ASSESSMENT OF ARBUSCULAR MYCORRHIZAL FUNGI IN FLAX PRODUCTION

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ABSTRACT

Arbuscular mycorrhizal fungi (AMF) play an important role in nutrient cycling and growth of flax (*Linum usitatissimum* L.). However, limited information is available regarding the symbiotic association between flax and AMF in field environments. A study was conducted to survey AMF communities colonizing flax grown in Saskatchewan. Additionally, field and growth chamber studies investigated the impact of AMF inoculation on nutrient uptake and growth of flax. Eighteen commercial flax fields were surveyed to assess mycorrhizal colonization of flax and to assess the impact of agricultural practices and soil abiotic factors on AMF activity. The flax root-associated AMF communities were explored using a 454 sequencing method, together with microscopic-based measurements of root AMF colonization and soil spore density. High levels of root colonization were detected in most flax fields. Of the 222 AMF operational taxonomic units (OTUs) identified in flax roots, 181 OTUs clustered as *Funneliformis-Rhizophagus*, 19 as *Claroideoglomus*, 14 as *Paraglomus*, six as *Diversisporales* and two as *Archaeospora*. Results suggest that tillage influenced the composition of AMF communities colonizing flax, and reduced AMF abundance and species richness. Additionally, AMF community characteristics were related to soil abiotic factors such as pH, EC, available phosphorus and nitrogen. Field experiments were conducted over two years (two sites per year) using a commercial AMF inoculant applied at three rates (0, 1X, and 2X the recommended rate) with or without P fertilizer (16.8 kg ha^{-1}). The response of flax cultivars to AMF inoculation was examined in a growth chamber experiment. In addition, 454 sequencing was employed to examine the impact of AMF inoculation on root-associated AMF communities. Under field conditions, only one site showed increased root colonization with AMF inoculation. Flax responded to AMF inoculation differently under different field conditions. At the two sites with intermediate initial soil P level, evidence of increased above-ground biomass and plant nutrient uptake with AMF inoculation was observed. However, such an effect was not detected when P fertilizer was combined with the inoculation. At a low P site and an irrigated site, P application accounted for all of the increases in plant nutrient uptake and biomass of flax, whereas no responses to AMF inoculation were detected. The 454

sequencing revealed different inoculation-induced changes in the diversity and composition of root-associated AMF communities between sites, which was possibly related to different field environments and native AMF communities. In the growth chamber, AMF inoculation resulted in general increases of plant nutrient uptake among cultivars, but only one cultivar showed enhanced biomass with inoculation. The diversity of AMF communities colonizing different flax cultivars was generally reduced by AMF inoculation. Community composition shifted under AMF inoculation, and the shifts appeared to be cultivar specific. These results suggested that benefits of AMF inoculation in flax production are limited and currently not predictable, and the degree of response is likely dependent on a myriad of soil and environmental conditions.

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LIST OF ABBREVIATIONS

AMF	Arbuscular mycorrhizal fungi
ANOVA	Analysis of variance
DGGE	Denaturing gradient gel electrophoresis
EC	Electrical conductivity
HSD	Tukey's honestly significant difference
JS	Jumpstart®
MAP	Monoammonium phosphate
MPN	Most probable number
MRPP	Multiresponse permutation procedure
NMS	Nonmetric multidimensional scaling
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PLFA	Phospholipid fatty acid
SD	Standard deviation
SSU	Small subunits
TOC	Total organic carbon
T-RFLP	Terminal restriction fragment length polymorphism

1. INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) are ubiquitous plant symbionts that form beneficial associations in most of agricultural crops, where they play key roles in transferring nutrients between soil and plants, and maintaining soil quality (Smith and Read, 1998). Intensively managed soils often contain low levels of AMF taxa abundance and diversity (Oehl et al., 2010; Verbruggen et al., 2012). Insufficient root colonization by AMF may lead to suboptimal plant performance (Tompson, 1987). A reduction of diversity can also limit those benefits derived from AMF (Verbruggen et al., 2013). Introducing non-indigenous AMF inoculant is an emerging technology in agriculture, which aims to restore AMF abundance and functioning, thereby enhancing crop production.

Although AMF are not host-specific, the responses of different plant-fungus combinations are highly fungus- and plant species- specific (Klironomos et al., 2008). Therefore, the efficacy of AMF inoculation may vary, depending on the genotypes of the host crop and the inoculant species. Flax (*Linum usitatissimum* L.) is an important oilseed crop in western Canada, which has lower tolerance to seed-placed phosphorus (P) fertilizer as compared with other annual crops (Kalra and Soper, 1968). Studies have shown that flax is highly dependent on AMF for soil P supply (Thingstrup et al., 1998; Grant et al., 2009; Monreal et al., 2011). Thus, AMF inoculation may provide a potential means for increasing flax production through enhanced uptake of P and other nutrients.

It has been suggested that a precondition for achieving crop growth enhancement via the application of AMF inoculant is that AMF are limited under agricultural settings (Verbruggen et al., 2013). However, little is known about the current status of AMF in flax fields, especially for AMF species that perform symbiotic functions in flax roots. The soil AMF communities in the Canadian prairies have been revealed by a limited number of investigations. These studies focused mainly on soil AMF in wheat fields, and identified the influences of soil type, land use, and management system on soil AMF abundance, community diversity and composition

(Talukdar and Germida, 1993; Dai et al., 2012, 2013, 2014; Bainard et al., 2014). Moreover, the increasing use of 454 sequencing technique is an emerging trend in large-scale AMF surveys, and this technique outperforms previous microscopic and molecular methods in terms of resolution and magnitude (Lindahl et al., 2013). In order to increase the understanding of AMF resources in flax fields, AMF communities colonizing flax roots in 18 flax fields across Saskatchewan were investigated using 454 sequencing. Relationships between flax root-associated AMF communities and soil biotic and abiotic factors were explored.

In addition, the impact of introducing a commercial AMF inoculant on flax growth was explored using a series of field and growth chamber experiments. The objectives of these experiments were to: i) evaluate the effect of AMF inoculation on flax growth in fields with different soil P availability; ii) examine if there is cultivar preference on the effect of AMF inoculation; and iii) explore how indigenous root-associated AMF communities change with inoculation. Specifically, field experiments conducted in 2012 and 2013 examined the potential response of flax to different combinations of a commercial AMF inoculant and P fertilizer under both dryland and irrigated conditions. A growth chamber experiment further examined the impact of crop variety on AMF colonization and subsequent response to AMF inoculation.

This thesis is comprised of six chapters. Following the Introduction (Chapter 1) is the main body of the study, which is contained within Chapters 2 through 5. Chapter 2 is a review of the literature pertaining to background knowledge in AMF ecology, the development of AMF inoculation, and tools used in AMF studies. Chapter 3 describes a survey of root-associated AMF communities in flax fields in Saskatchewan. Chapter 4 describes the effect of AMF inoculation on flax production, which was assessed under different field conditions in two years. This chapter also describes a growth chamber experiment that examined the responses of different flax cultivars to AMF inoculation. Chapter 5 is the synthesis and conclusions. Chapter 6 is the last chapter, which is comprised of a list of the literature cited.

2. LITERATURE REVIEW

2.1 AMF Ecology

2.1.1 Life cycle of AMF

Arbuscular mycorrhizal fungi (AMF) are obligate symbionts that occur in most ecosystems and plant species. The earliest fossil evidence of mycorrhizal symbiosis can be dated to 450 Ma ago (Cairney, 2000). Mycorrhizal symbiosis is thought to have played a key role in forming the original land flora (Wang et al., 2010). The life cycle of AMF passes through spore germination, pre-symbiotic mycelial growth, root colonization, development of intraradical structures, extraradical mycelial growth, and spore reproduction (Smith and Read, 1998). How the interaction between AMF and plant roots is triggered remains unclear. Once spores and roots are connected through extensive hyphal networks, however, a rapid channel for nutrient translocation is established. Aside from the intra-cellular arbuscules, which distinguish AMF from other mycorrhizae, other functional structures (e.g., coiled hyphae and vesicles) form within plant cortical cells (Bonfante and Genre, 2010).

The functional attributes of AMF have been described as a mutualism-parasitism continuum (Johnson et al., 1992), with parasitism occurring when the net costs of symbiosis exceed the net benefits. This paradigm is currently being reevaluated, with molecular evidence that the mycorrhizal pathway makes major contributions to phosphorus (P) uptake even if there are negative plant growth responses (Smith et al., 2004; Smith and Smith, 2011). The commonly accepted negative correlation between soil P levels and AMF root colonization is also being challenged. A recent study by Bainard et al. (2014) demonstrated that some AMF taxa have a negative correlation with soil P levels in soil but a positive correlation in roots, indicating the AMF-soil P interaction needs reconsideration. The revolution in thinking about AMF ecology is attributable to a rapid advancement of cellular and molecular technologies. New paradigms are developing. The roles of AMF, from cellular to ecosystem scales, are being reinterpreted (Smith and Smith, 2011).

2.1.2 Nutrient transfer of AMF

Arbuscular mycorrhizal fungi are well known for absorbing soil P through their highly

developed external mycelium. Conventional nutrient cycling models describing AMF contributions to plant growth typically focus on describing the exchange of C and P between plants and the fungi (Smith and Read, 1998). Based on this model, when plant C loss exceeds the P supply provided by AMF, plant growth depressions can occur, and the symbiotic relationship therefore shifts from beneficial to parasitic. With an increased understanding of the mycorrhizal pathway (Smith et al., 2009), however, simple explanations for growth depressions based on excessive C drain are, in some instances, untenable. Similarly, the multifaceted functions of AMF in the entire ecosystem cannot be denied even if there is no direct benefit in terms of increased plant growth or P uptake (Smith et al., 2010). In addition, the convention model cannot fully explain why there is also N supply by the AMF pathway, since plants can directly absorb soil N through direct root pathway. Fitter et al. (2011) explained the process by which AMF influence the nutrient pathway using what they termed a 'null-model'. According to the model, plants exude increasing amounts of C to the mycorrhizosphere in response to the local nutrient influx of both P and N supplied by AMF. In turn, AMF take up these sugars, effectively acting as scavengers. It does not matter, therefore, whether or not the plants need AMF to provide N. The point is that when they receive C, they exchange N. This null model is representative of a trend in interpreting AMF functioning, in studies ranging from plant or fungi perspectives to those at ecosystem scales (Smith and Smith, 2011). Much more extensive studies are needed for verification however, since the difficulties of model interpretation apparently increase with complex soil circumstances and different plant-fungus combinations in the field (Hamel, 2004).

2.1.3 Ecological function of AMF

Arbuscular mycorrhizal fungi account for 5 to 10% of soil microbial biomass (Fitter et al., 2011). They are, therefore, important components of soil food chains and soil microbial nutrient pools. Beyond those nutritional benefits for plant productivity, the ecological significance of AMF in ecosystem functioning and sustainability is commonly accepted (Smith and Smith, 2011), such as their participation in the formation of soil aggregates by exudation of glomalin (Swaby, 1984; Rillig et al., 2001), and in the development of natural plant communities through

their specificity to host plants (Stover et al., 2012). There also is potential for AMF to alleviate damage to plants due to soil abiotic stresses, such as drought, disturbance, salinity, heavy metals and toxicity (Gholamhaseini et al., 2013; Stover et al., 2012; Mohanmad and Mittra, 2013; Wang et al., 2011).

2.1.4 AMF phylogeny and taxonomy

Arbuscular mycorrhizal fungi belong to the phylum Glomeromycota, which consists of 230 species according to a recent phylogenetic classification (Schüßler and Walker, 2010). Four orders encompassing eleven families are classified in the current scheme. The taxonomy of AMF was established through "examining all available molecular-phylogenetic evidence within the framework of phylogenetic hypotheses and incorporating congruent morphological characters" (Redecker et al., 2013). Substantial debate indicates that the biological species concept of AMF is poorly resolved (Sharmah et al., 2010). Overall, AMF taxonomy is set up according to the small subunits (SSU) database, but SSU data frequently overlap sequence haplotypes among related species (Schüßler and Walker, 2010). The data are not, therefore, sufficiently qualified to supply species-level resolution (Schüßler and Walker, 2010). Additionally, numerous unknown *Glomus* sequences have been observed in recent metagenomic investigations (Verbruggen et al., 2010; Dai et al., 2012; Bainard et al., 2014). This raises the possibility that only a small fraction of the genetic diversity of AMF is represented by the described species (Helgason and Fitter, 2009).

2.2 Integrating AMF in Agriculture

2.2.1 Agroecosystems

An agroecosystem targets high crop productivity with intensive human intervention. Recent AMF community studies using advanced molecular tools highlight the relationship between soil abiotic and biotic factors and AMF community structure in agriculture systems. Soil types and land use intensity are considered major factors influencing AMF community composition over large agro-ecological scales (Oehl et al., 2010; Dai et al., 2012; Dai et al., 2013; Moebius-Clune et al., 2013). Conventional management practices (e.g., tillage, cropping strategy, and fertilization) modify the physicochemical characteristics of soil, and therefore

have a strong impact on AMF.

Cropping systems on the Canadian prairie traditionally have been based on the production of wheat (*Triticum aestivum* L.), which is often grown in rotation with other annual crops (Dai et al., 2012). When a non-mycorrhizal host (e.g., canola) is included in the rotation system, AMF taxa associated with previous crops undergo a deprivation of support and subsequent reduction in AMF diversity (Varma, 2008). For example, Monreal et al. (2011) observed there were evident increases in flax root biomass and root colonization when flax was grown following wheat rather than canola (*Brassica napus* L.), a non-mycorrhizal crop. In addition, plant hosts and the fungi partners seem to be mutually selective, with different plants hosting different AMF communities (Klironomos, 2008; Gosling et al., 2013; Bainard et al., 2014). In the study by Kahiluoto et al. (2000), flax exhibited a temporarily high demand for AMF-associated P supply at a low soil P level, whereas the same dependence on AMF was not found in barley (*Hordeum vulgare* L.).

Tillage disrupts fungal mycelia networks in soil, limiting the AMF species left from previous crops (Kabir et al., 1997). Studies have reported that tillage affects AMF biomass (Helgason et al., 2010; Zhang et al., 2012), hyphae density (Sheng et al., 2013), plant nutrient concentration (Kabir et al., 1998), root AMF community diversity (Mirás-Avalos et al., 2011), and soil AMF community composition (Helgason et al., 2010). There are, however, contradictions concerning the degree of such influences. For example, Jansa et al. (2002) found that although tillage decreased sporulation of some AMF species and influenced AMF community structure, community diversity was not affected. Yet, in a recent review, Verbruggen et al. (2013) suggested that tillage could be used in reducing resident AMF, to optimize the establishment of introduced AMF inoculants.

The activities of AMF are known to be sensitive to fertilization. Numerous studies report negative correlations between high levels of P and N fertilizers and AMF root colonization (Smith and Read, 1998). However, there are contrasting opinions on the issue of assessing root AMF biomass using root colonization. Critics argue that because the measurement of AMF colonization per unit root length is based on growth of both roots and AMF, it is not a valid

measurement of real AMF growth response, since an increase in root length by P fertilizer with constant AMF biomass can also result in a reduction in root colonization (Smith and Smith, 2011). In addition, the negative impact of P and N fertilization on AMF community diversity have been shown in many studies (Wang et al., 2010; Lakshmipathy et al., 2012; Yoshimura et al., 2012; Sheng et al., 2013); however, other studies argue the degree of such an impact. For example, Beauregard et al. (2013) reported that long-term P fertilization in a sandy loam soil reduced the length of external AMF mycelium, but greatly increased AMF community population size, species richness, and community diversity. A maize study conducted by Tial et al. (2013) found that N fertilization at agronomic rates had only minimal impact on overall AMF diversity. Furthermore, there is variation in the adaptability to fertilization for different AMF species (de Miranda and Harris, 1994; Wang et al., 2010).

2.2.2 AMF community assembly theories

Community assembly theories focus on interpreting "how the interplay of dispersal, environmental compatibility and species interactions determines the occurrence of species" (Verbruggen et al., 2012). Understanding how communities assemble is a central goal in ecology. Moreover, an optimized theoretical basis is urgently needed for managing AMF resources in agroecosystems, when introducing AMF inoculant and realizing the potentials of AMF in bioremediation, phytoremediation, and ecosystem re-establishment. In natural ecosystems, AMF communities are regulated by niche-related factors and neutral processes (Dumbrell et al., 2010). The life cycle of AMF in both root and soil environments complicates niche space regulation. Soil factors and plant species may play equal roles in AMF community assemblage (Johnson et al., 1992). The temporal niches for soil and root AMF communities also have been discussed recently (Bainard et al., 2014). It has been posited that AMF community composition is regulated by the neutral (stochastic) processes to the extent that "these govern distribution on survival or extinction of species with similar traits related to their performance in the environment and to competitive ability" (Hubbel, 2001). Following the neutral model, AMF taxa distribution is largely regulated by community size-dependent stochastic extinctions (Sloan, 2006). The effect of dispersal limitation on natural AMF

community distribution in Europe was elaborated by Dumbrell et al. (2010). In a large survey across Chernozem soil zones of the Canadian prairies (Dai et al., 2012), soil type (Chernozem Great Group) was found to be the major driving factor for soil AMF distribution, indicating the predominance of neutral processes in Canadian agroecosystems. Such dispersal limitation was not found, however, in other large-scale investigations (Moebius-Clune et al., 2013; Dai et al., 2013). This raises the possibility that agroecosystems are homogenized by niche-related factors, uniform plant covers, and agricultural practices.

2.2.3 AMF resource management

Intensive agricultural settings restrict AMF abundance and have further influence on AMF diversity. It is important to distinguish between the two types of limitation, to guide agricultural practices and AMF application (Verbruggen et al., 2013). Reduced root colonization (abundance) may lead to suboptimal plant growth, which can be alleviated through optimized management practices or introducing AMF inoculants. In contrast, loss of gene pool (diversity) eliminates the complementary benefits of different AMF species and the potential for highest yielding plant-fungal combinations. Diversity is difficult to recover with current AMF technology (Verbruggen et al., 2013). Oehl et al. (2010) reported that the diversity of soil AMF communities was reduced by intensive land use in central Europe. However, in an extensive investigation of different types of land use and ecozones on the Canadian prairies (Dai et al., 2013), the diversity of soil AMF resources in crop lands was reported to be maintained at the same level as natural communities.

2.2.4 Application of AMF in horticulture

The assumption regarding AMF inoculation is that where either AMF abundance or function is restricted in a system, application of fungal inoculant can be beneficial to plant growth (Verbruggen, 2013). With the loss of AMF gene pool in horticulture soil due to long-time management disruptions, inoculation may effectively optimize AMF performance and increase crop yield (Tawaraya et al., 2012). Multiple horticulture crops have shown increased yields by inoculation. These include onion (*Allium. fistulosum*) (Tawaraya et al., 2012), garlic (*A. sativum*) (Al-Karake, 2006), spicy pepper (*Capsicum annuum L. var.* Longum) (Hernádi

and Sasvári, 2012), and sunflower (*Helianthus annuus*) (Gholamhaseini, 2013). Additionally, inoculation with AMF decreased organophosphorus pesticide residues in carrot (*Daucus carota subsp. sativus*) and green onion (Wang et al., 2010).

It is common for studies to report inconsistent results with AMF inoculation. Such results are important for understanding the limitation of current AMF industry. First, AMF inoculants typically are marketed as bio-enhancers to increase the utilization of nonrenewable fertilizers, and therefore, a small rate of P fertilization is still needed. Tawaraya et al. (2012) examined the effect of AMF inoculation on onion with four soil available P levels. They found that inoculated plants grown in soil containing 300 mg P_2O_5 P kg^{-1} reached yields similar to those of non-inoculated plants grown in soil containing 1000 mg P_2O_5 P kg^{-1}. Second, inoculation may only be effective under specific conditions. Omirou et al. (2012) found that mycorrhizal inoculation improved root colonization and watermelon (*Citrullus lanatus*) responses under water stress only. Another study showed that AMF inoculation was able to compensate for the reduced N of sunflower leaves and seeds due to drought stress (Gholamhaseini, 2013). Third, there are functional differences among selected inoculant species. The study by Al-Karake (2006) found that garlic bulb production was significantly increased by *G. fasciculatum* inoculant under an intermediate soil P level. However, in a previous study using *G. mosseae* as the inoculant (Sari et al., 2002), neither mycorrhizal inoculation nor P supply was found to increase garlic growth.

2.2.5 AMF inoculation in agriculture

The development of AMF production technology has enabled massive production of AMF inoculant and its promotion in large-scale agroecosystems (Vosáta et al., 2012). Presumably, the agroecosystem situation is more complex than that of horticulture. Studies have shown that the efficacy of AMF inoculation is associated with various agricultural conditions, such as management practices (Sheng et al., 2013), crop types (Celebi, 2010), and weather (Knight, 2011) etc. Three factors generally dominate the establishment of AMF inoculant in agricultural systems: species compatibility (i.e., can the introduced species establish functional symbiosis?), field carrying capacity (i.e., does the habitat fit?), and priority effects (i.e., time factors and competition with local communities) (Verbruggen et al., 2013). Therefore, a critical evaluation

of the inoculation process requires not only traditional measurements of root colonization and crop growth properties, but also tracing genetic information regarding the establishment and persistence of AMF inoculants. For example, using LSU Glom1 primers and restriction fragment length polymorphism (RFLP), Pellegrino (2012) successfully traced two *F. mosseae* inoculants in the second year post inoculation. The contribution of inoculation to the stimulated root colonization and plant yield was confirmed.

Currently, massively commercial production of AMF inoculant is restricted to *Rhizophagus intraradices* (Vosáta et al., 2012). In order to remedy limitations of the single AMF inoculant, mixed inoculants including AMF and other microbial groups has been studied. A growth chamber study compared the use of single-species *R. irregularis* inoculant to mixed-inoculants (*R. irregularis, G. mosseae,* and *G. clarum*) in field pea (*Pisum sativum* L.) (Jin et al., 2013). The mixed inoculants promoted plant growth parameters without subsequent impact on root-associated AMF community composition. Wu et al. (2005) conducted a greenhouse study using AMF inoculant combined with three species of plant growth-promoting rhizobacteria in corn (*Zea mays* L). The combination significantly increased plant growth and nutrient uptake, and soil properties (organic matter and total N). Interestingly, there was a higher level of AMF infection when the bacterial inoculants were present, indicating the functional complementarity of microbial species. Given the inevitable limitations of AMF inoculant exhibited in horticulture and agriculture, it may be more feasible to apply AMF inoculant in confined field conditions (Vosátka et al. 2012).

2.3 Tools in AMF Studies

2.3.1 Traditional AMF studies

Before molecular tools were available, AMF studies centered on the determination of different forms of infective propagules, for example spores, extraradical hyphae, and infected roots, using microscopic techniques. Percent root colonization measures root AMF biomass as the proportion of the root length colonized by AMF, using a microscopic-based staining technique to reveal the intraradical structure of AMF. The staining quality depends on plant species and the quality of root materials (Carter and Gregorich, 2007).

Spore enumeration and morphology are traditional methods for estimating soil AMF biomass, taxonomic identification and diversity. Spores are commonly extracted using the density gradient centrifugation method together with spore sieving and decanting (Khan, 1999). Despite the concentration on spore morphological identification, the method is hampered by insufficient species-specific spore morphological characteristics, and therefore, may not necessarily reflect root AMF populations (Carter and Gregorich, 2007).

Several devices have been used specifically for measuring extraradical mycelium, such as the root exclusion chambers (Jakobsen et al., 1992), the membrane sandwich method (Giovannetti et al., 1993), and the inserted membrane techniques (Baláz and Vosátka, 2001). The phospholipid fatty acid marker (PLFA) method is widely used for estimation of extraradical AMF biomass at present (Podila and Varma, 2005).

2.3.2 Molecular methods in AMF studies

The development of DNA-based techniques enabled direct evaluation of AMF communities in environmental samples, and has greatly increased the understanding of community dynamics and functioning of AMF in different ecosystems. Polymerase chain reaction (PCR)-based denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) are both community fingerprinting technologies that have been widely used for the detection of the fungal 18S rRNA gene in AMF studies (Robinson-Boyer, 2009). The DGGE method separates DNA fragments based on different electrophoretic mobilities of partially melted double-stranded DNA molecules in polyacrylamide gels (Muyzer, 1999). This method has pitfalls in that DNA fragments from different origins may have similar migration behaviors (Ma et al., 2005), and therefore, there is a risk of underestimating the number of AMF species present. Most studies report a low recovery of AMF community profiles using DGGE and following sequence analyses (Ma et la., 2005; Yang et al., 2010; Beauregard et al., 2013). The T-RFLP approach uses fluorescently labelled primers (5') and restriction enzymes to target specific terminal restriction fragments (T-RFs) that represent different types of organisms (Marsh, 2005). The T-RFLP method also has the risk of underestimating AMF community diversity, because DNA fragments with

different sequences can give rise to similar T-RF sizes. Additionally, T-RFLP has limitation in identifying low abundance taxa (< 1%) (Verbruggen et al., 2012). Moreover, both DGGE and T-RFLP methods are limited by the strictly qualitative nature of data output (Robinson-Boyer et al., 2009).

2.3.3 454 sequencing

Next generation sequencing technology allows investigators to examine the all genetic information in environmental samples. In the 454 sequencing platform, both the *in vitro* sample preparation and sequencing processes have been tremendously simplified, enabling sequencing reactions to be conducted at a previously impossible scale (Rothberg and Leamon, 2008). For example, the 454 GS FLX system is able to examine about 150 amplicon libraries per run at 1% frequency with high statistical confidence (expect 750,000 output reads for two region gasket). In addition, sequencing power for long-reads DNA fragments is being continuously optimized. For example, the 454 GS Junior and 454 GS FLX allows researchers to sequence amplicons up to 550 bp, and 454 GS FLX+ is currently available for amplicons up to 800 bp (Roche, 2013). Expanding the range that can be sequenced has resulted in an improvement in the ability to identify microbial communities.

The sequencing process starts with amplicon library preparation (Sanschagrin and Yergeau, 2014). Genomic DNA is firstly amplified by PCR using fusion primers containing sequencing adapters, keys, barcodes and template specific sequences. An emulsion PCR follows to bind DNA libraries to spherical particles and subsequent clonally amplify DNA fragments. The next step is to load the particles on a disposable chip and insert the chip into a sequencing machine (Roche, 454 life Sciences, US). Parallel sequencing is performed on a picotiter plate platform. When individual nucleotides pass over the plate, a fluorescent signal is generated, which proportionally reflects the number of appearances of each nucleotide. In the end, sequences are retrieved, and barcodes are used to track sample records.

The 454 sequencing has made a significant breakthrough in terms of resolution and magnitude as compared with earlier methods (Lindahl et al., 2013). However, to what extent 454 sequencing data can be used quantitatively is doubted (Sanschagrin and Yergeau, 2014).

Firstly, as a PCR-based technique, it has all the biases associated with DNA extraction, selection of primers and cycling conditions (Schloss et al., 2011). In addition, next-generation sequencing technology was originally developed for genome sequencing, which does not require stringent quality filtering measures and full length coverage for individual genes (Schloss et al., 2011). Therefore, the limitation of 454 sequencing for species level resolution and the risk of overestimating rare species due to sequencing errors must be considered. Finally, 454 sequencing is still an emerging technology and the ability of the sequencing platform to generate similar quality data might be unstable (Schloss et al., 2011).

2.3.4 Application of 454 sequencing in AMF study

With the advantages in template preparation, sequencing capacity and intensity, 454 sequencing has been increasingly used in AMF studies, particularly in large-scale surveys. Dumbrell (2011) examined the spatial-temporal dynamics of soil AMF communities in a British grassland by taking six samples on each of 11 dates from summer to winter. They used a semi-nested PCR protocol with primer pairs NS31/AM1 and WANDA/AM1 to target 250bp SSU rDNA. The bioinformatics process reduced sequences from 142,004 to 108,245, and generated 70 OTUs. Dai et al. (2012) surveyed soil AMF communities in 76 wheat fields across major soil zones of the Canada prairies. They used a nested PCR protocol with primer pairs NS1/NS4 and AMV4.5NF/AMDGR targeting at 250 bp SSU rDNA. A stringent sequence cleaning process was performed reducing sequences from 90,364 to 7,086, and 33 OTUs were generated. In another AMF survey in the prairie soil, Dai et al. (2013) extensively sampled 317 sites based on different land use type and ecozones. The primer pair AMV4.5NF/AMDGR was used in PCR to target 250 bp SSU rDNA. They retained an average of 4,213 reads per sample after cleaning; however, since other non-AMF groups were not excluded, the real number of AMF sequences obtained was estimated to be an average of 669 reads per sample, with 122 AMF OTUs generated in total. Bainard et al. (2014) recently conducted a field experiment to examine the spatial and temporal structure of both soil and root AMF communities, with factors of three plants and four sampling times, and four replicates for each factor. A nested PCR protocol with universal eukaryotic primers NS1/NS4 and AMF specific primers AML1/AML2

was performed followed by 454 GS FLX+ to target 800 bp SSU rDNA. The bioinformatics process retained 133,089 sequences from 296,566 total reads and generated 40 OTUs.

Based on the experience with 454 sequencing, the choice of PCR primers might be a major factor that determines the quality of output sequences. The average number of AMF sequences retained per sample in the study by Bainard et al. (2014) was found to be more than those of previous studies. The primer pairs NS1/NS4 and AML1/AML2 used were reported to be highly AMF specific, with < 0.01% of sequences generated from non-AMF organisms. However, 454 sequencing is a step-by-step procedure, and each step in the process of amplicon preparation, sequencing and bioinformatics analysis may influence the final result (Lindahl et al., 2013). Therefore, it is still difficult to compare the effectiveness of different PCR protocols with the limited evidence available at present. In addition, the variable numbers of OTUs among studies cannot be fully attributed to the different sampling scales. The differences in sequencing intensity, the quality control process, the choice of classification mechanisms in bioinformatics analysis, and the length of DNA fragments all can influence the OTUs generated (Schloss et al., 2010). Thus, Schloss et al. (2010) argued that a standardized bioinformatics protocol, or at least a standardized format for reporting the result of 454 sequencing and bioinformatics process is in urgent need.

2.3.5 Bioinformatics analysis of raw data from 454 sequencing

As discussed above, the raw sequences generated by 454 sequencing contain a high proportion of unnecessary sequences due to PCR bias and sequencing errors (Schloss et al., 2011). The goal of bioinformatics analysis is to reduce the error rates to the lowest level, while retaining as many sequences as possible (Schloss et al., 2011). The process can be done using publicly available bioinformatics software (e.g., Mothur) (Schloss et al., 2009). However, since Mothur was originally used for analyzing 16S rRNA gene (Schloss et al., 2011), each quality filtering step of the standardized work-flow needs to be carefully evaluated and parameters might need to be adjusted for analyzing AMF data. To date, bioinformatics steps commonly used by AMF researchers include trimming possible erroneous reads (e.g., unexpected length, ambiguous bases, homopolymers, mismatches to primers, and low quality scores), aligning

sequences with the Silva eukaryotic database and screening sequences with peculiar regions, detecting and removing chimeras, identifying AMF sequences from other groups, and clustering sequences into OTUs based on different similarity levels (Bainard et al., 2014a; Bazghaleh et al., 2015). Despite computational limitations, it is possible for Mothur to analyze in parallel the whole set of samples by consistently tracking sample records using a group file and a name file. Mothur also provides a variety of approaches for calculating Alpha diversity and Beta diversity. Alternatively, the matrix with sample units and OTU abundances (shared file) generated in Mothur is applicable to following diversity and compositional analyses in other multivariate analysis platforms (e.g., PC-ORD; MjM Software Design).

Raw reads generated from a 454 sequencing platform can be greatly reduced using bioinformatics steps. However, considering the lack of knowledge in the rate of PCR biases and sequencing errors, and in the efficiency of adopted cleaning steps, it is hard to evaluate if the quality control process is stringent enough, if the number of OTUs generated is as expected, and hence if these OTUs can reflect the AMF community structure in the sampling environment (Lindahl et al., 2013). A mock community approach was used by Schloss et al. (2011) for estimating the efficiency of quality filtering steps (for 16S rRNA gene) in Mothur. The stringent cleaning steps reduced the general sequencing error rate by 30-fold, but it was still impossible to obtain the number of OTUs expected. Thus, there is a gap between the fast development of sequencing technology and the modest gain in controlling data quality (Schloss et al., 2011).

Although there are limitations associated with the different techniques currently available to assess AMF abundance, techniques are rapidly improving and it is now possible to estimate AMF community diversity and composition with greater confidence than ever before. These analytical techniques are providing an abundance of information that allows researchers to assess natural AMF communities and consequent function.

2.3.6 Analysis of AMF communities

There are various statistical approaches to interpreting data generated from AMF community studies. The general workflow includes sample adequacy measurement, univariate

measurements of abundance, species richness, and community diversity, and multivariate comparison of species distribution (community composition) (McCune and Grace, 2002). Rarefaction analysis is commonly used for evaluating sampling adequacy of AMF communities. A rarefied curve is derived from averaging the randomizations of the accumulation curve (Heck et al, 1975). The asymptote of this curve is the species richness expected at infinite effort. The more representative the sample, the lower the distance between the curve and the asymptote. Species is the strict sense of community diversity, which is measured as the number of species in a sample (McCune and Grace, 2002). The Chao1 and abundance-based coverage estimators (ACE) estimate species richness by adding a correction factor to the original species richness (Hughes et al., 2001). For example, Chao 1 calculates total species richness as:

$$S_{chao1} = S_{obs} + \frac{n_1^2}{2n_2} \quad \text{(Eq. 2.1)}$$

Where S_{obs} is the number of observed species, n_1 is the number of singletons, and n_2 is the number of doubletons. The ACE differs from Chao1 in that it incorporate data of fewer than 10 individuals (Chao and Lee, 1992). The ACE estimates species richness as

$$S_{\mathrm{ACE}} = S_{\mathrm{abund}} + \frac{S_{\mathrm{rare}}}{C_{\mathrm{ACE}}} + \frac{F1}{C_{\mathrm{ACE}}}\gamma^2_{ACE} \quad \text{(Eq. 2.2)}$$

Where S_{rare} is the number of rare samples with sampled abundances$\leq$10 and S_{abund} is the number of abundant species, that is, sampled abundances $>$10. $C_{ACE} = 1 - F_1/N_{rare}$, estimates the sample coverage, where $F1$ is the number of species with i individuals, and $N_{rare} = \sum_{i=1}^{10} iF_i$. Additionally, γ^2_{ACE} estimates the coefficient of variation of the F_i's in the equation, as described in Chao and Lee (1992). In addition to richness, community diversity incorporates equitability. Specifically, Shannon-Wiener index (H') (Shannon and Weaver, 1949) and Simpson's index (D) (Simpson, 1949) estimate community diversity as

$$H' = -\sum_{i=1}^{S_{\mathrm{obs}}} \frac{n_i}{N} \ln \frac{n_i}{N} \quad \text{(Eq. 2.3)}$$

$$D = 1 - \sum_{i=1}^{S_{\mathrm{obs}}} \frac{n_i^2}{N} \quad \text{(Eq. 2.4)}$$

Where S_{obs} is the number of species (species richness), n_i is the number of species i, and N is the total number of species, n_i / N represents the proportion of individuals belonging to species

I. According to McCune and Grace (2002), Simpson's index emphasizes common species, which is less affected by rare species as compared with Shannon-Wiener index and species richness. At last, various multivariate tools are used to identify community structure, depending on the nature of data and the questions of interest (Peck, 2010).

3. ASSESSMENT OF THE ARBUSCULAR MYCORRHIZAL FUNGI (AMF) COMMUNITIES COLONIZING FLAX IN SASKATCHEWAN

3.1 Preface

Arbuscular mycorrhizal fungi (AMF) play an important role in transferring soil nutrients (e.g., nitrogen and phosphorus) to their host plants, thereby increasing plant growth. Manipulating AMF communities, either through introducing exotic strains or by management of indigenous communities, provides a potential to increase agricultural productivity in a low-input manner. In this chapter, a survey of root colonizing AMF communities in flax fields in the Canadian prairies is described. This study provides background knowledge regarding indigenous AMF communities, and thereby can serve as a basis for further studies regarding AMF resource management and AMF inoculation in flax fields.

3.2 Abstract

Arbuscular mycorrhizal fungi (AMF) present in flax fields were investigated based on 454 sequencing analyses of abundance, species richness, diversity and structure of flax root-associated AMF communities, together with traditional measurements of root AMF colonization and soil spore density. A total of 222 AMF operational taxonomic units (OTUs) were identified in root samples from 18 flax fields, with 181 OTUs clustered as *Funneliformis-Rhizophagus*, 19 as *Claroideoglomus*, 14 as *Paraglomus*, six as *Diversisporales* and two as *Archaeospora*. Tillage appeared to be a major factor regulating AMF assemblages in flax roots. Comparatively higher abundance and richness was found in no-till fields. There also were differences in the composition of root associated AMF communities between fields under conventional tillage and no-till. There were negative correlations between root AMF community characteristics (abundance, species richness or community diversity) and soil pH, EC and available phosphorus. The composition of the dominant root AMF OTUs was correlated with soil available nitrogen and phosphorus. Root AMF colonization was associated with soil spore density. There was also variation in root AMF colonization among flax growth stages. These results indicate that tillage, together with soil abiotic factors (pH, EC, soil P and N) influenced AMF communities colonizing flax roots in some instances. This study provides a basis for further AMF application and AMF resource management in flax fields.

3.3 Introduction

Managing soil microbial resources is an emerging trend in agriculture. Arbuscular mycorrhizal fungi (AMF) are the most common and ubiquitous terrestrial symbionts that make great contributions in ecosystem function and sustainability (Gianinazzi et al., 2010). Mycorrhizal symbiosis enables a fast and efficient pathway for transferring soil nutrients (e.g., phosphorus and nitrogen) to host plants (Smith and Smith, 2011), and has the potential to increase plant productivity. The AMF association also provides ecological services, such as stabilizing soil structure, improving the resistance of host plants to harsh environments (e.g., low fertility, salinity and drought), and maintaining plant diversity (Smith and Read, 2008). Given that different AMF species may vary in their ability to benefit crop productivity (Wang et al., 2011), the abundance and diversity of AMF must be considered in agroecosystems.

Flax (*Linum usitatissimu* L.) is a traditional oilseed crop in Saskatchewan, accounting for 70% of the total flax production of Canada, and 25% of the world. Typically sown directly into stubble, flax is favored as an important annual crop in a three or four year rotation sequence (Flax Council of Canada and Saskatchewan Flax Development Commission). Compared with other annual crops, flax is poorly responsive to phosphate fertilizer, possibly due to poor root proliferation within the fertilization band (Strong and Soper, 1974). Researchers have suggested that flax is particularly dependent on the AMF association for soil P supply. For example, Grant et al. (2009) found that early-season biomass, P concentration, P uptake and flax seed yield were greater when flax was seeded after wheat (*Triticum aestivum* L.) rather than after canola (*Brassica napus* L.), which is a non-mycorrhizal crop. However, little is known about AMF resources in flax fields, particularly regarding the AMF taxa that perform symbiotic functions within flax roots.

Agricultural practices (e.g., tillage, fertilization and monocropping) intensively modify soil physicochemical properties, and significantly influence the diversity and composition of AMF communities (Verbruggen et al., 2012; Sheng et al., 2013). According to the investigation by Verbruggen et al. (2012) about soil and root AMF communities in 40 agricultural soils in the Netherlands, management intensity (P availability and grass-cropping history) was found to

influence the AMF community assemblage more strongly than soil type and chemical properties. In another study, Bainard et al. (2014) found that plant host identity and temporal factors both affected the AMF community composition in soil and roots. The root AMF community was more responsive to both of the factors and was correlated with soil pH, electrical conductivity (EC), P flux, and climatic variables, but the soil AMF community was found to be associated with soil pH and EC only. To date, the limited number of AMF surveys in the Canadian prairies have all focused on soil AMF, and mainly with large-scale factors (e.g., soil type, land use and management system) identified (Talukdar and Germida, 1993; Dai et al., 2012, 2013, 2014; Bainard et al., 2015).

The objective of this study was to investigate AMF resources in flax fields in Saskatchewan. Whereas other studies have focussed on the soil AMF community composition, we focused on the AMF community colonizing flax roots. It was hypothesized that the AMF communities colonizing flax root are similar, irrespective of external biotic and abiotic factors, including agricultural practices. To test this hypothesis, flax roots were collected from 18 flax fields across Saskatchewan and the AMF communities colonizing roots were assessed. Management history, and basic soil properties for each of the fields were assessed. The relationships between root AMF and soil biotic and abiotic factors were assessed based on traditional measurements of root AMF colonization and soil spore density, as well as 454 sequencing-based measurements of abundance, diversity and community composition of the AMF association. Considering the substantial effect of dispersal limitation (Verbruggen et al., 2012) and temporal niche (Bainard et al., 2014), field locations and flax growth stages were recorded.

3.4 Materials and Methods

3.4.1 Description of the flax fields surveyed

A total of 18 flax fields distributed throughout Saskatchewan were investigated, with field details shown in Table 3.1. Soils were classified according to the *Canadian System of Soil Classification* (Soil Classification Working Group, 1998). Cropping history and management practices (e.g., no-till or organic management) was provided by the farmers. Flax crops were between first flower and late flower (growth stage from 7 to 9, Turner, 1987) during sampling.

Fields coded with the same capital letters and different numbers (e.g., A1 and A2, G1 and G2, H1 and H2, I1 and I2, and M1and M2) represent sites sampled in close proximity (mostly adjacent fields), whereas other fields coded with different capital letters indicate that the fields were not continuous and had different cropping histories, although in some instances, the fields were managed by the same producer. A map of the sampling sites is provided in Appendix A.

3.4.2 Soil and root sampling and determination of soil properties

Sampling took place at the reproductive stage of flax, between the 8th and 11th of July, 2013. Five sampling points were randomly chosen within a ca. 100-m^2 area at each survey field, avoiding field edges. At each point, five to ten flax roots and surrounding soil were excavated to a depth of 15 cm using a flat-nosed shovel. Roots with adhering soil were collected and kept on ice during transportation. Soil samples were separated by hand from the bulk root samples. Roots were then further washed free of soil, bulked, and divided into two subsamples. A subsample for DNA extraction was immediately immersed in liquid N stored at -80 °C. The remaining root samples were temporarily stored at 4 °C for further determination of AMF colonization. Soil samples were homogenized and passed through a 2-mm sieve. A subsample of soil was air-dried and used to determine soil characteristics. Briefly, soil texture was determined by hand (Pennock, 2005). Soil pH (1:2 soil:water slurry) and EC (1:2 soil:water extraction) were determined using PC 700 Meter (OAKTON instruments, Vernon Hills, IL, USA). Total C was measured by dry combustion in a LECO C632 carbon analyzer (LECO Corp., ST. Joseph, MI, USA). Soil N was extracted with KCl solution (Keeny and Nelson, 1982). Soil P and K was extracted with modified Kelowna solution (Qian et al., 1994). Soil available NO_3-N, NH_4-N, P and K were analyzed using an auto-analyzer (Technicon AAII system, Tarrytown, NY, USA). The remaining soil samples were kept at 4 °C until further determination of spore density.

3.4.3 Determinations of spore density and root colonization

The AMF spores were extracted from 20 g soil using a wet sieving and decanting method (Gerdemann and Nicholson, 1963), followed with sucrose density gradient centrifugation (Mertz et al., 1979). The spore suspension was passed through a Millipore filter (2.5 μm) and

washed with distilled water. Spore numbers were counted under a stereomicroscope using up to 50-fold magnification. The density of AMF spores was expressed on a soil dry weight basis. Root AMF colonization was determined using the grid-line intersect method (Giovannetti and Mosse, 1980). Root samples were stained using the ink-vinegar method (Vierheilig et al., 1998) and assessed for percentage of AMF colonized root length under a stereo-microscope (x 50) using a grid-line, with three technical replicates for each sample.

3.4.4 DNA extraction and preparation of amplicon libraries

Root tissue samples (100 mg) were placed in screw-top microcentrifuge tubes (2 mL) with four ceramic beads (5 mm) and 400 µL Lysis Buffer (Buffer AP1, DNeasy Plant Mini Kit, catalogue No. 69104, Qiagen, Inc) and pulverized into powder using a Tissue Homogenizer according to manufacturer's recommendations (Precellys®24, Bertin Technology, US). Root DNA was extracted using a DNeasy Plant Mini Kit (catalogue No. 69104, Qiagen, Inc) following the manufacturer's maximum yields protocol and stored at -20 °C.

DNA samples were submitted to Génome Québec and McGill University Innovation Centre for amplicon library preparation and sequencing analysis. A nested PCR protocol was chosen targeting *ca.* 800-bp region of AMF 18S rDNA, with universal eukaryotic primer pair NS1/NS4 (White et al., 1990) in the first PCR; AMF-specific primer pair AML1/AML2 (Lee et al., 2008) in the second PCR; and ligation of adaptors and barcode sequences in the third PCR run. The primer pair AML1/AML2 was designed to cover all the Glomeromycotan orders and has been successfully used by Bainard et al. (2014a). The 5 µL reaction mixtures in the first PCR contained 1 µL of diluted DNA (1:10), 0.4 µM L^{-1} of each primer (NS1 and NS4), 0.25 U μL^{-1} *Taq* DNA polymerase, 22.5 mmol L^{-1} $MgCl_2$, 50 mM L^{-1} Tris-HCl (pH 8.3), 250 mM L^{-1} KCl, 1 mM L^{-1} dNTP, and 0.25 µL DMSO. Thermocycling conditions in the first PCR consisted of an initial denaturing step at 95 °C for 15 min, 33 cycles of 95 °C for 20 s, 50 °C for 40 s, and 72 °C for 1 min 30 s, and a final extension step at 72 °C for 3 min. The 5 µL reaction mixtures in the second PCR contained 1 µL of PCR product, 0.1 µM L^{-1} of each primer (AML1 and AML2), 0.25 U μL^{-1} *Taq* DNA polymerase, 22.5mmol L^{-1} $MgCl_2$, 50 mM L^{-1} Tris-HCl (pH 8.3), 250 mM L^{-1} KCl, 1 mM L^{-1} dNTP, and 0.25 µL DMSO. Thermocycling conditions in the

second PCR consisted of an initial denaturing step at 95 °C for 15 min, 35 cycles of 95 °C for 20 s, 60 °C for 30 s, and 72 °C for 45 s, and ended with a final extension step at 72 °C for 5 min. The 20 μL reaction mixtures in the third PCR contained 1 μL of PCR product, 1 μM of barcode templates (each), 1 U μL^{-1} *Taq* DNA polymerase, 90 mmol L^{-1} $MgCl_2$, 200 mM L^{-1} Tris-HCl (pH 8.3), 1000 mM L^{-1} KCl, 4 mM L^{-1} dNTP, and 1 μL DMSO. Thermocycling conditions in the third PCR consisted of an initial denaturing step at 95 °C for 10 min, 15 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 1 min, and a final extension step at 72 °C for 3 min. The first and final PCR products were purified by Agencourt AMPure XP (Beckman Coulter, Agilent Technologies, US). The quality of PCR products was confirmed using 2100 Bioanalyzer (Agilent Technologies, US). PCR products were quantified using PicoGreen® dsDNA quantitation assay (Life Technologies, US) before pooling. Final pools were quantified with Qubit® 2.0 Fluorometer (Life Technologies). Sequencing was conducted on the 454 GS FLX+ system (Roche, 454 Life Sciences, US).

3.4.5 Bioinformatics and phylogenetic analysis

Sequences of each sample unit were provided by the data server in a ".fastq" format, free of barcodes and primers. Data were analyzed using Mothur, which is a comprehensive software package for analyzing community sequence data (Schloss et al., 2009). Functions of Mothur relevant to the current study include trimming, screening, and aligning sequences; calculating distances; assigning sequences to operational taxonomic units (OTUs); and describing α and β diversity (Schloss et al., 2009). Specifically, the ".fastq" files were converted to fasta and quality files. Quality filtering was applied by removing sequences with uncommon length (> 900 bp or < 700 bp), low quality (average score < 30), ambiguous bases, and homopolymers (> 8). After this step, sequences were merged together, using a ".group" file to track the sample membership of each sequence. Sequences were aligned to SILVA eukaryotic database using the Needleman-Wunsch global alignment algorithm (Needleman and Wunsch, 1970). Sequences outside the desired range in the alignment were removed. Chimeric sequences were detected using the UCHIME algorithm (Edgar et al., 2011) and suspicious sequences were removed. Sequences at this point may still contain sequences belonging to groups other than

Glomeromycota, which were identified by comparison with the Silva eukaryotic reference using a k-nearest neighbor consensus and Wang approach (Wang et al., 2007); non-Glomeromycota sequences were removed. Sequences were assigned into OTUs with the furthest neighbour clustering method based on 95% similarity. To limit overestimation of species richness due to sequencing errors (Unterseher et al., 2011), singletons and doubletons were excluded in this study. Taxonomic identities were obtained by blasting representative sequences of each OTU in GenBank. Non-Glomeromycotan sequences and sequences with poor blasting results (< 95% similarity) were further removed. Representative OTU sequences and AMF references sequences were aligned using MUSLE in MEGA 5.2 (Tamura et al., 2007), and a neighbour-joining phylogenetic tree was constructed using the Kimura two-parameter model with a bootstrap value of 1000. The nomenclature used in this study was based on the AMF taxonomy classification proposed by Schüßler and Walker (2010).

A representative sequence of each AMF OTU analyzed in this study was deposited in GeneBank under accession numbers KP988325 - KP988546 (OTUs 1 to 222).

3.4.6 Data analysis

Soil texture was assigned to three categories, loam, clay loam and clay. Only Site E was classified as a loam and was excluded from following analyses. Root AMF colonization was reported as mean and standard deviation (SD) of three sampling replicates. Prior to analyses, root colonization was arcsine transformed. Differences of root colonization between different flax growth stages, soil textures, and management practices (conventional tillage and no-till) were tested using one-way ANOVA after testing for normality and homogeneity by Bartlett's and Dunnett' tests, using a significance level of 0.05. Within group differences were further tested using Tukey's honestly significant difference (HSD) test. The relationship between average root AMF colonization and spore density was assessed by Pearson's correlation coefficient.

Sequencing intensity was tested using rarefaction analysis in Mothur. Samples with curves unable to approach the asymptote indicate a deficiency of sampling effort (sequences are not enough to cover the diversity of AMF community), and therefore were excluded from further

analyses. The Shannon-Wiener index (*H'*), Simpson's index (*D*), and abundance-based coverage estimators (ACE) were calculated as measures of diversity by Mothur (McCune and Grace, 2002; Chao and Lee, 1992). The effects of tillage, soil texture and plant growth stage on these AMF community characteristics were examined either using Student's t-test or one-way ANOVA. The univariate relationships between AMF community characteristics and edaphic variables (pH, EC, total organic carbon (TOC), nitrate (NO_3^-), ammonium (NH_4^+), phosphate (PO_4^{3-}), and potassium (K)) were tested by Pearson's correlation.

Community compositional analyses were conducted in PC-ORD (*v*. 6, MjM Software Design, Gleneden Beach, OR, US). Elements of the main matrix (for rows) and the second matrix (for columns) were relativized by maximum. The multiresponse permutation procedure (MRPP) was used to test individual effect of tillage management, soil texture and plant growth stage on AMF community structure. Nonmetric multidimensional scaling (NMS) was performed on the 30 most abundant fungal OTUs (reads ≥ 50), to visualize the differences of root colonizing AMF communities and to relate soil physicochemical properties. A two-way cluster dendrogram was used to further reveal the compositional differences of root AMF communities (30 dominant OTUs) between conventional tillage and no-till sites.

A significance level of $p < 0.05$ was considered in all the tests. Unless otherwise noted, statistical analyses were conducted in R (*v.* 3.0.2, R development Core Team).

3.5 Results

3.5.1 Soil factors, AMF spore density and root colonization

The density of AMF spores varied from site to site (Table 3.2). Comparatively high spore density (46 to 51 spores soil g^{-1}) was found in the organically managed sites (Sites B and C), sites I2, M1 and M2. High levels of root colonization (> 80%) appeared in most of the flax fields except Sites D and L. Although traditional measurements of spore density and root AMF colonization were correlated ($r^2 = 0.602$, $p < 0.05$), neither of them was correlated with measured soil characteristics. In addition, among the abiotic and biotic factors investigated, AMF colonization varied among flax growth stages (Fig. 3.1). The difference in AMF colonization was significant between the first flower and the late flowering growth stages.

Table 3.1. Description of 18 flax fields in Saskatchewan sampled for AMF characterization.

Site†	Soil Zone	Previous Crop	Tillage	Management	Growth Stage	Location (Geographic position)
A1	Dark Brown	Wheat	Conventional	Conventional	Full Flower	Shauavon (49.7861N, 108.2472W)
A2	Dark Brown	Wheat	Conventional	Conventional	Full Flower	Shauavon (49.7950N, -108.2409W)
B	Dark Brown	Wheat	No-till	Organic	Early Flower	Bone Greek (49.8210N, 108.2182W)
C	Dark Brown	Wheat	No-till	Organic	Early Flower	Bone Greek (49.7491N, 108.2181W)
D	Dark Brown	Wheat	No-till	Conventional	Late Flower	Vanguard (49.9125N, 107.3017W)
E	Dark Brown	Wheat	Conventional	Conventional	Full Flower	Outlook (51.4774N, 107.0463W)
F	Dark Brown	Wheat	Conventional	Conventional	Full Flower	Saskatoon (52.1526N, 106.5401W)
G1	Dark Brown	Wheat	Conventional	Conventional	Full Flower	Buffalo Lake (51.7735N, 105.9245W)
G2	Dark Brown	Wheat	Conventional	Conventional	Full Flower	Buffalo Lake (51.8064N, 105.9008W)
H1	Dark Brown	Wheat	No-till	Conventional	Full Flower	Regina (50.3127N, 105.0305W)
H2	Dark Brown	Wheat	No-till	Conventional	Full Flower	Regina (50.3131N, 104.9848W)
I1	Dark Brown	Canola	Conventional	Conventional	Late Flower	Vibank (50.3080N, 103.8849W)
I2	Dark Brown	Canola	Conventional	Conventional	Late Flower	Vibank (50.3398N, -103.8621W)
J	Black	Wheat	Conventional	Conventional	Late Flower	Indian Head (50.4549N, 103.6674W)
K	Black	Wheat	Conventional	Conventional	Full Flower	Grenfell (50.5426N, 103.0026W)
L	Black	Wheat	Conventional	Conventional	Full Flower	Grenfell (50.4767N, 102.6708W)
M1	Black	Barley	Conventional	Conventional	Early Flower	Grenfell (50.5281N, 102.6592W)
M2	Black	Barley	Conventional	Conventional	Early Flower	Grenfell (50.5273N, 102.6589W)

† Adjacent fields were coded with same capital letters (e.g., A1 and A2, G1 and G2, and M1 and M2); other fields were coded with different capital letters.

Table 3.2. Soil physical and chemical characteristics, AMF spore density and root colonization in 18 flax fields in Saskatchewan.

Site†	Soil Texture	pH	EC (mS cm^{-1})	TOC%‡	NO_3^- (mg kg^{-1})	NH_4^+ (mg kg^{-1})	PO_4^{2-} (mg kg^{-1})	K (mg kg^{-1})	Spore (g^{-1} soil)	Colonization§ %
A1	Silty Clay Loam	8.42	0.472	1.39	3.7	5.9	12.5	200	30	83 ± 6.2
A2	Silty Clay	5.37	0.165	1.50	3.6	4.1	20.1	352	19	88 ± 1.1
B	Clay	7.18	0.333	1.17	2.7	2.5	8.7	254	46	85 ± 3.6
C	Clay	7.03	0.287	1.69	4.1	2.7	5.4	331	54	88 ± 4.8
D	Silty Clay	7.2	0.344	1.21	3.7	2.6	10.6	532	13	79 ± 1.5
E	Loam	8.02	0.224	1.35	24.7	2.4	16.5	285	29	86 ± 5.7
F	Clay Loam	6.48	0.35	2.27	4.8	2.5	23.7	685	15	82 ± 1.4
G1	Clay Loam	6.53	0.636	2.63	8.5	3.1	38.8	881	17	86 ± 2.9
G2	Silty Clay	7.19	0.432	2.11	5.2	2.6	16.2	423	30	89 ± 2.2
H1	Sandy Clay	7.33	0.318	2.28	3.4	2.9	15.2	708	24	89 ± 1.1
H2	Clay Loam	7.43	0.371	2.06	3.8	2.8	14.6	480	18	81 ± 0.1
I1	Sandy Clay Loam	7.61	0.44	1.99	6.2	3.0	34.7	410	27	86 ± 1.2
I2	Silty Clay	7.72	0.74	1.59	41.2	4.0	11.1	367	51	89 ± 3.3
J	Silty Clay Loam	7.49	1.022	3.03	2.3	2.1	20.8	415	26	81 ± 3.9
K	Silty Clay	6.29	0.484	3.37	17.0	3.6	146.8	956	35	87 ± 6.2
L	Silty Clay Loam	6.35	0.49	3.49	8.7	3.5	36.8	411	12	66 ± 2.2
M1	Silty Clay Loam	7.09	0.564	4.19	13.6	2.7	22.9	503	55	90 ± 2.0
M2	Silty Clay Loam	7.31	0.634	3.29	8.1	2.6	25.3	540	51	89 ± 4.2

† Adjacent fields were coded with same capital letters (e.g., A1 and A2, G1 and G2, and M1 and M2); other fields were coded with different capital letters.

‡ Soil total organic carbon.

§ Group average colonization % ± SD of 3 replicates.

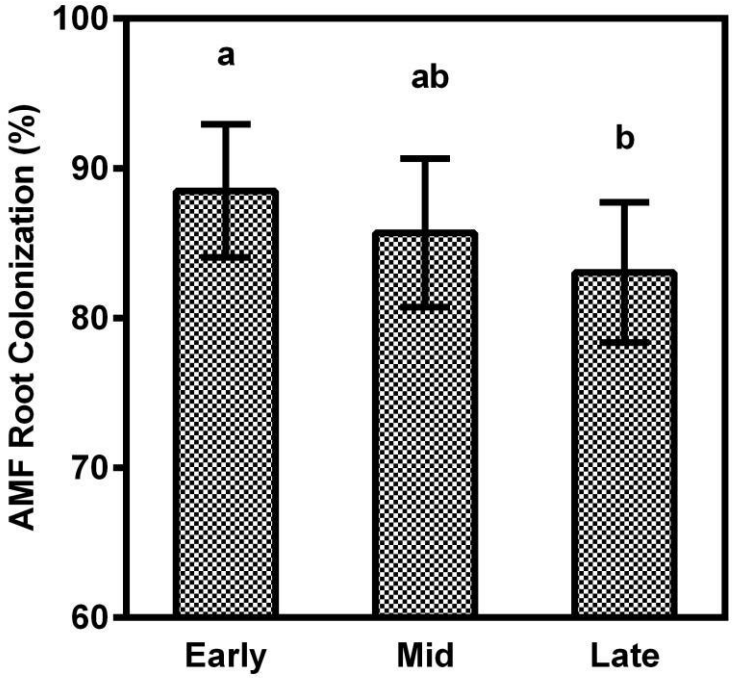

Figure 3.1. Root colonization of arbuscular mycorrhizal fungi (AMF) among fields under different flax growth stages [first flower (n = 4), full flower (n = 10) and late flower (n = 4)]. Means (± SD) labeled with different letters are significantly different based on Tukey's HSD test $p \leq 0.05$.

3.5.2 Sequencing results

The 454 GS FLX+ sequencing platform generated 96,814 raw sequences (average length = 785.5 bp) for the 18 samples collected in this study. A total of 8,109 AMF sequences (average length = 829.5 bp) were retained from bioinformatics analysis, and were clustered into 222 OTUs (Appendix B). The BLAST and phylogenetic analyses showed that these OTUs covered all four orders of the Glomeromycota, and most of the OTUs (82%) yielded good matches (≥ 95% identity) with known AMF taxa in GenBank. The majority of OTUs belonged to the family of Glomeraceae (181 OTUs), and commonly yielded high similarity with *F. mosseae* (accession No. NG_017178), *F. constrictum* (accession No. AJ534309), *R. intraradices* (accession No. AJ852526), *G. indicum* (accession No. GU059539), and *G. iranicus* (accession No. HM153424). Nineteen OTUs belonged to Claroideoglomeraceae, 18 of which showed good matches with *Claroideoglomus etunicatum* (accession No. Z14008). Fourteen OTUs belonged to Paraglomeraceae, 11 of which showed good matches with *Paraglomus occultum*

(accession No. NG_017179). Six OTUs belonged to Diversisporaceae, four of which shared high similarity with *Diversispora sp. P10* (EU332719). Two OTUs belonged to Acaulosporaceae, and both shared high similarity with *Acaulospora trappi* (accession No. AM114274).

3.5.3 Abundance, species richness and community diversity

Sequencing intensity for the samples from each flax field was examined using rarefaction curves. Three samples (Sites K, L and M1) failed in the rarefaction test and were excluded from further analyses. Abundance and species richness was highly correlated ($r = 0.83$, $p \leq 0.01$). There were considerable variations in the abundance (ranging from 2.89% to 12.27%) and species richness (ranging from 44 to 94) among flax fields (Table 3.3). The variations in the abundance and species richness were observed even for fields with close proximity (e.g., Sites A1 and A2, G1 and G2, and H1 and H2). Similarly, there was no noticeable difference for sites between different soil zones. Comparatively abundant and diverse root AMF communities were found in one of the organically managed sites (Site C). The two sites with canola as the previous crop (Site I1 and I2) were associated with comparatively low levels of root AMF abundance and diversity (ACE).

The significant effect of tillage on abundance and species richness of root associated AMF communities was revealed by ANOVA (Table 3.4). Flax roots in no-till fields typically hosted more abundant and richer AMF communities compared to that of conventional tillage systems. No significant impact of soil texture or flax growth stage was detected. In addition, a few of statistically significant correlations between measured soil physicochemical properties and root AMF communities with respect to abundance, species richness and diversity were detected (Table 3.4). Specifically, soil EC was negatively correlated with root AMF community diversity (*H'* and *D*), but not abundance. Soil pH was negatively correlated with abundance of AMF, but not species richness and diversity. Soil available P (i.e., PO_4^{3-}) was negatively correlated with species richness.

Table 3.3. Abundance, species richness, Shannon-Wiener diversity index (*H'*), Simpson's index (*D*), and ACE of root AMF communities in flax fields.

Site†	Soil Zone	Previous Crop	Tillage	Management	Growth Stage	Abundance‡	Species richness	*H'*	*D*	ACE
A1	Dark Brown	Wheat	Conventional	Conventional	Full Flower	273	57	3.64	0.97	70.36
A2	Dark Brown	Wheat	Conventional	Conventional	Full Flower	995	79	3.38	0.93	88.02
B	Dark Brown	Wheat	No-till	Organic	Early Flower	935	65	2.89	0.9	81.19
C	Dark Brown	Wheat	No-till	Organic	Early Flower	968	94	3.67	0.96	117.79
D	Dark Brown	Wheat	No-till	Conventional	Late Flower	678	82	3.56	0.95	100.87
E	Dark Brown	Wheat	Conventional	Conventional	Full Flower	245	59	3.39	0.95	136.51
F	Dark Brown	Wheat	Conventional	Conventional	Full Flower	234	50	3.29	0.95	70.23
G1	Dark Brown	Wheat	Conventional	Conventional	Full Flower	384	50	3	0.91	57.58
G2	Dark Brown	Wheat	Conventional	Conventional	Full Flower	486	65	3.41	0.95	80.6
H1	Dark Brown	Wheat	No-till	Conventional	Full Flower	485	58	3.25	0.94	96.52
H2	Dark Brown	Wheat	No-till	Conventional	Full Flower	712	69	3.45	0.95	90.93
I1	Dark Brown	Canola	Conventional	Conventional	Late Flower	252	44	3.11	0.93	59.94
I2	Dark Brown	Canola	Conventional	Conventional	Late Flower	262	46	3.21	0.94	59.77
J	Black	Wheat	Conventional	Conventional	Late Flower	421	65	2.82	0.84	127.92
M2	Black	Barley	Conventional	Conventional	Early Flower	392	55	3.38	0.95	68.06

† Adjacent fields were coded with same capital letters (e.g., A1 and A2, G1 and G2, and M1 and M2); other fields were coded with different capital letters.

‡Number of AMF sequences.

Table 3.4. Effect of categorical variables (tillage, soil texture, flax growth stage) on root AMF communities (Abundance, species richness and community diversity), and correlations between continuous soil properties (pH, EC, total organic carbon (TOC), nitrate (NO_3^-), ammonium (NH_4^+), phosphate (PO_4^{3-}) and potassium (K)) and AMF community characteristics.

Soil factors	Abundance	Species richness	*H'*	*D*	ACE
		ANOVA†			
Tillage	*	*			
Soil Texture‡					
Flax Growth Stage					
		Pearson's Correlation (r)§			
pH	-0.519*				
EC			-0.527*	-0.623*	
TOC					
NO_3^-					
NH_4^+					
PO_4^{3-}		-0.591*			
K					

† ANOVA analyses for each nonmetric variable. Only significant differences are shown.

‡ Due to lack of sufficient representation of loam soils, only differences in clay and clay loam soils were compared using a t-test.

§Pearson's correlation coefficient. Only significant correlations are presented.

3.5.4 AMF community composition

The MRPP analysis indicated that there was a significant effect of tillage on root AMF community structure among flax fields (p = 0.006). The NMS analysis (Figure 3.2) revealed diversely distributed root AMF communities (based on the top 30 most abundant OTUs) among flax fields, suggesting that many factors may influence the composition of root colonizing AMF communities, and the impact of these factors is currently difficult to predict. Moreover, there was only a weak dispersion of root AMF communities between conventionally tilled and no-till sites. The soil physicochemical properties correlated ($r^2 > 0.2$) with root AMF community composition (based on the top 30 most abundant OTUs) were projected in the NMS plot. Specifically, soil available P levels (i.e., PO_4^{3-}) were correlated with axis1 ($r^2 = 0.274$) and soil available N (NO_3^-) levels were correlated with axis 2 ($r^2 = 0.205$), suggesting that these factors had a limited impact on the composition of AMF communities colonizing flax roots.

No other soil physicochemical properties had a significant and predictable impact on community composition.

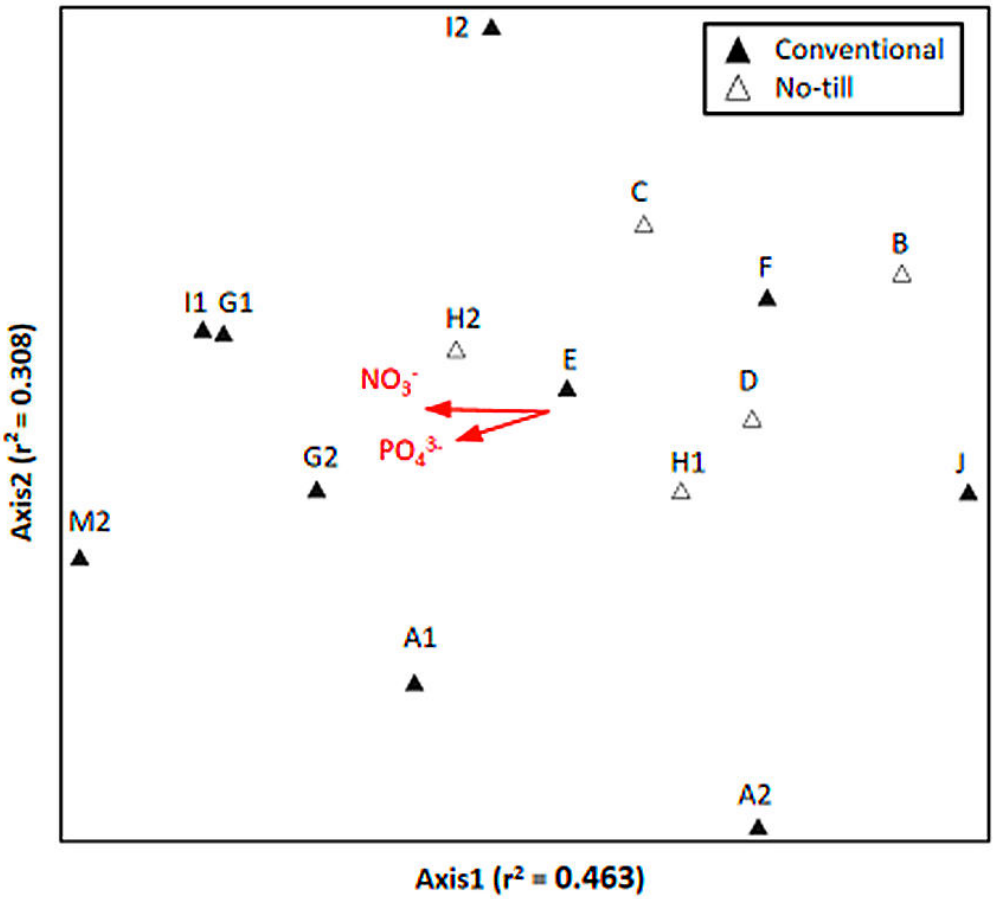

Figure 3.2. Nonmetric multidimensional scaling (NMS) ordination for the top 30 most abundant OTUs from the root AMF communities from 15 flax fields. Symbols are the ordination coordinates of samples from each flax field under different tillage managements. Environmental variables that were correlated with AMF community composition in the NMS analysis ($r^2 \geqslant 0.2$) were included in the joint plot. Final stress for two dimensional solution, 16.268 ($p = 0.03$); final instability, 0.0001.

The dominant OTUs (based on the top 30 most abundant OTUs) of root colonizing AMF communities in flax fields was further illustrated in a two-way cluster dendrogram (**Error! Reference source not found.**). Although root AMF communities were not fully clustered based on tillage management, tillage was still considered to be an important controlling factor for root AMF community composition in flax fields. In no-till fields, there was a greater number of dominant OTUs with relatively high diversity. Moreover, the organically managed sites (B and C) appeared to support diverse and abundant root colonizing AMF communities. Finally, the presence of the various OTUs differed among the different sites, and no single OTU

was detected at all sites within this grouping (i.e., within the top 30 OTUs in terms of abundance).

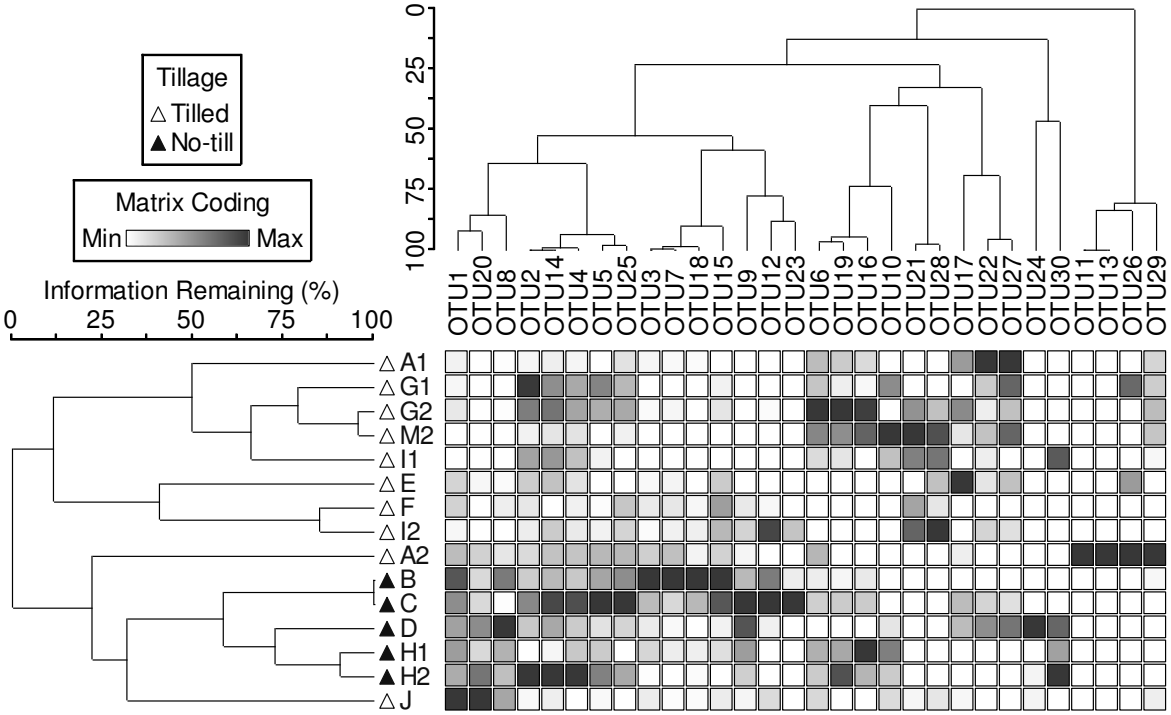

Figure 3.3. Two-way cluster dendrogram for 30 dominant OTUs of root AMF communities among 15 flax fields. The color of each rectangle indicates the abundance of each corresponding AMF OTU.

3.6 Discussion

In this study, tillage was detected as a driving factor for number of OTUs, species richness, and composition of AMF communities colonizing flax roots. Others have reported that tillage disturbs soil hyphal networks and reduces richness of soil AMF communities, and therefore influences the ability of AMF to absorb P (Sheng et al., 2013). The observed reduction in root AMF community diversity might be a consequence of reduced soil AMF diversity in conventional tillage systems. The negative impact of increasing land use intensity on soil AMF diversity has been shown in earlier investigations in Central Europe and Netherlands (Oehl et al., 2003; Oehl et al., 2010; Verbruggene et al., 2012). However, Dai et al. (2013) conducted a large-scale survey over 317 sites in the Canadian prairies and found no negative effect of crop production on the diversity of soil AMF in croplands as compared with natural areas. Further studies are needed to evaluate the possible loss of soil AMF in flax fields under different

agriculture managements.

Soil chemical factors have considerable influence on AMF community assemblages in both soil and roots. In a survey of AMF communities among different soil types (Oehl et al., 2010), soil pH was identified as the most influential parameter for soil AMF species composition. Bainard et al. (2014) examined the impact of soil abiotic factors and plant host identity on the spatial and temporal assemblage of AMF communities. They found soil pH and EC as the major driving factors in the spatial variation of AMF communities in both soil and roots; whereas the temporal shift in the composition of root-associated AMF communities were correlated with soil P flux and climatic variables. Results from the current survey of Saskatchewan flax fields revealed similar results. Specifically, negative correlations were detected between soil pH and abundance, soil EC and community diversity (*H'* and *D*), and soil available P content and species richness. Different from species richness and ACE index (incorporates species richness with a correction factor of less than 10 individuals), Shannon-Wiener and Simpson's diversity indices focus on describing the diversity of the middle and dominant population, respectively (McCune and Meffod, 1999). Thus, the inconsistent correlations suggest that there may be different responses for individual AMF species (e.g., dominant and rare) to specific soil abiotic factors, and further studies will be needed to fully understand these relationships.

Others have reported the distribution of soil AMF communities were associated with soil type and land use in the Canadian prairies (Talukda and Germida, 1993; Dai et al., 2012, 2013). According to the soil AMF survey by Dai et al. (2012), increasingly abundant and diverse soil AMF communities were found along the increasing moisture gradient in the Canadian Prairies, from Brown, to the Dark Brown, and the Black Chernozems. Bainard et al. (2015) recently examined the impact of soil type and land use on soil AMF community diversity and composition, using spore morphology and 454 sequencing methods separately in two surveys. The impact of land use practices (roadside versus agricultural) were identified; however, there was no effect of soil type on soil AMF community. Although sampling effort for different soil zones in the current study was limited, as can be seen in Fig. 3.1 and Fig. 3.2, there were no

obvious differences in the diversity and composition of root AMF communities among flax fields, irrespective of distances between sampling sites, suggesting that location, and the climatic controls associated with location, did not influence the root-associated AMF community in a predictable manner. One possible explanation is the homogenizing effect of crop production on AMF communities. For example, it is possible that flax favors colonization by selected AMF taxa, thus negating differences in taxa that exist in the soil. Similarly, management factors may have a greater effect on AMF community composition than factors related to soil characteristics and environment. For example, relatively high abundance, species richness and community diversity (*H'* and ACE) were observed in the root-associated AMF community from one of the organically managed fields, whereas the two fields with canola in the recent cropping history were both associated with comparatively low abundance, species richness and ACE index.

This study reported higher spore density levels compared to other AMF surveys (Talukdar and Germida, 1993; Oehl et al., 2010). Spores were sampled at the time of seeding for these two earlier studies, whereas sampling took place at the reproductive stage of flax in the current study, when soil AMF were well developed. Additionally, a correlation between spore density and AMF root colonization was observed, but there was no consistent trend of the two microscopy-based measurements with respect to either soil physicochemical properties, or abundance of root AMF determined by 454 sequencing. There may be deficiencies associated with using morphological and molecular approaches to verify each other (Shi et al., 2012). Furthermore, considering the high levels of AMF colonization observed among flax fields, the effect of AMF inoculation on flax may be limited unless the introduced AMF inoculant is greatly superior to native species for plant nutrient uptake.

Communities of AMF associated with flax roots grown in Saskatchewan have not been investigated previously using molecular methods. In this study, we used high-throughput 454 sequencing technology to reveal flax root-associated AMF communities. A wide range of phylogenic groups in the Glomeromycota were identified. The predominance of Glomeraceae family was shown in terms of abundance and diversity, followed by taxa within the

Claroideoglomeracea family. *F. mosseae*, *F. constrictum*, *R. irregularis*, *G. iranicus*, and *C. etunicatum* are AMF species that are commonly detected in the Canadian prairies (Talukdar and Germida, 1993; Dai et al., 2012). The presence of *G. indicum*, *P. occultum,* and *A. trappi* were also detected in the current study, which concurs with the recent survey by Dai et al. (2013).

The 454 sequencing method used in this study enabled qualitative and semi-quantitative analysis of AMF communities in flax roots. The ecological information provided by 454 sequencing was far beyond that provided by traditional measurements and thus captured a greater diversity of taxa than previously reported (Talukdar and Germida, 1993). It is important to note that the process from amplicon preparation to bioinformatics analysis involves a long series of steps and particular care must be taken at each step to avoid loss or distortion in final information.

A potential limitation in the sampling effort used in this study is that pooled samples without replicates were used to represent root-associated AMF communities in flax fields. According to Lindahl (2013), replication of composite samples could provide information about stochastic sampling effect, and thus it is possible that the current study failed to detect all possible members of the root colonizing community. Additionally, although coverage was good, the specificity of the primer pair AML1/AML2 was not as high as shown in the study by Bainard (2014), which has the potential to further limit the number of taxa detected. The current study used a different multiplex method in PCR, which might be the reason for this difference. The number of OTUs obtained in this study is comparatively higher than earlier 454 sequencing based AMF studies (Dai et al., 2012, 2013, 2014; Bainard et al., 2015). The differences in observations regarding AMF community composition between studies is not surprising given that different primers or bioinformatics approaches may yield different information. By choosing alternative clustering methods in Mothur or other bioinformatics tools with different methodologies, the OTU number may be reduced. Nonetheless, the different approaches do not necessarily invalidate the data, but rather may alter the scope of the AMF assemblage that were detected.

This study revealed that flax is well colonized by a range of AMF taxa in Saskatchewan soils, suggesting that AMF associations likely play an important role in flax nutrient uptake. Colonized flax roots were dominated by AMF taxa within the Glomeraceae family in terms of abundance and diversity, and a wide variety of taxa were found at different sites, suggesting that flax may benefit from these diverse communities. Soil management practices, particularly tillage, regulated root AMF abundance, community diversity and composition. The AMF community assembling in flax roots was also related to soil pH, EC, soil available N and P, to different extents.

4. THE EFFECT OF ARBUSCULAR MYCORRHIZAL INOCULATION ON GROWTH AND YIELD OF FLAX GROWN UNDER FIELD CONDITIONS

4.1 Preface

Chapter 3 described a study in which root-associated AMF communities colonizing flax were influenced by previous crop management such as tillage practices. The application of AMF inoculants provides the potential for restoring soil AMF abundance following intensive agricultural practices, and subsequently increasing crop production. Little is known, however, about the effect of AMF inoculation on flax (*Linum usitatissimum* L.) production. In a two-year field study, responses of flax to a commercial AMF inoculant were examined under field conditions with different P availability. Additionally, a growth chamber experiment was conducted to determine if the responses of flax to AMF inoculation are cultivar specific. The effectiveness of AMF inoculation in terms of enhancing plant biomass and nutrient uptake, root AMF colonization, soil infective propagules, and diversity and composition of root colonizing AMF communities, were assessed.

4.2 Abstract

Arbuscular mycorrhizal fungal (AMF) inoculation is an emerging technology for restoring soil AMF abundance and functioning and increasing crop production. The objective of this study was to examine the effect of AMF inoculation combined with phosphorus (P) fertilization on flax (*Linum usitatissimum* L.) growth and seed yield. A field study was carried out at sites with soils with low and intermediate initial P levels (Kelvington and Outlook, respectively) in 2012, and at a dryland site and an irrigated site (Outlook DL and Outlook IRR, respectively) in 2013. A commercial AMF inoculant (*R. intraradices*, MYKE®PRO, GR Premier Tech, Rivière-du-Loup, Québec, Canada) applied at 0, 1X, and 2X of the recommended application rate, with or without 16.8 kg ha^{-1} P fertilizer, was applied. Additionally, a growth chamber experiment was conducted to determine the responses of different flax cultivars to AMF inoculation. The effectiveness of inoculation was evaluated by measuring plant growth (i.e., above-ground biomass, seed yield and nutrient uptake) and AMF infectivity (i.e., root colonization and number of infective AMF propagules in the soil). Additionally, inoculation induced changes in root-associated AMF communities were explored using 454 sequencing.

Increases of above-ground biomass and plant nutrient uptake with AMF inoculation were found at two of four sites (Outlook and Outlook DL). Application of P fertilizer affected flax growth and AMF effectiveness differently according to different field conditions. At Outlook and Outlook DL, P fertilizer restricted early flax growth and the effectiveness of AMF inoculation. However, application of P fertilizer resulted in enhanced flax growth at Kelvington and Outlook IRR. Increased root colonization with AMF inoculation was only observed at Outlook. The pyrosequencing analysis revealed differences in AMF diversity between sites and compositional changes of root-associated AMF communities due to AMF inoculation. Presumably, the observed variations were related to soil environment and indigenous AMF communities.

Responses to AMF inoculation under controlled growth chamber conditions were limited and only one flax cultivar responded positively to AMF inoculation as enhanced biomass. Inoculation with AMF generally reduced diversity of root colonizing AMF communities across

cultivars. The shifts in community composition with inoculation appeared to be cultivar specific. This study revealed that the impact of AMF inoculation on flax growth is complex, and generally is closely related to initial available soil P levels, native AMF community, environmental conditions, and crop genotype.

4.3 Introduction

Arbuscular mycorrhizal fungi (AMF) are one of the most important soil microorganisms in the terrestrial ecosystem in terms of both abundance and functioning (Smith and Smith, 2011). Forming symbiotic associations with plant roots, AMF contribute directly to host plants by enhancing soil nutrient availability (e.g., P and N), and indirectly by enhancing resistance to pathogens and adverse environments (Fitter et al., 2011). Studies have shown the impact of intensive agricultural practices on soil AMF activity, which may lead to insufficient root AMF colonization and suboptimal plant performance (Boddington and Dodd, 2000; Wang et al., 2009; Mirás-Avalos et al., 2010). Commercial AMF inoculants have been promoted for use in large-scale agricultural systems, particularly for low-input systems, where crop growth is highly dependent on the nutrient supply by AMF (Vosátka et al., 2012).

Flax (*Linum usitatissimum* L.), an important oilseed crop in western Canada, is known for its low efficiency in utilizing P fertilizer (Kalra and Soper, 1968). Studies have shown that flax is highly dependent on AMF for P supply (Thingstrup et al., 1998; Monreal et al., 2011). In a study by Kahiluoto et al. (2000), AMF were found to be as effective as P fertilizer for enhancing flax growth in a soil with low initial P supply. Therefore, it is suggested that AMF inoculants can be used as a substitute for P fertilizer, and may be particularly well suited for flax production.

The potential of AMF inoculation to improve plant performance has been demonstrated for a number of crops, such as maize (Celebi et al., 2010), garlic (Al-Karaki, 2006), onion (Tawaraya et al., 2012), sunflower (Gholamhoseini, 2013), and spice pepper (Hernádi and Sasvári, 2012). However, a careful assessment of the feasibility of AMF inoculants is needed, since inoculants are not always beneficial (Schwartz et al., 2006). Moreover, there is strong functional diversity in different plant-fungus combinations (van der Heijden et al., 1998;

Klironoms, 2008), which requires an evaluation of the effect of AMF inoculation on the target crop species.

Several soil factors need to be considered with AMF inoculation. First, irrespective of differences in the sources of inoculants used, crops generally are found to be responsive to AMF inoculation in soil containing low or intermediate levels of P (Al-Karaki, 2006; Tawaraya et al., 2012). Soil moisture also has strong influence in AMF functioning. Yang et al. (2010) observed positive linear relationships between plant P and N concentrations and active soil AMF biomass in grassland plant communities under sufficient soil moisture conditions. In contrast, watermelon growth was improved by AMF inoculation only under water stress (Omirou et al., 2013). Furthermore, the effect of AMF inoculation differs not only with crop species; but also with crop cultivar (Khanizadeh et al., 1995). It has been suggested that there may be a process of mutual selection for both the fungus and plant genotype (Maherali and Klironomos, 2007).

The effectiveness of AMF inoculation is traditionally evaluated through crop performance (e.g., biomass and plant nutrient uptake) and AMF infectivity (e.g., root colonization and spore density) (Al-Karaki, 2006; Tawaraya et al., 2012). Currently, the advanced molecular technology enables tracing the establishment and persistence of AMF inoculation (Pellegrino et al., 2012). The pyrosequencing method, with advantages in identification and relative quantification of large sample sets, is increasingly used to study AMF communities (Lindahl et al., 2013). The objectives of this study were to examine: i) the effect of AMF inoculation on flax grown under field conditions; ii) the responses of different flax cultivars to AMF inoculation; and iii) inoculation induced changes in AMF community composition.

4.4 Materials and Methods

4.4.1 Field experiments

4.4.1.1 Experimental design

In 2012, a flax field study was carried out at Outlook and Kelvington, SK. The Outlook site was located at the Saskatchewan Irrigation Diversification Center, which was conventionally managed (i.e., dryland) to examine the impact of varying rates of AMF inoculant on flax growth

parameters. The Kelvington site was selected based on the low soil P content. In 2013, similar field experiments were conducted at the Saskatchewan Irrigation Diversification Center at Outlook using two different irrigation regimes; one site was under conventional dryland management (Outlook DL), and the other site was irrigated (Outlook IRR). Each site to be sown with flax received 60 kg N ha^{-1} as urea. The test flax variety was CDC Sorrel (Crop Development Centre, University of Saskatchewan). The seeding density was 500 seeds per square meter. A randomized complete block design was used. At Outlook and Kelvington (2012), the experiment was comprised of six treatments; six plots of each replicated block randomly received three rates [0 (Control), 7.5 (1X), and 15 (2X) kg ha^{-1}] of a commercial AMF inoculant (*R. intraradices*, MYKE®PRO GR, Premier Tech, Rivière-du-Loup, Québec, Canada) with and without adding 16.8 kg ha^{-1} P_2O_5 applied as monoammonium phosphate (MAP). The granular AMF inoculant was placed in the seed row. P was side-banded with flax seeds in rows at 23 cm row spacing. Treatments were replicated in four blocks, for a total of 24 plots, each measuring 6 m x 1.5 m. The experimental design was slightly modified at Outlook DL and Outlook IRR in 2013. Another fungal inoculant, Jumpstart® (JS) containing *Penicillium bilaii* was included at a rate equivalent to 80 g product per 330 kg flax seed, for comparing the effectiveness of the AMF inoculant with another commercial bio-fertilizer. Thereby, the number of treatments used in the 2013 field study was increased to eight [0 (Control), 1X AMF, 2X AMF and JS], each applied with or without P_2O_5, and made up a total of 32 plots in each site.

4.4.1.2 Soil and plant sampling

Soil samples for general soil characteristics were taken to a depth of 30 cm at five random locations in each site prior to seeding (ALS Laboratory Group, Saskatoon, Canada). The remaining soils were stored at 4 °C for diluent soil for measurement of soil infective propagules. Flax shoots were sampled at bud (midseason) and maturity stages by hand harvesting three 1-m rows. Midseason and final-harvest biomass and seed yield were determined. Shoots and seeds were subsequently ground into a powder, and wet digested with concentrated H_2SO_4 (Bowman, 1988). The N and P content of plant tissue was measured using a Technicon

AutoAnalyzer (Technizon Industrial Systems, Tarrytown, USA). Flax roots were sampled at midseason using a flat shovel and excavating roots and adhering soil to a depth of ca. 15 cm from midseason harvest rows. Excavated samples were stored in coolers during transportation. Soil samples were stored at 4 °C for assessment of soil infective propagules. Roots were washed using a gentle stream of water and hand manipulation. Subsamples of washed roots were immediately immersed in liquid N and stored at -80 °C for determination of root-associated AMF communities.

4.4.2 Growth chamber experiment

Soil was collected from the 0 to 15 cm depth of a field located near Central Butte, Saskatchewan in 2013. The cropping history of the field was summer fallow in 2013, spring wheat in 2012, and canola in 2011. The soil was air-dried, sieved through a 2 mm sieve, and mixed with silica sand in a ratio of 1:1 (w/w). Soil properties of the mixture were: pH (1:2 soil:water slurry), 8.5; electrical conductivity (EC, 1:2 soil:water extraction), 0.1 mS cm^{-1}; 11.2 mg kg^{-1} available inorganic N (NO_3^-); 8 mg kg^{-1} $NaHCO_3$-extractable P; 179 mg kg^{-1} CH_3COONH_4-extractable K; and 0.9% $K_2Cr_2O_7$-H_2SO_4 determined organic matter content (ALS Laboratory Group, Saskatoon, Canada).

Pots were packed with 1.5 kg of the soil and silica sand mix and watered for the recovery of microbial activity for a week prior to seeding. Modified Hoagland nutrient solution (KNO_3, 540 µg mL^{-1}; KH_2PO_4, 133 µg mL^{-1}; K_2SO_4, 176 µg mL^{-1}; $CaSO_4 \cdot 2H_2O$, 500 µg mL^{-1}; $MgSO_4 \cdot 7H_2O$, 103µg mL^{-1}) (Hoagland and Arnon, 1938) was used to compensate for soil nutrient levels. Six flax cultivars of different registration years were selected. Characteristics of flax cultivars are shown in Appendix C. Pots were arranged in a fully randomized manner using a 2 x 6 factorial design where one factor was AMF inoculation [non-inoculated (Control) and AMF inoculated (AMF)], and the other factor was flax cultivar [Linola ™ 2047, Bison, CDC Bethune, CDC Neela, Norlin, and Prairie Thunder], with four replicates for each treatment. Conditions of the growth chamber were: light intensity ranged from 262 to 525 µmol $m^{-2}s^{-1}$; 22:18 °C mean day/night temperatures; 55:70% mean day/night relative humidity; and 16:8 h day/night rhythm.

Prior to seeding, flax seeds were surface sterilized by treating with 95% (v/v) ethyl alcohol for 5 min followed by 10% (w/v) sodium hypochlorite for 15 min, after which the seeds were rinsed 10 times with sterile distilled water. Surface sterilized seeds were placed on sterile filter paper moistened with water and allowed to germinate in darkness. Seeding took place on October 31st, 2014. The AMF inoculant was applied by removing the top surface soil from the pots, spreading the AMF inoculant (0.1 g per pot) evenly over the exposed surface, and replacing the scalped soil. The application rate was calculated based on the actual amount of the AMF inoculant to which a field grown plant is exposed based on the recommended application rate (5.7 kg ha^{-1} in 2014), assuming a row spacing of 20 cm and a seed bed width of 2.5 cm. A total of 10~12 pregerminated seeds were sown in each pot, at a depth of ca. 1.5 cm. Following plant emergence, plants were thinned to three plants per pot. The soil was maintained at 70% moisture holding capacity, and the pots were re-randomized twice a week. After 45 d, flax shoots and roots were sampled. Shoots were air-dried, weighed, ground into powder, and measured for N and P content using the same protocol as described in Section 4.4.1.2. Roots were washed off soil. A subsample of roots was subsequently immersed in liquid N, and stored at -80 °C for molecular analysis. The remainder of the roots were stored at 4 °C for determination of AMF root colonization.

4.4.3 Determination of root colonization and soil infective propagules

The percentage root length colonized by AMF was measured using the same method as described in Section 3.4.3. Soil samples were pooled by treatment and sieved (2 mm). The number of infective propagules in the soil was assessed using the most probable number (MPN) method as described by Germida and de Freitas (2007) which employs a 10 fold dilution series to 10^{-5}, with five replicates per dilution. A sterile diluent soil was prepared by mixing soil used in the experiment with silica sand (1:1) and autoclaving. The 10^{-1} dilution was made by filling each cone-tainer (1.5'' x 8.25'') with a mixture of 450 g diluent soil and 50 g soil to be tested, following with repeated serial dilutions for the rest of dilutions. Pregerminated seeds were planted to each MPN tube (three seedlings per tube), and kept in a growth chamber for 35 d. Flax roots were examined for the presence or absence of AMF colonization. Finally, MPN

values were derived from a statistical table (Germida and de Freitas, 2007).

4.4.4 DNA extraction and preparation of amplicon libraries

Root samples from Outlook DL and Outlook IRR sites in the 2013 field study and the growth chamber study were used for molecular analyses. Four replicates of each treatment were pooled prior to DNA extraction. The DNA extraction and PCR was conducted using the same methods as described in Section 3.4.4.

4.4.5 Bioinformatics and phylogenetic analysis

The bioinformatics and phylogenetic analyses were conducted using the same protocol as described in Section 3.4.5.

4.4.6 Data analysis

Normality and homogeneity (Bartlett's and Dunnett's test, $p = 0.05$) were checked prior to conducting ANOVA. Arcsine transformation was applied to root AMF colonization prior to analyses. Backtransformed data are reported. The two way ANOVA was performed in R (*v*. 3.0.2, R development Core Team) to examine effect of AMF and JS inoculants combined with P fertilizer on root colonization, midseason and final-harvest biomass, seed yield, and corresponding nutrient (N and P) uptake in the flax field study, and AMF inoculation and flax cultivar on root colonization, biomass and shoot nutrient (N and P) uptake in the growth chamber study. Tukey's HSD test for multiple comparison of treatment means at the 5% level of significance was conducted.

Rarefaction curves, Shannon-Wiener diversity index (*H'*) and Simpson's diversity index (*D*) were calculated by Mothur (*v*. 1.33, Schloss et al., 2009). A paired Student t-test was conducted in R to identify the general effects of AMF inoculation on the diversity of root-associated AMF communities across different flax cultivars in the growth chamber study. Prior to community compositional analyses, samples were normalized using the "sub.sample" command in Mothur (size = 261 for flax field samples, and size = 506 for growth chamber samples). Rarefaction curves of the subsamples were reassessed. Nonmetric multidimensional scaling (NMS) was conducted in PC-ORD (*v*. 6, MjM Software Design, Gleneden Beach, OR, US) to visualize the OTU based compositional differences of root AMF communities among different treatments.

In addition, means of midseason flax growth properties (biomass, shoot N and P uptake) were included in a second matrix in the NMS analysis to identify possible correlations between flax growth and composition of AMF communities colonizing flax roots.

4.5 Results

4.5.1 Field results

4.5.1.1 Root AMF colonization and soil infective propagules

Soil properties of the four experimental sites are shown Table 4.1. The soil available P level was very low at Kelvington in 2012. The initial soil N content varied between the sites, which was further moderated by N fertilization at seeding.

Colonization of roots by AMF was increased by both rates of AMF inoculation at Outlook in 2012 (Table 4.2). The highest root colonization was detected in the 2X AMF treatment, which was significantly different from the control group. However, no difference in root colonization was detected among different AMF inoculation treatments when P fertilizer was applied. At Outlook DL (2013), compared with the uninoculated control, JS inoculation significantly reduced root colonization. There was no difference in root colonization associated with the addition of P fertilizer. At Kelvington (2012) and Outlook IRR (2013), neither AMF inoculation nor P fertilizer affected root AMF colonization.

The numeration of final-harvest infective AMF propagules in the soil (Table 4.3) indicated that there were different changes of soil AMF abundance under AMF inoculation among sites. At Outlook (2012) and Outlook DL (2013), the highest MPN was observed in 1X AMF and 2X AMF treatments, respectively. The two sites also showed generally reduced MPN values with P fertilization. At Kelvington (2012), soil AMF abundance was increased both with AMF inoculation and P fertilization. At Outlook IRR (2013), although the influence of AMF inoculation in the abundance of soil infective propagules was unclear, there were generally increases of MPN values with P fertilizer.

Table 4.1. Soil characteristics in the upper 15 cm of soil profiles in the flax field study.

	2012		2013	
Soil property	Outlook	Kelvington	Outlook DL	Outlook IRR
Soil zone	Brown	Black	Brown	Brown
Soil texture	Loam	Loam	Loam	Loam
pH	7.9	7.8	8.0	7.5
EC (mS cm^{-1})	0.1	0.2	0.2	0.2
NO_3 -N (kg ha^{-1})	4.5	12.3	84	51
Extracted P (kg ha^{-1})	37	3.4	24	32
Extracted K (kg ha^{-1})	> 600	> 600	455	365

Table 4.2. Effect of AMF inoculation and P fertilization on root AMF colonization (%) in a flax field study conducted at 4 sites in Saskatchewan.

Treatment	Root AMF colonization (%) in 2012		Root AMF colonization (%) in 2013	
	Outlook	Kelvington	Outlook DL	Outlook IRR
Control	85 (1.2) b ‡	86 (4.3)	92 (3.3) a	75 (9.1)
1 x AMF	92 (2.1) ab	92 (1.4)	87 (6.4) ab	84 (6.3)
2 x AMF	93 (1.9) a	90 (1.8)	80 (6.8) ab	74 (13.8)
JS†	—	—	77 (3.2) b	82 (9.0)
Control + P	89 (0.5) ab	91 (1.5)	88 (7.0) ab	77 (8.2)
1X AMF + P	86 (2.0) b	93 (1.1)	79 (3.4) ab	83 (5.0)
2X AMF + P	88 (3.4) b	90 (5.8)	82 (3.3) ab	76 (12.3)
JS + P	—	—	82 (5.1) ab	73 (5.0)
Interaction	**			
Inoculation effect	*		**	
P effect				

†The treatments of JS and JS + P were only applied in site Outlook DL and IRR in 2013.

‡Standard deviation in parentheses; values followed with different letters within a column are significantly different by Tukey's HSD test ($p \leq 0.05$).

Table 4.3. The most probable number (MPN) of infective propagules per 500 g soil at final harvest in the flax field study conducted at 4 sites in Saskatchewan.

Treatment	MPN in 2012		MPN in 2013	
	Outlook	Kelvington	Outlook DL	Outlook IRR
Control	2200	490	280	280
1x AMF	2800	950	460	220
2x AMF	1100	1100	790	120
JS	—	—	180	220
Control + P	1800	1800	210	350
1x AMF + P	2200	3500	350	490
2x AMF + P	330	3500	280	230
JS + P	—	—	350	220
Pre-seeding†	—	—	350	46

†Pre-seeding soil infective AMF propagules were only measured in Outlook DL and IRR site in 2013.

4.5.1.2 Flax growth

Flax responded to AMF inoculation and P fertilizer differently among the experimental sites. At Outlook (2012), the highest above-ground biomass and plant nutrient (N and P) uptake measured at midseason and final-harvest were observed in the 1X AMF treatment (Table 4.4). Significant increases in final-harvest plant N and P uptake with 1X AMF inoculation were observed, compared with the uninoculated controls. However, no difference of flax performance was detected with 2X AMF inoculation. The application of P fertilizer significantly reduced midseason biomass and plant N and P uptake relative to the control, and led to general decreases in midseason biomass and plant nutrient uptake across different AMF inoculation treatments. In contrast, at Kelvington (2012), midseason and final-harvest above-ground biomass, seed yield, and associated nutrient uptake were typically increased with P application, irrespective of the inoculation treatments (Table 4.5). At Outlook DL (2013), the 2X AMF treatment significantly increased final-harvest plant N and P uptake, as compared to the uninoculated controls (Table 4.6). However, the general increases of plant N and P uptake during midseason were only associated with JS inoculation. Adding P fertilizer generally reduced midseason biomass across different inoculation treatments. Similar to Kelvington, at Outlook IRR in 2013, P fertilizer was found to be the only driver for general increases in midseason biomass, plant N and P uptake across different inoculation treatments (Table 4.7).

Table 4.4. Effect of AMF inoculation and P fertilization on flax growth properties at Outlook in 2012.

Treatment	Midseason			Final-harvest			Seed		
	Biomass (kg ha^{-1})	N uptake (kg ha^{-1})	P uptake (kg ha^{-1})	Biomass (kg ha^{-1})	N uptake (kg ha^{-1})	P uptake (kg ha^{-1})	Yield (kg ha^{-1})	N uptake (kg ha^{-1})	P uptake (kg ha^{-1})
Control	3759 a†	89.2 a	14.9 ab	11927	95.7 b	14.4 b	1127	34.2	7
	103	*15.8*	*3.2*	*1810*	*15.8*	*2.3*	*140*	*1.5*	*0.37*
1X AMF	3974 a	101.2 a	18.4 a	15169	152.9 a	29.2 a	1018	32.6	6.43
	275	*6.4*	*1*	*1938*	*29.9*	*1.1*	*60*	*2.4*	*0.47*
2X AMF	3449 ab	75.3 ab	12.4 abc	13276	119.6 ab	20.5 ab	1125	33.7	6.84
	567	*9.1*	*2.1*	*1927*	*20.3*	*5.7*	*89*	*3.9*	*0.58*
Control + P	2598.4 b	59.7 b	9.3 c	14024	122.7 ab	17.1 ab	1126	36	7.3
	340	*14.4*	*1.1*	*2409*	*20.2*	*1.5*	*101*	*3.9*	*0.54*
1X AMF + P	3553.2 ab	78 ab	14.4 b	14218	128.8 ab	19.7 ab	1128.1	33.3	6.95
	509	*14*	*1*	*2692*	*18.1*	*4.9*	*117*	*2.3*	*0.58*
2X AMF + P	3096.4 ab	65.4 b	11.9 b	13083	116.3 ab	20.3 ab	1127.3	34.1	6.86
	343	*10*	*2.5*	*2674*	*10.8*	*4*	*138*	*2.1*	*0.49*
ANOVA									
Interaction			*						
Inoculation effect	*	*	**		*	*			
P effect	**	***	**						

†Standard deviation given in *italics*; values followed with different letters in a column are significantly different by Tukey's HSD test ($p \leq 0.05$).

Table 4.5. Effect of AMF inoculation and P fertilization on flax growth properties at Kelvington in 2012.

Treatment	Midseason			Final-harvest			Seed		
	Biomass (kg ha^{-1})	N uptake (kg ha^{-1})	P uptake (kg ha^{-1})	Biomass (kg ha^{-1})	N uptake (kg ha^{-1})	P uptake (kg ha^{-1})	Yield (kg ha^{-1})	N uptake (kg ha^{-1})	P uptake (kg ha^{-1})
Control	2021 b†	39.7 b	2.25 b	8294 b	97.5 ab	3.74 bc	894 bc	25.4 b	2.85 ab
	418	*7.5*	*0.6*	*1470*	*21.7*	*1*	*101*	*3*	*0.49*
1X AMF	2192 b	44.5 ab	2.45 b	7859 b	93.9 b	3.45 c	869 c	25.1 b	2.69 b
	482	*11.5*	*0.78*	*719*	*16.9*	*0.81*	*144*	*3.9*	*0.55*
2X AMF	2067 b	42.3 b	2.34 b	8366 b	94.5 b	3.77 c	871 c	24.5 b	2.68 b
	316	*7.8*	*0.37*	*1429*	*15.5*	*0.88*	*58.9*	*2.4*	*0.47*
Control + P	2774.4 ab	48.1 ab	3.2 ab	11763 a	115.4 ab	5.43 abc	960 abc	27 ab	3.09 ab
	400.4	*5.4*	*0.54*	*2184*	*14.7*	*1.78*	*65.8*	*2.2*	*0.31*
1X AMF + P	3343 a	54.6 ab	3.76 ab	12565 a	116.9 ab	6.3 ab	1060 ab	28.8 ab	3.46 ab
	417.9	*14.6*	*0.78*	*1440*	*20.1*	*1.3*	*79.8*	*2.1*	*0.35*
2X AMF + P	3376 a	63.2 a	4.3 a	13254 a	133.2 a	6.69 a	1070 a	31 a	3.7 a
	430.4	*15.5*	*1.13*	*1545*	*19.3*	*1.26*	*54.4*	*1.6*	*0.24*
ANOVA									
Interaction									
Inoculation effect									
P effect	***	**	***	***	***	***	***	***	***

†Standard deviation given in *italics*; values followed with different letters in a column are significantly different by Tukey's HSD test ($p \leq 0.05$).

Table 4.6. Effect of AMF inoculation and P fertilization on flax growth properties at Outlook DL in 2013.

Treatment	Midseason			Final-harvest			Seed		
	Biomass (kg ha^{-1})	N uptake (kg ha^{-1})	P uptake (kg ha^{-1})	Biomass (kg ha^{-1})	N uptake (kg ha^{-1})	P uptake (kg ha^{-1})	Yield (kg ha^{-1})	N uptake (kg ha^{-1})	P uptake (kg ha^{-1})
Control	4673	106.5 ab	15.4	11092	71.0 bc	6.82 b	2445	80	13.1
	246	*20.7*	*4.7*	*1252*	*16.9*	*0.49*	*222*	*7*	*1.1*
1X AMF	4388	102.7 ab	16.0	11539	66.7 c	6.13 b	2829	98.9	15.3
	546	*30.8*	*5.9*	*965*	*10.8*	*1.28*	*379*	*10.5*	*2.3*
2X AMF	4593	105.9 ab	15.2	10840	108.4 a	11.83 a	2759	92.9	14.9
	414	*3.4*	*4.5*	*787*	*7.5*	*3.62*	*204*	*7*	*0.9*
JS	4879	156.3 a	23.7	11547	71.5 ab	6.42 b	2671	96.5	16.3
	145	*23.3*	*2.3*	*2050*	*7.7*	*1.6*	*261*	*26.3*	*4.9*
Control + P	3889	109.0 ab	15.7	9665	61.9 b	6.66 b	2811	97.2	16.6
	222	*14.5*	*1.6*	*816*	*8*	*2.04*	*57.2*	*6.1*	*1.4*
1X AMF + P	4169	123.4 ab	17.2	10414	74 bc	5.88 b	2725	91.1	15.8
	436	*22.6*	*3.6*	*2350*	*8.6*	*2.32*	*460*	*15.3*	*2.4*
2X AMF + P	4776	96.5 b	16.1	11992	97.9 ab	9.15 b	2862	98.5	17.1
	454	*14*	*2*	*273*	*12.2*	*2.15*	*297*	*11.5*	*1.8*
JS + P	4155	134.0 ab	18.6	11167.0	90.6 ac	7.09 b	2792	100.1	17.5
	607	*28.6*	*2.6*	*1015.1*	*9.1*	*1.36*	*292*	*16.6*	*3.1*
ANOVA									
Interaction						*			
Inoculation effect		**	*		**	***			
P effect	*								

†Standard deviation given in *italics*; values followed with different letters in a column are significantly different by Tukey's HSD test ($p \leq 0.05$).

Table 4.7. Effect of AMF inoculation and P fertilization on flax growth properties at Outlook IRR in 2013.

Treatment	Midseason			Final-harvest			Seed		
	Biomass (kg ha^{-1})	N uptake (kg ha^{-1})	P uptake (kg ha^{-1})	Biomass (kg ha^{-1})	N uptake (kg ha^{-1})	P uptake (kg ha^{-1})	Yield (kg ha^{-1})	N uptake (kg ha^{-1})	P uptake (kg ha^{-1})
Control	3840†	77.3 ab	12.9	11978	82.3	10.5	1502	54	9.26
	385	*18*	*1.8*	*686*	*9.6*	*3.3*	*187*	*10.2*	*1.91*
1X AMF	4260	78.1 ab	14.8	11505	68.5	8.45	1589	57.7	8.55
	496	*20*	*4.6*	*1184*	*24*	*4.6*	*192*	*11.4*	*1.62*
2X AMF	3892	50.7 b	10.5	11957	61.1	8.83	1712	63.8	10.55
	712	*18.8*	*5.4*	*720*	*20.3*	*5.23*	*273*	*15*	*1.94*
JS	4081	74.9 ab	13.5	12042	75.5	8.86	1756	56.9	9.93
	713	*16.4*	*2.9*	*1383*	*22.1*	*2.76*	*289*	*19.4*	*3.13*
Control + P	4960	86.5 ab	16	12571	70.9	8.22	1292	45.6	7.31
	447	*27.3*	*4.6*	*1671*	*10.5*	*0.84*	*104*	*9*	*0.75*
1X AMF + P	4682	92.8 a	16.6	10939	63.6	8.69	1639	54.9	8.42
	657	*27.1*	*3.8*	*1102*	*8.9*	*4.79*	*121*	*10.6*	*1.77*
2X AMF + P	3996	82.1 ab	14.3	12136	64.6	7.23	1561	56.5	9.11
	301	*23.5*	*2.4*	*1355*	*11.2*	*1.87*	*194*	*18.6*	*3.6*
JS + P	4627	99.4 a	17.4	11628	87.8	11.3	1369	50.6	8.97
	475	*10.7*	*2.3*	*1774*	*9.4*	*1.35*	*191*	*6.6*	*2.09*
ANOVA									
Interaction									
Inoculation effect									
P effect	*	*	*						

†Standard deviation given in *italics*; values followed with different letters in a column are significantly different by Tukey's HSD test ($p \leq 0.05$).

4.5.1.3 Molecular analyses

After the cleaning steps in Mothur, 7,258 reads (average length = 826.8) were retained from a total of 82,548 reads (average length = 782.3) generated by the FLX+ platform for the 16 root samples representing each treatment in Outlook DL and IRR in the 2013 field study. A total of 149 OTUs were identified based on clustering at 95% similarity. The majority of the OTUs belonged to Glomeraceae family (110 OTUs); eighteen OTUs belonged to Diversisporaceae, fourteen to Claroideogomeraceae, six to Archaeosporaceae, and one to Paraglomeraceae (Appendix C). One sample (JS treatment, Outlook DL) was found to have only 73 reads left after cleaning, which was not enough to pass the rarefaction test, and therefore was removed from the following analyses.

The impact of AMF inoculation and P fertilization on the diversity of root colonizing AMF communities differed between the two sites in the 2013 field study (Table 4.8). At Outlook DL, comparatively lower abundance, species richness, and community diversity (*H'* and *D*) were detected in the control group. The highest *H'* and *D* were both observed in the 2X AMF treatment. It seemed that the diversity of root colonizing AMF community was stimulated with different inoculation treatments. However, such effects, especially for the 2X AMF and JS treatments, were eliminated when P was applied. At Outlook IRR, although inconsistent changes in abundance and species richness were observed, there was a tendency for reduced *H'* and *D* with 1X and 2X AMF inoculation relative to the control, irrespective of P application.

Table 4.8. Effect of AMF inoculation and P fertilization on abundance, species richness, Shannon-Wiener (*H'*) index, and Simpson's index (*D*) of root AMF communities at Outlook DL and IRR site in the 2013 flax field study.

Treatment	Outlook DL				Outlook IRR			
	Abundance†	Richness	*H'*	*D*	Abundance	Richness	*H'*	*D*
Control	343	49	2.59	0.856	261	36	3.032	0.936
1x AMF	723	57	3.001	0.907	371	51	3.003	0.909
2x AMF	462	81	3.698	0.958	441	42	2.637	0.864
JS	723	90	3.465	0.942	—	—	—	—
Control + P	599	53	2.67	0.88	359	43	3.081	0.93
1x AMF + P	529	68	3.201	0.927	301	31	2.77	0.907
2x AMF + P	287	53	3.003	0.9	495	50	2.883	0.891
JS + P	703	78	3.129	0.906	588	73	3.567	0.956

†AMF sequence number

Prior to compositional analysis, sampling intensity in the normalized samples was confirmed by generating rarefaction curves (Fig. 4.1). The NMS plot indicated variations in root AMF community structure under different inoculation treatments between the two sites (Fig. 4.2). At Outlook DL, distinctive AMF communities in roots receiving 2X AMF and JS inoculants were observed. There was no such difference when P fertilizer was added, indicating that P application may restrict the effect of AMF inoculation on indigenous AMF community. In contrast, distinctive root AMF communities in terms of community composition were observed with different inoculation treatments at Outlook IRR. Moreover, there was no obvious difference by P fertilizer. Additionally, the shifts in community composition with 2X AMF inoculation (with and without P) were against the vectors representing flax growth properties, indicating the risk of forming a root AMF community that did not favour flax growth with a high rate of AMF inoculation.

Root AMF community composition correlated with midseason biomass ($r^2 = 0.218$, axis1) and associated N ($r^2 = 0.398$, axis1) and P uptake ($r^2 = 0.266$, axis1). Changes in root AMF community composition with 2X AMF and JS inoculation at Outlook DL appeared to be associated with the decrease in the proportion of *Funneliformus* sequence (Fig. 4.3). At Outlook IRR, the dominance of *Rhizophagus* species was shown in root AMF communities under 2X AMF inoculation (with and without P fertilizer).

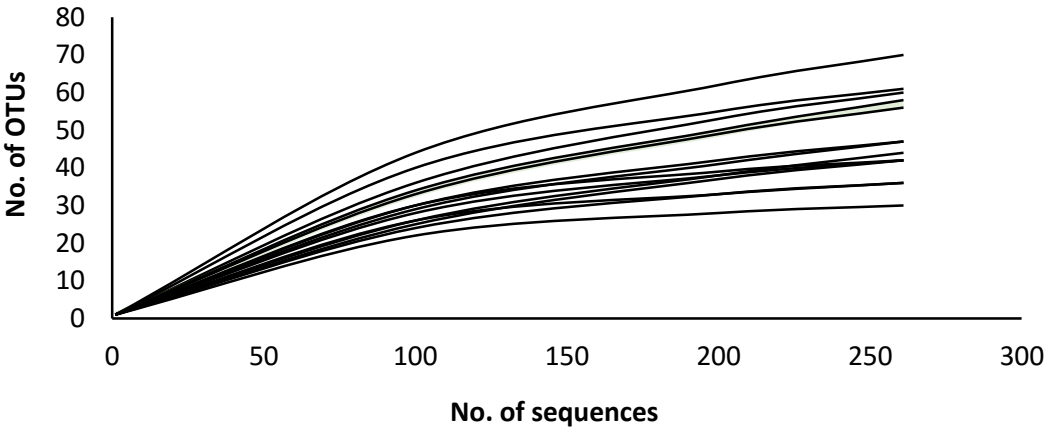

Figure 4.1. Rarefaction curves of subsampled reads (size = 260) of 15 root AMF samples from Outlook DL and IRR site in the 2013 flax field study.

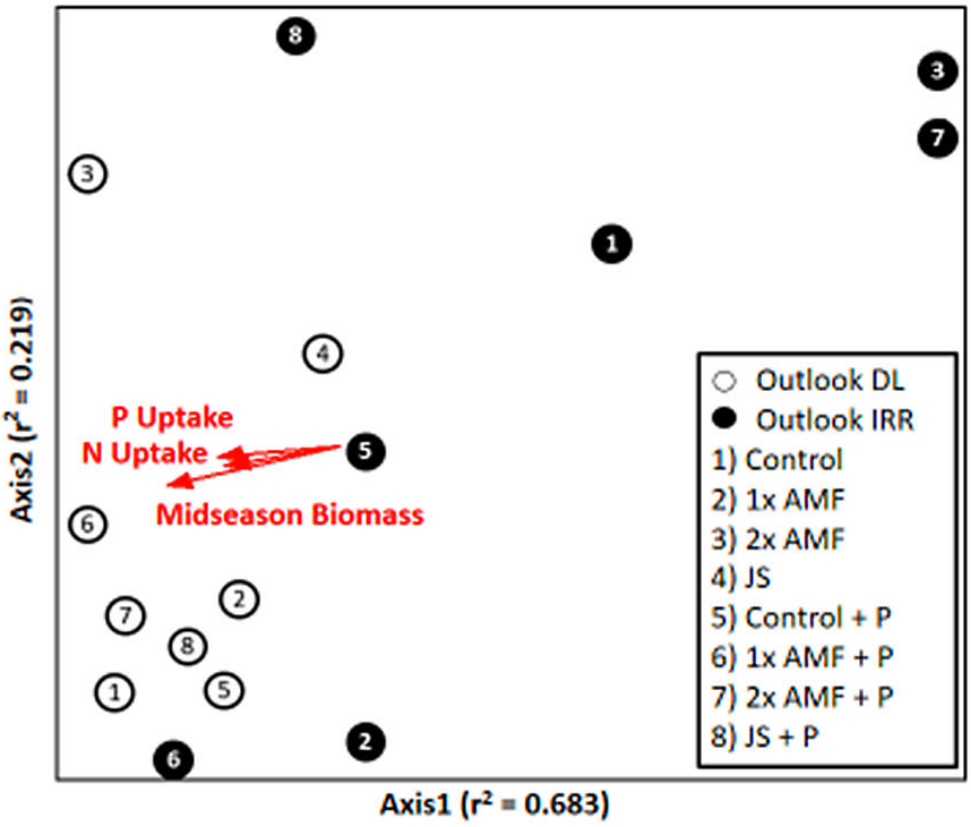

Figure 4.2. Nonmetric multidimensional scaling (NMS) ordination of root AMF communities from Outlook DL and IRR site in the 2013 flax field study. Symbols of different colors indicate ordination coordinates of samples of the two sites. Numbers of the symbols indicate different treatments of fungal inoculation and P fertilization in the field study. Plant growth properties correlated with AMF community composition in the NMS analysis ($r^2 \geqslant 0.2$) were included in the joint plot. Final stress for two dimensional solution, 10.379 (p = 0.04); final instability, 0.0001.

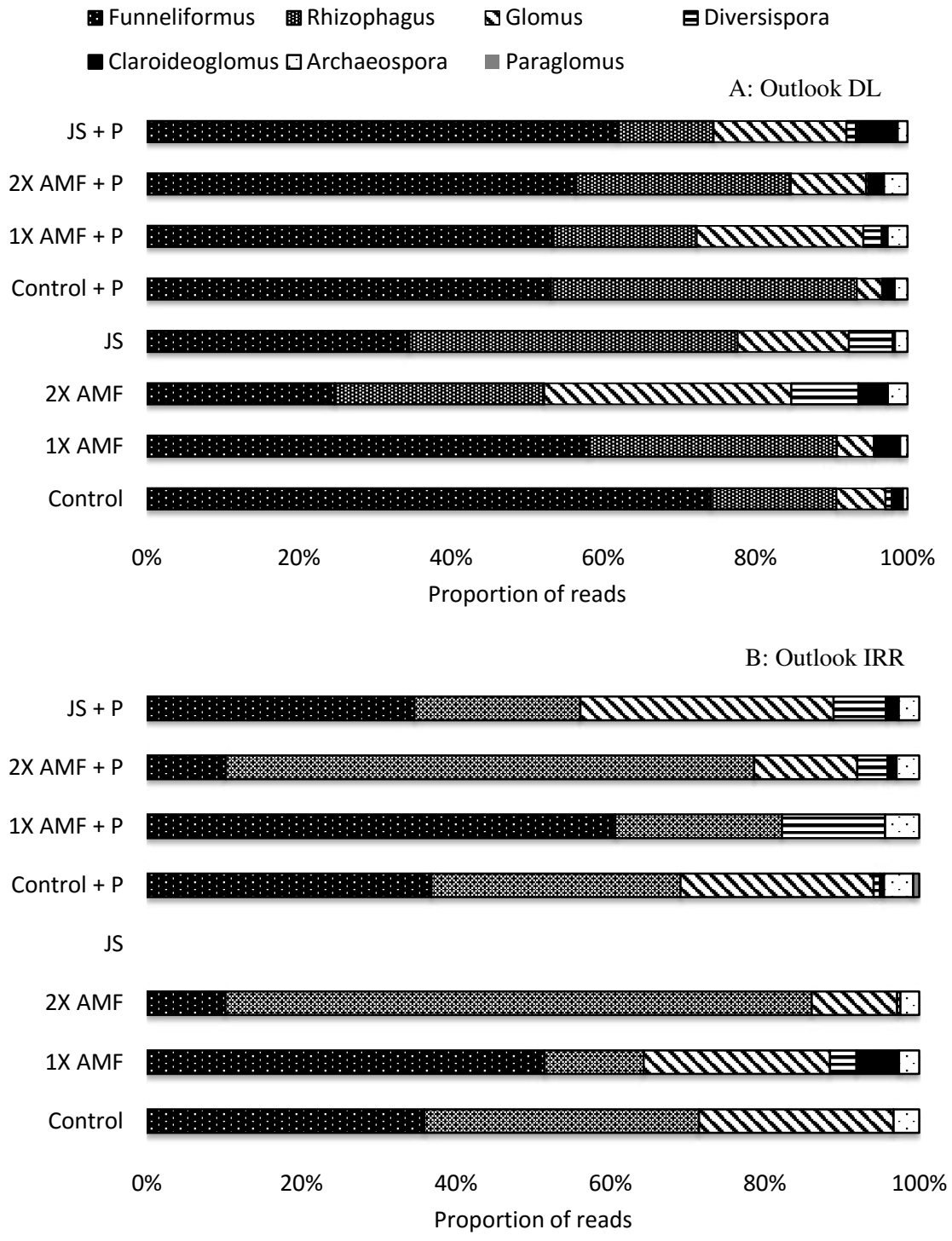

Figure 4.3. Distribution of Glomeromycota sequences at the genus level colonizing flax roots in Outlook DL (A) and IRR (B) site in response to different treatments of fungal inoculation and P fertilization.

4.5.2 Growth chamber results

4.5.2.1 Root AMF colonization, biomass and plant nutrient uptake

Root colonization in the growth chamber experiment supported findings in the flax field study; that is, relatively high levels of root AMF colonization were observed across flax cultivars (Table 4.9). Inoculation with AMF did not noticeably influence root colonization among cultivars. Irrespective of inoculation treatments, different levels of root AMF colonization were detected between Prairie Thunder and Bison ($p \leq 0.05$). Plant biomass generally varied among flax cultivars. Only biomass of CDC Neela was significantly increased by AMF inoculation. Although significant differences were not detected, AMF inoculation generally increased flax N and P uptake with AMF inoculation across cultivars.

4.5.2.2 Root colonizing AMF communities in the growth chamber experiment

A total of 11,727 reads (average length = 822.5) were retained from 77,093 raw sequences (786.8) generated by 454 sequencing. Among the 108 OTUs clustered by 92% similarity, thirty-six OTUs belonged to *Rhizophagus*, thirty-one belonged to *Funneliformus*, eighteen to *Glomus*, sixteen to *Charoideoglomus*, four to *Paraglomus* and three to *Diversispora* (Appendix D). Rarefaction curves indicated good coverage of AMF diversity in each sample.

The abundance and diversity of root colonizing AMF communities varied among flax cultivars (Table 4.10). The Student's t-test indicated that AMF inoculation generally reduced *D* ($p = 0.018$) among flax cultivars. The tendency of decreased richness and *H'* diversity was also observed in most of flax cultivars.

Compositional variation in root colonizing AMF communities associated with AMF inoculation and different flax cultivars was revealed in the NMS plot (Fig. 4.4). In the control group, there was variation of root AMF community composition among flax cultivars. Inoculation with AMF affected composition of root colonizing AMF communities differently according to different flax cultivars. The AMF community structure in Prairie Thunder shifted dramatically due to AMF inoculation. A correlation between root AMF community composition and biomass was detected ($r^2 = 0.394$, axis 1).

Table 4.9. Effect of AMF inoculation on root AMF colonization, biomass, and shoot N uptake and P uptake among flax cultivars in a growth chamber study.

Flax cultivar	Inoculation	Colonization (%)	Biomass (g)	N uptake (mg kg^{-1})	P uptake (mg kg^{-1})
Linola™2047	Control	87 (2.4)†	4.4 (0.2) abc‡	87.9 (9.6) b	4.5 (0.7) ab
	AMF	93 (0.9)	4.7 (0.6) abc	119.6 (36.0) ab	5.4 (1.0) ab
Bison	Control	94 (1.2)	3.6 (1.2) c	123.5 (15.0) ab	4.3 (1.4) b
	AMF	91 (1.6)	3.9 (0.1) bc	145.1 (33.0) ab	6.0 (0.6) ab
CDC Bethune	Control	87 (6.0)	5.1 (0.3) ab	119.7 (10.8) ab	4.3 (1.9) b
	AMF	86 (4.5)	5.0 (1.2) ab	116.7 (26.2) ab	5.1 (1.2) ab
CDC Neela	Control	88 (2.4)	3.9 (0.9) bc	117.0 (22.6) ab	4.9 (0.3) ab
	AMF	91 (3.7)	6.1 (0.1) a	160.9 (9.0) a	7.2 (0.4) ab
Norlin	Control	89 (4.4)	4.0 (0.7) bc	110.9 (12.6) ab	5.0 (0.4) b
	AMF	92 (0.2)	3.5 (0.4) c	116.4 (1.5) ab	4.7 (0.3) ab
Prairie Thunder	Control	83 (3.5)	4.2 (0.2) bc	117.3 (27.2) ab	5.5 (0.3) ab
	AMF	83 (1.1)	4.9 (0.5) abc	171.1 (32.2) a	8.1 (0.5) a
ANOVA					
Interaction			*		
Cultivar		**	***	*	*
Inoculation			*	**	***

†Standard deviation in parentheses; values followed with different letters within a column are significantly different according to Tukey's HSD test ($p \leq 0.05$).

Table 4.10. Effect of AMF inoculation and flax cultivar on abundance, species richness, Shannon-Wiener (*H'*) and Simpson's (*D*) diversity index of root AMF communities in the growth chamber study.

Cultivar	Inoculation	Abundance	Species richness	*H'*	*D*
Linola™2047	Control	586	43	2.431	0.835
	AMF	937	40	2.263	0.814
Bison	Control	1239	34	1.914	0.756
	AMF	763	33	1.932	0.754
CDC Bethune	Control	552	39	2.388	0.824
	AMF	1439	49	2.161	0.769
CDC Neela	Control	1096	45	2.375	0.832
	AMF	1327	35	2.1	0.764
Norlin	Control	506	54	3.003	0.92
	AMF	779	52	2.487	0.835
Prairie Thunder	Control	1326	55	2.412	0.834
	AMF	1177	55	2.394	0.801

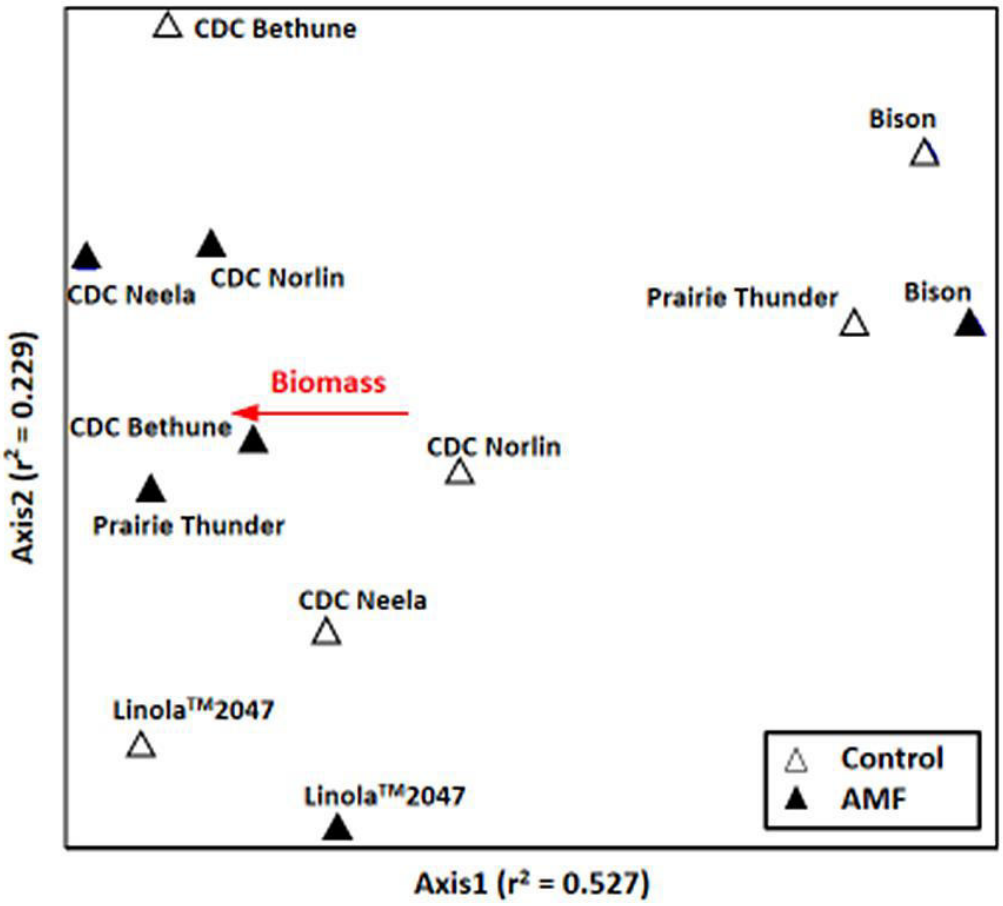

Figure 4.4. Nonmetric multidimensional scaling (NMS) ordination of AMF communities colonizing different flax cultivars, and with different AMF inoculation treatments. Plant growth properties correlated with AMF community composition in the NMS analysis ($r^2 \geqslant 0.2$) were included in the joint plot. Final stress for two dimensional solution, 7.935 ($p = 0.04$); final instability, 0.0001.

4.6 Discussion

The flax field study revealed that the impact of AMF inoculation on mid-season and final-harvest biomass and plant nutrient uptake varied in magnitude according to differences in field conditions, but ultimately no effect of AMF inoculation on seed yield or seed nutrient uptake was observed at any of the four field sites. Similarly, flax seed yield was unaffected by the other fungal inoculant, JS. Flax is less efficient in P absorption compared to other annual crops (Kalra and Soper, 1968), which may be due to a poorly proliferated root system within the P fertilizer band (Strong and Soper, 1974). Grant et al. (2009) reported that early season biomass and seed yield of flax were not affected by either annual or residual P application, although enhancement in early-season shoot P concentration and P uptake was observed.

In agreement with previous findings, the current study indicated that enhancing P supply

through biofertilizers, such as AMF or JS, does not necessarily translate to higher seed yield. At Outlook (2012) and Outlook DL (2013), where sites were under conventional dryland management with intermediate initial soil P levels, evidence of increased biomass and plant nutrient uptake with AMF inoculation were observed, but only in the absence of P fertilizer. In contrast, P fertilizer was found to be the only driver for the enhanced flax growth responses at the low P site (Kelvington in 2012) and the irrigated site (Outlook IRR in 2013). These findings agree with the study by Kahiluoto (2001), who reported that AMF may transiently impose a net cost to flax above a certain threshold P level. Results of this study also agree with previous studies in which functioning of AMF inoculation was affected by watering level (Omirou et al., 2013) and variable weather conditions (Sari et al., 2002). The midseason and final-harvest biomass reported in the current study were calculated based on the hand-harvest of three 1-m rows; and therefore, may not represent real field production, although it is assumed that the ranking of treatments would not vary, irrespective of harvest method.

When an agroecosystem is limited in abundance or functioning of AMF due to historical mismanagement, it is generally thought that AMF inoculation may be particularly beneficial in restoring system function and realizing yield increases (Verbruggen et al., 2012). However, little is known about how indigenous AMF communities respond to AMF inoculation under field environments, irrespective of previous management. In a growth chamber study conducted by Jin et al. (2013), although no effect on field pea growth was detected, inoculation of *G. irregulare* (currently called *R. intraradices*) significantly influenced root AMF community composition and reduced diversity of AMF communities colonizing field pea roots. Similar results were observed in the field study at Outlook IRR. Community composition seemed to vary with different rates of inoculation, irrespective of P fertilizer. The reduced community diversity might be due to the competition between the introduced AMF and native AMF in soil with 'reduced carrying capacity' (Verbruggen et al., 2012). However, there was a different trend of changes in root AMF community composition and diversity with AMF inoculation at Outlook DL. Distinctive root AMF community composition was found to be associated with 2X AMF and JS inoculation. No obvious differences were observed among

inoculation treatments when P fertilizer was applied, indicating that the activity of the introduced AMF species was possibly suppressed with P fertilizer. The increased root AMF community richness and diversity with AMF inoculation was not surprising, if the relatively low diversity in the control group means that there were unoccupied niches available for the introduced species (Verbruggen et al., 2012). Rather than a single increase in the proportion of the inoculant species *Rhizophagus*, we observed changes in the proportion of different Glomeromycota genera under inoculation at both sites. Further studies are needed to track the consequent influence of such changes in soil AMF diversity and composition. It was interesting to observe similar shifts in root AMF communities with the application of 2X AMF and JS. There were corresponding plant growth responses in both of the treatments, indicating that AMF community activity might be regulated by host plants.

It is important to examine the effect of AMF inoculation among different flax cultivars, since cultivars have shown different susceptibilities to AMF colonization (Azcón et al., 1981; Khanizadeh et al., 1995; Bazghaleh et al., 2015). In the growth chamber study, flax cultivars varied in the magnitude of increases in plant N and P uptake with AMF inoculation, but only CDC Neela showed significantly increased biomass with AMF inoculation. Further studies are needed to verify if different plant responses to AMF inoculation are associated with specific plant characteristics. For example, Neela was the most recently released variety, and presumably differs from varieties released earlier, although this study did not identify which characteristic(s) may have influenced the AMF response. The difference between growth chamber and field conditions and the low P soil used in the growth chamber experiment may affect AMF activity to a certain degree (Kahiluoto et al., 2001).

A general reduction across flax cultivars in root AMF community diversity following AMF inoculation was detected, indicating the competition between native AMF and the introduced species under limited field capacity (Verbruggen et al., 2012). Bazghaleh et al. (2015) reported that chickpea cultivar significantly influenced the composition of root fungal communities. In contrast, Santos-González et al. (2011) reported that soil, but not cultivar, was the factor influencing the structure of AMF assemblages associated with strawberry. In the current study,

AMF inoculation resulted in various shifts in the structure of root AMF communities, which seemed to be cultivar specific.

The nested PCR and 454 sequencing protocol successfully traced changes of root-associated AMF communities under AMF inoculation, which outperforms traditional biomass estimators (i.e., root colonization and soil infective propagules) in describing AMF activity. At Outlook DL, the shifts of community composition with 2X AMF and JS treatments were consistent with observed increases of flax growth properties, thereby suggesting a connection between AMF activity and plant growth responses in the field. The method also revealed shifts of root-associated AMF community composition with different inoculation treatments at Outlook IRR, where no difference in plant responses was detected, indicating that even if the AMF inoculant is successfully established, flax responses may not be expressed in some circumstances. Further studies are needed to examine the impact of irrigation on AMF functioning.

It has been suggested that AMF inoculation might drive plant growth responses through increased AMF abundance (Verbruggen et al., 2013). However, community composition was found to be much better than abundance for interpreting field results in the current study. The quantitative bias is still a focal problem regarding next generation sequencing technology (Lindahl et al., 2014).

To conclude, this study indicated that AMF inoculation may only result in a transient increase of flax growth, and this transient increase in plant growth may not necessarily translate to enhanced seed production and yield. Such an effect can be influenced by a number of factors, including inoculation rate, P fertilization, soil initial P level, soil moisture and weather conditions, and selected plant genotype. The pyrosequencing analysis revealed different changes of root AMF community diversity under AMF inoculation at different sites, which might be associated with native AMF community diversity. Community composition was an effective indicator of AMF inoculation induced changes of root-associated AMF community and provided valuable information for interpreting field results.

5. SYNTHESIS AND CONCLUSIONS

The overall goal of this study was to assess the potential for enhancing flax production using AMF inoculation. As a preliminary component of this study, the indigenous AMF communities colonizing flax roots in commercial flax fields were investigated. This survey was an important first step in identifying the impact of soil abiotic and biotic factors that influence the AMF assemblage in flax roots and evaluating the impact of introducing exotic AMF species to these indigenous communities. Following with the preliminary survey investigation, a series of field experiments were conducted to examine flax responses to the combinations of different rates of a commercial AMF inoculant and P fertilizer under different field conditions. The responses of root-associated AMF communities under AMF inoculation were monitored. Finally, a growth chamber experiment was conducted to examine the effect of AMF inoculation on different flax cultivars.

In the survey examining root-associated AMF communities in commercial flax fields, a high colonization level was detected in most of flax fields, suggesting an important role of mycorrhizal symbiosis on flax growth. Spore density varied among survey sites. There was some evidence to suggest that soil AMF abundance was affected by management type, e.g., of the two organically managed fields sampled, both contained high numbers of spores relative to conventionally managed fields. A correlation between root colonization and spore density was observed. However, no consistent trend of root colonization was detected with respect to soil properties and management practices. Similar results were found in the soil AMF survey conducted by Talukdar and Germida (1993), showing that there were no definite trends in AMF colonization with respect to the moisture, temperature gradient, plant available P and soil properties (organic matter, NO_3-N, and plant available P) of the four Chernozem soils in Saskatchewan.

The 454 sequencing method revealed a variety of root-colonizing AMF taxa in different flax fields. Flax roots were dominated by AMF taxa within the Glomeraceae family. The diversity and abundance of the Glomeraceae is featured for AMF communities of the prairie soils (Dai et al., 2013). Conventional management practices strongly modify the

physicochemical characteristics of soil, and thereby influence AMF communities. In the study by Bainard et al. (2014), an effect of host plant identity on the composition of root colonizing AMF communities was shown. Tillage appeared to be a driving factor for AMF communities colonizing flax roots, in terms of abundance, species richness and community composition. Cropping history (i.e., whether a non-AMF host crop such as canola is included in the rotation system) and management type (e.g., organic versus conventional) are other possible factors influencing AMF diversity. Negative correlations between root AMF community characteristics (i.e., abundance, species richness and community diversity) and soil pH, EC and available phosphate were detected. The structures of the root-associated AMF communities were correlated with soil available P and N.

The survey provided interesting data on the status of root-associated AMF communities in flax fields. The impacts of tillage and other agricultural practices on the abundance, diversity and structuring of flax root colonizing AMF communities were revealed. The diversity and composition of root-associated AMF communities were both correlated with soil available P. These results underscores the importance of available P levels, as responses to AMF inoculation, whether positive, negative or neutral, may be influenced by the initial available soil P content.

The field experiments showed inconsistent growth responses of flax to AMF inoculation. Increased root colonization with AMF inoculation was only detected at Outlook. At sites with intermediate initial P levels (Outlook and Outlook DL), evidence of transiently enhanced flax growth properties with AMF inoculation was observed. Both sites showed reduced early-season flax growth with P fertilization. Application of AMF inoculant in combination with P fertilizer also restricted the benefit of AMF inoculation on mid-season flax growth at Outlook. At the low P site (Kelvington) and the irrigated site (Outlook IRR), flax growth was enhanced by P fertilizer to different levels, but there was no effect of AMF inoculation on flax growth. In a greenhouse study conducted by Tydlová et al. (2011), inoculation with native AMF strains was found to significantly increased growth and shoot P concentration of two flax cultivars on reclaimed spoil bank clay. The complicated field environment and the competition with native

AMF possibly limited the effect of AMF inoculation on flax growth.

Analyzing root-associated AMF communities indicated that there were different trends in the changes to AMF community diversity and composition under AMF inoculation between Outlook DL and Outlook IRR. At Outlook DL, the diversity of root colonizing AMF communities appeared to be increased with both rates of AMF inoculation. A marked change of community composition was shown when the AMF inoculant was applied at 2X the recommended rate, which was consistent with results obtained from field experiments. A similar shift in the community composition was not observed when P fertilizer was applied, indicating that P fertilization may influence the impact of AMF inoculation on indigenous AMF community structure and AMF functioning. At Outlook IRR, the diversity of root-associated AMF communities was reduced by both rates of AMF inoculation. Community composition varied with different inoculation treatments, and there was no difference in treatment responses with P fertilizer. These findings agree with Verbruggen et al. (2013) who reported that AMF inoculation may not overcome the limited AMF population and functioning when reduced 'field carrying capacity' is the issue.

As was observed under field conditions, flax cultivars grown under growth chamber conditions were relatively unresponsive to AMF inoculation. Although there were different levels of increases of plant N and P uptake across flax cultivars, only one cultivar (i.e., CDC Neela) responded to AMF inoculation with increased biomass, and no inoculation-induced changes in root colonization were found. Inoculation with AMF generally reduced diversity of AMF communities colonizing different cultivars, and led to varying changes in community composition.

Overall, these findings indicate that the benefits of AMF application to flax production may be limited by a number of factors (e.g., soil initial P level, P fertilization, soil moisture and whether condition, native AMF community, and crop variety), and thus seed yield response increases are variable, and currently difficult to achieve. Further studies are needed for understanding the relationship between flax, AMF and soil P. Attention should be paid to the subsequent influence of AMF inoculation on soil AMF diversity. Furthermore, a breakthrough

is needed for current AMF technology to develop compatible inoculant strains to meet various field environments.

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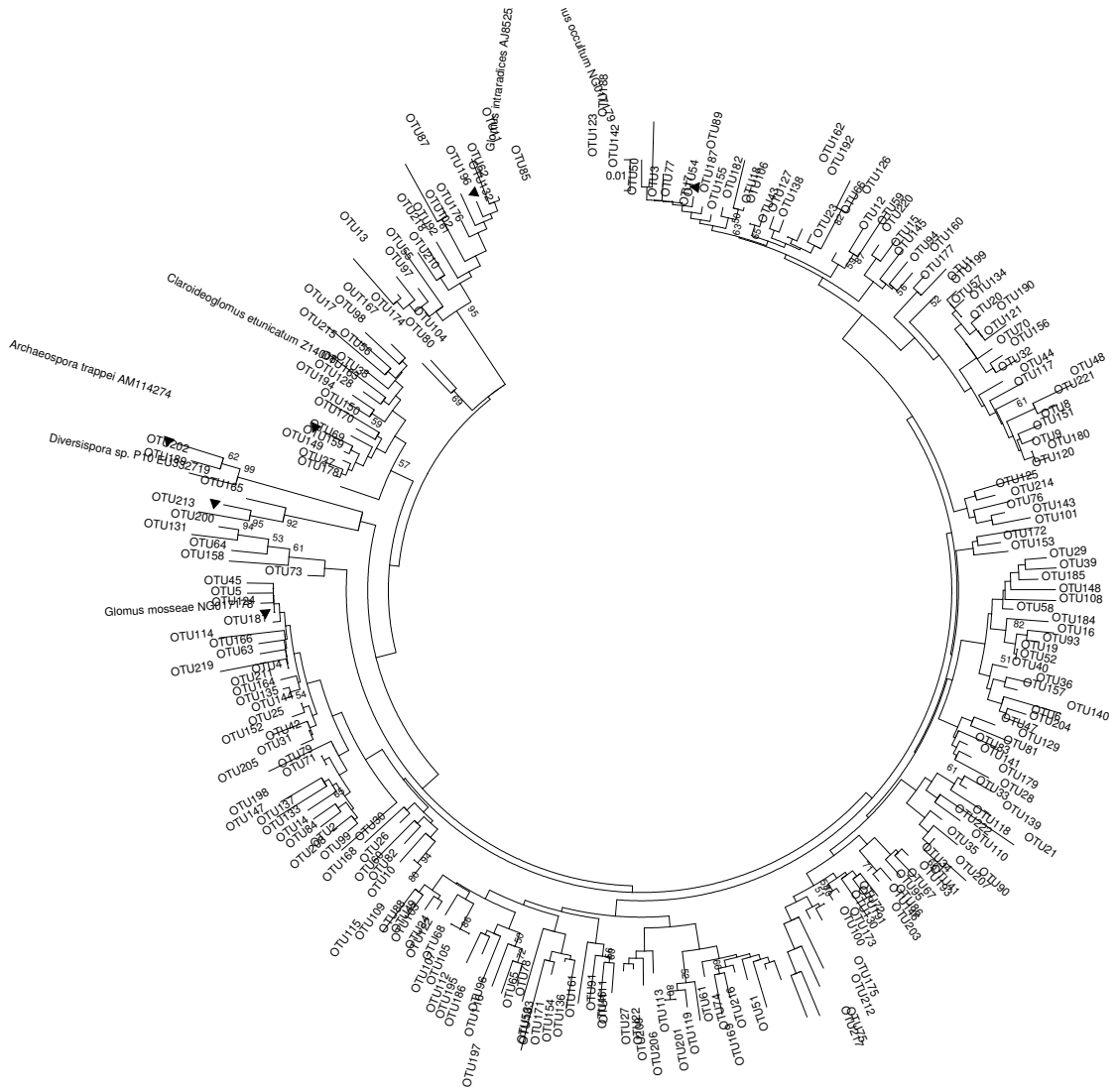
APPENDIX

(URL: http://www.google.ca/map)

Appendix A. The locations of the 18 flax fields in the survey of root inhabiting AMF communities.

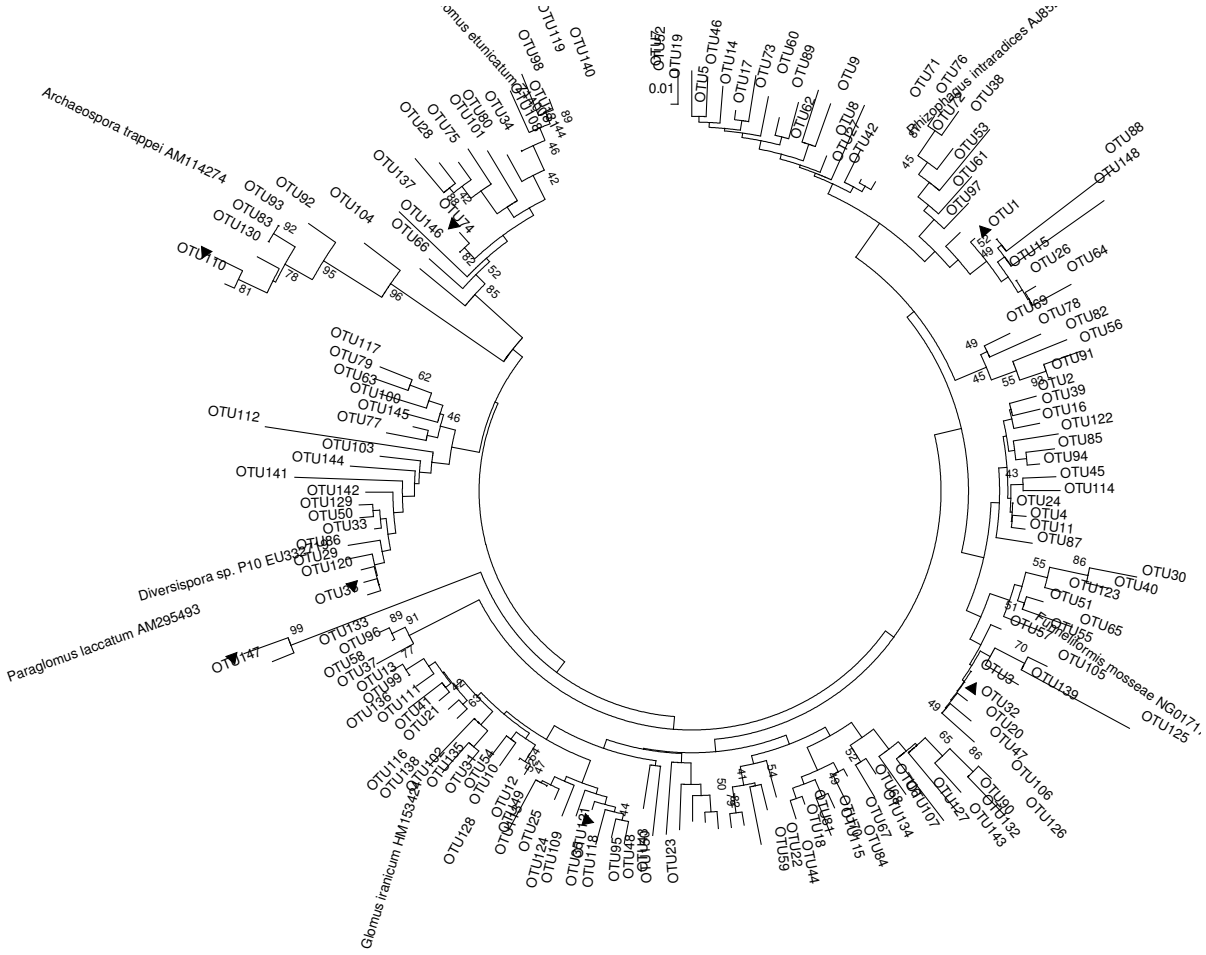


Appendix B. Phylogenetic analysis of the 222 arbuscular mycorrhizal fungal (AMF) operational taxonomic units (OTUs) from root samples of the flax field survey. Reference sequences (▲) are followed by their GenBank accession number. Values on branches are bootstrap values obtained with neighbor-joining algorithm; only values of ≥ 50 are shown.

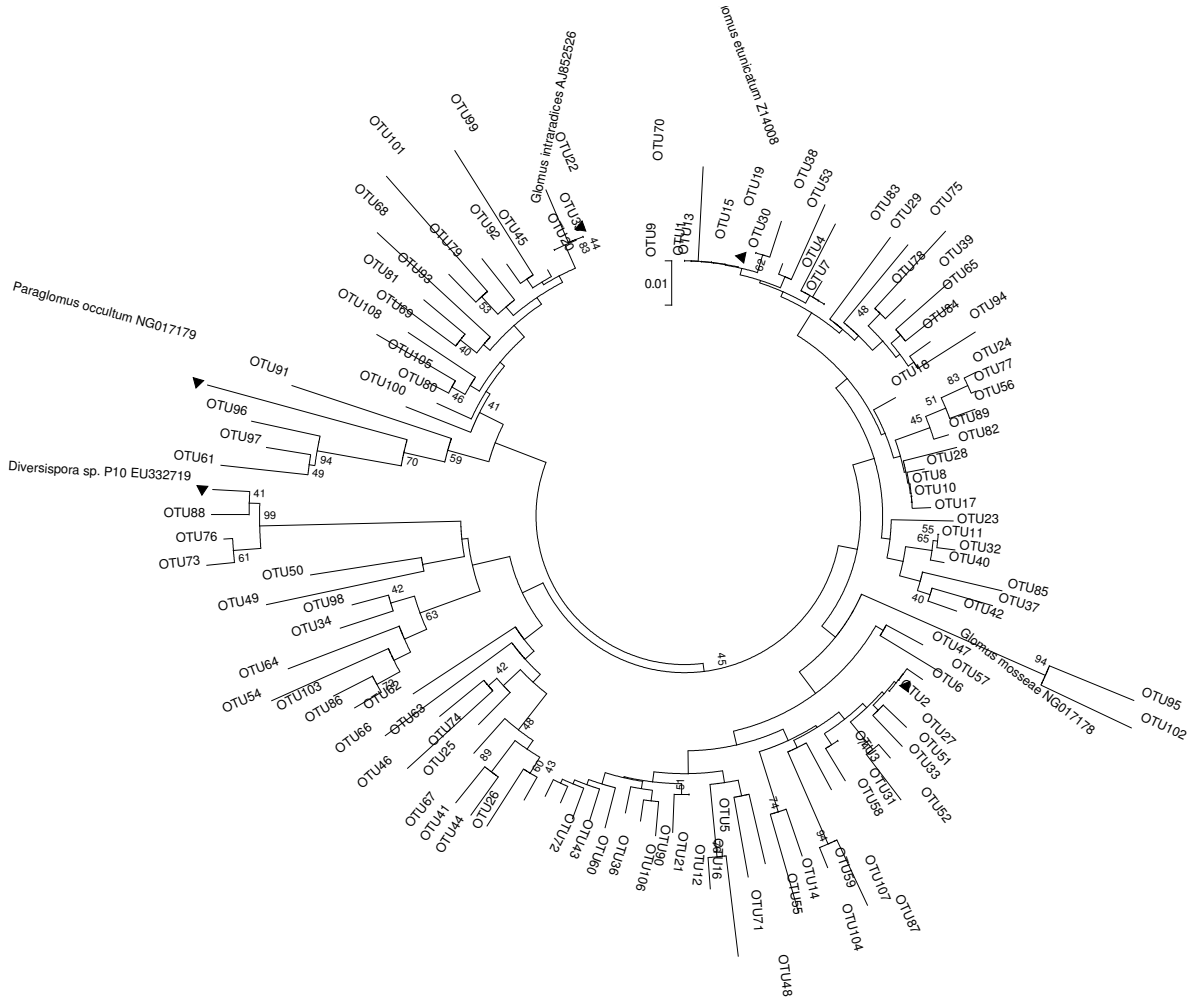
Appendix C. Characteristics of flax varieties in growth chamber study (adapted from Dribnecki et al., 2003; McGregor, 1953; Flax Council of Canada, 2006; Government of Saskatchewan, 2015).

Variety	Year of Registration	Maturity	Seed Color	Seed Size
LinolaTM2047	2003	Medium†	Yellow	Medium
Bison	1930	Late	Brown	Medium
CDC Bethune	1998	Late	Brown	Medium
CDC Neela	2013	Late	Brown	Medium
Norlin	1982	Medium	Brown	Medium
Prairie Thunder	2006	Medium	Brown	Medium

†Medium – 102.9 days according to Dribnecki et al., 2003.



Appendix D. Phylogenetic analysis of the 149 arbuscular mycorrhizal fungal (AMF) operational taxonomic units (OTUs) from flax root samples under different treatments at Outlook DL and IRR in 2013. Reference sequences (▲) are followed by their GenBank accession number. Values on branches are bootstrap values obtained with neighbor-joining algorithm; only values of ≥ 40 are shown.



Appendix E. Phylogenetic analysis of the 108 arbuscular mycorrhizal fungal (AMF) operational taxonomic units (OTUs) from flax root samples in the growth chamber study. Reference sequences (▲) are followed by their GenBank accession number. Values on branches are bootstrap values obtained with neighbor-joining algorithm; only values of ≥ 40 are shown.