. AM fungi are known to form beneficial symbiosis with plants roots. To establish and regulate this interaction, host plant and AM fungi adjust through metabolic changes. Consequently, AM fungi induced root exudation will also encourage

other microbial associations in the rhizosphere and increase the beneficial effects on plant (Narula et al. 2009).

Root-mediated rhizospheric interactions can be classified as positive or negative depending on the microbial groups the roots interact with and the type of exudates that are released (Bais et al. 2006; Uren 2007). As mentioned above, positive interactions include those with PGPB, AM fungi and beneficial fungal endophytes leading to the establishment of symbiotic relationships. They favor the growth of plants and/or chemical signaling for induced systemic resistance (ISR). Root-mediated negative interactions involve protective chemical signalling against pathogens/herbivores and toxic elements and include antimicrobial, phytotoxin, nematicidal and insecticidal compounds. Root exudates can influence the proliferation of many pathogenic fungi such as *Rhizoctonia*, *Fusarium*, *Sclerotium, Aphanomyces, Pythium, Verticillium* and *Phytophthora* (Narula et al. 2009). Susceptible cultivars of host plants can promote the proliferation of many of these fungi by releasing root exudates used by the fungi as food source. On the other hand plants can also produce toxic exudates to combat pathogenic growth, such as glycosides and hydrocyanic acid (Narula et al. 2009).

Root exudates are not necessarily stress-induced; they are naturally secreted during the normal development of the plant. However, some particular compounds such as phytoalexins and protectors against toxic aluminum (Al) are produced in response to the interaction with infectious organisms or when Al is present in its mobile active form (Al^{3+}), respectively (Uren 2007). Other root secretions that play important roles in modifying the rhizosphere include ectoenzymes (i.e. phosphatase), which are able to transform organic compounds into

usable plant nutrients, and mucilage which intervenes in water acquisition. Whiteley (1989) found evidence suggesting that mucilage may play a role in reducing the mechanical strength of soil in the proximity of the root tips of plants. This effect may vary depending on the composition and amount of exudate released by the root tips and the relationship between the solute potential of the exudate and the soil water potential, which can drive changes in the soil matric potential and influence microorganisms. The type of soil and its hydraulic characteristics will influence the remoulding of the soil by the root exudates as well (Whiteley 1989).

Plant species is a determinant of the structure of microbial communities. Litter quality, which can be translated as the amount of nutrients that plant residues release to the soil, can determine the abundance of particular groups of microorganisms (Breulmann et al. 2012). Soil fungi and bacteria play an important role in organic matter decomposition and nutrient cycling, but fungi are better at degrading residues with high C:N ratio. Therefore plants producing residues with high C:N ratio will favor the soil fungal community over bacteria, which prefer decomposing a litter with a low C:N ratio (Lange et al. 2014; Wardle et al. 2004). Studies by Germida et al. (1998) and Kaiser et al. (2001) found that the diversity of root-associated bacteria was mainly affected by plant type. Furthermore, in a two year study, Smalla et al. (2001) found that bacterial community shifts were plant-dependent and the relative abundance of bacteria increased in time when growing the same crop for two consecutive years. Removing particular plant groups from a field could drive significant changes in the soil community structure as well (Wardle et al. 1999).

2.4 Management of Soil Microbial Resources Through Crop Rotation in the Canadian Prairie

Certain agronomic practices are designed to manage biodiversity in the agroecosystem by enhancing beneficial microbial associations and repressing pests and disease outbreaks (Table 2.1). Among these practices, rotating crops is one of the more traditional and effective ways to diversify the microbial community, reduce the impact of diseases and weeds (Fiers 2012), and thus increase yields. The value of a cropping system depends on a number of factors including the genotype and crops included in the rotation (Garbeva et al. 2004), the sequence and frequency of the crops (Gan et al. 2003), the length of the rotation (Bennett et al. 2012), the management history (Ellouze et al. 2008), and soil characteristics (Bernard 2011). Overall, these factors impact the soil microbial community in different ways.

Table 2.1 General effects of agronomic practices on soil fungal diversity and abundance, disease incidence, soil fertility, crop nutrient use efficiency, and crop growth and yield reprinted from Ellouze et al. (2014).

Source of effect	Biodiversity level	Crop growth and productivity	Disease, pests and pathogens	Microbial abundance	Soil fertility	Nutrient use efficiency	References
Biodiversity management							
Crop rotation	+ †	+	-	+	NA‡	NA	(Blackshaw 2010; Dos Reis Martins 2012; Fiers 2012; Gan et al. 2003; Garbeva et al. 2004; Nelson 2012; Smith et al. 2008; Vilich 1993)
Cultivar mix	+	+	-	+	NA	NA	(Gan et al. 2003; Kiær et al. 2009; Vilich 1993)
Intercropping	+	±	-	+	NA	NA	(Blackshaw 2010)
Cover cropping	+	±	-	+	NA	NA	(Blackshaw 2010; Larkin et al. 2010; Smith et al. 2008)
Non-mycorrhizal crops	-	NA	+		NA	-	(Harinikumar and Bagyaraj 1988)
Transgenic crops	0	±	-	0	NA	NA	(Chun et al. 2012; Fließbach et al. 2012; Gschwendtner et al. 2011; Lin and Pan 2010; Lu et al. 2010; Zabaloy et al. 2012)

† **+** indicates positive to no effects, **0** indicates negligible effects, **-** indicates negative to no effects, and **±** indicates variable effect.
‡ NA: Not applicable

Table 2.1 Continuation

Source of effect	Biodiversity level	Crop growth and productivity	Disease, pests and pathogens	Microbial abundance	Soil fertility	Nutrient use efficiency	References
Biodiversity management							
Pesticide use	0[†]	+	-	0	NA	-	(Lupwayi et al. 2010; Muñoz-Leoz et al. 2012; Sheng et al. 2012)
Weed control	-	+	NA	-	NA	+	(Lupwayi et al. 2010; Sheng et al. 2012)
Inoculants	NA	±	+	NA	+	+	(Dhillion 1992; Linderman and Davis 2004; Siddiqui and Futai 2009; Singh et al. 2004; Verma and Arya 1998)
Soil management							
Organic amendments	+	+	NA	+	+	±	(Garbeva et al. 2004)
N fertilizers	±	+	NA	+	+	-	(Biederbeck et al. 1996; Grant and Wu 2008)
Mineral fertilization	-	+	NA	±	+	-	(Grant and Wu 2008; Lupwayi et al. 2010)
Tillage	±	±	±	±	±	±	(Brito et al. 2012; Jansa et al. 2002; Kabir 2005)

[†] **+** indicates positive to no effects, **0** indicates negligible effects, **-** indicates negative to no effects, and **±** indicates variable effect.
[‡] NA: Not applicable

Intercropping systems and crop rotations offer opportunities for a better management of soil fungi. Growing in alternation mixtures of different genotypes (Kiær et al. 2009; Vilich 1993), or crops such as wheat, barley, canola (Nelson 2012), clover, and alfalfa (Blackshaw 2010) can enhance crop production by reducing weeds and disease incidence at the system level. Also, changes in the frequencies of cultivars (Gan et al. 2003; Vilich 1993) over time can influence the incidence of stem and root rot diseases in the rotation system and improve yield stability. For example, corn grain yield could increase linearly in relation to the number of crops included in the rotation up to twice the yield of the monocrop when three rotation crops and three cover crops were included in the cropping system (Smith et al. 2008). Certain crop sequences are better than others and it can be complicated to determine what is the most profitable cropping system (Gan et al. 2003). Soil factors are also important to consider in the design of cropping systems (e.g., soil-water stable aggregation, soil organic C, and the carbohydrate composition of the surface layer) as these parameters affect the abundance, diversity, and distribution of the fungal community (Dos Reis Martins 2012).

In most cases, monoculture negatively affects microbial biomass and diversity (Castillo et al. 2006; Nayyar et al. 2009). Diversifying the crops in a rotation increases the taxonomic and functional diversity of soil fungal communities (Larkin and Honeycutt 2006). In addition, microbial activity and substrate utilization are significantly affected by crop rotation (Larkin et al. 2010). Different crops provide different organic residues, which can result in a diverse food base that promotes fungal diversity and activity, and increases soil fungal biomass and N mineralization (Swer et al. 2011). Interestingly, the biochemical composition of some plant tissues can modulate fungal associations. Diversifying crop rotations also decreases disease

pressure in agroecosystems by disrupting the life cycle of pathogens associated with a particular crop or plant genotype.

The length of the rotation and crop diversity are key factors for the success of a cropping system. Monocultures negatively affect fungal biodiversity by selecting for virulent pathogens, which then have a competitive edge and increase disease severity. In a continuous-pea rotation grown in the Canadian prairie, severe *Fusarium* root rot injury was related to a reduced soil microbial abundance and lower abundance of beneficial Gram positive bacteria and AM fungi (Nayyar et al. 2009). In some wheat fields, continuous cropping has increased the abundance of antagonistic microorganisms consequently reducing pathogens, and mitigating the impact of diseases (Garbeva et al. 2004), but as a general rule, at least three and possibly more crops should be included in cropping systems (Bennett et al. 2012) to maintain low disease pressure. In addition, short rotations are often more susceptible to diseases and produce lower yield than longer rotations (Bennett et al. 2012). Some plant pathogens can persist in the soil for several years as spores or other dormant structures, in absence of a host plant (Merz and Falloon 2009). Thus, selecting plants that are not alternate hosts for pathogenic fungi is important to reduce yield losses due to diseases. The inclusion of cover crops in cropping systems is particularly effective in reducing disease incidence (Larkin et al. 2010).

In semiarid cold and subtropical steppes, farmers have traditionally grown cereals in alternation with summer fallow. This consists of keeping the soil bare using tillage or herbicides during a growing season to increase soil water content and produce a good crop the following year. No till systems have been established for greater soil moisture

conservation (Gan et al. 2011; Smith et al. 2008) allowing the replacement of summer fallows by broadleaf crops such as field pea, lentil, chickpea, canola, and mustard. Crop diversification with broadleaf crops, especially pulses, has the benefit of increasing grain yield and protein content of the wheat crops following in rotation, partially due to residual soil N from biological fixation (Gan et al. 2003). Canola and mustard are non-mycorrhizal plants that do not associate with rhizobacteria. These crops also require the use of more N and S fertilizers; however, the value of these crops compensate for the larger investment in fertilizers. Despite the economic benefit of these crops, having non-mycorrhizal plants in the crop rotation may reduce AM fungal abundance and delay mycorrhizal formation in the following crop (Gavito and Miller 1998), which may impact AM dependent crop plants. Clearly, there are many factors to consider in the design of ecologically sustainable and economically viable crop rotation systems.

3.0 Fungal Diversity Associated with Pulses and its Influence on the Subsequent Wheat Crop in a Two Year Study

3.1 Preface

This 2-yr study characterized the fungal diversity associated with chickpea, pea, lentil and wheat and evaluated the effects of these previous crops on the fungal community present in the roots of wheat and its agronomic attributes. The microbial diversity associated with the different crops was characterized using advanced molecular and biochemical techniques.

3.2 Abstract

Evidence shows that soil fungal communities are affected by plant species. However, little is known about the influence of the crop rotation systems of the Canadian Prairie on fungal community structure. This study 1) characterized the taxonomic diversity of the overall fungal community in the rhizosphere and root endosphere of chickpea, pea, lentil and wheat in phase I of four 2-yr wheat-based rotations, and 2) evaluated the effects of these previous crops on the fungal communities and the performance of wheat in phase II of the rotation systems, in Swift Current, Saskatchewan. The host crop significantly affected the composition of the general fungal community inhabiting the roots. The non-AM fungal community associated with the roots of wheat was significantly different from that of pulses in phase I of the rotations, suggesting that host plants influence fungal diversity and distribution. The relative abundance of *Fusarium tricinctum* (OTU5) was highest in pea and lowest in wheat. *Fusarium redolens* (OTU16) was more abundant in the roots of lentil and pea than in chickpea and wheat. *Cryptococcus* sp. (OTU6) was most abundant in wheat roots. In phase I, crop identity did not affect the structure of the AM fungal community in roots at

mid bloom or in rhizosphere soil in the fall. The variations in the root and soil AM fungal communities seemed to be mainly driven by seasonal and local variation of year/site.

The overall fungal community in the endosphere of wheat in phase II of the rotations appeared to be selected by wheat or prevailing environmental conditions rather than phase-I crops. The AM and non-AM fungal communities in rhizosphere soil in the fall of rotation phase I and in phase-II wheat roots were different. In addition, previous crops did not affect the levels of root fungal colonization, soil microbial community structure and microbial activity patterns in the subsequent wheat crop. Contrary to what was expected, wheat monoculture did not reduce overall fungal richness and diversity associated with either wheat roots or rhizosphere soil, at least in a 2-yr rotation.

Root fungal colonization levels and microbial activity were highest in wet and cool years. In contrast, total soil fungal biomass and the ratio fungi: bacteria were higher in drier and warmer years. These results suggest that soil fungal communities seemed to be well adapted to the arid conditions of the Prairies, but moisture and temperature during the growing season influenced the activity and biomass of the microbial communities and its relationships with the host plants.

3.3 Introduction

During the last decades farmers of the Canadian Prairie have increased the frequency of pulse crops in their rotation systems. The ability of pulse crops to establish symbiotic associations with N_2-fixing rhizobia reduces their dependence on N fertilizers (Walley 2007). Nitrogen-fixing crops also reduce the dependence of cereal-based rotations on N fertilizers. Previous research has found uneven N rotation benefits from pulses. In studies by Stevenson and Kessel (1996) only 8% of the yield increase of wheat after pea could be attributed to its N benefits, whereas N contribution explained 60 to 70% of the rotation benefit in studies by Janzen et al. (1990). The addition of N fertilizer alone to continuous wheat does not substitute for a legume break crop due largely to differences in disease build up and soil acidification (Heenan 1993). Factors such as water availability and soil biology can influence crop productivity. Pulses in rotation systems often increase available soil water level (Gan et al. 2003; Micheni et al. 2015; Miller et al. 2003), reduce disease incidence (Fernandez et al. 2008), weed abundance and offer an alternative to summer fallow (Gan et al. 2003).

Rhizosphere microorganisms are able to influence plant fitness by modifying soil processes, such as nutrient cycling, and therefore, nutrient uptake and assimilation by the host plant (Marschner et al. 2001). Microbial communities present in the rhizosphere can also modify root architecture and mediate changes in the hormonal balance of the host, modifying plant performance. Endophytic fungi have a broad host range, but their interaction with each plant type is specific and they may have different impacts on host performance (Mandyam and Jumpponen 2005). Among the soil fungal community, AM fungi are known to promote plant growth and yield (Hoeksema et al. 2010), increase plant access to soil P (Yang et al.

2014) and provide protection against pathogens (Borowicz 2001; Vigo et al. 2000). Other fungal groups contain saprophytic fungi and pathogens involved in a wide range of interactions with AM fungi and other soil microorganisms. The structure and dynamics of the microbial communities can have a large influence on crop performance (Khan and Joergensen 2006; Mandal et al. 2007). However, little is known about the composition of the fungal communities associated with the pulse-cereal rotation systems of the Canadian Prairies and the influence of these crops on their abundance and dynamics.

Thus, the impact of chickpea, lentil, pea and wheat on specific soil microbial communities, in particular communities of AM and non-AM fungi was investigated. I hypothesized that previous pulse crops influence the composition of the fungal community associated with the rhizosphere and endosphere of a subsequent wheat crop, with feedback on wheat growth and productivity. These hypotheses were tested in a two-phase rotation study conducted in 2010-2011 and replicated in 2011-2012.

3.4. Material and Methods

3.4.1 Experimental design

The field experiment was conducted at the Semiarid Prairie Agricultural Research Centre (SPARC) of Agriculture and Agri-Food Canada, in Swift Current, (latitude: 50˚ 18’ N; longitude: 107˚ 41’ W), Saskatchewan. Treatments were: chickpea, pea, lentil or wheat in phase I followed by wheat in phase II of four 2-year rotations (Table 3.1). The plants used were AC Lillian hard red spring wheat (*Triticum aestivum* L.), CDC Frontier kabuli chickpea (*Cicer arietinum* L.), CDC Meadow yellow pea (*Pisum sativum* L.) and CDC Maxim CL red lentil (*Lens culinaris* L.), (Table 3.1). All seeds were purchased from “Certified seed growers”. The experiment was arranged in a randomized block design with four repetitions (16 plots; plot size: 4 x 12 m, borders: 2 x 12 m).

The field experiment was conducted in 2010-2011 (phases I-II) and replicated in 2011-2012, on another site located at the South Farm of SPARC (latitude: 50°17’N; longitude: 107°41’W, elevation 825 m). The previous stubble was hard red spring wheat for all the crop rotation treatments (Table 3.1).

Table 3.1. Crop rotation treatments applied in the field experiment conducted in 2010-2011 and replicated in 2011-2012 at two different locations on the South Farm of the Semiarid Prairie Agricultural Research Centre, in order to test the effect of crop on soil microbiology and crop nutrition and productivity.

Pre-test	Rotation phase I	Rotation phase II
Wheat	Chickpea	Wheat
Wheat	Lentil	Wheat
Wheat	Pea	Wheat
Wheat	Wheat	Wheat

The soil at this location is a very gently sloping Orthic Brown Chernozem of the Swinton soil association that has a silt loam texture. The top 0 to 15 cm layer of the soil has a pH of 6.5 and contained 9 kg ha^{-1} mineral N (Maynard and Kalra 1993), 36 kg ha^{-1} Olsen P (Olsen et al. 1954), 326 kg ha^{-1} available K (Hamm et al. 1970), and 53 kg ha^{-1} available S (Hamm et al. 1973). Plots were treated with different fertilizers, herbicides and fungicides pre and post emergence, as shown in Table 3.2, in order to suit crop needs and control pests. A modified Noble Hoe drill frame with 8 Morris "C" shanks supporting Atom Jet side band openers at 10 inch spacing, followed by 4.5 inch Morris steel Packer wheels was used for seeding. Wheat, chickpea, pea and lentil were seeded into wheat stubbles, with 10 inch of row spacing, at a rate of 65, 200, 162 and 56 kg ha^{-1} respectively, in the year/site 2010 of phase I, and at a rate of 65, 214, 186 and 56 kg ha^{-1}, respectively in the year/site 2011 of phase I of the rotations.

In phase II of the rotations, wheat was seeded at a rate of 65 kg ha^{-1}. Fertilization, pest and weed control were as shown in Table 3.2.

Table 3.2. Soil management in the field experiment conducted in 2010-2011 and replicated in 2011-2012 at two different locations on the South Farm of the Semiarid Prairie Agricultural Research Centre. One time application is represented by "x".

		Phase I								Phase II	
		2010				2011				2011	2012
Fertilizers/Inoculants/Pesticides	**Concentration**	**CHP**	**LEN**	**PEA**	**WHT**	**CHP**	**LEN**	**PEA**	**WHT**	**WHT**	**WHT**
Apron Maxx[†]	325 mL 100 kg^{-1}	x	x	x		x	x	x			
Vitaflo 280[‡]	330 mL 100 kg^{-1}				x				x	x	x
MAP[§]	17 kg P ha^{-1}	x	x	x	x	x	x	x	x	x	x
Urea	67 kg N ha^{-1}				x				x	x	x
Nitragin Soil Implant+ GC[¶]	5.6 kg N ha^{-1}	x	x	x		x	x	x			
Bonanza 10G[#]	9.0 kg ha^{-1}	x	x	x		x	x	x			
Pursuit 240[††]	0.007 kg ha^{-1}	x				x					
Roundup Weathermax[‡‡]	0.44 kg ha^{-1}	xx	x	x	x	xx	x	x	x	x	x
INTEGO Solo[§§]	0.02 kg ha^{-1}		x	x			x	x			
Horizon W. O.[¶¶]	0.055 kg ha^{-1}				x						
Buctril M[##]	0.28 kg ha^{-1}				x						
Assure II[†††]	0.0356 kg ha^{-1}	x				x					
Achieve™ SC[‡‡‡]	0.198 kg ha^{-1}								x	x	x
Brotex 240[§§§]	0.267 kg ha^{-1}								x	x	x
2,4-D LV Ester 600 [¶¶¶]	0.277 kg ha^{-1}								x	x	
Headline Duo One[###]	0.148 kg ha^{-1}	xx	x	x	x						
Bravo 500[††††]	1.98 kg ha^{-1}	xxx	x	x	x	xx	xx	xx	x		
Proline 480[‡‡‡‡]	0.19 kg ha^{-1}	x	x	x	x	xx					
Matador 120EC[§§§§]	0.010 kg ha^{-1}						x	x			
Reglone[¶¶¶¶]	0.415 kg ha^{-1}	x	x	x		x	x	x			

[†]Apron Maxx: 1.1 % Mefenoxam + 0.73 % Fludioxonil, [‡]Vitaflo 280: 15.59 % Carboxin + 13.25 % Thiram, [§]MAP: Monoammonium phosphate ($NH_4H_2PO_4$), [¶]Nitragin Soil Implant + GC: Peat-based granular inoculant which contains a minimum of 100 million (1×10^8) viable cells of *Mesorhizobium ciceri* per gram of product, [#]Bonanza 10G: 10% trifluralin granular, [††]Pursuit 240: 240 g L^{-1} imazethapyr, [‡‡]Roundup Weathermax: 48 % Glyphosate, N-(phosphonomethyl) glycine, [§§]INTEGO Solo:15-40 %Ethaboxam, [¶¶]Horizon W. O.: 22.3 % Clodinafop-propargyl, [##]Buctril M: 280 g L^{-1} Bromoxynil (present as mixed octanoate and heptanoate esters), [†††]Assure II: 10.3 % Quizalofop p-ethyl, [‡‡‡]Achieve™ SC: 40 g L^{-1} Tralkoxydim, [§§§]Brotex 240: 57.57-61.13 % Bromoxynil Octanoate, [¶¶¶]2,4-D LV Ester 600: 600 g L^{-1} 2,4-D iso-octyl ester, [###]Headline Duo One: 25.2 % Boscalid + 12.8 % Pyraclostrobin, [††††]Bravo 500: 40.3 % Chlorothalonil, [‡‡‡‡]Proline 480: 480 g L^{-1}Prothioconazole, [§§§§]Matador: 13.2 % Lambda-Cyhalothrin, [¶¶¶¶]Reglone: 20 % Diquat dibromide.

The study tested the effect of host crops on root fungal colonization, soil microbial biomass, soil dehydrogenase activity and fungal diversity, in phase I (i.e., the first year of the phase year rotation) of the rotations. The previous crop effects on the fungal diversity associated with the roots of wheat at anthesis, crop nutrition and productivity were assessed in phase II of the rotations. Productivity was estimated as harvest index (proportion of seed weight in the total biomass) and total yield (kg of grain per hectare). The samples for harvest index determination were always harvested by hand in order to collect both seed and straw, whereas the plot yield was obtained by harvesting the central six rows of the plot using a plot combine.

3.4.2 Soil and root sampling and processing of samples

Samples of roots, rhizosphere soil and bulk soil were collected at the mid bloom stage of pulses in phase I, and at wheat anthesis in phase II and assessments of fungal community structure and diversity were performed (Table 3.3). Rhizosphere and bulk soil samples were collected again after harvest. Three samples of roots and rhizosphere soil were taken on the row of each plot, avoiding the border, at a depth of approximately 0 to 30 cm. Samples were taken from the right hand side of the plots while sampling for plant biomass and yield determination was done on the left hand side of the plot when crops reached full maturity. Plants were dug out with a shovel from three randomly selected points in each plot and pooled together as a single sample, avoiding the borders. Roots with rhizosphere soil attached were collected in plastic Ziploc^{TM} bags and taken to the laboratory. Rhizosphere soil was brushed off from the root surfaces and stored at -80ºC until DNA extraction or PLFA analysis, as described in sections 3.4.3.1 and 3.4.4. Fresh roots were thoroughly washed and

subsamples were taken for root fungal colonization analysis, which was conducted immediately, as described in section 3.4.6. Root samples for molecular analysis of endosphere fungal communities were preserved at -20ºC and processed within a week as described in section 3.4.3. Three bulk soil cores per plot were randomly taken, on the row, at 7.5 cm depth, pooled as a single sample, and stored at 4ºC for microbial activity measurements as described in section 3.4.5. The variables listed in Table 3.3 were measured.

Soil nutrient content was measured prior to seeding time and post-harvest. Plant material for nutrient analysis was collected at the end of the growing season, oven dried at 40ºC, finely ground and analyzed for total N (Noel and Hambleton 1976) and P (Milbury et al. 1970) concentrations using a segmented flow auto-analyzer (Technicon, AAII System, Tarrytown, NY) and for total K concentration using atomic absorption spectrometry at the Analytical Chemistry Laboratory of the Semiarid Prairie Agricultural Research Centre in Swift Current, SK.

Table 3.3. Measurements conducted to determine fungal colonization, fungal diversity, and microbial community structure in roots and soil from pulse and wheat crops.

Description	Sampling time	Methods
Percentage of roots colonized by fungi	Mid bloom	Gridline intersect method (Giovannetti and Mosse 1980)
Fungal diversity in roots	Mid bloom	Amplicon pyrosequencing (Buee et al. 2009)
Soil fungal diversity	Fall	Amplicon pyrosequencing (Buee et al. 2009)
Soil microbial biomass and community structure	Mid bloom	PLFA/NLFA-FAME (Hamel et al. 2006)
Soil metabolic activity	Mid bloom and fall	Soil dehydrogenase activity (Casida et al. 1964)

3.4.3 Fungal diversity analysis

3.4.3.1 DNA Extraction

One hundred milligrams of fresh roots were frozen in liquid N and milled with three tungsten beads in a 2-mL micro-centrifuge tube by vigorously shaking for 1 min. Three root samples from each plot were used for DNA extraction and extracts from the same plot were pooled. Total DNA was extracted from each root sample using Qiagen DNeasy Plant Mini Kit (Catalog #69104-50, QIAGEN, Mississauga, ON,) following the manufacturer's recommendations. DNA was extracted from 1 g of rhizosphere soil using the Ultra Clean Soil DNA Isolation Kit (Catalog #12800-100, MoBio Laboratories, Inc., Carlsbad, USA). The DNA extracts were diluted 1:10 or 1:20 in DNA-free water prior to amplification by polymerase chain reaction (PCR).

3.4.3.2 PCR conditions

A combination of a forward fusion primer with adaptor and multiplex identifier (MID) (Milne I Fau - Lindner et al. 2009), a reverse primer, and Platinum PCR Supermix (catalogue no. 11306-016; Invitrogen, Mississauga, ON) was used in all PCR to amplify the ITS and 18S rDNA regions of general and AM fungi respectively, as shown in Table 3.4. The PCR were made in triplicates.

Thermal cycling was conducted in a Veriti 96-well fast thermal cycler (Applied Biosystems). PCR conditions were specific for each set of primers (Dai 2013; Kubartová et al. 2007). The ITS1F/ITS2 primer set (Gardes and Bruns 1993; White et al. 1990) was used to amplify the ITS region (about 500bp) of the overall fungal community in roots and rhizosphere soil samples (Table 3.4). Each PCR mixture consisted of 16 μL of Platinum Supermix (Invitrogen, Mississauga, ON), 1.6 μL of distilled water, 0.2 μL of each 20 μM primers, and 2 μL of DNA template diluted 1:10. PCR conditions were: denaturation at 95°C for 3 min followed by 35 cycles of 45 s at 94°C, 45 s at 55°C and 75 s at 72°C, and a final extension of 8 min at 74°C.

Primer set NS1/NS4 (Lee et al. 2008) and AMV4.5NF/AMDGR (Lumini et al. 2010) were combined in a nested PCR protocol to amplify a fragment (about 300bp) of the 18S rDNA gene of AM fungi (Table 3.4). NS1/NS4 set was used in the first step to amplify total eukaryotic DNA. Reactions contained 16 μL of Platinum Supermix (Invitrogen, Mississauga, ON), 2.6 μL of distilled water, 0.2 μL of each 20 μM forward and reverse primer, and 1 μL of DNA template diluted 1:10, for a final 20 μL reaction volume. PCR conditions were set at a first step of 3 min at 94ºC followed by 25 cycles of denaturation at 94ºC for 45 s, annealing

at 51^{o}C for 45 s, and elongation at 72^{o}C s for 1 min followed by a final elongation at 72^{o}C for 7 min. The PCR products were used as template for the next PCR reaction with the AMV4.5NF/AMDGR primer set (Table 3.4). Reactions contained 16 μL of Platinum Supermix (Invitrogen, Mississauga, ON), 0.2 μL of each primer (20 μM), 2.6 μL of ultrapure water and 1.0 μL of 1:10 diluted PCR product. The PCR conditions were denaturation for 10 min at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s of annealing at 55°C, 1 min of elongation at 72°C, and a final elongation for 9 min at 72°C.

Table 3.4. Primer sets, adaptors and key sequences used to amplify ITS and 18S rDNA gene regions of target fungal communities in phases I and II of the rotation systems.

Primer set	Sequence	Target group
ITS-1F / ITS2	CTTGGTCATTTAGAGGAAGTAA / GCTGCGTTCTTCATCGATGC	General fungi
NS1 / NS4	GTAGTCATATGCTTGTCTC / CTTCCGTCAATTCCTTTAAG	Eukaryotes
AMV4.5NF / AMDGR	AAGCTCGTAGTTGAATTTCG / CCCAACTATCCCTATTAATCAT	AM fungi
Adaptor/Key (Forward)	CCATCTCATCCCTGCGTGTCTCCGAC/TCAG	
Adaptor/Key (Reverse)	CCTATCCCCTGTGTGCCTTGGCAGTC/TCAG	

3.4.3.3 Gel electrophoresis

A subsample containing 5 µL of each PCR products was mixed with 1 µL of Cyan/Yellow dye for visualization in 0.8 % agarose gel electrophoresis. The first column of the gel was loaded with 5 µL of 100 bp DNA ladder (Promega, Madison, USA) and the others were loaded with the dyed PCR products. The power was adjusted at 120V, 300 mA for 30 minutes. Bands were visualized using UV radiation and photographed.

3.4.3.4 DNA purification and library assembly

Each set of PCR products was pooled and purified with Agencourt AMPure XP (Agencourt Bioscience Corp.) The concentration and the purity of the tagged amplicons were measured using a Bioanalyzer 2100 Expert (Agilent Technologies, Inc). Pools of 16 samples corresponding to phase I of the rotations were sent to the Laboratories for Advanced Genome Sequencing in Vancouver Prostate Center (2660 Oak St, Vancouver, BC, V6H 3Z6) for pyrosequencing with the GS-FLX Titanium technology in 2010. In 2011 and 2012, pools of 16 samples were sent to Génome Québec Innovation Centre (740, Dr. Penfield Avenue, Room 7104, Montréal, QC, H3A 0G1) for sequencing with the same technology.

3.4.3.5 Bioinformatics and phylogenetic analysis

All AM fungal sequences were quality-checked, trimmed, and unique sequences were aligned, filtered and preclustered using Mothur v.1.33.0 (Open software, University of Michigan, USA) (See Appendix 7.1). One replicate of raw sequences from phase I (year/site 2010) was excluded from the analyses due to the poor quality of the sequencing data. The 18S rDNA sequence reads between 200 and 260 bp in length were considered for further analysis (Lumini et al. 2010). Ambiguous base pairs, homo-polymers longer than 8 bp,

chimeras, and low quality reads were excluded from downstream analysis. After obtaining a file containing the most abundant operational taxonomical units (OTUs) from each sample, the command "merge.files" was used to combine all the fasta-formatted files in a common file in order to generate a database. The sequences of the combined file were re-aligned and re-clustered based on 97% similarity into operational taxonomic units (OTUs) using CD-HIT Suite (http://cd---hit.org) and a list of the representative AM fungal OTUs per cluster was created. The representative sequence of each OTU cluster was used for BLAST search against the NCBI nucleotide collection database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences that matched with more than 95% similarity and 90% query coverage were considered. The reads contained into fasta-formatted files, corresponding to each sample, were classified into OTUs using the command "classify.otu" and organized into an OTU database in Microsoft Excel. Neighbor-joining phylogenetic trees for AM fungi were designed in MEGA 5 (Tamura et al. 2011) to verify OTU clusters.

General fungal sequences corresponding to the ITS region libraries were screened and trimmed using the Mothur command "trim.seqs". Only sequences longer than 200 bp were used for further analysis. Reads were aligned and filtered using the UNITE database as template (http://unite.ut.ee/repository.php), using the commands "align seqs" and "filter.seqs" respectively. Filtered fasta-formatted files were edited manually in BioEdit to remove unnecessary gaps. Sequences were clustered at 95 % similarity using the high-throughput search and clustering software USEARCH (http://drive5.com/usearch/).

Representative OTUs were classified using the Ribosomal Database Project Classifier (https://rdp.cme.msu.edu/classifier/classifier.jsp) (Wang et al. 2007). Biodiversity data were

resampled to the median value of the number of reads and biodiversity indices were calculated using R-statistical software v.3.1.2 (R Core Team 2014).

3.4.4 Fatty acid analysis

Microbial fatty acid markers in the phospholipidic (PLFA) and neutral (NLFA) fractions of rhizosphere soil lipid extracts were quantified to assess the effect of crop plant species and crop rotation on active soil microbial biomass and community structure. Rhizosphere soil samples collected at mid bloom and after harvest were analyzed. Four grams of soil (dry weight equivalent) were extracted in an accelerated solvent extractor using dichloromethane and methanol, both HPLC grade, in a proportion 2:1. The phospholipidic and neutral fatty acid fractions of soil lipid extracts were separated using silica columns. The columns were prepared using 5.75" Pasteur pipettes packed with silica gel approximately 2/3 of the length on the pipette barrel. Columns were conditioned with six washes of dichloromethane before adding the samples. The neutral fraction eluted with the addition of dichloromethane after the samples were loaded. Methanol was used to elute the PLFA fraction. The PLFA and NLFA fractions were methylated and purified following the method described by Hamel et al. (2006) and quantified by gas chromatography. The biomarkers i15:0, a15:0, 15:0, i16:0, i17:0 and 17:0 were chosen to represent bacterial biomass (Frostegård et al. 1993). Saprophytic fungi were represented by the PLFA biomarker 18:2 (Frostegård et al. 1993) and AM fungi were represented by the PLFA biomarker 16:1ω5c (Olsson et al. 1995). Also the NLFA marker 16:1ω5c was identified in the neutral fraction of soil lipids and used as a marker specific to AM fungi (Olsson 1999) because the specificity of PLFA 16:1ω5c is usually low (Hamel et al. 2006). The ratio fungi: bacteria was calculated as an indicator of soil health.

3.4.5 Soil dehydrogenase activity

Reactions catalyzed by soil dehydrogenases are related to the total oxidative activities of soil microorganisms, which are essential for soil organic matter decomposition (Raiesi and Beheshti 2014). Thus, soil microbial activity was assessed through the measurement of soil dehydrogenase activity at the mid-bloom stage of pulses in phase I, at anthesis of wheat in phase II, and in the fall in both phases.

Fresh bulk soil equivalent to 20 g of oven dried soil was mixed with 0.2 g of calcium carbonate ($CaCO_3$). Three samples of the mixture equivalent to 6 g of dry soil were placed into three 16 x 125 mm test tubes and double-distilled water (dd H_2O) was added to bring soil to field capacity. One millilitre of 2,3,5-triphenyltetrazolium chloride (TTC) solution (3%) was added to each tube, except the controls. Controls only contained the soil-$CaCO_3$, mix and dd H_2O. An extra 2.5 mL of dd H_2O was added to all tubes. The contents of the tubes were vortex, inverted a few times, and incubated for 24 h at 37ºC.

After incubation, 10 mL of methanol was added to each tube. The contents of the tubes were vortex for 1 min. The suspension was filtered through Whatman No. 5 filter paper, using Buchner funnels and vacuum. While the filtration occurred 40 mL of methanol were added to the funnels. Before filtering the controls, 3% TTC was added.

The intensity of the reddish colour of the filtrate was measured on a UV-VIS spectrophotometer (ShimatzuMini-1240) set at λ = 485 nm. The amount of triphenyl formazan (TPF) produced was estimated using a calibration curve prepared from standards (Casida et al. 1964).

3.4.6 Quantification of root colonization by endophytic fungi

Fresh clean roots finely cut into 1-cm pieces were placed in staining cassettes. Samples were prepared in duplicates. Cassettes were placed in a boiling 10 % potassium hydroxide (KOH) solution for 10 to 12 min, rinsed with tap water and transferred into a boiling ink-vinegar staining solution (5 ml of Shaeffer Black Ink in 95 ml of household vinegar) for 3 min. The cassettes were removed from the stain and sequentially soaked into water and in a destaining solution made of tap water and a few drops of vinegar (Vierheilig et al. 1998). Root colonization was analyzed at 400X magnification. AM and non-AM fungal structures were quantified using the gridline intersect method (Giovannetti and Mosse 1980) and photomicrographs were taken.

3.4.7 Statistical analysis

ANOVA was conducted using JMP10 software (SAS Institute Inc., Cary, NC, USA) to test the significance of the effect of previous crop species, year/site, and the interaction of these factors on fungal root colonization, soil dehydrogenase activity, relative abundance of fungi in the rhizosphere and roots endosphere, biodiversity indices and crop performance, at $\alpha = 0.05$. The data were checked for normality and homoscedasticity and they were square root or log-transformed if required to meet the assumption of ANOVA. Multiple mean comparisons were made using the Least Significant Difference (LSD) test and pair-mean comparisons were made using the Student's t test. Non parametric data were analyzed using the Kruskal-Wallis test.

To test the effect of previous crop, year and their interaction on the structure of the fungal communities of rhizosphere soil and crop root endosphere a two-way factorial permutation

test was performed. The test was based on multivariate analysis of variance (Per-MANOVA) using the Bray–Curtis measure of ecological distance (Bray and Curtis 1957) on the PLFA and pyrosequencing data. The DNA read data were resampled to the median value of the number of reads, and Shannon diversity and Chao richness indices were calculated using R-statistical software v.3.1.2, Vegan package (R Core Team 2014). The relative abundances of the AM fungal OTUs were estimated as the proportion, in percentage, of the total Glomeromycota reads. The relative abundances of the non-AM fungal OTUs were calculated as the proportion, in percentage, of the total fungal reads. In order to compare the crop rotation systems based on the relative abundance of their AM communities, AM fungal OTUs were pooled by genera and pairwise comparisons were made with Multiresponse Permutation Procedures (MRPP) using Sørensen distance in PC-ORD v.6 (McCune 2002). The same analysis was used to compare the previous crop rhizosphere soil fungal community in phase I in fall to the root-associated fungal community of wheat roots at anthesis in phase II.

The non-AM fungal OTU that accounted for more than 2 % of the total fungal reads were used for community structure analyses in each phase. Rare OTUs were excluded from the analyses, since in large enough sample size the rare sequences will potentially inflate estimates of diversity (Huse et al. 2010). PerMANOVA was used to analyze treatment effect in both sets of non-AM fungal community representative sample and the complete OTU dataset (Table B1). Pairwise comparison made with Multiresponse Permutation Procedures (MRPP) in PC-ORD v.6 (McCune 2002) were used to compare crop rotation systems based on the structure of their fungal communities. Non-metric multidimensional scaling (NMS) (Legendre and Legendre 1998) was performed in PC-ORD v.6 using Sørensen distance

measure to visualize the effect of treatments on fungal community structure. Spearman correlation test and regression analyses were performed using JMP10 software (SAS Institute Inc., Cary, NC, USA) to detect the relationships within fungal phylotypes, and between fungal phylotypes and crop performance.

3.5 Results

3.5.1 Molecular analysis of AM fungal communities associated with pulses and wheat

A total of 73 AM fungal OTUs were detected. *Glomus* and *Claroideoglomus* were the most diversified genera with 20 and 17 OTUs, respectively. Eleven OTUs corresponded to *Funneliformis*, five to *Rhizophagus*, six to *Diversispora*, five to *Paraglomus*, five to *Archaeospora*, and four to *Septoglomus*. Unfortunately, pyrosequencing failed to yield good quality sequences in phase I, year 2010. Therefore, only phase I, year 2011 was used for further analyses.

The AM fungal community in the endosphere of wheat appears to be selected by the host plant rather than the crop sequence (Fig.3.1). The AM community associated with the rhizosphere soil in the fall of phase I of pulses (Sf_P-I) and the AM community associated with the following wheat roots at anthesis in phase II (Ra_P-II) were different (Table 3.5). However, the community associated with the roots of wheat grown after wheat was similar to the soil community of the previous fall (Fig. 3.1, Table 3.5).

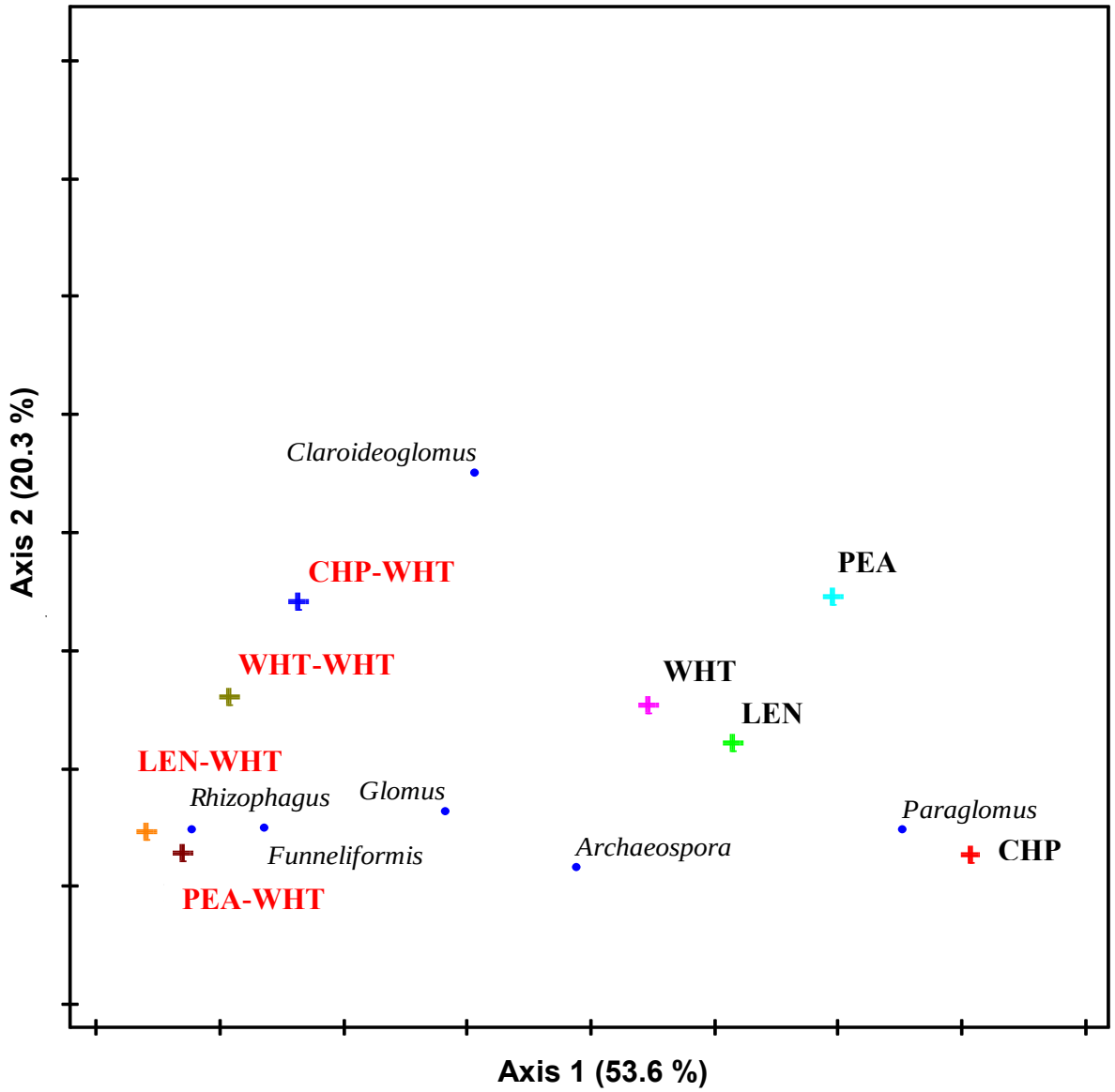

Figure 3.1. Ordination relating the crops grown in phase I of the rotations: chickpea (CHP), lentil (LEN), pea (PEA) and wheat (WHT), with the wheat grown in phase II, based on the AM genera distribution in rhizosphere soil collected in the fall of phase I (Sf_P-I) and in roots of wheat at anthesis in phase II (Ra_P-II), respectively (n = 4), according to non-metric multidimensional scaling (NMS) using Sørensen distance measure. A tri-dimensional solution was recommended. Final stress = 14.1. The percentages between parentheses represent the contribution of each axis to the ordination solution. Crops in Sf_P-I are represented in bold letters and wheat Ra_P-II are represented in bold red letters. Refer to Table 3.5 for information in multiple pairwise comparisons of AM community associated with the crop rotation treatments

Table 3.5. Summary statistics of MRPP and multiple pairwise comparisons of the relative abundance of the AM communities in rhizosphere soil collected in the fall of phase I (year/site 2011) and in the roots of wheat at anthesis in phase II (year/site 2012) (n = 4). Significant differences are indicated in bold.

Distance matrix	Observed δ	Expected δ	Variance of δ	Skewness of δ	*T*	*A*	*p*
Sørensen	0.4999	0.6155	1.02×10^{-3}	-0.5006	-3.6239	0.1876	**0.0014****
Crop comparisons							
Chickpea (2011)		vs	Lentil (2011)				0.1993
Chickpea (2011)		vs	Pea (2011)				0.4870
Chickpea (2011)		vs	Wheat (2011)				0.1869
Chickpea (2011)		vs	Chickpea-Wheat (2012)				**0.0161***
Chickpea (2011)		vs	Lentil-Wheat (2012)				**0.0081****
Chickpea (2011)		vs	Pea-Wheat (2012)				**0.0105***
Chickpea (2011)		vs	Wheat-Wheat (2012)				**0.0098****
Lentil (2011)		vs	Pea (2011)				0.6618
Lentil (2011)		vs	Wheat (2011)				0.9203
Lentil (2011)		vs	Chickpea-Wheat (2012)				0.2858
Lentil (2011)		vs	Lentil-Wheat (2012)				**0.0094****
Lentil (2011)		vs	Pea-Wheat (2012)				**0.0197***
Lentil (2011)		vs	Wheat-Wheat (2012)				0.0864
Pea (2011)		vs	Wheat (2011)				0.9268
Pea (2011)		vs	Chickpea-Wheat (2012)				0.1685
Pea (2011)		vs	Lentil-Wheat (2012)				**0.0068****
Pea (2011)		vs	Pea-Wheat (2012)				**0.0080****
Pea (2011)		vs	Wheat-Wheat (2012)				**0.0188***
Wheat (2011)		vs	Chickpea-Wheat (2012)				0.5647
Wheat (2011)		vs	Lentil-Wheat (2012)				**0.0218***
Wheat (2011)		vs	Pea-Wheat (2012)				0.0706
Wheat (2011)		vs	Wheat-Wheat (2012)				0.1465
Chickpea-Wheat (2012)		vs	Lentil-Wheat (2012)				0.0567
Chickpea-Wheat (2012)		vs	Pea-Wheat (2012)				0.7697
Chickpea-Wheat (2012)		vs	Wheat-Wheat (2012)				0.8212
Lentil-Wheat (2012)		vs	Pea-Wheat (2012)				0.4398
Lentil-Wheat (2012)		vs	Wheat-Wheat (2012)				**0.0080****
Pea-Wheat (2012)		vs	Wheat-Wheat (2012)				0.9891

* Significant effect at $p \leq 0.05$, ** Significant effect at $p \leq 0.01$.

In phase II, the interaction between the previous crop and year/site was detected (Table 3.6); hence the simple effects of previous crop and year/site were examined (Table 3.7). Pea, as previous crop, might be affecting the composition of the AM fungal communities of wheat roots (Fig. 3.2), but results were not conclusive since only the replicate year/site 2011 of phase II showed significant effects (Table 3.7).

Table 3.6. Probability of effect (*p*) of crop and year/site on the Glomeromycota community based on the reads abundance of AM fungal genera in the roots and rhizosphere soil of pulses and wheat in phases I and II of the rotations, according to PerMANOVA. Significant effects are indicated in bold.

Source of variation	**Phase I (*N* = 16)**[†]	**Phase II – Wheat (*N* = 32)**
Roots	– – – – – – – – – – Probability (*p*) – – – – – – – – – – – –	
Crop	0.675	0.122
Year/Site	NA[‡]	0.142
Crop*Year/Site	NA	**0.050***
Rhizosphere soil		
Crop	0.751	0.895
Year/Site	NA	**0.003****
Crop*Year/Site	NA	0.800

[†] Note that only the data of phase I from 2011 was analyzed, due to the poor quality of the read data obtained in 2010.
[‡] Not applicable.
* Significant effect at $p \leq 0.05$.
** Significant effect at $p \leq 0.01$.

Table 3.7. Probability of effect (*p* values) of previous crop on the Glomeromycota community, based on the reads abundance of AM fungal genera in the roots and rhizosphere soil of wheat in phases II of the rotations, and according to PerMANOVA (*n* = 4). Significant effects are indicated in bold.

	Phase II			
	Year/Site 2011		**Year/Site 2012**	
Source of variation	Roots	Soil	Roots	Soil
Crop	**0.041***	0.826	0.075	0.720

* Significant effect at $p \leq 0.05$.

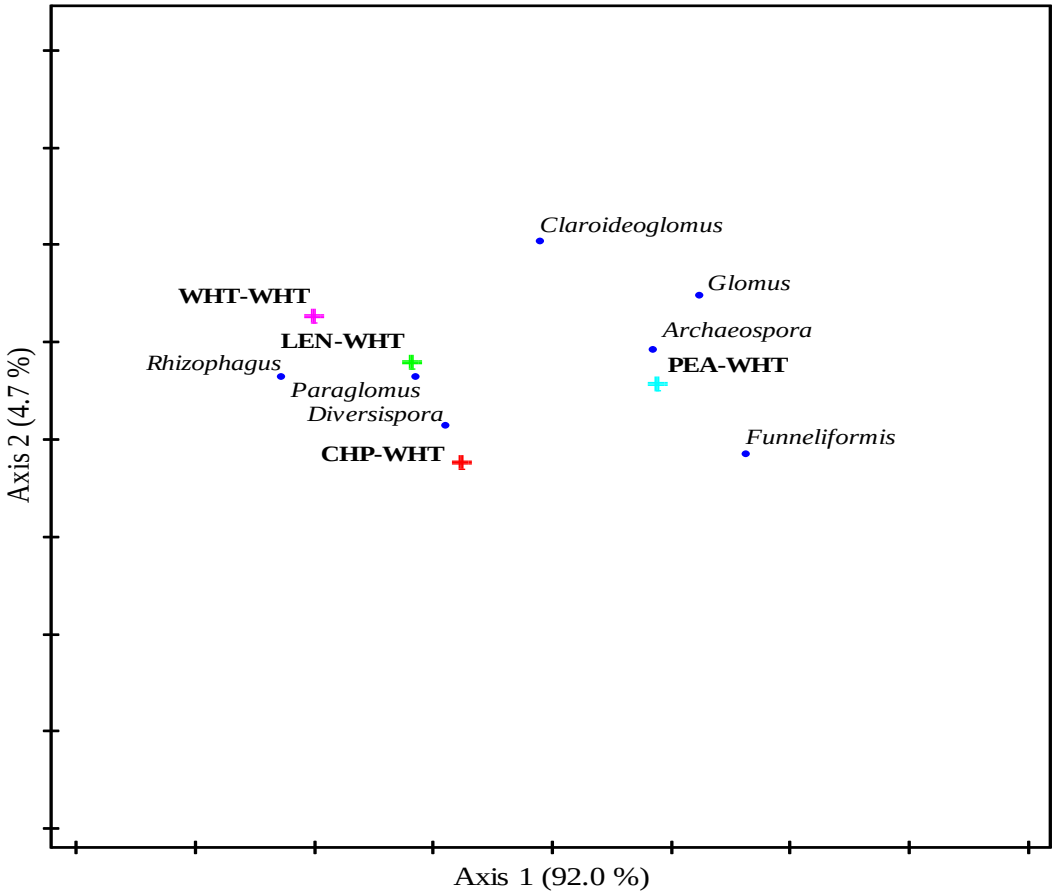

Figure 3.2. Ordination relating crop rotation systems based on the AM fungal genera detected in the roots of wheat at anthesis in phase II, year/site 2011 (*n* = 4), according to non-metric multidimensional scaling (NMS) using Sørensen distance measure. A bi-dimensional solution was recommended. Final stress = 3.1. The percentages between parentheses represent the contribution of each axis to the ordination solution. CHP: chickpea, LEN: lentil, PEA: pea, WHT: wheat.

The abundance of some individual AM genera in wheat roots at anthesis was affected by previous crop and year/site (Table 3.8), despite the lack of effects at the community level (Table 3.6). *Archaeospora* was more abundant after pea than after lentil (Table 3.8). *Diversispora* was less abundant after chickpea than any other previous crops (Table 3.8). *Paraglomus* was more abundant in year/site 2011 than in year/site 2012, in phase II of the rotations (Table 3.8).

The composition of AM genera in the rhizosphere soil of phase II in fall was not influenced by the crop rotations, but by year/site of cultivation (Table 3.9). *Claroideoglomus* and *Paraglomus* were more abundant in year/site 2011 than in year/site 2012, whereas *Glomus* and *Rhizophagus* were more abundant in year/site 2012 (Table 3.9).

Table 3.8. Relative abundance of AM genera in the roots of wheat grown in phase II of the rotations, detected by pyrosequencing. Within a column, significant probabilities of treatment effect, according to Kruskal-Wallis tests, are indicated by different letters in bold.

Phase II	**AM genera**						
ROOTS	*Archaeospora*	*Claroideoglomus*	*Diversispora*	*Funneliformis*	*Glomus*	*Paraglomus*	*Rhizophagus*
Crop rotations (*n*=8)	Relative abundance (%)						
Chickpea-Wheat	0.01 **ab**	26.6 ns	0.01 **b**	17.0 ns	14.5 ns	0.14 ns	41.6 ns
Lentil-Wheat	0.00 **b**	5.1 ns	7.95 **a**	10.5 ns	8.2 ns	0.36 ns	67.0 ns
Pea-Wheat	0.15 **a**	7.3 ns	5.42 **a**	36.2 ns	26.5 ns	0.96 ns	23.5 ns
Wheat-Wheat	0.06 **ab**	13.5 ns	0.67 **a**	18.2 ns	9.5 ns	0.47 ns	57.5 ns
Year/Site (*n*=16)							
2011	0.04 ns[‡]	8.7 ns	0.12 ns	19.0 ns	12.1 ns	0.70 **a**	59.3 ns
2012	0.07 ns	17.5 ns	6.90 ns	21.9 ns	17.3 ns	0.26 **b**	35.5 ns
Source of variation[†]	Probability (*p*)						
Crop	**0.0344***	0.2963	**0.0440***	0.3056	0.2881	0.4221	0.0786
Year/Site	0.8043	0.5209	0.0643	0.7062	0.3859	**0.0289***	0.0675

[†] Interaction Crop x Year/Site could not be tested by Kruskal-Wallis test for non-parametric data.
[‡]Not significant.
* Significant effect at $p \leq 0.05$.

Table 3.9. Relative abundance of AM genera in the rhizosphere soil of wheat in the fall of phase II of the rotation, detected by pyrosequencing. Within a column, significant probabilities of treatment effect, according to Kruskal-Wallis tests, are indicated by different letters in bold.

Phase II	**AM genera**							
SOIL	*Archaeospora*	*Claroideoglomus*	*Diversispora*	*Funneliformis*	*Glomus*	*Paraglomus*	*Rhizophagus*	*Septoglomus*
Crop rotations (*n*=8)	Relative abundance (%)							
Chickpea-Wheat	0.50 ns‡	40.1 ns	12.5 ns	7.1 ns	20.8 ns	9.46 ns	9.6 ns	0.00 ns
Lentil-Wheat	0.84 ns	20.0 ns	1.50 ns	17.1 ns	28.0 ns	9.26 ns	17.3 ns	6.05 ns
Pea-Wheat	0.77 ns	31.4 ns	13.0 ns	20.8 ns	21.4 ns	8.39 ns	4.2 ns	0.00 ns
Wheat-Wheat	0.03 ns	35.6 ns	4.3 ns	22.5 ns	15.0 ns	5.22 ns	17.3 ns	0.03 ns
Year/Site (*n*=16)								
2011	1.02 ns	42.1 **a**	13.7 ns	10.1 ns	6.60 **b**	15.7 **a**	8.9 **b**	1.94 ns
2012	0.05 ns	21.5 **b**	11.9 ns	23.7 ns	36.0 **a**	0.52 **b**	16.0 **a**	1.10 ns
Source of variation†	Probability (*p*)							
Crop	0.3928	0.4044	0.5611	0.2912	0.7970	0.2865	0.2132	0.0707
Year/Site	0.1679	**0.0418***	0.2030	0.1647	**0.0001****	**0.0264***	**0.0388***	0.3416

† Interaction Crop x Year/Site could not be tested by Kruskal-Wallis test for non-parametric data.
‡Not significant.
* Significant effect at $p \leq 0.05$.
** Significant effect at $p \leq 0.01$.

3.5.2 Molecular analysis of non-AM fungal communities associated with pulses and wheat

The non-AM fungal community contained different fungi with a wide range of possible effects on crop performance. A total of 313 fungal OTUs were detected. Rare OTUs were excluded from the analyses. Reeder and Knight (2009) reviewed techniques for minimizing pyrosequencing biases, and suggested that the majority of pyrotags that make up the rare phylotypes represents the accumulation of small sequencing errors (Huse et al. 2010). Therefore, the OTUs with more than 2 % of occurrence in the dataset were used to characterize the fungal community (Table 3.10) in phases I and II of the rotations.

Host plant identity had a significant influence on the structure of the non-AM fungal community inhabiting the roots of rotation phase I plants at mid bloom (Table 3.11). Among the pulses, the fungal communities associated with the roots of chickpea and pea were different (Table 3.12, Fig. 3.3). The fungal community associated with wheat roots was significantly different from that of pulses (Table 3.12, Fig. 3.3). No significant crop effects were detected on the general soil fungal community in fall of phase I. (Table 3.11).

Table 3.10. Non-AM fungal OTUs with more than 2 % occurrence in the roots and rhizosphere soil of crops in phases I and II of the rotations. Relative abundance was estimated as the number of reads of a specific OTU in relation with the total fungal reads detected by pyrosequencing in each sample.

Sample	OTU Identity	Classification (Identity ≥ 95 %)	Relative abundance (%)
		Phase I – Pulses/Wheat	
Roots	5	*Fusarium tricinctum*	18.7
	6	*Cryptococcus sp.*	12.1
	3	*Clonostachys rosea*	11.0
	0	*Cryptococcus fuscescens*	7.9
	9	*Lachnum sp.*	7.4
	313	Unidentified taxa	6.3
	12	*Thanatephorus cucumeris*	4.9
	27	*Mortierella sp.*	3.8
	16	*Fusarium redolens*	3.3
	38	*Thielaviopsis basicola*	2.1
Soil	0	*Cryptococcus fuscescens*	16.3
	313	Unidentified taxa	12.6
	8	*Mortierella sp.*	10.4
	27	*Mortierella sp.*	7.9
	15	*Sistotrema sp.*	4.9
	38	*Thielaviopsis basicola*	2.4
	14	*Pseudogymnoascus roseus*	2.4
		Phase II – Wheat	
Root	13	*Microdochium bolleyi*	19.8
	4	*Fusidium griseum*	8.6
	1	*Periconia macrospinosa*	8.0
	43	*Setophoma terrestris*	6.6
	2	*Microdochium bolleyi*	5.1
	313	Unidentified taxa	4.1
	75	*Olpidium brassicae*	3.5
	5	*Fusarium tricinctum*	3.0
	39	*Davidiella tassiana*	2.9
	16	*Fusarium redolens*	2.8
	30	*Mortierella sp.*	2.7
	62	*Hymenoscyphus scutula*	2.7
	38	*Thielaviopsis basicola*	2.4
	9	*Lachnum sp.*	2.4
	55	*Setophoma terrestris*	2.1
	11	*Ophiosphaerella sp.*	2.0
Soil	0	*Cryptococcus fuscescens*	14.0
	313	Unidentified taxa	10.2
	10	*Alternaria eichhorniae*	9.7
	39	*Davidiella tassiana*	9.3
	5	*Fusarium tricinctum*	5.1
	76	*Podospora intestinacea*	3.4
	8	*Mortierella sp.*	3.3
	27	*Mortierella sp.*	2.9
	29	*Sistotrema sp.*	2.4
	9	*Lachnum sp.*	2.0

Table 3.11. Summary statistics of the effects of crop and year/site on the most abundant non-AM fungal community found at mid bloom in the roots of the crops grown during phases I and II of the rotation, according to PerMANOVA. Significant probabilities are indicated in bold.

	Phase I – Pulses/Wheat (*N* = 16)		**Phase II – Wheat (*N* = 32)**	
	Roots	**Soil**	**Roots**	**Soil**
Source of variation[†]	– – – – – – – – – – – – Probability (*p*) – – – – – – – – – – – –			
Crop	**0.046***	0.508	0.912	0.096
Year/Site	NA[‡]	NA	**0.001****	**0.001****
Crop*Year/Site	NA	NA	0.998	0.999

[†] Note that interaction Crop x Year/Site could not be tested in one of phase I year/site replicates due to poor quality of the reads in 2010 samples.
[‡]Not applicable
* Significant effect at $p \leq 0.05$
** Significant effect at $p \leq 0.01$.

Table 3.12. Summary statistics for MRPP and multiple pairwise comparisons of the structure of the non-AM fungal community inhabiting the roots of the crops in phase I of the rotations ($n = 4$). The significant probabilities are indicated in bold.

Distance matrix	**Observed δ**	**Expected δ**	**Variance of δ**	**Skewness of δ**	***T***	***A***	***p***
Sørensen	0.5701	0.6969	1.72×10^{-3}	-0.8011	-3.0544	0.1819	**0.0076****
– – – – – – – – – – **Crops – Phase I** – – – – – – – – –							
Chickpea		vs.	Lentil		0.0269	-0.0018	0.3945
Chickpea		vs.	Pea		-2.8345	0.1733	**0.0159***
Chickpea		vs.	Wheat		-1.9130	0.1175	**0.0500***
Lentil		vs.	Pea		0.5867	-0.0277	0.6882
Lentil		vs.	Wheat		-2.2589	0.1722	**0.0310***
Pea		vs.	Wheat		-4.0185	0.3879	**0.0065****

* Significant effect at $p \leq 0.05$
** Significant effect at $p \leq 0.01$.

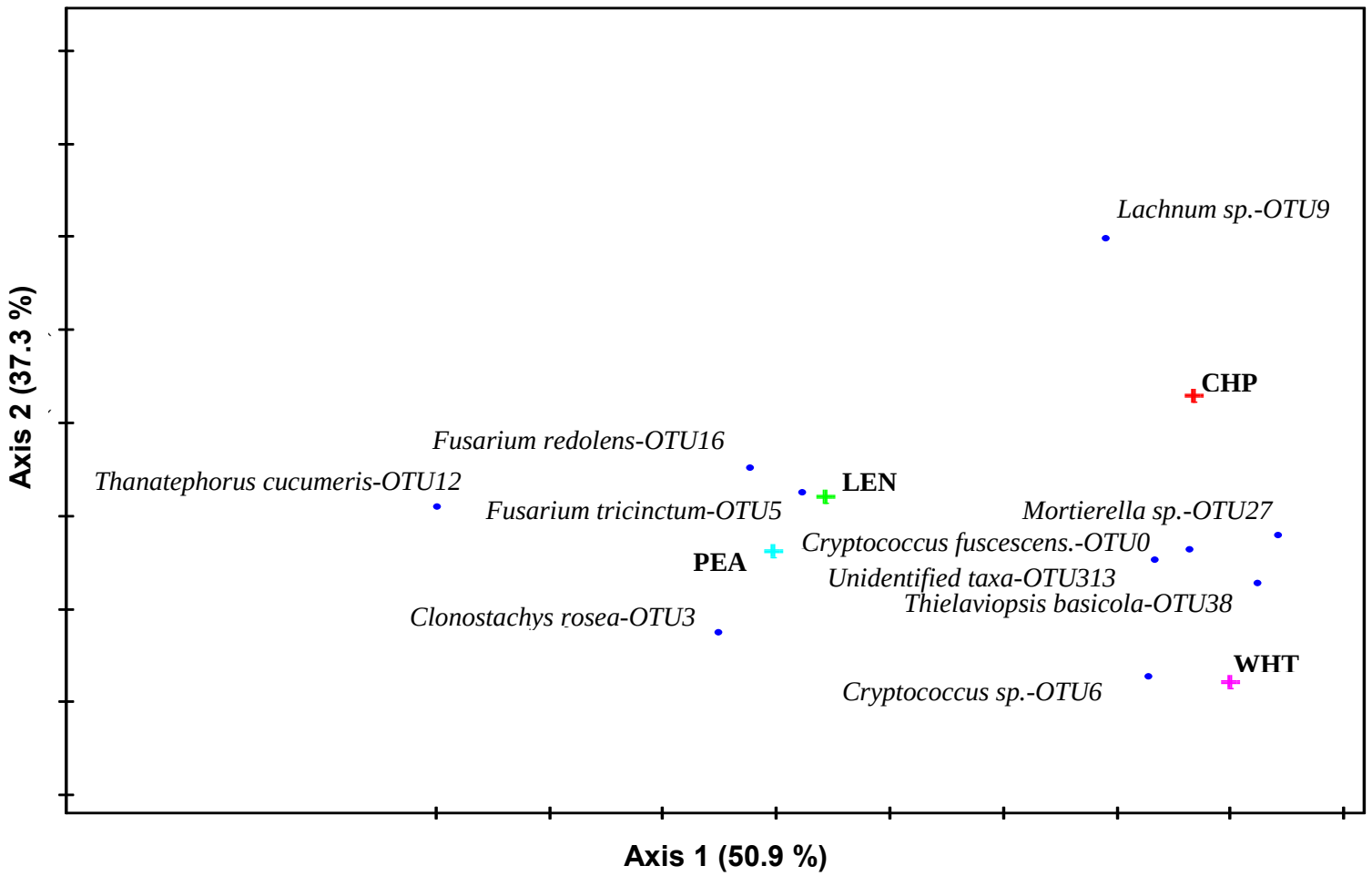

Figure 3.3. Ordination representing the crop effect on the root-associated non-AM fungi in phase I of the rotations, according to non-metric multidimensional scaling (NMS) and PerMANOVA (p = 0.046) using Sørensen distance measure. A bi-dimensional solution was recommended. Final stress = 13.9. The percentages between parentheses represent the contribution of each axis to the ordination solution. The mean of each treatment is represented (n = 4). CHP: chickpea, LEN: lentil, PEA: pea, WHT: wheat.

In phase I of the rotations, the abundance of some fungal phylotypes in the roots of the crops varied depending on the plant species to which they were associated. The relative abundance of *Fusarium tricinctum* (OTU5) was highest in pea and lowest in wheat (Fig. 3.4 A). *Fusarium redolens* (OTU16) was more abundant in the roots of lentil and pea than in the roots of chickpea and wheat (Fig. 3.4 B). *Cryptococcus* sp. (OTU6) was the most abundant OTU found in wheat (Fig. 3.4 C).

In phase II, the non-AM fungal communities associated with the roots of wheat at anthesis and with the rhizosphere soil in the fall were similar among treatments as shown in Table 3.11. The differences in the root and rhizosphere soil fungal communities seemed to be driven by year/site (Table 3.11).

To verify the possible influence of the previous crop fungal community on the subsequent cereal crop, the non-AM fungal communities of fall soil in phase I were compared with those of wheat roots grown in the same plots on the subsequent year. The fungal communities in phase I soil and phase II roots were different, according to MRPP analysis (Table 3.13), suggesting that wheat is selecting a specific fungal community, despite the fungal communities present in the previous fall soil. Results are represented in an ordination in Figure 3.5.

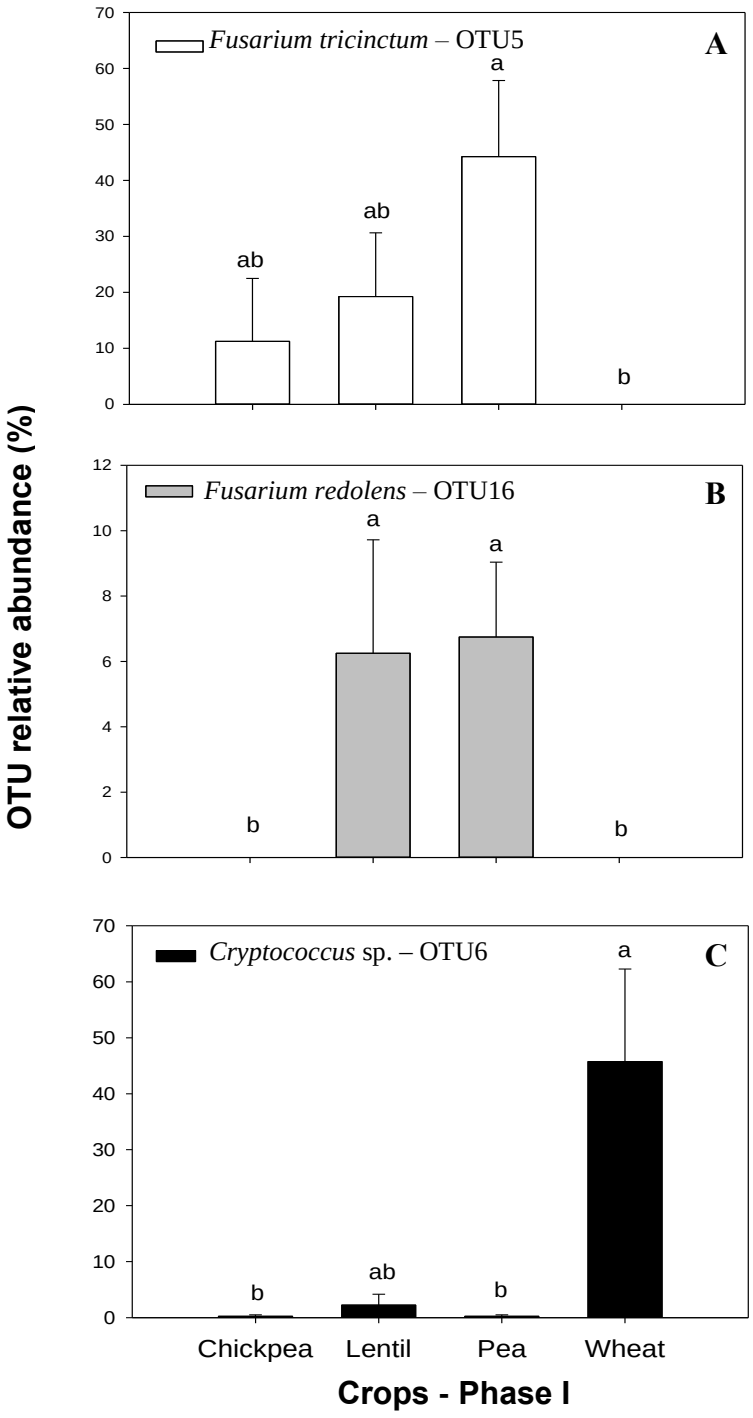

Figure 3.4. Relative abundance of OTU5 (A), OTU16 (B) and OTU6 (C) in the roots of chickpea, lentil, pea and wheat in phase I of the rotations. Different letters represent significant differences between crops ($p \leq 0.05$), according to Kruskal-Wallis test (n = 4). Relative abundance is the proportion of reads per OTU related to the total number of fungal OTUs per sample

Table 3.13. Summary statistics of MRPP and pairwise comparisons of the non-AM fungal community in rhizosphere soil collected in the fall of phase I (year/site 2011) and in the roots of wheat at anthesis in phase II (year/site 2012) (n =4). Bold numbers show significant differences between the root and soil communities.

Distance matrix	**Observed δ**	**Expected δ**	**Variance of δ**	**Skewness of δ**	***T***	***A***	***p***
Sørensen	0.3604	0.5520	0.0012	-0.6316	-5.5754	0.3472	**<.0001****
– – – – – – – – – – – – – Crop comparisons– – – – – – – – – – – – –							
Chickpea (2011)	vs	Lentil (2011)					0.4779
Chickpea (2011)	vs	Pea (2011)					0.9273
Chickpea (2011)	vs	Wheat (2011)					0.7799
Chickpea (2011)	vs	Chickpea-Wheat (2012)					**0.0069****
Chickpea (2011)	vs	Lentil-Wheat (2012)					**0.0072****
Chickpea (2011)	vs	Pea-Wheat (2012)					**0.0066****
Chickpea (2011)	vs	Wheat-Wheat (2012)					**0.0076****
Lentil (2011)	vs	Pea (2011)					0.6875
Lentil (2011)	vs	Wheat (2011)					0.6934
Lentil (2011)	vs	Chickpea-Wheat (2012)					**0.0092****
Lentil (2011)	vs	Lentil-Wheat (2012)					**0.0093****
Lentil (2011)	vs	Pea-Wheat (2012)					**0.0089****
Lentil (2011)	vs	Wheat-Wheat (2012)					**0.0105***
Pea (2011)	vs	Wheat (2011)					0.8850
Pea (2011)	vs	Chickpea-Wheat (2012)					**0.0132***
Pea (2011)	vs	Lentil-Wheat (2012)					**0.0137***
Pea (2011)	vs	Pea-Wheat (2012)					**0.0114***
Pea (2011)	vs	Wheat-Wheat (2012)					**0.0144***
Wheat (2011)	vs	Chickpea-Wheat (2012)					**0.0068****
Wheat (2011)	vs	Lentil-Wheat (2012)					**0.0069****
Wheat (2011)	vs	Pea-Wheat (2012)					**0.0066****
Wheat (2011)	vs	Wheat-Wheat (2012)					**0.0073****
Chickpea-Wheat (2012)	vs	Lentil-Wheat (2012)					0.7158
Chickpea-Wheat (2012)	vs	Pea-Wheat (2012)					0.9058
Chickpea-Wheat (2012)	vs	Wheat-Wheat (2012)					0.9109
Lentil-Wheat (2012)	vs	Pea-Wheat (2012)					0.3306
Lentil-Wheat (2012)	vs	Wheat-Wheat (2012)					0.9373
Pea-Wheat (2012)	vs	Wheat-Wheat (2012)					0.4213

* Significant effect at $p \leq 0.05$

** Significant effect at $p \leq 0.01$.

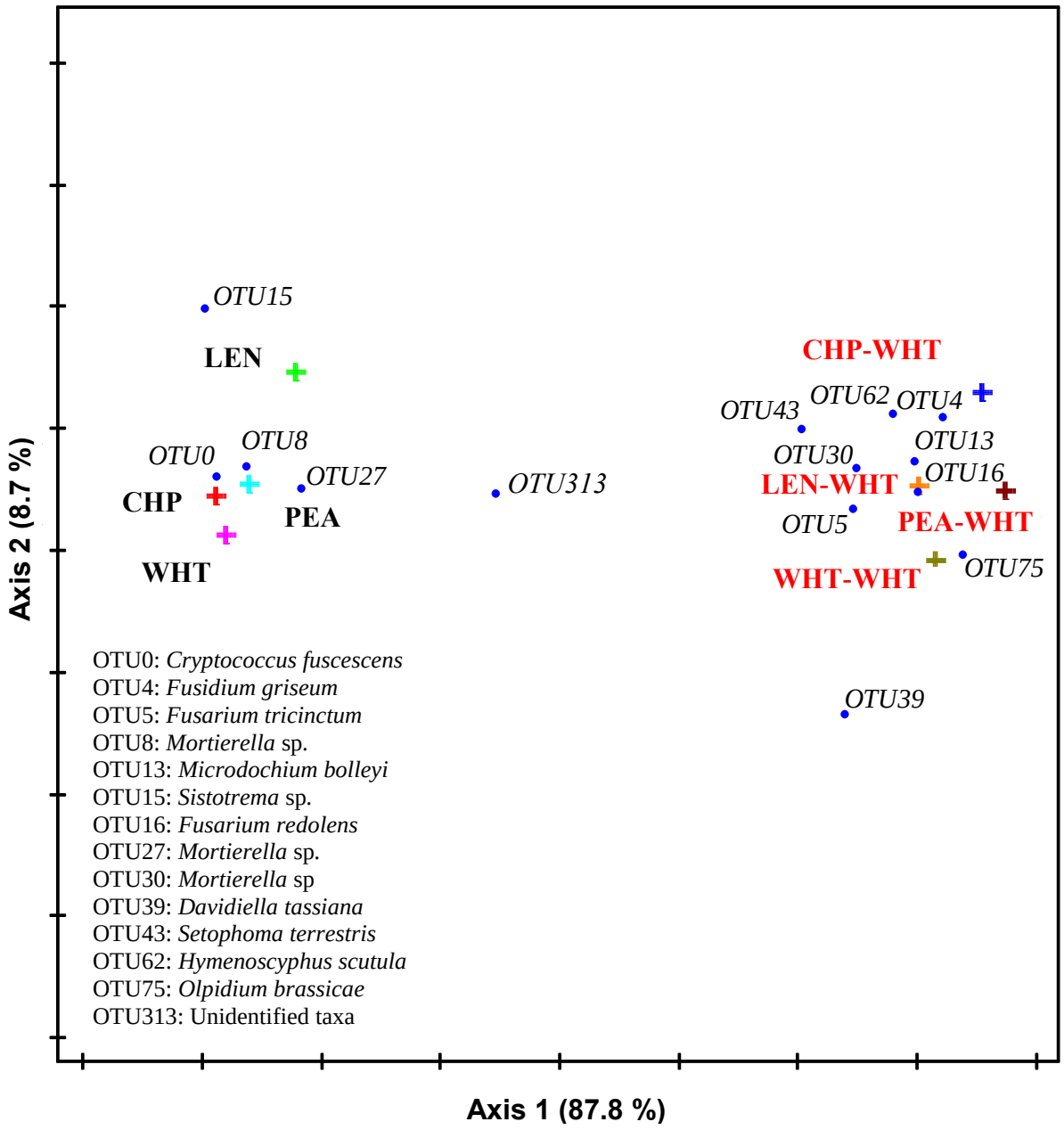

Figure 3.5. Ordination relating the phase I crops (2011) and the subsequent wheat grown in phase II (2012) of the rotations, based on the non-AM fungal communities structure in rhizosphere soil collected during the phase I fall (Sf_P-I) and in roots of wheat at anthesis in phase II (Ra_P-II), according to non-metric multidimensional scaling (NMS) using Sørensen distance measure. A bi-dimensional solution was recommended. Final stress = 11.2. The percentages between parentheses represent the contribution of each axis to the ordination solution. Crops in Sf_P-I are represented in bold letters and wheat Ra_P-II are represented in bold red letters. The means of each treatment are represented (n = 4). CHP: chickpea, LEN: lentil, PEA: pea, WHT: wheat.

3.5.3 Richness and diversity indices

Wheat monoculture did not reduce overall fungal richness or diversity. In fact, AM fungal richness in the fall rhizosphere soil of phase II was higher after wheat than after pulses (Table 3.14). The AM species richness in wheat roots in phase II was affected by year/site, and it was higher in 2011 than in 2012 (Table 3.14). No crop effects were influencing the diversity indices estimated in roots and soil AM communities in phase I of the rotations (Table 3.14).

The richness and diversity of non-AM fungal communities was only influenced by year/site of cultivation (Table 3.15) in phase II of the rotations. The non-AM species richness in wheat roots and rhizosphere soil in fall, and the Shannon diversity in wheat roots were higher in 2012 than in 2011 (Table 3.15).

Table 3.14. Chao richness and Shannon diversity indices of the AM fungal communities in roots and rhizosphere soil, in phases I and II of the rotations. Within a column, significant differences between crop means, according to the Least Significant Difference (LSD) test ($p \leq 0.05$) are indicated in bold by different letters. Significant differences between year/site means, according to Student's t test ($p \leq 0.05$) are also indicated in bold by different letters.

	Phase I (*N* =16)				**Phase II – Wheat (*N* =32)**			
AM fungi	**Chao richness (S)**		**Shannon diversity (H)**		**Chao richness (S)**		**Shannon diversity**	
Crops	**Roots**	**Soil**	**Roots**	**Soil**	**Roots**	**Soil**	**Roots**	**Soil**
Chickpea	26.2 ns[†]	7.5 ns	1.68 ns	0.71 ns	9.6 ns	11.0 **b**	0.87 ns	1.55 ns
Lentil	23.2 ns	6.7 ns	2.06 ns	0.70 ns	12.0 ns	11.3 **b**	0.96 ns	1.43 ns
Pea	27.5 ns	7.5 ns	1.75 ns	0.67 ns	13.6 ns	8.6 **b**	1.25 ns	1.37 ns
Wheat	16.5 ns	8.5 ns	1.10 ns	0.88 ns	14.1 ns	16.0 **a**	0.88 ns	1.48 ns
Year/Site								
2011	NA[‡]	NA	NA	NA	14.7 **a**	11.7 ns	0.84 ns	1.46 ns
2011/2012	NA	NA	NA	NA	9.9 **b**	11.7 ns	1.14 ns	1.45 ns
Source of variation	Probability (*p*)							
Crop	0.2714	0.9504	0.2930	0.9033	0.3898	**0.0208***	0.3746	0.5964
Year/Site	NA	NA	NA	NA	**0.0230***	1.0000	0.0878	0.6925
Crop*Year/Site	NA	NA	NA	NA	0.8713	1.0847	0.2282	0.2590

[†] Not significant.
[‡] Not applicable.
* Significant effect at $p \leq 0.05$.
** Significant effect at $p \leq 0.01$.

Table 3.15. Chao richness and Shannon diversity indices of the non-AM fungal communities in roots and rhizosphere soil, in phases I and II of the rotations. Within a column, significant differences between crop means, according to the Least Significant Difference (LSD) test ($p \leq 0.05$) are indicated in bold by different letters. Significant differences between year/site means, according to Student's t test ($p \leq 0.05$) are also indicated in bold by different letters.

	Phase I (*N* =16)				**Phase II – Wheat (*N* =32)**			
Non-AM fungi	**Chao richness (S)**		**Shannon diversity**		**Chao richness (S)**		**Shannon diversity (H)**	
Crops	**Roots**	**Soil**	**Roots**	**Soil**	**Roots**	**Soil**	**Roots**	**Soil**
Chickpea	19.3 ns†	34.0 ns	1.58 ns	2.61 ns	37.8 ns	57.2 ns	2.21 ns	2.44 ns
Lentil	21.0 ns	34.3 ns	1.22 ns	2.39 ns	39.1 ns	53.6 ns	2.42 ns	2.53 ns
Pea	19.3 ns	34.0 ns	1.20 ns	2.71 ns	41.0 ns	58.6 ns	2.23 ns	2.52 ns
Wheat	15.0 ns	45.8 ns	1.40 ns	2.80 ns	41.0 ns	70.0 ns	2.25 ns	2.86 ns
Year/Site								
2011	NA‡	NA	NA	NA	26.5 **b**	30.6 **b**	1.97 **b**	2.51 ns
2011/2012	NA	NA	NA	NA	52.9 **a**	89.0 **a**	2.58 **a**	2.67 ns
Source of variation	Probability (*p*)							
Crop	0.6584	0.7439	0.8472	0.6811	0.8329	0.4390	0.7939	0.1260
Year/Site	NA	NA	NA	NA	**<.0001****	**<.0001****	**0.0011****	0.2409
Crop*Year/Site	NA	NA	NA	NA	0.5928	0.4379	0.7569	0.3763

† Not significant.
‡ Not applicable
* Significant effect at $p \leq 0.05$.
** Significant effect at $p \leq 0.01$.

3.5.4 Root colonization

AM and non-AM fungi were observed and quantified in the roots of the different crops. Hyaline non septate mycelia, arbuscules and globous spores, usually stained in dark blue, are characteristic of AM fungi (Fig 3.6 D, E, F). Non-AM fungi are also known as dark septate endophytes (DSE) due to the dark brownish color of the melanized septate hyphae and storage structures that characterize them, allowing their microscopic identification and separation from AM fungi (Fig 3.6 A, B, C, D).

Pulses had higher levels of AM fungal root colonization than wheat in phase I of the rotations (Table 3.16). Among the pulses, pea had the highest AM root colonization levels (Table 3.16). Levels of AM fungi and DSE root colonization were higher in 2010 (Table 3.16). No crop effect affecting the DSE root colonization levels was detected (Table 3.16), either in phase I nor II of the rotations

In phase II, the interaction between previous crop and year/site had a significant effect on the level of wheat root colonization by AM fungi (Table 3.16). In 2012, wheat grown after pea had the highest level of AM fungal colonization, but it was low in 2011. In contrast, wheat grown after lentil had the highest AM fungal colonization level in 2011, but it was low in 2012 (Fig. 3.7).

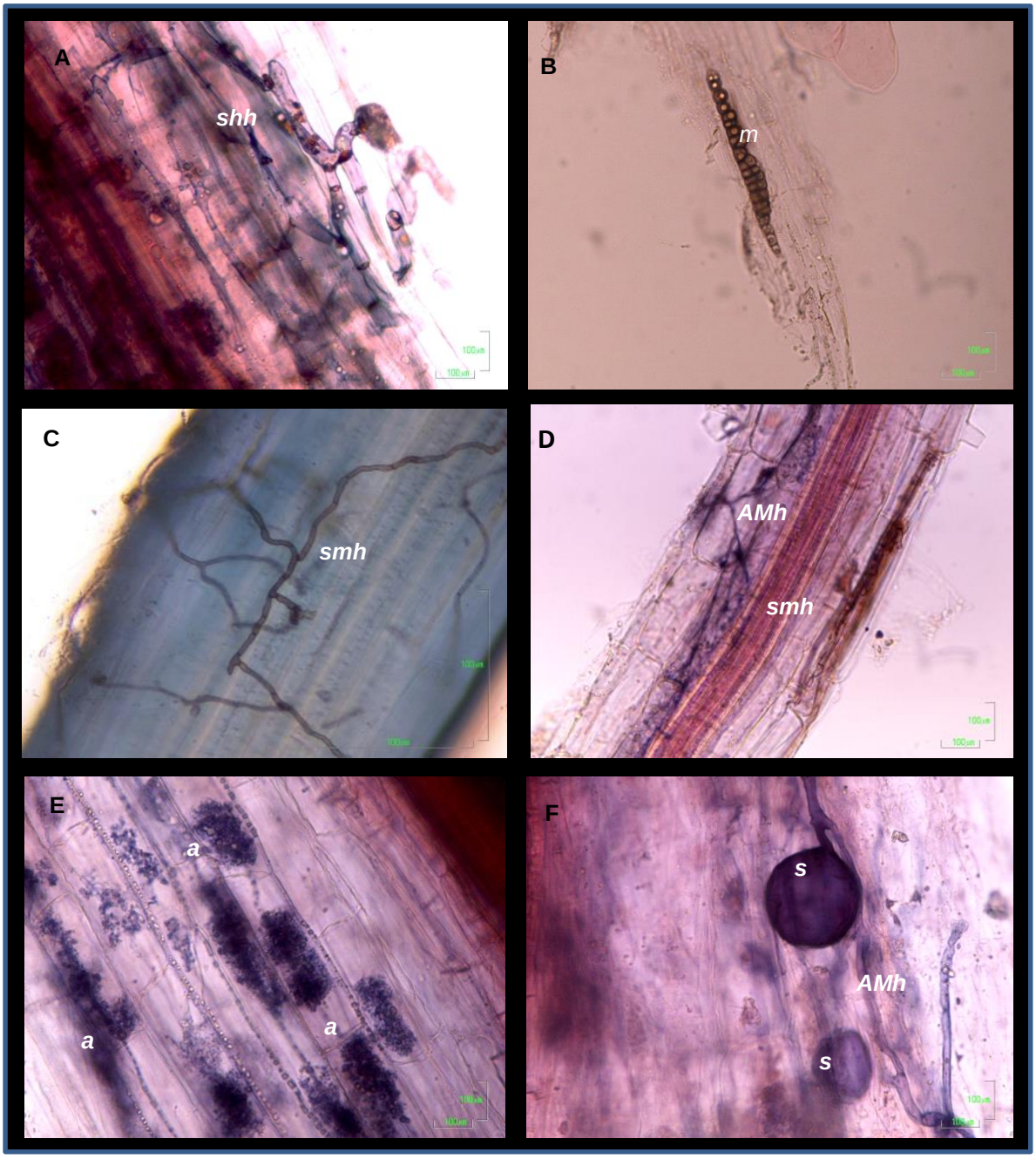

Figure 3.6. Root colonization by non-AM (A, B, C, D) and AM fungi (D, E, F). Note the two types of fungal colonization in microphotograph D. (A) Septate hyaline hyphae (*shh)* in the cortex of pea roots (B) Microsclerotia (*m)* in lentil roots. (C) Septate melanized non-AM hyphae (*smh*) colonizing a wheat root. (D) AM fungal hyphae (*AMh*) and septate melanized non-AM hyphae (*smh)* colonizing a wheat root. (E) Arbuscules (*a*) in lentil roots. (F) Hyphae (*AMh)* and AM spores (*s*) in pea roots.

Table 3.16. Level of root colonization by AM and non-AM fungi in phases I and II of the rotations and probability of treatment effect, according to ANOVA. Crop means associated with different letters are different at $p \leq 0.05$, according to LSD test ($n = 4$). Year/site 2010/2011 and 2011/2012 represent replicates of the phases I and II respectively, and their means are connected with the same letter when no significant differences at $p \leq 0.05$ were found, according to Student's t test .

	Endophytic root colonization levels			
	Phase I		**Phase II - Wheat**	
	AM	**Non-AM**	**AM**	**Non-AM**
Crops	---------- Percentage of colonized roots (%) -------			
Chickpea	53.9 **b**	15.3 ns†	33.8 ns	30.9 ns
Lentil	53.8 **b**	23.1 ns	29.4 ns	26.6 ns
Pea	65.3 **a**	19.9 ns	29.8 ns	28.0 ns
Wheat	40.7 **c**	22.4 ns	31.8 ns	25.7 ns
Year/Site				
2010/2011	59.0 **a**	28.6 **a**	31.4 ns	36.0 **a**
2011/2012	49.6 **b**	12.2 **b**	33.3 ns	24.0 **b**
Source of variation	-------- ----- Probability (p) ----------			
Crop	**0.0002****	0.1030	0.5208	0.8489
Year/Site	**0.0022****	**<.0001****	0.2295	**0.0310***
Crop*Year/Site	0.9853	0.5732	**0.0132***	0.9643

† Not significant.
* Significant effect at $p \leq 0.05$.
** Significant effect at $p \leq 0.01$.

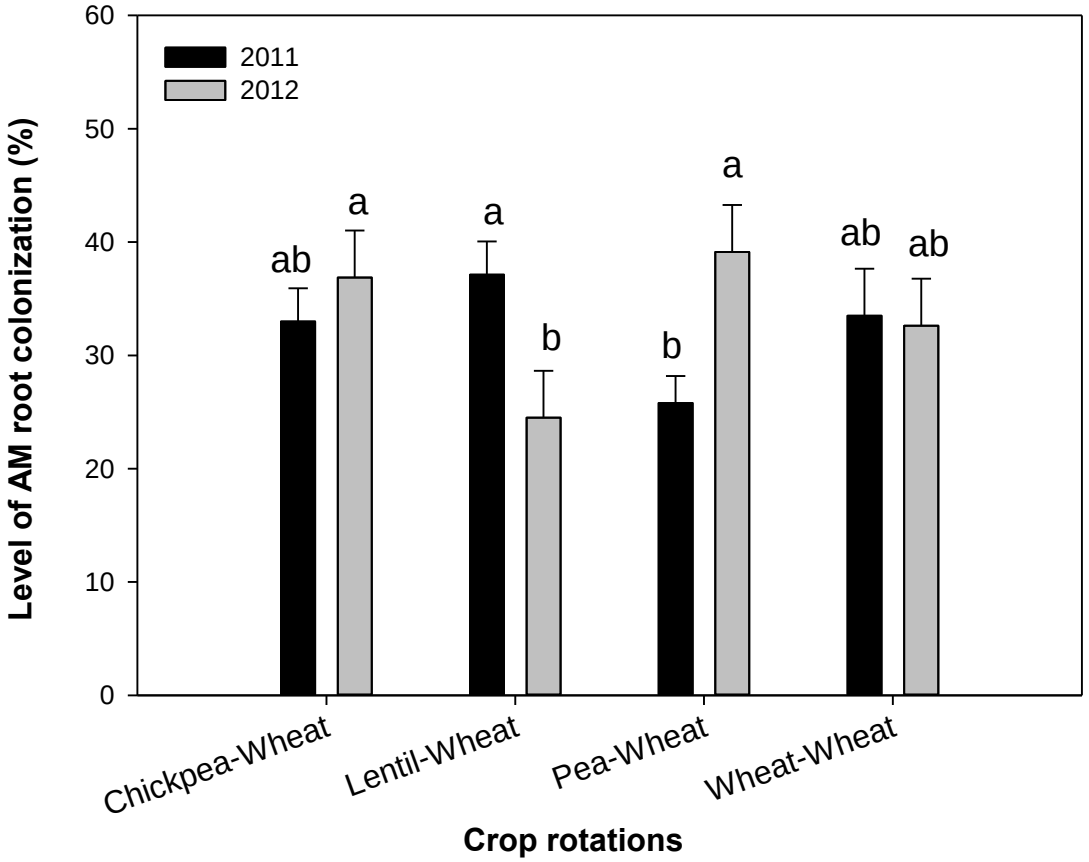

Figure 3.7. Levels of root colonization by AM fungi of wheat grown in phase II of the rotations as influenced by the interaction of previous crop and year/site (p = 0.0132). Means with standard error are not significantly different according to LSD test (n = 4) when associated with the same letter.

3.5.5 Fatty acid profile and activity of the overall soil microbial community associated with pulses and wheat.

Fatty acid profiles and dehydrogenase activity were used to obtain a general overview of treatment effects on the structure of soil microbial communities. The relative biomass of bacteria and fungi in the rhizosphere soil were estimated by the concentration of phospholipid fatty acid (PLFA) indicators of these microbial groups. In phases I and II of the rotations, microbial community structure (Table 3.17) was not significantly affected by crop. Soil microbial communities seemed to be mostly influenced by changes in year/site of cultivation (Table 3.17). The year/site effects on the PLFA indicators are described in Table 3.18. Soil microbial activity was not influenced by crop, or previous crop, during phases I and II of the rotations (Table 3.19).

Higher microbial activities coincidently occurred in wet and cool years (See Section 3.5.7). In phase I the concentrations of the PLFA and NLFA biomarkers 16:1ω5c, corresponding to AM fungal biomass, the bacteria indicator a15:0, the total fungal PLFA and the ratio fungi: bacterial were higher in 2011 than in 2010 (Table 3.18). Contrastingly, in phase I of the rotation, microbial activities at mid bloom and in the fall were higher in 2010 than in 2011 (Table 3.19). In phase II, the concentration of the PLFA 16:1ω5c, the bacteria indicators i16:0 and i17:0, the total bacterial and fungal PLFA and the ratio fungi: bacterial were higher in 2012 than 2011 (Table 3.18). In contrast, microbial activity was higher in the fall of 2011 than 2012 (Table 3.19).

Table 3.17. Summary statistics from PerMANOVA (N = 32) performed on the microbial community structure data of phases I and II of the rotations, as estimated by PLFA analysis. Significant effects are indicated in bold.

Source of variation	d.f.	SS	MS	F value	*p value*
Phase I					
Crop	3	0.3365	0.11218	0.7803	0.584
Year/Site	1	0.8737	0.87373	6.0771	**0.003****
Crop*Year/Site	3	0.3164	0.10547	0.7335	0.710
Residual	24	3.4506	0.14377	0.69327	
Total	31	4.9773	1		
Phase II					
Crop	3	0.1974	0.06579	0.6264	0.718
Year/Site	1	1.7359	1.73588	16.527	**0.001****
Crop*Year/Site	3	0.1548	0.05161	0.4913	0.862
Residual	24	2.5208	0.10503	0.54694	
Total	31	4.6089	1		

** Differences are significant at $p \leq 0.01$.

Table 3.18. Abundance of bacterial and fungal PLFA, and NLFA c16:1ω5 in the soil of phases I and II of the rotations, as influenced by year/site. Significant effects of year/site on soil microbial biomarkers according to Kruskal-Wallis tests (n = 16) are indicated in bold.

		Phase I			Phase II - Wheat		
Type of microorganism	**Microbial biomarker**	**2010**	**2011**	***p***	**2011**	**2012**	***p***
AM fungi	NLFA c16:1ω5	0.0208	0.1745	**0.0458***	0.0447	0.0535	0.4695
AM fungi	PLFA c16:1ω5	0.0184	0.1955	**0.0195***	0.0310	0.6689	**<.0001****
Fungi	PLFA c18:2	0.0311	0.0438	0.0899	0.1201	0.1309	0.4930
Bacteria	PLFA i15:0	0.0401	0.0160	0.1000	0.0513	0.0732	0.1753
Bacteria	PLFA a15:0	0.0780	0.1473	**0.0382***	0.1127	0.1293	0.4427
Bacteria	PLFA 15:0	0.0825	0.0523	0.4510	0.1060	0.1177	0.3076
Bacteria	PLFA i16:0	0.0000	0.0001	0.3173	0.0012	0.9913	**<.0001****
Bacteria	PLFA i17:0	0.1131	0.1430	0.7345	0.4576	0.0627	**0.0237***
Bacteria	PLFA c17:0	0.0394	0.0409	0.2913	0.0597	0.0790	0.1366
Total bacterial PLFA		0.3530	0.2996	0.5718	0.7884	1.4533	**0.0116***
Total fungal PLFA		0.0496	0.2393	**0.0116***	0.1512	0.7999	**<.0001****
Ratio fungi: bacteria		0.1833	1.3161	**0.0110***	0.3000	0.6145	**0.0076****

*Significant effects at $p \leq 0.05$.
** Significant effects at $p \leq 0.01$.

Table 3.19. Metabolic activity of the soil microbial community at mid bloom and in the fall, in phases I and II of the rotations, and probability of treatment effects. The effect of crop and year/site was considered significant at $p \leq 0.05$, according to LSD test and Student's t test respectively.

Crops/Previous crops	**Phase I**		**Phase II - Wheat**	
	– – – Triphenyl formazan production (*mg TPF.kg*$^{-1}$) – – – –			
Crops (*n* = 8)	**Mid bloom**	**Fall**	**Anthesis**	**Fall**
Chickpea	1.70 ns †	2.50 ns	1.05 ns	1.25 ns
Lentil	1.55 ns	2.40 ns	1.00 ns	1.20 ns
Pea	1.59 ns	2.30 ns	0.89 ns	1.06 ns
Wheat	1.99 ns	1.98 ns	0.87 ns	1.03 ns
Year/Site (*n* =16)				
2010/2011	2.78 **a**	3.60 **a**	0.84 ns	1.30 **a**
2011/2012	0.64 **b**	1.04 **b**	1.06 ns	0.97 **b**
Source of variation	– – – – – – – – – – – – – Probability (*p*) – – – – – – – – – – – – –			
Crop	0.2389	0.9856	0.6150	0.3726
Year/Site	**<.0001****	**<.0001****	0.0555	**0.0035****
Crop*Year/Site	0.4664	0.0631	0.5737	0.2429

† Not significant

** Significant effects at $p \leq 0.01$

3.5.6 Impact of previous crop and year/site on wheat performance and its relationship with the microbial community

Wheat after pulses performed better than the monoculture. Wheat after a pulse had higher plant density and produced more seed biomass and total yield (Table 3.20). Previous crop and year/site of cultivation had significant effects on wheat performance. Plant density, plant mature biomass, harvest index, yield, number of seeds, and heads per plant, and plant nutrition were influenced by the previous crops as shown in Tables 3.20 and 3.21. Overall, phase II wheat productivity was higher in 2011 than in 2012; however the number of seeds per plant and wheat head per square meter were higher in 2012 (Table 3.21).

Straw and seed N were influenced by the interaction between previous crop and year/site. The straw N content of wheat was highest after chickpea in 2011, but it was highest after pea in 2012 (Fig. 3.8). Wheat seed N content was highest after chickpea in 2011, but in 2012, no previous crop effect on wheat seed N was detected (Fig. 3.9).Interestingly, seed P content was highest in the monoculture (Table 3.21).

Table 3.20. Least square means and probabilities of treatment effect on wheat productivity attributes in phase II of the rotations. Within a column, significant effects are indicated in bold. Crop rotation and year/site means associated with different letters are different at $p < 0.05$ according to LSD test and Student's t test, respectively.

	Plant density (plant m^{-2})	**Mature plant biomass(g m^{-2})**	**Harvest index**	**Yield (kg ha^{-1})**	**Seeds.plant^{-1}**	**Wheat head density (heads m^{-2})**
Crop rotation (*n* =8)						
Chickpea-Wheat	128 **a**	677 **ab**	0.372 ns	2706 **a**	65.3 **b**	347 **bc**
Lentil-Wheat	103 **b**	749 **a**	0.356 ns	2707 **a**	88.6 **a**	422 **a**
Pea-Wheat	94 **b**	726 **a**	0.357 ns	2615 **a**	92.2 **a**	394 **ab**
Wheat-Wheat	105 **b**	573 **b**	0.355 ns	2121 **b**	70.0 **b**	317 **c**
Year/site (*n* =16)						
2011	132 **a**	710 ns†	0.375 **a**	2759 **a**	63.5 **b**	345 **b**
2012	83 **b**	653 ns	0.345 **b**	2316 **b**	94.6 **a**	394 **a**
Source of variation	Probability (*p*)					
Previous crop	**0.0092****	**00127***	0.2838	**0.0004****	**0.0097****	**0.0028****
Year/Site	**<.0001****	0.1370	**0.0003****	**<.0001****	**<.0001****	**0.0150***
Previous crop*Year/Site	0.9689	0.4300	0.5982	0.2004	0.4618	0.1831

† Not significant.
* Significant effects at $p \leq 0.05$.
** Significant effects at $p \leq 0.01$.

Table 3.21. Least square means and probabilities of treatment effect on wheat straw and seed nutrient content in phase II of the rotations. Within a column, significant effects are indicated in bold. Crop rotation and year/site means associated with different letters are different at $p < 0.05$ according to LSD test and Student's t test, respectively.

	Straw N	**Straw P**	**Seed N**	**Seed P**
	Nutrient content ($mg\ g^{-1}$)			
Crop rotation (*n = 8*)				
Chickpea-Wheat	3.60 ns[†]	0.71 ns	24.46 **a**	4.60 **ab**
Lentil-Wheat	3.03 ns	0.46 ns	21.90 **b**	4.38 **b**
Pea-Wheat	2.90 ns	0.46 ns	21.95 **b**	4.45 **b**
Wheat-Wheat	3.10 ns	0.65 ns	22.01 **b**	4.80 **a**
Year/site (*n = 16*)				
2011	3.08 **b**	0.65 ns	22.45 ns	4.58 ns
2012	3.23 **a**	0.48 ns	22.70 ns	4.53 ns
Source of variation	Probability (p)			
Previous crop	0.7183	0.1673	**0.0331***	**0.0073****
Year/Site	**0.0410***	0.9682	0.1441	0.5455
Previous crop*Year/Site	**0.0027****	0.0966	**0.0080****	0.1628

[†] Not significant.
* Significant effects at $p \leq 0.05$.
** Significant effects at $p \leq 0.01$.

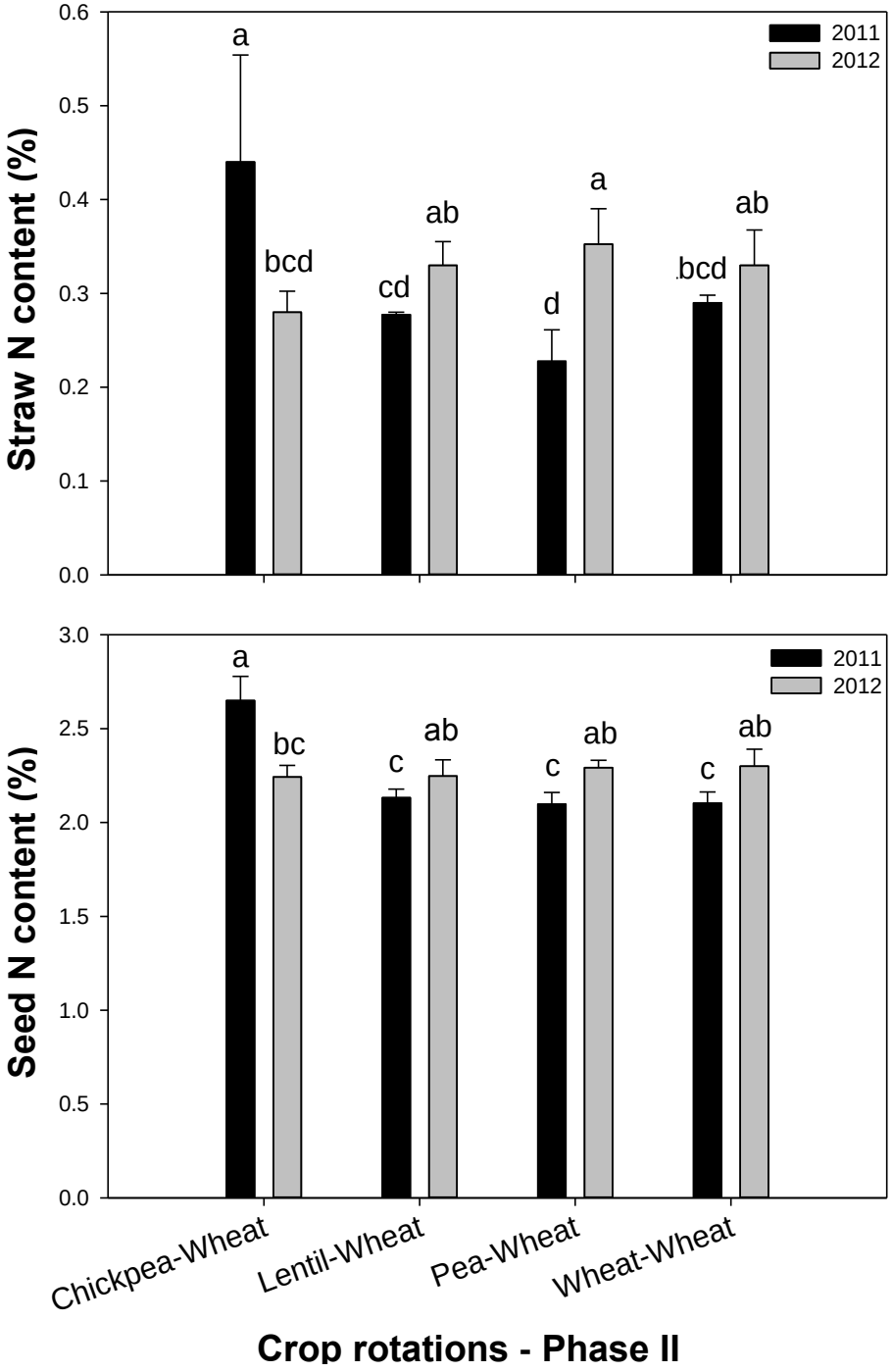

Figure 3.8. Least square means and standard errors of wheat straw (p = 0.0027) and seed N (p = 0.0008) concentrations, as influenced by crop x year/site. Bars with the same letter are not significantly different according to LSD test (n = 8).

The analysis of the relationships between fungal phylotypes in phase II revealed an inverse relationship between the relative abundances of *Rhizophagus* (OTU2) and *Fusarium redolens* (OTU16) in the roots of wheat (Fig. 3.9 A), suggesting the possible antagonistic function of some AM taxa and pathogenic fungi. The possibility of competition for space and resources within the roots could explain this relationship. *Rhizophagus* (OTU2) was very abundant in wheat roots. The abundance of *Rhizophagus* (OTU2) was also negatively correlated with wheat seed C concentration (Fig. 3.9 C), which is reasonable, based on the energetic cost of this symbiosis for the plant. The abundance of *Fusarium redolens* (OTU16) was negatively related with plant mature biomass, mature seed weight and yield of wheat (Table 3.22, Fig. 3.9 D, E, F). The relative abundance of *Rhizophagus* (OTU2) was also negatively correlated with the relative abundance of *Funneliformis* (OTU11) (Fig. 3.9 B). The proportions of these AM fungi in the roots might be also determined by differences in the phenology of each fungal species. Other fungal interactions are presented in Table 3.22.

Table 3.22. Significant Spearman correlations (N = 32) between the agronomic performance of wheat and the relative abundance of root-associated AM and non-AM fungal reads, detected by pyrosequencing. Wheat was grown in the South Farm of the Semiarid Prairie Agricultural Research Centre near Swift Current, SK in 2011 and 2012.

OTUs	vs.	Correlation (r)	Probability (p)
AM fungi			
Funneliformis (OTU11)	Seed carbon content	0.3595	0.0165
Glomus (OTU13)	Dehydrogenase activity (Mid bloom)	0.4199	0.0105
Rhizophagus (OTU2)	Seed carbon content	-0.4682	0.0450
	Fusarium redolens (OTU16)	-0.4217	0.0076
	Funneliformis (OTU11)	-0.6295	<.0001
Non-AM fungi			
Fusarium redolens (OTU16)	Mature biomass	-0.4203	0.0222
	Mature seed biomass	-0.4582	0.0102
	Yield	-0.4931	0.0620
	Dehydrogenase activity (Mid bloom)	0.4370	0.0501
	Rhizophagus (OTU2)	-0.4217	0.0076
Microdochium bolleyi (OTU2)	Seeds/plant	-0.5643	0.0047
	Dehydrogenase activity (Fall)	0.4845	0.0237
	Periconia macrospinosa (OTU1)	0.7126	<.0001
Periconia macrospinosa (OTU1)	Plant density	0.5669	0.0001
	Harvest index	0.4464	0.0014
	Dehydrogenase activity (Fall)	0.4513	0.0028

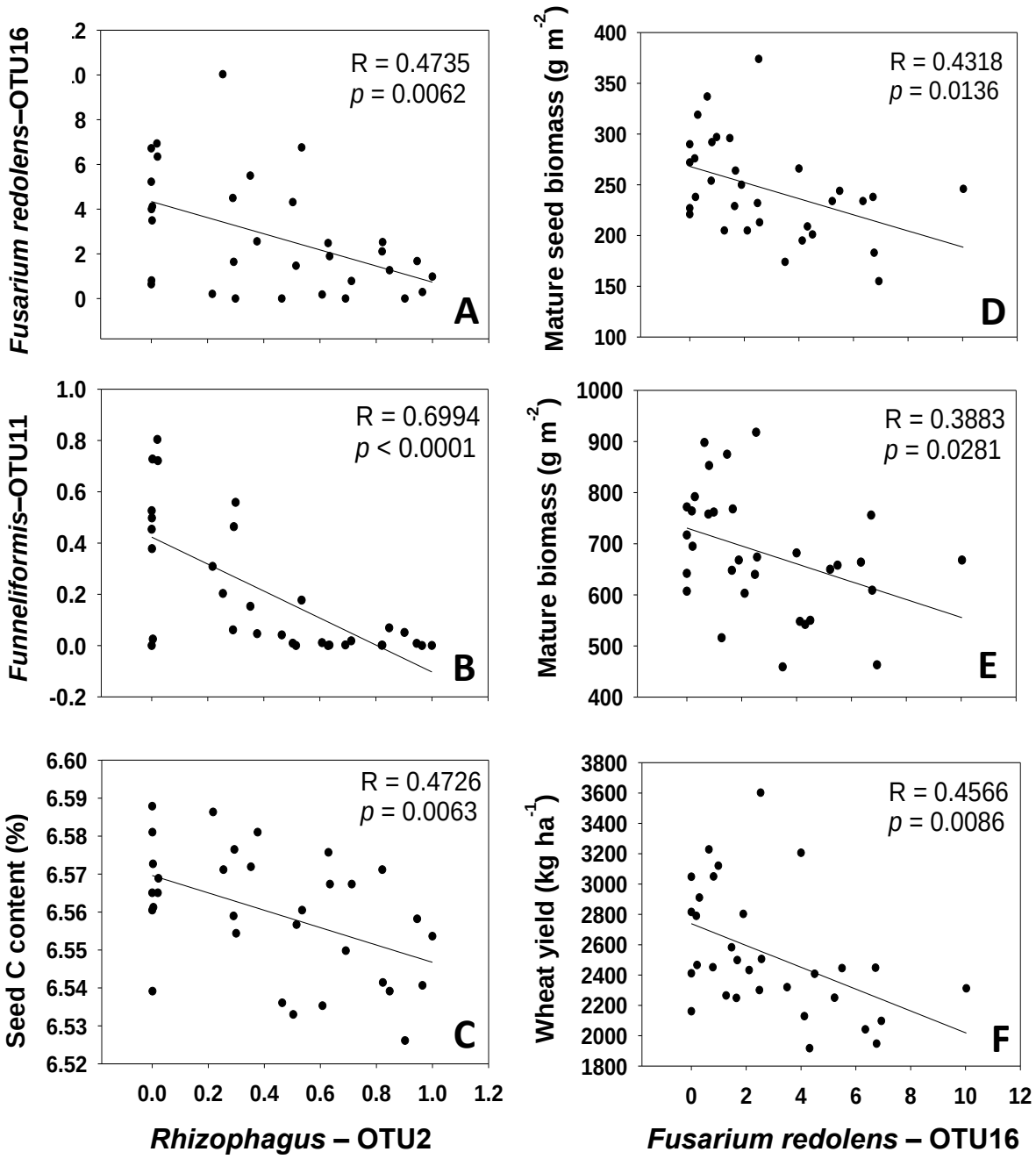

Figure 3.9. Significant linear regressions (N = 32) between the relative abundance of the root-associated *Rhizophagus* (OTU2) and that of other fungi and seed C content in wheat (A, B, C) and the relationship between *Fusarium redolens* (OTU16) and agronomic attributes of wheat (D, E, F).

3.5.7 Impact of climate, soil water and fertility on wheat performance

Weather data was received from Environment Canada. The growing season was hotter and drier in 2011 than in 2010 (Fig. 3.10). In phase II, the climate at the time of sampling was particularly different in the years/site replicates. July 2012 was hotter and drier than July 2011 (Fig. 3.11).

In phase II, spring soil water content was influenced by crop rotation and year/site of cultivation. Interestingly, there was higher spring soil water content after wheat than after the pulses (Table 3.23). Spring soil water was higher in 2011 than in 2012 (Table 3.23).

Previous pulse crops significantly increased the level of available soil N in the spring of phase II, which was positively correlated with wheat yield (Table 3.24 and Fig. 3.12).

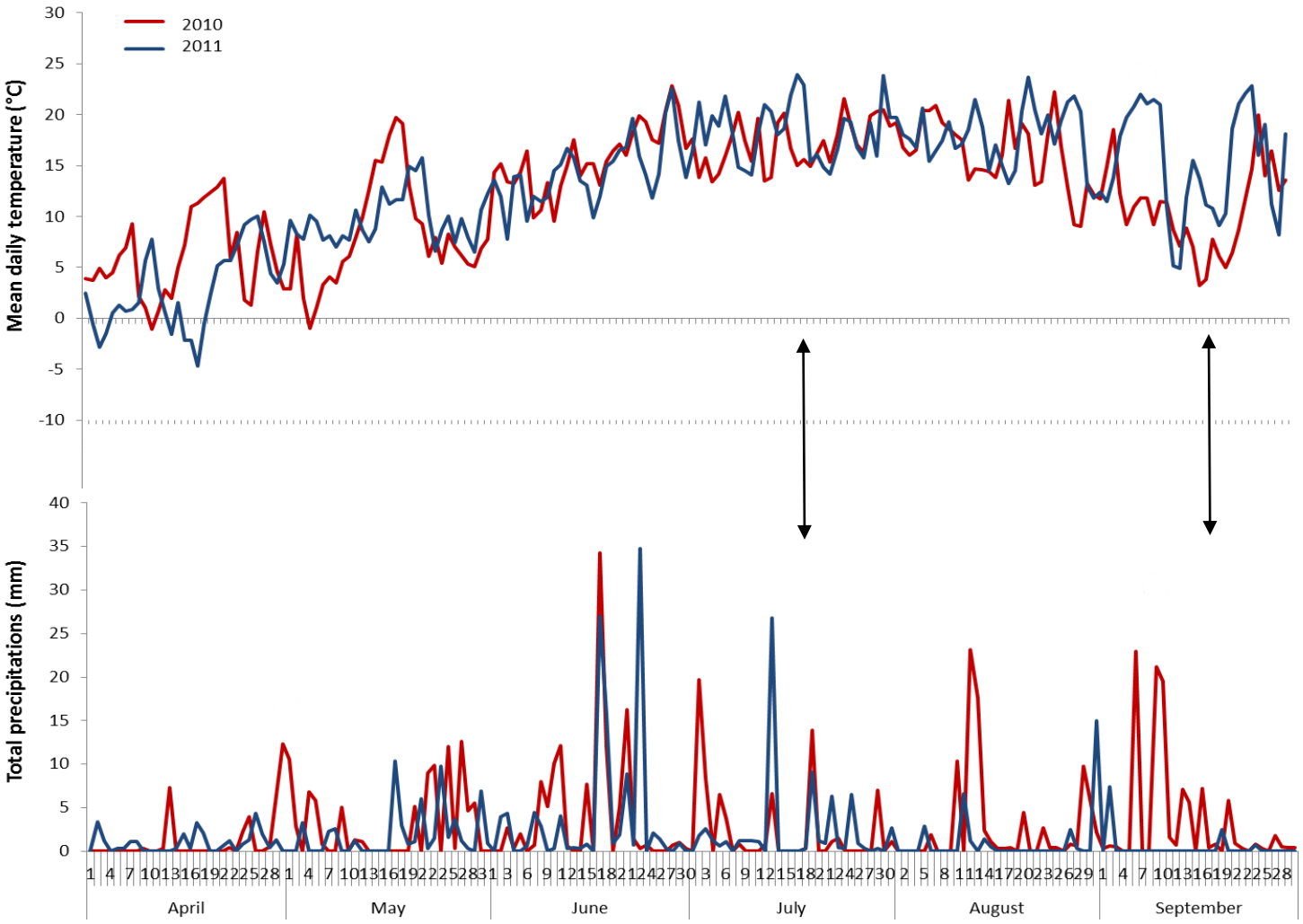

Figure 3.10. Daily average temperature and precipitation during the growing season 2010 and 2011. Data collected at the Swift Current CDA Station, Swift Current, Saskatchewan. Double arrows indicate sampling time at mid bloom and after harvest.

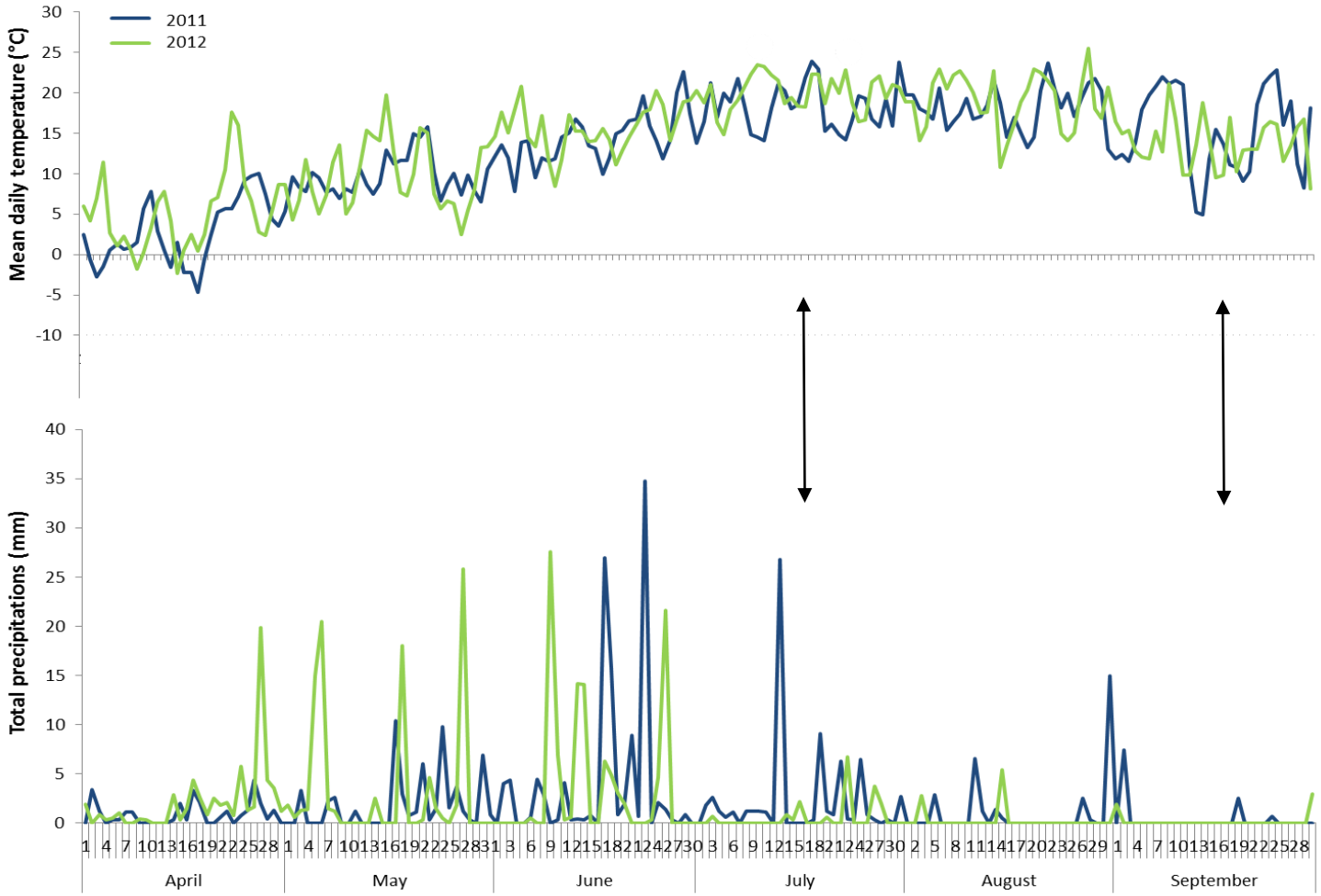

Figure 3.11. Daily average temperature and precipitation during the growing season 2011 and 2012. Data collected at the Swift Current CDA Station, Swift Current, Saskatchewan. Double arrows indicate sampling time at mid bloom and after harvest.

Table 3.23. Least square means and probabilities of treatment effect on spring soil total N, P and gravimetric soil water levels in phase II of the rotations. Significant probabilities are indicated in bold. Crop rotation and year/site means associated with different letters are different at $p < 0.05$ according to LSD test and Student's t test, respectively.

	Spring soil N	**Spring soil P**	**Spring soil water**
	Depth (cm)		**Depth (cm)**
Treatments	**0-30**	**0-30**	**0-60**
Crop rotation (*n = 8*)	– – – – – – – – – – *kg ha^{-1}* – – – – – – – – – – –		– – – – – *mm* – – – – –
Chickpea-Wheat	12.5 **a**	63.0 ns†	72.9 **c**
Lentil-Wheat	11.5 **a**	49.3 ns	73.9 **c**
Pea-Wheat	8.0 **a**	54.9 ns	77.3 **b**
Wheat-Wheat	4.7 **b**	58.4 ns	82.8 **a**
Year/site (*n = 16*)			
2011	9.2 ns	55.5 ns	86.1 **a**
2012	9.2 ns	57.3 ns	67.4 **b**
Source of variation	– Probability (*p*) –		
Previous crop	**0.0021****	0.6355	**0.0001****
Year/Site	0.9791	0.8587	**<.0001****
Previous crop*Year/Site	0.9340	0.2945	0.5406

† Not significant. * Significant effects at $p \leq 0.05$. ** Significant effects at $p \leq 0.01$.

Table 3.24. Spearman pairwise correlations (N = 32) between the agronomic performance of wheat and soil N, P and water content in spring. Wheat was grown in the South Farm of the Semiarid Prairie Agricultural Research Centre near Swift Current, SK in 2011 and 2012.

Variables	vs. Variables	Correlation (r)	Probability (p)
Spring soil P (0-15 cm)	Spring soil N (0-15 cm)	0.0099	0.9570
Spring soil water (0-60 cm)	Spring soil N (0-15 cm)	-0.2584	0.1534
Spring soil water (0-60 cm)	Spring soil P (0-15 cm)	-0.0258	0.8887
Yield	Spring soil N (0-15 cm)	0.3538	**0.0470***
Yield	Spring soil P (0-15 cm)	-0.0323	0.8607
Yield	Spring soil water (0-60 cm)	0.3144	0.0797

* Significant effects at $p \leq 0.05$,

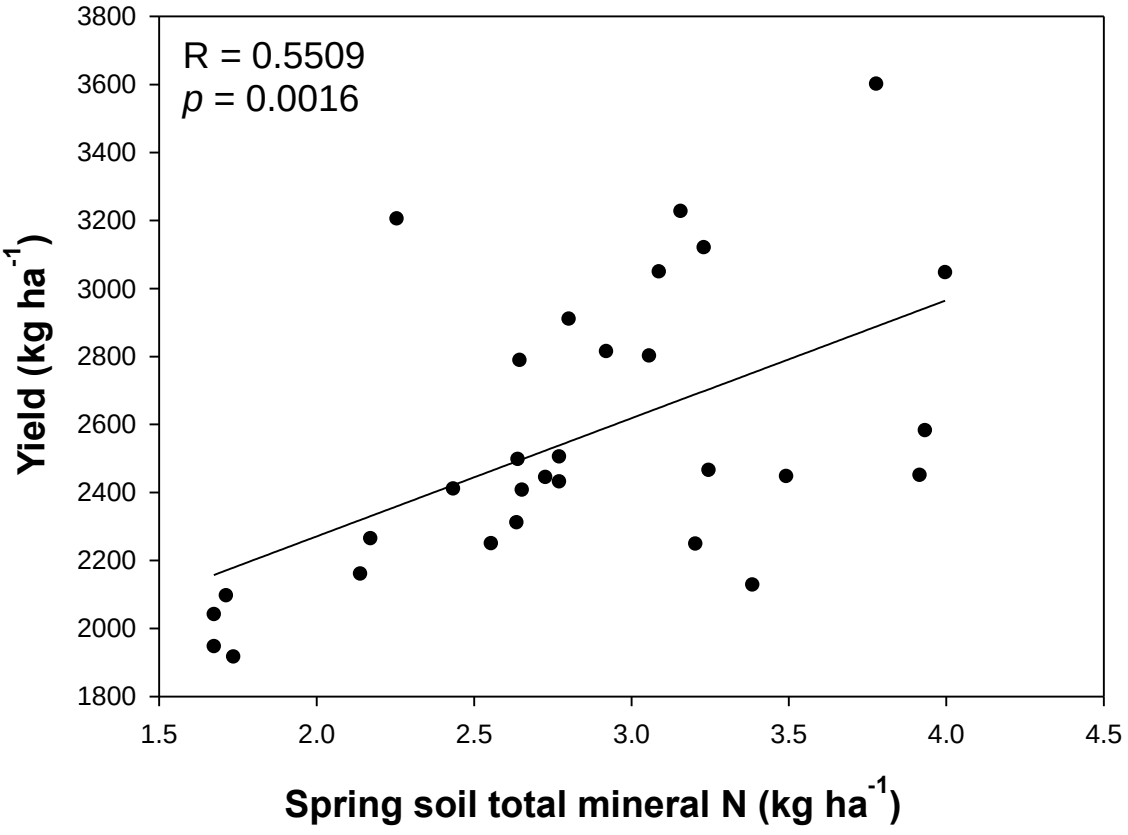

Figure 3.12. Linear regression between wheat yield and spring soil N content at 0-30 cm depth (N = 30). Soil N data was square root transformed and plotted as such, to meet normality test requirements.

3.6 Discussion

In this study, 1 the impact of chickpea, lentil, pea and wheat on specific soil microbial communities, in particular communities of AM and non-AM fungi was investigated, and 2) whether these previous crops influenced the composition of the fungal community of the rhizosphere and root endosphere of a subsequent wheat crop, as well as the performance of this crop.

Crop species had a significant effect on the composition of the fungal community inhabiting its roots. The non-AM fungal community associated with the roots of wheat was significantly different from that of pulses in phase I of the rotations, suggesting that host plants greatly influence the diversity and distribution of their associated root fungal communities, as previously reported (Bainard et al. 2012; Ellouze et al. 2008; Li et al. 2014; Mathimaran et al. 2007). Fungi may have a broad host range, however many of these interactions with plants are specie-specific and they may have different impacts on the performance of different hosts (Mandyam and Jumpponen 2005; Marschner et al. 2001; Morgan et al. 2005). Some potentially pathogenic fungal phylotypes, such as *Fusarium redolens* (OTU16) and *Cryptococcus* sp. (OTU6) were detected in all crops but their relative abundance depended on the host they associated with. Previous studies showed that plant root exudates are able to modify the soil environment leading to the selection of microorganisms that are tolerant or well adapted to the new conditions imposed by root exudation (Schenk et al. 2012). Plants use root exudates to regulate their interactions with the soil microbial community. Plants produce signal molecules triggering specific pathways, promoting and regulating symbioses with beneficial fungi, or defense barriers against pathogenic species (Schenk et al. 2012).

Wheat, as the host plant in phase II of the rotations, seemed to strongly select a specific fungal community as there was little evidence of the previous crops influencing the fungal community of wheat roots. There was a clear separation between the soil fungal community existing after the harvest of the pulses and the fungal community inhabiting the roots of the following wheat plants, but the composition of the fungal community establishing in the roots of a subsequent wheat crop was not significantly influenced by the previous crop. Previous studies by Fernandez et al. (2008) found that *Fusarium* spp. in previous crop residues can survive 10 to 11 months after harvest and can parasitize the cereal that follows. In contrast, my study showed that wheat grown after pulses, which were associated with a high relative abundance of *Fusarium* spp., was a poor host for these pathogenic root-associated phylotypes. In similar field studies, Nayyar et al. (2009) found that pea-wheat rotations were less affected by root rot than continuous pea suggesting that the depression in soil productivity under continuous pea was mostly attributed to root rot caused by *Fusarium* spp. Since wheat seems to be a poorer host than pulses for *Fusarium* spp., using wheat breaks alternating with pulses in rotation systems can increase the soil health and reduce the negative impact of *Fusarium* spp. Wheat seemingly has a strong influence on microbial root and rhizosphere inhabitants. The similarities between the levels of roots colonization, soil microbial biomass and microbial activity patterns among the different wheat-based rotations at phase II in my study, demonstrated that wheat controls the soil microbiome to a large extent. The specificity of the relationships between plants and microbial communities has been documented (Li et al. 2014; Mathimaran et al. 2007). However, my studies offer new information on the biodiversity associated with crops based on an extensive assessment of the fungal communities across different years and sites, which provides strength and repeatability

to the results.

The similarities between the composition of the fungal communities associated with the different wheat based rotations can be only partially attributed to the strong selective power of wheat as host plant over soil microorganisms. The homogenizing power of the winter on the soil microbial communities cannot be overlooked. Fluctuations in soil moisture, temperature, pH, and nutrient cycling have a great influence on soil microbes and these factors are largely responsible for the variations observed in fungal community structure overwinter (Dumbrell et al. 2011). Therefore, in order to totally comprehend the source of the variations in the fungal communities from the previous fall to the next growing season, in-depth analyses of the spring and mid-season soil fungal communities would be required.

My research showed that AM fungi were less affected by crops species than non-AM fungi in phase I of the rotation. This was unexpected since previous research showed that AM fungal communities are usually greatly influenced by plant species (Germida et al. 1998) and a higher number of plants co-occurring in the same area (plant diversity) may increase the AM fungal diversity (Torrecillas et al. 2012). On the other hand, studies conducted by Su et al. (2011) found that AM fungal composition was similar among five different plants species and that most AM fungi had little host specificity, except *Acaulospora* sp., *Glomus constrictum, G. diaphanum* and *Glomus* sp. which showed a certain degree of host preference. It is more likely that season and host plant species co-affect AM fungal community structure, but their effects may be more or less noticeable depending on the sampling dates, plant phenology and soil environment fluctuations.

In the phase I of the rotation, the AM fungi community structure was only affected by

year/site. Assemblages of AM fungi have shown to be highly susceptible to changes in temperature, moisture and daylight duration, which can drive important temporal changes in their community composition and structure (Dumbrell et al. 2011; Hernandez and Allen 2013). The variability of soil moisture and temperature may alter the diurnal patterns of mycelia growth and spore production of AM fungi, thereby modifying soil C sequestration, nutrient cycling and host plant success (Hernandez and Allen 2013). Net growth of AM fungal hyphae may be impacted by a variety of environmental events such as heat waves, drought and also longer term weather fluctuation produced by global climate change (Hernandez and Allen 2013)

The difference between the AM fungal community of wheat after pea and wheat after other crop plants in phase II (Fig. 3.2), suggests that a pea crop influences the soil system quite differently from chickpea, lentil or wheat. This effect was not consistent in the 2012 replicate; probably due to environmental variations preventing or masking the effects of the previous crops. One important feature of pea is that it has an extremely short growing season. This has an impact on soil water level and aids crop residue recycling. Seasonal and temporal fluctuations and the micro-variations in the soil environment among experimental sites and years may be driving shifts in some of the fungal communities in phase II of the rotations. The climate and available soil water at the time of sampling was particularly different between years/sites. Previous research has shown that pea exudates have a much stronger effect on the rhizosphere than wheat exudates, resulting in the establishment of dramatically different rhizosphere communities in these two plants (Turner et al. 2013). Further research is required to define how pea influences the composition of wheat microbial community.

The year 2011 was warmer and drier than 2010. Total fungal biomass and the ratio fungi: bacteria were higher in 2011 than 2010. This could be indicating that fungal communities are better adapted to the semiarid conditions of the Prairie than bacteria. However, other parameters such as fertilizer application rates, soil moisture and pH should also be considered as possible sources of microbial community variations. In contrast, other studies found that warming significantly decreased the fungi: bacteria ratio and induces a community shift towards bacteria (Zhang et al. 2014). Lekberg et al. (2013) found that fungal communities are particularly stable, and reported that fungal structures persist in roots during times of low C-allocation with little or no seasonal variation. In my studies, climate had a significant influence on the functionality of microbial communities. Higher microbial biomasses and lower activities in 2011, compared with 2010, were detected, suggesting that hot and dry conditions do not decrease soil microbial biomass, but do affect the metabolic activity of soil microorganisms.

Pulses were better previous crops than wheat, at improving wheat development and productivity in phase II of the rotations. Studies by Gan et al. (2003) reported that soil water content at spring seeding time was 10 % greater in dry pea and lentil stubbles than in wheat stubble. In contrast, my study showed higher spring soil water contents in wheat monoculture than after a pulse. The high N: C ratio of wheat residues makes decomposition slower than pulses' and the standing wheat stubble could possibly trap more snow during the winter months, which could explain the higher water content after wheat in the following spring. The significant increase in wheat yield in phase II of the rotation seemed to be driven by the N left in the soil by the previous pulse crops. Previous studies (Campbell et al. 1992; Gan et al. 2003) found that residual NO_3–N, located 60 cm below soil surface after a pulse crop, was

the main factor contributing to the increased yield and grain protein content of the subsequent wheat crop. This effect is mainly attributed to the low C:N ratio of pulse residues which enhances organic matter decomposition rates and soil N availability (Cox et al. 2010; Walley 2007). In Manitoba, field pea has the largest contribution to soil N (Przednowek et al. 2004). In low organic matter soils, chickpea as the previous crop largely promoted wheat yield, enhanced the soil organic matter level and left a positive N balances in the soil (Aslam et al. 2003). Lentil has been successfully used as a previous crop for wheat in the long term rotation experiment in Swift Current (Campbell et al. 2011; Campbell et al. 1992; Gan et al. 2003; van Kessel and Hartley 2000). In general, field studies found a positive N contribution to the soil by lentil and pea, while the benefits of chickpea are inconsistent (Przednowek et al. 2004; Walley 2007). However, my studies showed that pea, chickpea and lentil had a similar positive N contribution to soil N levels.

This research showed that all pulses were better previous crops than wheat monoculture at improving wheat growth and productivity, mostly due to their positive contribution to soil N, and highlighted the importance of the host plant in the selection for specific fungal communities. Fungal community was strongly affected by the host plant but also by year/site. Further research on parameters contributing to year/site variations such as fertilizer rates, soil moisture, pH, micronutrients, etc, would aid to a better understanding of fungal diversity shifts in the agroecosystem.

4.0 Influence of Four Year Crop Rotation Sequences on the Structure and Function of the Rhizosphere and Root Endosphere Fungal Community of Wheat

4.1 Preface

This field study tested the effect of eight 4-yr crop rotations on soil and root microbial dynamics, community structure and wheat performance. A greenhouse experiment was also conducted to define the influence of the soil microbiota selected under the first three phases of these rotation systems on wheat growth.

4.2 Abstract

Crop rotation may impact soil fungal communities and increase wheat productivity in wheat-based agroecosystems. In this study, (1) I determined the relative efficiency of eight legume-cereal rotation systems involving pea (P), lentil (L), chickpea (C) and wheat (W), based on the performance of wheat in the last phase of eight 4-yr crop rotations, (2) assessed the influence of the eight 4-yr crop rotation systems on plant-associated fungi and (3) the relationships among themselves and with wheat performance.

In the field, rotation systems diversified with the inclusion of legumes, such as L-W-L-W and P-W-P-W, promoted wheat yield, whereas wheat monoculture had the lowest yield and plant density. Contrary to what was predicted, the highly diversified rotation L-W-C-W resulted in one of the lowest yield and seed C content, suggesting that the selection of the crops to be included in diversified rotation is important. Lentil and pea alternating with wheat largely contributed to wheat performance, whereas chickpea contributed little benefit to the rotation. Diversified rotations had higher levels of mycorrhizal root colonization than continuous wheat in the third year of the rotations (phase III). Microbial community analyses

revealed that the changes in the root-associated AM community were mainly driven by year/site of cultivation, whereas the non-AM fungal community was influenced by both, crop rotation sequence and year/site. The roots of wheat in the last year of all rotations (phase IV) hosted similar fungal communities, suggesting that wheat as host plant has a strong effect on the fungal community that overrides the effect of rotation.

The greenhouse study aim to answer the question: how a specific microbial community selected by different crop rotations can affect wheat growth and productivity, in a controlled environment? In contrast with the field results, the greenhouse study revealed that microbial communities selected by rotations that had chickpea in phase III best promoted wheat productivity. Therefore, under favorable conditions such as the greenhouse environment, the community selected by chickpea in the field had a positive influence on wheat performance. The greenhouse experiment also revealed that diversified crop rotations promoted beneficial microbial communities as compared to wheat monoculture. In the field, spatial variability, land management and climate influence specific groups of microorganisms, potentially masking the direct effects of the soil microbiota on crop performance.

The broad range of relationships I found between the attributes of fungal communities and wheat performance indicators revealed how complex and dynamic fungi-host interactions can be in agroecosystems. Correlation analyses are not conclusive on finding the cause of a particular observation, but they can suggest possible avenues of evidence for true interactions or functions that would need to be confirmed using a different experimental and statistical approach. In this study, soil bacterial and fungal biomass were positively correlated with wheat yield and number of seeds per plant, underlining the key role of soil microorganisms in

plant performance. The negative relationships between the levels of colonization of wheat roots by AM versus non-AM fungi; and more specifically, the negative relationship between the abundance of *Fusarium redolens* (OTU16) and the levels of AM fungal colonization in the roots of wheat, could suggest a possible antagonistic interactions between AM fungi and wheat pathogens. On the other hand, the co-occurrence of *Claroideoglomus* and *Glomus* phylotypes, respectively, could suggest possible synergistic effect between fungi belonging to the same genus. However the mechanisms involved in these interactions are not clear and further research would be required to confirm them and describe them in detail. Additionally, conventional soil fertilization could cause shifts in the fungal communities, limiting AM fungal proliferation and possibly favoring the growth and colonization of other fungal species, perhaps pathogenic species.

4.3 Introduction

Maintaining and improving soil health is essential for agricultural productivity and sustainability. Increasing fertilizer inputs to meet crop demands and increase yields often masks soil quality loss. A wise management of the agricultural land may aim to establish desirable microbial populations, such as xenobiotic degraders, N_2-fixers, AM fungi and pathogen antagonists, which ultimately would lead to the sustainability of the agroecosystem (Insam 2001).

Microbial diversity influences soil ecosystem functioning and it is known to provide resilience to disturbance (Garbeva et al. 2004). Microbes play an essential role in soil aggregate stability, soil formation and nutrient cycling (Huang et al. 2005). They mediate biochemical processes through different interactions with plants and amongst themselves. Root-microbe interactions could broadly be classified as positive or negative depending on the net balance of the interaction and the type of exudates that plants release (Bais et al. 2006; Uren 2007). Positive interactions include those with plant growth promoting bacteria (PGPB), AM fungi and beneficial fungal endophytes leading to the establishment of mutualistic relationships. These plant associated microorganisms favor the growth of plants and trigger plant induced systemic resistance (ISR) increasing plant health. Plant roots also release protective chemical signals against pathogens/herbivores. These include antimicrobial, phytotoxin, nematicidal and insecticidal compounds (Bais et al. 2006). Understanding the complexity of plant-microbe interactions can support the development of strategies to increase the sustainability of agricultural systems.

Crop plant species and genotypes, and soil management through rotation are major factors affecting soil biology. The identity and frequency of the crops in rotation systems affect the water use efficiency of cropping systems, the availability of soil water and nutrients, and the incidence of diseases (Gan et al. 2003). The changes in soil properties driven by crop rotation can also create shifts in the soil microbial communities (Egamberdieva 2008). Thus, studying soil microbial diversity in relation with soil processes is of great importance to find ways to improve the productivity and sustainability of cropping systems (Acosta-Martínez et al. 2007). Cutforth et al. (2013) found that farmers can increase the overall yield and water use efficiency of crop rotations on the Canadian semiarid prairie by growing deeper-rooted crops such as wheat and canola for one or two years following a shallow-rooted pulse crop. Little is known about the influence of pulse frequency and sequence in wheat-based rotation systems of the Canadian Prairie and how it affects soil microbial attributes and overall soil health.

The length of a rotation and its level of plant diversity and sequence are key factors for the success of a cropping system (Ellouze et al. 2014). Short rotations are more susceptible to diseases and produce lower yield than longer rotations (Bennett et al. 2012). Studies with more than 2-yr in the rotation and diverse combinations of crops may help in understand the dynamics and functionality of the microbial communities associated with wheat in diversified rotation systems. Environmental factors also influence the yield and quality of field crops. Soil water and biogeochemical properties, nutrient availability and climate fluctuations during the growing season also influence the soil, cause shifts in microbial community and could mask or increase the effects of specific crop rotations.

The overarching goal of this study is to improve the productivity and stability of crop

yields in pulses and cereal based cropping systems with the establishment of better beneficial microbial associations through management. Here, I hypothesized that 1) the structure of the fungal communities inhabiting wheat root in the last year of the rotations are affected by the different 4-year crop rotation sequences, 2) that different rotation systems will have different impact on wheat performance (more diversified rotations versus monoculture) and that 3) microbial community selected by different rotation systems will influence the productivity and quality of the wheat crops produced.

4.4 Materials and Methods

4.4.1 Experimental design

A field experiment was conducted to evaluate the effects of crop rotations on soil microbial diversity and wheat growth, nutrition and productivity. Eight crop rotations (Table 4.1) were tested in 32 plots arranged in a completely randomized block design with four replicates at each site. Plots were 4-m wide and 12-m long, with borders of 2 x 12 m. The field experiment was conducted from 2010 to 2013, and repeated from 2011 to 2014 at another location (year/site), at the South Farm of the Semiarid Prairie Agricultural Research Centre (SPARC) in Swift Current, SK, Canada (latitude: 50° 17'N; longitude: 107° 41'W, elevation 825 m). Data were collected in the last two phases of the rotations, up to 2013; thus only phase III (Table 4.1) was replicated in time and location. The plants used were AC Lillian hard red spring wheat (*Triticum aestivum* L.), CDC Frontier kabuli chickpea (*Cicer arietinum* L.), CDC Meadow yellow pea (*Pisum sativum* L.) and CDC Maxim CL red lentil (*Lens culinaris* L.). All seeds were purchased from "Certified seed growers".

The soil is a very gently sloping Orthic Brown Chernozem of the Swinton soil association

that has a silt loam texture. The top 0 to 15 cm layer of the soil had a pH of 6.5 and contained 9 kg ha^{-1} mineral N (Maynard and Kalra 1993), 36 kg ha^{-1} Olsen P (Olsen et al. 1954), 326 kg ha^{-1} available K (Hamm et al. 1970), and 53 kg ha^{-1} available S (Hamm et al. 1973). Plots were treated with different fertilizers, herbicides and fungicides pre and post emergence (Table 4.2) in order to suit crop needs and aim to control weeds and pathogens. In year/site 2012, the phase III crops wheat, chickpea, pea and lentil were seeded at a rate of 83, 214, 186, and 56 kg ha^{-1} respectively, and in year/site 2013 of phase III, these crops were seeded at 91, 188, 206 and 56 kg ha^{-1} respectively. In the phase IV (2013), wheat was seeded at a rate of 100 kg ha^{-1}. The management of plots in the phases I and II of the rotations was described in detail in Chapter 3.4.

Table 4.1. Crop rotations applied in the field experiment conducted in 2010-2013 (phase I-IV) and replicated in 2011-2014 at two different locations in the South Farm of the Semiarid Prairie Agricultural Research Centre, in order to test the effect of crop rotation on soil microbiology and wheat development, nutrition and productivity.

Pre-test (2009/2010)	**Phase I (2010/2011)**	**Phase II (2011/2012)**	**Phase III (2012/2013)**	**Phase IV[†] (2013)**
Wheat	Chickpea	Wheat	Chickpea	Wheat
Wheat	Chickpea	Wheat	Wheat	Wheat
Wheat	Lentil	Wheat	Chickpea	Wheat
Wheat	Lentil	Wheat	Lentil	Wheat
Wheat	Pea	Wheat	Lentil	Wheat
Wheat	Pea	Wheat	Pea	Wheat
Wheat	Pea	Wheat	Wheat	Wheat
Wheat	Wheat	Wheat	Wheat	Wheat

[†] Note: Sampling was conducted until 2013. Hence only one year/site replicate of phase IV was analyzed.

Table 4.2. Agrochemicals used in the field experiments of phase III in 2012, replicated in 2013, and phase IV in 2013, at two different locations on the South Farm of the Semiarid Prairie Agricultural Research Centre. One time application is represented by "x".

		Phase III								Phase IV
		2012				2013				2013
Inocula/Fertilizers/Pesticides	**Concentration**	**CHP**	**LEN**	**PEA**	**WHT**	**CHP**	**LEN**	**PEA**	**WHT**	**WHT**
Apron Maxx[†]	325 mL 100 kg^{-1} seeds	x	x	x		x	x	x		
Vitaflo 280[‡]	330 mL 100 kg^{-1} seeds				x				x	x
MAP[§]	17 kg P ha^{-1}	x	x	x	x	x	x	x	x	x
Urea	67 kg N ha^{-1}				x				x	x
Nitragin Soil Implant+ GC[£]	5.6 kg N ha^{-1}	x	x	x		x	x	x		
Bonanza 10G[δ]	9.0 kg ha^{-1}	x	x	x		x	x	x		
Roundup Weathermax[€]	0.88 kg ha^{-1}	x	xx	xx	xx	xx	xx	xx	xx	xx
INTEGO Solo[∂]	0.02 kg ha^{-1}		x	x			x	x		
Horizon W. O.[¶]	0.055 kg ha^{-1}				x				x	x
Buctril M[&]	0.277 kg ha^{-1}				x				x	x
Assure II[¢]	0.0356 kg ha^{-1}					x				
Achieve™ SC[Þ]	0.198 kg ha^{-1}				x					
Brotex 240[ł]	0.267 kg ha^{-1}	x	x	x	x	xx	xx	xx	xx	x
Headline Duo One[Φ]	0.293 kg ha^{-1}	xxx	xx	xx		xx				
Bravo 500[ɤ]	1.98 kg ha^{-1}	xx				xxx	xx	xx		
Proline 480[ϕ]	0.19 kg ha^{-1}					x				
Matador 120EC[Ψ]	0.101 kg ha^{-1}			x				x		
Reglone[π]	0.415 kg ha^{-1}	x	x	x		x	x	x	x	

[†]Apron Maxx: 1.1 % Mefenoxam + 0.73 % Fludioxonil, [‡]Vitaflo 280: 15.59 % Carboxin + 13.25 % Thiram, [§]MAP: Monoammonium phosphate ($NH_4H_2PO_4$), [£] Nitragin Soil Implant + GC: Peat-based granular inoculant which contains a minimum of 100 million (1×10^8) viable cells of *Mesorhizobium ciceri* per gram of product, [δ]Bonanza 10G: 10% trifluralin granular, [¥]Pursuit 240: 240 g L^{-1} imazethapyr, [€]Roundup Weathermax: 48 % Glyphosate, N-(phosphonomethyl) glycine, [∂]INTEGO Solo:15-40 %Ethaboxam, [¶]Horizon W. O.: 22.3 % Clodinafop-propargyl, [&]Buctril M: 280 g L^{-1} Bromoxynil (present as mixed octanoate and heptanoate esters), [¢]Assure II: 10.3 % Quizalofop p-ethyl, [Þ]Achieve™ SC: 40 g L^{-1} Tralkoxydim, [ł]Brotex 240: 57.57-61.13 % Bromoxynil Octanoate, [Σ]2,4-D LV Ester 600: 600 g L^{-1} 2,4-D iso-octyl ester, [Φ]Headline Duo One: 25.2 % Boscalid + 12.8 % Pyraclostrobin, [ɤ]Bravo 500: 40.3 % Chlorothalonil, [ϕ]Proline 480: 480 g L^{-1}Prothioconazole, [Ψ]Matador: 13.2 % Lambda-Cyhalothrin, [π]Reglone: 20 % Diquat dibromide.

4.4.2 Fungal community attributes

The level of root colonization by AM and non-AM fungi was determined using the staining methodology described by Vierheilig et al. (1998) and the gridline intersect method (Giovannetti and Mosse 1980) (See Chapter 3, section 3.4.6). Rhizosphere soil fungal community structure were assessed by 454 pyrosequencing (Buee et al. 2009) in the fall of phase III (Sf_P-III) and in the roots of wheat at anthesis in phase IV (Ra_P-IV) of the rotations. Refer to Chapter 3, section 3.4.2 for details on roots and rhizosphere soil sample collection, processing and preservation.

The small subunit rRNA gene was targeted with primers AML1 / AML2 (Table 4.3). These primers are known to amplify a 800 bp region in all subgroups of AM fungi (Lee et al. 2008). A nested PCR was used. Firstly, primers NS1/NS4 (Table 4.3) were used to amplify total eukaryotic DNA. PCR was carried out in a Veriti 96-well fast thermal cycler (Applied Biosystems) using the following cycle regime: denaturation at 94ºC for 3 min, 25 cycles of 45 s for denaturation at 94ºC, 45 s for primer annealing at 51ºC, and 1 min of extension at 72ºC, followed by a final extension period of 10 min at 72ºC. The PCR products were diluted 1:10 in microbial DNA-free water and used for amplification with the primers AML1/AML2 (Table 4.3). PCR conditions were: a first denaturation step of 3 min at 94ºC, 30 cycles of denaturation-annealing-extension of 30 s at 94ºC, 40 s at 58ºC and 55 s at 72ºC respectively, followed by a final extension of 10 min at 72ºC.

The internal transcribed space (ITS) sequences representing general fungal groups were amplified using the primer set ITS1F / ITS2 (Table 4.3). Refer to Chapter 3, Section 3.4.3 for PCR conditions. The PCR products were purified using the AMPure PCR Purification Kit

(Agencourt Bioscience, Beverly, MA), and analyzed with an Agilent Bioanalyzer (Agilent Technologies, Edmonton, AB) to evaluate concentration and purity. AM and non-AM fungal read data were made and analyzed according to the procedures explained in Chapter 3, section 3.4.3.5.

Soil dehydrogenase activity (Casida et al. 1964) was measured every year, at mid-bloom and after harvest. Microbial biomass was assessed by quantifying phospholipid fatty acid indicators of different microbial groups (Hamel et al. 2006). The procedures to conduct these measurements were explained in detail in Chapter 3, sections 3.4.4 and 3.4.5.

Table 4.3. Primer sets, adaptors and key sequences used to amplify the ITS and 18s rDNA gene regions of target fungal communities in phases III and IV of the rotation.

Primer set	Sequence	Target group
ITS-1F / ITS2	CTTGGTCATTTAGAGGAAGTAA / GCTGCGTTCTTCATCGATGC	General fungi
NS1 / NS4	GTAGTCATATGCTTGTCTC / CTTCCGTCAATTCCTTTAAG	Eukaryotes
AML1 / AML2	ATCAACTTTCGATGGTAGGATAGA / GAACCCAAACACTTTGGTTTCC	AM fungi
Adaptor/Key (Forward)	CCATCTCATCCCTGCGTGTCTCCGAC/TCAG	
Adaptor/Key (Reverse)	CCTATCCCCTGTGTGCCTTGGCAGTC/TCAG	

4.4.3 Nutrient and water content in plants and soil

Soil water, total N, P and K contents at depths 0-15, 15-30, 30-60, 60-90, and 90-120 cm were measured at seeding and after harvest. Plant material was collected at the end of the growing season, oven dried at 40ºC, finely ground and analyzed for total N (Noel and Hambleton 1976) and P (Milbury et al. 1970) concentrations using a segmented flow auto-analyzer (Technicon, AAII System, Tarrytown, NY) and for total K concentration (Anonymous 1987)using atomic absorption spectrometry at the Analytical Chemistry Laboratory of the Semiarid Prairie Agricultural Research Centre in Swift Current, SK.

4.4.4 Agronomic attributes of the crops and their relationship with the microbial community

To understand the influence of the microbial community on the performance of the different rotations, several agronomic measurements were made (Table 4.4) and correlated with microbial attributes. Plant density was assessed in each plot as the number of plants per square meter. Once crops reached maturity, shoots were cut at ground level and oven dried at 40ºC until constant weight, which was recorded as biomass. Productivity was estimated as harvest index, which is the ratio of seed mature biomass to total plant biomass, total yield, number of heads per square meter, seeds per square meter, and thousand kernels weight. The samples for harvest index determination were always harvested by hands in order to collect both seed and straw, whereas the plot yield was obtained by harvesting the central six rows of the plot using a plot combine. Nutrient concentrations in plant tissues were determined as described in Section 4.4.3.

Table 4.4. Agronomic measurements taken every year in the cropping systems at the South Farm, in Swift Current, SK.

For pulses crops	For re-cropped cereal
Plant density	Plant emergence and density
Above-ground biomass at maturity	Above-ground biomass at maturity
Seed yield	Grain yield
Seed weight	Seed weight /Number of heads/Number of seeds per m^2
Seed N, P, C	Seed N, P, C
Straw N, P, C	Straw N, P, C

4.4.6 Influence of the field soil microbiota selected under eight three year crop sequences on wheat grown under greenhouse conditions

The influence of the soil microbiota on plant growth as selected under the first three phases of the rotation systems was experimentally tested in the field, and evaluated in an inoculation assay, in the greenhouse at the Semiarid Prairie Agricultural Research Centre (SPARC), Agriculture and Agri-Food Canada (AAFC), Swift Current, SK. Bulk soil was collected at the South Farm (See soil description in section 4.4.1), sieved through a 1 cm pore space sieve to mix soil particles homogenously, remove big stones and large organic matter and then pasteurized with the Pro-Grow Electric Sterilizer at 90^{o}C for 2 hours, and used as substrate in 1-L cardboard pots. Nylon nets were autoclaved and placed at the bottom of the pots before adding the soil. Pots were watered abundantly and left to drain overnight. Rhizosphere soil, which contained the microbial community selected by the crop rotation as of the fall of phase III, was used to inoculate wheat in the greenhouse. Each pot was

inoculated with 50 g of rhizosphere soil from the field plots. There were as many pots as there were field plots. Controls did not receive any inocula. Wheat seeds were surface sterilized by immersion and shaking in a 2 % sodium hypochlorite (NaOCl) solution for 30 s, rinsing in sterile distilled water for 1 min, immersion in 80 % ethanol for 30 s, and rinsing in sterile distilled water (Sauer and Burroughs 1986). Four seeds were placed directly on top of the inoculant, covered with a 2-3 cm layer of pasteurized soil, and carefully watered avoiding splashing that could cause contamination between pots. Pots were arranged in four blocks on the greenhouse bench and rotated within blocks once a week. Two weeks after germination, plants were thinned to two plants per pot. TUNE-UP (20-20-20) water soluble fertilizer was applied to the plants in week 5 according to the recommendations of the manufacturer, at a rate of 1g L^{-1}. This provided 200 µg per g soil of each of N, P and K.

Plants were harvested at the early milk stage (73 stage on the Zadoks two-digit code system) (Zadoks et al. 1974). The above-ground biomass, root biomass, number of heads per plant, shoot nutrient content and level of endophytic fungal root colonization were quantified (Giovannetti and Mosse 1980).

4.4.7 Statistical analysis

ANOVA was conducted to test the significance of crop phase III rotation, year/site and the interaction of these factors on overall microbial communities and crop attributes using JMP11 software (SAS Institute Inc., Cary, NC, USA). The data were checked for normality and homoscedasticity and they were square root or log-transformed if required to meet the assumptions of ANOVA. Multiple mean comparisons were made using the Least Significant Difference (LSD) test and pair-mean comparisons were made using the Student's t test. Non-

parametric data were analyzed using the Kruskal-Wallis test. DNA read data were resampled to the median value of the number of reads and Shannon diversity and Chao richness indices were calculated using R-statistical software v.3.1.2 (R Core Team 2014).

Two-way factorial permutation-based analysis of variance (Per-MANOVA) using the Bray–Curtis measure of ecological distance (Bray and Curtis 1957) was performed on DNA reads data in order to test the effects of previous crop, year and their interaction on the structure of the fungal communities of rhizosphere soil and root endosphere. The relative abundance of the AM fungal OTUs was estimated as the proportion, in percentage, of the total Glomeromycota reads. The relative abundance of the non-AM fungal OTUs was calculated as the proportion, in percentage, of the total fungal reads.

To compare the crop rotation systems based on AM community profile, AM fungal OTUs were pooled by genera and pairwise comparison made with multiresponse permutation procedures (MRPP) using Sørensen distance in PC-ORD v.6 (McCune 2002). The same analysis was used to compare crop rotation systems based on the structure of their non-AM fungal communities. The sequences that did not match representative sequences at 95% similarity were grouped under the name of "unidentified taxa-OTU313". Rare OTUs were removed from the analyses since in large enough sample size the rare sequences will potentially inflate estimates of diversity (Huse et al. 2010) and they contains little information but may influence the outcome of the analysis (Legendre and Legendre 1998). The OTUs that had more than 2% occurrence (Table 4.6) were used as a representative sample to characterize the non-AM fungal community in phases III and IV of the rotations. The comparison between the previous fall soil fungal community structure, selected by eight

3-year rotations, and the root-associated fungal community of wheat was tested with multiresponse permutation procedures (MRPP) using Sørensen distance in PC-ORD v.6 (McCune 2002). Non-metric multidimensional scaling (NMS) (Legendre and Legendre 1998) was performed in PC-ORD v.6 to visualize the effect of treatments on the fungal community structure.

Redundancy analysis (RDA) was performed in PC-ORD v.6 to test the relationship between the rhizosphere fungal community and the agronomic attributes of phase IV wheat. Spearman correlation test and regression analyses were performed in JMP10 software (SAS Institute Inc., Cary, NC, USA) to detect relationships between soil microbial biomass, metabolic activity, fungal community composition, agronomic attributes, crop performance and environmental conditions.

4.5 Results

4.5.1 Molecular analysis of the AM fungal communities associated with different crop rotation systems

The molecular analysis of the AM fungal community associated with wheat roots at anthesis in phase IV of the rotations revealed the presence of 234 OTUs corresponding to the genera *Rhizophagus*, *Glomus*, *Funneliformis*, *Paraglomus*, *Claroideoglomus* and *Diversispora*. *Rhizophagus* represented almost half of the Glomeromycota reads abundance present in wheat roots (Fig. 4.1). *Diversispora* was considered rare, as it represented approximately 1 % of the Glomeromycota relative abundance (Fig. 4.1). Crop rotation sequence did not have a significant effect on the relative abundance of Glomeromycota reads in the fall soil in phase III or in the roots of the following wheat (Table 4.5). The variation in

the AM community of the soil in the fall of phase III was only related to the different environmental conditions of year/site (Table 4.5).

Wheat as host plant seemed to have a large selective effect on its associative microbial community. In general the AM fungal community associated with the roots of wheat in phase IV was similar to that found in the previous fall soil. Only the wheat root community of C-W-W-W, L-W-C-W and L-W-L-W differed from the AM communities found in the soil the previous fall (Fig. 4.2).

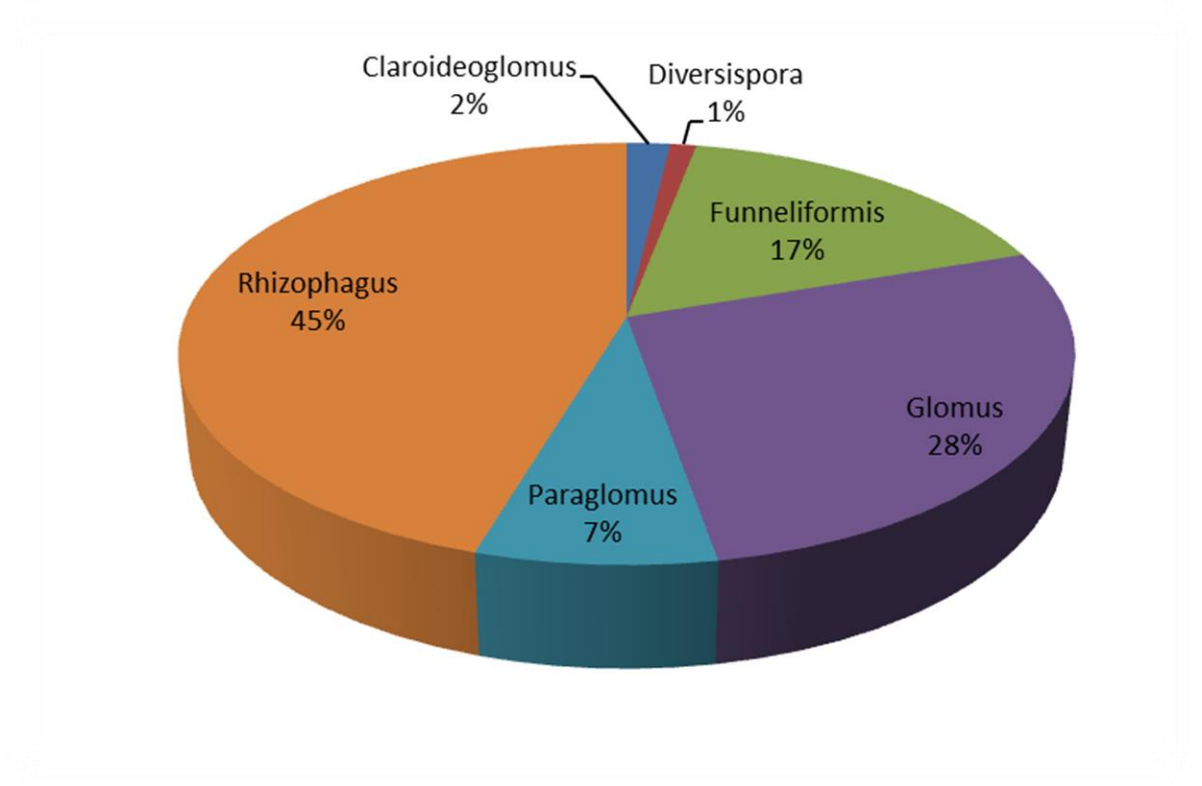

Figure 4.1. Relative abundance of Glomeromycota reads by genera, as detected by pyrosequencing, in the roots of wheat at anthesis in phase IV of the rotations.

Table 4.5. Probability of effects of crop rotation and year/site on the AM fungal OTU community in rhizosphere soil collected in the fall of phase III and wheat roots at anthesis in phase IV, according to PerMANOVA. Significant differences are indicated in bold.

	Phase III – Pulses & Wheat	**Phase IV - Wheat**
Source of variation	Probability (*p*)	
Crop rotation	0.556	0.894
Year/Site	**0.001****	NA[†]
Crop rotation*Year/Site	0.989	NA

[†] Not applicable
** Significant effect at $p \leq 0.01$.

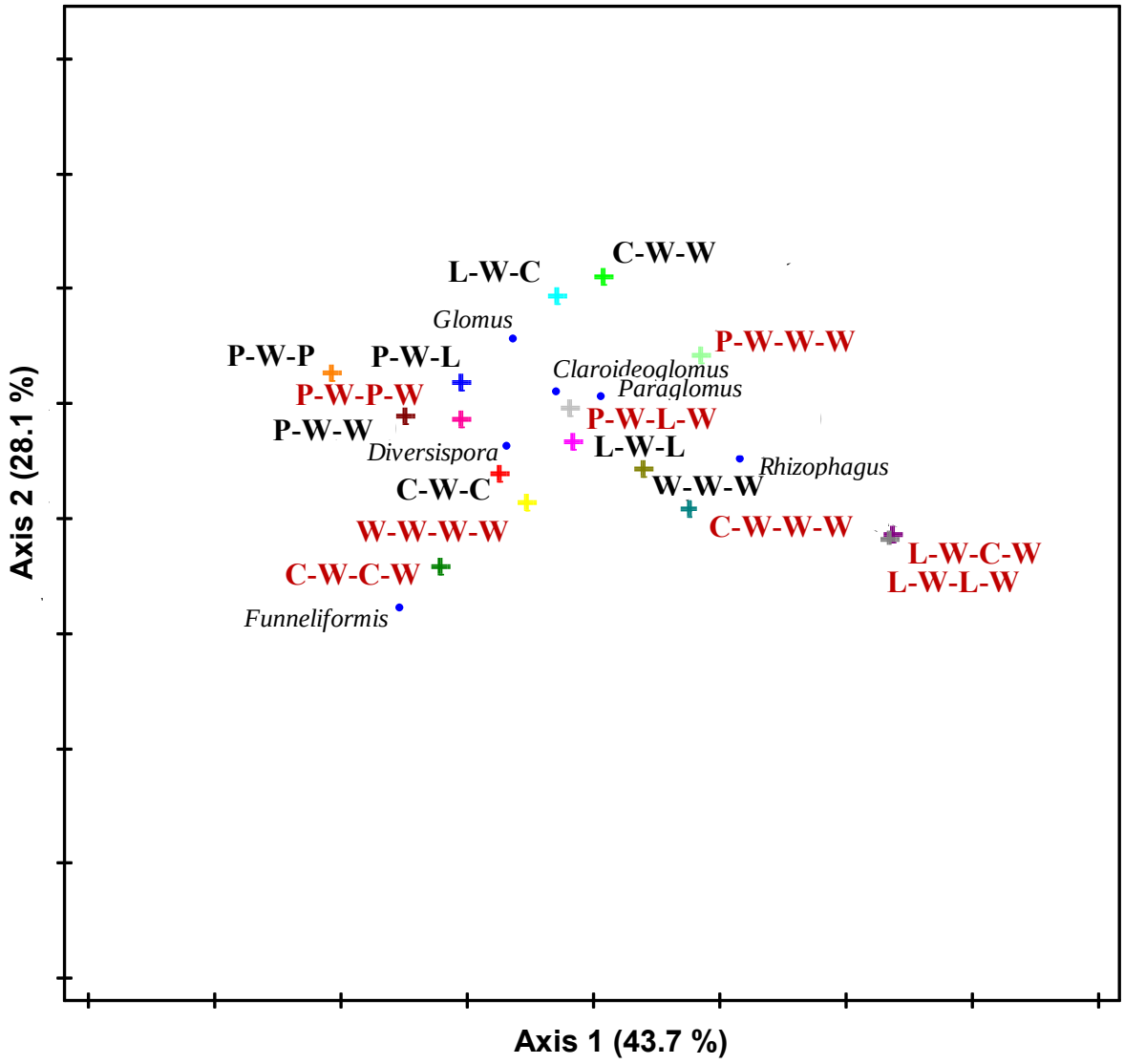

Figure 4.2. Nonmetric multidimensional scaling ordination of the crop rotations in phase III, represented in bold black letters, and phase IV, in bold red letters, based on the relative abundance of AM reads found in rhizosphere soil collected in the fall of phase III (Sf_P-III) and in roots of wheat at anthesis in phase IV (Ra_P-IV). Means of each treatment are represented (n = 4). "C" stand for chickpea, "L" for lentil, "P" for pea and "W" for wheat. Sørensen distance measure was used in the ordination. A bi-dimensional solution was recommended. Final stress = 17.5. The percentages between parentheses represent the contribution of each axis to the ordination solution. For information on the p values, refer to appendix 7.4.

4.5.2 Molecular analysis of the non-AM fungal communities associated with different crop rotation systems

A total of 312 fungal OTUs were detected and identified. The sequences that did not match representative sequences at 95% similarity were grouped under the name of "unidentified taxa-OTU313". Some of the most abundant phylotypes found in rhizosphere soil in phase III corresponded to the pathogenic species *Alternaria eichhorniae*, *Davidiella tassiana* and *Fusarium redolens* (Table 4.6). In phase IV, wheat roots at anthesis associated with a diverse fungal community, which also included some pathogenic phylotypes, such as *Microdochium bolleyi*, *Olpidium brassicae*, *Phaeosphaeria nodorum* and *Fusarium redolens* (Table 4.6).

Host plants, more than the rotation treatments, appeared to influence the structure of the general fungal communities found in the fall rhizosphere soil in phase III and in the roots of wheat in phase IV (Table 4.7). The soil fungal communities in the phase III fall sampling were strongly influenced by crop rotation, year/site and the interaction between these factors (Table 4.7). However in phase IV when wheat was grown in all the rotation treatments, the fungal communities in the roots were similar, despite the large variations detected in soil the previous fall (Table 4.7).

Table 4.6. Relative abundance of reads of the non-AM fungi with more than 2 % occurrence in rhizosphere soil collected in the fall of phase III and in roots of wheat at anthesis, as detected by pyrosequencing.

OTU identity	Classification (Identity ≥ 95 %)	Relative abundance (%)
	– – – – – – – – – – – – Phase III – Pulses & Wheat – – – – – – – – – –	
10	*Alternaria eichhorniae*	15.2
5	*Fusarium tricinctum*	13.3
313	Unidentified taxa	7.5
39	*Davidiella tassiana*	5.5
16	*Fusarium redolens*	5.0
21	*Podospora* sp.	3.8
	– – – – – – – – – – – – – – Phase IV – Wheat – – – – – – – – – –	
13	*Microdochium bolleyi*	11.0
5	*Fusarium tricinctum*	10.0
75	*Olpidium brassicae*	9.6
4	*Fusidium griseum*	7.5
23	*Phaeosphaeria nodorum*	6.0
16	*Fusarium redolens*	5.8
43	*Setophoma terrestris*	4.5
313	Unidentified taxa	4.0
30	*Mortierella* sp.	3.7
9	*Lachnum* sp.	2.4
253	*Ceratobasidium* sp.	2.4
65	*Hymenoscyphus* sp.	2.0

Table 4.7. Probability of effects of crop rotation and year/site on the total fungal OTU community in rhizosphere soil collected in the fall of phase III and in roots of wheat at anthesis, according to PerMANOVA. Significant differences are indicated in bold.

	Phase III – Pulses & Wheat	**Phase IV - Wheat**
Source of variation	– – – – – – – – – – Probability (p)	– – – – – – – – – –
Crop rotation	**0.001****	0.521
Year/Site	**0.001****	NA
Crop rotation*Year/Site	**0.027***	NA

† Not available value
* Significant effects at $p \leq 0.05$
** Significant effects at $p \leq 0.01$

The interaction between crop rotation and year/site was found to affect the rhizosphere soil fungal communities (Sf_P-III) in phase III (Table 4.7). Hence, crop rotation effect was tested in each year/site separately and significant effects were found (Fig. 4.3). In the replicate year/site 2012, the non-AM fungal community under the rotation C-W-C was similar to those under L-W-C, L-W-L and P-W-P, but different from those under other rotations (Fig. 4.3 A). In year/site 2013, the soil fungal community under the rotation C-W-C was only different from that under L-W-L and P-W-W (Fig. 4.3 B). C-W-C and the monoculture of wheat were associated with similar soil fungal communities in the year/site 2012, but not in 2013 (Fig 4.3). The rotations L-W-C and P-W-P were associated with different non-AM soil fungal communities in 2012 (Fig 4.3 A), but these were similar in 2013 (Fig. 4.3 B). The rotations L-W-L and P-W-W were associated with similar soil fungal communities in 2012 (Fig 4.3 A), but not in 2013 (Fig 4.3 B). The fungal community selected in soil under the rotation P-W-P was similar to that of P-W-W and the wheat monoculture in 2012, but not in 2013 (Fig. 4.3).

The roots of wheat in phase IV of the rotations selected a specific community, similar in all the rotations treatments (Fig. 4.4), and different from the previous fall rhizosphere soil communities (Fig 4.5). Note that the community in phase IV was only analyzed in the year/site 2013. Accordingly, only phase III replicate in year/site 2012 was used in the comparison between the rhizosphere soil fungal community structure and the root community at wheat anthesis.

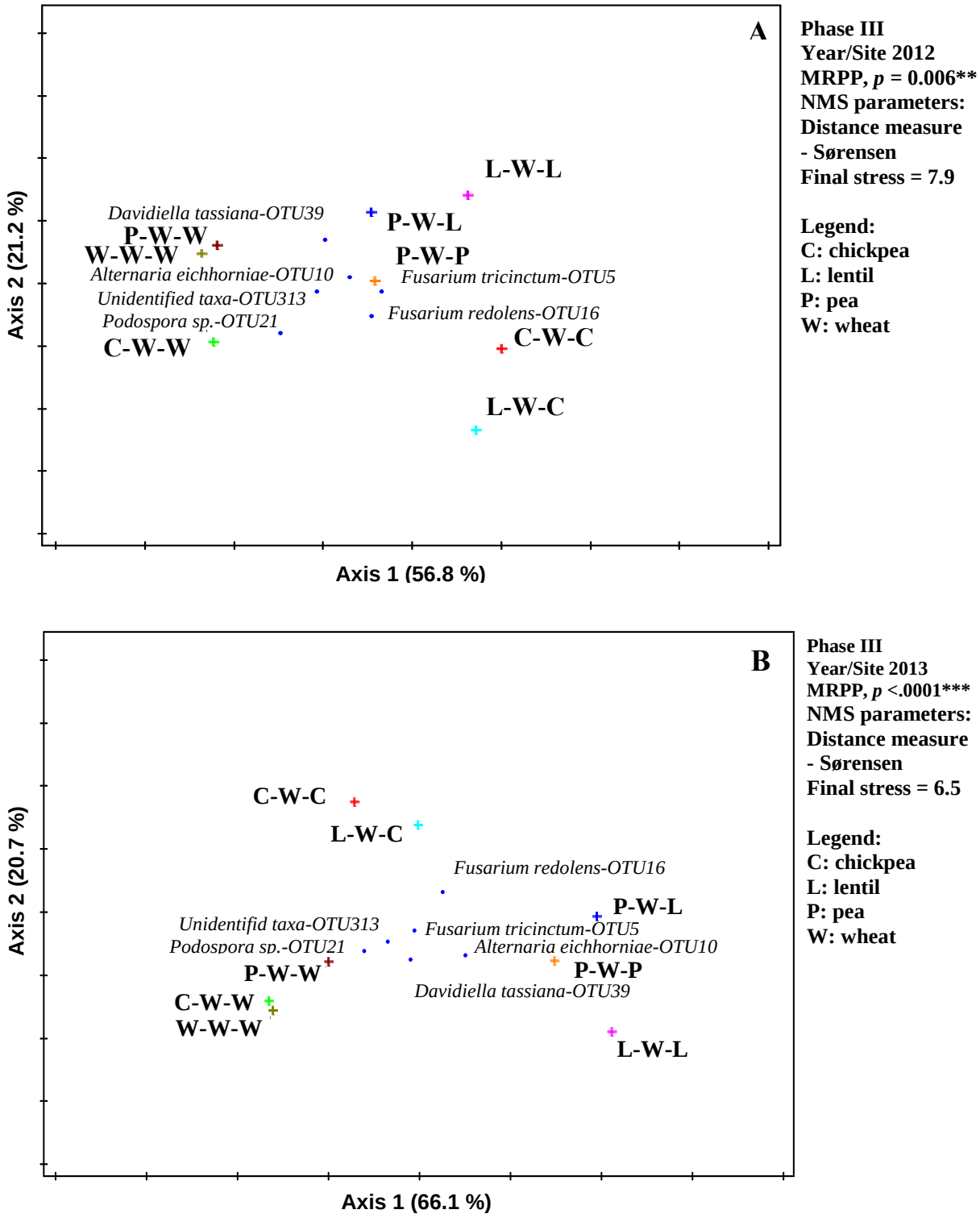

Figure 4.3. Ordinations of the crop rotations based on the structure of their non-AM fungal community in the rhizosphere soil collected in the fall of phase III (Sf_P-III), in year/site 2012 (A) and 2013 (B), according to nonmetric multidimensional scaling (n = 4).

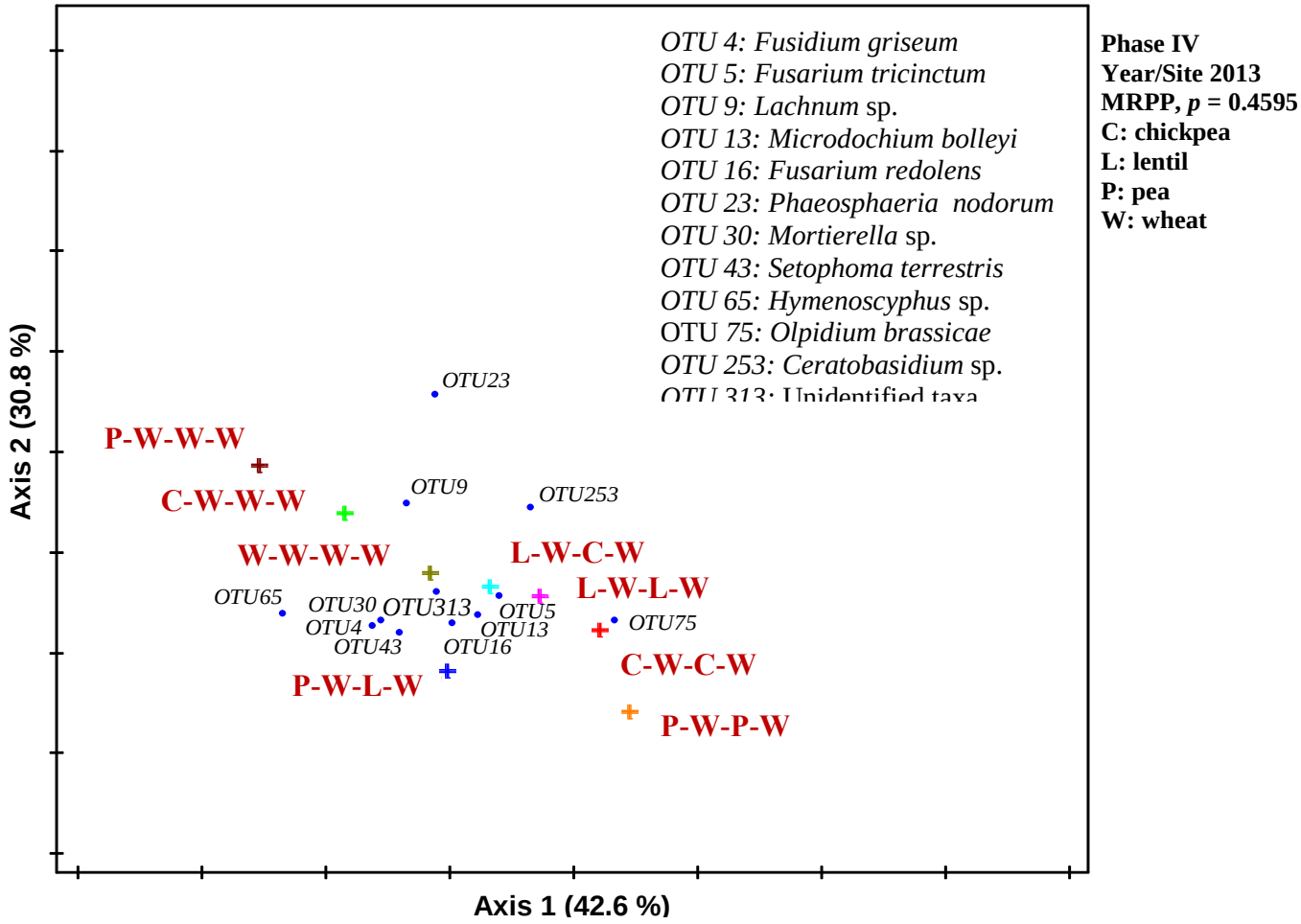

Figure 4.4. Nonmetric multidimensional scaling ordinations of the crop rotations based on the structure of the non-AM fungal community in the roots of wheat at anthesis in phase IV (Ra_P-IV) in the year/site 2013 (n = 4). Sørensen distance measure was used in the ordination. A tri-dimensional solution was recommended. Final stress = 12.9. The percentages between parentheses represent the contribution of each axis to the ordination solution.

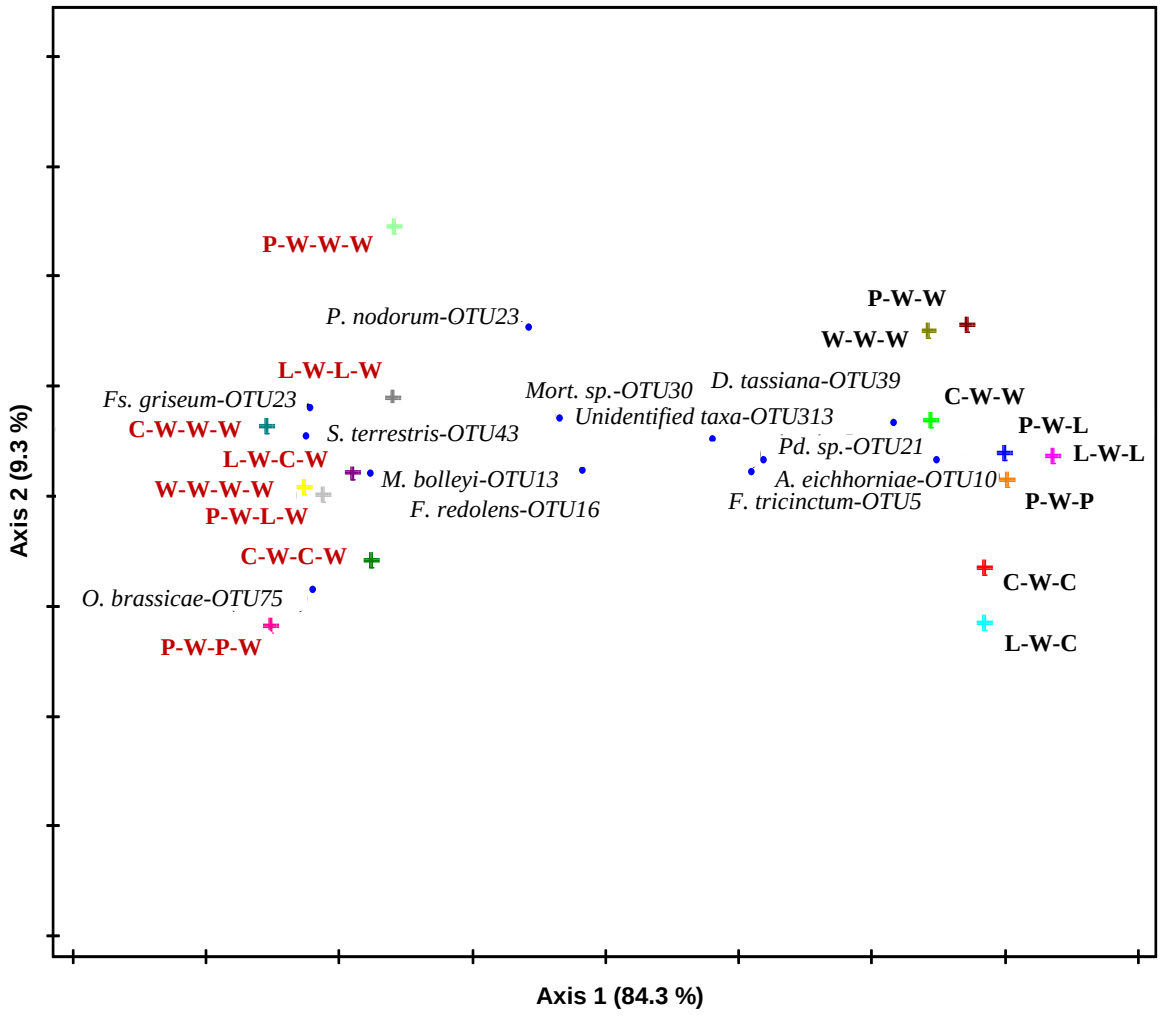

Figure 4.5. Nonmetric multidimensional scaling ordination of the crop rotations based on the structure of their non-AM fungal communities of the fall rhizosphere soil in phase III (Sf_P-III) and the roots of wheat at anthesis in phase IV (Ra_P-IV) (*n* = 4). Sf_P-III is represented in bold letters and Ra_P-IV in red bold letters. "C" stand for chickpea, "L" for lentil, "P" for pea and "W" for wheat. "*A*" stands for *Alternaria, "D*" for *Davidiella*, "*F*" for *Fusarium*, "*Fd*" for *Fusidium*, "*M"* for *Microdochium*, "*Mor"* for *Mortierella*, "*O*" for *Olpidium,* "*Pd*" for *Podospora*, and "*S*" for *Setophoma.* (MRPP, p <.0001, n = 4). Sørensen distance measure was used in the ordination. A tri-dimensional solution was recommended. Final stress = 12.9. The percentages between parentheses represent the contribution of each axis to the ordination solution.

4.5.3 Richness and diversity indices

High species richness and diversity were not exclusive to diversified rotations. In fact wheat monoculture did not decrease the richness or diversity of AM and non-AM fungal species in both rhizosphere soil and wheat roots (Table 4.8 and 4.9). Crop rotation and year/site of cultivation had significant effects on the richness and diversity of the AM and non-AM fungal communities found in rhizosphere soil in phase III of the rotations (Table 4.8 and 4.9).

In phase III, AM species richness was higher in the rotations L-W-L, C-W-W, C-W-C and W-W-W, in that order (Table 4.8), and lower in P-W-P and L-W-C. Shannon diversity was higher in 2012 than in 2013 (Table 4.8).

Shannon diversity of the non-AM rhizosphere soil community was influenced by both crop rotation and year/site in phase III of the rotations. The 3-year monoculture of wheat had the highest Shannon diversity and the rotation L-W-L had the lowest (Table 4.9). Furthermore, non-AM fungal diversity and species richness were affected by year/site. Diversity was higher in 2012 than in 2013 and, conversely, Chao richness was higher in 2013 than in 2012 (Table 4.9).

No significant effects of crop rotation or year/site were affecting the diversity indices in phase IV of the rotations (Tables 4.8 and 4.9).

Table 4.8. Least square means of the Chao richness and Shannon diversity indices of the AM fungal communities selected by cropping systems in the rhizosphere soil collected in the phase III fall, and in roots of wheat at anthesis in phase IV. Within a column, significant differences between crop rotations and year/site means are indicated in bold by different letters, according to LSD test and Student's t test respectively.

AM fungi	**Phase III – Soil (*N* = 64)**		**Phase IV - Wheat roots (*N* = 32)**	
Crop rotations	**Chao richness (S)**	**Shannon diversity (H)**	**Chao richness (S)**	**Shannon diversity (H)**
C-W-C-W	78.6 **a**	3.23 ns	26.0 ns	1.56 ns
C-W-W-W	79.5 **a**	3.13 ns	25.7 ns	1.70 ns
L-W-C-W	60.0 **d**	2.90 ns	24.7 ns	1.61 ns
L-W-L-W	79.6 **a**	3.14 ns	21.2 ns	1.49 ns
P-W-L-W	69.2 **c**	2.92 ns	34.2 ns	2.12 ns
P-W-P-W	59.7 **d**	2.70 ns	25.5 ns	1.88 ns
P-W-W-W	73.2 **bc**	3.00 ns	34.7 ns	2.01 ns
W-W-W-W	77.6 **ab**	3.05 ns	24.0 ns	1.79 ns
Year/Site				
2012/2013	74.3 ns[†]	3.22 **a**	NA[‡]	NA
2013	70.0 ns	2.80 **b**	NA	NA
Source of variation	Probability (*p*)			
Crop rotation	**0.0272***	0.0574	0.8184	0.7421
Year/Site	0.2644	**<.0001****	NA	NA
Crop rotation*Year/Site	0.2787	0.2065	NA	NA

[†] Not significant. [‡] Not applicable.

* Significant effects at $p \leq 0.05$. ** Significant effects at $p \leq 0.01$.

Table 4.9. Least square means of the Chao richness and Shannon diversity indices of the non-AM fungal communities selected by cropping systems in the rhizosphere soil collected in the phase III fall, and in roots of wheat at anthesis in phase IV. Within a column, significant differences between crop rotations and year/site means are indicated in bold by different letters, according to LSD test and Student's t test respectively.

Non- AM fungi	**Phase III – Soil (*N* = 64)**		**Phase IV - Wheat roots (*N* = 32)**	
Crop rotations	**Chao richness (S)**	**Shannon diversity (H)**	**Chao richness (S)**	**Shannon diversity (H)**
C-W-C-W	97.8 ns[†]	3.09 **bcd**	69.7 ns	2.81 ns
C-W-W-W	116.8 ns	3.39 **ab**	68.7 ns	2.72 ns
L-W-C-W	104.3 ns	2.96 **de**	66.7 ns	2.55 ns
L-W-L-W	104.1 ns	2.50 **f**	66.2 ns	2.48 ns
P-W-L-W	102.1 ns	2.71 **ef**	64.7 ns	2.59 ns
P-W-P-W	101.2 ns	2.95 **de**	61.7 ns	2.53 ns
P-W-W-W	112.8 ns	3.31 **abc**	67.2 ns	2.82 ns
W-W-W-W	112.0 ns	3.48 **a**	73.0 ns	2.85 ns
Years/Site				
2012/2013	92.4 **b**	3.22 **a**	NA[‡]	NA
2013	120.4 **a**	2.80 **b**	NA	NA
Source of variation	Probability (*p*)			
Crop rotation	0.5992	**<.0001****	0.9224	0.5541
Year/Site	**<.0001****	**0.0015****	NA	NA
Crop rotation*Year/Site	0.7848	0.0567	NA	NA

[†] Not significant.
[‡] Not applicable.
* Significant effects at $p \leq 0.05$.
** Significant effects at $p \leq 0.01$.

4.5.4 Root colonization

Crop rotation system had a significant effect on the AM and non-AM root colonization levels in phase III of the rotations (Table 4.10). AM colonization was higher in pulses than wheat. The year/site of cultivation also influenced AM root colonization levels, which were higher in 2013 (Table 4.10). In phase III of the rotations, non-AM fungal root colonization was lower in chickpea than in other plant species (Table 4.10).

In phase IV of the rotations, wheat roots were similarly colonized by AM and non-AM fungi despite the variations in the crop sequences observed in previous years (Table 4.10).

Table 4.10. Levels of AM and non-AM root colonization during phases III and IV of the rotations. Within a column, means associated with different letters are different, according to Kruskal-Wallis test.

	Phase III (*n* = 8)		**Phase IV – Wheat (*n* = 4)**	
	----- Number of colonized roots (%) -----			
Crop rotations	**AM**	**Non-AM**	**AM**	**Non-AM**
C-W-C-W	68.8 **a**	13.0 **b**	28.5 ns	29.0 ns
C-W-W-W	35.5 **b**	17.7 **a**	37.8 ns	20.5 ns
L-W-C-W	71.8 **a**	12.8 **b**	29.0 ns	23.8 ns
L-W-L-W	68.0 **a**	27.7 **a**	34.8 ns	21.3 ns
P-W-L-W	68.6 **a**	28.7 **a**	26.3 ns	27.8 ns
P-W-P-W	75.3 **a**	16.4 **a**	24.5 ns	29.8 ns
P-W-W-W	31.6 **b**	24.1 **a**	33.3 ns	22.3 ns
W-W-W-W	37.7 **b**	20.1 **a**	38.8 ns	20.5 ns
Year/Site				
2012/2013	65.2 **a**	18.3 ns[†]	NA[‡]	NA
2013	49.1 **b**	21.7 ns	NA	NA
Source of variation according to PerMANOVA	---------- Probability (*p*) ----------			
Crop rotation	**0.001****	**0.025***	0.5797	0.6479
Year/Site	**0.001****	0.322	NA	NA
Crop rotation*Year/Site	**0.043***	0.180	NA	NA

[†] Not significant
[‡] Not applicable
* Significant effect at $p = 0.05$
** Significant effect at $p = 0.01$

4.5.5 Impact of 4-year crop rotations on wheat performance and overall fungal community

Crop rotation had a large influence on wheat performance in the last phase of the rotations (Table 4.11). Wheat plant density was highest after P-W-W and L-W-L and lowest in the wheat monoculture. Wheat yield was highest after L-W-L and after P-W-P and lowest in the monoculture of wheat. Seed C content was highest in the rotation P-W-L-W and lowest in C-W-W-W. The monoculture of wheat was the first wheat crop to reach maturity and had the highest number of seeds per plant (Table 4.11).

Different relationships were established between the overall fungal communities associated with wheat roots and the agronomic data collected in phase IV (Fig 4.6). The increase in yield in the rotations including pulses before wheat seemed related with the remaining N left by the pulses in soil the previous fall. The rotations having at least three consecutive years of wheat monoculture seemed to be in relationship with spring water and P in the soil (Fig. 4.6).

Table 4.11. Least square means of agronomic attributes of wheat in phase IV of the rotations. Within a column, means associated with different letters are different, according to LSD test (n = 4). Significant probabilities are indicated in bold.

Crop rotations	Plant density (plants m^{-2})	Days to maturity	Yield (kg ha^{-1})	1000 seeds weight (g)	Seeds per plant	Seed C (mg g^{-1})
C-W-C-W	63.0 **ab**	100.2 **bc**	2333 **bc**	35.8 **bc**	109 **abc**	434.2 **a**
C-W-W-W	58.8 **bc**	102.0 **ab**	2335 **bc**	34.5 **e**	121 **ab**	427.4 **c**
L-W-C-W	66.3 **ab**	99.2 **c**	2188 **c**	34.8 **de**	96 **bc**	428.3 **bc**
L-W-L-W	78.8 **a**	99.5 **c**	2684 **a**	36.9 **a**	93 **cd**	435.0 **a**
P-W-L-W	64.0 **ab**	100.5 **bc**	2520 **ab**	36.8 **a**	112 **abc**	436.2 **a**
P-W-P-W	54.3 **bc**	102.5 **a**	2651 **a**	37.1 **a**	131 **ab**	432.1 **ab**
P-W-W-W	79.8 **a**	101.0 **b**	2231 **c**	35.5 **cd**	79 **d**	434.6 **a**
W-W-W-W	44.0 **c**	102.7 **a**	1913 **d**	36.7 **ab**	134 **a**	429.2 **bc**
Source of variation	– Probability (p) –					
Crop rotations	**0.0348***	**0.0030****	**0.0005****	**0.0014****	**0.0431***	**0.0337***

Note: In the phase IV, only data from 2013 was collected, hence the factors year/site and the interaction between year/site and crop rotation were not tested.
* Significant effect at p = 0.05.
** Significant effect at p = 0.01.

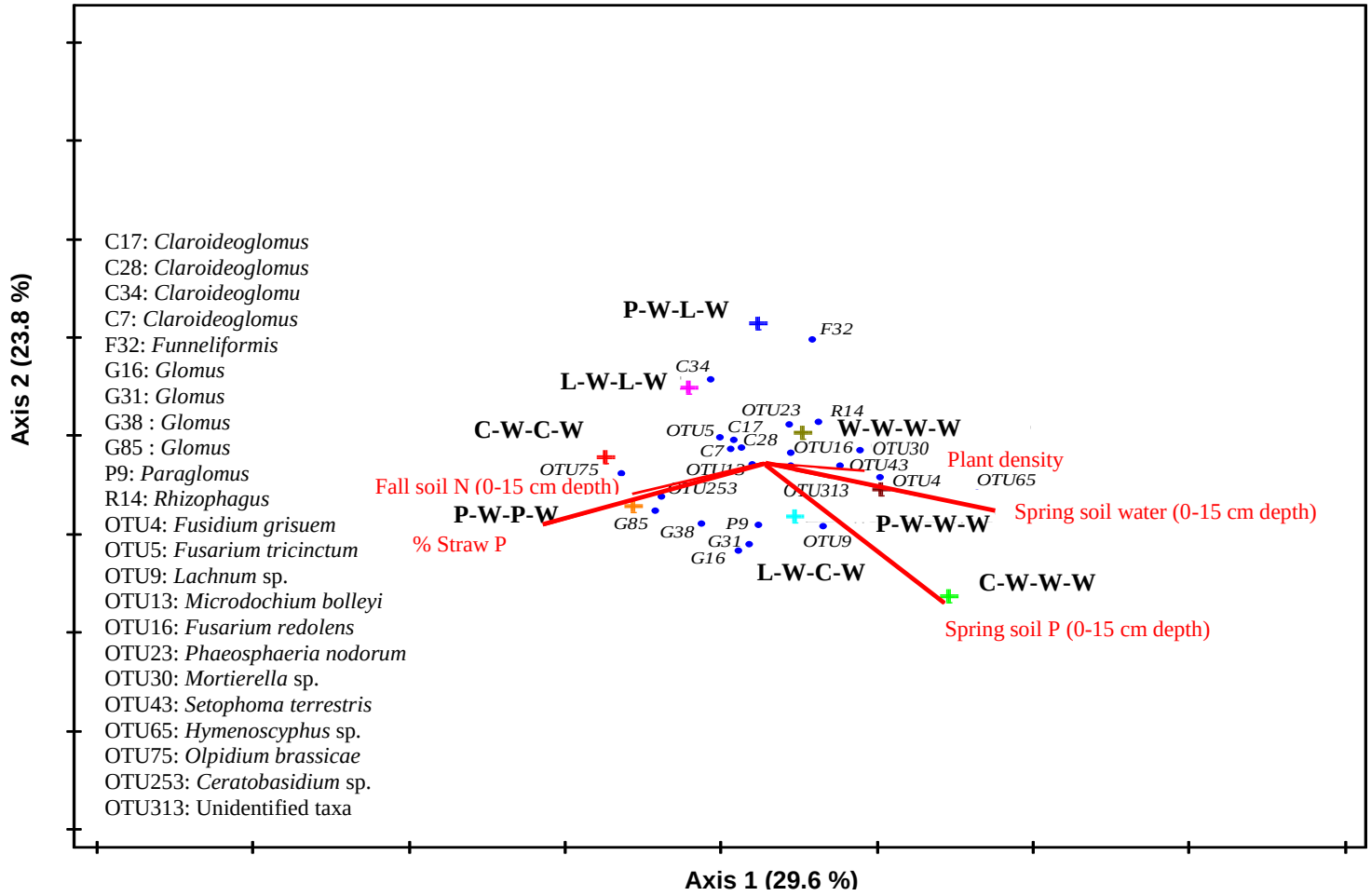

Figure 4.6. Non-metric multidimensional scaling ordination using Sørensen distance measure showing the relationship between the root fungal community and the agronomic attributes of phase IV wheat. The relationship is significant according to redundancy analysis (RDA) (n = 4, p = 0.0280).A tri-dimensional solution was recommended. Final stress = 16.0. The percentages between parentheses represent the contribution of each axis to the ordination solution.

Microbial attributes, environment and wheat performance were related in different ways as presented by Pearson correlations in Table 4.12. Positive correlations between soil microbial biomass and wheat yield components were detected (Table 4.12). Also negative correlations between the levels of AM and non-AM fungal colonization of wheat roots were found (Table 4.12). More specifically, the abundance of *Fusarium redolens* (OTU16), a pathogen of wheat, was negatively correlated with the levels of AM fungal colonization in the roots of wheat. The relative abundance of another *Fusarium* phylotype: *Fusarium tricinctum* (OTU5), a saprophyte, was also negatively correlated with the AM fungal neutral lipids (NLFA) 16:1ω5c (Fig. 4.7A). Also, the abundance of *Hymenoscyphus* sp. (OTU65), a genus that contains pathogenic and saprophytic fungi, was negatively related with wheat straw P content and with the AM fungal PLFA biomarker 16:1ω5c.

The potential detrimental effect of pathogenic species of wheat was reflected as negative relationships between the fungal phylotypes and wheat performance. For example, the abundance of *Microdochium bolleyi* (OTU13) was negatively correlated with wheat straw C content; suggesting that the proliferation of the pathogen in the roots can drain much of the C the plant is producing, and could consequently affecting overall plant performance. The abundance of another pathogenic phylotype: *Olpidium brassicae* (OTU75), which is the fungal vector for most, if not all, necroviruses and has been previously detected and isolated in wheat roots (Barr and Slykhuis 1976), was positively correlated with soil dehydrogenase activity both, at anthesis and in the fall, and negatively correlated with wheat straw N content (Table 4.14).

Conventional fertilizer application in the field might favor some groups of fungi over others. If these fungal groups have naturally developed antagonistic relationships, their interaction might be compromised with possible detrimental effects on wheat productivity. For example, in the field study soil P content at seeding was negatively correlated with the levels of AM root colonization, but positively correlated with the levels of non-AM fungal colonization of wheat roots. Many of the non-AM fungi detected by pyrosequencing in the roots of wheat were pathogenic pylotypes, such as *Microdochium bolleyi*, *Phaeosphaeria nodorum* and *Fusarium redolens* (See Table 4.7). Also, the abundance of *Rhizophagus* (OTU14), a beneficial AM fungi, was negatively correlated with soil N content in spring (Table 4.12). Furthermore, by thinking we have covered, and possibly exceed all plant nutritional requirements, we might be affecting the abundance of healthy beneficial fungal communities in the roots and enabling pathogen proliferation due to reduced competition and availability of space and resources within the roots.

Some fungi might be well established and adapted to the arid conditions of the Saskatchewan prairie as suggested by the negative correlation between the relative abundance of *Claroideoglomus* OTUs and the amount of soil water (0-7.5 cm depth) in spring. Also within the *Claroideoglomus* taxa, positive correlations were found among different OTUs. This positive relationship between OTUs of the same genus was also observed among the *Glomus* phylotypes (Table 4.12). Neither *Claroideoglomus* nor *Glomus* OTUs showed positive relationships with wheat attributes. Instead, *Claroideoglomus* (OTU17) abundance was negatively correlated with plant density and *Glomus* (OTU16) had a negative relationship with wheat harvest index (Table 4.12), suggesting that AM fungi can have a

wide variety of niches and not all are beneficial for the plant. However, plants have developed complex mechanisms to select beneficial fungal associations (Bonfante and Genre 2010) and rely on them to improve their nutrition, as suggested by the positive relationship of *Paraglomus* (OTU9) relative abundance with wheat straw and seed P content (Fig. 4.7 B), and with the AM fungal colonization levels of wheat roots.

Table 4.12. Significant Spearman correlations between the microbial community associated with wheat and other fungal, agronomic or environmental attributes in phase IV of the rotations (N = 32).

Microbial community	Crop, fungi and environment	Correlation	Probability
PLFA iso c15:0	Wheat yield	0.3861	0.0290
	Wheat seeds.plant^{-1}	0.4125	0.0190
PLFA ante c15:0	Wheat yield	0.3563	0.0453
	Wheat seeds.plant^{-1}	0.4212	0.0164
	PLFA iso c15:0	0.9289	<.0001
PLFA c15:0	Wheat yield	0.4131	0.0188
	Wheat seeds.plant^{-1}	0.3640	0.0406
	Microbial activity (Mid bloom)	0.4192	0.0169
	PLFA iso c15:0	0.9296	<.0001
	PLFA ante c15:0	0.9758	<.0001
PLFA c16:0	Wheat yield	0.4106	0.0196
	Wheat seeds.plant^{-1}	0.4791	0.0055
	PLFA iso c15:0	0.8566	<.0001
	PLFA ante c15:0	0.8651	<.0001
	PLFA c15:0	0.8453	<.0001
PLFA iso c17:0	Wheat yield	0.4124	0.0190
	Wheat seeds.plant^{-1}	0.4106	0.0196
	PLFA iso c15:0	0.9153	<.0001
	PLFA ante c15:0	0.9560	<.0001
	PLFA c15:0	0.9575	<.0001
	PLFA c16:0	0.8629	<.0001
PLFA c17:0	Wheat yield	0.3695	0.0374
	Wheat seeds.plant^{-1}	0.4058	0.0212
	Wheat seed N content	-0.3644	0.0403
	PLFA iso c15:0	0.8526	<.0001
	PLFA ante c15:0	0.8453	<.0001
	PLFA c15:0	0.9659	<.0001
	PLFA c16:0	0.8590	<.0001
	PLFA iso c17:0	0.8801	<.0001

Table 4.12 (continued)

Microbial community	Crop, fungi and environment	Correlation	Probability
PLFA c16:1ω5	Wheat seeds.plant^{-1}	0.3787	0.0326
	PLFA iso c15:0	0.8221	<.0001
	PLFA ante c15:0	0.8394	<.0001
	PLFA c15:0	0.8306	<.0001
	PLFA c16:0	0.9047	<.0001
	PLFA iso c17:0	0.8699	<.0001
NLFA c16:1ω5	PLFA iso c15:0	0.3594	0.0434
	PLFA ante c15:0	0.4567	0.0086
	PLFA c15:0	0.4454	0.0106
	PLFA c16:0	0.4648	0.0074
	PLFA iso c17:0	0.4282	0.0145
	PLFA c16 1ω5	0.5418	0.0014
	PLFA c17:0	0.5150	0.0026
PLFA c18:2	Wheat seeds.plant^{-1}	0.5039	0.0033
	PLFA iso c15:0	0.5979	0.0003
	PLFA ante c15:0	0.5152	0.0025
	PLFA c15:0	0.4789	0.0056
	PLFA c16:0	0.5101	0.0029
	PLFA iso c17:0	0.5240	0.0021
	PLFA c16 1ω5	0.4837	0.0050
	PLFA c17:0	0.4914	0.0043
Non-AM root colonization	Wheat yield	0.3894	0.0276
AM arbuscules	Wheat seed weight	-0.3532	0.0474
	AM fungal root colonization	0.7655	<.0001
	Non-AM fungal root colonization	-0.4419	0.0113
AM spores	AM fungal root colonization	0.4854	0.0049
AM fungal root colonization	Spring soil P (0-7.5 cm depth)	-0.3652	0.0398
	AM hyphae	0.8835	<.0001
	Non-AM fungal root colonization	-0.4411	0.0115
	AM arbuscules	0.8202	<.0001
Non-AM hyphae	Spring soil P (0-7.5 cm depth)	0.3615	0.0420
	Non-AM fungal root colonization	0.6725	<.0001
	AM spores	-0.4432	0.0111

Table 4.12 (continued)

Microbial community	Crop, fungi and environment	Correlation	Probability
Microsclerotia	AM fungal root colonization	-0.4292	0.0142
	Non-AM fungal root colonization	0.5468	0.0012
	AM arbuscules	-0.4196	0.0171
	AM hyphae	-0.5342	0.0016
Microbial activity (Mid bloom)	Wheat seed N content	-0.5922	0.0004
Microbial activity (Fall)	Wheat straw N content	-0.4630	0.0076
	Spring soil water (0-7.5 cm depth)	-0.4879	0.0046
Claroideoglomus-OTU17	Plant density	-0.3515	0.0485
	Spring soil water (0-7.5 cm depth)	-0.4925	0.0042
Claroideoglomus-OTU28	Spring soil water (0-7.5 cm depth)	-0.4115	0.0193
	Claroideoglomus-OTU17	0.8415	<.0001
Claroideoglomus-OTU34	AM fungal root colonization	-0.4365	0.0125
	AM hyphae	-0.4287	0.0144
	Claroideoglomus-OTU17	0.4792	0.0055
Claroideoglomus-OTU7	Spring soil water (0-7.5 cm depth)	-0.4277	0.0146
	Claroideoglomus-OTU17	0.8550	<.0001
	Claroideoglomus-OTU28	0.7347	<.0001
	Claroideoglomus-OTU34	0.3975	0.0243
Funneliformis-OTU32	Microbial activity (Mid bloom)	-0.4212	0.0164
Glomus-OTU16	Harvest index	-0.4111	0.0194
	Wheat straw P content	0.3657	0.0396
Glomus-OTU31	PLFA c18:2	-0.3966	0.0246
	Claroideoglomus-OTU34	-0.4343	0.0130
	Glomus-OTU16	0.7961	<.0001
Glomus-OTU38	*Glomus*-OTU16	0.6584	<.0001
	Glomus-OTU31	0.6766	<.0001
Glomus-OTU85	Non-AM fungal root colonization	-0.3609	0.0424
Paraglomus-OTU9	Wheat straw P content	0.4682	0.069
	Wheat seed P content	0.5290	0.0019
	AM fungal root colonization	0.3818	0.0310
	AM hyphae	0.3716	0.0363
	Claroideoglomus-OTU34	-0.4283	0.0145
	Funneliformis-OTU32	-0.4504	0.0097
Rhizophagus-OTU14	Spring soil N (0-7.5 cm depth)	-0.3772	0.0333

Table 4.12 (continued)

Microbial community	Crop, fungi and environment	Correlation	Probability
Fusidium griseum-OTU4	Microbial activity (Mid bloom)	-0.3782	0.0328
	Spring soil P (0-7.5 cm depth)	0.3636	0.0408
	NLFA c16:1ω5	-0.3772	0.0333
	Claroideoglomus-OTU7	-0.3886	0.0279
Fusarium tricinctum-OTU5	NLFA c16:1ω5	-0.3889	0.0278
	Spring soil water (0-7.5 cm depth)	-0.3998	0.0234
	Glomus-OTU85	0.3946	0.0254
Lachnum sp.-OTU9	AM fungal root colonization	0.4466	0.0104
	AM hyphae	0.4483	0.0101
	AM arbuscules	0.5018	0.0034
	Spring soil water (0-7.5 cm depth)	0.3533	0.0473
	Microdochium bolleyi-OTU13	-0.3589	0.0437
Microdochium bolleyi-OTU13	Wheat straw C content	-0.5113	0.0023
Fusarium redolens-OTU16	AM fungal root colonization	-0.4076	0.0206
	AM hyphae	-0.4485	0.0100
	AM arbuscules	-0.4168	0.0177
	Phaeosphaeria nodorum-OTU23	-0.4716	0.0064
P. nodorum-OTU23	Microsclerotia	-0.5210	0.0022
Mortierella sp.-OTU30	AM spores	-0.4732	0.0062
	Fusidium griseum-OTU4	0.5861	0.0004
	Fusarium redolens-OTU16	0.5477	0.0012
Setophoma terrestris-OTU43	PLFA c18:2	-0.3872	0.0286
	Fusidium griseum-OTU4	0.4062	0.0211
	Fusarium tricinctum-OTU5	-0.4769	0.0058
	Mortierella sp.-OTU30	0.4967	0.0038
Hymenoscyphus sp.-OTU65	PLFA c15:0	-0.3585	0.0439
	PLFA c16:1ω5	-0.4163	0.0178
	Straw P content	-0.3879	0.0283
	Spring soil P (0-7.5 cm depth)	0.4570	0.0086
	Claroideoglomus-OTU7	-0.3803	0.0318
	Fusidium griseum-OTU4	0.6559	<.0001
	Lachnum sp.-OTU9	0.4163	0.0178
	Mortierella sp.-OTU30	0.5501	0.0011
	Setophoma terrestris-OTU43	0.4827	0.0051

Table 4.12 (continued)

Non-AM-Fungi	Crop, fungi and environment	Correlation	Probability
Olpidium brassicae-OTU75	Microbial activity (Mid bloom)	0.3781	0.0329
	Microbial activity (Fall)	0.3864	0.0289
	AM spores	0.3857	0.0293
	Wheat straw N content	-0.3665	0.0391
	Fusidium griseum-OTU4	-0.4106	0.0196
	Mortierella sp.-OTU30	-0.5088	0.0029
	Setophoma terrestris-OTU43	-0.3629	0.0412
	Hymenoscyphus sp.-OTU65	-0.6039	0.0003
Ceratobasidium sp.-OTU253	Spring soil N (0-7.5 cm depth)	0.4349	0.0129
	Hymenoscyphus sp.-OTU65	-0.4472	0.0103

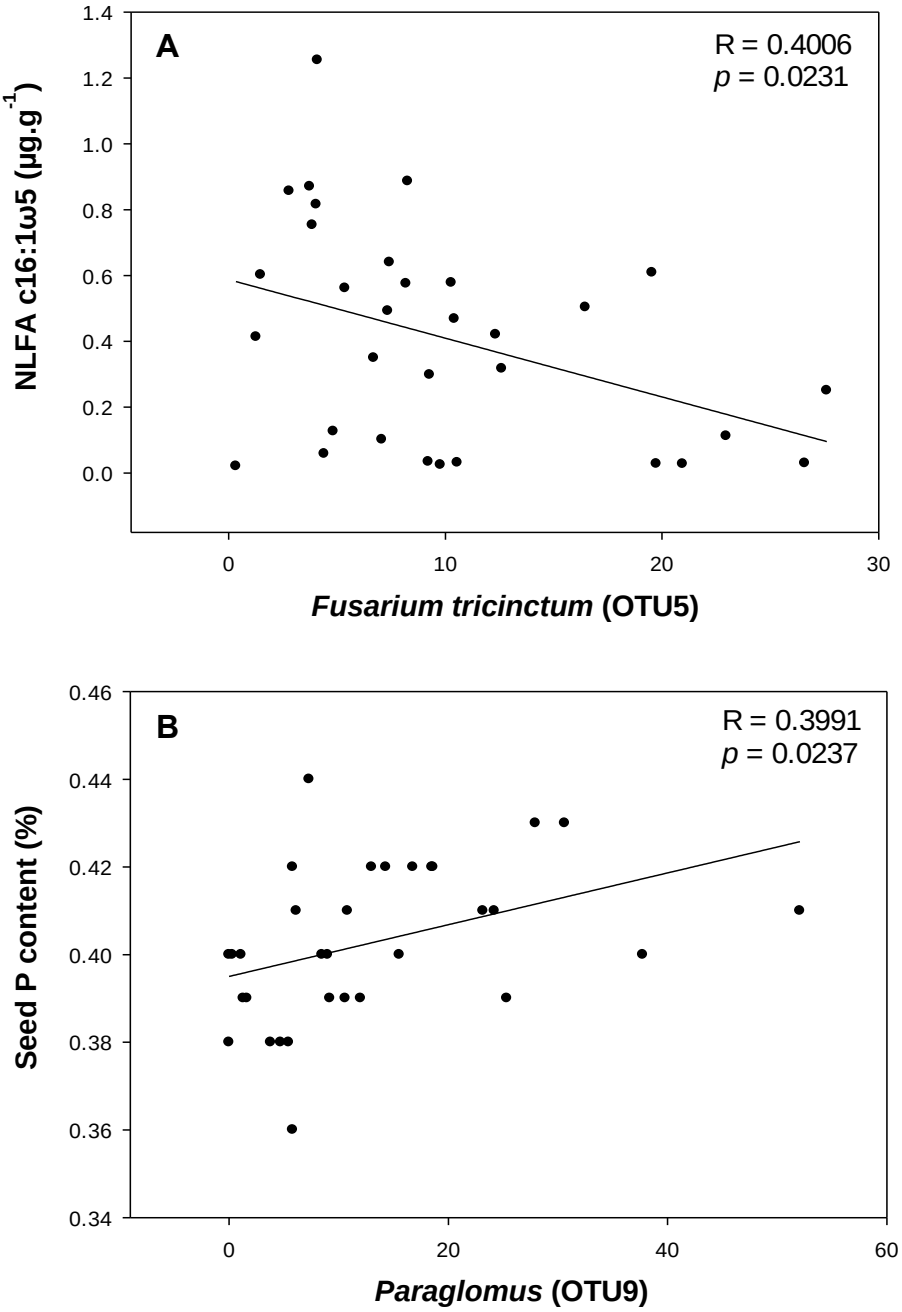

Figure 4.7. Relationship between the relative abundance of *Fusarium tricinctum* (OTU5) and the AM fungal biomarker NLFA c16:1ω5 (A), and between *Paraglomus* (OTU9) and the seed P content of wheat (B), according to linear regression (N = 32).

4.5.6 Impact of the soil microbiota selected by eight three year crop rotations on wheat grown in the greenhouse

Crop rotations selected specific soil microbial communities which had significant effects on wheat biomass and wheat productivity represented by the number of heads per plant (Table 4.13). There was an interaction between the crop rotations and year/site effects on wheat above ground biomass. Hence each year/site was analyzed separately. Inoculation with soil from year/site 2013 had a significant effect on the above ground biomass of wheat. Soil from the rotations C-W-C and L-W-C increased wheat biomass above the control (Fig. 4.8). Wheat inoculated with soil from P-W-W and from the wheat monoculture had the lowest above ground biomass, which was as low as that of the control group (Fig. 4.8). The number of heads per plant was higher than the control with all soil inoculants, except those from P-W-L and the wheat monoculture soil (Table 4.13). These effects were not detected when wheat was inoculated with soil from year/site 2012 (Fig. 4.8).

Wheat nutrient content was only influenced by the microbial community selected by year/site (Table 4.13). Plant N and P contents were higher with the microbial community selected in 2012 than in 2013. The opposite situation occurred for the K content of wheat; it was higher with the microbial community selected in 2013 than 2012 (Table 4.13).

Table 4.13. Least square means of agronomic attributes and levels of fungal root colonization of wheat inoculated with the microbial communities selected by eight different crop rotations, and grown in the greenhouse at SPARC. Different letters within a column indicate significant differences at $p \leq 0.05$, according to LSD test, or non-parametric pair comparisons Kruskal Wallis test ($N = 72$).

Crop rotations	**Plant biomass (g m^{-2})**	**Heads.plant^{-1}**	**Plant N (mg g^{-1})**	**Plant P (mg g^{-1})**	**Plant K (mg g^{-1})**	**Plant C (mg g^{-1})**	**Fungal root colonization (%)**
C-W-C-W	12.3 ns†	7.9 **a**	13.7 ns	2.8 ns	28.7 ns	428.0 ns	15.8 **a**
C-W-W-W	12.2 ns	6.9 **ab**	14.4 ns	2.7 ns	25.9 ns	426.6 ns	16.9 **a**
L-W-C-W	12.4 ns	7.9 **a**	14.0 ns	3.0 ns	32.2 ns	431.1 ns	15.9 **a**
L-W-L-W	11.2 ns	6.3 **ab**	12.9 ns	2.7 ns	36.3 ns	425.5 ns	16.1 **a**
P-W-L-W	10.8 ns	5.4 **abc**	13.3 ns	2.8 ns	28.4 ns	433.0 ns	15.8 **a**
P-W-P-W	11.6 ns	6.6 **ab**	13.4 ns	2.6 ns	27.9 ns	427.3 ns	18.7 **a**
P-W-W-W	11.9 ns	6.8 **ab**	17.0 ns	3.1 ns	28.9 ns	425.1 ns	16.1 **a**
W-W-W-W	10.7 ns	4.4 **bc**	16.0 ns	3.1 ns	28.8 ns	426.3 ns	17.1 **a**
Control	11.9 ns	2.4 **c**	12.6 ns	2.8 ns	33.7 ns	425.0 ns	1.1 **b**
Year/Site							
2012/2013	11.3 **b**	5.9 ns	15.8 **a**	3.1 **a**	25.0 **b**	429.2 ns	14.39 ns
2013	12.1 **a**	6.2 ns	12.5 **b**	2.6 **b**	35.1 **a**	425.9 ns	15.28 ns
Source of variation according to PerMANOVA	Probability (p)						
Crop rotation	0.2090	**0.0380***	0.4920	0.7980	0.7830	0.2480	**0.0010****
Year/Site	**0.0300***	0.9290	**0.0090****	**0.0190***	**0.0010****	0.0600	0.5160
Crop rotation*Year/Site	**0.0070****	0.2290	0.7480	0.9390	0.7480	0.6990	0.9600

† Not significant

* Significant effect at $p \leq 0.05$.

** Significant effect at $p \leq 0.01$.

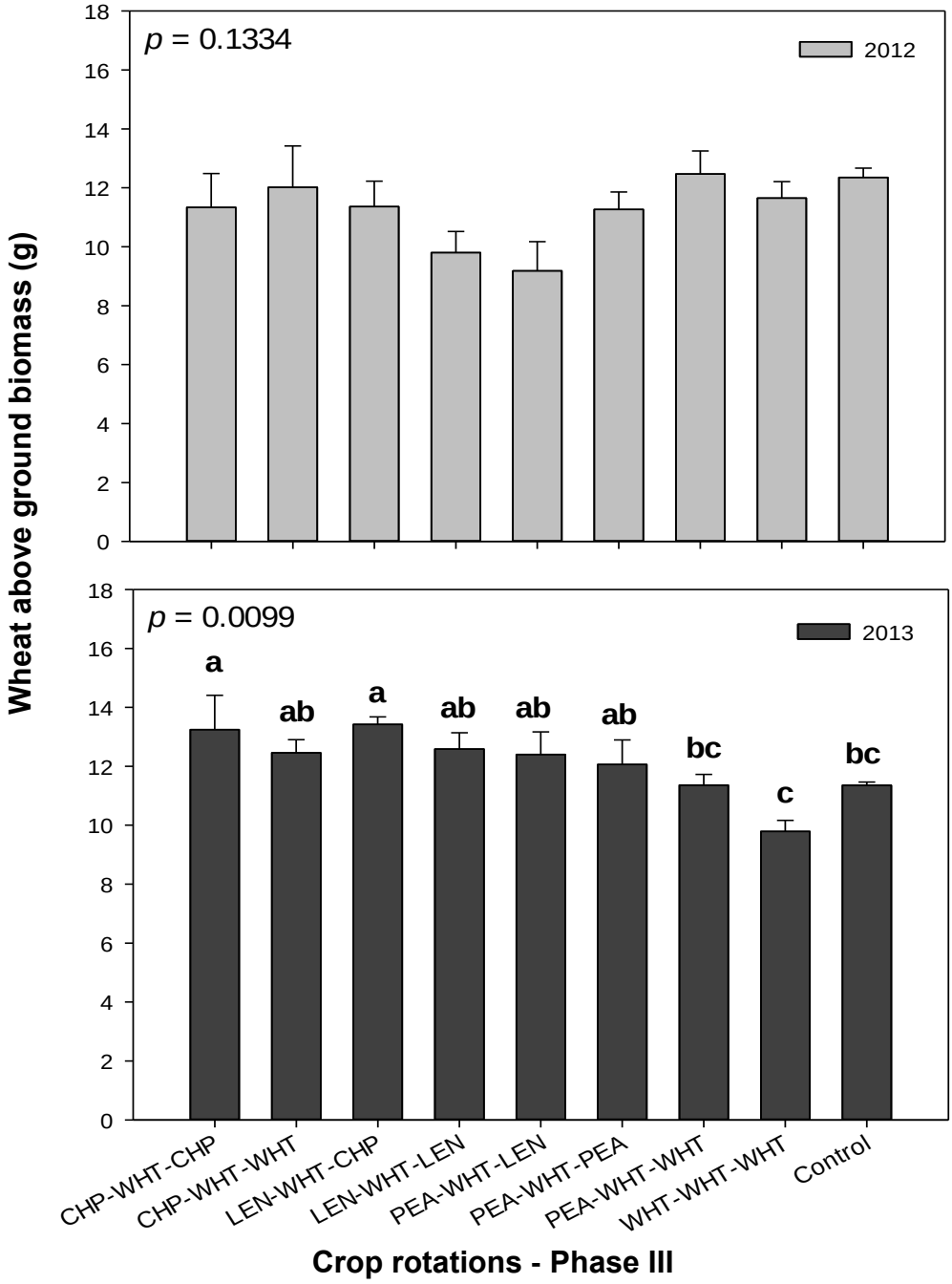

Figure 4.8. Least square means of the above ground biomass of wheat inoculated with the microbiota selected by different rotation systems at phase III in the fall of 2012 and 2013. Significant differences according to LSD test (n = 4), are indicated by different letters. Error bars represent standard errors of the mean.

4.5.7 Climate, soil water and fertility

Weather data was received from Environment Canada. The analysis showed that July was significantly warmer in 2012 than in 2013 (Fig. 4.9). At the beginning of the growing season, 2012 was also a wetter year than 2013 (Fig. 4.9) and accordingly, soil water content was higher during the spring of 2012 (Table 4.14). The year/site replicates of phase III of the rotations were affected by these climate variations as previously described.

In phase IV of the rotation no significant crop rotation effect was detected on soil water, N or P in spring (Table 4.15).

Table 4.14. Least square means of soil N, P and water levels in spring in phase III of the rotations and probabilities of crop rotation and year/site effects. Within a column, significant differences according to Kruskal-Wallis test ($p \leq 0.05$) are indicated in bold.

	Spring soil water (0-30 cm depth)	**Spring soil water (0-60 cm depth)**	**Spring soil N (0-30 cm depth)**	**Spring soil P (0-30 cm depth)**
Crop rotations	*mm*		*kg ha^{-1}*	
C-W-C	34 ns[†]	31 ns	7.0 ns	41.5 ns
C-W-W	35 ns	36 ns	4.8 ns	44.8 ns
L-W-C	32 ns	28 ns	5.4 ns	45.8 ns
L-W-L	35 ns	35 ns	4.3 ns	49.9 ns
P-W-L	33 ns	27 ns	6.2 ns	49.5 ns
P-W-P	35 ns	31 ns	4.6 ns	37.8 ns
P-W-W	34 ns	32 ns	4.3 ns	51.3 ns
W-W-W	34 ns	31 ns	5.9 ns	44.8 ns
Year/Site				
2012/2013	36 **a**	36 **a**	5.6 ns	49.0 **a**
2013	33 **b**	28 **b**	5.0 ns	42.3 **b**
Source of variation according to PerMANOVA	Probability (*p*)			
Crop rotation	0.330	0.131	0.278	0.624
Year/Site	**0.001****	**0.001****	0.253	**0.038***
Crop rotation*Year/Site	0.487	0.209	0.102	0.658

[†] Not significant

* Significant effect at $p \leq 0.05$.

** Significant effect at $p \leq 0.01$.

Table 4.15. Least square means of soil N, P and water levels in spring in phase IV of the rotations and probabilities of crop rotation effect. Within a column, significant differences according to LSD test ($p \leq 0.05$) are indicated in bold. Note that only the experimental replicate year/site 2013 was analyzed in the phase IV of the rotations.

	Spring soil water (0-30 cm depth)	**Spring soil water (0-60 cm depth)**	**Spring soil N (0-30 cm depth)**	**Spring soil P (0-30 cm depth)**
Crop rotations	*mm*		*kg ha^{-1}*	
C-W-C-W	30.3 ns†	31.1 ns	9.7 ns	37.9 ns
C-W-W-W	32.8 ns	28.4 ns	6.8 ns	53.0 ns
L-W-C-W	27.9 ns	26.3 ns	3.5 ns	50.1 ns
L-W-L-W	28.5 ns	26.0 ns	6.2 ns	41.2 ns
P-W-L-W	29.4 ns	26.0 ns	7.7 ns	35.9 ns
P-W-P-W	27.8 ns	26.9 ns	6.6 ns	45.1 ns
P-W-W-W	34.0 ns	33.2 ns	3.5 ns	43.8 ns
W-W-W-W	34.3 ns	24.8 ns	4.3 ns	35.9 ns
Source of variation	Probability (p)			
Crop rotation	0.3262	0.7919	0.1280	0.5792

† Not significant

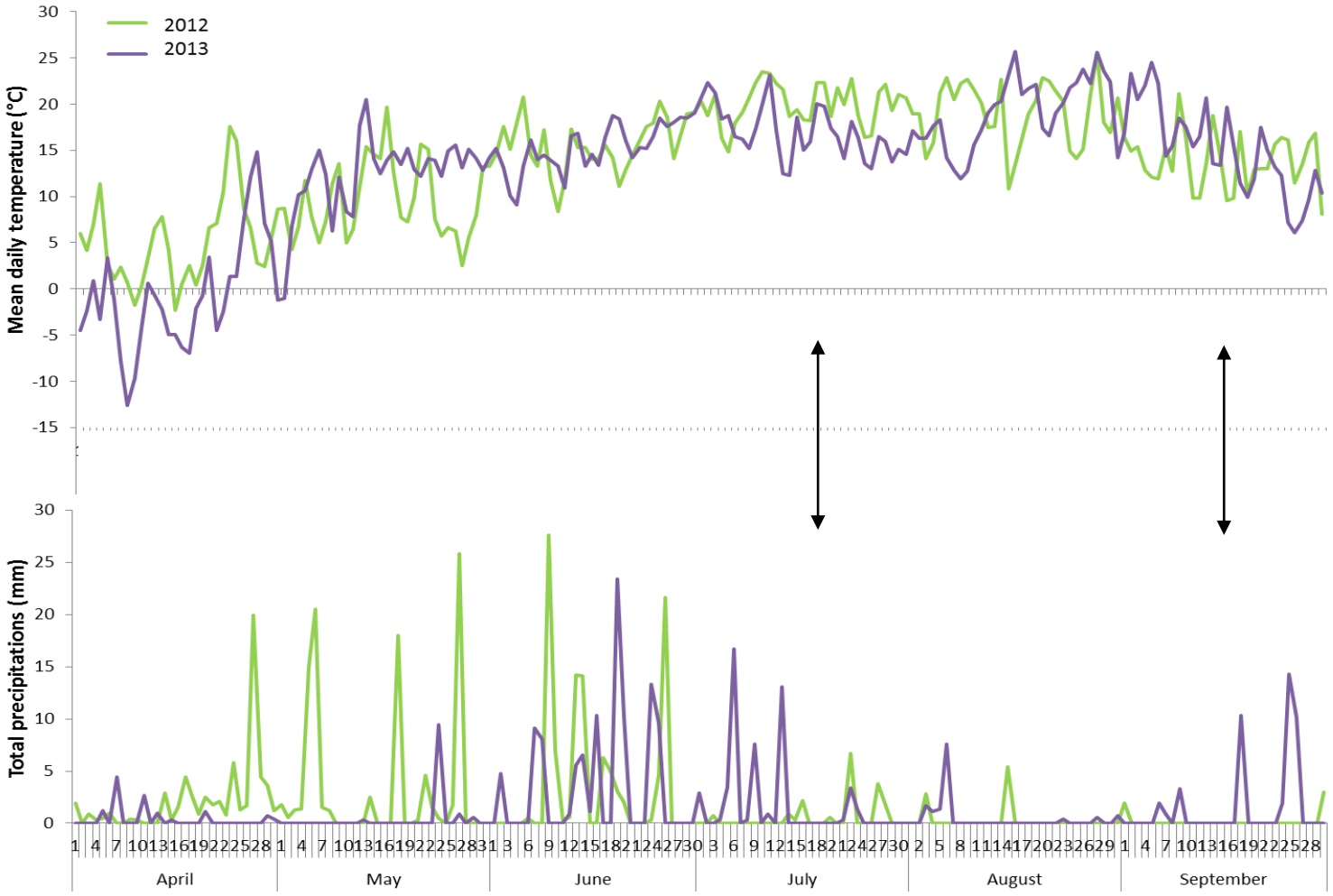

Figure 4.9. Daily variations of average temperature and total precipitation during the growing season of 2012 and 2013. Data collected at the Swift Current CDA Station, Swift Current, Saskatchewan. Double arrows correspond to the sampling times at wheat anthesis and after harvest.

4.6 Discussion

4.6.1 Effect of eight four year crop rotations on the overall fungal communities associated with wheat grown in the Canadian Prairie and their influence on crop performance.

The goals of this study were 1) to determine whether or not the structure of the fungal communities inhabiting wheat root in the last year of the rotations was affected by the different 4-year crop rotation sequences, 2) to assessed how different rotation systems impact wheat performance and 3) to determine how the microbial community selected by previous crop rotations influences the productivity and quality of the wheat crops produced. Rotation systems diversified with the inclusion of a pulse crop in alternate years generally promoted wheat yield, whereas wheat monoculture had the lowest yield and plant density. Agroecosystem diversification is known to enhance soil fertility and reduce the inputs needed to grow crops (Altieri 1999). Some highly diversified rotations, such as P-W-L-W had higher yield than the monoculture of wheat. However, the diversified rotation L-W-C-W had one of the lowest yields, suggesting that careful selection of the pulse crops to be included in diversified rotation is essential to improve the performance of the agroecosystem. Lentil and pea alternating with wheat improved wheat performance, whereas chickpea contributes little benefit or nothing to the rotation. This is consistent with studies conducted in Manitoba, by Przednowek et al. (2004) where the rotation benefits of pulse crops for the subsequent cereal were examined and results showed that field pea made the largest contribution to soil N, whereas the benefit of chickpea was unreliable. Lentil has been successfully used as a previous crop for wheat in long term rotations in Swift Current (Campbell et al. 2011;

Campbell et al. 1992; Gan et al. 2003; van Kessel and Hartley 2000). The soil N pools after lentil can increase up to 23 kg N ha^{-1} in a year, compared with 8 kg N ha^{-1} for fertilized wheat (Campbell et al. 2000; van Kessel and Hartley 2000; Walley 2007). The magnitude of the N contribution of pulses to the soil varies with years and environmental conditions. However, a positive N contribution of both pea and lentil, to soil N is expected (Walley 2007).

In addition to the effects of crop rotations on wheat performance in the field, my results showed that the specific microbial community selected by the crop rotations has the potential to influence wheat productivity in the greenhouse. In the field, the influence of microorganisms may be masked by the effect of previous crops (e.g. nutrient and water legacy) and modified by the conditions of the soil environment (e.g. micronutrients, pH, soil moisture and temperature). However, in the greenhouse, the selected microbiota was able to establish and colonize the host plants positively influencing plant performance in comparison with the non-inoculated control. There are several mechanisms through which microorganisms can improve plant fitness. Microbes are able to modify soil processes, such as nutrient cycling and mobilization by producing enzymes involved in the hydrolysis of N and P compounds from the soil organic matter, e.g., by the release of enzymes, thus, facilitating uptake and assimilation by the host plant (Marschner et al. 2001). Particularly, AM fungi can enhance plant access to water and nutrients due to the ability to explore extended soil area and to translocate them through the extraradical hyphal network. Microbial communities can also modify root architecture and mediate changes in the hormonal balance of the host, modifying plant performance. Associative fungi have a broad host range, but their

interaction with each plant type is specific and they may have different impacts on host performance (Mandyam and Jumpponen 2005).

In contrast with the field results, the greenhouse experiment results showed that the microbial communities selected by rotations that had chickpea in phase III best promoted wheat productivity, suggesting that, under certain conditions, chickpea could be an excellent previous crop for wheat. In previous studies, a positive influence of chickpea on the establishment of a subsequent wheat crop was observed. The inclusion of chickpea in the rotations increased the soil microbial diversity levels in the subsequent wheat crop (Ellouze et al. 2013), and reduced N fertilizer requirements (Marcellos et al. 1993; Thomas et al. 2010). Chickpea N-fixation provided benefits equivalent to the application of 60 kg N ha^{-1} to the following wheat (Marcellos et al. 1993). Thomas et al. (2010) reported that chickpea could provide soil N benefits of 20 kg N ha^{-1} to a subsequent wheat crop. In both studies chickpea reduced the N fertilizer requirements for the following cereal crops, resulting in a wheat yield increase compared with unfertilized continuous wheat.

The addition of N fertilizer alone to continuous wheat does not substitute for a legume break crop due largely to differences in disease build up and soil acidification (Heenan 1993). Chickpea has also been successfully used as a break crop to reduce the level of *Fusarium* inoculum in wheat-based cropping systems (Cruz et al. 2012) and soil acidification. Chickpeas produces volatile compounds with bioactivity against *Fusarium* spp., such as *trans*-2-hexen-1-ol and 1-hexanol, which showed potential in reducing the negative impact of these fungi in wheat (Cruz et al. 2012).

The importance of host identity as a determinant of the structure of the AM fungal communities was revealed in my field study. The lack of crop rotation effect on the relative abundance, Shannon diversity and Chao richness of the AM fungal OTUs, and the similar levels of AM fungal colonization in wheat roots at anthesis of phase IV, suggest that host plant species is an important factor determining the composition, distribution and abundance of the AM fungal community. Ngosong et al. (2010) found that wheat had a considerable impact on soil microbial biomass and community structure. Previous research has also shown that the composition, diversity and size of AM fungal communities were strongly affected by the identity of the host plant (Li et al. 2014; Mathimaran et al. 2007).

Wheat monoculture did not reduce AM fungal diversity, at least in a period of 3-4 years, as indicated by the high Chao richness and Shannon diversity indices estimated in the fall soil of phase III of the rotations, and the lack of crop rotation effect on the diversity and richness associated with wheat roots at anthesis in phase IV. These results contrast with several studies, where more diversified crop rotations promote microbial biodiversity (Ellouze et al. 2014; Lehman et al. 2012; Nayyar et al. 2009). Other studies found that the AM fungal richness and diversity remain unchanged through crop rotations and years (Higo et al. 2015). The higher AM colonization found in the roots of pulses versus the lowest AM root colonization levels found in wheat could supports opposite conclusions. However, the root morphology of pulses and wheat is quite different and root colonization is far from being the perfect tool to estimate AM fungal diversity, although it gives a fairly accurate evaluation of fungal proliferation in the roots.

Variations in conditions between years also affected the overall fungal diversity and non-AM fungal community composition in phase III of the rotations. Sampling was conducted in July, which was warmer in 2012 than in 2013. The growing season in 2012 was wetter than in 2013. In previous studies, fungal spore counts and density have been positively correlated with soil temperature and moisture (Sharma and Jha 2014). Temporal shifts in soil and root fungal community composition have been reported among diverse plant species under various environmental conditions (Higo et al. 2015). A drier year is sometimes less favorable to some fungi (Dai et al. 2013) and this could explain the lower Shannon diversity indices detected in 2013. Thus, longer term studies may be needed to clarify relationships among rotations of pulses and wheat crops, and the biodiversity of the AM fungal community.

4.6.2 Relationship between microbial attributes and the agronomic parameters of wheat in 4-year rotation systems

The microbial communities associated with wheat at anthesis in phase IV of the rotation were related in diverse and complex ways with wheat performance and among themselves. Soil bacterial and fungal biomass were positively correlated with wheat yield and the number of seeds per plant, which highlights the key role of microorganisms in plant performance, through improvement of soil and plant health. An abundant and diversified microbial community positively influences wheat productivity. Greenhouse studies by Chen et al. (2000) showed a close relationship between soil microbial biomass and plant yield. Field studies have also shown the positive relationship that exists between soil microbial biomass at anthesis and the yield of wheat (Khan and Joergensen 2006; Mandal et al. 2007), maize,

millet, groundnut, mungbean and rapeseeds (Khan and Joergensen 2006). The importance of soil microorganisms for maintaining soil health and fertility has been well demonstrated.

AM fungi promote plant growth and yield (Hoeksema et al. 2010), increase soil P availability to plants (Yang et al. 2014), and antagonize plant pathogens (Borowicz 2001). Many of the most abundant OTUs found in the roots of wheat belonged to pathogenic species. *Microdochium bolleyi*, *Fusarium tricinctum*, *Olpidium brassicae*, *Phaeosphaeria nodorum*, *Fusarium redolens*, *Setophoma terrestris*, *Ceratobasidium* sp. can impact crops negatively in Saskacthewan (Esmaeili Taheri et al. 2011; Fernandez et al. 2008; Vujanovic et al. 2012). The antagonistic relationship between AM fungi and certain wheat pathogens is documented (Filion et al. 1999; St-Arnaud et al. 1995). AM fungi have been used as biocontrol agents against common wide spectrum soil-borne pathogens associated with field crops (Suprapta 2012), such as *Rhizoctonia solani* (Yao et al. 2002), and *Phytophthora* species (Cordier et al. 1996; Suprapta 2012; Trotta et al. 1996). In my study, a negative relationship between the levels of colonization of wheat roots by AM versus non-AM fungi at anthesis was detected. More specifically, the abundance of *Fusarium redolens* (OTU16), a pathogen of wheat, was negatively correlated with the levels of AM fungal colonization in the roots of wheat. The relative abundance of another *Fusarium* phylotype: *Fusarium tricinctum* (OTU5), a saprophyte, was also negatively correlated with the AM fungal NLFA marker 16:1ω5c, which could suggest antagonistic interactions between AM fungi and different non-AM fungi, some of them identified as pathogens of wheat. However, more research is required to confirm or clarify the role of these fungal phylotypes on crop production.

Fertilizers are thought to cover the nutritional needs of crop plants. However, the importance of AM fungi as crop requirement is sometimes overlooked or underestimated. It is possible that excessive fertilization facilitates pathogen proliferation by reducing the antagonistic effect of AM fungi. Previous research has shown the detrimental effects of high soil P content on AM fungi (Khakpour and Khara 2012; Lin et al. 2012). Studies by Graham and Abbott (2000) proved that AM fungi could be expected to produce non-beneficial effects on wheat in the field depending on the P status of the soil and the aggressiveness of the AM fungi inhabiting the soil. Accordingly, in my field study, soil P content at seeding was negatively correlated with AM root colonization levels at anthesis, but positively correlated with the non-AM fungal colonization of wheat roots. The abundance of *Rhizophagus* (OTU14) was also negatively correlated with soil N content in spring. Whether high nutrient concentration in the soil can increase the proliferation of pathogenic species by affecting the antagonistic role of AM fungi, or by affecting the ability of AM fungi to compete with pathogens colonizing the roots, is a question that needs to be answered. A meta-analysis conducted by Borowicz (2001) explained that plants under attack by pathogenic fungi benefited most from AM fungi.

The increase in plant tolerance to pathogens mediated by AM fungi has been attributed to several mechanisms, such as the increased surface for nutrient uptake with AM mycelia, allowing the host to tolerate root loss to diseases (Borowicz 2001). AM fungi cause anatomical or architectural changes in the root system when pathogenic infection is present; it causes a more profusely branched root system (Gutjahr et al. 2009; Wehner et al. 2010). Direct interference competition, chemical interactions and indirect exploitation competition

have been also suggested as mechanisms by which AM fungi can reduce the abundance of pathogenic fungi in roots (Filion et al. 1999; St-Arnaud et al. 1995). These mechanisms are based on the assumption that AM fungi and pathogens utilize common resources in the roots, such as space (Borowicz 2001) and carbon (Graham and Abbott 2000).

The AM fungal taxa have developed different adaptations to soil conditions. While the abundance of many AM fungi is increased by warm and wet environment (Sharma and Jha 2014), *Claroideoglomus* seems to be well adapted to the dry climate of the Canadian Prairie. The positive correlation between the relative abundances of *Claroideoglomus* OTUs 7, 17, 28 and 34 and similarly, the positive relationship between the *Glomus* OTUs 16, 31 and 38, suggest that environmental conditions can randomly favor the proliferation and diversity of more than one AM genus in the agroecosystem. The co-occurrence of *Claroideoglomus* and *Glomus* phylotypes, could also be due to a synergistic effect between fungi belonging to the same genus. Fungi of the *Glomeraceae* family are known to be particularly resilient to ecosystem disturbance, partially due to their ability to form anastomosis between hyphae, which improves the functionality and integrity of the AM fungal mycelial network (De La Providencia et al. 2005).

The broad range of relationships I found between the AM fungal phylotypes and wheat performance reveals how complex and dynamic fungi-host interactions can be in the agroecosystem. For example, some phylotypes of *Claroideoglomus* and *Glomus* had negative relationships with wheat plant density and harvest index, whereas the abundance of *Paraglomus* (OTU9) was positively correlated with wheat straw and seed P contents and with the AM colonization level of wheat roots at anthesis. These results indicate that not all AM

fungi are beneficial for the plants. Unexpected negative correlation between the abundance of *Glomus* and wheat productivity has been reported before (Dai et al. 2014). Studies using controlled conditions are required in order to explain the negative impact of co-occurring *Glomus* and *Claroidoglomus* species on wheat performance. On the other hand, *Paraglomus* has been identified as a highly dominant colonizer of crop roots in the Canadian Prairie (Bainard et al. 2014). So more likely, beneficial fungi might be "allowed" to colonized the roots extensively, whereas pathogens or detrimental AM species might be controlled through antagonistic competition or sophisticated and not yet totally understood plant defensive mechanisms.

5.0 Discussion and Synthesis

Earth's population is over 7 billion and continues to grow. The demand for wheat, which increases with the growing population, is expected to double by 2050 to meet rising demands (Ray et al. 2013). The needed increase in productivity can be partially achieved using improved genetics, but better agronomic practices are required to reduce the gap between the target and actual yields. An efficient management of soil quality through crop rotation has raised attention as a strategy to improve crop yields and healthier microbial associations in sustainable agriculture. Studies on how crop rotation impacts microbial biodiversity, community dynamics and their interaction with the soil environment are required to understand and manipulate these interactions to the benefit of the agroecosystem.

My research aimed to answer the question: which crop rotation sequences best improve wheat yield and quality, but also reduce the abundance of fungal pathogens and promote associations with beneficial fungi? I characterized the fungal communities associated with diversified crop rotations of economically important crops in Canada. The selection of root-associated fungal communities by crop plants is a key finding of my studies. My research also highlights the potential importance of using beneficial fungal associations to repress disease in the field, as revealed by the negative relationship between AM fungi and the pathogens of wheat. An appropriate management system using crop rotations can increase beneficial AM fungi and be used as a sustainable way to control disease, improve crop productivity and soil health, while reducing costly agrochemical inputs.

Chapter 3 shows that pulses are better previous crops than wheat monoculture at improving wheat growth and productivity. The results in Chapter 4 indicate that diversified

4-yr rotations positively influence wheat performance, but the inclusion of chickpea in the crop sequence contributed little benefits, despite that it may selects a beneficial soil microbial community, as shown by the greenhouse assay.

These findings contribute to knowledge on wheat based crop rotations systems, the contribution of pulses to wheat productivity, and the crop rhizosphere microbiome. My research delivers a comprehensive analysis on the structure and diversity of the fungal community associated with pulses and wheat in different crop rotations in the Canadian Prairie and provides information on the influence of diversified rotations versus monoculture on overall crop performance in wheat-based rotation systems.

5.1 Pulse sequence and frequency influence wheat performance, but not the soil microbial communities in wheat based cropping systems.

The selection of the crops to be included in diversified field rotations in wheat based agroecosystems is key to improved wheat productivity. Lentil and pea alternating with wheat largely contribute to wheat performance, whereas chickpea contributed little benefits to wheat in 4-yr rotations.

Wheat monoculture did not reduce fungal diversity, at least in a period of 3-4 years. Wheat grown in eight different crop rotations had similar AM and non-AM fungal community composition, Shannon diversity and Chao richness, and levels of fungal root colonization. Also soil microbial biomass and metabolic activities were similar.

Wheat in 2-yr and 4-yr term rotations selected specific fungal communities, generally different from those in the previous fall soil. My results contradict other studies where more

diversified crop rotations increased soil microbial diversity (Ellouze et al. 2014; Lehman et al. 2012; Nayyar et al. 2009). However, they agree with the research conducted by Higo et al. (2015), where phylotype richness and diversity of AM fungal communities remain unchanged through crop rotations and years and that the composition, diversity and size of fungal communities are strongly affected by the identity of the host plant (Li et al. 2014; Mathimaran et al. 2007).

In conventional agricultural systems, *Rhizophagus*, *Funneliformis*, *Glomus*, *Claroideoglomus* and *Paraglomus* phylotypes are commonly found even in tilled soils (Bainard et al. 2014; Higo et al. 2015; Higo et al. 2013) suggesting that these taxa are very resilient to disturbance. Also the advantage of forming hyphal anastomosis (De La Providencia et al. 2005) and the high sporulation rates of *Glomerales* (Oehl et al. 2003) contributes to the endurance of these communities in the agroecosystems. However, different plant root exudates from different crops at different phases of the rotation may drive shifts in the fungal community composition and select specific fungal root associations, as shown in my studies. Further investigation into the physiological mechanisms mediating fungal community selection would help to elucidate the benefits or cost of particular AM and non-AM fungal associations on the performance of the subsequent wheat crop.

5.2 Dynamics of *Fusarium* and AM fungi in two and four year wheat based rotation systems.

Fusarium is a large genus containing some of the most economically important species of plant pathogens affecting agriculture throughout the world. Many of these filamentous fungi

produce a number of mycotoxins that can affect human and animal health if entering the food chain (Summerell et al. 2010). *Fusarium redolens* has been frequently isolated from the necrotic and discolored roots and crown tissues of chickpea, pea, lentil and durum wheat in Saskatchewan (Esmaeili Taheri et al. 2011) and it is known as one of the most frequent *Fusarium* species in the soils of the prairie in Manitoba (Gordon 1952).

Chapter 3 reveals a negative correlation between the relative abundance of *F. redolens* and the AM fungal taxa *Rhizophagus* in wheat roots. Although this does not represents conclusive evidence of an antagonistic relationship between the AM fungi and *Fusarium* taxa, it suggests this possibility, supporting the conclusion of others that at least some *Rhizophagus* species can reduce root disease development (Yergeau et al. 2006). The use of *Rhizophagus* species as biocontrol for pathogens and to boost crop yields has been documented in field (Akköprü and Demir 2005) and *in vitro* experiments (Filion et al. 1999; St-Arnaud et al. 1995). *Rhizophagus irregularis* (syn. *Glomus intraradices*) is formulated as Myke Pro, an inoculant (Primer Tech, QC, Canada) that is proven effective at producing a 10% increase in cereal yield, and reducing plant disease incidence, as stated by the Saskatchewan Wheatland Conservation Area research program (Le Quéré 2005).

The AM fungal mycelium has strong and selective effects on the growth of other microorganisms, which creates a new environment called the mycosphere (Filion et al. 1999). There might be direct interactions between the extraradical mycelia of AM fungi and soil borne pathogens (St-Arnaud et al. 1995), or this mycelium could contribute to the proliferation of specific microorganisms, some of them with antagonistic properties provided

through mechanisms such as antibiosis, competition for resources and parasitism (Filion et al. 1999).

In Chapter 4, the negative relationships between the levels of colonization by AM and non-AM fungi found in wheat roots in 4-yr rotation systems, and the negative correlation between AM extraradical fungal biomass and the abundance of *Fusarium tricinctum* in the roots of wheat, also suggests possible antagonistic interactions between AM fungi and *Fusarium*. The mechanisms involved in such microbial interactions are complex, mostly unexplored. The interaction between AM fungi and *Fusarium* spp. is an observation deserving further investigation.

5.3 Future research

My research shows that selection of plant species in crop rotation systems can increase the performance of wheat based rotations in the Canadian Prairie. The use of more than four phases of diversified rotations in the field can generate more information about the influence of crop sequence on the dynamics and composition of the microbial community associated with wheat. Whether or not all varieties of pulse crops species have the same effect on the wheat based cropping system needs to be determined. Also, the physiological mechanisms mediating the selection of specific fungal communities by the host plant requires more investigation.

Further analysis would be required to clarify the effects of AM fungal communities on disease suppression and the mechanisms involved in this type of interaction. More research is also needed on how high soil nutrient levels drive shifts in fungal communities. We need to

find out in particular the consequences of reduced AM fungal abundance on plant susceptibility to disease and on the dynamics of plant pathogens.

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7.0 APPENDICES

7.1 Appendix A: AM sequence processing in phases I and II of the rotations

Table A1. AM fungi data processing using Mothur v.1.33.0 (Open software, University of Michigan, USA) (http://www.mothur.org/wiki/Main_Page) (Edgar 2004).

Mothur command	Action
trim.seqs	Screens sequences based on the 'qual' file that comes from 454 sequencers, removes primers, long homo-polymers and cull sequences based on sequence length.
unique.seqs	Returns only the unique sequences found in a 'fasta' file and a file that indicates those sequences that are identical to the reference sequence.
chimera.uchime	Reads a 'fasta' file and reference file and outputs potentially chimeric sequences.
remove.seqs	Takes a list of sequence names and either a 'fasta', 'name', 'group', 'list', 'count' or 'align.report' file to generate a new file that does not contain the sequences in the list.
align.seqs	Aligns the fasta-formatted candidate sequence file to a fasta-formatted template alignment (e.g.: SILVA database (http://www.arb-silva.de).
screen.seqs	Enables user to keep sequences that fulfill certain user defined criteria.
filter.seqs	Removes columns from alignments based on a criteria defined by the user.
pre.cluster	Implements a pseudo-single linkage algorithm with the goal of removing sequences that are likely due to pyrosequencing errors.
dist.seqs	Calculates uncorrected pairwise distances between aligned DNA sequences.
cluster.seqs	Assign sequences to OTUs using the Furthest neighbor method, at 97 % similarity.
get.oturep	Generates a 'fasta' file containing only a representative sequence for each OTU.
split.abund	Reads a 'fasta' file and a 'list' or a 'names' file and splits the sequences into rare and abundant groups.

Table A2. Average number of AM fungal reads post-cleaning produced by 454 pyrosequencing in phases I and II of the rotations.

Phase†	Sample type	Total reads	Total fungi	Ascomycota	Basidiomycota	Glomeromycota
I	Roots	3854	3106	1216	783	1107
	Soil	8029	6427	4828	654	945
II	Roots	4463	4401	293	164	3963
	Soil	5987	4846	3328	340	1178

† Phase I includes four treatments of pulses and wheat randomized in four blocks at the South Farm of the Semiarid Prairie Agricultural Research Centre, near swift Current, SK in 2010. Phase II: Represents the second year of the rotations and involves wheat growing after different pulses versus wheat monoculture in 2011 and replicated in 2012. The primer sets used for PCR amplification of the 18S rDNA gene of AM fungi were NS1/NS4 and AMV4.5-NF/AMDGR.

Table A3. Summary statistics for MRPP and multiple pairwise comparisons of the relative abundance of AM fungal community inhabiting the roots of wheat in phase II of the rotations, year/site 2011 (n = 4). Significant differences between the AM root-associated communities are indicated in bold.

Distance matrix	Observed δ	Expected δ	Variance of δ	Skewness of δ	*T*	*A*	*p*
Sørensen	0.3592	0.4603	$1.66E^{-03}$	-0.9457	-2.4789	0.2197	**0.0212***
---------- **Crop rotations** ----------							
Chickpea-Wheat	vs.	Lentil-Wheat			0.5285	-0.0496	0.6508
Chickpea-Wheat	vs.	Pea-Wheat			-1.4415	0.1585	0.0869
Chickpea-Wheat	vs.	Wheat-Wheat			-1.4714	0.1131	0.0858
Lentil-Wheat	vs.	Pea-Wheat			-2.2731	0.2280	**0.0342***
Lentil-Wheat	vs.	Wheat-Wheat			0.1817	-0.0126	0.4482
Pea-Wheat	vs.	Wheat-Wheat			-3.5177	0.4173	**0.0102***

* Significant effect at $p \leq 0.05$.

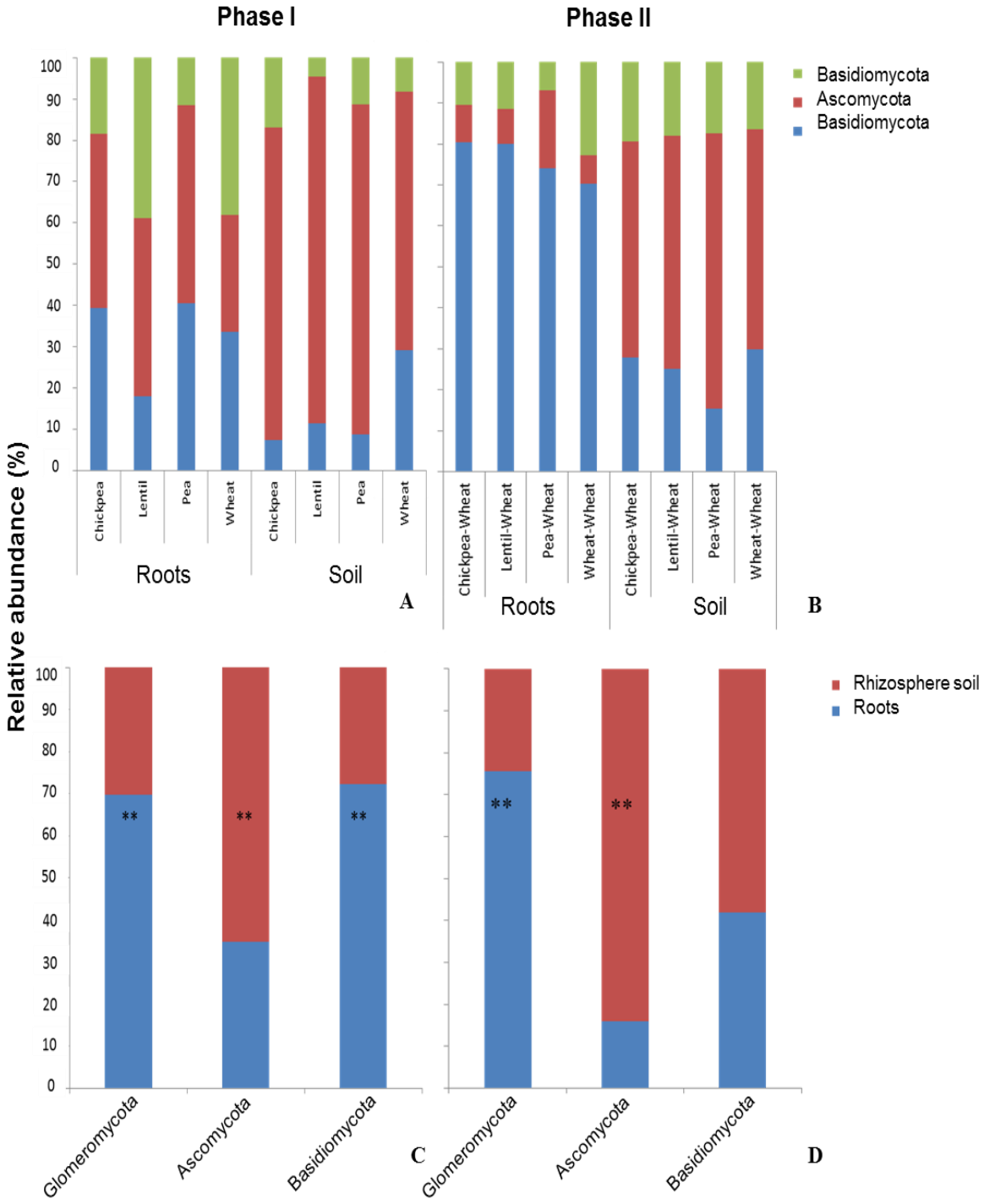

Figure A1. Relative abundance of Ascomycota, Basidiomycota and Glomeromycota in roots and rhizosphere soil in rotation phase I (A, C) and II (B, D). Significant differences between the rhizosphere soil and root fungal communities according to Student's t test (n = 4) are indicated by * ($p \leq 0.05$) and ** ($p \leq 0.01$).

7.2 Appendix B: Non-AM sequence analysis in phases I and II of the rotations

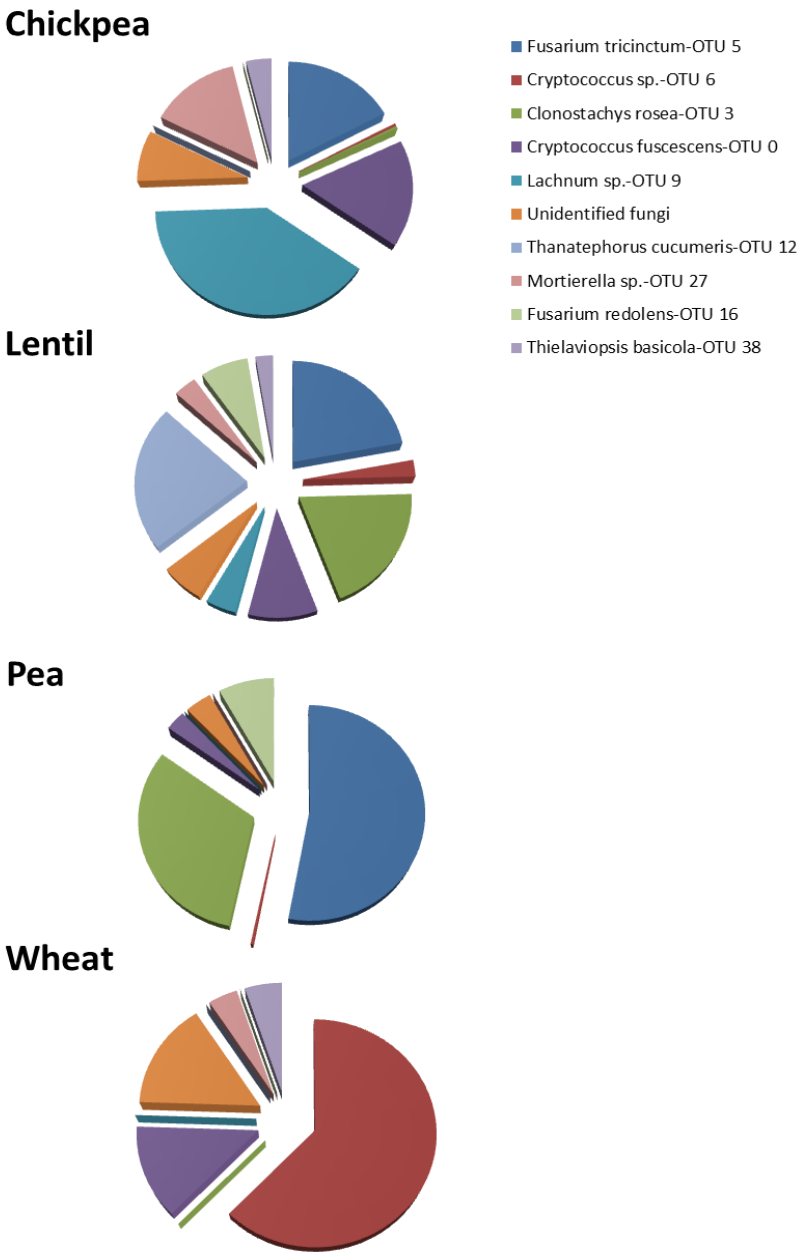

Figure B1. Effect of plant species on the non-AM fungal community structure in the roots of chickpea, lentil, pea and wheat grown in phase I of the rotations (n = 4).

Table B1. Summary statistics of the effects of crop and year/site on the complete data set of non-AM fungal OTUs and the most abundant non-AM fungal OTUs found at mid bloom in the roots of the crops grown during the phases I and II of the rotation, according to PerMANOVA. Significant probabilities are indicated in bold.

Source of variation	**Phase I – Pulses/Wheat (*N* = 16)**		**Phase II – Wheat (*N* = 32)**	
	Roots	**Soil**	**Roots**	**Soil**
Complete OTU dataset	– – – – – – – – – – – – Probability (*p*) – – – – – – – – – – – –			
Crop	**0.002****	0.758	0.896	0.051
Year/Site	NA†	NA	**0.001****	**0.001****
Crop*Year/Site	NA	NA	0.978	0.127
OTU subsample (occurrence ≥ 2%)				
Source of variation	– – – – – – – – – – – – Probability (*p*) – – – – – – – – – – – –			
Crop	**0.046***	0.508	0.912	0.096
Year/Site	NA	NA	**0.001****	**0.001****
Crop*Year/Site	NA	NA	0.998	0.999

†Not available

* Significant effect at $p \leq 0.05$

** Significant effect at $p \leq 0.01$.

7.3 Appendix C: Soil microbial fatty acid markers in phases I and II of the rotations

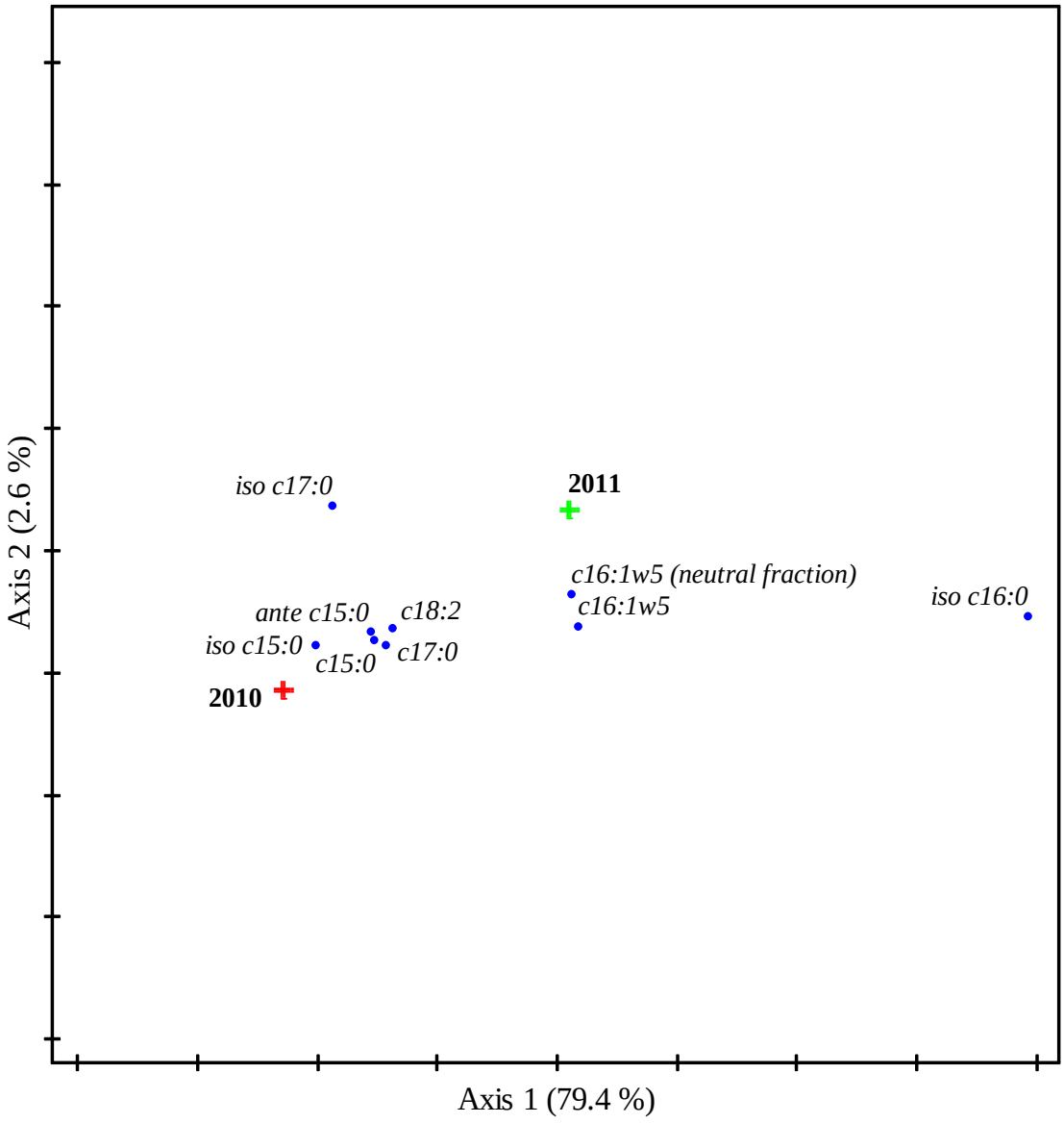

Figure C1. Nonmetric multidimensional scaling ordination of the year/site 2010 and 2011 of rotation phase I, based on the soil microbial PLFA (*n* = 4).

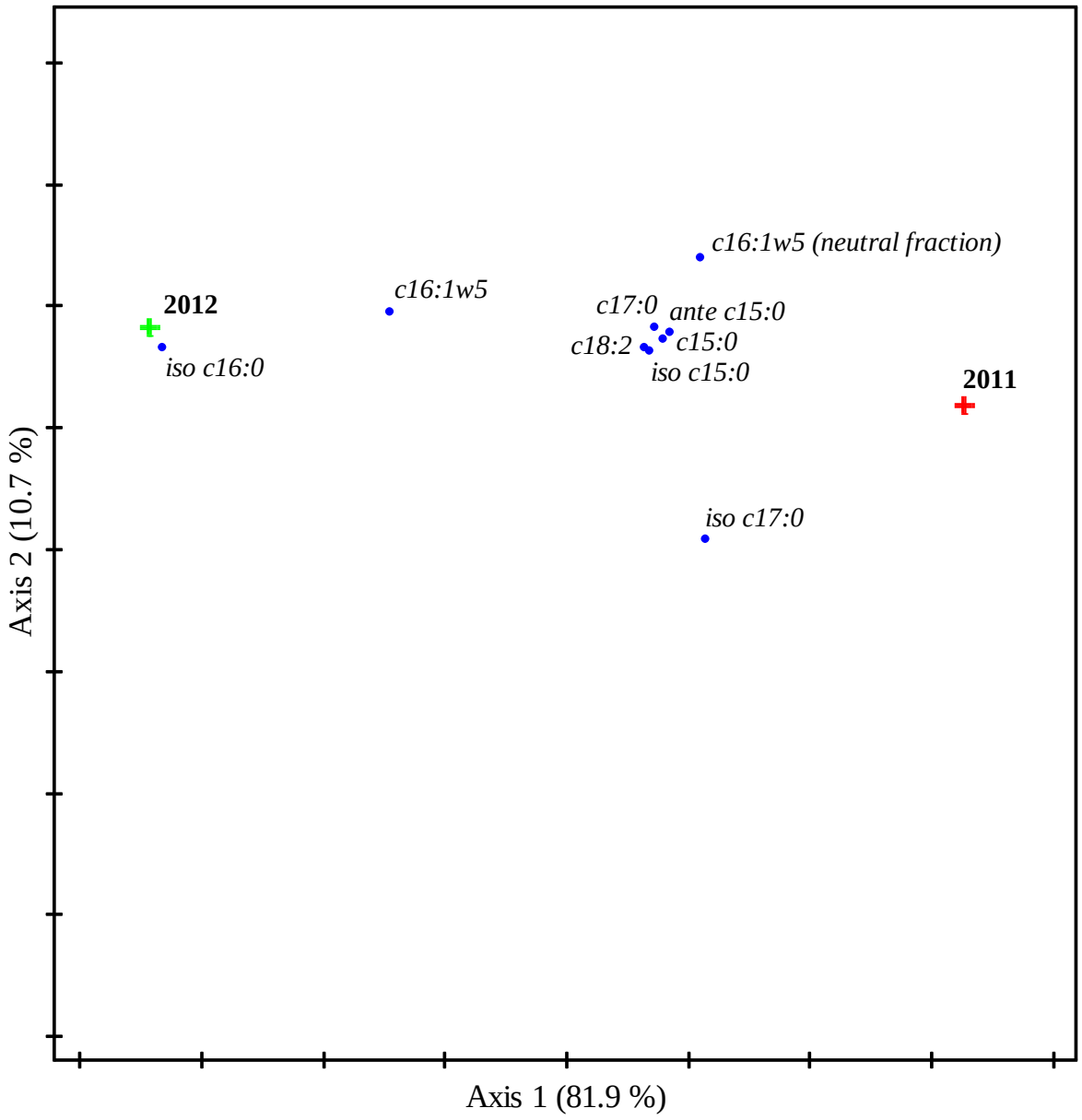

Figure C2. Nonmetric multidimensional scaling ordination of the year/site 2011 and 2012 of rotation phase II, based on the soil microbial PLFA (*n* = 4).

7.4 Appendix D: Characterization of the AM and non-AM fungal community associated with wheat roots in 4-year rotation systems

Table D1. Summary statistics for multi-response permutation procedures and multiple pairwise comparisons of the relative abundance of AM fungal community found in Ra_P-IV of wheat and that detected in Sf_P-III (n = 4). Significant differences between the root and soil communities are indicated by * ($p \leq 0.05$) and ** ($p \leq 0.01$) and presented in bold letters.

Distance matrix	**Observed δ**	**Expected δ**	**Variance of δ**	**Skewness of δ**	***T***	***A***	***p***
Sørensen	0.4425	0.4712	$0.20314E^{-3}$	-0.3018	-2.0178	0.0610	**0.0292***
Multiple comparisons							
CHP-WHT-CHP-soil		vs.	CHP-WHT-WHT-soil		-1.4595	0.0774	0.0769
CHP-WHT-CHP-soil		vs.	LEN-WHT-CHP-soil		0.2856	-0.0157	0.5531
CHP-WHT-CHP-soil		vs.	LEN-WHT-LEN-soil		1.3266	-0.0691	0.9243
CHP-WHT-CHP-soil		vs.	PEA-WHT-LEN-soil		0.8621	-0.0420	0.8017
CHP-WHT-CHP-soil		vs.	PEA-WHT-PEA-soil		0.3195	-0.0188	0.5506
CHP-WHT-CHP-soil		vs.	PEA-WHT-WHT-soil		0.5835	-0.0306	0.6697
CHP-WHT-CHP-soil		vs.	WHT-WHT-WHT-soil		0.1549	-0.0074	0.4864
CHP-WHT-CHP-soil		vs.	CHP-WHT-CHP-WHT-roots		-0.7556	0.0500	0.2061
CHP-WHT-CHP-soil		vs.	CHP-WHT-WHT-WHT-roots		-1.4814	0.0758	0.0824
CHP-WHT-CHP-soil		vs.	LEN-WHT-CHP-WHT-roots		-3.5131	0.2857	**0.0080****
CHP-WHT-CHP-soil		vs.	LEN-WHT-LEN-WHT-roots		-2.7928	0.2140	**0.0163***
CHP-WHT-CHP-soil		vs.	PEA-WHT-LEN-WHT-roots		-1.6025	0.0811	0.0660
CHP-WHT-CHP-soil		vs.	PEA-WHT-PEA-WHT-roots		-1.6049	0.0813	0.0613
CHP-WHT-CHP-soil		vs.	PEA-WHT-WHT-WHT-roots		-0.1651	0.0106	0.3471
CHP-WHT-CHP-soil		vs.	WHT-WHT-WHT-WHT-roots		-0.0744	0.0034	0.4499
CHP-WHT-WHT-soil		vs.	LEN-WHT-CHP-soil		0.7361	-0.0360	0.7504
CHP-WHT-WHT-soil		vs.	LEN-WHT-LEN-soil		-1.0325	0.0509	0.1507
CHP-WHT-WHT-soil		vs.	PEA-WHT-LEN-soil		0.5267	-0.0359	0.6822
CHP-WHT-WHT-soil		vs.	PEA-WHT-PEA-soil		-2.2021	0.1400	**0.0292***
CHP-WHT-WHT-soil		vs.	PEA-WHT-WHT-soil		-0.7306	0.0455	0.2069

Table D1 (continued)

Multiple comparisons			***T***	***A***	***p***
CHP-WHT-WHT-soil	vs.	WHT-WHT-WHT-soil	-1.8997	0.0755	0.0355
CHP-WHT-WHT-soil	vs.	CHP-WHT-CHP-WHT-roots	-1.2040	0.1025	0.1178
CHP-WHT-WHT-soil	vs.	CHP-WHT-WHT-WHT-roots	-1.9627	0.0812	0.0315*
CHP-WHT-WHT-soil	vs.	LEN-WHT-CHP-WHT-roots	-3.8940	0.2957	0.0069**
CHP-WHT-WHT-soil	vs.	PEA-WHT-LEN-WHT-roots	-1.0052	0.0594	0.1530
CHP-WHT-WHT-soil	vs.	PEA-WHT-PEA-WHT-roots	-2.2232	0.1100	**0.0336***
CHP-WHT-WHT-soil	vs.	PEA-WHT-WHT-WHT-roots	0.1674	-0.0114	0.5249
LEN-WHT-CHP-soil	vs.	PEA-WHT-WHT-soil	-0.5050	0.0220	0.2929
LEN-WHT-CHP-soil	vs.	WHT-WHT-WHT-soil	0.0572	-0.0034	0.4819
LEN-WHT-CHP-soil	vs.	CHP-WHT-CHP-WHT-roots	1.6369	-0.0802	0.9718
LEN-WHT-CHP-soil	vs.	CHP-WHT-WHT-WHT-roots	0.2905	-0.0169	0.5719
LEN-WHT-CHP-soil	vs.	LEN-WHT-CHP-WHT-roots	0.7857	-0.0359	0.7837
LEN-WHT-CHP-soil	vs.	LEN-WHT-LEN-WHT-roots	0.1726	-0.0081	0.5553
LEN-WHT-CHP-soil	vs.	PEA-WHT-LEN-WHT-roots	-0.7216	0.0479	0.2232
LEN-WHT-CHP-soil	vs.	PEA-WHT-PEA-WHT-roots	-0.6096	0.0394	0.2392
LEN-WHT-CHP-soil	vs.	PEA-WHT-WHT-WHT-roots	-3.1057	0.1967	**0.0112***
LEN-WHT-CHP-soil	vs.	WHT-WHT-WHT-WHT-roots	-2.3277	0.1530	**0.0302***
LEN-WHT-LEN-soil	vs.	PEA-WHT-LEN-soil	-0.4621	0.0220	0.2968
LEN-WHT-LEN-soil	vs.	PEA-WHT-PEA-soil	-0.5066	0.0218	0.2936
LEN-WHT-LEN-soil	vs.	PEA-WHT-WHT-soil	1.0810	-0.0740	0.8883
LEN-WHT-LEN-soil	vs.	WHT-WHT-WHT-soil	0.3591	-0.0139	0.6031
LEN-WHT-LEN-soil	vs.	CHP-WHT-CHP-WHT-roots	0.6034	-0.0278	0.6948
LEN-WHT-LEN-soil	vs.	CHP-WHT-WHT-WHT-roots	0.1576	-0.0076	0.4814
LEN-WHT-LEN-soil	vs.	LEN-WHT-CHP-WHT-roots	0.2323	-0.0151	0.4984
LEN-WHT-LEN-soil	vs.	LEN-WHT-LEN-WHT-roots	0.5519	-0.0321	0.6515
LEN-WHT-LEN-soil	vs.	PEA-WHT-LEN-WHT-roots	-0.5481	0.0357	0.2524
LEN-WHT-LEN-soil	vs.	PEA-WHT-PEA-WHT-roots	-0.9923	0.0433	0.1577
LEN-WHT-LEN-soil	vs.	PEA-WHT-WHT-WHT-roots	-2.9036	0.2302	**0.0152***
LEN-WHT-LEN-soil	vs.	WHT-WHT-WHT-WHT-roots	-2.4376	0.1615	**0.0245***
PEA-WHT-LEN-soil	vs.	PEA-WHT-PEA-soil	-1.8206	0.0879	**0.0459***
PEA-WHT-LEN-soil	vs.	PEA-WHT-WHT-soil	-1.3587	0.0770	0.0972
PEA-WHT-LEN-soil	vs.	WHT-WHT-WHT-soil	0.1360	-0.0073	0.5279

Table D1 (continued)

Multiple comparisons			***T***	***A***	***p***
PEA-WHT-LEN-soil	vs.	CHP-WHT-CHP-WHT-roots	0.2219	-0.0178	0.5518
PEA-WHT-LEN-soil	vs.	CHP-WHT-WHT-WHT-roots	-0.1687	0.0117	0.3563
PEA-WHT-LEN-soil	vs.	LEN-WHT-CHP-WHT-roots	-2.9663	0.1952	**0.0140***
PEA-WHT-LEN-soil	vs.	LEN-WHT-LEN-WHT-roots	-2.2957	0.1566	**0.0280***
PEA-WHT-LEN-soil	vs.	PEA-WHT-LEN-WHT-roots	-0.0489	0.0030	0.4531
PEA-WHT-LEN-soil	vs.	PEA-WHT-PEA-WHT-roots	0.6050	-0.0295	0.7050
PEA-WHT-LEN-soil	vs.	PEA-WHT-WHT-WHT-roots	0.6553	-0.0442	0.7246
PEA-WHT-LEN-soil	vs.	WHT-WHT-WHT-WHT-roots	0.9518	-0.0572	0.8291
PEA-WHT-PEA-soil	vs.	PEA-WHT-WHT-soil	0.6221	-0.0425	0.6885
PEA-WHT-PEA-soil	vs.	WHT-WHT-WHT-soil	-1.4502	0.0839	0.0876
PEA-WHT-PEA-soil	vs.	CHP-WHT-CHP-WHT-roots	-0.4135	0.0282	0.2873
PEA-WHT-PEA-soil	vs.	CHP-WHT-WHT-WHT-roots	-1.6054	0.1011	0.0729
PEA-WHT-PEA-soil	vs.	LEN-WHT-CHP-WHT-roots	-3.6992	0.3199	**0.0072****
PEA-WHT-PEA-soil	vs.	LEN-WHT-LEN-WHT-roots	-3.4189	0.2672	**0.0087****
PEA-WHT-PEA-soil	vs.	PEA-WHT-LEN-WHT-roots	-1.8403	0.0917	**0.0441***
PEA-WHT-PEA-soil	vs.	PEA-WHT-PEA-WHT-roots	-0.3923	0.0200	0.2963
PEA-WHT-PEA-soil	vs.	PEA-WHT-WHT-WHT-roots	-0.5386	0.0308	0.2905
PEA-WHT-PEA-soil	vs.	WHT-WHT-WHT-WHT-roots	-0.0276	0.0013	0.4396
PEA-WHT-WHT-soil	vs.	WHT-WHT-WHT-soil	-0.4217	0.0272	0.2688
PEA-WHT-WHT-soil	vs.	CHP-WHT-CHP-WHT-roots	-0.1955	0.0139	0.3614
PEA-WHT-WHT-soil	vs.	CHP-WHT-WHT-WHT-roots	-1.3709	0.0847	0.0965
PEA-WHT-WHT-soil	vs.	LEN-WHT-CHP-WHT-roots	-3.3449	0.2781	**0.0101***
PEA-WHT-WHT-soil	vs.	LEN-WHT-LEN-WHT-roots	-3.0093	0.2404	**0.0131***
PEA-WHT-WHT-soil	vs.	PEA-WHT-LEN-WHT-roots	-0.9959	0.0620	0.1582
PEA-WHT-WHT-soil	vs.	PEA-WHT-PEA-WHT-roots	-0.0468	0.0025	0.3979
PEA-WHT-WHT-soil	vs.	PEA-WHT-WHT-WHT-roots	-0.5441	0.0302	0.2789
PEA-WHT-WHT-soil	vs.	WHT-WHT-WHT-WHT-roots	0.2381	-0.0115	0.5626
WHT-WHT-WHT-soil	vs.	CHP-WHT-CHP-WHT-roots	-1.1884	0.0799	0.1207

Table D1 (continued)

Multiple comparisons			***T***	***A***	***p***
WHT-WHT-WHT-soil	vs.	CHP-WHT-WHT-WHT-roots	-0.4919	0.0265	0.2590
WHT-WHT-WHT-soil	vs.	LEN-WHT-CHP-WHT-roots	-2.9850	0.2035	**0.0126***
WHT-WHT-WHT-soil	vs.	LEN-WHT-LEN-WHT-roots	-2.2431	0.1493	**0.0285***
WHT-WHT-WHT-soil	vs.	PEA-WHT-LEN-WHT-roots	-1.1714	0.0755	0.1232
WHT-WHT-WHT-soil	vs.	PEA-WHT-PEA-WHT-roots	-0.9702	0.0530	0.1618
WHT-WHT-WHT-soil	vs.	PEA-WHT-WHT-WHT-roots	-0.1546	0.0075	0.4364
WHT-WHT-WHT-soil	vs.	WHT-WHT-WHT-WHT-roots	-1.0156	0.0334	0.1535
CHP-WHT-CHP-WHT-roots	vs.	CHP-WHT-WHT-WHT-roots	0.4861	-0.0371	0.6227
CHP-WHT-CHP-WHT-roots	vs.	LEN-WHT-CHP-WHT-roots	-1.1227	0.0945	0.1261
CHP-WHT-CHP-WHT-roots	vs.	LEN-WHT-LEN-WHT-roots	-1.0976	0.0760	0.1361
CHP-WHT-CHP-WHT-roots	vs.	PEA-WHT-LEN-WHT-roots	0.4124	-0.0306	0.6261
CHP-WHT-CHP-WHT-roots	vs.	PEA-WHT-PEA-WHT-roots	0.6336	-0.0536	0.7015
CHP-WHT-CHP-WHT-roots	vs.	PEA-WHT-WHT-WHT-roots	0.1613	-0.0127	0.5282
CHP-WHT-CHP-WHT-roots	vs.	WHT-WHT-WHT-WHT-roots	1.3744	-0.1231	0.9326
CHP-WHT-WHT-WHT-roots	vs.	LEN-WHT-CHP-WHT-roots	0.5652	-0.0298	0.7056
CHP-WHT-WHT-WHT-roots	vs.	LEN-WHT-LEN-WHT-roots	0.9636	-0.0589	0.8452
CHP-WHT-WHT-WHT-roots	vs.	PEA-WHT-LEN-WHT-roots	0.9302	-0.0595	0.8231
CHP-WHT-WHT-WHT-roots	vs.	PEA-WHT-PEA-WHT-roots	0.5495	-0.0431	0.6439
CHP-WHT-WHT-WHT-roots	vs.	PEA-WHT-WHT-WHT-roots	0.7571	-0.0420	0.7602
CHP-WHT-WHT-WHT-roots	vs.	WHT-WHT-WHT-WHT-roots	1.3051	-0.0780	0.9264
LEN-WHT-CHP-WHT-roots	vs.	LEN-WHT-LEN-WHT-roots	1.6901	-0.1102	0.9840
LEN-WHT-CHP-WHT-roots	vs.	PEA-WHT-LEN-WHT-roots	-1.7693	0.1260	0.0612
LEN-WHT-CHP-WHT-roots	vs.	PEA-WHT-PEA-WHT-roots	-1.8209	0.1462	0.0566
LEN-WHT-CHP-WHT-roots	vs.	PEA-WHT-WHT-WHT-roots	-0.6999	0.0442	0.2254
LEN-WHT-CHP-WHT-roots	vs.	WHT-WHT-WHT-WHT-roots	-0.4467	0.0285	0.2530
LEN-WHT-LEN-WHT-roots	vs.	PEA-WHT-LEN-WHT-roots	-1.3244	0.0956	0.1006
LEN-WHT-LEN-WHT-roots	vs.	PEA-WHT-PEA-WHT-roots	-1.7465	0.1293	0.0626
LEN-WHT-LEN-WHT-roots	vs.	PEA-WHT-WHT-WHT-roots	-0.2301	0.0166	0.3241

Table D1 (continued)

Multiple comparisons			***T***	***A***	***p***
PEA-WHT-PEA-WHT_roots	vs.	WHT-WHT-WHT-WHT_roots	0.8350	-0.0565	0.7954
PEA-WHT-WHT-WHT_roots	vs.	WHT-WHT-WHT-WHT_roots	0.8783	-0.0757	0.8395

Table D2. Probability of effects of crop rotation and year/site on the AM fungal community based on all OTUs and most abundant OTUs (> 2 %) in Sf_P-III and Ra_P-IV according to PerMANOVA. Significant differences are indicated in bold.

Source of variation	**Phase III – Pulses & Wheat**	**Phase IV - Wheat**
Complete OTU data set (234 OTUs)	Probability (*p*)	
Crop rotation	0.509	0.904
Year/Site	**0.001****	NA†
Crop rotation*Year/Site	0.949	NA
Most abundant OTUs (occurrence > 2%)	Probability (*p*)	
Crop rotation	0.556	0.894
Year/Site	**0.001****	NA
Crop rotation*Year/Site	0.989	NA

† Not applicable

** Significant effect at $p \leq 0.01$.

Table D3. Probability of effects of crop rotation and year/site on the non-AM fungal community based on all and most abundant OTUs (> 2 %) detected in Sf_P-III and in Ra_P-IV, according to PerMANOVA. Significant differences are indicated in bold.

Source of variation	Phase III – Pulses & Wheat	Phase IV - Wheat
Complete OTU data set (313 OTUs)	Probability (*p*)	
Crop rotation	**0.001****	0.541
Year/Site	**0.001****	NA†
Crop rotation*Year/Site	**0.001****	NA
Most abundant OTUs (occurrence > 2%)	Probability (*p*)	
Crop rotation	**0.001****	0.521
Year/Site	**0.001****	NA
Crop rotation*Year/Site	**0.027***	NA

† Not applicable
* Significant effects at $p \leq 0.05$
** Significant effects at $p \leq 0.01$

7.5 Appendix E: Root colonization by AM fungi in phase III of the rotations

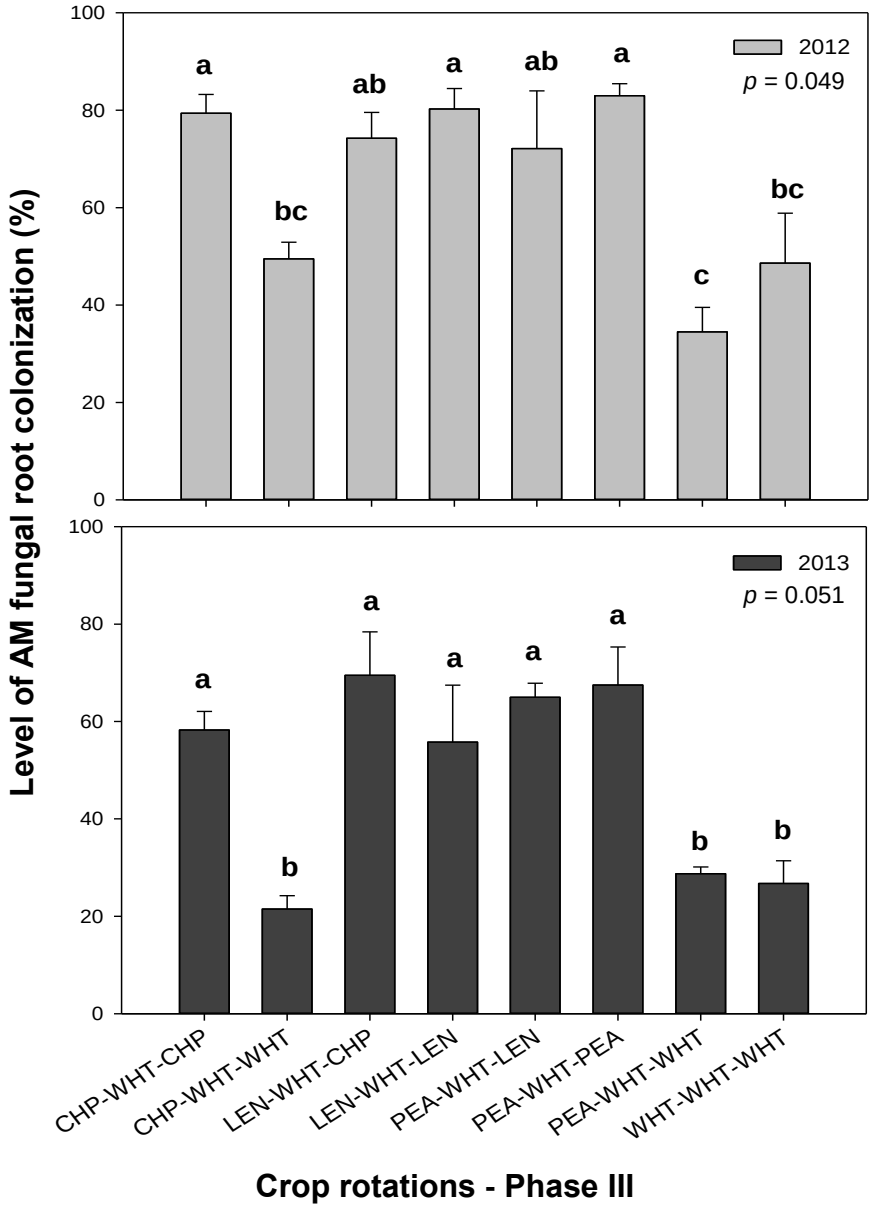

Figure E1. Least square means of the levels of root colonization by AM fungi, as influenced by crop rotation and year/site of cultivation. Means with standard errors are significantly different when associated with different letters according to Kruskal-Wallis test (n = 4).