Mycorrhizal Communities associated with Soil Aggregates in the Rhizosphere of Willows (*Salix* Spp.) Inoculated with *Rhizophagus intraradices* and *Hebeloma cylindrosporum* Inoculants

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M.A.K. Wijesinghe

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ABSTRACT

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Madantha A. K. Wijesinghe **Advisor: Professor Kari E. Dunfield**

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Mycorrhizae improve plant growth and ecosystem sustainability by forming a symbiotic association with higher plants including the economically important willows (*Salix* spp.). With the recognition of the importance of mycorrhizae in terrestrial ecosystems, inoculation of plants with mycorrhizae is becoming a common practice in agricultural and land reclamation industries. However, little information is available on important ecological parameters such as soil aggregation, soil organic carbon content (SOC) and mycorrhizal communities associated with willow rhizospheres in response to mycorrhizal inoculation in willow systems. The main objectives of this research were to explore how commercial mycorrhizal inoculation of willows impacts (1) percent water-stable soil aggregates (%WSA); (2) indigenous arbuscular mycorrhizal fungi (AMF) and ectomycorrhizal fungi (EMF) community composition associated with different size soil

aggregates; (3) SOC, and (4) willow growth. These objectives were examined under both greenhouse and field conditions.

Using molecular approaches, we observed the existence of spatial variability in indigenous AMF communities among different soil aggregate size classes. This spatial variability varied with type of soil and could be reduced by inoculating with a commercial AMF inoculant. The indigenous EMF diversity was low and did not show spatial heterogeneity among soil aggregates, but it did vary with soil type. In addition, other parameters, including %WSA, SOC within the willow rhizosphere, and willow plant growth also varied by soil type and changed in association with commercial mycorrhizal inoculation.

In the field experiment, soil extracts from willow nursery soil had comparable plant growth promotion with commercial mycorrhizal inoculation, and altered the AMF community associated with bulk soil and soil aggregates in the willow rhizosphere. These results suggest that historically superior willow growth in the willow nursery soil could be due to pre-existing soil microbiological factors.

Overall this research indicates that AMF and EMF inoculation has the potential to influence the composition of the AMF community, but had little influence on the composition of the EMF community in the systems tested.

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Mycorrhizal fungi have been proven to improve plant growth in various direct and indirect ways by establishing a symbiotic association with over 80% of terrestrial plants. Due to the beneficial effects of mycorrhizal fungi on plant growth, inoculation of seedlings with commercial mycorrhizal inoculants is becoming popular in agricultural and land reclamation industries. Further, willow (*Salix* spp.) is an economically important tree which benefits from a dual mycorrhizal symbiosis. However, the impacts of inoculation of willows with commercial mycorrhizae on willow plant growth and some important soil health indicators in willow rhizosphere are unknown.

The purpose of this PhD research project was to explore the impacts of inoculation of willows with a commercial mycorrhizal inoculant on plant growth and indicators of soil health such as soil aggregation and organic carbon content, and to assess small scale spatial variability of indigenous mycorrhizal communities in the willow rhizosphere.

A combination of greenhouse and field experiments had the following main objectives:

1) To determine if inoculation with commercial mycorrhizae affected the spatial distribution of indigenous arbuscular and ectomycorrhizal fungi (AMF and EMF) communities among different soil aggregate sizes under controlled and field conditions.

2) To determine if mycorrhizal inoculation, soil type, or species of willow (*Salix viminalis* and *S. miyabeana*) impacted indicators of soil health (%WSA and SOC) under controlled environmental conditions.

3) To determine if inoculating with a combination of mycorrhizal inoculants or an unsterilized soil extract impacts indicators of soil health, plant growth, and the AMF and EMF communities in the rhizosphere of willows grown in the field.

The research presented in this thesis is part of a multidisciplinary research project, which also explores the impacts of mycorrhizal inoculation on nitrogen cycling, denitrifying bacterial communities, root colonization by mycorrhizae, and quality and quantity of woody willow biomass. Results presented in this thesis will broaden the knowledge of mycorrhizal inoculation impacts on soil aggregation, soil organic carbon status, and aggregate level spatial heterogeneity of indigenous AMF and EMF communities in willow rhizosphere.

1.1.THESIS FORMAT

This Ph.D. thesis consists of five chapters. The first chapter is an introduction and literature review of the topics of mycorrhizae, spatial heterogeneity of microbes, soil aggregation, soil organic carbon, and willows (*Salix* spp.). This chapter will provide background information to justify the objectives of my research.

The next three chapters are in manuscript format. The second chapter is a greenhouse study that assessed the community structure of AMF and EMF associated with four different soil aggregate size classes in the rhizosphere of willow inoculated with either *Rhizophagus intraradices* (an AMF inoculant), *Heboloma cylindrosporum* (an EMF inoculant), or a combination of AMF+EMF in two different soils.

The third chapter is an experiment that measured percent water-stable aggregates (%WSA) and soil organic carbon content (SOC) in rhizospheres of two willow species. The willows were planted in six different soils, inoculated with either AMF, EMF, or a combination of AMF and EMF inoculants, and maintained under greenhouse conditions.

The fourth chapter outlines a field study where willow cuttings were inoculated with a combination of commercial AMF and EMF inoculants or an unsterilized soil extract and left to grow for a year. Estimates of willow biomass and impacts of inoculation on soil health parameters, such as soil aggregation, soil organic carbon, and the diversity of the AMF and EMF communities were assessed.

The fifth chapter provides a summary and discussion of the results of all research chapters. This summary also recommends future research directions based on experimental results.

1.2. LITERATURE REVIEW

Soil microbial communities are diverse (Curtis et al., 2002; Robe et al., 2003) and comprise much of the biodiversity of the earth (Green et al., 2004; Roesch et al., 2007). It has been reported that up to 10 billion microorganisms, and thousands of different species, coexist in one gram of soil (Knietsch et al., 2003). These soil microorganisms are ubiquitous in the environment and play a critical role in a range of important ecological functions, including maintenance of ecosystem health and ecological stability, biogeochemical cycling, organic matter decomposition, and release of mineral nutrients (Artursson et al., 2006; Biro et al., 2000; Green et al., 2004; Kennedy, 1999; Nannipieri et al., 2003; Rutigliano et al., 2004). By influencing these key soil processes, microorganisms can be important determinants of the primary productivity of soil. Soil microbial communities vary mainly with soil factors such as soil type (Silveira et al., 2006; Singh et al., 2006) and soil physico-chemical properties (Girvan et al., 2003).

1.2.1. MYCORRHIZAL FUNGI

Mycorrhizal fungi form symbiotic associations with about 80% of plant species and are found in most habitats in the world (Smith and Read, 2008). The two main groups of mycorrhizae, arbuscular mycorrhiza (AMF) and ectomycorrhiza (EMF), are distinguished by their physiological and structural characteristics. The main structural differences between these two fungi are the AMF colonize intracellularly, do not have a fungal mantle and hartig net, and hyphae are aseptate, where as EMF colonize only intercellularly, have a fungal mantle and hartig net, and hyphae are septate (Smith and Read, 2008).

Both mycorrhizal types make a critical link between soil and plants (Wilson et al., 2009) and improve plant growth directly and indirectly. AMF directly influence plant growth by improving nutrient status and water relationships (Koide and Mosse, 2004; Roldan et al., 2008; Smith and Read, 2008), and by suppressing diseases (Lowe et al., 2012) and pests (Hol and Cook, 2005; Shreenivasa et al., 2007). In addition to these direct effects, mycorrhizae make large indirect contributions to soil sustainability by improving soil aggregation (Bearden and Petersen, 2000;Bedini et al., 2009; Caravaca et al., 2002; Celik et al., 2004; Rillig, 2004; Rillig and Allen, 1999), improving carbon sequestration through C cycling (Finlay, 2008; Fitter et al., 2000; Zhu and Miller, 2003), and changing rhizosphere microbes (Andrade et al., 1998; Bakhtiar et al., 2001; Carlsen et al., 2008; Chandanie et al., 2005). Despite structural differences from AMF, EMF are also known to improve water relations (Bingham and Simard, 2012; Lehto and Zwiazek, 2011), nutrient absorption (Jansa et al, 2011; Alvarez et al, 2009), and resistance to diseases (Zhang et al., 2011) in plants. Hence, pre inoculation of planting material with commercial mycorrhizae is a common practice in horticultural and land reclamation industries (Mummey et al., 2009) to get the benefits from introduced mycorrhizae in addition to the benefits obtain from indigenous mycorrhizal fungi present in the system. Further, it is clear that these fungi are important and common component in the terrestrial ecosystem. However, their importance on ecosystem functions is poorly addressed. Therefore, currently, the focus of mycorrhizal research has switched from exploration of their ecology and influence on plant communities to exploration of their contribution to ecosystem processes (Rillig, 2004).

1.2.1.1.MYCORRHIZAL INOCULANTS

Several mycorrhizal inoculants are used in agriculture and land reclamation industries. Arbuscular mycorrhizal inoculants used in horticultural crops have proven to increase seedling establishment both under greenhouse and field conditions (Lovato et al., 1995), improve acclimatization of micro propagated plants (Vestberg et al., 2002), induce early flowering (Sohan et al., 2003), and increase resistance to soil-borne diseases (Azcon-Aguilar et al., 2002) in plant nurseries.

Inoculation of tree plants with EMF improves plant growth and production (Garbaye et al., 1988) by improving water relations and nutrient absorption (Plamboecket al., 2007; Jansa et al., 2011; Lehto and Zwiazek, 2011; Bingham and Simard, 2012), and by inducing resistance to soil-borne pathogens (Zhang et al., 2011). Both arbuscular and ectomycorrhizal strains are commonly used as inoculants to improve plant growth and establishment. Some of the arbuscular mycorrhizal species used as mycorrhizal inoculants are as follows (note that new mycorrhizal names are provided in brackets): *Glomus etunicatum (Claroideoglomus etunicatum), G. fasciculatum, G. intraradices (Rhizophagus irregularis), G. manihotis, G. mosseae (Funneliformis mosseae),G. claroideum (Claroideoglomus claroideum),G. deserticola, G. aggregatum, G. clarum, G. macrocarpum,G. fasciculatum, G.geosporum, Gigaspora margarita, Gigaspora gigantea, Gigaspora rosea, Paraglomus occultum, Acaulospora scrobiculata, Acaulospora denticulata, Acaulospora morrowiae, Diversispora spurca, Scutellospora calospora,* and *Scutellospora pellucida* (Aggangan et al., 2011; Corkidi et al., 2004; Herrera-Peraza et al., 2011; Klironomos, 2003; Kruger et al., 2012; Mummey et al., 2009; Vázquez et al., 2000). Some EMF species that have been used as mycorrhizal inoculants include

Cenococcum geophillum, Cortinarius purpurascens, Entoloma nidorosum, Entoloma spp., *Hebeloma crustuliniforme, Hebeloma pusillum, Hebeloma westraliense, Laccaria bicolor, Laccaria laccata, Paxillus involutus, Pisolithus albus, Pisolithus tinctorius, Rhizopogon parksii, Suillus luteus*, and *Thelephora terrestris* (Assigbetse et al., 2005; Loree et al., 1989; Selosse et al., 1998; Theodorou, 1971; Thomson et al., 1996).

According to expectations, inoculation with the above-listed commercial mycorrhizal inoculants should improve plant growth. However, it has been reported that the response of plants to mycorrhizal inoculation can range from extremely positive to negative, depending on the plant species and type of mycorrhiza (Klironomos, 2003; Piotrowski et al., 2004). Numerous companies world-wide produce commercial mycorrhizal inoculants (Gianinazzi and Vosátka, 2004). Though they list the concentration of spores and the colony-forming units of the mycorrhizal fungi in their products, the actual performance of most of these mycorrhizal inoculants are not well known (Tarbell and Koske, 2007).

Mycorrhizal inoculation could also impact the native microbial community (Roesti et al, 2006; Srivastava et al., 2007). Srivastava et al. (2007) suggested that any microbial inoculant can change the rhizosphere (soil surround the roots of plant), and thus alter the residing micro floral communities. For instance, when cowpea and pea plants were inoculated with AMF and *Pseudomonas,* Srivastava et al. (2007) observed a drastic change in the residential microbial community when analyzed by DGGE. Associated AMF can change root exudates quantitatively and qualitatively, thereby altering the microbial community structure of the rhizosphere (Pinior et al., 1999; Vierheilig et al., 2000; Scervino et al., 2005). Furthermore, when mycorrhiza is

introduced to plant roots, it also can alter the native AMF community structure through positive (facilitation) or negative (competition) interactions (Callaway and Walker, 1997). Pre-inoculation of seedlings with AMF has caused changes in the AMF communities that were colonized in *Leucanthemum vulgare* roots (Mummey et al., 2009). However, the impact of inoculating willows with commercial mycorrhizae on indigenous mycorrhizal community in the willow rhizosphere, and their small-scale spatial heterogeneity, is completely unknown.

1.2.2. SPATIAL HETEROGENEITY OF MYCORRHIZAL FUNGI AMONG SOIL AGGREGATE SIZE CLASSES

Soil environments are diverse, and are expected to have diverse soil microbial communities. Spatial distribution is one of the most fundamental ecological parameters for any group of organisms (Mummey and Rillig, 2008). To understand the functions mediated by microbes and their controlling factors requires a detailed analysis of microbial communities at the scales where they actually operate(Mummey et al, 2006). Communities of AMF can have spatial heterogeneity (Rosendahl and Stukenbrock, 2004) and can be structured at sub-meter scale levels (Mummey and Rillig, 2008). Information on spatial distribution of mycorrhizal fungi communities is rare; however, it has been identified as an important new focus of mycorrhizal ecology in order to understand the ecosystem functions they mediate(Martínez-García and Pugnaire, 2011).

Soil aggregates provides a spatially segregated micro environment in the soil (Mummey et al., 2006). Different micro environmental conditions within soil aggregates provide small-scale heterogeneity in microbially mediated ecosystem functions (Sey et

al., 2008). Previous studies have shown that microbially mediated soil functions, such as denitrification and enzymatic activities, can vary among different soil aggregate size classes (Drury et al., 2004; Gupta and Germida, 1988; Hoffmann et al., 2007; Lensi et al., 1991; Mendes et al., 1999; Seech and Beauchamp, 1988; Sexstone et al., 1985; Sey et al., 2008). Additionally, microbial biomass and diversity of some bacterial groups have also been shown to vary across soil aggregate size classes (Gupta and Germida, 1988; Kong et al., 2010; Mendes et al., 1999; Mendes and Bottomley, 1998; Miller et al., 2009; Miller and Dick, 1995; Mummey et al., 2006; Ranjard et al., 2000). Though mycorrhizal fungi are involved in many ecosystem functions such as C sequestration, nutrient cycling, and organic matter decomposition (Smith and Read, 2008), there is no information on mycorrhizal communities associated with soil aggregates and their spatial heterogeneity among soil aggregates.

1.2.3. INDIRECT CONTRIBUTIONS OF MYCORRHIZAE TO SOIL SUSTAINABILITY

1.2.3.1. SOIL AGGREGATION

Soil structure is expressed as degree of stability of soil aggregates (Bronic and Lal, 2005; Six et al., 2000). Soil structure is important in ecosystem sustainability and moderates environmental and water quality (Bronic and Lal, 2005). Poorly structured soils erodes easily and it has been reported that over one-third of arable lands in the world have been lost due to soil erosion over the past 40 years (Pimentel et al., 1995). As such, a current focus in sustainable agriculture is towards managing well-aggregated soils (Piotrowski et al., 2004).

Tisdal and Oades (1982) showed that soil aggregation is a complex, hierarchical process mediated by soil biotic and abiotic factors. In this process, soil particles cement together to form micro-aggregates (<250 μm in diameter) which are further bound together to form different sizes of macro aggregates (>250 μm in diameter) (Jastrow et al., 1996). The amount of aggregation in a soil varies according to soil characteristics. Organic carbon content, for example, is one of the main factors that impacts soil aggregation. Tisdall and Oades (1982) described the importance of soil organic matter in binding soil aggregates to withstand slaking by rapid wetting. Micro aggregates are stabilized by simple organic compounds such as polysaccharides (Spaccini et al., 2002), while macro aggregates are stabilized by complex humidified organic compounds (Denef et al., 2001). A recent study by Benbi and Senapati (2010) showed a linear relationship between soil organic carbon and percentage of macro aggregates, while applying different organic residues to soils of rice–wheat systems in north India.

Mycorrhizal fungi are known to improve soil aggregation. Among the two main types of mycorrrhizae, EMF and AMF, the significance of AMF on soil aggregation has been well documented. Extraradical mycelia of AMF entangle soil particles to stabilize the soil aggregates while extensively growing in the soil (Rillig, 2004; Tisdall and Oades, 1982). Furthermore, the ability of AMF mycelia to repel water helps to maintain water-stable soil aggregates (Rillig et al., 2010). Soil aggregates can also be stabilized by glomalin, a putative protein produced by AMF (Rillig, 2004; Wright and Upadhyaya, 1996). Wright and Upadhyaya (1998) showed that the soil aggregate water stability was positively and linearly correlated with concentrations of glomalin-related soil protein. Some microbes, such as *Paenibacillus* bacteria, associated with AMF mycelium, and

spores have also been proven to improve soil aggregation (Bezzateet al., 2000; Budi et al., 1999; Hildebrandt et al., 2002; Mansfeld-Giese et al., 2002). The effect of EMF alone or in combination with any other bio-inoculant on soil structure has, however, been less studied. Ambriz et al. (2010) investigated effects of AMF and EMF alone and in combination on soil aggregation by inoculating *Fraxinus* seedlings grown in sterilized soil. They observed that water-stable aggregation (%WSA) varied with type of mycorrhizal inoculation and the size of soil aggregate fraction. They also found %WSA of the 2mm and 1mm size soil aggregate classes was lower in AMF-inoculated systems compared to EMF-inoculated systems and a combination of AMF- and EMF-inoculated systems.

1.2.3.1.1. EFFECT OF PLANT ROOTS ON SOIL AGGREGATION

Plant roots have a major impact on soil aggregation. Tisdall and Oades (1982) proposed a conceptual model which showed the importance of plant roots in the formation of macro soil aggregates from micro aggregates by physical entanglement. Bronick and Lal (2005) explained how plant roots and their rhizospheres can affect soil aggregation through physical, biological and chemical measures. Physically, soil aggregates can form as a result of enmeshing and realigning of soil particles by plant roots**.** Chemically, root exudates, such as polygalacturonic acid, help to bind soil particles to form soil aggregates (Bronick and Lal, 2005). Furthermore, roots and their rhizosphere host a large population of micro- and macro-organisms which help in biological soil aggregation. Caracvaca et al. (2002) and Thomas et al. (1993) showed higher soil aggregate stability in rhizospheric soil than in non-rhizospheric soil. Jastrow

(1987) reported a strong and linear relationship of root biomass to formation of water-stable aggregates using soils of Prairie Peninsula. Harris et al. (1966) and Chan and Heenan (1996) showed higher macro-aggregate stability in barley plants with fibrous roots.

1.2.3.2 SOIL ORGANIC CARBON (SOC)

Soil is the largest pool of terrestrial organic carbon in the biosphere and stores more carbon than do plants and the atmosphere together (Schlesinger, 1997). The soil organic carbon pool is an important component of terrestrial ecosystems and is crucial for regulating the carbon fluxes between biosphere and atmosphere (Zhu and Miller, 2003). It has potential to mitigate the problem of the current rise in atmospheric CO_2 concentration (Godblod et al., 2006). Further, an understanding of SOC storage is crucial for understanding the ecosystem processes as it affects and is affected by plant production and plays a major role in maintaining soil fertility and productivity (Jobbagy and Jackson, 2000).

Mycorrhizal fungi are an important component of the SOC pool (Zue and Miller, 2003). They can improve SOC by various direct and indirect ways. Directly they consume about 4–20% of the host plant's carbon (Graham, 2000). They exhibit rapid turnover of hyphae which are recalcitrant due to the chitin found in their cell walls (Staddon et al., 2003). AMF also produces glomalin, a putative protein which is fairly stable in soil (Steinberg and Rillig, 2003). Indirectly, mycorrhizae also contribute to the SOC pool by increasing the sink demand for plant carbohydrates (Graham, 2000), up regulating photosynthesis by improving water and mineral nutrient status of plants

(Miller et al., 2002), improving soil aggregation (Oades and Waters, 1991), and translocating C away from the root zone which has high losses of C through respiratory activities (Treseder and Allen, 2000; Zhu and Miller, 2003). Zhu and Miller (2003) reported that the amount of SOC derived from AMF ranges from 54–900 kg/ha. Hogberg and Hogberg (2002) showed that extrametrical mycelia of EMF together with associated roots contribute half of the dissolved organic carbon in a pine forest soil in Sweden. Godbold et al. (2006) showed that external mycelia of mycorrhizae provide the dominant route (62%) for C to enter the soil organic matter pool.

1.2.3. WILLOWS (*SALIX* SPP.)

Willow (*Salix* spp.) is among the earliest recorded pre-ice age flowering plants (Newsholme, 2002). It originated in the subtropics, and then extended to tropical areas and to temperate regions. Willow (*Salix* spp.) is considered an economically important tree. Mainly, willows are used in biomass production for bio-fuel and fibre industries (Kuzovkina and Quigley, 2005). Average growth rate of new willow clones exceed 15 oven dried tons (odt) ha^{-1} $year^{-1}$ on 3- to 4-year rotations with low inputs, showing the potential of willow as a bio-energy crop. The biomass value of willows vary from $ 40-90 odt^{-1} (Buchholz and Volk, 2011). Willows are also becoming more prevalent in environmental restoration, phytoremediation, and erosion control (Kuzovkina et al., 2004; Kuzovkina and Quigley, 2005). These multiple uses of willows are mainly due to their unique physiological characteristics. Some of those characteristics are their superior growth rate (Christersson et al., 1993; Wilkinson, 1999) and their extensive fibrous root systems which expand the area for absorption of water and nutrients (Gray and Sotir,

1996; Rytter and Hansson, 1996). Furthermore, willows can uptake nutrients efficiently (Elowson, 1999; Ericsson, 1981), tolerate water-logged conditions (Aronsson and Perttu, 2001; Jackson and Attwood, 1996; Kuzovkina and Quigley, 2005), and propagate easily (Gray and Sotir, 1996). In addition, vigorous re-establishment after coppicing (Ceulemans et al., 1996; Philippot, 1996) and accumulation of high concentrations of toxic metals within vegetative biomass (Klang- Westin and Eriksson, 2003)are added advantages of their usage in restoration of contaminated sites. Interestingly, willows are known to benefit from symbioses with both AMF and EMF (Paradi and Baar, 2006). Despite the importance of willows in bio-fuel and fibre production, and its emerging influences in soil ecology, there are gaps in many ecological aspects in the literature, such as their role in soil aggregation, carbon sequestration, and their associations with mycorrhizal communities. The main goal of this research was to study how commercial mycorrhizal inoculation impacts soil aggregation, C sequestration, and the spatial variability of mycorrhizal communities associated with the willow rhizosphere.

1.4. CONCLUSIONS

This literature survey, focused on the recent findings about mycorrhizal fungi, spatial heterogeneity of mycorrhizal communities, soil aggregation, and SOC, shows the importance of these parameters for the function and sustainability of terrestrial ecosystems. Furthermore, it indicates that the soil aggregation and soil microbial communities vary with soil factors and bio-inoculation. The reported literature also reveals the importance of willow trees and mycorrhizal fungi for ecosystem sustainability. Although mycorrhizae and willows are two of the important components used in maintaining and improving terrestrial ecosystem sustainability, less information is available about their combined influence on important ecological parameters such as soil aggregation, SOC, and mycorrhizal communities associated with willow rhizosphere.

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CHAPTER 2: MYCORRHIZAL COMMUNITIES ASSOCIATED WITH SOIL AGGREGATE SIZE CLASSES IN THE RHIZOSPHERE OF MYCORRHIZAL INOCULATED WILLOWS (*SALIX* SPP.)

2.1. INTRODUCTION

Soil structure is a key factor in soil health (Bronick and Lal, 2005). It is important for soil function through the maintenance of soil physical, chemical (Emerson et al., 1986; Martin et al., 1955; Oades, 1984) and biological properties (Coleman, 1986; Elliott and Coleman, 1988), and hence is crucial to the success of a sustainable agricultural ecosystem and erosion resistance (Piotrowski et al., 2004).

Soil aggregate stability is commonly used as an indicator of soil structure (Six et al., 2000).Soil aggregation is a complex, hierarchical process mediated by a combination of soil biotic and abiotic factors (Tisdall and Oades, 1982). In this hierarchical model, soil particles are cemented together to form micro-aggregates (<250 µm in diameter), which are bound together to form different sizes of macro- aggregates (>250 µm in diameter) (Jastrow et al., 1996). Soil aggregates provide a wide diversity of spatially segregated micro environments (Young and Ritz, 1998) characterized by complex distribution patterns of substrates, nutrients, water, oxygen, and pH (Mummey et al., 2006). These different micro environmental conditions select for different microbial communities (Blaud et al., 2012) and provide small-scale heterogeneity of microbially mediated ecosystem functions (Sey et al., 2008). Further, microbial communities and their functions are mainly influenced by soil structure (Young and Crawford, 2004), the size of the aggregate and location within the aggregate (Ranjard et al., 2000; Poly et al., 2001; Mummey and Stahl, 2004).

Previous studies have shown that microbially mediated soil functions, such as denitrification and enzyme activities, can vary among different soil aggregate size classes (Drury et al., 2004; Gupta and Germida, 1988; Hoffmann et al., 2007; Lensi et al., 1991; Mendes et al., 1999; Seech and Beauchamp, 1988; Sexstone et al., 1985; Sey et al., 2008). In addition, microbial biomass and diversity of some bacterial groups have also been shown to vary across soil aggregate size classes (Gupta and Germida, 1988; Kong et al., 2010; Mendes et al., 1999; Mendes and Bottomley, 1998; Miller et al., 2009; Miller and Dick, 1995; Mummey et al., 2006; Ranjard et al., 2000).

Mycorrhizal fungi form a symbiotic association with most of the higher plants in terrestrial ecosystems (Schussler et al., 2001). They make contributions to improve plant growth through a variety of direct and indirect methods, including improvement of nutrient status and water relationships (Smith and Read, 2008), suppression of diseases (Zhang et al., 2011, Lowe et al., 2012) and pests (Hol and Cook, 2005; Shreenivasa et al., 2007), and influence on many soil functions such as organic matter decomposition, water balance and nutrient cycling (Smith and Read, 2008). However, information on the spatial distribution of mycorrhizal fungal communities is rare; this distribution has been identified as an important new focus of mycorrhizal ecology (Martínez-García and Pugnaire, 2011).

Willow (*Salix* spp.) is an economically important tree which is used in environmental restoration (Kuzovkina and Quigley, 2005) and is distributed worldwide, covering a range of soil types (Argus, 1997). The possibility of duel symbiosis with both arbuscular and ectomycorrhizae make willows an interesting study species.

Inoculation of planting material with mycorrhizal fungi is common practice in horticultural and land reclamation industries (Mummey et al., 2009). However, impacts of inoculation of willow planting materials with commercial mycorrhizae on important ecological aspects such as soil aggregation and aggregate-scale distribution of mycorrhizal communities in the willow rhizosphere are unknown. Further, soil aggregation and microbial communities are reported to vary with soil characteristics (Tisdall and Oades, 1982; Silveira et al., 2006; Singh et al., 2006).

The objective of this greenhouse study was to assess the impact of inoculating willow with a combination of arbuscular and ectomycorrizal inoculants on the diversity of rhizosphere mycorrhizal fungal communities among soil aggregate classes in two soil types. I hypothesized that mycorrhizal communities would differ according to aggregate size class, and that both soil and inoculation type would affect this community.

2.2. MATERIALS AND METHODS

2.2.1. SOIL AND PLANT MATERIALS

Soils were collected from two fields that had not been cultivated for at least a growing season. Physical and chemical properties of these soils are reported in Table 2.1. Since willows normally grow in marginal lands, to simulate marginal soils we collected our soils from abandoned farms. Willow cuttings (25 cm long) were retrieved from mature willow stands of *Salix viminalis* (Alpha clone) belonging to LandSaga Biogeographical Inc., Guelph, ON, Canada.

2.2.2. GREENHOUSE EXPERIMENT

This experiment was designed as a two-way factorial experiment using 2 soil types and 4 mycorrhizal fungal inoculations (n = 8) for a total of 64 plants.

Willow plant cuttings were surface sterilized (70% ethanol, 3 min; 1.5% sodium hypochlorite, 5 min) and planted in 66 mL cone-tainers (Steuwe and Sons Inc., Tangent, OR) containing gamma-irradiated (sterilized) peat-based growth medium (Sunshine Mix #4, SunGro Horiculture, Bellevue, WA). We used sterilized growth medium to minimize native mycorrhizal competition and enhance the mycorrhizal colonization in willow roots.

Willows were inoculated with either *Rhizophagus intraradices* (10 propagules per cone-tainer) (AMF), *Hebeloma cylindrosporum* (3750 propagules per cone-tainer) (EMF), a combination of both AMF and EMF (AMF+EMF), or sterile water (none). Inoculants were supplied by Mikro-Tek Inc., Timmins, ON, Canada. The plant cuttings were allowed to root in the cone-tainers kept in a Phytotron-Greenhouse (University of Guelph) with the following conditions: 22–24 / 16–18ºC day/night temperature, ambient air

relative humidity, and supplemental light intensity at 300–500 μM m^2 sec^{-1}. After three months, cuttings were transplanted into plastic pots (2.8 L) filled with unsterilized field soil (Selkirk clay and Guelph Loam) and re-inoculated, in the same way as described above, to ensure colonization with inoculated mycorrhizae. Samples from both the soils were sent to the laboratory services division, University of Guelph, Guelph, ON, Canada for chemical characterization (Table 2.1).

The pots were arranged in completely randomized design in the greenhouse, and irrigated with a drip irrigation system at a rate of 120 mL pot^{-1} day^{-1}. No fertilizer was added to the plants. Willows were harvested after 29 weeks of growth; shoot and root samples were separated. Small roots in the soil samples were removed by hand and soil from each pot were divided into two parts and stored in plastic bags separately under 4°C and -20°C for further analysis.

2.2.3. QUANTIFICATION OF WATER-STABLE AGGREGATES

A subsample of soil (about 200 g) from each soil sample was obtained and allowed to air dry. Any remaining roots in the soil were removed using a forcep. Air dried soil was sieved using 2 mm and 1 mm sieves to obtain 1–2 mm size fraction. Four grams of the 1–2 mm soil fraction was placed on the 250 μm sieve and wet sieved (35 times per minute) for three minutes using a wet sieving apparatus. The percentage of water stable aggregates (%WSA) was estimated using the method described by Kemper and Rosenau (1986). Briefly, the oven dry (110°C) weights of the soil fractions that remained in the sieve (water-stable aggregates (W1)) and passed through the sieve (water-unstable aggregates (W2)) were obtained, and percent water-stable aggregates

were calculated by Formula 1. According to protocol, the oven dry weights of water-stable aggregates were corrected for sand fraction by dispersing and washing using 0.5% sodium hexametaphosphate solution.

1. WSA% = W1/(W1+W2) x 100

2.2.4. AGGREGATE SIZE CLASS SEPARATION AND DNA EXTRACTION

Soil from the eight replicates of each treatment was partially pooled by combining replicates 1, 2, and 3; replicates 4, 5, and 6; and replicates 7 and 8 to reduce the number of samples and allow for an adequate amount of soil for dry sieving analysis. Pooled soils (n=3) were dry sieved into four size classes (>4 mm, 4–2 mm, 2–0.5 mm and <0.5 mm) as described in Seech and Beauchamp (1988). Dry sieving was chosen to avoid desorption of DNA from soil particles, as described in Levy-Booth et al. (2009). A 0.25 g subsample per pooled soil sample was randomly removed from each homogenized aggregate size class, and DNA was extracted using the Mo Bio Power Soil DNA extraction kit (Mo Bio Laboratories, Solana Beach, CA). Extracted DNA was stored at -20°C for subsequent analysis.

Table 2.1. Characteristics of soils used for the experiment

Soil Character	Selkirk Clay	Guelph Loam
NH_4-N (ppm)	4.8	2.4
NO_3-N (ppm)	57.5	40.3
P (ppm)	28	5
K (ppm)	222	62
Mg (ppm)	681	396
Org. matter (%)	5.3	3.8
pH	6.5	7.6
Clay (%)	49.9	11.2
Bulk density (field)	0.9	1.1
Textural class	Clay	Loam

2.2.5. QUANTIFICATION OF AMF USING REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION

Abundance of AMF in soil aggregate classes was determined by real-time quantitative polymerase chain reaction (qPCR) using Bio-Rad i^{TM}Q5 Multicolor Real-Time PCR detection system (Bio-Rad Laboratories Inc.). Amplification was performed using iQ SYBR Green Supermix (2X reaction buffer with dNTPs, iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR Green I, fluorescein and stabilizers) (Bio-Rad Laboratories, Hercules, CA, USA), and primers AML1 (5'- ATC AAC TTT CGA TGG TAG GAT AGA-3') and AML2 (5'- GAA CCC AAA CAC TTT GGT TTC C-3') targeting a small sub-unit rRNA gene (10 pmol ul^{-1}). We choose primers LR1 and LR2 as these primers are suitable for surveying relative abundance of all known AMF using a single PCR reaction (Lee et al., 2008). The thermal cycling program used was as follows: 3-min denaturation at 95ºC followed by 40 cycles of 30-s denaturation at 95ºC, 40-s primer annealing at 58ºC and 55-s extension at 72ºC , followed by 10 min of final extension at 72ºC. A melt curve analysis was performed for each reaction mixture as a final cycle at 58–96ºC to determine the product specificity. The threshold cycles (ct) were calculated byBio-Rad-iQ5 software to indicate significant fluorescence signals raising the above background during early cycles of the exponential phase of PCR amplification. A minimum of two negative controls (adding water without templates) were included in the qPCR run to check for any contamination in the reaction components. Standard curves were constructed from the AML gene using known quantities of pure culture, linearized plasmid. A 10-fold dilution series (from 10^1–10^6) was run in triplicate along with the unknown samples.

2.2.6. DIVERSITY OF AMF COMMUNITIES

The diversity of AMF communities was assessed by PCR denaturant gradient gel electrophoresis (PCR-DGGE). Due to the complexity of this analysis, and to focus on the impact of AMF inoculation, only three mycorrhizal inoculation types (non inoculated control, AMF, and AMF+EMF treatments), two soil types (Selkirk clay and Guelph loam),were analysed for all aggregate size classes.

2.2.6.1. PCR AMPLIFICATION OF A PARTIAL LSU rDNA REGION

Analysis of DNA sequences of the large sub-unit (LSU) of the ribosomal RNA genes was used to study the diversity of AMF using nested polymerase chain reaction (PCR) as described previously (Miras-Avalos et al., 2011). Primers LR1, LR2, FLR3, and FLR4 were chosen, as these sets of primers have been shown to give successful results from PCR-DGGE (Miras-Avalos et al., 2011; Rodríguez-Echeverría et al., 2009). Briefly, in the first PCR, primers LR1 and FLR2 (Trouvelot et al., 1999; Van Tuinen et al., 1998) were used to amplify sequences of the LSU of ribosomal DNA (rDNA) of general fungi. A 20 µl reaction mixture contained 2 µl of 10X PCR buffer (Promega, Madison, WI, USA), 25 mM $MgCl_2$, 200 nM deoxy nucleotide triphosphate (dNTPs), 500 nM of each primer, 1.25 U of Go Taq Flexi polymerase (Promega, Madison, WI, USA), and 1 µl of template DNA. The mixture was amplified following the PCR conditions used by Van Tuinen et al. (1998). PCR products were analyzed in 1% agarose gel electrophoresis under 100 V for 20 min and visualized under UV transillumination to confirm product integrity and yield.

After confirmation, the PCR products were diluted 100 X and used as a template for nested PCR, to select the sequences of LSU of rDNA of AMF (Gollotte et al., 2004). Primers FLR3F and FLR4R were used under the same PCR conditions as described above. A GC clamp was attached to the forward primer to stabilize the PCR products during denaturant gradient gel electrophoresis (DGGE) (Green et al., 2009).

2.2.6.2. CHARACTERIZATION OF AMF COMMUNITY BY DGGE

DGGE was performed for all the PCR products using D-Code system (Bio-rad, Hercules, CA, USA) as described by Muyzer et al. (1993). Each PCR mixture (30 µl) was loaded into the wells in the stack gel above the 8% acrylamide gel which contained 30–70% denaturant gradient gel (where 100% denaturant is defined as 7 M urea and 40% (v/v) formamide; Muyzer and Smalla, 1998). The gels were run at 20 V (with pump off) until the temperature reached 60°C and then at 75 V (with pump on) at 60ºC for 17 h. After electrophoresis, gels were stained with SYBR Green, visualized under UV transillumination, and digital images were captured by Gene Snap (Syngene, Cambridge, UK) for statistical analysis. Prominent DGGE bands were excised and amplified by PCR using FLR3F and FLR4R primers and under same PCR conditions. The PCR products were submitted to the Genomic Facility, University of Guelph, ON, Canada, for sequencing (3730 DNA Analyzer, Applied Biosystems, CA, USA).

2.2.6.3. ECOLOGICAL INDICES-AMF

The population diversity, richness, and evenness of the AMF community were examined using Shannon's index of general diversity ($H' = - \Sigma (ni/N)\log(ni/N)$),

Margalef's richness index (d = (S-1)/log(N)), and Pielou's evenness index (J = H'/log(S)). Three densitometric curves of each track in DGGE gels were created by GeneTools (Syngene, Cambridge, UK) in which the intensity of the band is transformed into a peak height and peak volume on the curve, and were used to calculate the H', d and J (Smith et al., 2010). The H', d and J are calculated using the peak height of each individual band (ni), the total peak heights of all bands in the track (N), and the total number of bands (S).

2.2.6.4. PHYLOGENETIC ANALYSIS-AMF

MEGA 4 software (Tamura et al., 2007) was used for phylogenetic analysis. The phylogenetic tree was constructed by neighbour-joining analysis with 1000 bootstrap values.

2.2.7. DIVERSITY OF EMF COMMUNITIES

2.2.7.1. PCR AMPLIFICATION OF INTERNAL TRANSCRIBED SPACER (ITS) REGION

Analysis of DNA sequences of the ITS region of the ribosomal RNA genes was used to study the diversity of EMF using nested polymerase chain reaction (PCR), as described previously (Landeweert et al., 2003). Briefly, primers ITS1F and ITS4B (Gardes and Burns, 1993) were used to amplify sequences of the ITS region of ribosomal DNA (rDNA) of Basidiomycetes fungi. A 20 µl reaction mixture contained 2 µl of 5X

buffer (Promega, Madison, WI, USA), 1.2 µl of 25 mM $MgCl_2$, 0.4 µl of 10 mM deoxy nucleotide triphosphate (dNTPs), 1 µl of 10 pM per µl each primer, 0.25 µl of Go Taq Flexi polymerase (Promega, Madison, WI, USA), and 1 µl of template DNA. The mixture was amplified using the following thermal cycle: 1.25-min denaturation at 95ºC followed by 13 cycles of 35-s denaturation at 95ºC, 55-s primer annealing at 55ºC and 45- s extension at 72ºC , followed by 13 cycles of 35-s denaturation at 95ºC, 55- s primer annealing at 55ºC, 1.2- min extension at 72ºC and 9 cycles of 35-s denaturation at 95ºC, 55 -s primer annealing at 55ºC, 3 -min extension at 72ºC and 10- min of final extension at 72ºC. PCR products were analyzed in 1% agarose gel electrophoresis under 100 V for 20 minutes and visualized under UV transillumination to confirm product integrity and yield.

2.2.7.2. CHARACTERIZATION OF EMF COMMUNITY BY DGGE

The same method that was described in 2.2.6.2. was followed to characterize the EMF communities.

2.2.7.3. ECOLOGICAL INDICES-EMF

The population diversity, richness, and evenness of the EMF communities were examined as described above for AMF communities.

2.2.8. STATISTICAL ANALYSIS

2.2.8.1. UNIVARIATE ANALYSIS

The variance analysis (ANOVA) was employed to fit the model for response variables (%WSA, diversity indexes (H', d and J) and AML copy number) by PROC

GLM of SAS (SAS Institute Inc., Cary, NC, 9.2) to determine the variation due to treatments. Significance of the effects of independent variables on response variables was examined using the F test. Least square means and their standard errors were generated. Multiple mean separation was done by Tukey's t test. In the variance analysis, it assumed that the model effects are linear, additive, and the experimental errors are distributed in a random, independent, and normal manner, with the mean of zero and a common variance (homogeneous). These assumptions were tested by using residual analysis and normality test.

2.2.8.2. MULTIVARIATE ANALYSIS

The digital images of the DGGE gels were analyzed using Genetools software (Syngene, Cambridge, UK). When the DGGE gels were opened with Genetool, yellow lines are visible along the bands. The yellow marks that appeared on the gel in Genetools were corrected by tallying with the bands seen in the original gel. The corrected DGGE profiles in Genetools were then archived to the Gene Directory (Syngene, Cambridge, UK), and lanes of the gels were compared with each other by cluster analysis via the unweighted pair group mathematical averages (UPGMA: Dice coefficient of similarity) under 3% percentage tolerance. The number of bands, intensity and position of each bands of each lane were compared to each other in the cluster analysis. The similarity matrix obtained from cluster analysis was imported to Microsoft Excel software and then analyzed by factor analysis, a method of principle component analysis, using SAS (SAS Institute Inc., Cary, NC, 9.2).

2.3. RESULTS

2.3.1. WATER-STABLE AGGREGATES

Percent WSA differed depending on the presence and type of mycorrhizal inoculant (Table 2.2). AMF inoculation increased the %WSA significantly compared to the un inoculated control in both the soils. Also, in the Guelph loam soil, the highest % WSA was recorded in pots that were inoculated with AMF alone (Figure 2.1). However, in pots that were inoculated with a combination of AMF and EMF or EMF alone there were no differences from the control pots. Furthermore, %WSA significantly varied along with the soil type (Table 2.2). On average overall the treatments the Selkirk clay soil showed significantly higher %WSA (72.2%) than the Guelph loam soil (62.9%) ($p<0.05$). Interestingly, only the AMF inoculated treatment had %WSA that was not significantly higher in Selkirk clay compared to the Guelph Loam.

Table. 2.2. ANOVA for percent water stable aggregates in two soil types (Selkirk clay soil and Guelph loam soil) inoculated with one of four mycorrhizal combinations (AMF (*Rhizophagus intraradices*), EMF (*Hebeloma cylindrosporum*), AMF+EMF, and non inoculated control).

Source	DF	Type 1 SS	Mean squares	F value	Pr > F
Soil	1	994.2216	994.2216	17.61	0.0001
Myco	3	770.3088	256.7696	4.54	0.0077
Soil x Myco	3	1080.5058	360.1686	6.38	0.0011
Error	49	2765.8071	56.4450		
Total corrected	63	5898.2313			

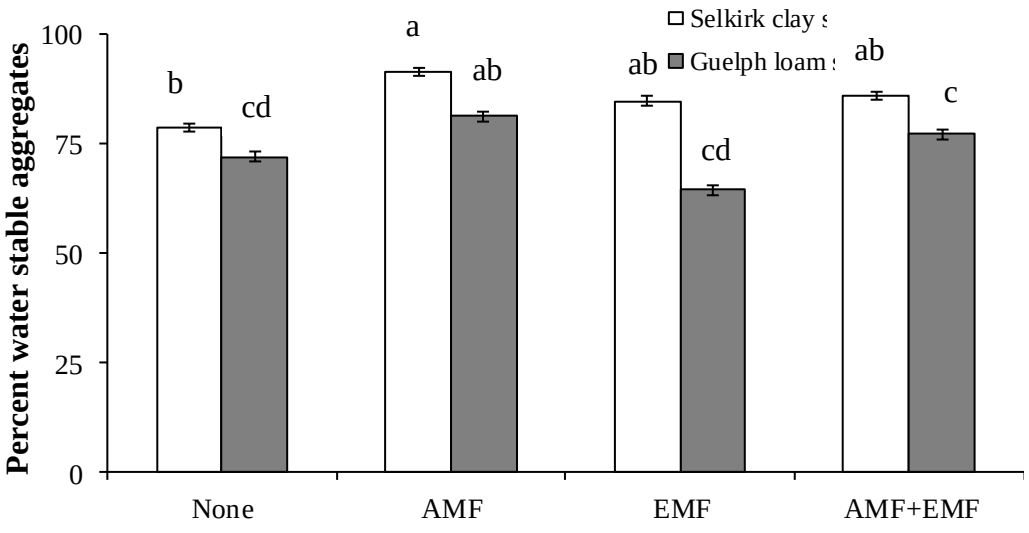


Figure 2.1. Percent water-stable aggregates (1–2 mm) in the rhizosphere of willows grown in two soils (Selkirk clay and Guelph loam). Willow plants were inoculated with arbuscular mycorrhiza (AMF), ectomycorrhiza (EMF), and a combination of both (AMF+EMF). Non-inoculated control (None) was also included (n=8). Least square means are presented; bars indicate standard error of the mean.Means followed by the same letter are not significantly different according to Tukey's mean separation ($p<0.05$).

2.3.2. QUANTIFICATION OF AMF

An estimate of the abundance of AMF in each aggregate size class was obtained by quantifying the number of AML gene copies per gram of aggregated soil. Across all the treatments, the AMF abundance in Selkirk clay soil was five times higher than that in the Guelph loam soil. Therefore AML gene copies in the two soils were analysed separately. Although AML gene copies statistically differed along with aggregate size class and type of mycorrhizal inoculation(Tables 2.3a and 2.3b), there were few differences in the mean AML copy number. Inoculation with AMF only significantly increased the mean AML copies in the 2-4 mm size class in both soils, and inoculation with AMF + EMF increased mean AML copies in the 0.5–2 mm and the 2-4 mm size classes in Selkirk soils, indicating that native AMF were present in both soils (Figure 2.2).

AMF abundance was highest in the 2–4 mm aggregate size of willows inoculated with AMF alone when grown in the Guelph soil, but in the Selkirk soil both the AMF and AMF+EMF inoculation yielded similar abundance in the 2-4 mm size class. In addition the AMF+EMF treatment yielded a high abundance of AML genes in the 0.5-2 mm size class in this soil.

Table. 2.3 ANOVA for AMF abundance in four mycorrhizal inoculants (AMF (*Rhizophagus intraradices*), EMF (*Hebeloma cylindrosporum*), AMF+EMF, and non inoculated control) and four aggregate size classes (>4, 4-2, 2-0.5, <0.5 mm) in Selkirk clay (a) and Guelph loam (b) soils.

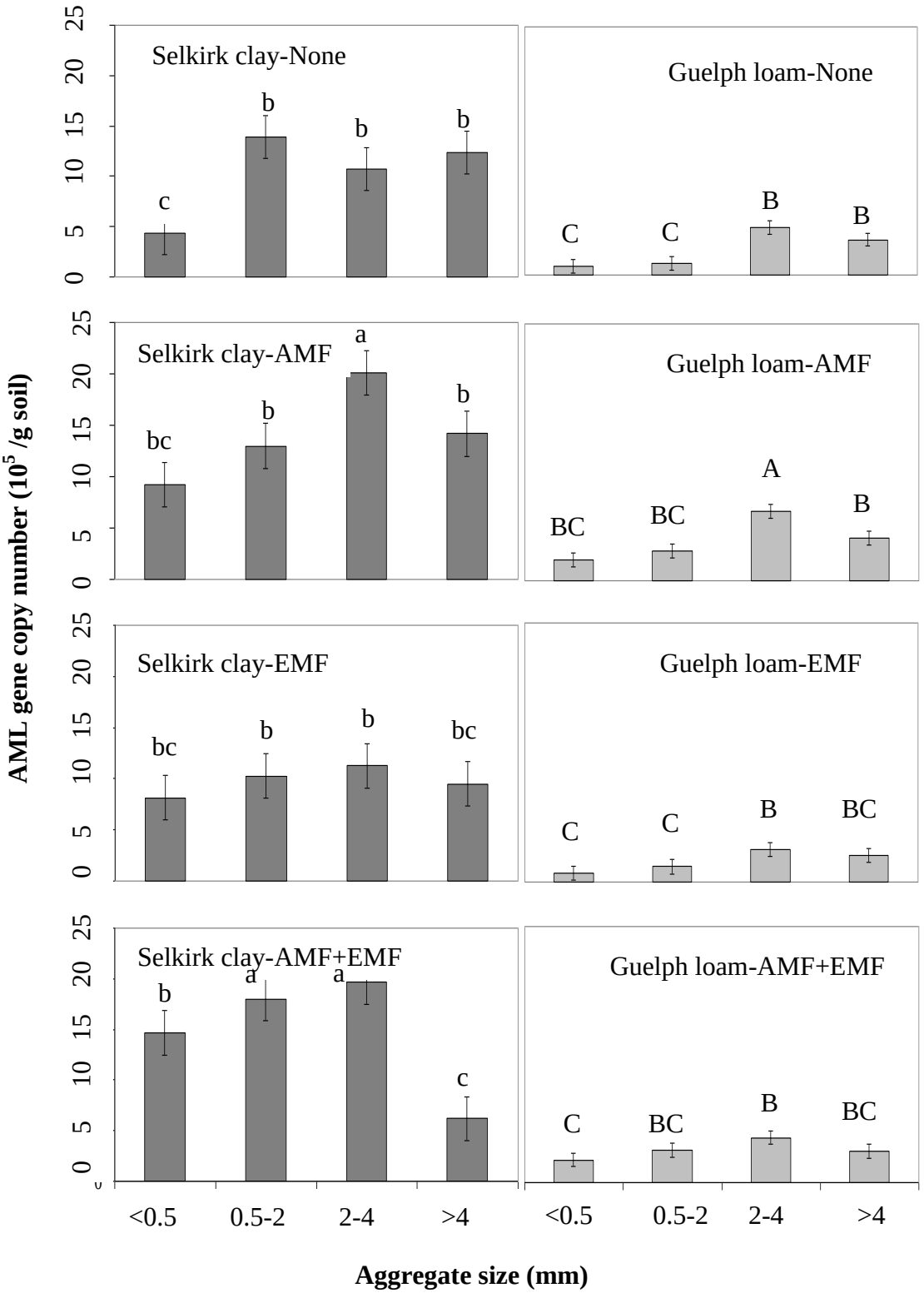
a) Selkirk clay

Source	DF	Type 1 SS	Mean Squares	F value	Pr > F
Myco	3	429.9158	143.3053	5.67	0.0031
Aggregate size	3	577.7705	192.5902	7.62	0.0006
Myco x Agg. size	9	718.8011	79.8668	3.16	0.0077
Error	30	758.2294	25.2743		
Corrected total	47	2499.7164			

b) Guelph loam

Source	DF	Type 1 SS	Mean Squares	F value	Pr > F
Myco	3	937.7265	312.5755	10.67	0.0023
Aggregate size	3	2427.3671	809.1224	27.62	0.001
Myco x Agg. size	9	2678.7165	297.6352	10.16	0.0026
Error	30	878.8442	29.2948		
Corrected total	47	6985.9718			

Figure 2.2. AML gene copy number (10^5) per gram of soil of four different aggregate size classes (<0.5 mm, 0.5–2 mm, 2–4 mm, and >4 mm) in the willow rhizosphere in Selkirk clay and Guelph loam soils. Willow plants were inoculated with arbuscular mycorrhiza (AMF), ectomycorrhiza (EMF) and a combination of both (AMF+EMF). A non-inoculated control (None) was also included. Soil from eight replicates of each treatment were partially pooled, and aggregate size classes were separated before extracting DNA (n=3). Least square means are presented; error bars indicate standard error of the mean. Means followed by the same letter within a soil type are not significantly different according to Tukey's mean separation ($p<0.05$).


Selkirk clay-None
Guelph loam-None
Selkirk clay-AMF
Guelph loam-AMF
Selkirk clay-EMF
Guelph loam-EMF
Selkirk clay-AMF+EMF
Guelph loam-AMF+EMF
AML gene copy number (10^5 /g soil)
0
5
10
15
20
25
<0.5
0.5-2
2-4
>4
Aggregate size (mm)

2.3.3. AMF DIVERSITY

Analysis of the AMF community using DGGE showed differences according to soil type, mycorrhizal inoculant, and aggregate size class (Figures 2.3 A and 2.3 B). Visual observation of the complex banding pattern in the DGGE gels showed distinguishable AMF communities in each sample.

Factor analysis of AMF communities within each mycorrhizal inoculant treatment showed the AMF community was significantly affected by soil type, and that unique communities were associated with each aggregate size class, but communities tended to be more similar when inoculated with AMF (Figure 2.4). In the Guelph loam soil, differences between the communities decreased in the aggregates that were <4 mm in size. The Selkirk clay soils showed a similar trend, and tended to group together when inoculated with AMF alone, irrespective of aggregate size (Figure 2.5).

Factor analysis was supported by ecological indices that showed differences in the structure of the AMF community according to soil type, mycorrhizal inoculation, and soil aggregate size(Tables 2.4). Shannon's diversity index and Marglef's richness index was highest in the <0.5 mm aggregate size class in Selkirk clay soil inoculated with AMF (0.83 and 2.5, respectively) and in the 2–0.5mm class in non-inoculated Guelph loam soil (0.86 and 2.7, respectively)(Table 2.5). The lowest diversity and richness were recorded in the >4 mm aggregate size of the Guelph loam soil inoculated with AMF in combination with EMF (0.29 and 0.43, respectively) and in the >4 mm aggregate size inoculated with AMF alone (0.32 and 0.46, respectively). On average, the Shannon's diversity index was significantly higher when willows were inoculated with AMF (0.67) compared to the control treatment (0.62) ($p<0.05$).

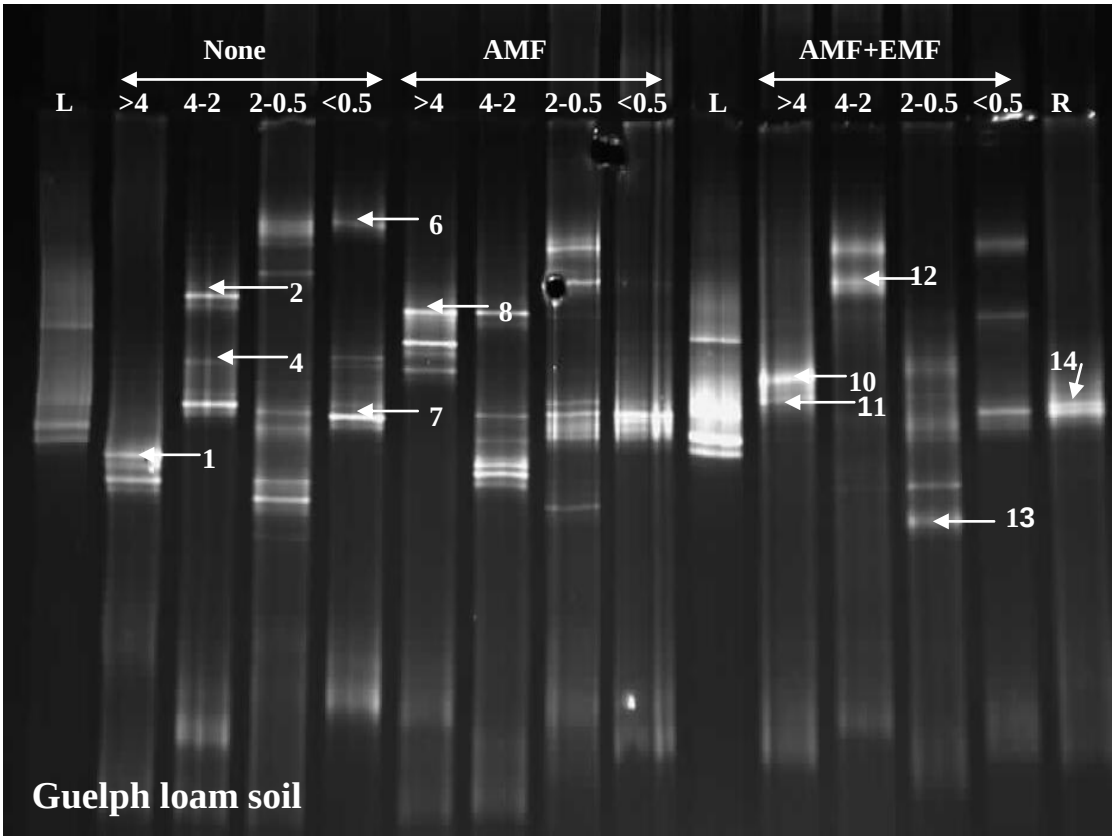


Figure 2.3(A). Sample DGGE gels indicating the AMF community associated with different aggregate size classes (>4 mm, 4–2 mm, 2–0.5 mm and <0.5 mm) in the rhizosphere of willow plants in Guelph loam soil. Willow plants were inoculated with arbuscular mycorrhiza alone (AMF) and in combination with EMF (AMF+EMF). A non-inoculated control (None) was also included. Soil from eight replicates of each treatment were partially pooled, and aggregate size classes were separated before extracting DNA (n=3). A 100 bp ladder (L) and DNA from pure culture of the commercial inoculant *Rhizophagus intraradices* (R) was included. Numbers by the arrows identify DGGE bands that were sequenced and included in phylogenetic analysis (Figure 2.6).

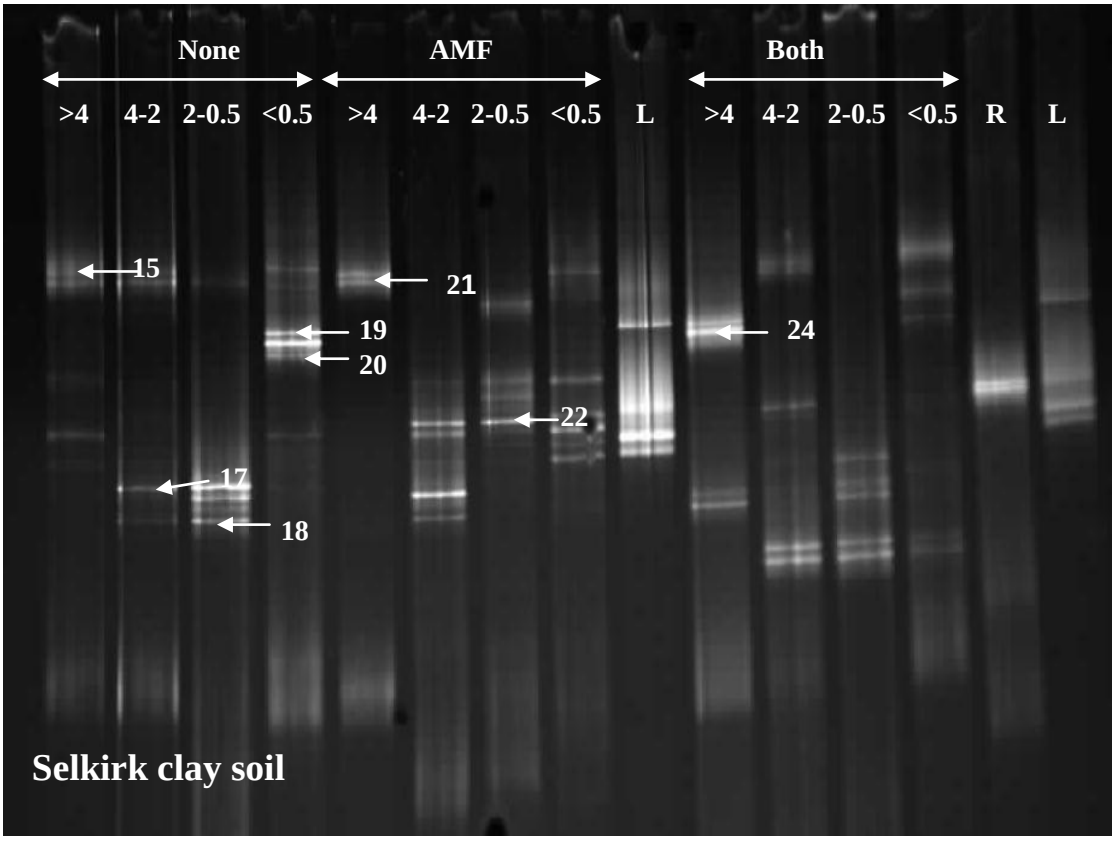


Figure 2.3 (B). Sample DGGE gels indicating the AMF community associated with different aggregate size classes (>4 mm, 4–2 mm, 2–0.5 mm and <0.5 mm) in the rhizosphere of willow plants in Selkirk clay soil. Willow plants were inoculated with arbuscular mycorrhiza alone (AMF) and in combination with EMF (AMF+EMF). A non-inoculated control (None) was also included. Soil from eight replicates of each treatment were partially pooled, and aggregate size classes were separated before extracting DNA (n=3). A 100 bp ladder (L) and DNA from pure culture of the commercial inoculant *Rhizophagus intraradices* (R) was included. Numbers by the arrows identify DGGE bands that were sequenced and included in phylogenetic analysis (Figure 2.6).

Table. 2.4. ANOVA for ecological indices (Shannon index of general diversity (H'), Margalef's richness index (d), Pielou's evenness index (J)) forAMF communities in four different aggregate sizes (>4, 4–2, 2–0.5, and <0.5mm) in the rhizosphere of willows inoculated with different mycorrhizal types (AMF, AMF+EMF, and control) and planted in two soils (Selkirk clay and Guelph loam).

a) Shannon index of general diversity (H')

Source	DF	SS	MS	F value	Pr>F
Myco	2	799.1905	399.5952	27.79	<0.0001
Soil	1	90.4901	90.4901	6.29	0.0243
Agg. Size	3	1321.6529	440.5510	30.64	<0.0001
Myco x Soil	2	816.6243	408.3122	28.40	<0.0001
Soil x Agg. Size	2	572.2735	286.1368	19.90	<0.0001
Myco x Agg. size	2	757.6813	378.8406	26.35	<0.0001
Myco x Soil x Agg. Size	6	2203.3083	367.2181	25.54	<0.0001
Error	51	733.3292	14.3790		
Corrected total	71	7390.5708			

b)Margalef's richness index (d)

Source	DF	SS	MS	F value	Pr>F
Myco	2	869.5648	434.7824	27.2128	<0.0001
Soil	1	83.1744	83.1744	5.2059	0.0243
Agg. Size	3	1417.8087	472.6029	29.5800	<0.0001
Myco x Soil	2	1016.2346	508.1173	31.8028	<0.0001
Soil x Agg. Size	2	546.0916	273.0458	17.0898	<0.0001
Myco x Agg. size	2	788.6965	394.3482	24.6821	<0.0001
Myco x Soil x Agg. Size	6	2448.1876	408.0313	25.5385	<0.0001
Error	51	814.8326	15.9771		
Corrected total	71	7998.5987			

c) Pielou's evenness index (J)

Source	DF	SS	MS	F value	Pr>F
Myco	2	59.3106	29.6553	1.7898	0.1675
Soil	1	24.8722	24.8722	1.5011	0.2241
Agg. Size	3	547.6661	182.5554	11.0180	<0.0001
Myco x Soil	2	18.6541	6.2180	0.3753	0.6766
Soil x Agg. Size	2	91.8357	45.9179	2.7713	0.0178
Myco x Agg. size	2	227.9949	113.9975	6.8802	0.0005
Myco x Soil x Agg. Size	6	470.6580	78.4430	4.7344	0.0005
Error	51	845.0161	16.5689		
Corrected total	71	2306.7916			

Table 2.5. Means of ecological indices (Shannon index of general diversity (H'), Margalef's richness index (d), Pielou's evenness index (J)) for AMF communities in four different aggregate sizes (>4, 4–2, 2–0.5, and <0.5mm) in the rhizosphere of willows inoculated with different mycorrhizal types (AMF, AMF+EMF, and control) and planted in two soils (Selkirk clay and Guelph loam).

Soil type	Mycorrhiza	Agg.size	H'	D	J
Selkirk clay	AMF	>4 mm	0.32 KL	0.46 L	1.00 AM
		4-2 mm	0.57 FG	1.62 EF	0.84 JK
		2-0.5 mm	0.56 G	1.32 GHI	0.91 DEFGHI
		<0.5 mm	0.83 A	2.55 A	0.95 BCD
	Both	>4 mm	0.54 G	1.16 IJ	0.91 DEFGHI
		4-2 mm	0.56 G	1.26 HI	0.93 CDEFG
		2-0.5 mm	0.65 D	1.74 DE	0.96 ABCD
		<0.5 mm	0.63 DEF	1.96 CD	0.90 FGHI
	Control	>4 mm	0.53 GH	1.38 GHI	0.88 IJ
		4-2 mm	0.43 IJ	0.97 JK	0.90 EFGHI
		2-0.5 mm	0.64 DE	1.53 EFG	0.88 GHIJ
		<0.5 mm	0.57 FG	1.54 EFG	0.82 K
Guelph loam	AMF	>4 mm	0.56 G	1.31 GHI	0.94 BCDEF
		4-2 mm	0.76 BC	2.18 BC	0.93 CDEFG
		2-0.5 mm	0.82 AB	2.23 B	0.97 ABC
		<0.5 mm	0.47 I	0.78 K	0.99 AB
	Both	>4 mm	0.29 L	0.43 L	0.97 ABC
		4-2 mm	0.36 JK	0.84 K	0.77 L
		2-0.5 mm	0.59 EFG	1.44 FGH	0.96 ABC
		<0.5 mm	0.45 I	0.93 JK	0.88 HIJ
	Control	>4 mm	0.48 HI	0.87 K	0.95 BCDE
		4-2 mm	0.75 C	1.87 D	0.94 BCDEF
		2-0.5 mm	0.86 A	2.70 A	0.94 BCDEF
		<0.5 mm	0.67 D	1.96 CD	0.87 IJ
	SE		0.023	0.128	0.027

Means followed by the same letter within one column are not significantly different according to Tukey's mean separation, $P<0.05$. $n = 3$

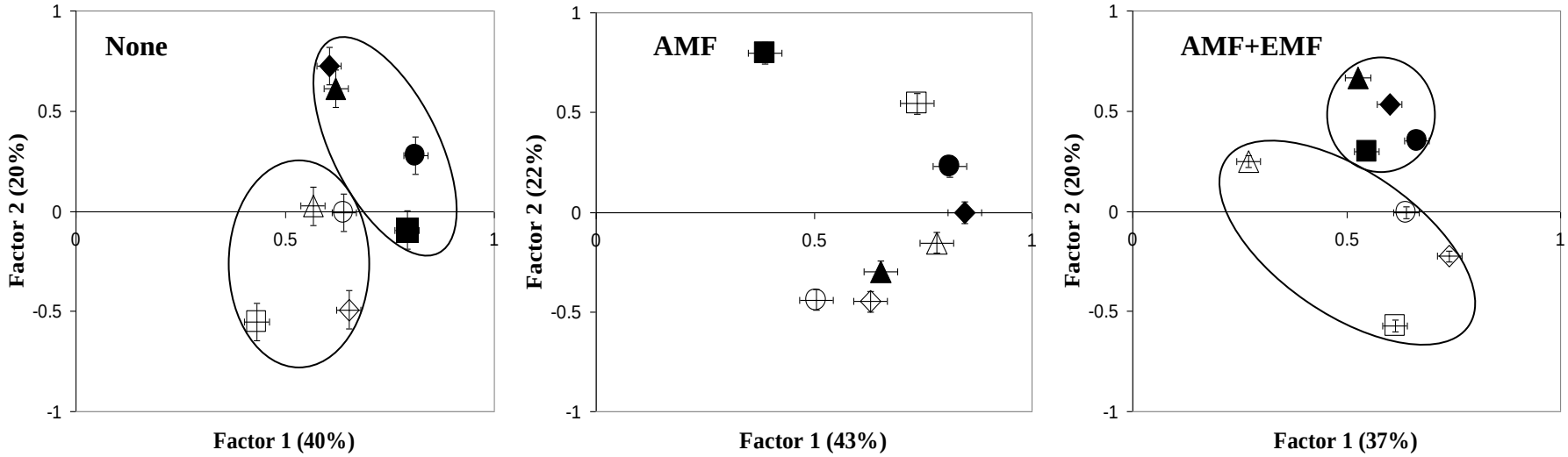


Figure 2.4. Arbuscular mycorrhizal (AM) fungal community composition associated with soil aggregates in the rhizosphere of non-inoculated willow plants (None) and willow plants inoculated with AMF alone (AMF) and in combination with EMF (AMF+EMF).Ordination was based on AM fungal communities detected from four different aggregate sizes: >4 mm (squares), 4–2 mm (triangles), 2–0.5 mm (diamonds), and <0.5 mm (circles) of Selkirk clay (closed symbols) and Guelph loam (open symbols) soils. Symbols are the average (± 1 SE) ordination coordinates of 3 replicates in each sample. Error bars indicates standard deviation of the mean.

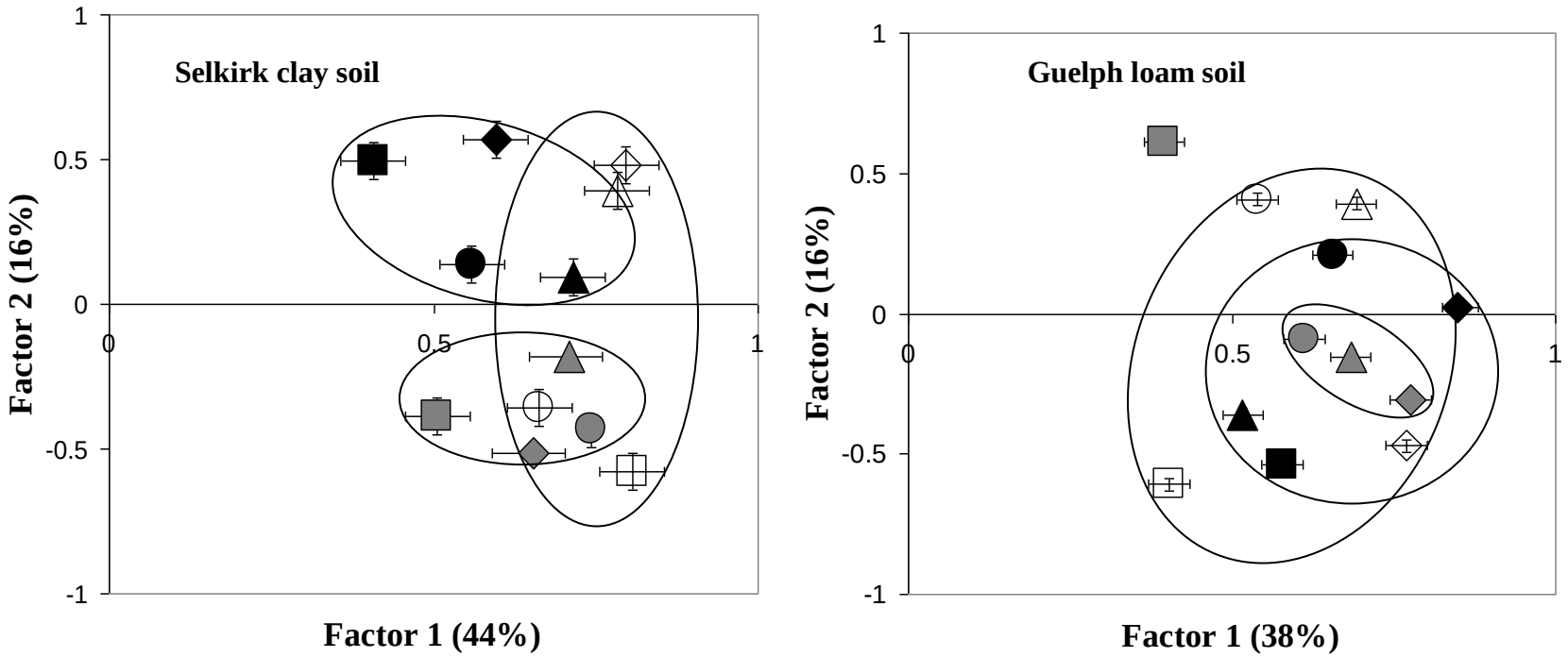


Figure 2.5. Arbuscular mycorrhizal (AM) fungal community composition associated with soil aggregates in the rhizosphere of willows planted in clay and loam soils. Willow plants were inoculated with AMF alone and in combination with EMF. A non-inoculated control was also included. Ordination was based on the AM fungal community detected from four different aggregate sizes: >4 mm (squares), 4–2 mm (triangles), 2–0.5 mm (diamonds), and <0.5 mm (circles) in willow rhizospheres of three mycorrhizal treatments: non-inoculated (open symbols), inoculated with AMF alone (gray symbols), and inoculated with AMF in combination with EMF (black symbols). Symbols are the mean (± 1 SE) ordination coordinates of 3 replicates in each sample. Error bars indicate standard deviation of the mean.

2.3.4. PHYLOGENETIC ANALYSIS

Phylogenetic analysis showed that all of the sequences of DGGE bands obtained (numbered arrows in Figures 2.3A and 2.3B) were closely related to different species of the phylum *Glomeromycota*. Most were related to different species of *Glomeraceae*(9/24) and*Claroideoglomeraceae*(5/24) while 3 of 24 grouped most closely to members of the *Ambisporaceae*(Figure 2.6). One band (DGGE 14) was obtained from the commercial inoculant (*Rhizophagus intraradices*) used in this study. This band was also identified in each of the aggregate size classes except for the>4mm size class (Figures 2.3A and 2.3B). Several other sequences clustered closely with *Claroideoglomus claroideum,Claroideoglomus etunicatum, G. microaggregatum, Funneliformis mosseae,* and several uncultured *Glomus* sequences, indicating that there was a high species diversity associated with our system.

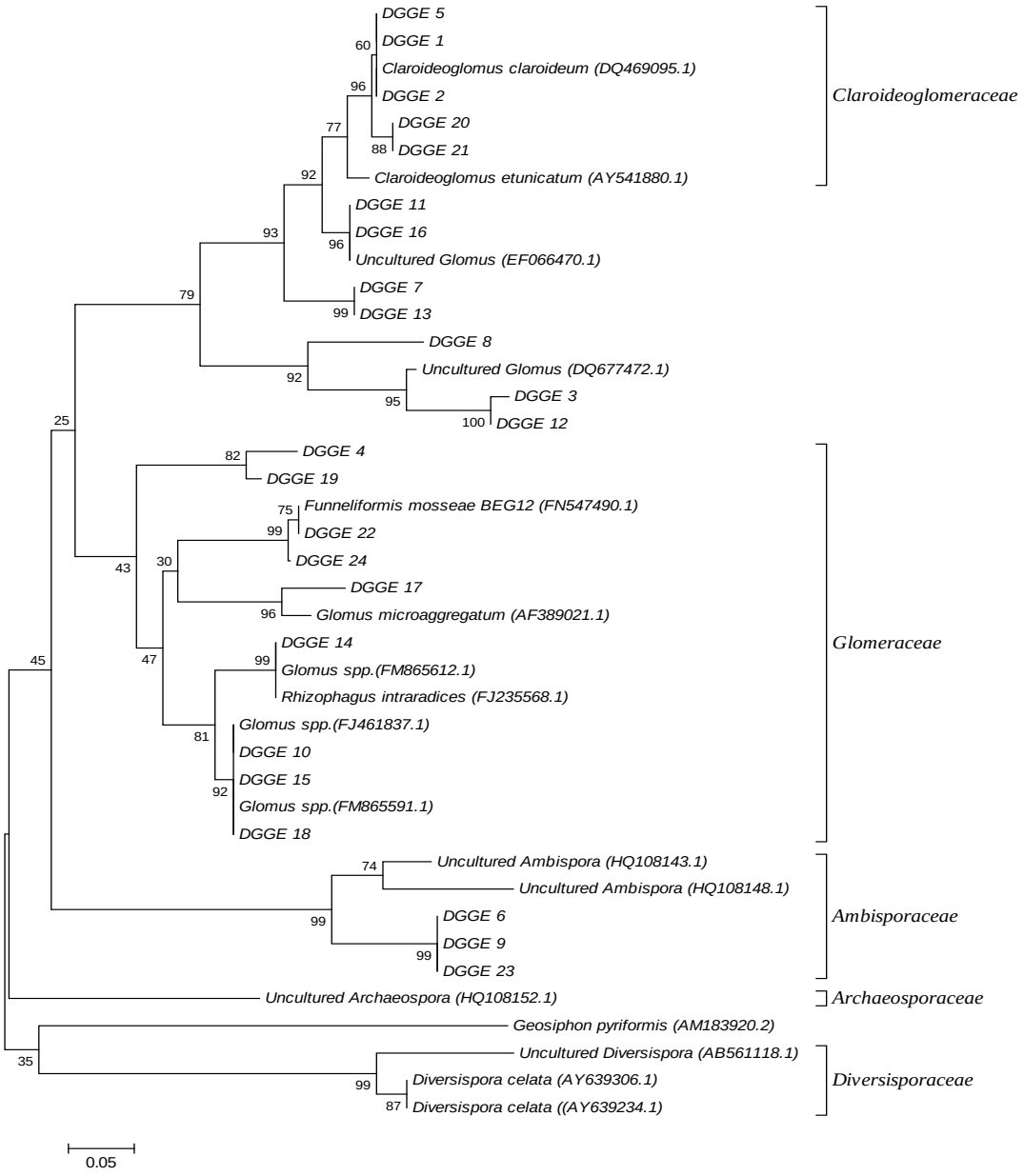


Figure 2.6. Phylogenetic tree showing phylogenetic position of different sequences of AMF obtained from band isolates using PCR–DGGE (DGGE 1-24) (Figures 2.3 A and 2.3 B) and their close relatives obtained from NCBI (Latin binomials) using Megablast. Other *Glomeromycota* were also included. Bootstrap values above the branches are obtained from neighbour-joining analysis (bootstrap value 1000); these are shown only when >50% in at least one of the analyses.

2.3.5. EMF DIVERSITY

Analysis of EMF communities using DGGE showed that EMF communities varied with soil type, but not with soil aggregates or mycorrhizal inoculation.

Banding patterns in each DGGE gel showed similar EMF communities in all the treatments in each soil and different EMF communities between the two soils.

Factor analysis of EMF communities within each mycorrhizal inoculant treatment clearly showed that EMF community was significantly affected by soil type in the pots inoculated by all the types of mycorrhizal inoculants and in the non-inoculated soils (Figure 2.7). Analysis of EMF communities in the rhizosphere of non-inoculated willows grown in the both soils separately indicated that uniform communities were associated among all the aggregate size class and these EMF communities were not affected by any type of mycorrhizal inoculation (Figure 2.8). Ecological indices also showed the differences between EMF communities were associated primarily with soil type. Further interaction effect of soil type and mycorrhizal inoculant on Margalef's richness index (d) was significant (Tables 2.6 and 2.7). Few differences in EMF communities were associated with aggregate size class or mycorrhizal inoculant. Sequences obtained from DGGE bands from the Guelph loam soil were similar (>97%) to *Hebeloma collariatum, H. repandum*, and *H.cylindrosporum*. We were able to obtain some sequences similar (>97%) to *Hebeloma collariatum, Hebeloma cylindrosporum, Hymenogaster griseus, Hymenogaster populetorum*, and *Hymenogaster boozeri* from the Selkirk clay soil.

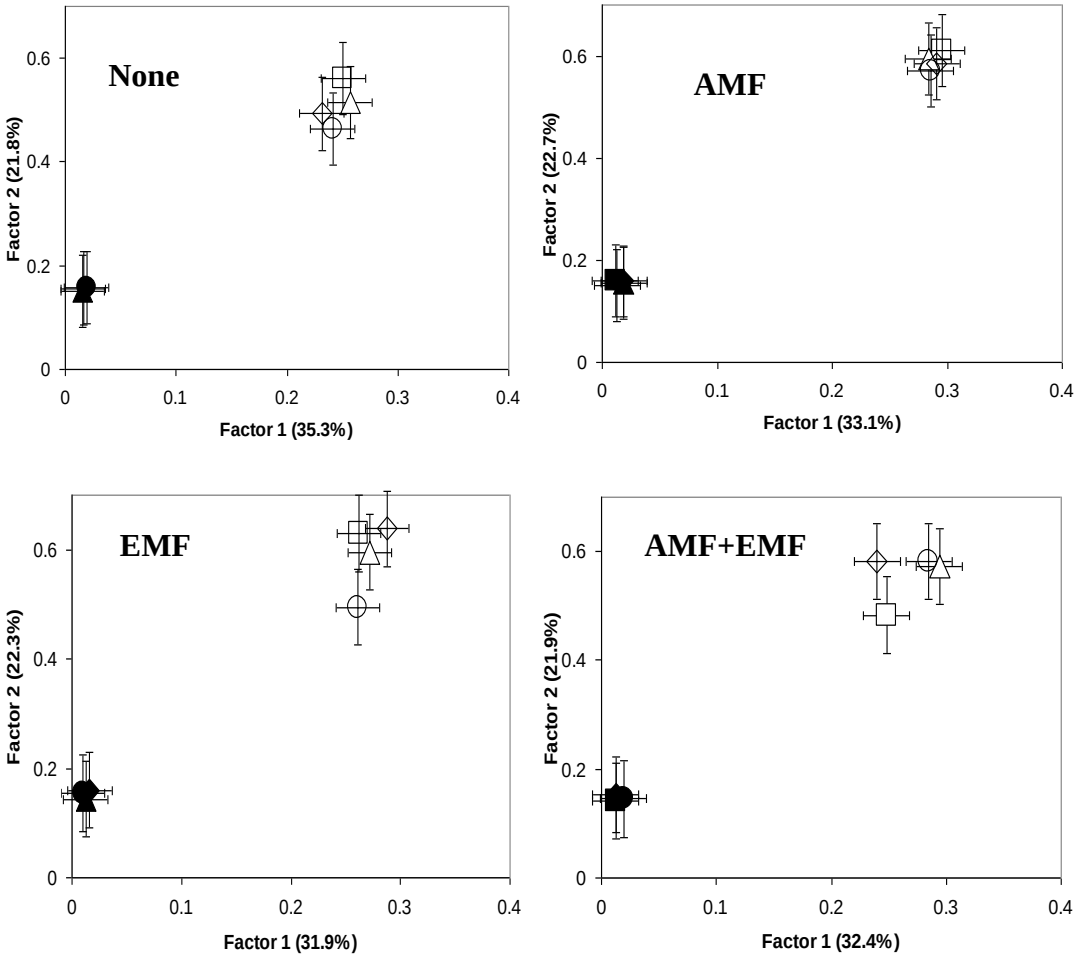


Figure 2.7. Ectomycorrhizal (EM) fungal community composition associated with soil aggregates in the rhizosphere of non-inoculated willow plants (None) and willow plants inoculated with AMF alone (AMF), EMF alone (EMF) and in combination with EMF (AMF+EMF).Ordination was based on the EMF community detected from four different aggregate sizes: >4 mm (squares), 4–2 mm (triangles), 2–0.5 mm (diamonds), and <0.5 mm (circles) of Selkirk clay (closed symbols) and Guelph loam (open symbols) soils. Symbols are the average (± 1 SE) ordination coordinates of 3 replicates in each sample. Error bars indicate standard deviation of the mean.

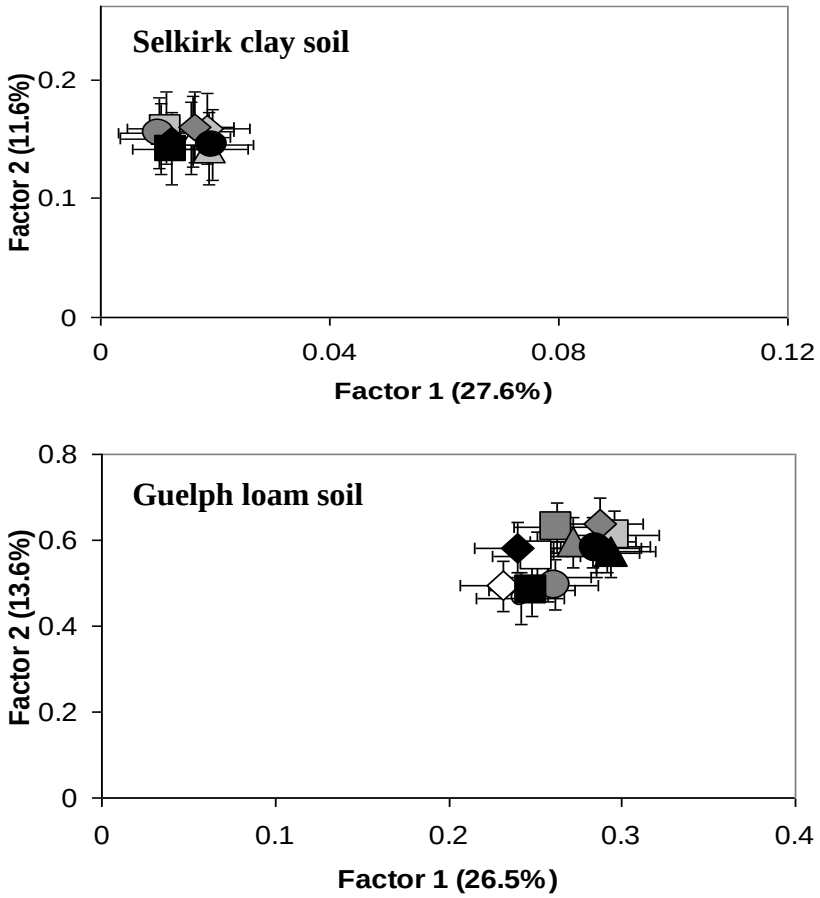


Figure 2.8. Ectomycorrhizal (EM) fungal community composition associated with soil aggregates in the rhizosphere of willows planted in clay and loam soils.Willow plants were inoculated with AMF alone, EMF alone, and AMF in combination with EMF. A non-inoculated control was also included. Ordination was based on the EM fungal community detected from four different aggregate sizes: >4 mm (squares), 4–2 mm (triangles), 2–0.5 mm (diamonds), and <0.5 mm (circles) in willow rhizospheres of three mycorrhizal treatments: non-inoculated (open symbols), inoculated with AMF alone (light gray symbols), inoculated with EMF alone (dark gray symbols), and inoculated with AMF in combination with EMF (black symbols).Symbols are the mean (± 1 SE) ordination coordinates of 3 replicates in each sample. Error bars indicate standard deviation of the mean.

Table. 2.6. ANOVA for ecological indices (Shannon index of general diversity (H'), Margalef's richness index (d), Pielou's evenness index (J)) for EMF communities in four different aggregate sizes (>4, 4–2, 2–0.5, and <0.5mm) in the rhizosphere of willows inoculated with different mycorrhizal types (AMF, EMF, AMF+EMF, and control) and planted in two soils (Selkirk clay and Guelph loam).

a) Shannon index of general diversity (H')

Source	DF	Type 1SS	Mean Squares	F value	Pr>F
Myco	3	45.5953	15.1984	2.31	0.9271
Soil	1	5188.0329	5188.0329	787.04	0.0013
Agg. Size	3	30.4607	10.1536	1.54	0.9601
Myco x Soil	3	41.3806	13.7935	2.09	0.8832
Soil x Agg. Size	3	32.1849	10.7283	1.63	0.9739
Myco x Agg. size	9	112.0725	12.4525	1.89	0.8493
Myco x Soil x Agg. Size	9	88.5086	9.8343	1.49	0.3848
Error	62	408.6976	6.5919		
Corrected total	95	6146.9332			

b)Margalef's richness index (d)

Source	DF	Type 1SS	Mean Squares	F value	Pr>F
Myco	3	18.7803	6.2601	1.17	0.9852
Soil	1	4710.1336	4710.1336	881.96	<0.0001
Agg. Size	3	16.6074	5.5358	1.04	0.8946
Myco x Soil	3	1164.5346	388.1782	72.68	0.0499
Soil x Agg. Size	3	825.0921	275.0307	51.50	0.05
Myco x Agg. size	9	73.1035	8.1226	1.52	0.8743
Myco x Soil x Agg. Size	9	64.7222	7.1914	7.19	0.6821
Error	62	331.1130	5.3405	5.34	
Corrected total	95	7524.0867			

c) Pielou's evenness index (J)

Source	DF	Type 1SS	Mean Squares	F value	Pr>F
Myco	3	307.1617	102.3872	9.55	0.1675
Soil	1	8468.4412	8468.4412	790.04	0.0241
Agg. Size	3	108.4100	36.1367	3.37	0.2845
Myco x Soil	3	105.6063	35.2021	3.28	0.2766
Soil x Agg. Size	3	245.4801	81.8267	7.63	0.178
Myco x Agg. size	9	761.6737	84.6304	7.89	0.1745
Myco x Soil x Agg. Size	9	1020.5493	113.3944	10.58	0.1538
Error	62	664.5824	10.7190		
Corrected total	95	11793.0491			

Table 2.7. Means of indices of diversity (Shannon index of general diversity (H'), Margalef's richness index (d), Pielou's evenness index (J)) for EMF communities in four different aggregate sizes (>4, 4–2, 2–0.5, and <0.5 mm) in the rhizosphere of willows inoculated with different mycorrhizal types (AMF, EMF, AMF+EMF, and control) and planted in two soils.

Soil type	Myco. Trt.	Agg. size	H'	d	J
Guelph loam	Control	>4mm	0.13 b	0.33 c	0.28 b
		4-2mm	0.16 b	0.43 c	0.27 b
		2-0.5mm	0.15 b	0.49 c	0.27 b
		<0.5mm	0.10 b	0.33 c	0.29 b
	AMF	>4mm	0.10 b	0.31 c	0.28 b
		4-2mm	0.17 b	0.40 c	0.20 b
		2-0.5mm	0.20 b	0.39 c	0.29 b
		<0.5mm	0.13 b	0.42 c	0.30 b
	EMF	>4mm	0.22 b	0.36 c	0.28 b
		4-2mm	0.11 b	0.38 c	0.30 b
		2-0.5mm	0.10 b	0.38 c	0.29 b
		<0.5mm	0.13 b	0.50 c	0.29 b
	Both	>4mm	0.19 b	0.39 c	0.29 b
		4-2mm	0.10 b	0.37 c	0.27 b
		2-0.5mm	0.10 b	0.38 c	0.30 b
		<0.5mm	0.19 b	0.38 c	0.27 b
Selkirk clay	Control	>4mm	0.68 a	1.29 b	0.96 a
		4-2mm	0.71 a	1.35 ab	0.90 a
		2-0.5mm	0.70 a	1.41 ab	0.93 a
		<0.5mm	0.70 a	1.38 ab	0.92 a
	AMF	>4mm	0.65a	1.73 a	0.94 a
		4-2mm	0.70 a	1.73 a	0.90 a
		2-0.5mm	0.64 a	1.71 a	0.95 a
		<0.5mm	0.68 a	1.58 a	0.89 a
	EMF	>4mm	0.70 a	1.56 a	0.93 a
		4-2mm	0.61 a	0.98 b	0.91 a
		2-0.5mm	0.58 a	0.97 b	0.88 a
		<0.5mm	0.72 a	1.00 b	0.89 a
	Both	>4mm	0.58 a	1.86 a	0.91 a
		4-2mm	0.68 a	1.60 a	0.92 a
		2-0.5mm	0.76 a	1.36 ab	0.93 a
		<0.5mm	0.72 a	1.85 a	0.94 a
SE			0.016	0.183	0.027

Means followed by the same letter within one column are not significantly different according to Tukey's mean separation, $P<0.05$. $n = 3$

2.4. DISCUSSION

2.4.1. SOIL VARIATION

Type of soil is the main determining factor for many soil parameters including soil aggregation (Tisdall and Oades, 1982) and soil microbial communities (Silveira et al., 2006), and affected soil aggregation in this experiment. Water-stable aggregation is often used as a predictor of soil health, because it is dependent on a combination of biotic and abiotic factors (Tisdall and Oades, 1982). Our results showed %WSA was higher in Selkirk clay soil than in Guelph loam soil, most likely due to its higher percentage of clay content (Wagner et al., 2007). Further higher organic matter content in Selkirk clay soil also may be attributed to its high %WSA, because water stability of soil aggregates depends primarily on organic materials (Benbi and Senapati, 2010; Goldberg et al., 1988;Tisdall and Oades, 1982).

AMF distribution in soil also can be affected by soil texture (Lekberg et al., 2007) and other soil properties (Fitzsimons et al., 2008). We have quantified AMF abundance by quantifying AML gene copy number using AML1 and AML2 primers as these primers are specific to AMF (*Glomeromycota*) and provide an opportunity to study relative abundance of all the known AMF by single PCR reaction (Lee et al., 2008). We found that the abundance of AMF in two soils, with different texture and properties, were different. Further, we also found that the aboveground biomass of willows grown in the Selkirk clay soil was significantly higher than the biomass of those grown in the Guelph loam soil, which can be related to differences in the soil physico-chemical properties between the two soils (Chapter 3). Though we did not measure root parameters, it is reasonable to expect differences in root parameters such as root exudates between the two

soils due to the differential growth of plants, which may relate to the significant differences in the abundance of the AMF community in the two soils. Abundance of AMF in the Selkirk clay soil was much higher than that in the Guelph loam soil in our experiment, suggesting a high proliferation of AMF in this clay soil. In general, AMF proliferation is higher in low fertility soils than in fertile soil with a high amount of nutrients (Smith and Read, 2008). However, this was not observed in our study. The soil nutrient content of Selkirk clay soil was higher than that of Guelph loam soil in the present study (Table 2.1).

The complex banding pattern seen in the DGGE gels showed that AMF communities in both the soils were highly diverse. In contrast, EMF communities in both the soils were less diverse. However, factor analysis indicated that both the AMF and EMF communities associated with soil aggregates were different depending on the type of soil. The present study agrees with previous studies that have shown that the diversity and distribution of AMF in soil can be affected by soil texture (Lekberg et al., 2007) and other soil properties (Fitzsimons et al., 2008).

2.4.2. SPATIAL DISTRIBUTION AMONG SOIL AGGREGATE SIZE CLASSES

Spatial distribution is a fundamental ecological parameter for any group of organism (Mummey and Rillig, 2008). Communities of AMF have been shown to have spatial heterogeneity (Rosendahl and Stukenbrock, 2004), and they can be spatially structured at sub meter scales (Mummey and Rillig, 2008). However, no study has investigated the spatial heterogeneity of AMF or EMF or any other fungal types in soil aggregates. For the first time we showed that AMF communities were spatially separated in different sized soil aggregates. Results from the factor analysis of DGGE gels clearly

showed the differences of AMF communities among aggregate size classes in both the soils. For example, AMF communities in large aggregates (>4mm) tended to be less diverse, and separated from the communities associated with smaller aggregates. Ecological indices supported this trend, however, care must be taken when interpreting these results, since there are some issues in measuring ecological indices using DNA fingerprinting methods (Bent et al, 2007).

In addition, our findings suggest that AMF abundance varied with aggregate size class. Other studies have also indicated that the abundance of certain types of soil microbes have varied across soil aggregate sizes and soil particle fractions(Gupta and Germida, 1988; Mendeset al., 1999; Miller and Dick, 1995; Gupta and Germida, 1988; Schutter and Dick, 2002). One reason for this spatial heterogeneity could be the differences in micro habitats among different aggregate sizes (Young and Ritz, 1998; Mummey et al., 2006). Our results suggest that it is important to analyze AMF communities at the aggregate level in order to understand their ecological role .

Importantly, the results obtained for EMF communities were quite different than those for the AMF. The EMF communities were similar among soil aggregate size classes in each soil, suggesting no spatial heterogeneity in the EMF communities among soil aggregates sizes examined in this study.

2.4.3. MYCORRHIZAL INOCULATION

Mycorrhizal fungi, mainly AMF, play a major role in soil aggregation (Tisdall and Oades, 1982). Our results show that the soil associated with willows inoculated with AMF had the greatest amount of water stable aggregates. Other studies have also shown a positive effect of AMF on soil aggregation in different rhizospheres (Bethlenfalvayet al.,

1999; Choet al., 2009; Enkhtuya and Vosatka, 2005;Enkhtuya et al., 2003;Milleret et al., 2009;Rillig et al., 2002; Schreiner and Bethlenfalvay, 1997;Wilson et al., 2009). AMF can improve soil aggregation by a variety of means: entangling soil particles to stabilize the soil aggregates(Rillig, 2004; Tisdall and Oades, 1982)and increasing the water repellence by extraradical mycelium of AMF (Rillig et al., 2010). The soil aggregates may also be stabilized by a putative protein called glomalin produced by AMF (Wright and Upadhyaya, 1996).

Soil aggregation in the rhizosphere of willow inoculated with EMF alone was lower than that in rhizospheres inoculated with AMF(Figure 2.1). Information on the effect of EMF alone or in combination with AMF on soil aggregation is rare. Ambriz et al. (2010) observed that aggregation varied with type of mycorrhizal inoculation and the size of soil aggregate fraction when they inoculated *Fraxinus* seedlings grown in sterilized soil with mycorrhizae (AMF (*Rhizophagus intraradices*)and EMF (*Pisolithus tinctorius*), and a combination of AMF and EMF)). In contrast to our findings, they found that the amount of water stable aggregates was lower in AMF-inoculated systems compared to EMF-inoculated systems. In addition, in our experiment, the inoculation with a combination of AMF and EMF showed lower amount of water stable aggregates than with AMF alone. This reduction may be due either to a suppressive or antagonistic effect of EMF against AMF or to an incompatibility of the inoculated AMF and EMF communities. Early studies by Read et al. (1976) and Lodge and Wentworth (1990) suggested AMF can be temporarily suppressed or displaced by EMF. This negative effect may be either common for all AMF and EMF or specific only for some species or specific hosts. This effect should be explored further when AMF and EMF are

inoculated in combination, especially to develop strategies for using willows in restoration systems.

Any bio-inoculant can change the microbial communities in the rhizosphere (Srivastava et al., 2007). Our results showed that both the abundance and diversity of AMF associated with soil aggregates in the willow rhizosphere can be altered by mycorrhizal inoculation. Other authors have shown that AMF inoculation can affect the microbial communities (Srivastava et al., 2007;Mummey et al., 2009) by changing rhizosphere parameters such as quantity and quality of root exudates (Pinior et al., 1999; Vierheilig et al., 2000; Scervinoet al., 2005), which in turn affect the microbial communities (Srivastava et al., 2007). However, no studies have described the impact of an inoculant on the distribution of the AMF community across soil aggregates. AMF could be enumerated in all pots, including uninoculated and EMF only treatments, indicating a significant native AMF population present in both soils. However, our results clearly indicate that the abundance of AML gene copies increased in the majority of aggregate sizes when pots were inoculated with AMF alone and in combination with EMF. No significant change in AMF abundance was associated with many of aggregate sizes in the pots inoculated with EMF alone, suggesting that EMF inoculation does not alter AMF abundance within soil aggregates.

Factor analysis showed that the structure of the AMF communities in all aggregate size classes in the rhizosphere of willow inoculated with *Rhizophagus intraradices* tended to be more similar to each other than to those in non-inoculated willows, showing reduction of spatial heterogeneity of AMF communities in aggregate size classes with mycorrhizal inoculation. Further, ecological indices showed that the

diversity of AMF communities was increased due to AMF inoculation. A study by Antunes et al. (2009) which examined the effect of commercial mycorrhizal inoculation on indigenous AMF diversity in a fine sandy loam soil, reported no significant changes in AMF community structure. Since AMF community composition could have important implications for ecosystem functions (Rillig, 2004), reduction of spatial heterogeneity and increase of AMF associated with soil aggregates should be taken into consideration when inoculating soil with mycorrhizae or any other bio-inoculants, as their effect could be either positive or negative.

In contrast, EMF communities were not affected by inoculation with any of mycorrhizal inoculants, suggesting EMF communities associated with soil aggregates in willow rhizosphere are fairly stable.

2.4.4. COMMUNITY STRUCTURE

Phylogenetic analysis of the sequences obtained from our study indicated that all the sequences belonged to the phylum *Glomeramycota*, and most were *Glomus* spp. *Glomus* is reported to be a highly infective and fast-growing group of AMF (Antuneset al., 2009), which becomes more abundant under environmental stresses like in agro ecosystems (Helgasonet al., 1998; Oehl et al., 2004), especially those with tillage disturbances (Jansaet al., 2003). Though we did not disturb the soil during our experiment, both of the soils used in our experiment were collected from agricultural farms which had been neglected only for a few months. Therefore, it is possible to expect *Glomus* dominancy in these soils. Further, we were able to detect our inoculant strain *Rhizophagus intraradices* in the DGGE profiles of all soil samples, except in the >4mm aggregate size class.

Interestingly, the sequenced AMF bands grouped with several known species of *Glomus, Claroideoglomus,Funneliformis,* and *Rhizophagus* and with some uncultured *Glomus* sequences, and three bands grouped close to the *Ambispora* spp., suggesting that the diversity in this system is fairly high. In contrast, we were only able to detect a few sequences of EMF from both the soils, belonging to the inoculant strain, *Hebeloma* and also a *Hymenogaster* spp.

2.5. CONCLUSIONS

From the results of our greenhouse experiment, it could be concluded that inoculation with commercial mycorrhizae can impact important ecological aspects such as soil aggregation and spatial heterogeneity of AMF communities across the soil aggregates in the willow rhizosphere. However, these impacts vary with type of soil and type of mycorrhiza used for inoculation. Further both parameters: soil aggregation and mycorrhizal communities in willow rhizosphere vary with the soil type.

For the first time, we showed aggregate scale spatial heterogeneity in AMF communities associated with different aggregate size classes. This information directs to analyze AMF communities at aggregate scale to understand the ecological functions they mediate. Further, this spatial heterogeneity of the AMF community can be increased by inoculation with a *Rhizophagus intraradices* inoculant, but not with a combination of *R. intraradices* and *Heboloma cylindrosporum* inoculants. This indicates that the change in mycorrhizal diversity should be taken into consideration when introducing commercial inoculants because mycorrhizal diversity may affect plant communities and ecosystem processes. Since reports on heterogeneity of AMF communities in soil are sparse, this spatial heterogeneity should be further studied. Interestingly, our results did not show aggregate scale spatial heterogeneity in EMF communities. Further, EMF communities associated with soil aggregates were not altered by mycorrhizal inoculation, suggesting EMF communities associated with soil aggregates are uniform and fairly stable.

These conclusions were based on an experiment conducted under greenhouse conditions. This topic should be further explored under real field conditions over a long term.

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CHAPTER 3: SOIL AGGREGATION AND ORGANIC CARBON IN THE RHIZOSPHERE OF WILLOW(*SALIX*SPP.) INOCULATED WITH ARBUSCULAR MYCORRHIZA (*RHIZOPHAGUS INTRARADICES*) AND ECTOMYCORRHIZA (*HEBELOMA CYLINDROSPORUM*)

3.1. INTRODUCTION

Mycorrhizae form a symbiotic association with higher plant roots and are functional components of terrestrial ecosystems (Schussler et al., 2001). Mycorrhizae make a critical link between soil and plants (Wilson et al., 2009), and they improve plant growth in both direct and indirect ways (Roldan et al., 2008; Smith and Read, 2008). In addition, mycorrhizae are involved in some important ecosystem functions, such as soil aggregation (Rillig, 2004) and carbon sequestration (Finlay, 2008; Fitter et al., 2000; Zhu and Milller, 2003). Given the multifunctional nature of mycorrhizal effects on sustainability of the ecosystem, pre inoculation of seedlings with mycorrhiza is becoming a common practice in agriculture (especially horticulture) and land reclamation industries (Mummey et al., 2009).

Soil aggregation is a complex, hierarchical process mediated by soil biotic and abiotic factors (Tisdall and Oades, 1982). It is a key component of soil structure which is important in maintaining physical, chemical (Emerson et al., 1986; Martin et al., 1955; Oades, 1984) and biological properties of soil (Coleman, 1986; Elliott and Coleman, 1988). Therefore soil aggregation is crucial for maintaining soil fertility and productivity, hence for soil sustainability (Piotrowski et al., 2004). Soil structure can be expressed as a degree of stability of soil aggregates (Bronic and Lal, 2005; Six et al., 2000).

Mycorrhizae, especially AMF, are known to improve soil aggregation (Bearden and Petersen, 2000; Bedini et al., 2009; Caravaca et al., 2002; Celik et al., 2004; Rillig and Allen, 1999). However, the AMF–host species combination and the species of fungi associated with a single host both can affect the percent water-stable aggregates (%WSA) in the rhizosphere (Piotrowski et al., 2004; Schreiner and Bethlenfalvay, 1997). Though several studies have examined %WSA in relation to AMF, the effect of EMF alone or in combination with AMF on soil structure has been much less explored (Ambriz et al., 2010). Ambriz et al. (2010) observed that %WSA varied with type of mycorrhizal inoculation and the size of soil aggregate fraction when *Fraxinus* seedlings grown in sterilized soil were inoculated with mycorrhizal fungi.

Soil carbon is an important component of terrestrial ecosystems (Zhu and Miller, 2003). It affects and is affected by plant production and plays a major role in maintaining soil fertility and productivity (Jobbagy and Jackson, 2000). AMF can influence soil carbon storage (Miller and Jastrow, 1992) through a variety of mechanisms: protecting relatively labile soil carbon inside the soil aggregates (Cambardella and Elliott, 1994; Jastrow and Miller, 1997), and decreasing losses of carbon through microbial respiration by translocating carbon away from the root zone (Treseder and Allen, 2000; Zhu and Miller, 2003). Further mycorrhizal networks constitute about 20–30% of total soil microbial biomass carbon (Leake et al., 2004), and glomalin carbon represents up to 5% of total soil carbon (Zhu and Miller, 2003). EMF also contributes to SOC by allocating about 10–20% of photosynthetic carbon for the growth of their vegetative mycelium (Smith and Read, 2008). Extrametrical mycelia of EMF together with associated roots contribute half of the dissolved organic carbon in a pine forest soil

(Hogberg and Hogberg, 2002). However, less information is available on SOC in relation to EMF and commercial mycorrhizal inoculation. Therefore it is worthwhile to examine how SOC is influenced by inoculation of plants with AMF and EMF alone and in combination.

We used willow plants in our experiment because of their economic importance worldwide (Kuzovkina and Quigley, 2005; Rooney et al., 2009). Further, willow is one of the few plants in terrestrial ecosystems known to benefit from symbiosis with both EMF and AMF (Paradi and Baar, 2006). Therefore, their growth can be enhanced by inoculating willows with different mycorrhizal combinations. Loree et al. (1989) reported that EMF inoculation increased the willow plant growth. Moreover, willows grow all over the world, in various types of soils (Kuzovkina and Quigley, 2005). Further, soil aggregation and plant growth vary with the soil properties (Tisdall and Oades, 1982; Miller and Donahue, 1990) and plant species. However, no reports are available on the impact of the inoculation of willows with different combinations of mycorrhizae on soil aggregation, SOC, plant growth or soil sustainability.

In this greenhouse study, I hypothesized that soil aggregation, organic carbon and plant growth would be affected by inoculating willows with commercial mycorrhizal inoculants, and results would vary with soil type and plant species.

3.2. MATERIALS AND METHODS

3.2.1. EXPERIMENTAL SETUP

A three-way factorial experiment (6 different soils by 2 types of willow species by 4 types of mycorrhizal inoculations) with eight replicates (n= 384) was established. Six different soils from southern Ontario were used: Selkirk clay, Ridgetown clay, Delhisand, Norfolk sand, Guelph loam, and Elora loam. The soils represent a range of textural and fertility characteristics (Table 3.1). These soils belonged to the major soil textural classes (sandy, clay, and loamy soils). Two soil types from each major textural class were chosen. Soil was taken from locations which were fallow for at least one growing season or which had been cultivated but had not been fertilized for at least one growing season. We used abandoned or non-fertilized soils for the experiment because willows are normally planted in marginal lands. Two species of willow hybrids, *Salix viminalis* and *S. miyabeana*, obtained from LandSaga Inc. (Guelph, ON)were included. Four mycorrhizal inoculations were applied: (1) AMF (*Rhizophagus intraradices*), (2) EMF (*Hebeloma cylindrosporum*), (3) a combination of AMF+EMF, and (4) a non-inoculated control. Mycorrhizal inoculants were supplied by Mikro-Tek Inc. (Timmins, ON). A set of non-planted bare soil pots from each type of soil also was included (n=8) to study the effect of plant roots on soil aggregation.

The experiment was conducted at the phytotron greenhouse at the University of Guelph, ON, Canada. The experimental setup, inoculation, and experimental conditions are the same as for the experiment explained in Chapter 2.

Shoots of willows were harvested after 29 weeks of growth in potted soil, by coppicing the plant about 5 cm above the soil surface. Soil was separated manually from roots and air dried. The air dried soils were used to determine %WSA and SOC.

Table 3.1. Characteristics of soils used in the experiment

Soil Character	Selkirk Clay	Ridgetown Clay	Guelph Loam	Elora Loam	Delhi Sand	Norfolk Sand
NH_4-N(ppm)	4.8	3.1	2.4	1.7	3.6	3
NO_3-N(ppm)	57.5	28.6	40.3	54.9	5.8	23.1
P (ppm)	28	26	5	15	26	69
K (ppm)	222	107	62	89	24	185
Mg (ppm)	681	142	396	314	42	133
Org.matter (%)	5.3	2.3	3.8	3.2	0.3	1.5
pH	6.5	7.2	7.6	7.7	8.2	6
Clay content	49.9	42.3	11.2	13.0	2.8	6.5
Texture	Clay	Silty Clay	Loam	Loam	Sand	Sand
Bulk density (field)	0.9	1	1.1	1	1.2	1.1

3.2.2. QUANTIFICATION OF WATER-STABLE AGGREGATES

The same procedure was followed to estimate the %WSA as described in Chapter 2.

3.2.3. DETERMINATION OF SOIL ORGANIC CARBON

A subsample of each air-dried soil was ground and sieved with a 0.5 mm sieve to obtain a 0.5 mm fraction of soil. Care was taken to remove all the root fragments from the soil samples (by hand picking) before grinding to obtain root-free air-dried soil. Total soil carbon was determined by direct combustion (Krom and Berner, 1983) of 0.2–0.3 g of soil in a clean C-free combustion boat at 1200^{o}C using a Leco carbon analyzer (Leco CR-12, Model # 781-700, Leco Instruments Ltd., St. Joseph, MI, USA). Soil inorganic carbon was determined by placing the soil in a Muffle furnace (Lindberg, Watertown, WI, 53094, USA) for 20 h at 575^{o}C to remove the entire organic C. Then SOC was estimated by subtracting soil inorganic C from the total C (Krom and Berner, 1983).

3.2.4. WILLOW ABOVEGROUND BIOMASS PRODUCTION

The willow shoots from coppicing were oven dried for 14 d at 60^{o}C and willow aboveground biomass was determined (raw data for willow aboveground biomass was obtained from Angela Straathof, SES, University of Guelph).

3.2.5. STATISTICAL ANALYSIS

The variance analysis (ANOVA) was employed to fit the model for response variables (%WSA and SOC) by PROC GLM of SAS 9.2 (SAS Institute Inc., Cary, NC) to determine the variation due to treatments. Significance of the effects of independent variables on response variables was tested using an F test. Least square means and their standard errors were generated. Multiple mean separation was done by Tukey's t test. Throughout the experiment, the type 1 error rate was kept as 0.05. ANOVA assumptions were tested as described in chapter 2.

Correlation and regression analyses were performed by SAS 9.2 (SAS Institute Inc., Cary, NC) to evaluate any relationships between %WSA and SOC, between aboveground willow biomass and SOC, and between aboveground willow biomass and %WSA.

Percent change in soil and plant parameters (%WSA, SOC, and willow above ground biomass) in response to each mycorrhizal inoculation was calculated by formula 2.

2. Percent change of the parameter = T - C/ C x 100

   Where, T - Treatment mean value of the parameter

   C - Mean value of the parameter in control

3.3. RESULTS

3.3.1. SOIL AGGREGATION

Mycorrhizal inoculation, soil type, plant species, and interaction between soil type and mycorrhizal inoculation significantly affected %WSA, with soil type being the largest contributor to differences (Table 3.2). In most of the soils (4/6), AMF inoculation significantly increased %WSA compared to the control (Figures 3.1). However, inoculation with AMF had no effect in the Norfolk sand and Ridgetown clay soils. In the Elora loam, %WSA also significantly increased when plants were inoculated with AMF and EMF in combination ($p<0.05$). However, this trend was not observed in other soils. EMF inoculation did not show any response on %WSA in any of the soils. Further, on average, the%WSA was higher in *Salix viminalis* (50.94%) than in *S. miyabeana* (49.36%) ($p<0.05$).

Since the bare soil treatment was left uninoculated , it could not be included in the main statistical analysis. However, when analyzed in comparison to the non-inoculated control treatment (none) treatment it was possible to assess the effect of the un inoculated willow plant on soil aggregation. The %WSA in the no plant treatment in each soil was significantly lower than the non-inoculated control (rhizosphere) treatment(Figure 3.2).

Table. 3.2. ANOVA for percent water stable aggregates in six soil types (Delhi sand, Norfolk sand, Guelph loam, Elora loam, Selkirk clay, and Ridgetown clay soil), four mycorrhizal inoculants (AMF (*Rhizophagus intraradices*), EMF (*Hebeloma cylindrosporum*), AMF+EMF, and non inoculated control), and two willow species (*Salix viminalis and S. Miyabeana*).

Source	DF	Type I SS	Mean Square	F Value	Pr> F
Soil	5	242442.1384	48489.0783	1019.95	<0.0001
Myco	3	2996.4462	998.9962	21.01	<0.0001
Plant	1	106.9654	107.0067	2.25	0.0491
Soil x Myco	15	4150.2424	276.6795	5.82	<0.0001
Soil x Plant	5	663.1837	132.5077	2.79	0.096
Myco x Plant	3	365.1072	121.8082	2.56	0.0536
Soil x Myco x Plant	15	1896.8463	126.4742	2.66	0.0529
Error	329	15640.8212	47.5405		
Corrected total	383	268261.7836			

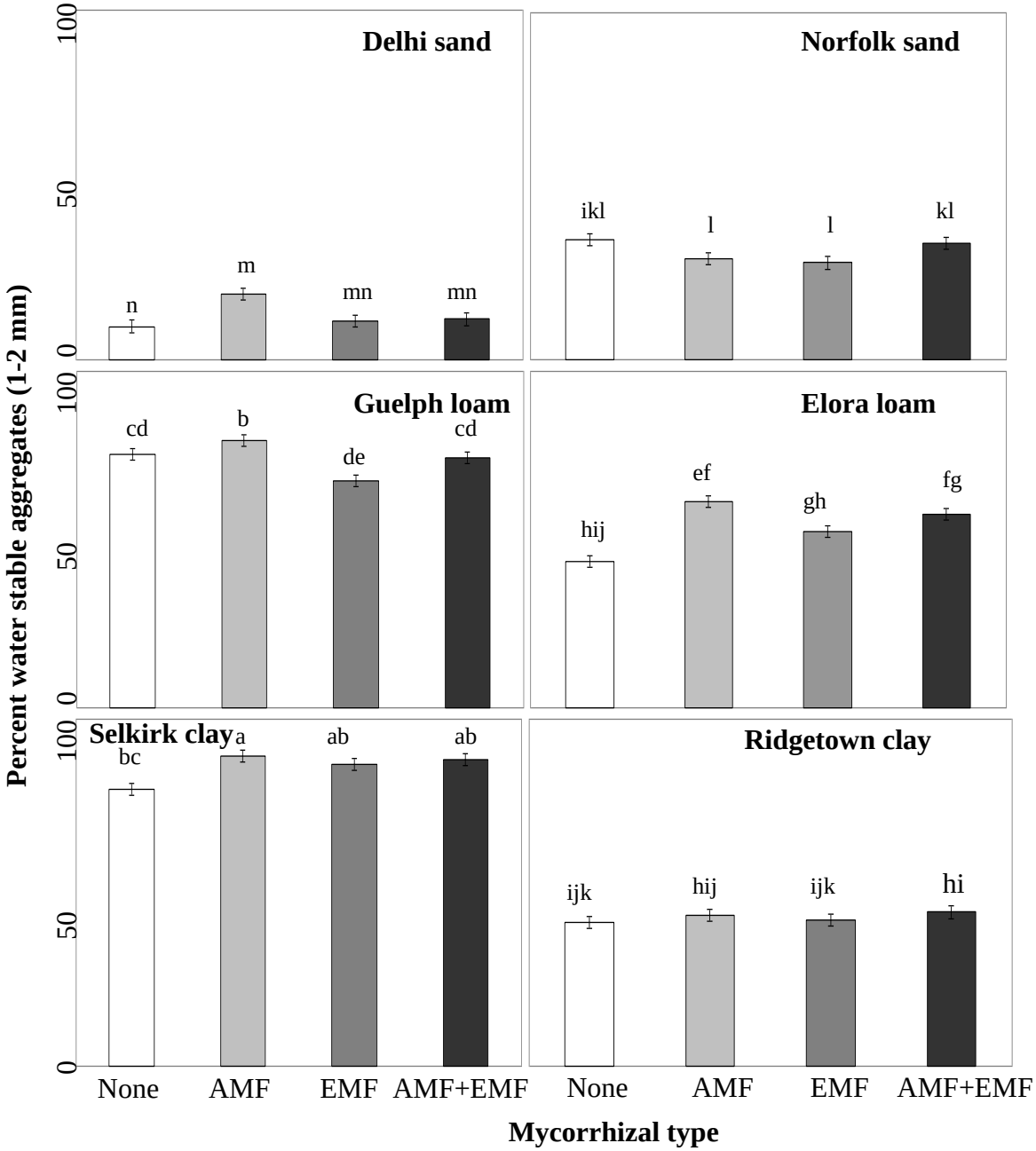

Figure 3.1. Percent water-stable aggregates (1–2 mm) in the rhizosphere of willows grown in six soils. Willow plants were inoculated with arbuscular mycorrhiza (AMF), ectomycorrhiza (EMF), and a combination of AMF+EMF. A non-inoculated control (None) was also included (n=16). Least squares means are presented; error bars indicate standard error of the mean.Means followed by the same letter are not significantly different according to Tukey's mean separation, P<0.05.

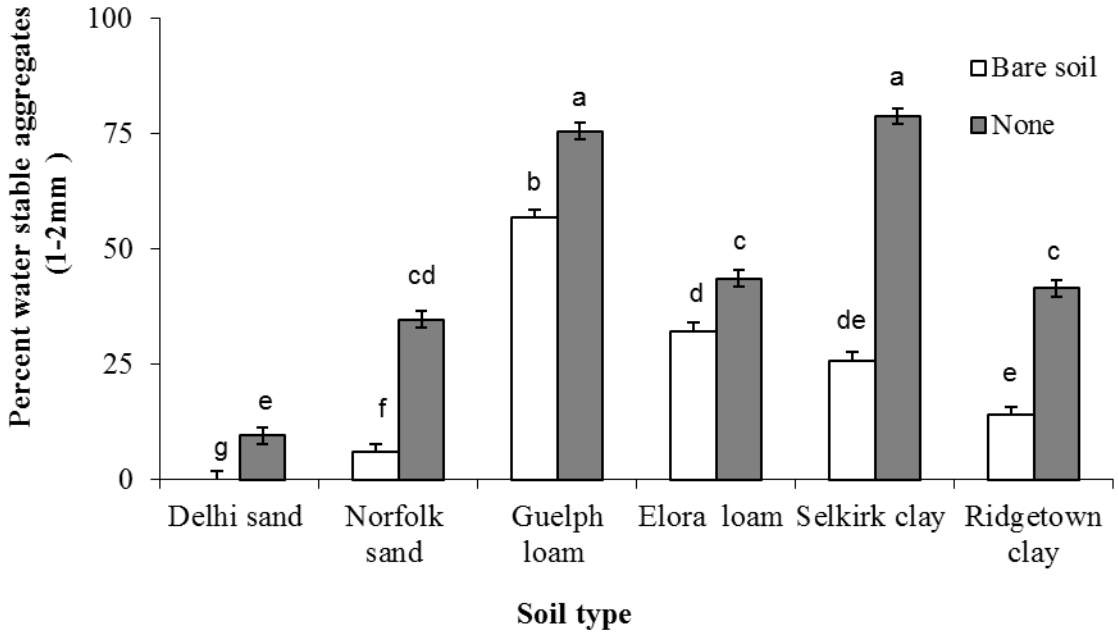

Figure 3.2. Percent water-stable aggregates (1–2 mm) in the non-inoculated control treatment (None) and no-plant treatment (bare soil) in six different soils (n=8). Least squares means are presented; error bars indicate standard error of the mean. Means followed by the same letter are not significantly different according to Tukey's mean separation, $P<0.05$.

3.3.2. SOIL ORGANIC CARBON CONTENT

The SOC significantly varied with mycorrhizal inoculation, soil type, and the interaction between soil type and mycorrhizal inoculation (Table 3.3). In the Norfolk sand, Elora loam, and Ridgetown clay soils, AMF treatment significantly increased the SOC compared to the non-inoculated willow plant rhizosphere (Figures 3.3). Interestingly, inoculation with EMF alone was shown to increase SOC significantly only in the Norfolk sand. Inoculation with combination of AMF and EMF increased SOC only in Ridgetown clay soil.

We analyzed only two treatments: none-inoculated control (none) and no plant (bare soil) together to study the effect of the plant roots on SOC. The SOC of the no plant treatment in Norfolk sand, Guelph loam, and Selkirk clay soils was significantly lower than their non-inoculated control (rhizosphere) (Figure 3.4) although the general pattern remained the same, with the highest SOC in Selkirk, and lowest in Delhi sand.

Correlation and regression analysis showed that a significant ($r=0.80955$), linear and positive relationship exists between SOC and %WSA when all data points were analysed (Figure 3.5).

Table. 3.3. ANOVA for soil organic carbon in six soil types (Delhi sand, Norfolk sand, Guelph loam, Elora loam, Selkirk clay, and Ridgetown clay soil), four mycorrhizal inoculants (AMF (*Rhizophagus intraradices*), EMF (*Hebeloma cylindrosporum*), AMF+EMF, and non inoculated control), and two willow species (*Salix viminalis and S. Miyabeana*).

Source	DF	Type 1SS	Mean Square	F Value	Pr> F
Soil	5	87001.82	17400.3684	272.84	<0.0001
Myco	3	1331.565	443.8551	6.96	0.0003
Plant	1	0.4983	0.4983	0.007	0.9298
Soil x Myco	14	2482.76	177.3383	2.78	0.0456
Soil x Plant	5	41.1321	8.2264	0.13	0.9854
Myco x Plant	3	289.5425	96.5141	1.51	0.1223
Soil x Myco x Plant	14	1365.7725	97.5551	1.52	0.1234
Error	90	5739.7677	63.7752		
Corrected total	137	98252.8675			

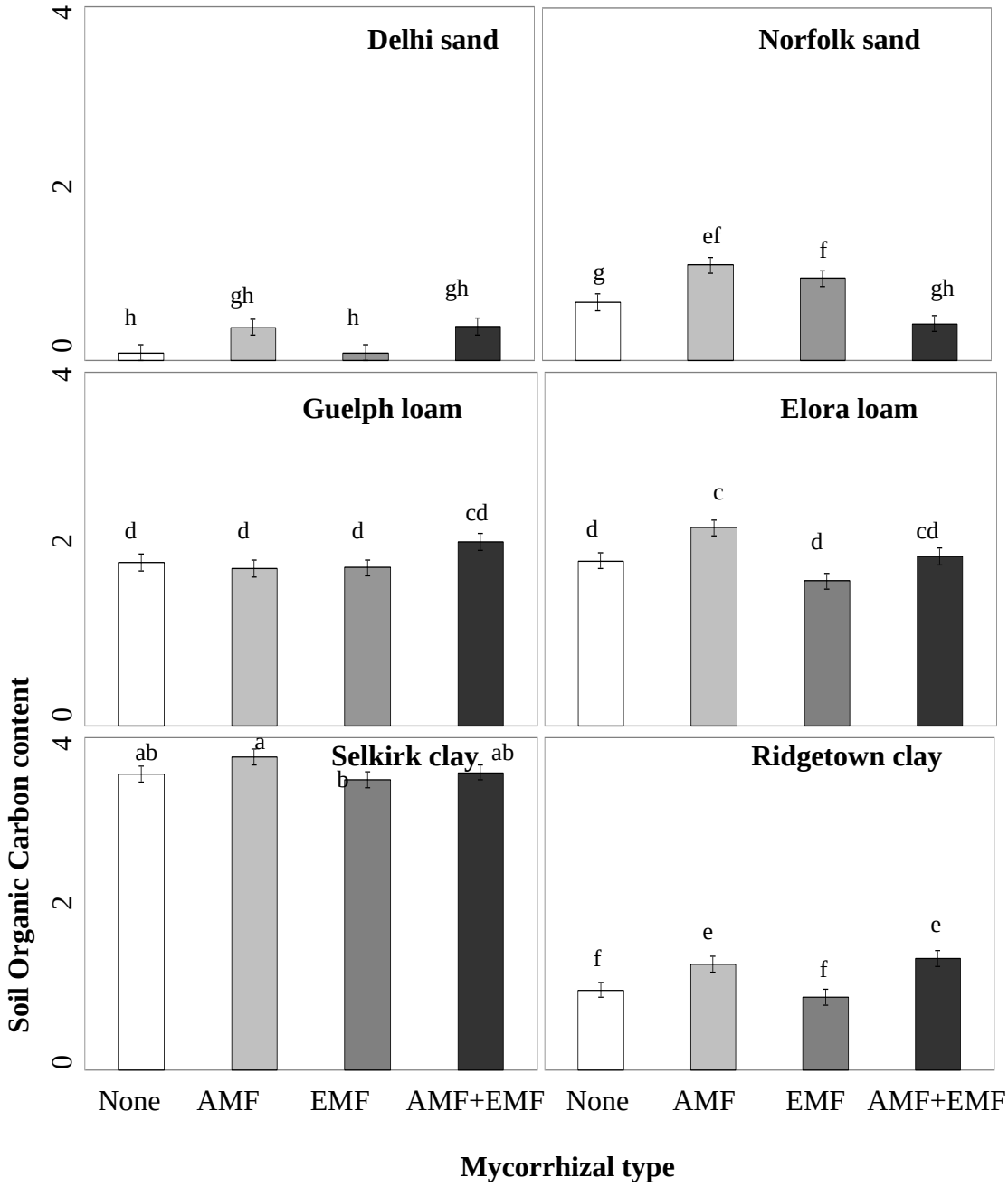

Figure 3.3. Soil organic carbon (SOC) content in the rhizosphere of willows grown in six soils. Willows were inoculated with arbuscular mycorrhizae (AMF), ectomycorrhizae (EMF), and a combination of both (AMF+EMF). A non-inoculated control (None) was also included(n = 3).Least square means are presented,error bars indicate standard error of the mean. Means followed by the same letter are not significantly different according to Tukey's mean separation, $P<0.05$.

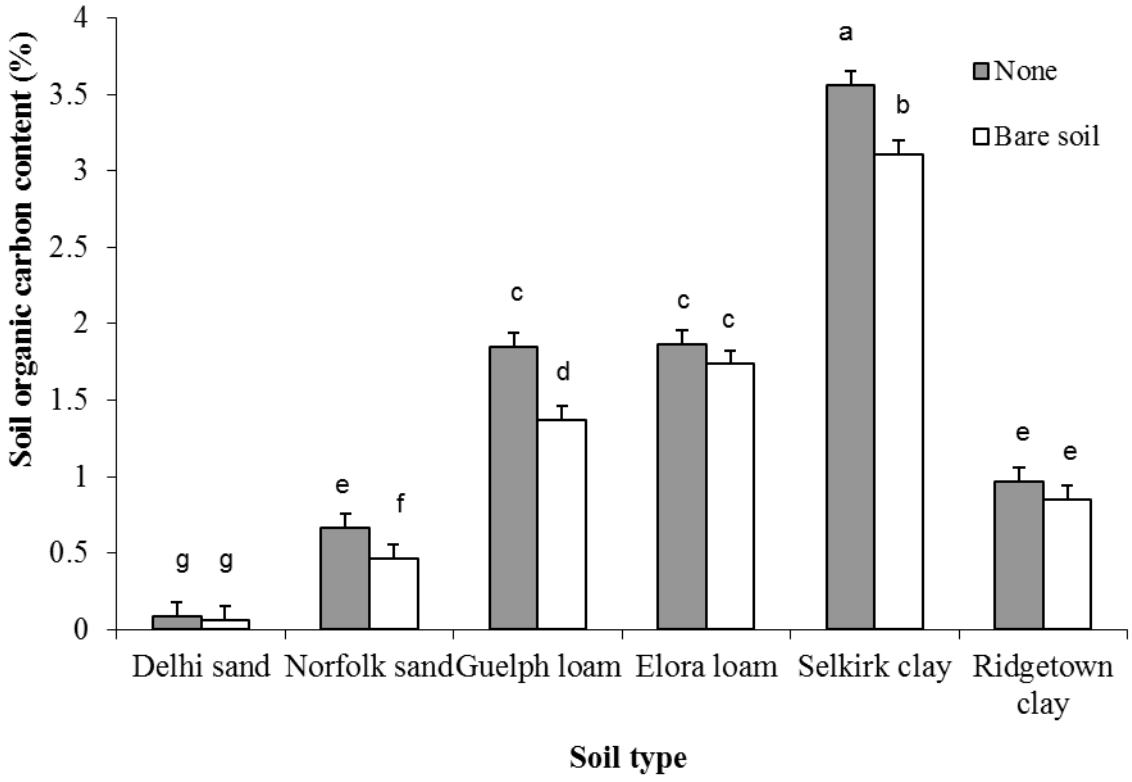

Figure 3.4. Soil organic carbon content in the non-inoculated control treatment and no-plant treatment in six different soils(n=8). Least squares means are presented; error bars indicate standard error of the mean. Means followed by the same letter are not significantly different according to Tukey's mean separation, $P<0.05$.

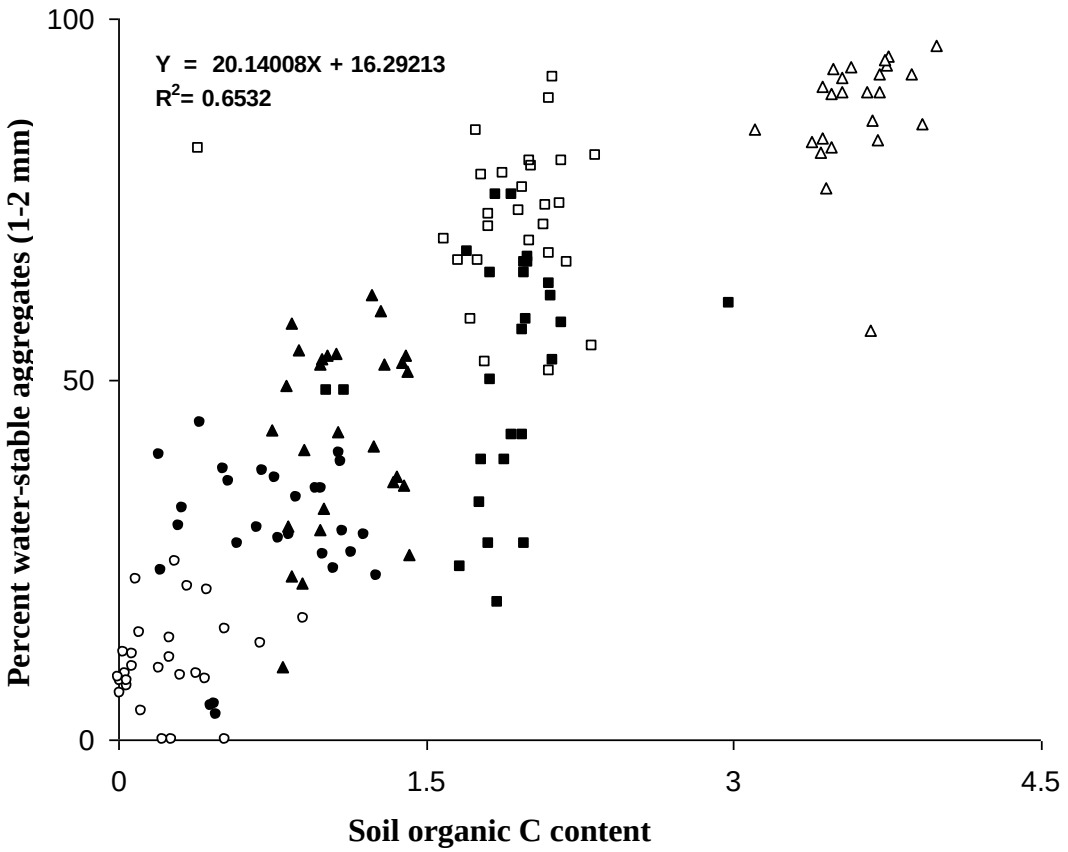

Figure 3.5. Correlation between soil organic carbon (SOC) content and %WSA in the rhizosphere of willows planted in different soils (Delhi sand (○), Norfolk sand (●), Selkirk clay (△), Ridgetown clay (▲), Guelph loam (□), and Elora loam (■)). Two willow species (*Salix viminalis* and *S. miyabeana*) were planted in six soils and were inoculated with arbuscular mycorrhizae (AMF), ectomycorrhizae (EMF), and a combination of both (AMF+EMF). A non-inoculated control (None) was also included. Points represent values for each pot (n = 144) (r=0.80955).

3.3.3. ABOVEGROUND BIOMASS PRODUCTION

Soil type, mycorrhizal inoculation, plant species, and the interaction between soil type and mycorrhizal inoculation significantly affected the willow aboveground biomass(Table 3.4). *Salix viminalis* produced the higher biomass (11.36 g) than *S. miyabeana* (9.04 g). On average Selkirk clay produced higher biomass (21.14 g) and Delhi sand (5.25 g) produced the lowest biomass. Only two of the soils showed any impact of inoculation on plant biomass, and the trend was not consistent (Figure 3.6). In the Selkirk clay soil, when willows were inoculated with AMF alone and EMF alone, but not in combination, the biomass was significantly higher compared to the control. In Ridgetown clay soil, when willows were inoculated with EMF alone and with EMF in combination with AMF, but not with AMF alone, the biomass significantly increased compared to the control treatment.

Table. 3.4. ANOVA for willow aboveground biomass in six soil types (Delhi sand, Norfolk sand, Guelph loam, Elora loam, Selkirk clay, and Ridgetown clay soil), four mycorrhizal inoculants (AMF (*Rhizophagus intraradices*), EMF (*Hebeloma cylindrosporum*), AMF+EMF, and non inoculated control), and two willow species (*Salix viminalis and S. Miyabeana*).

Source	DF	Type I SS	Mean Square	F Value	Pr> F
Soil	5	9923.2465	1984.6438	332.49	<0.0001
Myco	3	39.0970	13.3227	2.28	0.0089
Plant	1	503.2879	503.2983	84.32	<0.0001
Soil x Myco	15	201.5446	13.4429	2.25	0.0051
Soil x Plant	5	44.1708	8.8354	1.48	0.1959
Myco x Plant	3	10.2437	3.4125	0.57	0.6338
Soil x Myco x Plant	15	140.8746	9.3926	1.57	0.0794
Error	319	1904.1261	5.9729		
Corrected total	373	12766.5483			

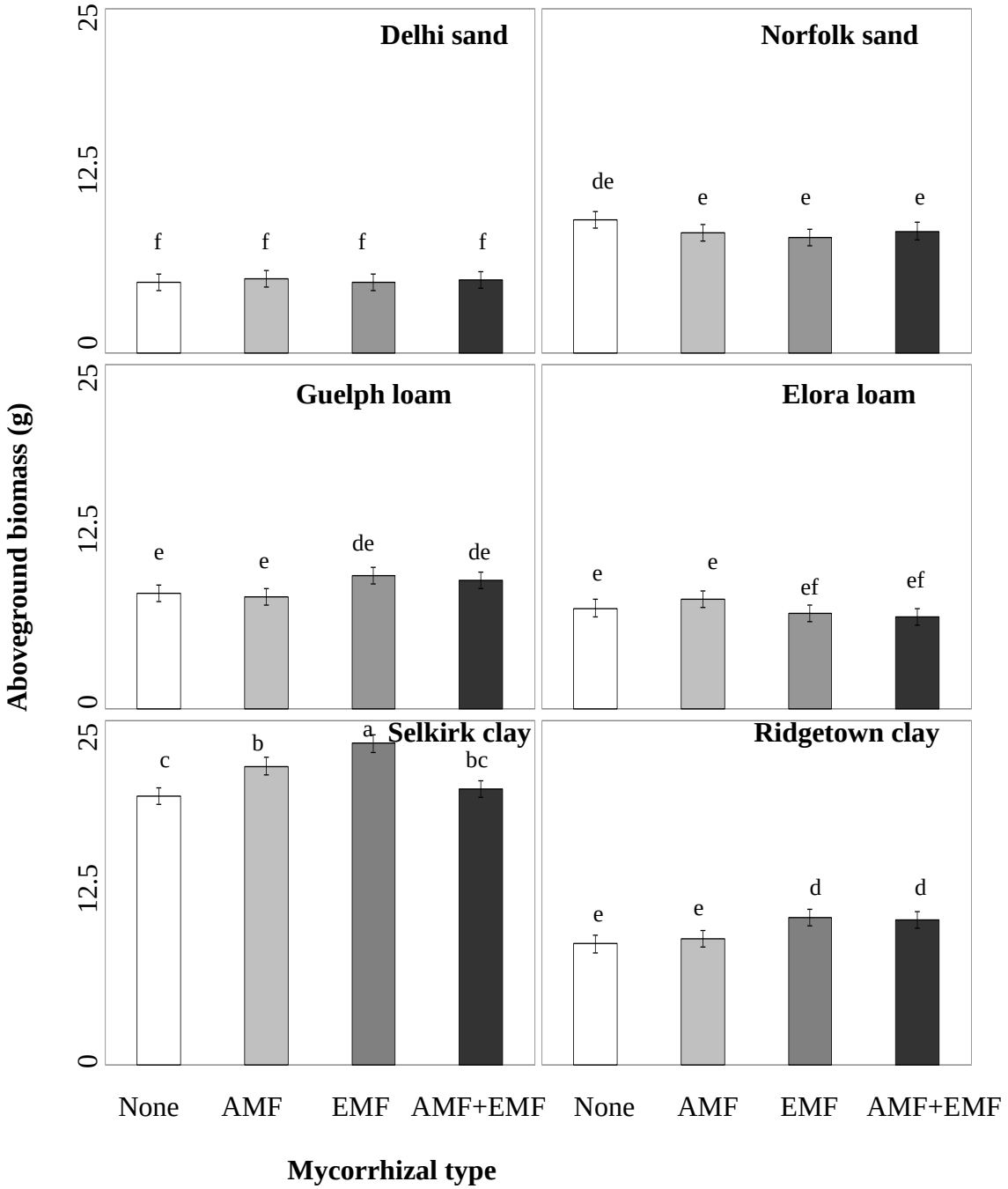

Figure 3.6. Aboveground biomass production (oven dry weight) of willows grown in six soils. Willow plants were inoculated with arbuscular mycorrhizae (AMF), ectomycorrhizae (EMF), and a combination of both (AMF+EMF). A non-inoculated control (None) was also included (n=16). Least square means are presented; error bars indicate standard error of the mean. Means followed by the same letter are not significantly different according to Tukey's mean separation, $P<0.05$.

3.4. DISCUSSION

3.4.1. SOIL TYPE

Water-stable aggregation is crucial for maintaining soil sustainability (Piotrowski et al., 2004), and may be used as a predictor of soil health. The present study shows that %WSA significantly varied with type of soil.

Soil aggregation is mainly determined by different binding agents present in soil, such as soil organic matter, inorganic materials, soil microbes (especially bacteria and mycorrhizal fungi), and plant roots (Benbi and Senapati, 2010; Goldberg et al., 1988; Tisdall and Oades, 1982). Clay content of soil is also a primary determinant of soil aggregation (Wagner et al., 2007). The amount of organic matter, clay, and inorganic materials (such as NH_4, NO_3, P, K, and Mg) in our soils differed from each other (Table 3.1). Further AMF and EMF communities (Chapter 2) and the abundance of the total bacterial population (16S rRNA gene) and denitrifying genes (*amoA*, *nosZ*, and *nirS*) (Straathof, 2010) varied among our soils. Differences between other soil microbes are expected among our soils because soil microbial communities mainly vary with type and physico-chemical properties of soil (Silveira et al., 2006; Singh et al., 2006; Reeve et al., 2010). These differences may be related to the differences in aggregation among the soils used in our experiment. Further our results showed that bare soil from pots maintained without willow plants showed a lower amount of water stable aggregates than the soils from pots containing a willow plant, showing the importance of plant roots in soil aggregation and further confirming the conceptual model (Tisdall and Oades, 1982).

The soil organic carbon content is another important soil health parameter which affects soil and plant productivity (Jobbagy and Jackson, 2000). The soil contains the

largest carbon pool in the biosphere. It stores more carbon than in plants and the atmosphere together (Schlesinger, 1997) and has a potential to manage elevating CO_2 levels in the atmosphere (Godbold et al., 2006). As expected, soil organic carbon significantly varied with type of soil in our experiment, as we collected soils from locations with varying cropping histories, soil textures, and soil organic matter contents.

Since, soil aggregation and SOC showed same trend in our experiment, correlation and regression analysis was performed to see any relationship between these two parameters. Our results showed a, positive, and linear relationship between water stable aggregation and soil organic carbon, suggesting interdependency between two parameters. Benbi and Senapati (2010) showed the same relationship between the above parameters, corroborating our results. Soil organic matter and soil carbon facilitates soil aggregation by acting as a cementing agent between soil primary particles and providing a nucleus for the formation of soil aggregates (Bronick and Lal, 2005). On the other hand, soil aggregates protect labile SOC encrusted within soil aggregates from decomposition (Plante and McGill, 2002).

3.4.2 MYCORRHIZAL INOCULATION

Mycorrhizal fungi are known to influence soil aggregation (Tisdall and Oades, 1982). Results of the present study show that water stable aggregation significantly affected by mycorrhizal inoculation. However, the effect varied with type of mycorrhizal inoculant and soil properties. The %WSA was significantly increased after inoculation with AMF in a majority of the soils in the willow rhizosphere. Soils inoculated with AMF had the highest amount of aggregation, compared to the other mycorrhizal

treatments in Norfolk sand and Ridgetown clay soils. The positive effect of AMF on soil aggregation has been well documented (Bethlenfalvay et al., 1999; Cho et al., 2009; Enkhtuya and Vosatka, 2005; Enkhtuya et al., 2003; Milleret et al., 2009; Rillig et al., 2002; Schreiner and Bethlenfalvay, 1997; Wilson et al., 2009). As seen in the earlier chapter describing only the Selkirk and Guelph soils, soil aggregation can be improved by AMF through various means. However, our results showed that soil aggregation was not affected by inoculation with EMF. Less information is available on the effect of EMF alone or in combination with AMF on soil aggregation. In contrast to our results, Ambriz et al., (2010) showed that EMF+AMF and EMF alone could increase aggregation in the rhizosphere of inoculated *Fraxinus* seedlings compared to AMF alone. Though statistically not significant, it is interesting to note that the majority of our soils (Selkirk clay, Guelph loam, Elora loam, and Delhi sand) showed lower aggregation in combination of AMF and EMF treatment, than the AMF alone treatment. It has been suggested that in some soils there can be a suppressive or antagonistic effect of EMF against AMF, or an incompatibility (Lodge and Wentworth, 1990; Read et al., 1976). This may be the case in these soils, however, this effect should be explored further, as inoculation with a combination of AMF+EMF is common in land reclamation industries. It is clear from our study that AMF and EMF inoculants behave in a different manner in different soils, and that many factors affect soil aggregation, suggesting a need for further investigation.

Further, as expected, aggregation was much lower in non-plant treatments (bare soils) compared to all rhizosphere treatments, showing the importance of plant roots in soil aggregation. This agrees with previous findings which showed greater aggregate

stability in rhizosphere soil than in non-rhizosphere soil (Caravaca et al., 2002; Thomas et al., 1993). Plant roots play an important role in the formation of macro aggregates by causing physical entanglement of micro aggregates (Tisdall and Oades, 1982). Jastrow (1987) reported on the relation of root biomass to formation of water-stable aggregates. Improved %WSA by plant roots may be attributed to the physical, chemical, and biological effects of roots and their exudates on soil aggregation (Bronick and Lal, 2004) by rhizo-deposition, root length, root distribution pattern, root turnover, and macro and microorganisms associated with roots (Haynes and Beare, 1997). Polygalacturonic acid in root mucilage increases soil aggregation by increasing the bonding strength and decreasing the wetting rate in soil particles (Czarnes et al., 2000).

Inoculation with AMF alone was shown to increase the organic carbon content in half of the soils used in this study.AMF associations have the potential to influence soil carbon content because plants allocate 2–20% of their assimilate to their AMF partner (Jakobsen and Rosendahl, 1990; Pearson and Jakobsen, 1993; Smith and Read, 2008). The carbon allocated to the AMF partner then enters into the soil organic carbon through a variety of mechanisms. The extrametrical mycelia of AMF can make up about 20–30% of soil biomass carbon (Leake et al., 2004). Through growth and turnover of extrametrical mycelium containing chitin which is resistant to decomposition, AMF directly enters the soil carbon pool (Zhu and Miller, 2003). In addition, exudates of AMF hyphae contain glomalin which has a long residence time (about 6–42 years) in soil and which contributes directly and extensively to the SOC pool (Rillig et al., 2001).

Both AMF hyphae and glomalin, which stabilize the soil aggregates, indirectly contribute to the SOC pool (Zhu and Miller, 2003) by protecting relatively labile soil

carbon inside the soil aggregates (Cambardella and Elliott, 1994; Jastrow and Miller, 1997). AMF hyphae also enhance carbon sequestration in the soil matrix by translocating carbon away from the root zone which has high microbial respiration (Treseder and Allen, 2000; Zhu and Miller, 2003). Further, past research findings show that AMF can contribute about 15% to the SOC pool in soils of grasslands (Miller and Kling, 2000) and of tropical rainforest (Rillig et al., 2001). Similar to AMF, EMF is also reported to increase SOC by allocating about 10–20% of photosynthetic C for the growth of EMF vegetative mycelia (Smith and Read, 2008). Hogberg and Hogberg (2002) showed that extrametrical mycelia of EMF together with associated roots contribute half of the dissolved organic carbon in a pine forest soil in Sweden. Surprisingly, inoculation with EMF did not increase SOC in our experiment, except in Norfolk sand, contradicting the above findings. However, most of the mycelia of EMF are in the form of a mantle which tightly binds to roots; some EMF produce little mycelia other than the mantle, while some produce dense long mycelia with a high biomass (Agerer, 2001). If the external mycelia (other than the mantle) of *Hebeloma cylindrosporum* (our EMF inoculant) and of the other EMF proliferating in our soils are less, we can expect less or no contribution of EMF to SOC in the short time period of our study. However, this is the first report that shows SOC in relation to the commercial mycorrhizal (AMF and EMF) inoculation and soil types, further studies are recommended to explore why EMF inoculation does not increase SOC in the majority of these soils.

The main purpose of commercial mycorrhizal inoculation is to improve plant growth. However, our results did not show significant positive effect of arbuscular mycorrhizal inoculation on willow plant growth in a majority of soils. Inoculating

willows with EMF increased willow plant growth in only two of the six soils tested. Loree et al. (1989) also found the same when they inoculated *Salix viminalis* and *S.dasyclados* with EMF. However, inoculation with AMF increased willow growth only in the Selkirk clay soil.

3.5. CONCLUSIONS

This study has shown that mycorrhizal inoculation affects soil aggregation, SOC, and willow growth. Further, the effect of mycorrhizal inoculation depended on the soil properties and type of mycorrhizal inoculant. Therefore, it is important to consider the soil properties and type of mycorrhizal inoculant when using mycorrhizal fungi as an inoculant.

AMF inoculation increased soil aggregation and SOC in majority of the soils. However, higher %WSA and SOC was observed when soil was inoculated with AMF alone than with a combination of AMF and EMF. This topic should be further explored. Increased SOC due to AMF inoculation is interesting as SOC has potential to mitigate the problem of increasing atmospheric CO_2 levels. Therefore our results suggest a need for further studies on managing atmospheric CO_2 levels by increasing SOC through AMF inoculation. The positive linear relationship between %WSA and SOC in our study suggests that %WSA could be increased by increasing SOC and vice-versa in the willow rhizosphere.

These conclusions were based on an experiment conducted under greenhouse conditions. These topics should be further explored under real field conditions in multiple locations over a long term.

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CHAPTER 4: MYCORRHIZAL COMMUNITIES, SOIL HEALTH INDICATORS, AND GROWTH OF WILLOWS IN RESPONSE TO MYCORRHIZAL INOCULATION UNDER FIELD CONDITIONS

4.1. INTRODUCTION

Willow (*Salix* spp.) is an economically important tree with multiple uses. It is an important source of biomass for the biofuel and fibre industries. Further, its importance is increasing for use in environmental restoration, phytoremediation, and erosion control (Kuzovkina and Quigley, 2005). These multiple uses of willow are mainly due to its unique physiological characteristics such as superior growth rate (Christersson et al., 1993; Wilkinson,1999); an extensive fibrous root system which provides a strong attachment to the soil (Gray and Sotir, 1996; Rytter and Hansson, 1996) and efficient nutrient uptake (Elowson,1999; Ericsson,1981); tolerance to waterlogged conditions (Aronsson and Perttu, 2001; Jackson and Attwood, 1996; Kuzovkina et al., 2004); the ability to propagate quickly (Gray and Sotir, 1996); vigorous re-establishment after coppicing (Ceulemans et al., 1996; Philippot, 1996); and accumulation of high concentrations of toxic metals (Klang-Westin and Eriksson, 2003). Willow is one of the few plants in terrestrial ecosystems which is known to benefit from symbioses with both arbuscular and ectomycorrhizal fungi (AMF and EMF, respectively) (Paradi and Baar, 2006). The unique physiological characteristics of willow may be attributed to this dual mycorrhizal colonization (Mrnka et al, 2012). However, many ecological questions related to this interesting symbiotic relationship have yet to be explored. Our previous research (Chapters 2 and 3) showed that the some of the important ecological parameters such as soil aggregation, SOC, mycorrhizal communities associated with willow

rhizosphere were significantly affected by commercial mycorrhizal inoculation. However, this study was conducted under controlled environmental conditions for a short period of time (29 weeks). Therefore, the main objective of the current study was to explore how inoculating willows with commercial mycorrhizal inoculants impact soil aggregation, SOC, mycorrhizal communities in the willow rhizosphere and willow growth under field conditions.

This study was conducted at LandSaga hybrid willow nursery, Ignatius farm, Guelph, ON, Canada. Our collaborators, LandSaga Biogeographical Inc., have a long-term field site established since 1992, where 9 hybrid willow clones were propagated in a randomized complete block design. Superior plant growth has been observed in one block of willows (Super Block) compared to the other 11 blocks in the study. Previous analysis determined similar soil fertility in the Super Block compared to the other blocks, leading to the hypothesis that the superior plant growth in the Super Block may be due to an unidentified plant growth-promoting factor (Hendrickson, unpublished data). The effect of this factor could be either direct or indirect such as improvement of soil aggregation or soil carbon, or changes in mycorrhizal communities in favour of plant growth. Preliminary experiments conducted by LandSaga in a greenhouse showed superior growth of willow cuttings planted in the soil collected from the Super Block after one growing season compared to willow cuttings planted in commercial potting media, suggesting that the unidentified plant growth-promoting factor could be soil-borne. The second objective of this field study was to compare the effect of an unsterile aqueous soil extract from the Super Block to a commercially available AMF and EMF mycorrhizal inoculants on plant growth, soil aggregation, SOC, and native mycorrhizal

communities, and, further, to determine whether there was a plant growth-promoting factor in the Super Block that is due to a microbiological characteristic.

In this experiment the hypothesis was that co-inoculation of willows with AMF and EMF would increase %WSA, SOC, diversity of mycorrhizal communities and decrease the aggregate scale spatial variability mycorrhizal communities in the willow rhizosphere. A second hypothesis was that the Super block soil extract was microbiological in nature and would promote willow growth.

4.2. MATERIALS AND METHODS

4.2.1. EXPERIMENTAL DESIGN

The experiment was conducted at the LandSaga hybrid willow nursery at Ignatius Farm, Guelph, ON, Canada. The soil was a sandy loam with pH 7.2; organic matter content, 1.5%; extractable phosphorus, 27 mg/L; total nitrogen, 0.06%; extractable magnesium, 140 mg/L; and extractable potassium, 72 mg/L. The experiment was set up in a randomized block design with four blocks. Each block was divided into three plots assigned to three different treatments: (1) non-inoculated control, (2) commercial mycorrhizae (*Rhizophagus intraradices* (AMF) and *Hebeloma cylindrosporum*(EMF)), and (3) unsterile Super Block soil extract. Soil extract was obtained by mixing soil collected from the Super Block with water in a 1:10 ratio and passing it through a 100 µm filter. Since the diameter of most bacterial cells, fungal spores, and fungal mycelia are less than 100 µm (Atlas, 1995; Madelin and Johnson, 1992), those organisms can pass through a 100 µm filter. Hence, the filtrate should contain bacteria, fungal hyphae, and spores, as well as water-soluble plant growth-promotion factors. Twenty-five cm long rooted willow (*Salix miyabeana*) cuttings were obtained from LandSaga Biogeographical Inc., Guelph, ON, Canada, for the experiment. Forty-two cuttings were planted in each plot. Two weeks after planting, willow plants were treated with either commercial mycorrhizae (250 ml of solution, containing 125 propagules of *R. intraradices* and 46,875 propagules of *H. cylindrosporum*, per plant), and either an unsterile Super Block soil extract (250 ml per plant) or 250 ml of water per plant according to the experimental

design. No other amendment (fertilizers or pesticides) was added to the plants during the experiment, and weed control was performed by hand.

4.2.2. SOIL SAMPLING

One year after planting, soil was collected randomly from the rhizosphere (at 5–15 cm depth) of 15 plants from the centre of each plot by using a sterile soil corer (two soil corers from a plant). The soil samples taken from each plot were composited in a polythene bag and put directly into a cooler to minimize the changes in the microbial community. Upon return from the field, each soil sample was divided into two subsamples. One set of subsamples was placed in the freezer at -20^{o}C and the other was placed on paper for air drying.

4.2.3. SOIL AND PLANT PARAMETERS

Water-stable aggregates and SOC were assessed as described in Chapter 2 and 3, respectively. In order to avoid destructive sampling of willows, plant height after 18 months growth was taken as an indicator of plant biomass production. Height of the willow plants (from the collar (base of the plant near the soil surface) to the tip of the plant) was measured.

4.2.4. SOIL SEPARATION AND DNA EXTRACTION

Field soil from each plot was homogenized, and a sub-sample (about 20 g of bulk rhizosphere soil) was stored in the refrigerator at -20^{o}C. A second sub-sample (250 g) was dry-sieved into four aggregate size classes (>4 mm, 4–2 mm, 2–0.5 mm and <0.5

mm) as described in Seech and Beauchamp (1988). Dry-sieving was chosen to avoid desorption of DNA from soil particles, as described in Levy-Booth et al. (2009). A 0.25 g sub-sample was randomly removed from each homogenized aggregate size class and from each bulk rhizosphere soil sample and used for DNA extraction (Mo Bio Power Soil DNA extraction kit, Mo Bio Laboratories, Solana Beach, CA). Extracted DNA was stored at -20°C for subsequent analysis.

4.2.5. ASSESSMENT OF AMF COMMUNITIES

The diversity and abundance of AMF communities in the bulk soil and aggregate size classes were assessed as described in Chapter 2.

4.2.6. PHYLOGENETIC ANALYSIS

MEGA 4 software (Tamura et al., 2007) was used for phylogenetic analysis. The phylogenetic tree was constructed by neighbour-joining analysis with 1000 bootstrap values.

4.2.7. ASSESSMENT OF EMF COMMUNITIES

The diversity of EMF communities in the bulk soil and aggregate size classes were assessed as described in Chapter 2.

4.2.8. STATISTICAL ANALYSIS

4.2.8.1. UNIVARIATE ANALYSIS

The variance analysis (ANOVA) was employed to fit the model for response variables (%WSA, SOC, plant heights, ecological indices [i.e., Shannon's index of general diversity (H'), Margalef's richness index (d), and Pielou'sevenness index (J)], and AML copy number) by PROC GLM of SAS 9.2 (SAS Institute Inc., Cary, NC) to determine the variation due to treatments. Significance of the effects of independent variables on response variables was tested using F test. Least square means and their standard errors were generated and compared. Multiple mean separation was done by Tukey's t test. ANOVA assumptions were tested as described in chapter 2.

4.2.9.2. MULTIVARIATE ANALYSIS

Multivariate analysis was performed to analyze DGGE gel data as described in Chapter 2.

4.3. RESULTS

4.3.1 SOIL AND PLANT PARAMETERS

Inoculation treatments significantly affected %WSA, AML gene copies, and willow plant growth (plant height) (Table 4.1).

Water-stable aggregates in the willow rhizosphere were significantly higher (17%) when willows were inoculated with mycorrhizae (AMF + EMF) ($p < 0.05$), compared to the non-inoculated treatment (Table 4.2). However, inoculation of willow plants with the soil extract showed no significant effect on %WSA. The average SOC was 2.37% in the willow rhizosphere in the control treatment. There were no significant changes in the SOC content in the willow rhizosphere due to inoculation with either of the treatments (Table 4.2).

Plant growth was determined by plant height of 18-month-old willow plants, and was significantly greater in willows that had been inoculated with mycorrhizae or that received the soil extract compared to in the non-inoculated control plants ($p<0.05$). However, there was no significant difference between the mycorrhizal and soil extract treatment on plant growth.

Table. 4.1. ANOVA for percent water stable aggregates (a), soil organic carbon (b), and AML gene copies (c) in willow rhizosphere and willow plant growth (d) in three treatments (mycorrhiza, soil extract, and non-inoculated control).

a)

Source	DF	Type1 SS	Mean Square	F value	Pr> F
Trt	2	1564.3824	782.1912	23.57	0.0007
Error	6	199.0840	33.1807		
Corrected total	11	1779.7705			

b)

Source	DF	Type1 SS	Mean Square	F value	Pr> F
Trt	2	1.1176	0.5588	14.91	0.0931
Error	6	0.2248	0.0375		
Corrected total	11	1.5294			

c)

Source	DF	Type1 SS	Mean Square	F value	Pr> F
Trt	2	3.2637	0.2794	55.43	0.0031
Error	6	0.2248	0.0150		
Corrected total	11	1.5294			

d)

Source	DF	Type1 SS	Mean Square	F value	Pr> F
Trt	2	186.6300	93.3150	7.17	0.0496
Error	6	78.1351	13.0225		
Corrected total	11	339.4858			

Table 4.2. Percent water-stable aggregates (1–2 mm), soil organic carbon content, and abundance of mycorrhizal fungi (AML gene copy number) in the willow rhizosphere and the plant height of willows grown over a field season. Willow plants were inoculated with a combination of arbuscular mycorrhizae and ectomycorrhizae (AMF+EMF), or a soil extract. A non-inoculated control (None) was also included (n=4).

Treatments	%WSA	SOC	AML gene copy no.	Plant height (cm)
None	63.8 b	2.37 a	7.2×10^5 b	29.7 b
AMF+EMF	80.5 a	2.45 a	15.2×10^5 a	35.3 a
Soil extract	66.4 b	2.3 a	4.6×10^5 b	34.6 a
SE	2.88	0.04	0.69×10^5	0.98

Means followed by the same letter within one column are not significantly different according to Tukey's mean separation, $p<0.05$, $n = 4$.

4.3.2. AMF ABUNDANCE

Quantitative PCR of the bulk rhizosphere soil (Table 4.2) indicated that the abundance of AMF in the willow plots inoculated with mycorrhizae was significantly higher than in non-inoculated willow plots ($p<0.05$). However, no significant change in AMF abundance was found in willow plots that were inoculated with the soil extract. AMF abundance associated with aggregate size classes were significantly affected by the type of inoculant and the soil aggregate size class (Table 4.3 and Figure 4.1).

Table. 4.3. ANOVA for AMF abundance in three treatments (mycorrhiza, soil extract, and non-inoculated control) and four aggregate size classes.

Source	DF	Type1 SS	Mean Square	F value	Pr> F
Aggregate size	3	9.8011	3.2670	4.76	0.0105
Trt	2	799.7016	399.8508	582.93	<0.0001
Trt x Agg.	6	4.4057	0.7343	1.07	0.04096
Error	22	15.0905	0.6859		
Corrected total	35	829.7805			

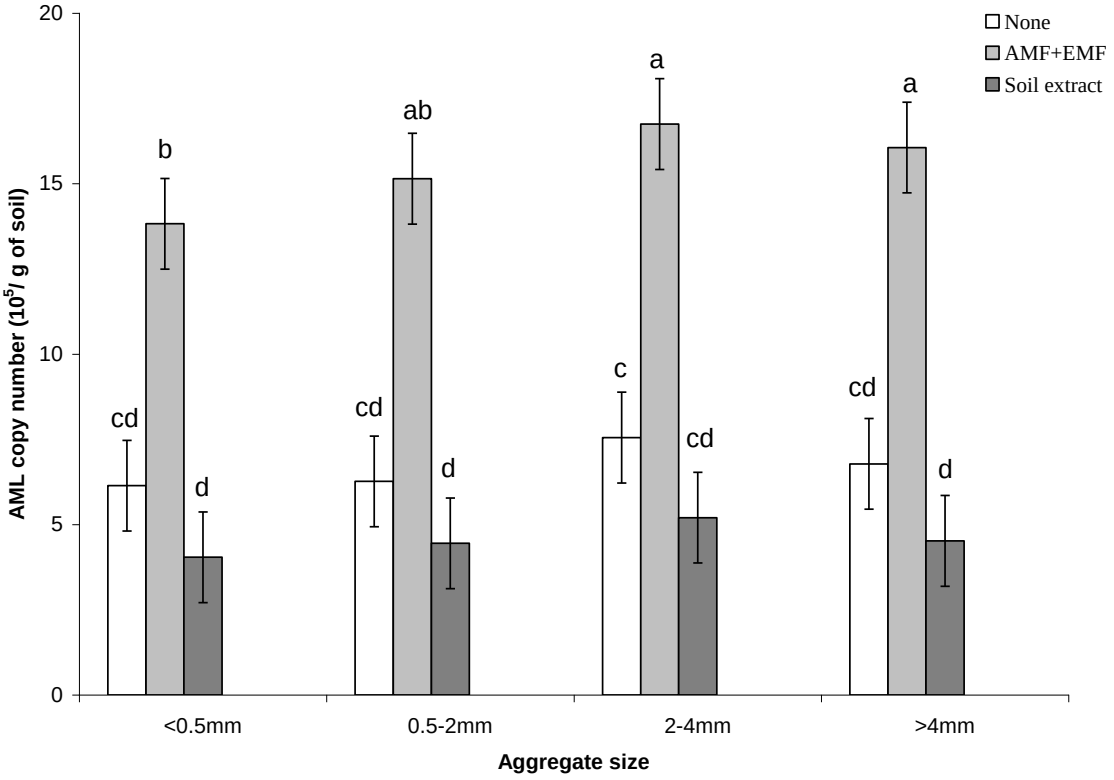

Figure 4.1. AML gene copy number (10^5) per gram of soil in the willow rhizosphere. Willow plants were inoculated with commercial mycorrhizae (AMF+EMF) and soil extract. A non-inoculated control (None) was also included. Soil from each replicate of each treatment was separated into four aggregate size classes before extracting DNA (n=4). Least square means are presented; error bars indicate standard error of the mean.Means followed by the same letter are not significantly different according to Tukey's mean separation, $p<0.05$, $n = 4$.

4.3.3. AMF DIVERSITY

4.3.3.1. AMF DIVERSITY IN BULK RHIZOSPHERE SOIL

Arbuscular mycorrhizal community structure associated with the bulk rhizosphere soil of willows varied according to inoculation type (Figure 4.2). Factor analysis of the similarity matrix obtained from DGGE gels showed that AMF communities associated with different treatments were distinguished from each other (Figure 4.3). Ecological indices (Shannon's index of general diversity (H') and Margalef's richness index (d)) showed significant differences among the treatments (Table 4.4) confirming the results from factor analysis. AMF diversity (H') and richness (d) were significantly higher in mycorrhizal-inoculated plots compared to plots inoculated with the soil extract (Table 4.5). Further, soil extract significantly increased AMF diversity and decreased richness. Control plots, in which willows were not inoculated, had the lowest AMF diversity.

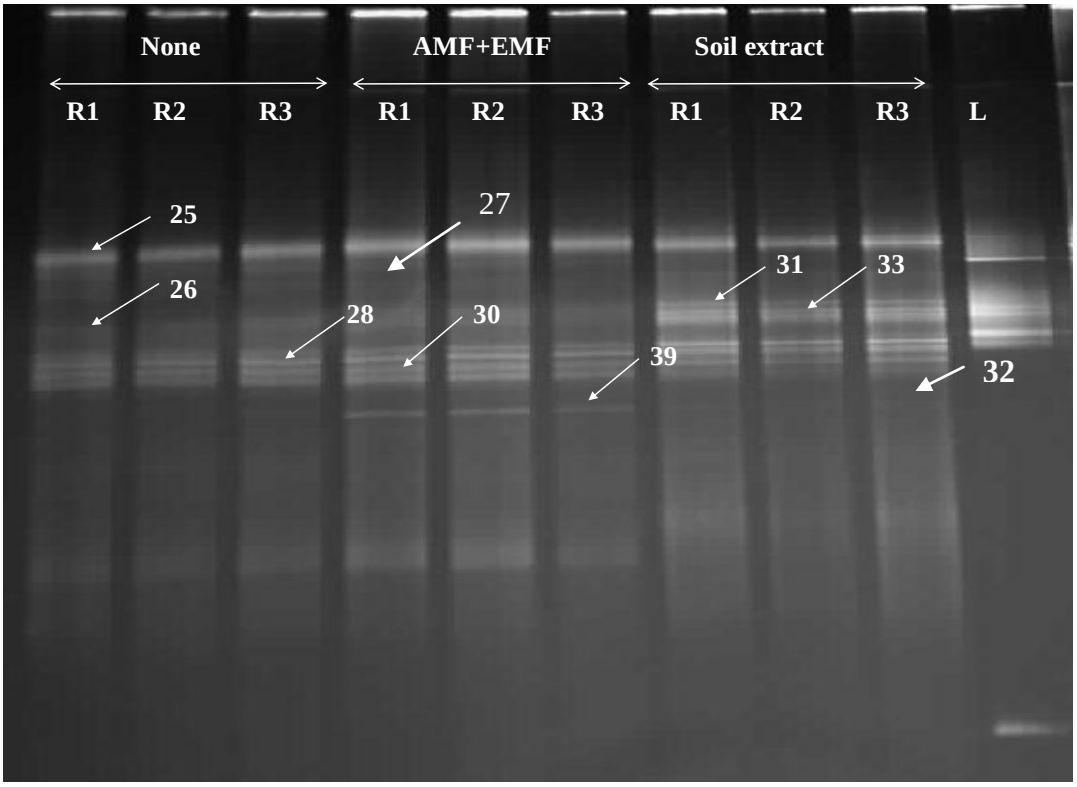

Figure 4.2. Sample DGGE gel indicating the AMF community associated with bulk soil of willow rhizosphere. Willow plants were inoculated with commercial mycorrhizae (AMF+EMF) and soil extract. A non-inoculated control (None) was also included. Numbered arrows identify DGGE bands that were sequenced and included in phylogenetic analysis (Figure 4.6).

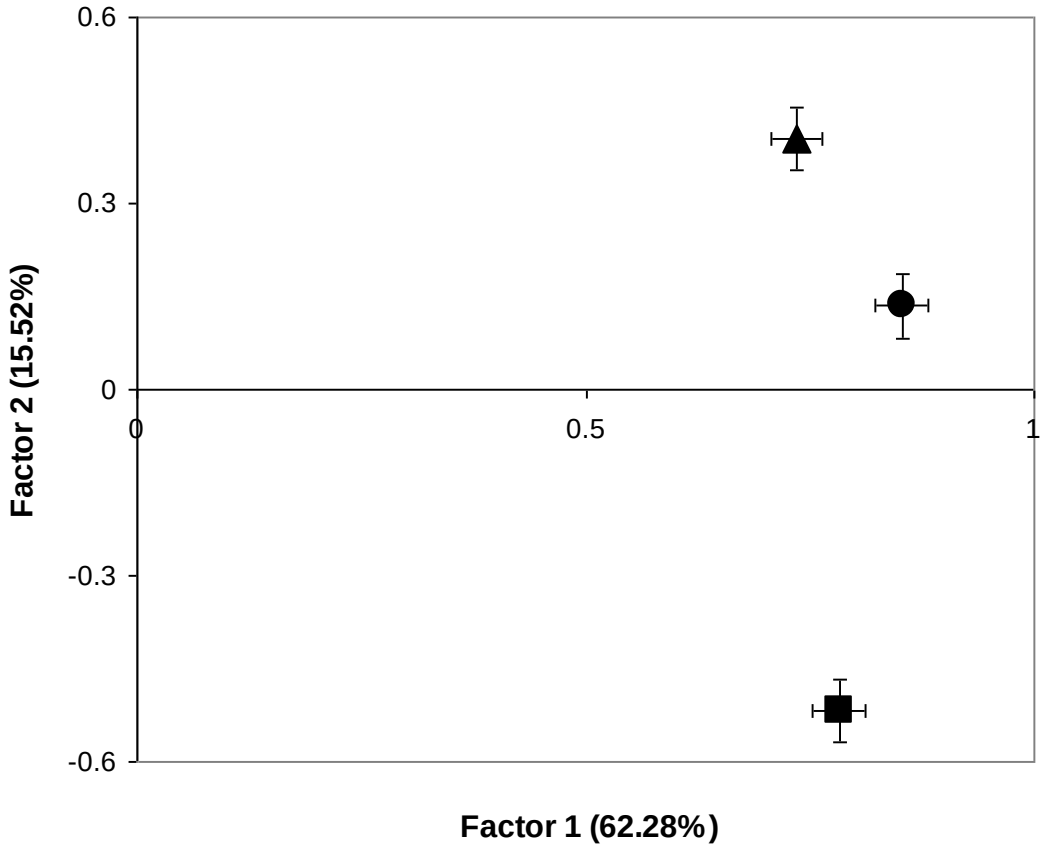

Figure 4.3. Arbuscular mycorrhizal (AM) fungal community composition associated with the bulk rhizosphere soil of willows. Willow plants were inoculated with commercial mycorrhizae (AMF+EMF) and soil extract. A non-inoculated control (None) was also included. Ordination was based on the AM fungal community detected from the willow rhizosphere of three treatments: None (triangles), AMF+EMF (circles), and soil extract (squares). Symbols are the mean of (± 1 SE) ordination coordinates of 4 replicates in each treatment. Error bars indicate standard deviation of the mean.

Table. 4.4. ANOVA for ecological indices ((Shannon index of general diversity (H'), Margalef's richness index (d), Pielou's evenness index (J)) for AMF communities in three treatments (mycorrhiza, soil extract, and non-inoculated control) in bulk rhizosphere.

H'

Source	DF	Type1 SS	Mean Square	F value	Pr> F
Trt	2	748.5692	374.2846	163.88	0.033
Error	6	13.7034	2.2839		
Corrected total	11	774.3827			

d

Source	DF	Type1 SS	Mean Square	F value	Pr> F
Trt	2	56.5894	28.2947	6.60	0.0483
Error	6	25.7094	4.2849		
Corrected total	11	89.9274			

J

Source	DF	Type1 SS	Mean Square	F value	Pr> F
Trt	2	39.2715	19.6358	8.86	0.0381
Error	6	13.2946	2.2158		
Corrected total	11	63.2958			

Table 4.5.Means of indices of diversity (Shannon's index of general diversity (H'), Margalef's richness index (d), and Pielou's evenness index (J)) for AMF communities associated with bulk rhizosphere soil of willows grown over a field season. Willow plants were inoculated with a combination of arbuscular mycorrhizae and ectomycorrhizae (AMF+EMF), or an extract of field soil. A non-inoculated control (None) was also included (n=4).

Treatment	**H'**	**d**	**J**
None	0.8838 c	3.118 b	0.8726 b
AMF+EMF	0.9618 a	3.867 a	0.8913 ab
Soil extract	0.9124 b	2.862 c	0.910 a
SE	0.020	0.148	0.013

Means followed by the same letter within one column are not significantly different according to Tukey's mean separation, $p<0.05$; n= 4

4.3.3.2. AMF DIVERSITY IN SOIL AGGREGATES

AMF diversity differed depending on aggregate size class and inoculant treatment (Figure 4.4). Factor analysis of DGGE gels showed separation of AMF communities associated with certain soil aggregate sizes in the non-inoculated treatment (Figure 4.5). Ecological indices supported the factor analysis results (Table 4.6). Interestingly, inoculation with the soil extract, resulted in the highest measures of AMF diversity, however, minimized the differences in the AMF communities between aggregate size classes (Table 4.7). AMF communities associated with different aggregate sizes in willows inoculated with mycorrhizal inoculant tended to group with the communities of plots that were inoculated with the soil extract. Margalef's richness index indicated that aggregates >4 mm had the highest richness compared to other aggregate size classes in both mycorrhizal and soil extract treatments.

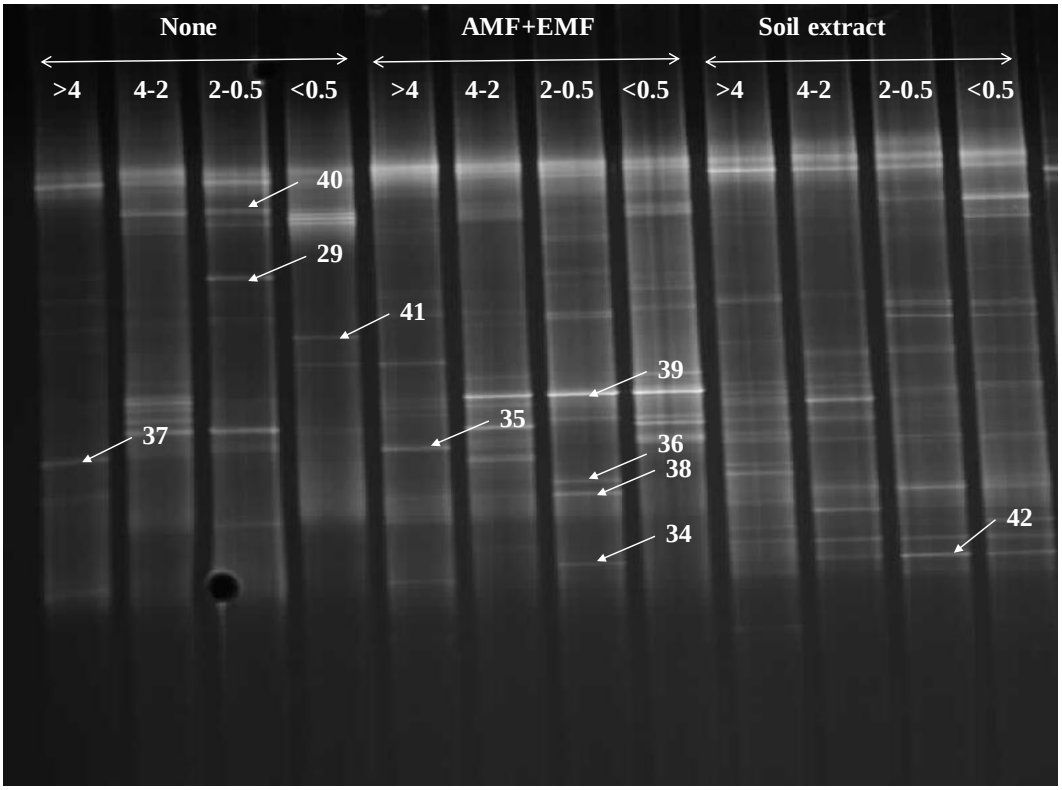

Figure 4.4. Sample DGGE gel indicating the AMF community associated with different aggregate size classes (>4 mm, 4–2 mm, 2–0.5 mm and <0.5 mm) of willow rhizospheres. Willow plants were inoculated with commercial mycorrhizae (AMF+EMF) and soil extract. A non-inoculated control (None) was also included. Soil from each replicate of each treatment was separated into four aggregate size classes before DNA was extracted (n=4). Numbered arrows identify DGGE bands that were sequenced and included in phylogenetic analysis (Figure 4.6).

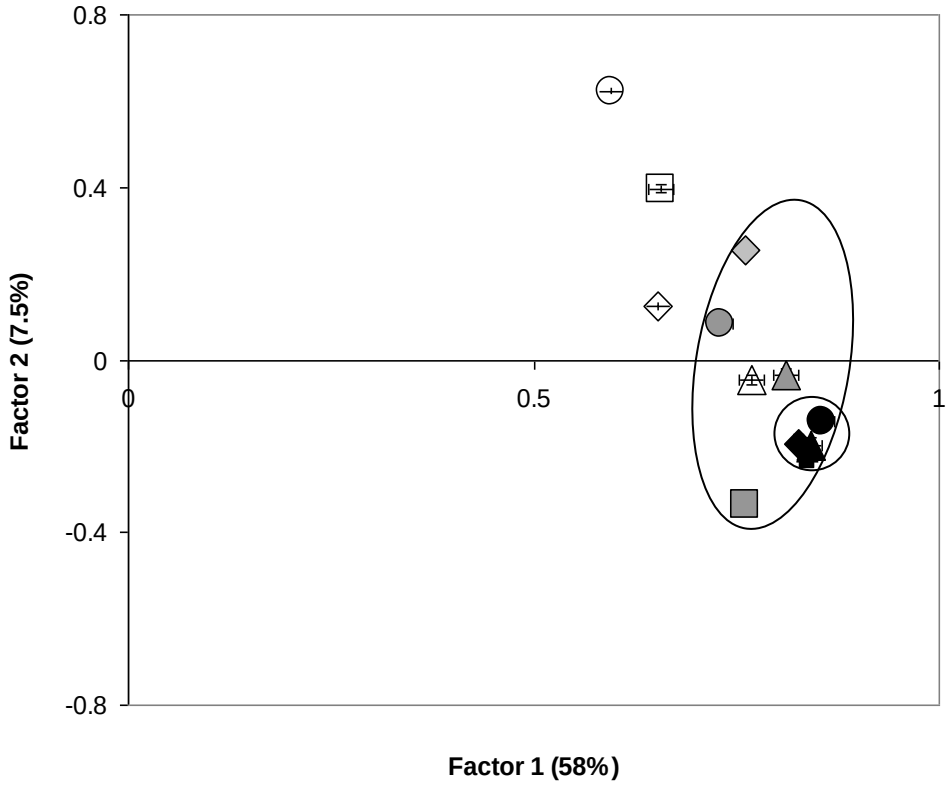

Figure 4.5. Arbuscular mycorrhizal (AM) fungal community composition associated with soil aggregates in the rhizosphere of willow plants. Willow plants were inoculated with commercial mycorrhizae (AMF+EMF) and soil extract. A non-inoculated control (None) was also included. Soil from each replicate of each treatment was separated into four aggregate size classes before DNA was extracted. Ordination was based on the AM fungal community detected from four different aggregate sizes: >4 mm (squares), 4–2 mm (triangles), 2–0.5 mm (diamonds), and <0.5 mm (circles) in the willow rhizosphere of three treatments: none (open symbols), AMF+EMF (gray symbols), and soil extract (black symbols).Symbols are the mean of (± 1 SE) ordination coordinates of 4 replicates in each sample. Error bars indicate standard deviation of the mean.

Table. 4.6. ANOVA for ecological indices ((Shannon index of general diversity (H'), Margalef's richness index (d), Pielou's evenness index (J)) for AMF communities in three treatments (mycorrhiza, soil extract, and non-inoculated control) and four soil aggregates (>4, 4-2, 2-0.5, <0.5 mm).

H'

Source	DF	Type1 SS	Mean Square	F value	Pr> F
Trt	2	123.1552	61.5776	30.58	0.0019
Aggregate size	3	20.1972	6.7324	3.34	0.0362
Trt *Agg.	6	67.5798	11.2633	5.59	0.0265
Error	33	66.4587	2.0139		
Corrected total	47	292.3909			

d

Source	DF	Type1 SS	Mean Square	F value	Pr> F
Trt	2	289.6661	144.8331	29.39	<0.0001
Aggregate size	3	1231.0366	410.3455	83.28	<0.0001
Trt *Agg.	6	809.7525	134.9587	27.39	<0.0001
Error	33	162.5910	4.9273		
Corrected total	47	2520.0462			

J

Source	DF	Type1 SS	Mean Square	F value	Pr> F
Trt	2	1041.0686	520.5343	28.47	0.0162
Aggregate size	3	17952.8199	5984.2732	327.28	<0.0001
Trt *Agg.	6	3942.7481	657.1246	35.94	<0.0001
Error	33	603.3951	18.2847		
Corrected total	47	23563.0317			

Table 4.7. Means of indices of diversity (Shannon's index of general diversity (H'), Margalef's richness index (d), and Pielou'sevenness index (J)) for AMF communities associated with four different aggregate sizes (>4 mm, 4–2 mm, 2–0.5 mm, and <0.5mm) in the rhizosphere of willows. Willow plants were inoculated with a combination of arbuscular mycorrhizae and ectomycorrhizae (AMF+EMF), or an extract of field soil. A non-inoculated control (None) was also included (n=4).

Treatment	**Agg. Size**	**H'**	**d**	**J**
None	4 mm	0.86gh	4.07efg	0.73 f
	2-4 mm	1.04cdef	4.60def	0.83 bcdef
	2-0.5 mm	0.98defg	3.64fg	0.85 abcde
	0.5 mm	0.94efgh	3.20gh	0.88 abc
AMF+EMF	4 mm	1.06bcde	5.67bcd	0.80 bcdef
	2-4 mm	0.98defg	4.88def	0.77 def
	2-0.5 mm	1.08bcd	4.83def	0.83 bcdef
	0.5 mm	1.09abcd	5.05de	0.84 abcdef
Soil extract	4 mm	1.18ab	7.29a	0.81 bcdef
	2-4 mm	1.16ab	5.72bcd	0.79 cdef
	2-0.5 mm	1.22a	6.49abc	0.86 abcd
	0.5 mm	1.18ab	6.77ab	0.84 abcdef
SE		0.030	0.077	0.015

Means followed by the same letter within one column are not significantly different according to Tukey's mean separation, $p<0.05$; n= 4.

4.3.3.3. PHYLOGENETIC ANALYSIS

Phylogenetic analysis showed that all the sequences of DGGE bands (from DGGE 25 to DGGE 42) obtained from this study (Figures 4. 2 and 4.4) were closely related to different species of *Glomeraceae* and *Claroideoglomeraceae* (Figure 4.6). DGGE band 39 had 99% similarity to *Rhizophagus intraradices* sequences found in the Genbank, and the sequence was identical to the sequence obtained from the DNA of a pure culture of the commercial AMF inoculant (*Rhizophagus intraradices*) used in this study. DGGE 39 band was more prominent in the mycorrhizal treatment than in other treatments (Figure 4.2) and it was less prominent in the >4 mm aggregate size class than in other size classes in the mycorrhizal treatment (Figure 4.4). Several bands, DGGE 17, 25, 26, 27, 28, 29, 32, and 41, were grouped with *Claroideoglomeraceae*, and 31, 35, 36, 38, 39, and 40 belong to the *Glomeraceae.* DGGE bands 30, 33, 34, and 37 belong to *Glomeromycota* but not to any of the families of *Glomeromycota* according to the new AMF phylogeny introduced by Kruger et al. (2012).

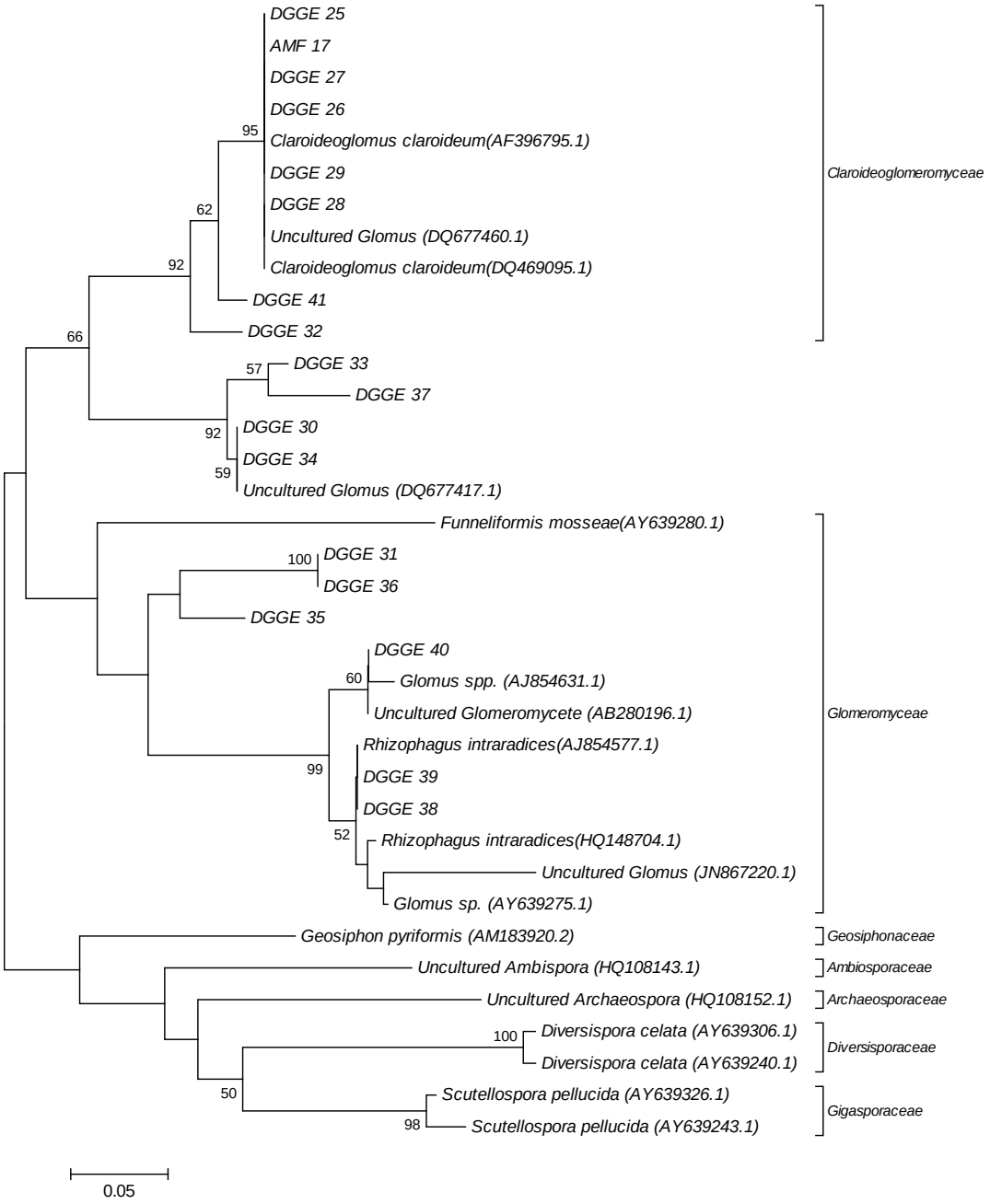

Figure 4.6. Phylogenetic tree showing phylogenetic position of different sequences of AMF obtained from band isolates using PCR–DGGE (DGGE 25-42) (Figures 4. 2 and 4.4) and their close relatives obtained from NCBI (Latin binomials) using Megablast. Bootstrap values above the branches are obtained from neighbour-joining analysis (bootstrap value 1000); these are shown only when >50% in at least one of the analyses.

4.3.4. EMF DIVERSITY

There was a low EMF diversity in the bulk rhizosphere and soil aggregates. Factor analysis of the DGGE gel showed that the EMF community in the bulk rhizosphere soil was significantly changed by both inoculations (Figure 4.7). Ecological indices also showed significant differences due to treatments (Table 4.8). Shannon's index of general diversity (H') and Margalef's richness index (d) were significantly lower in the control treatment than in other treatments (Table 4.9). Moreover, Pielou's evenness index (J) was significantly lower in the soil extract treatment compared to the other two treatments.

Factor analysis of DGGE gels showed that there was no significant difference among EMF communities associated with the different soil aggregates within each treatment (Figure 4.8). Further, analysis also showed that EMF communities associated with the soil aggregates were altered by inoculation with soil extract. There was no change in EMF communities in the soil aggregates when inoculated with mycorrhizae.

The EMF sequences obtained in the study were similar to only *Hebeloma cylindrosporum* and another *Hebeloma* spp.

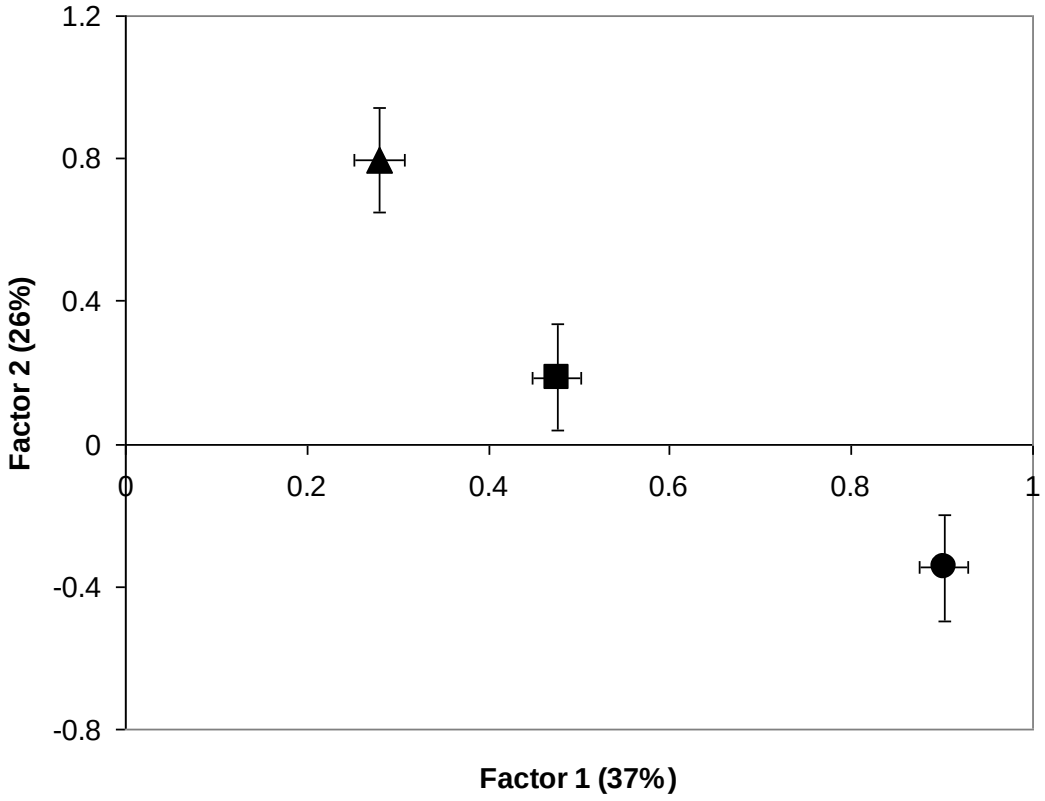

Figure 4.7. Ectomycorrhizal (EM) fungal community composition associated with the bulk rhizosphere soil of willows. Willow plants were inoculated with commercial mycorrhizae (AMF+EMF) and soil extract. A non-inoculated control (None) was also included. Ordination was based on EM fungal community detected from the willow rhizosphere of three treatments: None (triangles), AMF+EMF (circles), and soil extract (squares). Symbols are the mean of (± 1 SE) ordination coordinates of 4 replicates in each treatment. Error bars indicate standard deviation of the mean.

Table. 4.8. ANOVA for ecological indices ((Shannon index of general diversity (H'), Margalef's richness index (d), Pielou's evenness index (J)) for EMF communities in three treatments (mycorrhiza, soil extract, and non-inoculated control) in bulk rhizosphere.

H'

Source	DF	Type1 SS	Mean Square	F value	Pr> F
Trt	2	640.3351	320.1675	34.53	0.0028
Error	6	55.6368	9.2728		
Corrected total	11	715.9719			

d

Source	DF	Type1 SS	Mean Square	F value	Pr> F
Trt	2	427.4192	213.7096	29.34	0.0041
Error	6	43.7034	7.2839		
Corrected total	11	495.1226			

J

Source	DF	Type1 SS	Mean Square	F value	Pr> F
Trt	2	453.4969	226.7484	18.29	0.0018
Error	6	74.3844	12.3974		
Corrected total	11	544.8813			

Table 4.9.Means of indices of diversity (Shannon's index of general diversity (H'), Margalef's richness index (d), and Pielou's evenness index (J)) for EMF communities associated with bulk rhizosphere soil of willows grown over a field season. Willow plants were inoculated with a combination of arbuscular mycorrhizae and ectomycorrhizae (AMF+EMF), or an extract of field soil. A non-inoculated control (None) was also included (n=4).

Treatment	H'	d	J
Control	0.27b	0.64b	0.90a
AMF+EMF	0.45a	1.29a	0.94a
Soil extract	0.47a	1.31a	0.78b
SE	0.027	0.184	0.047

Means followed by the same letter within one column are not significantly different according to Tukey's mean separation, $p<0.05$; n= 4

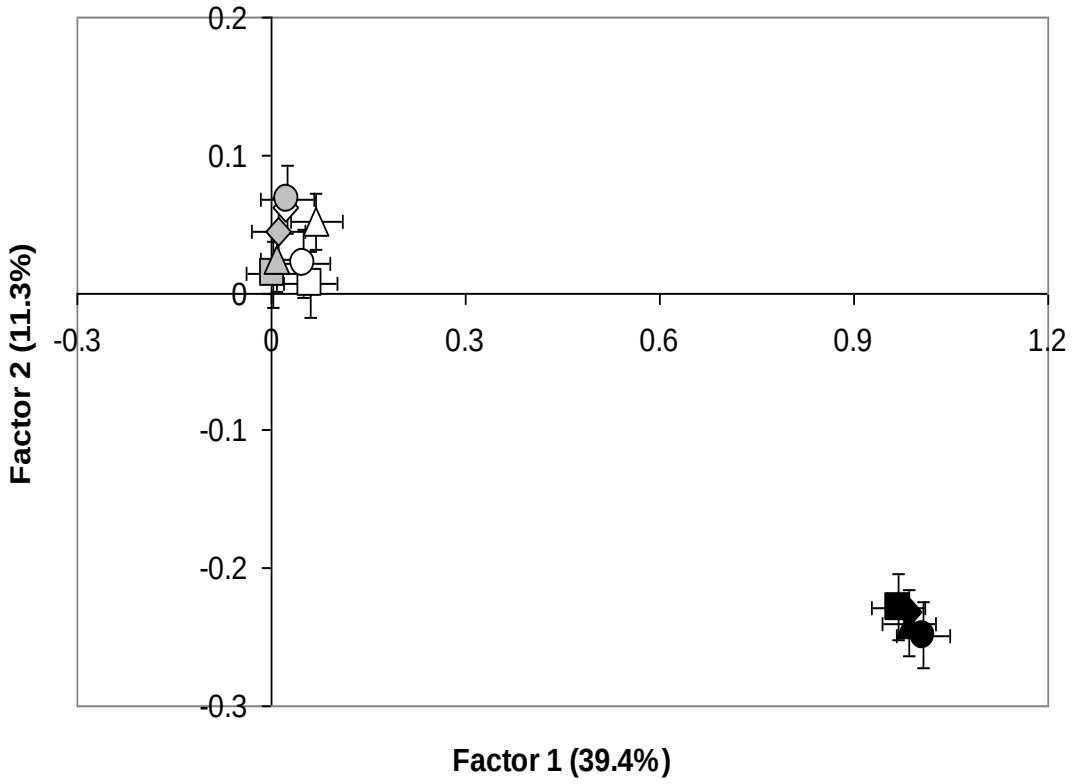

Figure 4.8. Ectomycorrhizal (EM) fungal community composition associated with soil aggregates in the rhizosphere of willow plants. Willow plants were inoculated with commercial mycorrhizae (AMF+EMF) and soil extract. A non-inoculated control (None) was also included. Soil from each replicate of each treatment was separated into four aggregate size classes before DNA was extracted. Ordination was based on the EM fungal community detected from four different aggregate sizes: >4 mm (squares), 4–2 mm (triangles), 2–0.5 mm (diamonds), and <0.5 mm (circles) in willow rhizosphere of three treatments: none (open symbols), AMF+EMF (gray symbols), and soil extract (black symbols).Symbols are the mean of (± 1 SE) ordination coordinates of 4 replicates in each sample. Error bars indicate standard deviation of the mean.

4.4. DISCUSSION

Our results showed that the Super Block soil extract was comparable to commercial mycorrhizae with respect to the plant growth promotion. Increased plant height of young willow plants in the present study showed that inoculation of plants with mycorrhizae and soil extract had a positive effect on plant growth. A positive effect of co-inoculation of AMF and EMF on plant growth under controlled environmental conditions has been reported (Ambriz et al., 2010; Chen et al., 2000; Founoune et al., 2002; Ramanankierana, 2007). An increase in willow plant growth due to co-inoculation of AMF and EMF was observed in only one soil type (Ridgetown clay soil) in our greenhouse experiment (Chapter 3). Mycorrhizal responsiveness to plant growth can vary from highly positive to negative (Klironomos, 2003). Some researchers showed improved plant growth (Bagyara and Sreeramulu, 1982; Bidondo et al., 2011; Janos, 1980; Karagiannidis et al., 2002; Latef, 2011), whereas others showed no response (Simpson and Daft, 1990) or even a reduction in plant growth (Antunes and Cardoso, 1990) due to mycorrhizal inoculation. The improved plant growth in response to mycorrhizal inoculation could be due to some of the direct and indirect effects of mycorrhizae on plant growth. In our experiment we observed improved %WSA and changes in mycorrhizal communities in response to the mycorrhizal inoculation. These effects could be responsible for the improvement of willow growth in our experiment. Improved plant growth in the soil extract treatment may be due to the plant growth-promoting factors that were responsible for the superior willow growth in the Super Block from where the soil extract was obtained. Our results suggest that those factors

can be microbiological and transferable through soil extract, because we used unsterilized soil extract. Some soil microbes can act as plant growth-promoting agents through various mechanisms such as providing biological control of diseases and pests; inducing systemic resistance to pests and diseases (Compant et al., 2005); and improving drought tolerance (Mayak et al.,2004a), soil fertility and accessibility of nutrients to plants (Rodriguez et al., 2007; Vessey, 2003), resistance to salt stress (Mayak et al., 2004b), tolerance to heavy metals (Burd et al., 2000), and nutrient and water relations in plants (Smith and Read, 2008). We observed the beneficial effect of the soil extract only on plant growth, but not on other soil characteristics measured, suggesting another plant growth-promoting mechanism may be involved.

The results of the field experiment showed that initial willow plant growth can be improved by co-inoculating with *Rhizophagus intraradices* and *Hebeloma cylindrosporum* inoculants under field conditions. Further plant growth promoting factor in Super block is microbiological and it should be further explored to see what type of microbes and what type of mechanisms involved in plant growth promotion

The amount of water stable aggregates in the rhizosphere of field-grown willow plants was increased by 17% within one year after inoculating with mycorrhizae. In our greenhouse study, an increase in the amount of water stable aggregates in the willow rhizosphere when inoculated with the same combination of AMF and EMF was seen only in the Selkirk clay and Elora loam soils (Chapter 3). Other studies have also shown a positive effect of AMF on soil aggregation (Bethlenfalvayet al., 1999; Choet al., 2009;Enkhtuya and Vosatka, 2005;Enkhtuya et al., 2003; Milleret et al., 2009;Rillig et al., 2002;Schreiner and Bethlenfalvay, 1997;Wilson et al., 2009). The mechanisms of

improved soil aggregation by mycorrhizal inoculation could be the entanglement of soil particles and stabilization of the soil aggregates by the extraradical mycelium of AMF and glomalin produced by AMF (Rillig et al., 2010;Rillig, 2004; Tisdall and Oades, 1982; Wright and Upadhyaya, 1998). The impact of EMF on soil aggregation has been proposed by Tisdall (1994), Rillig (2004), and Rillig and Mummey (2006). Ambriz et al. (2010) observed the increase in %WSA in 1 mm and 2 mm sized soil aggregates when they co-inoculated *Fraxinus* seedlings grown in sterilized soil with EMF and AMF under a controlled environment in a growth chamber. To our knowledge this is the first report exploring the impact on soil aggregation of dual inoculation of willow plants with AMF and EMF under field conditions. This shows the possibility of increasing soil structural stability by inoculating willows with commercial mycorrhizal fungi. This information is may be important to environment restoration projects in managing degraded poor-structured soils.

The results of this field experiment showed that there was no significant effect of mycorrhizae or the soil extract on SOC in the willow rhizosphere. However, our greenhouse study showed that the SOC was significantly affected when willows were co-inoculated with AMF and EMF (Chapter 3). The difference in the findings from the two studies is not clear, however, previous reports by Treseder and Allen (2000) and Zhu and Miller (2003) indicate that AMF hyphae can translocate C away from the root zone. In both cases we sampled soil from the root zone, however in greenhouse pots were root bound, therefore there would be no opportunity for carbon translocation outside of the root zone. This could explain some of differences we see in the two studies.

A high level of AMF abundance was observed in the field soil. Further, the abundance of AMF was increased in the willow rhizosphere, when plants were co-inoculated with AMF and EMF. This result agrees with the finding from our greenhouse study which showed increased AMF abundance in willow rhizosphere due to co-inoculation of plants with AMF and EMF (Chapter 2). Other authors have previously shown that AMF can alter the rhizosphere microbial community structure (Mummey et al., 2009).AMF has also been shown to alter root exudates quantitatively and qualitatively (Pinior et al., 1999; Scervinoet et al., 2005; Vierheilig et al., 2000), which in turn affects soil microbial communities. Srivastava et al. (2007) suggested any microbial inoculant including AMF can alter the rhizosphere microbial communities by changing the rhizospheric conditions. However, no information is available on co-inoculation of plants with both of the commercial mycorrhizal inoculants (AMF and EMF) related to the rhizosphere microbial community composition under field conditions.

In addition, diversity of AMF in the willow rhizosphere was also altered with both inoculant treatments. Further, AMF communities were quite different between mycorrhizal and soil extract treatments, though both inoculants significantly influenced the AMF diversity. Since diversity of AMF influences plant diversity and productivity (O' Connor et al., 2002; Van der Heijdenet al., 2006, 1998a, b), it is important to consider this change in AMF diversity when inoculating plants with mycorrhizal inoculants.

The AMF diversity associated with different aggregate sizes varied in the willow rhizosphere, of both un inoculated and inoculated plants as observed in the greenhouse experiment (Chapter 2). As shown in the greenhouse experiment (Chapter 2), the spatial heterogeneity of indigenous AMF communities among different sized soil aggregates

under field conditions also changed when willow plants were inoculated with both the inoculants. Interestingly, co-inoculation with AMF and EMF reduced the spatial heterogeneity in AMF diversity among aggregate size classes. With the soil extract inoculation, this spatial heterogeneity was further reduced, and AMF communities became more similar among different sized soil aggregates. Further, ecological indices showed that diversity of AMF communities associated with soil aggregate size classes were increased due to both the inoculations. This increased diversity could be one reason for reducing the aggregate-scale spatial heterogeneity of AMF communities. In this field experiment we showed that an aggregate-scale spatial heterogeneity exists under field conditions and AMF communities operate at aggregate-scale. To understand the ecosystem functions mediated by microbes and their controlling factors requires the analysis of soil microbes at the scale where they actually operate (Mummey et al., 2006) Therefore, our results directs the exploration of AMF communities at the aggregate scale to understand the ecological functions mediated by AMF and their controlling factors in details.

The diversity of EMF found in the bulk rhizosphere of willows in our experiment was very low. There were few bands visible, however, the diversity was different in the in the rhizosphere of both of the inoculations (mycorrhizae and soil extract). There was no spatial heterogeneity of EMF communities among different soil aggregate sizes, which agrees with results obtained in the greenhouse study (Chapter 2). EMF associated with soil aggregates in willow rhizosphere was also very low. However, factor analysis showed that EMF communities associated with the aggregates were altered by inoculating with the soil extract, but not with the mycorrhizal inoculation.

Phylogenetic analysis of the sequences obtained in our study indicated that all the sequences found in our study belonged to the families *Glomeramyceae* and *Claroideoglomeraceae*. Our inoculant strain *Rhizophagus intraradices* was detected in the DGGE profiles of most of the soil samples, and that band was more prominent in aggregate sizes that are less than 4 mm in mycorrhizal treatment and in bulk rhizosphere soil of mycorrhizal treatment. Importantly, AMF sequences obtained from our field soil grouped with several known species of *Glomeraceae* and *Claroideoglomeraceae* and some uncultured *Glomus* species, which suggests a diverse AMF community present in our soil.

On the other hand, sequences of EMF obtained from the experiment showed very low diversity of EMF associated with our willow plants. All the sequences belonged to two different species of *Hebeloma.* We were able to find our EMF inoculant (*Hebeloma cylindrosporum*) in most of the soil samples.

4.5. CONCLUSIONS

This study has shown that co-inoculation of willows with AMF and EMF can increase willow plant biomass and soil aggregation in the willow rhizosphere under field conditions. Mycorrhizal inoculation also increased the diversity of native AMF and EMF communities in the bulk rhizosphere soil and diversity of AMF communities in soil aggregates of willow rhizosphere. Further, we showed that the existence of spatial variability in AMF communities among different sized soil aggregates and that spatial variability could be reduced by inoculating with mycorrhizae and soil extract. This change in mycorrhizal communities should be taken into consideration when plants are inoculated with mycorrhizae, as mycorrhizal diversity affects the plant growth, diversity, and other ecological functions.

However, we could not find a spatial heterogeneity among EMF communities associated with soil aggregates in willow rhizosphere. Soil extract from Super Block is comparable to the commercial mycorrhizae with respect to the willow plant growth promotion, supporting our original hypothesis. Increased plant growth and change in diversity of AMF, when inoculated with unsterile soil extract, suggests that the plant growth promotion factor in Super Block could be microbiological.

In addition, we have shown that the rhizosphere of young willows grown in the field conditions has a fairly high level of AMF communities and a very low level of EMF communities.

We have made these conclusions based on results from data obtained one year after establishment of a field experiment carried out in one location. Additional research should be carried out over several growing seasons and carried out at multiple locations.

Further studies should also be carried out to identify what microbiological agents are responsible for the superior plant growth in Super Block.

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CHAPTER 5: SUMMARY OF CONCLUSIONS AND RECOMMENDATIONS

Willow is an economically important tree which benefits from a dual mycorrhizal symbiosis. Therefore, the inoculation of willow cuttings with commercial mycorrhizal inoculants has been suggested as a sustainable practice. However, the effect of inoculating willows with exotic, commercially available mycorrhizal species, is not well documented. Mycorrhizae have the potential to increase plant growth and benefit soil health, through increased soil aggregation, and soil organic carbon, however, their influence in the willow rhizosphere is not known. Our results show that soil aggregation, SOC, AMF communities in willow rhizosphere and willow growth can be changed by commercial mycorrhizal inoculation. However, it also showed that the impacts of mycorrhizal inoculation varies with soil properties, type of mycorrhizal inoculant, and plant species. This information is very important when inoculating willows or other plant species and can influence soil health and plant growth.

This was the first documentation of aggregate-scale spatial heterogeneity in indigenous AMF communities. The diversity and abundance of the AMF community varied with aggregate size class, soil type and was reduced by inoculating with AMF. Through phylogenetic analysis we observed a complex diverse AMF community dominated by *Glomus* spp. Interestingly, our commercial mycorrhizal inoculant *Rhizophagus intraradices* could be detected in all aggregates less than 4 mm in diameter. In contrast, the indigenous EMF communities associated with soil aggregates in a soil type were uniform and were not altered by mycorrhizal inoculation.

Arbuscular mycorrizal inoculation was found to influence soil health parameters, including, increasing the percent water-stable aggregates and soil organic carbon in most soils, however, this was dependent on the type of soil and soil properties, mainly clay and organic matter content. Interestingly, these parameters were higher when willows were inoculated with AMF alone than when inoculated with a combination of AMF and EMF. This implies the occurrence of some interactions between different mycorrhizal inoculants when co-inoculating, and highlights the complex interactions that occur within the rhizosphere micro biota.

Findings from our initial 29 week greenhouse experiment were confirmed in the field. The existence of aggregate-scale spatial heterogeneity in the AMF communities was observed under field conditions, but no variability was observed in the EMF communities. This spatial heterogeneity of AMF could be reduced by co-inoculating willows with AMF and EMF. The willow plant growth and percent water-stable aggregates in the willow rhizosphere also significantly increased in response to co-inoculation of willow with AMF and EMF under field conditions within a year, whereas no significant change occurred in the SOC.

Interestingly, unsterile Super Block soil extract was shown to be comparable to the commercial mycorrhiza with respect to the willow plant growth promotion and reduction of spatial heterogeneity of the indigenous AMF communities among aggregate size classes in willow rhizosphere.

Based on the findings of this research project, several recommendations can be made for further studies to broaden the knowledge gained from the present study. These include:

1. An experiment should be designed to study more closely the aggregate-scale spatial heterogeneity of mycorrhizal fungi using different sizes of micro and macro soil aggregates and different types of commercial inoculants in different environments.

2. The resulting lower %WSA and SOC when inoculated with a combination of AMF+EMF compared to inoculation with AMF alone in the present study may be either (1) due to the interactions between two organisms, other rhizosphere microbes, or other soil properties, or (2) host specific. Further studies are recommended to investigate whether this is common to all the species of mycorrhizae and to find the causes of this reduction by exploring morphology and physiology of mycorrhizal species.

3. The significant increase in soil organic carbon in response to AMF inoculation is very interesting. SOC has the potential to mitigate the problem of elevating atmospheric CO_2 levels. Therefore, SOC should be studied intensively to explore ways of managing atmospheric CO_2 levels through inoculating soil or planting materials with AMF.

4. Further studies are recommended for characterization of Super Block soil extract to find what kind of microbiological factors are associated with plant growth promotion.