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The effect of mycorrhizal fungi associated with willows growing on marginal agricultural land

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Résumé

Cette thèse porte sur une expérience réalisée sur des terres agricoles d'une superficie totale d'environ 1,4 hectare, durant trois saisons de croissance, de 2010 à 2012. Les performances de *Salix miyabeana* SX61 et SX64, deux cultivars de saule hautement productifs en biomasse, ont été testées dans trois types de sols très différents mais tous marginaux pour l'agriculture. Pour chacun des trois sites, la culture des deux cultivars de saule a été menée avec ou sans fertilisation à l'azote (75 kg/ha) et avec ou sans inoculation mixte par des champignons symbiotiques de types mycorhizien arbusculaire (AM) *Rhizoglomus irregulare* et ectomycorhizien (EM) *Hebeloma longicaudum*. Cette approche est différente de la plupart des expériences d'inoculation par mycorhizes portant sur des arbres ou même sur la plupart des légumes, car les boutures de saule sont plantées directement dans le sol plutôt qu'en transplantant de jeunes plants cultivés en sol stérile, en serre ou en pépinière. De cette manière, tous les plants de saule (contrôle et traitement) ont été mis en contact avec la microflore du sol dès leur premier stade de développement. L'inoculum ajouté aux plants traités s'est donc retrouvé en compétition directe avec les microorganismes indigènes déjà présents dans le sol, ce qui reflète plus fidèlement les pratiques agricoles courantes et augmente de la même façon la pertinence et d'application pratique des résultats. De plus, ces résultats ne feront pas l'objet d'un biais caché qui pourrait influencer les expériences d'inoculation dont les plants de contrôle poussent dans des sols complètement stériles pendant leur période de croissance initiale. Bien que potentiellement d'un rôle important, les organismes indigènes existant dans les sols marginaux pour l'agriculture sont relativement sous-étudiés. Afin d'apporter un éclairage sur la question, l'étude présentée dans cette thèse porte en grande partie sur les communautés mycorhiziennes natives du sol. Celles-ci ont été identifiées par clonage et séquençage. Ainsi, l'ADN de 36 échantillons de racines et d'extraits de rhizosphère, récoltés en fin de première saison de croissance (2010), a été extrait, amplifié par PCR puis cloné et séquencé, en utilisant le gène universel de champignon ITS et le gène 18S spécifique au type AM. Un séquençage plus exhaustif a été effectué, par Illumina MiSeq, sur l'ADN extrait de 96 échantillons de rhizosphère prélevés à la fin de la saison 2011, en ciblant le gène ITS. Ceci nous a permis de dresser un profil des principales souches

mycorhiziennes présentes sur nos divers sites. Nous avons observé une hausse de croissance marquée suite à la fertilisation à l'azote, ainsi qu'une hausse substantielle de croissance initiale pour le champ humide et plus sablonneux, bien que ces résultats soient limités à une observation qualitative étant donné que l'expérience n'était pas élaborée pour tester directement l'effect de la fertilisation. De plus, et contrairement aux études publiées sur l'inoculation par des mycorhizes en milieu stérile, partiellement stérile ou en serre, notre expérience d'inoculation en champ non stérile n'a procuré aux boutures de saule aucun bénéfice en termes de croissance. Ceci suggère une certaine dominance des souches mycorhiziennes natives du sol, qu'il importe dès lors d'investiguer, de même que leur grande diversité révélée dans nos résultats de séquençage. Nos résultats suggèrent également une forte spécificité des mycorhizes natives pour les types de sol rencontrés, qui nécessite d'être mieux comprise, même si de nombreuses souches identifiées sont probablement des mycorhizes présentes sur les deux cultivars.

Mots-clés : champignons mycorhiziens, saules, inoculation, expérience sur terrain agricole, rhizosphère, champignons indigènes

Abstract

This thesis involved a farm-scale field experiment that ran for three growing seasons from 2010 to 2012, and covered almost 1.4 hectares in total. The experiment used two cultivars of shrub willows grown for biomass, *Salix miyabeana* Seeman SX61 and SX64, on three very different marginal fields, with and without a nitrogen fertilization treatment of 75 kg/ha, and with and without a mixed symbiotic fungal inoculation treatment of the arbuscular mycorrhizal (AM) species *Rhizoglomus irregulare* and the ectomycorrhizal (EM) species *Hebeloma longicaudum*. This experiment was unique from most mycorrhizal inoculation experiments with trees or even most vegetables, because willow cuttings are planted directly into farm soil (instead of started as seedlings in a nursery or greenhouse). Both the control willows and treated willows interacted from the beginning with intact, unsterilized soil. The inoculum had to compete throughout with the native microorganisms already present, providing a robust proxy for agricultural systems planting with seed and increasing the practical relevance and applicability of these results. The results will also not contain a hidden bias that we believe could affect inoculation experiments whose control plants grow in completely sterile soil during their initial growth period. The fact that our experiment includes the imperfectly understood wild organisms that exist in unsterile farm soil, explains why a large part of this thesis is also an exploration of the native mycorrhizal community. PCR-cloning-sequencing targeting the universal fungal Internal transcribed spacer (ITS) as well as an AM specific 18S fragment, was used on DNA extracted from 36 root and rhizospheric soil samples obtained at the end of the first growing season in 2010. Then more comprehensive Illumina MiSeq sequencing targeting the fungal ITS was used on DNA extracted from 96 rhizospheric soil samples obtained at the end of the second growing season in 2011. We did observe a marked increase in growth with nitrogen fertilization on marginal land, as well as a substantial increase in initial growth correlated with a higher-moisture, higher-sand-percentage-soil field, though these findings are limited to qualitative observation as the experiment was not designed to test them specifically. Notably, this experiment conducted in unsterilized farm soil showed no growth benefit from inoculating with mycorrhizal fungi, in direct contrast with lab and greenhouse results obtained in the past. Furthermore, the inoculum

did not appear to compete successfully with numerous indigenous fungi. Interestingly, the majority of the indigenous fungi present have never been cultured or studied. Also, even though many of the fungi are likely mycorrhizal and all associated with cuttings of the same two willow cultivars, the fungal community in one of the test fields is very different from those fungi in the other two fields. This difference could correlate to a marked difference in soil type.

Keywords: mycorrhizal fungi, willow, inoculation, agricultural field experiment, rhizospheric soil, indigenous fungi

Table of contents

Résumé ... i

Abstract ... iii

Table of contents ... v

List of tables ... viii

List of figures ... x

List of acronyms ... xii

List of abbreviations ... xiii

Acknowledgements ... xv

Chapter I: Introduction and review ... 1

I.1. Willow as a potential crop for Quebec's marginal lands ... 1
I.2. Current knowledge of willows and mycorrhizal fungi ... 2
I.3. Theoretical framework for the thesis ... 5
I.4. Central questions ... 7
I.5. Objectives and hypotheses ... 8

Chapter II: Investigating the effect of a mixed mycorrhizal inoculum on the productivity of biomass plantation willows grown on marginal farm land ... 12

Abstract ... 12
II.1. Introduction ... 13
II.2. Materials and methods ... 16
II.2.1. Experimental design ... 16
II.2.2. Sampling and measurements ... 18
II.2.3. Molecular fungal community analysis ... 19
II.2.4. Statistical analysis ... 21
II.3. Results ... 22
II.3.1. Sequence library results ... 23
II.4. Discussion ... 24

II.5. Bibliography 26
Supplementary material to Chapter II 41
SII.1: Additional diameter analyses 41
SII.2: Additional height analyses 46
SII.3: Additional mass analyses 53
Chapter III: Exploration of willow-associated fungal communities in short-rotation coppice fields 58
Abstract 58
III.1. Introduction 59
III.2. Materials and methods 60
III.2.1. Experimental design 60
III.2.2. Harvest of soil and plant materials 60
III.2.3. Soil DNA extraction, ITS amplification, and Illumina MiSeq sequencing 61
III.2.4. Sequence processing 61
III.2.5. Graphical and statistical analysis 62
III.3. Results 63
III.4. Discussion 65
III.5. Bibliography 68
Supplementary material to Chapter III 77
SIII.1: Additional tables and figures 77
IV. Conclusion 83
IV.1. Findings from field experiments 83
IV.2. Hypotheses revisited 84
IV.3. Discussion 85
IV.4. New ideas, and support from the scientific literature 86
IV.5. Summary 88
Bibliography 89
Appendix A1: Field experiment timeline i
Appendix A2: Experimental plan ii

Appendix A3: Selected pictures iv
Appendix A4: Moisture probe data vi
Appendix A5: Root staining and microscopy vii
Appendix A6 Mortality charts ix
Appendix A7: Primer investigation xi
Appendix A8: PCR-cloning-sequencing results xiii
Appendix A9: Phylogenetic trees xvii

List of tables

Table I.I: Examples of native mycorrhizal fungi found in willow roots, by habitat................ 11
Table II.I: Characterization of the three field sites, including a soil analysis at two depths 32
Table II.II: 2011 Stem basal area /ha ANOVA results (LOG transformed)............................ 34
Table II.III: 2011 Stem basal area per hectare (m^2/ha) ANOVA predicted values and test results 35
Table II.IV: Arbuscular mycorrhizal sequences found in rhizosphere samples from biomass plantation willows, by inoculation treatment.. 39
Table II.V: Ectomycorrhizal sequences (and sequences of other fungi) found in rinsed root samples from biomass plantation willows, by inoculation treatment...................................... 40
Table SII.1.I: 2010 Stem basal area ANOVA results (LOG transformed).............................. 41
Table SII.1.II: 2010 Stem basal area per hectare (m^2/ha) ANOVA predicted values and test results 42
Table SII.1.III: 2012 Stem basal area ANOVA results (LOG transformed) 43
Table SII.1.IV: 2012 Stem basal area per hectare (m^2/ha) ANOVA predicted values and test results 44
Table SII.2.I: 2010 Height ANOVA results (LOG transformed) 46
Table SII.2.II: 2010 Height (cm) ANOVA predicted values and test results 47
Table SII.2.III: 2011 Height ANOVA results (LOG transformed) 48
Table SII.2.IV: 2011 Height (cm) ANOVA predicted values and test results 49
Table SII.2.V: 2012 Height ANOVA results (LOG transformed)... 51
Table SII.2.VI: 2012 Height cm ANOVA predicted values and test results 52
Table SII.3.I: 2011 Oven dry tons /ha ANOVA results (LOG transformed) 53
Table SII.3.II: 2011 Oven dry tons /ha ANOVA predicted values and test results................. 54
Table SII.3.III: 2012 Oven dry tons /ha ANOVA results (LOG transformed)........................ 56
Table SII.3.IV: 2012 Oven dry tons /ha ANOVA predicted values and test results 57
Table III.I: Characterization of the three field sites, including a soil analysis at two depths... 71
Table III.II: Identity of main fungal OTUs (comprising ~94% of the community) using the ITS gene, found in willow rhizosphere samples 72

Table III.III: Identity of dominant fungal OTUs recognized with species names, using the ITS gene, found in willow rhizosphere samples. 73
Table SIII.1.I: Identity of fungal OTUs recognized with species names, using the ITS gene with MiSeq sequencing, found in willow rhizosphere samples... 77
Table SIII.1.II: Identity of fungal OTUs comprising ~97% of the community, using the ITS gene with MiSeq sequencing, found in willow rhizosphere samples 79
Table A5.I: Moisture probe data 2010 (in fields at least three months at 30 cm depth)............ vi
Table A8.I: Arbuscular mycorrhizal (AM) species found in rhizospheric soil (and one root sample from a same treatment) of *Salix* grown for biofuel xiii
Table A8.II: Fungal species found in the roots of short-rotation coppice *Salix* Fall 2010 (planted early Summer 2010), ITS PCR-cloning-sequencing xv

List of figures

Figure II.1: Cumulative growth of *Salix miyabeana* (SX61 and SX64) during establishment on marginal land, treated with mycorrhizal inoculum 33
Figure II.2: Cumulative growth of *Salix miyabeana* (SX61 and SX64) during establishment on marginal land, in three different fields. Stem basal area predicted values (untransformed) and test results (LOG transformed) by field for all three years 36
Figure II.3: Cumulative growth of *Salix miyabeana* (SX61 and SX64) during establishment on marginal land. Stem basal area predicted values (untransformed) and ANOVA results (LOG transformed) for the year a fertilization treatment was applied (2011), as well as the year after (past growth, plus any residual nitrogen in the soil) 37
Figure II.4: Cumulative growth of *Salix miyabeana* (SX61 and SX64) during establishment on marginal land. Oven dry tons per ha predicted values (untransformed) by field for 2011 38
Figure III.1: ITS, rhizospheric soil, 2011, ~96 willows, showing proportion of sequences in each Order (and Phylum), all fungal sequences normalized by sample and averaged by field 74
Figure III.2: Principal coordinate analysis by field 75
Figure III.3: Principal coordinate analysis by fertilization treatment, ITS gene with MiSeq sequencing, rhizospheric soil, 2011 76
Figure SIII.1.1: ITS gene with MiSeq sequencing, rhizospheric soil, 2011, ~96 willows, 50 OTUs that made up the bulk of the sequence reads 80
Figure SIII.1.2: Principal coordinate analysis by inoculation treatment 81
Figure SIII.1.3: Principal coordinate analysis by willow cultivar, ITS gene with MiSeq sequencing, rhizospheric soil, 2011 82
Figure A1.1: Field Experiment Timeline i
Figure A2.1: Experimental design (rocky field shown, used as example) iii
Figure A6.1: Willow Mortality ix
Figure A6.2: Number living on average per block-row x
Figure A7.1: *Glomus vesiculiferum* 18S rRNA gene, isolate Att14-8, clone pWD193-2-3 (Schüßler group) xii

Figure A8.1: 18S for AM PCR-cloning-sequencing, 2010, rhizospheric soil (and root for Sandy) xiv
Figure A8.2: ITS PCR-cloning-sequencing, rinsed roots, 2010 xvi
Figure A9.1: Arbuscular mycorrhiza maximum likelihood tree created using Mega5 xvii
Figure A9.2: Total root fungi maximum likelihood tree created using Mega5 xxi

List of acronyms

AM	arbuscular mycorrhiza
ANOVA	analysis of variance
BMP	Brazilian Microbiome Project
DNA	deoxyribonucleic acid
EM	ectomycorrhiza
ITS	internal transcribed spacer
NCBI	National Center for Biotechnology Information
OTU	operational taxonomic unit
PCoA	principal coordinates analysis
PCR	polymerase chain reaction
SRC	short-rotation coppiced
TBI	tree-based intercropping
TRFLP	terminal restriction fragment length polymorphis

List of abbreviations

°C	degrees Celsius
cm	centimeter
etc.	*et cetera* (and so on)
et al.	*et alia* (and company)
ha	hectare
kg/ha	kilogram per hectare
km	kilometer
LOG	base 10 logarithm
m	meter
min	minute
mL	milliliter
N	nitrogen
ODT/ha	oven dry tons per hectare
ON	Ontario
P	phosphorus
s	second
SBA/ha	stem basal area (m^2) per hectare
µL	microliter

I dedicate this work to the world my children will grow up in, one more piece of knowledge to add to the rest we have gained. And to my wife.

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Chapter I: Introduction and review

I.1. Willow as a potential crop for Quebec's marginal lands

Wild willows often grow in wet or periodically flooded habitats (Newsholme 2003). They are hardy, though, and some species within the genus can be found in dryer alpine environments or seemingly sterile sand and gravel beds (Newsholme 2003). Perhaps what characterize most willow habitats are conditions that are either harsh enough or transient enough to allow this otherwise early-succession plant to dominate (Fralish and Franklin 2002).

Short rotation coppicing (SRC) is the cultivation of regenerative, fast-growing trees such as shrub willow and poplar, and harvesting them by cutting them down to the ground every few years (Shield *et al.* 2015). The trees regrow from their stumps, and can be harvested dozens of times over multiple decades before a field needs to be replanted (Shield *et al*. 2015). Coppice agriculture is not new. It was practiced for thousands of years, but was made largely obsolete with the industrial extraction of coal and later oil (Shield *et al.* 2015). Modern agriculture has begun to re-embrace the strategy, as a potential source for biofuel amid increasing environmental concerns with fossil fuels (Shield *et al.* 2015).

Already cleared, marginal lands are available in Canada and Europe for willow growing, due to their early history of intense farming before prairie settlement and modernization (Labrecque and Teodorescu 2005, González-García *et al.* 2012). Willow is also easier for farmers as a flexible alternative crop since the smaller stems and roots allow changing back to other crops afterwards, or if prices change, with plowing and land preparation using tractors (instead of bulldozers as for larger trees) (Labrecque and Teodorescu 2005, González-García *et al.* 2012). Moreover, the economics of annual or semi-annual willow harvesting is closer to traditional crops, rather than the financing needed to wait a decade or two for larger trees to grow (Labrecque and Teodorescu 2005, González-García *et al.* 2012).

Arbuscular mycorrhiza (AM) are a commonly overlooked fungal group, because they inhabit the soil around plant roots and most of their structures are microscopic (they do not form mushrooms above ground) (Simon *et al.* 1993, Schüßler *et al.* 2001). Incredibly

important, however, AM are considered integral ecosystem components, contributing to the nutrient acquisition of a majority of plant species, and thus play a role in driving plant community structure, productivity, and diversity (van der Heijden *et al.* 1998, 2008). Ectomycorrhiza (EM) are associated with a much smaller percentage of plant species, but many of those plants are tree types that dominate the world's forested areas (Tedersoo *et al.* 2010). Physically linked to, and therefore extending the reach of plant root networks, both AM and EM fungal hyphae supply water and mineral nutrients to plants in exchange for sugars (Johnson 2009, Tedersoo *et al.* 2010).

This thesis investigates if growing SRC shrub willows on marginal agricultural land could be augmented with the inoculation of mycorrhiza, both AM and EM.

I.2. Current knowledge of willows and mycorrhizal fungi

Unlike most cereal crops, which associate with only AM fungi, willow trees have been shown to form relationships with both AM and EM fungi (van der Heijden 2001). This was borne out by the field explorations listed in Table I.I, such as those of Hashimoto and Higuchi (2003) and Becerra *et al.* (2009). Both research efforts found AM and EM fungi associated with willows in the wild. However, both investigated riparian ecosystems, and while AM were found, EM fungi dominated both in colonization and in number of species present. In fact, as shown in Table I.I, Paradi and Baar (2006) found only EM fungi in their investigation of older riparian willow stands.

Milne *et al.* (2006) and Ryberg *et al.* (2011) investigated dryer sub alpine willow stands. Milne *et al.* (2006) did find both AM and EM colonization, but AM colonization was low and they only quantified and identified EM fungi. Ryberg *et al.* (2011) did find significant EM colonization but did not test for AM. Puettsepp *et al.* (2004) investigated naturally colonized SRC willows, and also found significant EM colonization without testing for AM.

It appears from these studies that the genus *Salix* has a greater affinity with EM fungi, and there are even those who point to wild willows being colonized by EM fungi to the point of seeming to exclude AM fungi (Becklin *et al.* 2012). However, I am very cautious to accept this. Out of the six field explorations we found two did not even test for AM fungi. The others

did not use molecular techniques to identify AM community members, simply stating that colonization percentages were low. It has been shown that colonization level does not necessarily correlate with the level of benefit a plant may receive from its mycorrhizal symbiont. Furthermore, AM fungi, with their ability to retreat into a spore bank within the soil while waiting for an optimum time of year or environmental conditions, would be more likely to be underestimated or under sampled in root surveys. We must know more about what variables influence mycorrhizal type and the benefits they confer to willows.

These selected studies point to variables such as soil manipulation, length of stand establishment and level and type of fertilization having an effect on mycorrhizal species and their benefit to the willows. We looked for more such findings, specifically in mycorrhizal inoculation experiments involving willows.

Several studies were found that began investigating the effects of mycorrhizal inoculum on *Salix*, as well as other closely related biomass crops such as *Populus* species. *G. intraradices* inoculation was shown to increase phosphorus uptake in *Salix* through increased growth (Fillion *et al.* 2011). However, in another study inoculation did not change biomass production of a *Salix* cultivar or one of *Populus* clone (Bissonnette *et al.* 2010).

Interestingly, in an investigation of six different EM including *H. longicaudum* plus the AM, *G. intraradices*, and two bacteria for inoculating *Populus* species in the nursery, only a combination of the EM fungus *Paxillus involutus* plus the bacteria *Burkholderia cepacia* boosted both P and N uptake (and markedly increased biomass). Others, including *H. longicaudum* did boost N uptake and biomass, but at levels that were statistically significant but not very biologically noteworthy (Quoreshi and Khasa 2008).

These few inoculation experiments with willows or related poplar species are inconclusive but do seem to point to the benefit of a diverse inoculum. Because of the low number of inoculation experiments using willows, we must look at mycorrhizal inoculation experiments using other plant species.

Studying plant productivity and diversity responses to inoculation, the particular species and even strain of mycorrhizal fungi is found to have a big effect on the level of benefits seen or not (Vogelsang *et al.* 2006). Even more relevant to our goals, when several

different nursery inoculations in conifer and hardwood tree seedlings were examined after three or five year outplanting field trials, many inoculated fungal species were outcompeted by wild species. However, some combinations did provide statistically measurable growth and survival effects (Quoreshi *et al*. 2008). A clearer positive effect was seen in a large study of farm trials using a commercial AM inoculant for growing potatoes (Hijri 2016), showing that it is important to consider a wider representation of the conditions found in the field before concluding on the effect of inoculation in one crop.

An extensive meta-analysis of existing literature (combining 1994 studies from 183 papers; Hoeksema *et al*. 2010) looked in detail at mycorrhizal inoculum effects. The authors found that the host plant functional group and N-fertilization were more important for determining an inoculum's effect than its identity or even possibly competition in the field. Because willow's functional group, non-N-fixing woody plants, was shown to benefit from inoculum, we would predict a strong effect from inoculation even if there were confounding phosphorus variations. Hoeksema *et al*.'s (2010) results would also predict that we see more of an inoculum benefit in our non-N-fertilized treatment groups.

Even including other plant families than willow, inoculation experiments can be difficult to interpret. The complex interactions that appear to be involved in functioning plant-mycorrhizal ecologies, and apparently those that need to be better understood to create a useful inoculum, are only beginning to be figured out.

Use of 18S-targeting TRFLP and PCR-cloning-sequencing approaches to explore AM diversity in *Populus* tree-based intercropping (TBI), demonstrated that there is indeed quite a complex community even in agricultural settings (Chifflot *et al*. 2008).

Moebius-Clune *et al*. (2013) looked at changes in AM phylogeny across New York State cornfields, and their findings were striking. They found one of the most influential variables determining AM phylogenetic distance was soil texture (Moebius-Clune *et al*. 2013). Magnesium, organic matter, potassium and other soil variables had an effect (to a lesser extent), and they posited that soil texture acted so strongly through its influence on moisture availability (Moebius-Clune *et al*. 2013).

It is logical to suppose that those families or genera of mycorrhizal fungi more prevalent when moisture or another variable is limiting are better adapted to those conditions and therefore better able to help their host under such limitation. While we generally cannot directly measure the physical and metabolic traits of all the mycorrhizal fungi we find, or even yet correlate functional genes to those traits, phylogenetic distance should serve as a proxy. The type of substrates broken down, the soil chemistry preferred, in short, the part they play in an ecosystem is being determined in bacteria on a phylogenetic basis already (Fierer *et al*. 2007). It is consistent with evolutionary theory that organisms closely related to one another are more likely to share similar physical and metabolic traits. Complex chemical pathways, the suites of proteins tied to an adaptation, cell wall components and exudates, all can be supposed to be resilient to evolutionary change. Therefore, although unique contrary species can certainly exist, within a class or order of bacteria we can begin to predict general characteristics and even likely sample site environmental parameters (Fierer *et al*. 2009).

This introduction began with background about willows and mycorrhizal organisms, and then linked research that informs our efforts to use mycorrhizal inoculation in SRC willow agriculture. It should be apparent that any inoculation attempts we design or conduct are very much at the forefront of such technology and are highly experimental by nature. No established inoculum species have been developed for the production of willows and there would be a good chance native mycorrhizal fungi would dominate over inoculated strains. However, this would provide an opportunity for fundamental ecological exploration of the soil. By identifying mycorrhizal fungi in as wide a phylogenetic sweep as possible, such an experiment would directly continue the type of research currently limited to bacteria, and by focusing on mycorrhizal fungi perhaps uniquely complement such work.

I.3. Theoretical framework for the thesis

Our work here stems from the possibility that issues of fertility and water stress that arise with farming on marginal land can be addressed or mitigated with the inoculation of mycorrhizal fungi. One of the main benefits conferred by mycorrhizal symbiosis is improved acquisition of water resources, the very resource that has been shown to be the primary limiting factor in growing willows on marginal, especially sandy, land (Aronson and Perttu

2001). Also, willows are a perennial crop, not needing replanting for two or three decades. Inoculation could therefore only be needed once at planting.

Because willows are relatively water-hungry plants, most willow farmers do not try to grow them in areas that need extra irrigation (Labrecque and Teodorescu 2001; Guidi *et al*. 2013). Effectively, therefore, the most important reoccurring cost willow farmers must budget for is fertilization. Since excess nitrogen (N) can eventually return to the atmosphere as gas, but not phosphorous (P) or potassium (K), most agricultural and formerly agricultural soils have excesses of P and K (Caslin et al 2010). The largest input required the first few coppicing cycles is therefore N (Caslin et al 2010). Mycorrhizal fungi should be key to willows better accessing nutrients that are provided to them, as well as those added by farmers in the past and that have built up in forms difficult for plants to access.

To develop this idea, understanding the influence of wild mycorrhizal fungi found in natural stands of willows (and some SRC stands as well) is critical. Recent developments in soil microbiology offer new tools to exploit for our research. Because most of the structures that make up mycorrhizal fungi are microscopic, their study has had a renaissance with the development of DNA identification techniques. This type of data gives our experiment, which was designed and driven by practical hopes for improving willow agriculture (with some lessons available for agriculture and silviculture in general), a larger tie-in to fundamental questions of ecology and basic soil science.

There is a substantial discrepancy between what we know of the relatively small number of bacteria and fungi that are cultivable and the vast lists of organisms modern molecular tools turn up. In most environments, these lists of organisms present us with a picture more of a formless mass than of an ecosystem, and researchers are only just beginning to tease out taxonomic generalizations (Fierer *et al*. 2007). It follows then we often do not know which organisms depend on one another within complex chemical ‘disassembly lines’ as decomposers, or which are predators or parasites of the decomposers. We usually do not know which are generalists, or which are part of the ecology associated with specific above ground ecosystems, soil types, or even particular plant species. Finally, the very definition of a species or a strain of organisms is called into question, when lateral transfer of genes can

hypothetically change the metabolism or lifestyle of a line of microbes in a single generation (Richards *et al*. 2011, Croll *et al*. 2009).

I.4. Central questions

All of these circumstances make the field of microbial ecology exciting, but can lead to the paradoxical situation where an environmental microbial community analysis (especially of soil) becomes an uninspiring list of accession numbers without significance in the short term. The beauty of focusing on mycorrhizal organisms, however, is that their place in the ecosystem is known. The link to the classical above ground ecosystems is obvious. Four specific questions central to our work emerge.

1) Within the huge range of soil conditions willows can grow in, how much of the mycorrhizal community makeup is determined by the soil texture and chemistry? Hrynkewicz *et al*. (2012) found a difference in EM communities between agricultural willows and nearby native willows, but did not resolve whether those differences were from soil chemistry/texture linked to agricultural history or different willow species. With three different soil profiles in our fields, but the same two willow cultivars in each field (in fact cuttings of two biomass cultivars within the same species), we aim to examine influence of soil parameters in depth. Since our two willow cultivars are not as different as those in the Hrynkewicz *et al.* study, and as we are not testing undisturbed vs. agricultural soil *per se*, if we find similar differences in the community structure that would suggest the soil parameters were the most important factor.

2) Do the mycorrhizal communities change after the first year as the willows transition from seedling/cutting development to established tree stands? By isolating fungi from environmental SRC willow samples, Corredor *et al*. (2012) see a change from a first planting year to the next. Corredor *et al*. (2012) even noted a shift from pathogenic fungi to possible beneficial fungi, but their techniques could not identify obligate mycorrhizal fungi such as AM. Would we find a similar pattern using both EM and AM? We can identify both AM, by 18S gene PCR-cloning-sequencing, as well as potential EM, by ITS gene PCR-cloning-sequencing. ITS PCR-cloning-sequencing could also identify pathogens, and if fewer are

present (or conversely, if more known EM or AM are present) our methods might demonstrate this.

3) Does nitrogen fertilization alter the recruited mycorrhizal community? Treseder (2004) found fewer AM caused by nitrogen fertilization, and Whiteside *et al*. (2012) showed both a lower AM diversity with nitrogen fertilization and began piecing together the relationships behind the trend. For the first time in a natural environment, Whiteside *et al*. (2012) directly observed AM transporting nitrogen to host plants. There had been more work by researchers linking phosphorus uptake to AM up to that point, though Toussaint *et al*. (2004) had shown this nitrogen link earlier. This presents a puzzle. We might predict a greater mycorrhizal colonization with nitrogen fertilization as the host plant becomes more phosphorus limited. Johnson (2009) proposes a host/fungi economy that would predict this. However, with Whiteside *et al*.'s (2012) new findings, we must also consider the possibility that mycorrhizal fungi involved in a more nitrogen dominated economy could be lost if we fertilized with nitrogen. Since we used nitrogen fertilization, this is another issue we can help resolve.

4) What would answers to any of these questions mean for a willow farmer that wishes to use mycorrhizal fungi? More academically what would answers mean for understanding ecosystems in general, their resilience in the face of human disturbance, and their potential for restoration or mimicry within agricultural settings?

I.5. Objectives and hypotheses

The overall objective of this project was to better inform agriculture through the lens of soil microbial ecology, focusing on mycorrhizal fungi. Our hypothesis was that mycorrhiza are vital to plant health, and that we would find improved agricultural methods through paying attention to this neglected part of the plant-soil system.

Specifically, with the experiment and data gathered in our work shown in chapter II, the objective was to use inoculation to improve the growth and survival of willows in agricultural fields, particularly fields of marginal agricultural productivity. Our hypothesis was that inoculation would aid in water and nutrient acquisition. We also hypothesized that

agricultural fields, with annual soil disturbance, unnatural fertilizer inputs, and lack of perennial rhizospheric habitats would be poor in native mycorrhizal species, therefore our control plots would show less growth. Furthermore, we believed that the plots in more marginal fields would show a more pronounced effect from inoculation, because the plants would be in greater need of help.

A subobjective in the work described in chapter II was to test the effect nitrogen fertilization would have on mycorrhizal inoculation. Since most SRC willow plantations are fertilized, we wanted to see if inoculation would have an effect under common agricultural conditions, but we also wanted to see if inoculation could lesson the need for fertilization. We suspected that inoculation could show a greater beneficial growth effect relative to uninoculated controls if the willows were unfertilized (particularly in marginal fields). We hypothesized that inoculated species would help mobilize nitrogen and pass it on to the willows, therefore encouraging growth over uninoculated willows which would be nutrient limited. In the unfertilized treatments, this effect would be the most evident, as the control willows would be the most nitrogen limited. An alternative hypothesis, however, was that if the willows were phosphorus limited, inoculated willows that were also fertilized with nitrogen would be able to better take advantage of the nitrogen (the mycorrhiza would help mobilize and provide phosphorus), and we would see the opposite of our first expectation. In this unusual case, inoculated willows in the nitrogen fertilized treatment plots would show the most benefit compared to controls.

Finally, chapter II describes the two different willow cultivars we used. The objective of this was to increase the robustness of any results by giving them wider applicability. The two cultivars would also give an insurance against unexpected disease or pest attack on the willows.

With the data gathered for chapter III, our objective was to improve the state of knowledge of field rhizospheric soil fungal diversity. We predicted soil sampled from our experimental fields would start with inoculated species dominating a low-diversity system, followed by gradual integration and competition of wild organisms from nearby weeds and farther forests. We also predicted that since we were focusing on rhizospheric fungi and all three fields would have clones of the same two willow cultivars, that similar species profiles

would be seen throughout. Perhaps inoculated fungi would dominate in the more marginal plots, because the willow shrubs would have greater need of mycorrhizal assistance. Conversely, the harsher conditions could gradually facilitate the takeover of the rhizosphere by better-adapted native mycorrhizal species.

A subobjective in chapter III was to observe the relative success of AM vs. EM species in colonizing the willow rhizospheres. We predicted both the AM and EM in our inoculum would do well in the willow rhizospheres of our experiment, as both are known to use willow hosts. We anticipated that it would be hard to differentiate between our inoculated AM and any indigenous field AM, due to similarities in genetic sequences. We expected the EM, with greater genetic diversity and lower likelihood of indigenous inhabitants, would be identifiable. Following the literature, we suspected that our AM would do particularly well in the first months or even the first year of growth, but that eventually our EM would have time to become established and be competitive within the rhizosphere. We hoped the different types of mycorrhizal species in our inoculum would complement each other, and both would give benefits to our willow plants.

Table I.I: Examples of native mycorrhizal fungi found in willow roots, by habitat

Willow species	**AM presence (root colonization %)**	**EM genus (species when available and space allows)**
Riparian		
Salix humboldtiana (Becerra *et al*. 2009)	Present (unidentified) (zero to 14 % of root length colonized)	*Inocybe* sp. (unidentified species) *Tomentella* sp. (plus 5 other unidentified morphotypes)
Salix sachalinensis (Hashimoto and Higuchi 2003)	Present (unidentified) (0 - 1 % of root length colonized)	(4 unidentified morphotypes)
Salix alba (Paradi and Baar 2006)	Not present	*Cortinarius sp.* *Hebeloma sp.* *Pezizales sp.*-3 unique *Thelephora sp.*-2 unique *Tuber sp.*-5 unique
Subalpine		
Salix lapponum (Milne *et al*. 2006)	Present (unidentified) (0 - 1 % of root length colonized)	*Hebeloma sp*. *Laccaria proxima* *Pezizales sp*. *Thelephora terrestris* *Thelephoraceae sp*.
Salix polaris and *Salix herbacea* (Ryberg *et al*. 2011)	Not determined	*Cenococcum geophilum* *Cortinarius sp*.-8 unique *Clavulina sp*. *Hebeloma sp*. *Inocybe egenula* *Laccaria sp*.-2 unique *Lactarius sp*.-2 unique *Russula sp*.-3 unique *Sebacina sp*.-2 unique *Sistotrema alboluteum* *Tomentella sp*.-10 unique
SRC fields		
Salix viminalis L. (Puettsepp *et al*. 2004)	Not determined	*Hebeloma helodes* *Laccaria sp*. *Phialophora finlandia* *Tomentella lilacinogrisea*
Salix dasyclados Wimm. (Puettsepp *et al*. 2004)	Not determined	*Cortinarius cedriolens* *Cortinarius saturninus* *Hymenoscyphus ericae* *Phialophora finlandia* *Tomentella. lilacinogrisea*

Note: AM, arbuscular mycorrhiza; EM, ectomycorrhiza; SRC, short-rotation coppiced. These examples are drawn from those papers that used both physical examination for identification and sampling, followed by molecular sequencing, to identify only those mycorrhizal fungi that are confirmed willow symbionts.

Chapter II: Investigating the effect of a mixed mycorrhizal inoculum on the productivity of biomass plantation willows grown on marginal farm land

(Extensive proof-reading and suggested edits were contributed by WG Nissim, but the work and writing were by TJ Pray. The framework for the project was proposed by M St-Arnaud and M Labrecque, in the initial proposal to the *Programme de soutien à l'innovation en agroalimentaire* of the *Ministère de l'agriculture, des pêcheries et de l'alimentation du Québec*, but final design and implementation were done by TJ Pray. As thesis advisors, M St-Arnaud and M Labrecque gave advice and input throughout the project, as well as suggested edits and proof-reading of the document, but the final product represents the contributions and decisions made by TJ Pray.)

Abstract

A large field experiment found no productivity difference between inoculated and uninoculated *Salix miyabeana* Seeman (SX61 and SX64), shrub willow cultivars grown for biomass. Productivity was measured using stem diameter (converted to stem basal area per hectare, or SBA/ha), height, and above ground mass (converted to oven dry tons per hectare, or ODT/ha). The inoculum species, *Rhizoglomus irregulare* and *Hebeloma longicaudum*, are likewise those most likely to be commercially available, and represent both arbuscular and ectomycorrhizal inoculum types. The negative result is one that should be of particular interest to farmers, as the experiment was conducted at farm scale with 21,600 willows over three growing seasons and with typical farm equipment. Also, the soil, like typical farm soil, was not sterile. The wild fungal organisms, present naturally, provided a truer control than a laboratory or greenhouse experiment could. The experiment used a hierarchical design with inoculation treatments randomized first, cultivars randomized second, and fertilization treatments randomized third, that was repeated across three fields (given the descriptive names in our experiment of Sandy, Rocky, and Dry). The experiment tested the inoculum's effect across three different marginal soil types, two different biomass willow cultivars (SX61 and

SX64), and with and without nitrogen fertilization. We did not observe a productivity difference between cultivars, but nitrogen fertilization of 75kg per hectare gave a predicted mean increase of 27% SBA/ha in 2011, using data from across all three fields. Sandy field showed a predicted mean increase of 55% SBA/ha over Rocky field in 2011. Complementary molecular data suggests a diversity of native fungi was and still is present in the field soils, which could have either outcompeted or masked the effect of the introduced species. Finally, though there are no obvious molecular signatures of the inoculum in this preliminary data, community profile differences between inoculated and uninoculated rhizosphere samples do suggest the inoculum survived long enough to have a community impact (but one that again, did not result in productivity differences during the experiment).

II.1. Introduction

Growing short-rotation coppiced (SRC) willows for energy purposes is particularly promising on agriculturally marginal land. Such land would neither come as a sacrifice to pristine, old-growth wilderness, nor would it take away from significant food production as much of this marginal land has gone or is going out of production within current competitive agricultural marketplaces (Liu *et al*. 2012). However, the main driver of efforts to develop biomass plantations, global warming concerns, can also fuel worries about their fertilizer use (Don *et al*. 2012). In general, SRC willows can be grown on many types of agricultural land (though wetter land is much better than dry) (Labrecque and Teodorescu 2001; Guidi *et al*. 2013). Due to high biomass yields, though, they remove nutrients at a high rate (Kopp *et al.* 1993). This means that poor sites are not suitable for SRC cultivation unless fertilizers are supplied. A relevant study showed that fertilizer represents up to 10–20% of the cost of production over several rotations of a willow SRC crop (Buchholz and Volk 2011). Estimates based on nutrient off-take measurements vary between 50 – 130kg N, 60 – 83kg K, and 8 – 16kg P per hectare per year are required by willow SRC, but generally farmers only need to add nitrogen (N) the first several years as potassium (K) and phosphorus (P) usually build up excessively in farm soil (Caslin et al 2010). This paper addresses whether the central challenge of fertility that arises with SRC farming in a carbon-negative way on marginal land could be addressed or mitigated by the inoculation of mycorrhizal fungi.

Arbuscular mycorrhiza (AM), which penetrate the root cells of their host plant, and ectomycorrhiza (EM), which interact just as intimately with their host plant but at a root interface that does not penetrate, are the two main types of mycorrhizal fungi (e.g., Wang and Qiu 2006). In exchange for sugars the plant produces, both AM and EM provide nutrients they have harvested from surrounding soil (particularly those trapped in mineral form, or difficult to degrade organic molecules) with their extensive fungal networks and specialized degradation enzymes (Whiteside *et al.* 2012, Smith *et al.* 2011). There is also extensive evidence of AM and EM providing their hosts with protection from disease and aiding with water stress in drought (Liu *et al.* 2007, St-Arnaud and Vujanovic 2007, Lekberg and Koide 2005).

Biomass shrub willows—though small and with multiple stems instead of a trunk—are nevertheless a tree species in the genus *Salix*, and are typically found with EM symbionts in samples of their roots and rhizosphere (the narrow soil zone under the influence of roots) (Puettsepp *et al.* 2004, Paradi and Baar 2006, Ryberg *et al*. 2011). *Salix* species, however, have been found associated with both EM and AM (separately or at the same time) (van der Heijden 2001, Hashimoto and Higuchi 2003, Milne *et al.* 2006, Becerra *et al.* 2009). Also, minor association with AM cannot be ignored, as some evidence suggests mycorrhiza can provide significant benefits for their host even at low levels of root colonization (Baxter and Dighton 2001, Fransson *et al.* 2013).

Because agricultural land (or marginal, formerly agricultural land) does not have a diverse collection of trees growing on it, researchers have conducted experiments inoculating trees with mycorrhiza, reasoning that the specific EM species that would colonize biomass willows could be missing (Corredor *et al*. 2012). Similarly, as agricultural land is often exhausted of organic carbon and repeatedly left barren of plant hosts (when crops are harvested in late summer or fall), even AM that could use crop plants for hosts might be low in number and/or diversity (Six *et al.* 2006, Douds *et al.* 2012).

For this reason, researchers have conducted experiments inoculating trees with mycorrhiza. Some of these experiments showed a benefit from inoculation, but many others were inconclusive (Garbaye and Churin 1997, Baum *et al.* 2002, Duponnois *et al.* 2007, Quoreshi *et al.* 2008, Chapdelaine *et al*. 2008). The same is true for experiments specifically

using *Salix* (or closely related *Populus*) and AM or EM inoculum (Quoreshi and Khasa 2008, Fillion *et al.* 2010, Bissonnette *et al.* 2010).

In most of these studies, the controlled conditions used are significantly different from what willow growers will actually encounter in their fields. Even when young trees are outplanted into natural settings in some of these experiments, the seedlings are first raised in containers. This means that the control conditions, with completely sterile potting soil, could stunt the trees' early growth and do not reflect a natural control with its random mix of native mycorrhiza. Experiments with sterile control soils, and sterile soils plus one or a few inoculum species throughout the measurement time period, while valuable for basic research, are inherently limited. Agricultural activities almost never deal with sterile soils, so for practical purposes farmers and agricultural researchers need to know what their treatments do in relation to unsterile controls.

This study addresses the sterile control problem by planting directly into intact farm soil. This is possible in practical terms because willow cuttings do not need to be started in pots, and in theoretical terms because of the sheer number of willow cuttings planted. The myriad combinations of native mycorrhiza, as well as plant pathogens, etc., that would threaten a smaller experiment with too many confounding variables, are better dealt with through replication across our large fields. If a few willows are negatively affected by small areas of soil with disease pathogens, or conversely if a few willows have increased growth due to a small area of particularly beneficial mycorrhiza, their growth measurements will not significantly shift the mean results from many more growth measurements we were able to take with a field-scale investigation. Furthermore, the high variability in growth of plants in fields that are naturally patchy in soil structure and nutrient concentration can be accounted for statistically with enough data points from several different blocks. This "random" variation was part of our ANOVA models, and our field sizes allowed us to do this. Biomass farmers, and those who advise farmers, should take note of this study's results as they relate closely to real-world conditions. Even the equipment used to set up and implement the experiment was true to modern farming realities.

If such energy intensive products as fertilizers can be limited through mycorrhizal inoculation and likewise marginal agricultural land can be better taken advantage of, the use of

biofuel grown with carbon taken from the atmosphere would inarguably be a net climate benefit. Knowing whether inoculation is effective or not is a key step for this industry, and more generally those working in agriculture. Our experiment aimed to do that. It tested whether or not a mycorrhizal inoculant can have a positive effect on the growth of SRC willows. Furthermore, our experiment was also designed to allow us to test its effect across two different cultivars of willow, three different marginal fields, and whether fertilized with nitrogen or not.

II.2. Materials and methods

II.2.1. Experimental design

Three similarly designed experimental fields, 108 m by 43.2 m, or ~4,670 m^2 each, were established in the summer of 2010 at three sites on the Allard family farm (company Agro Énergie), in Saint-Roch-de-l'Achigan, QC, Canada (N45.848783, W73.674546). The farm is ~60 m above sea level, flat and open, but sparsely wooded at the edges of farm fields. It lies within the St. Lawrence River watershed, ~25 km north-northeast of Montreal, QC, Canada. A different soil type defines each experimental field, and details are summarized in Table II.I. One location, referred to in this paper as Sandy field, is almost pure sand with a low pH of 6. Another named Dry field is sandy-loam with a close to neutral pH of 7. The last is silty-loam with a high pH of 8, but covered almost ~30% at the surface with small and medium sized rocks (~1-5 cm), and therefore named Rocky field. Fields had been planted in the past with the standard North American rotation of corn and soybeans (corn the most recent), but Sandy field had also been periodically planted with carrots before that.

The experimental design was a modified split-plot design and had twelve full blocks repeated in each field. In this hierarchical design, inoculation treatments were randomized before cultivar, and fertilization treatments randomized last. Local soil conditions and weather determined our partner farmer's best practices for preparing a weed-free, flat, and loose soil bed (a mix of plowing and disking). Small cuttings (~30 cm long, and ~1-2 cm thick) of two cultivars of willow, *Salix miyabeana* Seeman SX64 and SX61 were planted (SX61 is also referred to as *Salix sachalinensis*, but those involved in the breeding program now believe it

was mislabeled—personal communication). Planting was done using a modified 3-row cabbage planter. Rows were 1.8 m apart, and willows were planted every 36 cm, for a density of 16,103 trees per hectare. Flagged stakes marked every 18 m, showing the edges of experiment blocks (six, 12-row-wide blocks running down each half of the field for the 12 total in each field—see appendix for diagram). The modified aspect of the split-plot design came with the randomization of cultivar. Instead of being randomized with each block, the random selection from the first block was continued for the entire length of the field to facilitate timely and accurate planting at this large scale (again, see appendix for diagram). The three farmers sitting on the planter were informed at the beginning of each new group of three rows which of the two willow types to feed into the rotating planter cylinders. However, during planting water delivery of the inoculant was turned off and on by hand as the planter stopped and started each block section, allowing true randomization between each experimental block. Smaller flags on wire stakes subdivided the twelve blocks every 9 m and every six rows, but these subdivisions directed nitrogen fertilization, which was not applied until the second growing season (this was applied by hand, due to the relatively small size of each treatment plot—see next paragraph for application rates). Therefore, during the first year each experimental block had four treatment subplots within them, which then became eight treatment subplots within each block from the second year on. Similar blocks, though with a different randomly assigned treatment pattern, were set up in the three different fields.

Inoculation was done using a mixed inoculum of AM and EM fungi (patent pending) provided by the firm BioSyneterra Solutions Inc, with water-suspended delivery to each cutting at planting as roughly ~350 propagules of the AM and ~250 propagules of the EM in 50 mL of water. The AM strain used was a *Rhizoglomus irregulare* DAOM197198 (syn. *Rhizophagus irregularis*, *Glomus irregulare* and *G. intraradices*; Sieverding *et al*. 2015, Stockinger *et al*. 2009) from Pont Rouge, QC, Canada. The EM strain used was a proprietary *Hebeloma longicaudum* strain. Viability of the inoculum was checked by taking a sample of the inoculum suspension on the day of the planting and inoculating several potted willows in autoclaved farm soil. Viability was confirmed qualitatively, with root staining and microscopic visualization of AM and EM structures, as well as macroscopic identification of EM fructifications. Some 7,200 cuttings were planted on each site, for a total of 21,600

seedlings. The experiment ran for two and a half years (through three growing seasons). Only during the second growing season did half of the trees receive nitrogen fertilization (75 kg/ha N, as pelleted chicken feather compost scattered by hand in May).

II.2.2. Sampling and measurements

Before planting had taken place, a baseline soil analysis was conducted. Soil was collected using a 1000 cm^3, screw-boring hand sampler, combining seven samples taken along a diagonal across each field. Two depths were sampled, 0-20 cm and 20-40 cm. All of the soil from each sample type was mixed thoroughly, a subsample taken back to the laboratory, air dried and sent to a commercial service (Agridirect, Longueuil, QC, Canada) for chemical analysis.

We measured the diameter of the largest stem 10 cm off the ground, height of the longest stem, and the number of stems to assess shrub growth. Growth measurements were taken every other tree, in October 2010, along the middle row of the three-row treatment groups, starting and stopping ~10 trees from the edges of treatment groups (block edges this first year) to leave a buffer between treatments and reduce edge effects. This meant that 16 trees were measured in each treatment group, and with four treatment groups the first year and 12 blocks repeated in each field, that totaled 2,304 trees measured (the actual total was slightly lower as one block within the Sandy field was not measured in year one due to excessive mortality concentrated in that block, presumably as roots hadn't developed enough to compensate for the higher drainage in an almost 100% sand patch).

At the end of the second growing season, in November 2011, a total of 1,152 trees were measured for growth, with 576 also selected randomly to be cut and weighed. The subselection was necessary due to time constraints, and was randomized at the level of block and treatment row in order to keep equal numbers of willows in each treatment. A representative 2-3 kg of stem pieces (no leaves) were further subsampled from each field to measure moisture percentage each day willows were cut down. The 1,152 trees measured were again those from the middle row, now with a ~10 tree buffer at the edges and middle of blocks to accommodate the fertilization treatment subgroups (4 trees per subgroup, with every third tree selected for measurement). Although a block in the Sandy field had been dropped from

the analysis in the first year due to high mortality, it was kept this second year. This was because many of the trees that had died were actually concentrated in the middle of the block, and there were enough trees left to give an accurate representation of all the treatments with the increased fertilization subdivision added.

At the end of the third growing season, in 2012, only 288 trees total were measured (and weighed, see Figure II.1 and the appendix). This was the minimum of two trees per treatment subgroup per block, that could still be analyzed using the ANOVA model, and was collected as an extra effort in addition to the original project and grant to double check that the previous results held true farther into a growth cycle between coppicing.

Thirty-six whole root samples were also dug up and collected in October 2010 after the first growing season and consisted of three replicates of the twelve treatment combinations then in place (randomly chosen from the ten trees in the middle of each treatment group, within three randomly chosen blocks in each field). Because nitrogen fertilization was not applied until the second season, the twelve treatment combinations consisted of the two willow cultivar types in each of the three fields, inoculated and not. After shaking off unattached or excess soil, each plant's root system was separately bagged and stored at -30 °C in the laboratory's freezers the same day.

Root systems were later thawed and vigorously rinsed and agitated by hand in distilled water, the dirty rinse water allowed to settle and the sediment set aside and refrozen as 36 rhizospheric soil samples. After cutting up the roots into 1 cm pieces, they were well mixed and further homogenized (with a washed and sterilized commercial food-beverage blender in milli-Q/0.1TE buffer solution) and frozen as 36 root samples for the next molecular analysis steps.

II.2.3. Molecular fungal community analysis

DNA from all the first-year root and rhizosphere samples (72 total—36 rhizospheric soil and 36 root) was extracted using MoBio Laboratories PowerSoil Extraction kit according to the manufacturer's instructions, modified such that instead of the standard homogenization and lysis with a vortexer in the first step, an MP Biomedicals FastPrep machine at setting 4 was used for 25 s and six repetitions.

For a first round of cloning-sequencing, all of the replicates of both willow cultivars were pooled together by field, keeping separate inoculated from uninoculated replicates. Also, paradoxically, the DNA extracted from rhizospheric soil was used to look for AM, and the DNA from root samples to look for EM. This was because preliminary efforts revealed a tendency of the AM specific primers (AML1/AML2; Lee *et al*. 2008) to amplify willow DNA as well. Conversely in order to look for EM, universal fungal primers were used to maximize the likelihood of amplifying mycorrhizal species by targeting the roots themselves. Root extract from the sandy field was used instead of rhizospheric soil extract, however, to look for AM when that field's rhizospheric soil extract did not produce any AM sequences. All of this left six pooled samples for AM PCR-cloning and six for EM PCR-cloning (three inoculated and three uninoculated for each).

Initial tests confirmed previous experience that attempting to directly PCR the sample DNA with the AM specific primer pair AML1/AML2 did not yield amplicons, so a nested PCR reaction was run with the first six samples used for AM detection. The initial PCR used the primer pair NS1/NS41 in a 25 µL reaction with reagents from the MoBio PCR CoreKit. Initial denaturation was at 94 °C for 3 min, followed by 30 cycles of 94 °C for 45 s, 55 °C for 1 min, and 72 °C for 1 min (protocol modified from Hassan *et al.* 2011). The reaction ended with 10 min at 72 °C. One µL of product from this first PCR was then amplified in a second 25 µL reaction using the AML1/AML2 primer pair. Initial denaturation was at 94° C for 3 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 45 s, and 72 °C for 45 s, and ending with 10 min at 72° C (Hassan *et al.* 2011). Ependorf MasterCycler Pro thermocyclers were used, and PCR products visualized on 1% agarose gels according to the directions for Biotium's GelRed dye and BioRad's Molecular Imager Gel Doc XR.

The other six samples used for EM detection could be amplified in one step (unnested) using the same MoBio reagents and the primer pair ITS1F/ITS2. Initial denaturation was at 94 °C for 3 min, followed by 30 cycles of 94 °C for 45 s, 60 °C for 1 min, and 72 °C for 1 min (modified from Bell *et al.* 2014). The reaction ended with 10 min at 72 °C. The same thermocyclers and visualization method was used as in the first six samples.

PCR products were cloned according to directions using the TOPO TA Cloning Kit for Sequencing, from Life Technology. Forty-eight clones for each sample were reamplified and

the products sequenced at the McGill University and Génome Québec Innovation Centre in Montreal, QC, Canada, using the common Sanger-sequencing method.

Sequences were grouped by CD-HIT (cd-hit.org/) into OTUs of 98% similarity. One sequence from each OTU group was then randomly selected to be analyzed using the BLAST search tool in the National Center for Biotechnology Information (NCBI) database. The closest matching organism in the database could then be matched to that OTU. Only those OTUs that were matched with AM organisms were tabulated (the 18S gene is a highly conserved region and the AML1/AML2 primer pair is known to amplify other eukaryotic organisms as well).

II.2.4. Statistical analysis

A full-factorial ANOVA was performed on the measured growth data. Growth was presented in this paper as stem basal area per hectare (SBA m^2/ha) (modified from McKnight 1965). We determined SBA by calculating transectional stem areas from diameter measurements, multiplied by mean stem number, and then divided by the average area of land one willow occupied in hectares. SBA was calculated for each measured tree before running the ANOVA, thereby normalizing diameter measurements for different mortality rates in each field. The actual average density of willows was quite different between fields, after mortality suffered during dry conditions in the weeks following planting (almost ~25% in Dry field, ~15% in Sandy, and ~5% in Rocky; but scattered enough in each field to be roughly equivalent across treatment combinations and blocks). Finally, because of fertilizer spillover from a nearby field during the third year, an entire row of the Sandy field had to be dropped from the analyses and the blocks in that field redrawn to maintain the correct number of each treatment combination (reducing the number of blocks in that field by four).

Growth was also measured the second and third year by cutting down and weighing above-ground biomass (see notes in Figure II.1, as well as appendix), and reported as oven dry tons per hectare (ODT/ha). This was calculated from wet mass measurements taken in the field, using a conversion of 0.53 in 2011 and 0.61 in 2012, found from drying samples brought back to the lab, and then the same density values for each field were used to calculate per ha, as with SBA/ha.

In the model for our ANOVA, experimental blocks needed to be nested in field since block 1 of Dry field was not the same as block 1 of Sandy field and so on. The model was modified accordingly, keeping a full-factorial combination of all variables besides those instances where field and (now nested) blocks combined. Furthermore, all combinations with block had to be designated as random components, since they were not a variable we were interested in testing but necessary for the ANOVA to take into account for random heterogeneity within the fields. ANOVA residuals did indicate heteroscedasticity, which was corrected by LOG transforming our measured data.

II.3. Results

The experiment found no productivity difference between uninoculated and inoculated plantation willows. As Figure II.1 shows, the first two years where the most trees were measured showed no difference between inoculation treatment predicted mean SBA/ha values. Similarly, while the third year had fewer measured trees and did seemingly see a small difference between SBA/ha mean predicted values, this difference was not statistically significant. This result held true whether tree height was measured instead, or even whole trees cut down and weighed (see supplementary material, SII.2.I-VI, and SII.3.I-IV).

Table II.II shows the results of the ANOVA on 2011 SBA/ha data in more depth. This ANOVA confirms that the field sites used were different enough to affect the willows' productivity, and that the nitrogen fertilization treatment was significant as well. Table II.III provides the effect size, showing fertilization gave a predicted mean increase of 27% SBA/ha in 2011 (13.92-10.95=2.97, 2.97/10.95=0.27). Even more strikingly, Sandy field showed a predicted mean increase of 55% SBA/ha over Rocky field in 2011 (15.96-10.31=5.65, 5.65/10.31=0.55). Similarly significant biological and statistical effect sizes were seen in other years, and with the height and weight data, though fertilization was only applied in 2011 and therefore showed less effect in 2012 (SII.1.I-IV, SII.2.I-VI, and SII.3.I-IV). The overall patterns in Figure II.1, and Tables II.II and II.III are representative of those seen over the three years with height and weight as seen in the supplementary data (SII.2.I-VI, and SII.3.I-IV). As seen in Figure II.2, the different fields did show different effects in proportion to one another. While Sandy field showed markedly higher cumulative growth the first two years, cumulative

growth in the Dry field caught up and equaled that in Sandy field by the third year (Figure II.2).

Figure II.3 shows the effect of nitrogen fertilization on growth using SBA/ha again, but in graphical form, as well as making it easier to see the difference in effect between 2011 and 2012. Figure II.4, meanwhile, shows this fertilization effect on mass using ODT/ha (Oven dry tons per hectare) instead of SBA/ha. Figure II.4 also focuses on 2011, the year of chief fertilization effect, and because of an interaction between field and fertilization as indicated by the ANOVA (see S.II.3, Table SII.3.I) breaks down the effect by field. The effect of fertilization is even more marked in this case, as fertilization gave a predicted mean increase of 51% ODT/ha in 2011 in Sandy field (5.76-3.82=1.94, 1.94/3.82=0.51).

II.3.1. Sequence library results

Table II.IV shows the results of sequencing AM species, pooled by inoculated and uninoculated treatment groups. Wild AM fungi (in our uninoculated group) included three *Diversispora* OTUs, one OTU of the closely related *Archeospora*/*Ambispora*, and three Glomeromycota OTUs. The inoculum only added a single unique OTU identified as uncultured *Archaeospora* with one sequence (possibly an artifact), but did appreciably increase the relative *Glomus* sequence numbers and eliminate the *Diversispora* numbers entirely. Also, somewhat intriguingly, the inoculum increased relative *Ambispora* sequence numbers.

Table II.V shows the EM fungi (found in root samples), as well as putative EM and non-EM fungi. Five OTUs were mycorrhizal according to NCBI database notes: *Pulvinula constellatio*, *Hymenogaster griseus*, uncultured Sebacinales, uncultured ectomycorrhizal fungus, and uncultured *Salix* associated fungus. The inoculum did not have a clear effect, with *P. constellatio* increasing in number but the uncultured ectomycorrhizal fungus decreasing appreciably and the most numerous sequence, the uncultured *Salix* associated fungus, unaffected. One OTU identified as *Hebeloma* cf. *crustuliniforme* was not labeled as EM in the NCBI notes, but is known to be ectomycorrhizal (Aanen *et al.* 2000). It is only present in the inoculated samples, and is so closely related to the EM species inoculated (*Hebeloma*

longicaudum) that the five sequences found could very well be the inoculum surviving at low numbers.

II.4. Discussion

Willow growers with access to marginal land should be interested in our data as they represent farm-scale, real-world results across different field types as well as with nitrogen fertilization and without. The success of the less drained, wetter Sandy field is promising for those with similar land, though the apparent early growth benefit of Sandy field could simply have resulted from the more clay-rich Dry and Rocky fields being harder for the willow roots to penetrate. At the least a farmer with a rock-strewn, clay-heavy field like our Rocky field might decide not to try willows after seeing our results, especially if it is deficient in phosphorous minerals as ours was. Likewise, a farmer might not try to eliminate his fertilizer costs (or the climate impacting effect of such fertilizers) if our data indicate a 51% increase in growth is possible in particular fields with a fairly modest nitrogen addition of 75 kg/ha. However, though such results are of interest they must be seen as ancillary to those concerning inoculation. The entire design of the experiment is geared towards looking at the effect of inoculation. Field selection and characterization, as well as fertilization treatment were chosen to test inoculation, not drainage or fertilizer effect per se. Neither drainage nor fertilization were changed systematically enough to have any idea of the effects' rate across different treatment values, as well as upper or lower bounds.

The likeliest conclusion to draw from the experiment is that inoculation with the two mycorrhizal species used, *R. irregulare* and *H. longicaudum*, does not appreciably benefit the growth of biomass willows cultivated in marginal agricultural fields. Neither cultivar of willow tested showed a significant difference across inoculation treatments. Neither did the full factorial ANOVA show any mixed effects between inoculation and field site or inoculation and fertilization treatment. So, inoculation could not be shown to benefit within even one of the range of soil and fertility conditions tested.

The AM species used, *R. irregulare*, has been shown in sterile soil greenhouse experiments to benefit willow growth (van der Heijden 2001). That it did not in this experiment, strongly suggests wild mycorrhizal species mask the treatment. In fact, several

wild AM species were found (Table II.IV), as well as several wild probable EM species (Table II.V). This is not that surprising, as corn and soybean crops that would have been present in previous years could host AM species. Annual monocrop systems are not ideal for diverse mycorrhizal communities (Verbruggen and Kiers 2010), but many AM species are known to survive (Beauregard *et al.* 2013, Moebius-Clune *et al.* 2013). Such wild strains could benefit uninoculated control willows just as much as the inoculum might benefit treatment willows, or even outcompete the inoculum strain with the same result. The wild EM species are more unexpected however, because willow specific EM fungi are not likely to be associated with annual agricultural crops (Tedersoo *et al*. 2010). In this case, though, wild willows bordered the sandy site and all three sites were fairly close (within a quarter km) of established willow plantations. Such locally adapted, willow-specific EM strains could easily have outcompeted or masked the introduced strain.

The pooled sequence libraries for the inoculation treatments do indicate that the inoculum was viable and competitive enough to be detected after the first year of growth in the field (the possibility that it was not had to be considered as an alternative hypothesis to explain the data). Furthermore, viability of the inoculum was confirmed with a small greenhouse test, as mentioned in the methods. The AM species (Table II.IV) show roughly twice the number of relative *Glomus* species in the inoculated plots. The EM species (Table II.V) are more difficult to interpret, but there are some *Hebeloma* sequences in the treated plots that are not present in the untreated plots.

The experimental results suggest that inoculation with AM fungi in general will not benefit willows grown in agricultural settings as such willows already have the potential to interact with numerous wild AM strains. A diversity of AM species appears to be ubiquitous to different degrees in agricultural soil (Moebius-Clune *et al*. 2013).

However, we cannot conclude that another strain of EM fungi would not benefit willows grown in such a setting. The *Hebeloma* species selected had been found associated with a very closely related species of tree (*Populus*), but greenhouse or pot experiments to confirm positive interaction with biomass willows had not been conducted prior to this experiment. Not many EM fungi are commercially available at application rates needed on an agricultural scale, and the EM inoculum was the only one available at the time of our

experiment's setup. Because EM fungi are more host specific than AM fungi (Newton and Haigh 1998, Kilronomos 2000) and are less likely to be present in agricultural fields (Dickie and Reich 2005, Oehl *et al.* 2003), we encourage researchers to test other species and strains as they become available for agricultural application.

For willow growers, our results caution against investing in mycorrhizal inoculation unless a strain has proven benefits in field tests with willows (or in pot tests using unsterilized farm soil). We would still advise growers to apply mycorrhizal inoculation if planting on semi-sterile soil, such as heavily fungicide-applied sands, mine tailings, etc., but our results suggest this inoculation could simply be a diluted soil slurry from a healthy agricultural field nearby.

(to be published with co-authors Werther Guidi Nissim[2], Michel Labrecque[1], Marc St-Arnaud[1])

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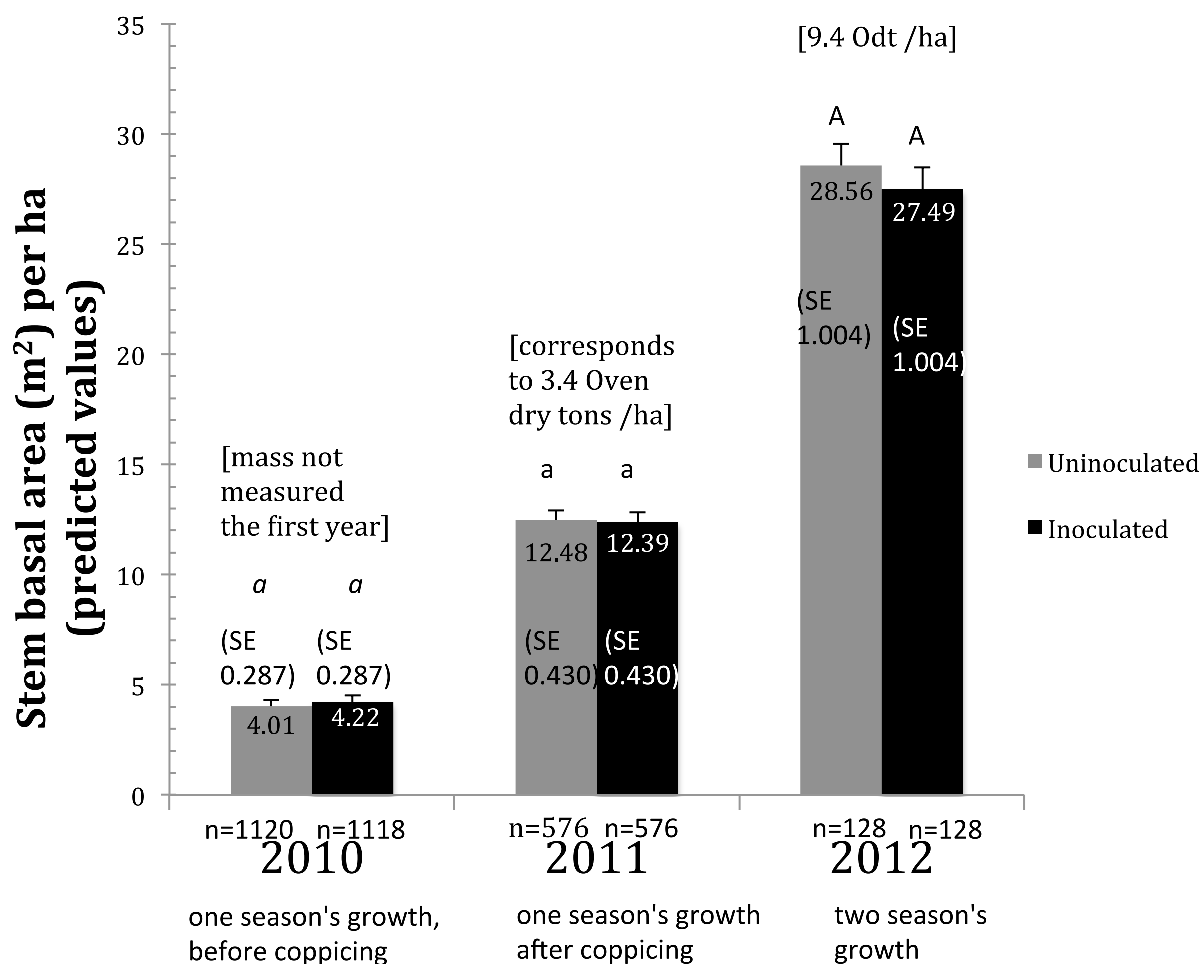
Table II.I: Characterization of the three field sites, including a soil analysis at two depths

Depth	pH	Nitrate	Phosphorus	Organic matter	Clay	Silt	Sand	Soil Type
Dry field (N45.825276, E-73.624675)								
(0-20 cm)	7.1	5.77 ppm	130 kg/ha	4.0%	21.0%	40.9%	38.1%	Medium loam
(20-40 cm)	7.3	7.35 ppm	81 kg/ha	3.9%	19.9%	32.7%	47.4%	(edging towards sandy)
Defining qualitative features: heavily drained with a 0.5m deep ditch along its west side and a 2m deep channel along its south side.								
Rocky field (N45.827205, E-73.626789)								
(0-20 cm)	7.9	6.87 ppm	63 kg/ha	3.5%	29.5%	39.2%	31.3%	Medium loam
(20-40 cm)	7.9	5.77 ppm	39 kg/ha	2.8%	24.1%	43.5%	32.4%	(edging towards clay)
Defining qualitative features: ~30% of its surface covered with small and medium sized rocks (~1-5 cm).								
Sandy field (N45.825629, E-73.617733)								
(0-20 cm)	6.0	5.35 ppm	256 kg/ha	2.1%	2.5%	10.4%	87.1%	Loamy sand
(20-40 cm)	6.1	5.91 ppm	192 kg/ha	2.0%	3.4%	6.8%	89.8%	(very close to pure sand)
Defining qualitative features: far removed from any drainage, it often had standing water for a week at a time after any rainfall, between the field and the forest boardering its south side and the southern half of its east side.								

Note: all samples tested <0.2% total nitrogen—below detection level

Figure II.1: Cumulative growth of *Salix miyabeana* (SX61 and SX64) during establishment on marginal land, treated with mycorrhizal inoculum



Note: The values presented are the predicted values, or least square means, of three full-factorial ANOVAs (each year calculated separately) based on fall calliper measurements (diameters converted to area, multiplied by 5.84—the average number of counted stems—and then converted to per ha based on field density). Error bars are standard error (SE), modeled across all experimental blocks in three soil types using two cultivars at two fertilization levels (except for 2010 which did not yet have a fertilization treatment). We applied an inoculum of *Rhizoglomus irregulare* (arbuscular myc.) and *Hebeloma longicaudum* (ectomycorrhizal), at roughly 250 and 350 propagules per plant respectively. ANOVA residuals did indicate heteroscedasticity, which was corrected by LOG transforming our measured data, but the predicted values and SE shown in this graph are not transformed. Within each year, similar letters above the bars indicate that the ANOVA did not find significant differences ($p < 0.05$) between predicted means for inoculation treatment.

Table II.II: 2011 Stem basal area /ha ANOVA results (LOG transformed)

Source	Nparm	DF	DFDen	F Ratio	Prob > F
field	2	2	33	21.0017	<.0001*
inoc	1	1	33	0.0175	0.8955
field*inoc	2	2	33	1.1145	0.3401
fert	1	1	33	105.1391	<.0001*
field*fert	2	2	33	1.8694	0.1702
inoc*fert	1	1	33	0.0187	0.8920
field*inoc*fert	2	2	33	0.2658	0.7682
cultivar	1	1	33	0.0465	0.8305
field*cultivar	2	2	33	0.4076	0.6686
inoc*cultivar	1	1	33	0.8624	0.3598
field*inoc*cultivar	2	2	33	0.2301	0.7957
fert*cultivar	1	1	33	0.1629	0.6891
field*fert*cultivar	2	2	33	0.4619	0.6341
inoc*fert*cultivar	1	1	33	2.0731	0.1593
field*inoc*fert*cultivar	2	2	33	0.602	0.5536

Note: All combinations of the block treatment, by itself and with the other treatment variables were part of the model, but block was treated differently since it was nested in the field variable and labeled as a random attribute. It therefore does not appear in this table. An asterisk (*) next to the p-value denotes a 5% statistical significance.

Table II.III: 2011 Stem basal area per hectare (m^2/ha) ANOVA predicted values and test results

field	Least Sq Mean	Std Error	Tukey's test	
Dry	11.029889	0.66402733	Dry	B
Rocky	10.307269	0.66402733	Rocky	B
Sandy	15.964387	0.66402733	Sandy	A

inoculation	Least Sq Mean	Std Error	Student's T-test	
no	12.478451	0.43045506	not inoculated	A
yes	12.389246	0.43045506	inoculated	A

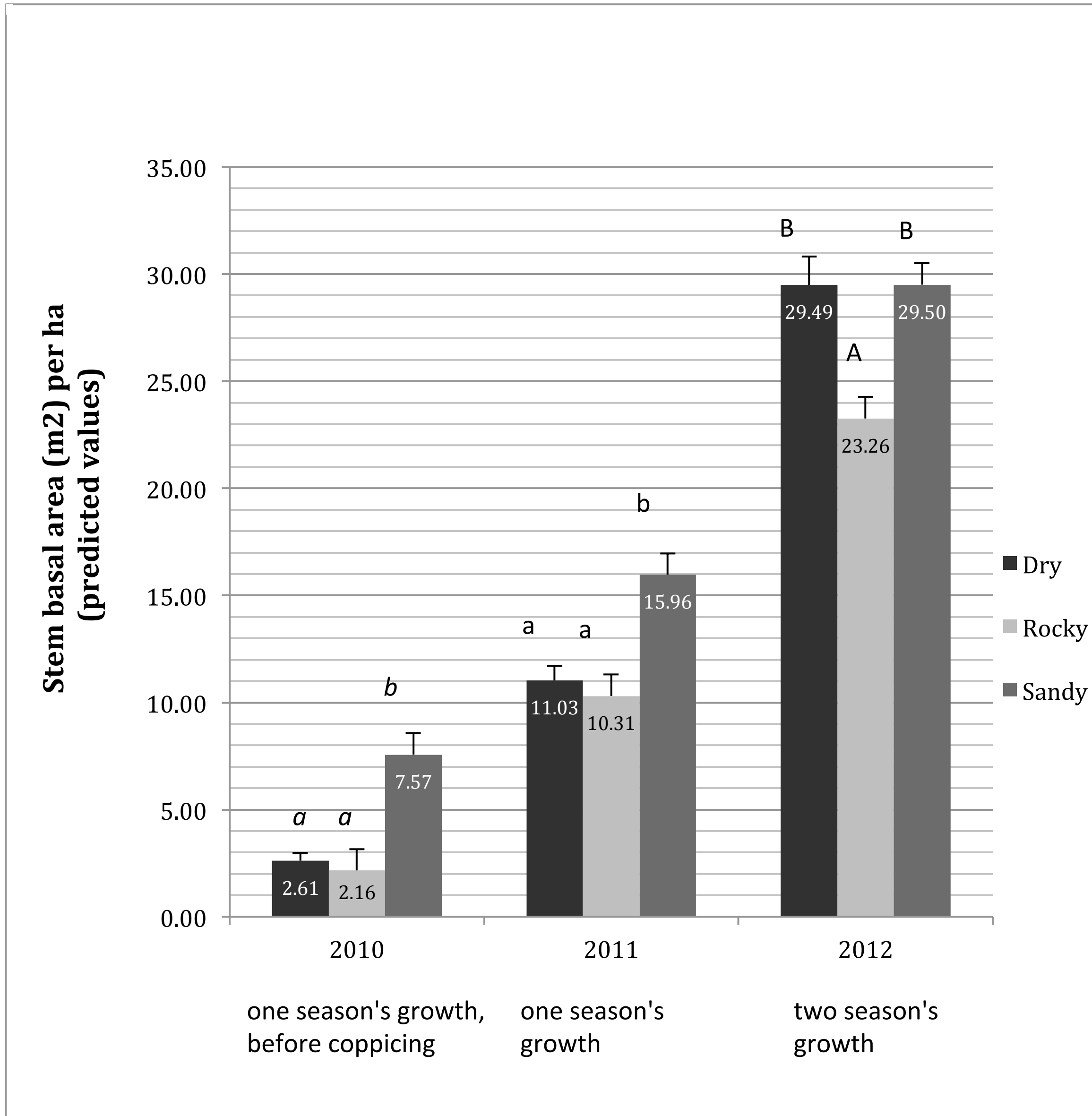
(these least sq mean values were used to generate the 2011 bars for Figure II.1)

nitrogen	Least Sq Mean	Std Error	Student's T-test		
fertilized	13.921206	0.4122257	fertilized	A	
unfertilized	10.946491	0.4122257	unfertilized		B

cultivar	Least Sq Mean	Std Error	Student's T-test	
SX64	12.486569	0.44855227	SX64	A
SX61	12.381127	0.44855227	SX61	A

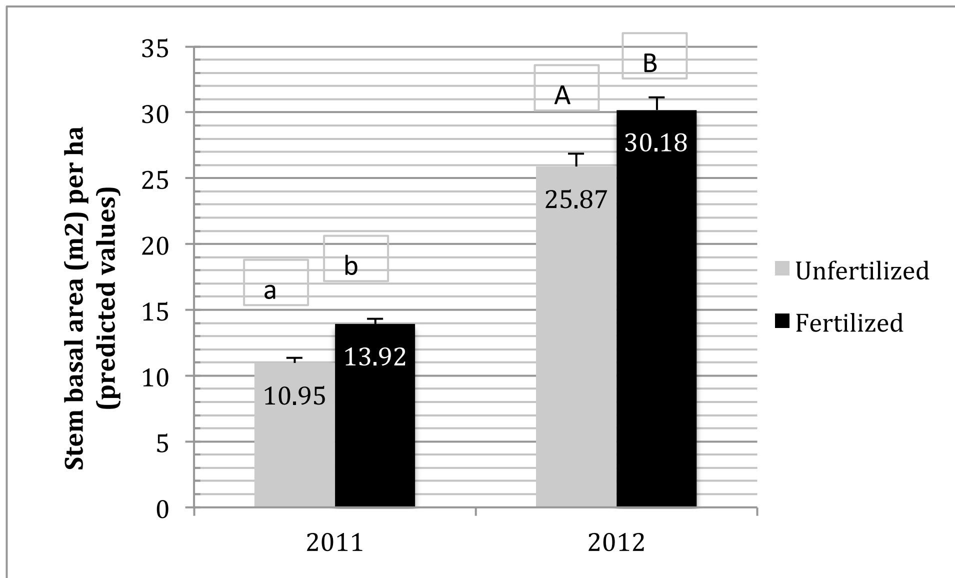
Note: different letters indicate better than 0.05 p-value difference between means. Data transformed for the analysis but not before generating this table, to allow comparison with other agricultural and forestry studies.

Figure II.2: Cumulative growth of *Salix miyabeana* (SX61 and SX64) during establishment on marginal land, in three different fields. Stem basal area predicted values (untransformed) and test results (LOG transformed) by field for all three years



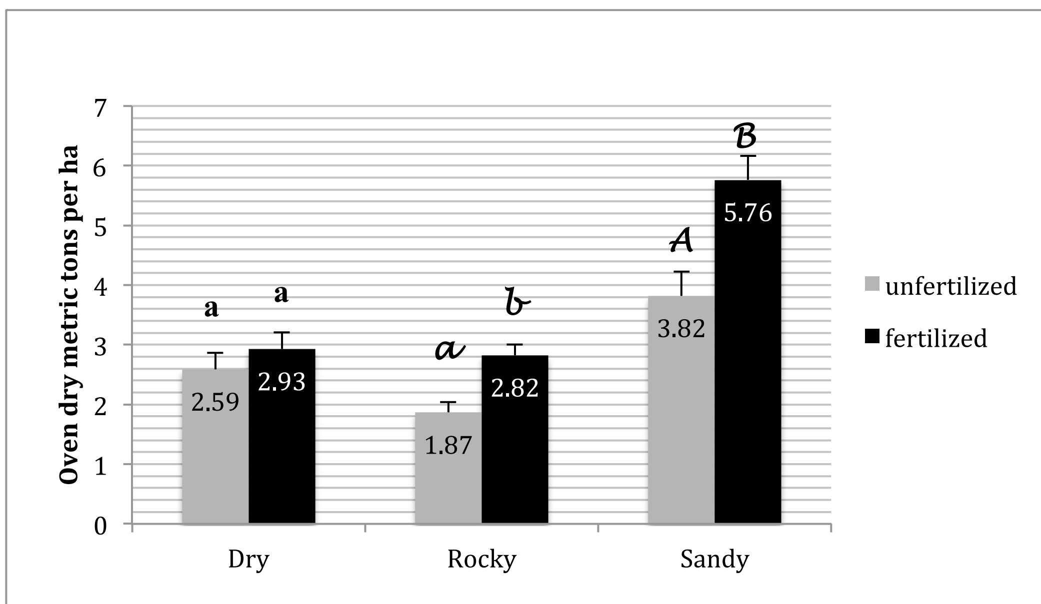
Note: 2012 represents only one cultivar type (SX61) because of treatment interaction, but 2010 and 2011 are the full model. Error bars are standard errors. Different letters above the bars indicate that the Tukey's test found a significant difference ($p < 0.05$) between predicted means for each field (a separate analysis each year).

Figure II.3: Cumulative growth of *Salix miyabeana* (SX61 and SX64) during establishment on marginal land. Stem basal area predicted values (untransformed) and ANOVA results (LOG transformed) for the year a fertilization treatment was applied (2011), as well as the year after (past growth, plus any residual nitrogen in the soil)



Note: Error bars are standard errors. Different letters above the bars indicate that the ANOVA found a significant difference ($p < 0.05$) between predicted means for fertilization treatment.

Figure II.4: Cumulative growth of *Salix miyabeana* (SX61 and SX64) during establishment on marginal land. Oven dry tons per ha predicted values (untransformed) by field for 2011



Note: Error bars are standard errors. Different letters above the bars indicate that the Student's t-test found a significant difference ($p < 0.05$) between predicted means for fertilization treatment (each field analyzed separately). The ANOVA (performed on LOG transformed values), including all variables in the model, found a likely interaction between field and fertilization, prompting the breakdown by field.

Table II.IV: Arbuscular mycorrhizal sequences found in rhizosphere samples from biomass plantation willows, by inoculation treatment

	uninoculated	**inoculated**	**Name and GI of closest match in NCBI database**
OTU-10	**1**	**-**	*Diversispora celata*: 224586636
OTU-11	**32**	**-**	*Diversispora* sp. W4538: 342298391
OTU-12	**2**	**-**	Uncultured *Diversispora*: 398649715
OTU-13	**19**	**47**	*Glomus* sp. MC27: 334683211
OTU-14	**31**	**39**	Uncultured *Glomus*: 401664149
OTU-15	**10**	**78**	Uncultured *Ambispora*: 308084344
OTU-16	**-**	**1**	Uncultured *Archaeospora*: 308084350
OTU-17	**9**	**-**	Glomeromycota sp. MIB 8442: 328541374

Note: This table represents the combined data from six cloning reactions, 48 colonies picked and sequenced from each reaction (these six cloning reactions in turn each represent six pooled DNA extracts from separate trees, 18 inoculated and 18 uninoculated, with two different willow cultivars and three different field soil types). Four of the six cloning reactions used pooled DNA from extracted rhizospheric soil, but two had to use pooled DNA from extracted rinsed roots, when the rhizospheric soil samples from the sandy field did not find any AM sequences. Out of the total 384 sequenced clones these 269 AM sequences represent 70% specificity for our primers AML1 and AML2, nested following amplification with primers NS1 and NS41. OTUs are base on 98% similarity. Those OTUs not shown were non-specific amplifications, many eukaryotic, and numbered 35 (for 43 OTUs total).

Table II.V: Ectomycorrhizal sequences (and sequences of other fungi) found in rinsed root samples from biomass plantation willows, by inoculation treatment

	uninoculated	**inoculated**	Name and GI of closest match in NCBI database	
OTU-2	**5**	**3**	*Cladosporium cladosporioides*: 356484684	Ascomycota
OTU-3	**2**	**2**	*Epicoccum nigrum*: 404474360	Ascomycota
OTU-6	**8**	**8**	*Magnusiomyces capitatus*: 357934165	Ascomycota
OTU-9	**9**	**24**	**Pulvinula constellatio*: 10178659	Ascomycota
OTU-11	**2**	-	*Trichurus spiralis*: 237872399	Ascomycota
OTU-17	-	**2**	Uncultured *Geopora*: 295291451	Ascomycota
OTU-18	**2**	**2**	Uncultured *Hyaloscyphaceae*: 193850652	Ascomycota
OTU-21	**-**	**5**	*Hebeloma* cf. *crustuliniforme* 2 UE-2011: 359751813	Basidiomycota
OTU-22	**-**	**3**	**Hymenogaster griseus*: 387145960	Basidiomycota
OTU-28	**2**	-	Uncultured Basidiomycota: 334683052	Basidiomycota
OTU-30	**1**	**3**	*Uncultured Sebacinales: 264716693	Basidiomycota
OTU-33	**16**	**1**	*Uncultured ectomycorrhizal fungus: 404247775	environmental samples
OTU-34	**71**	**70**	*Uncultured fungus (from *Salix* rhiz.): 402535072	environmental samples
OTU-35	**4**	**3**	Uncultured soil fungus: 195964332	environmental samples
OTU-36	**1**	**5**	*Olpidium brassicae*: 87159723	Fungi incertae sedis
OTU-40	**2**	**-**	*Entrophospora* sp. JJ38: 15809596	Glomeromycota

Note: Names marked with an asterisk (*) are EM species according to NCBI entry notes. This table represents the combined data from six cloning reactions, 48 colonies picked and sequenced from each reaction (these six cloning reactions in turn each represent six pooled DNA extracts from separate trees, 18 inoculated and 18 uninoculated, with two different willow cultivars and three different field soil types). The universal fungal primers, ITS1F and ITS2, can amplify soil fungi that were not completely rinsed free from the root samples, but concentrating on those present in higher numbers that are most likely associated with the willow roots was necessary. Therefore presented here are the 16—out of 40 total—OTUs that contained two or more sequences (and therefore almost 90% of the 288 total sequenced clones). OTUs are based on 98% similarity.

Supplementary material to Chapter II

SII.1: Additional diameter analyses

Table SII.1.I: 2010 Stem basal area ANOVA results (LOG transformed)

Source	Nparm	DF	DFDen	F Ratio	Prob > F
field	2	2	32	71.3478	<.0001*
inoc	1	1	32	0.2942	0.5913
field*inoc	2	2	32	0.2974	0.7448
cultivar	1	1	32	6.8584	0.0134*
field*cultivar	2	2	32	1.5426	0.2293
inoc*cultivar	1	1	32	0.2683	0.6080

Note: All combinations of the block treatment, by itself and with the other treatment variables were part of the model, but block was treated differently since it was nested in the field variable and labeled as a random attribute. It therefore does not appear in this table. An asterisk (*) next to the p-value denotes a 5% statistical significance.

Table SII.1.II: 2010 Stem basal area per hectare (m^2/ha) ANOVA predicted values and test results

field	Least Sq Mean	Std Error	Tukey's test		
Dry	2.6053323	0.38832364	Dry		B
Rocky	2.1631002	0.38830761	Rocky		B
Sandy	7.5695565	0.40557407	Sandy	A	

inoculation	Least Sq Mean	Std Error	Student's T-test	
no	4.0063284	0.28720369	not inoculated	A
yes	4.2189976	0.28721333	inoculated	A

(these least squares mean values were used to generate the 2010 bars for Figure II.1)

cultivar	Least Sq Mean	Std Error	Student's T-test		
SX64	4.5770967	0.25273457	SX64	A	
SX61	3.6482293	0.25273457	SX61		B

Note: different letters indicate better than 0.05 p-value difference between means. Data transformed for the analysis but not before generating this table, to allow comparison with other agricultural and forestry work.

Table SII.1.III: 2012 Stem basal area ANOVA results (LOG transformed)

Source	Nparm	DF	DFDen	F Ratio	Prob > F
field	2	2	29	11.4172	0.0002*
cultivar	1	1	29	0.1088	0.7439
field*cultivar	2	2	29	3.9184	0.0312*
fert	1	1	29	10.1083	0.0035*
field*fert	2	2	29	0.2931	0.7481
cultivar*fert	1	1	29	0.2028	0.6558
field*cultivar*fert	2	2	29	1.7832	0.1860
inoc	1	1	29	0.6511	0.4263
field*inoc	2	2	29	1.2736	0.2950
cultivar*inoc	1	1	29	0.2813	0.5999
field*cultivar*inoc	2	2	29	0.2105	0.8114
fert*inoc	1	1	29	1.6048	0.2153
field*fert*inoc	2	2	29	0.5363	0.5906
cultivar*fert*inoc	1	1	29	0.3318	0.5690
field*cultivar*fert*inoc	2	2	29	0.0610	0.9409

Note: All combinations of the block treatment, by itself and with the other treatment variables were part of the model, but block was treated differently since it was nested in the field variable and labeled as a random attribute. It therefore does not appear in this table.

Table SII.1.IV: 2012 Stem basal area per hectare (m^2/ha) ANOVA predicted values and test results

inoculation	Least Sq Mean	Std Error	Student's T-test	
no	28.559201	1.0040098	not inoculated	A
yes	27.488155	1.0040098	inoculated	A

(these least squares mean values were used to generate the 2012 bars for Figure II.1)

nitrogen	Least Sq Mean	Std Error	Student's T-test		
fertilized	30.177806	0.97665161	fertilized	A	
unfertilized	25.869550	0.97665161	unfertilized		B

By SX64

field	Least Sq Mean	Std Error	Tukey's test		
Dry	34.709104	1.8646605	Dry	A	
Rocky	26.283434	1.8646605	Rocky		B
Sandy	24.899135	2.2837334	Sandy		B

By SX61

field	Least Sq Mean	Std Error	Tukey's test		
Dry	29.489316	1.3162810	Dry	A	
Rocky	23.258381	1.3162810	Rocky		B
Sandy	29.502699	1.6121084	Sandy	A	

By Dry

cultivar	Least Sq Mean	Std Error	Student's T-test	
SX64	34.709104	2.1116928	SX64	A
SX61	29.489316	2.1116928	SX61	A

Table SII.1.IV: 2012 Stem basal area per hectare (m^2/ha) ANOVA predicted values and test results (continued)

By Rocky

cultivar	Least Sq Mean	Std Error	Student's T-test	
SX64	12.486569	0.44855227	SX64	A
SX61	12.381127	0.44855227	SX61	A

By Sandy

cultivar	Least Sq Mean	Std Error	Student's T-test		
SX64	24.899135	1.4917259	SX64		B
SX61	29.502699	1.4917259	SX61	A	

Note: different letters indicate better than 0.05 p-value difference between means. Data transformed for the analysis but not before generating this table, to allow comparison with other agricultural and forestry work.

SII.2: Additional height analyses

Table SII.2.I: 2010 Height ANOVA results (LOG transformed)

Source	Nparm	DF	DFDen	F Ratio	Prob > F
field	2	2	32	63.3900	<.0001*
inoculation	1	1	32	0.2956	0.5904
field*inoculation	2	2	32	0.0614	0.9406
species	1	1	32	4.4930	0.0419*
field*species	2	2	32	0.7960	0.4598
inoculation*species	1	1	32	0.3374	0.5654
field*inoculation*species	2	2	32	0.8143	0.4519

Note: All combinations of the block treatment, by itself and with the other treatment variables were part of the model, but block was treated differently since it was nested in the field variable and labeled as a random attribute. It therefore does not appear in this table.

Table SII.2.II: 2010 Height (cm) ANOVA predicted values and test results

field	Least Sq Mean	Std Error	Tukey's test			
Dry	102.18433	4.7197366	Dry		B	
Rocky	84.01693	4.7193538	Rocky			C
Sandy	161.26136	4.9292043	Sandy	A		

inoculation	Least Sq Mean	Std Error	Student's T-test	
no	114.28638	3.8472487	not inoculated	A
yes	117.35537	3.8474574	inoculated	A

cultivar	Least Sq Mean	Std Error	Student's T-test		
SX64	121.10819	3.3643152	SX64	A	
SX61	110.53356	3.3643152	SX61		B

Note: different letters indicate better than 0.05 p-value difference between means. Data transformed for the analysis but not before generating this table, to allow comparison with other agricultural and forestry work.

Table SII.2.III: 2011 Height ANOVA results (LOG transformed)

Source	Nparm	DF	DFDen	F Ratio	Prob > F
field	2	2	33	44.0406	<.0001*
inoc	1	1	33	0.7392	0.3961
field*inoc	2	2	33	0.0904	0.9138
fert	1	1	33	40.6618	<.0001*
field*fert	2	2	33	0.9828	0.3849
inoc*fert	1	1	33	0.0969	0.7575
field*inoc*fert	2	2	33	0.3688	0.6944
cultivar	1	1	33	0.2922	0.5924
field*cultivar	2	2	33	4.5066	0.0186*
inoc*cultivar	1	1	33	0.2426	0.6256
field*inoc*cultivar	2	2	33	0.5059	0.6076
fert*cultivar	1	1	33	0.1325	0.7182
field*fert*cultivar	2	2	33	1.1733	0.3219
inoc*fert*cultivar	1	1	33	0.3443	0.5614
field*inoc*fert*cultivar	2	2	33	1.5840	0.2203

Note: All combinations of the block treatment, by itself and with the other treatment variables were part of the model, but block was treated differently since it was nested in the field variable and labeled as a random attribute. It therefore does not appear in this table.

Table SII.2.IV: 2011 Height (cm) ANOVA predicted values and test results

inoculation	Least Sq Mean	Std Error	Student's T-test	
no	255.52951	3.7838672	not inoculated	A
yes	258.10764	3.7838672	inoculated	A

nitrogen	Least Sq Mean	Std Error	Student's T-test	
fertilized	268.02257	3.7049795	fertilized	A
unfertilized	245.61458	3.7049795	unfertilized	B

By SX61

field	Least Sq Mean	Std Error	Tukey's test	
Dry	241.98958	5.8055002	Dry	B
Rocky	217.35938	5.8055002	Rocky	C
Sandy	307.94792	5.8055002	Sandy	A

By SX64

field	Least Sq Mean	Std Error	Tukey's test	
Dry	252.03646	7.8390971	Dry	B
Rocky	230.96354	7.8390971	Rocky	B
Sandy	290.61458	7.8390971	Sandy	A

By Dry

cultivar	Least Sq Mean	Std Error	Student's T-test	
SX64	252.03646	7.6478295	SX64	A
SX61	241.98958	7.6478295	SX61	A

Table SII.2.IV: 2011 Height (cm) ANOVA predicted values and test results (continued)

By Rocky

cultivar				
cultivar	**Least Sq Mean**	**Std Error**	**Student's T-test**	
SX64	230.96354	3.7392427	SX64	A
SX61	217.35938	3.7392427	SX61	B

By Sandy

cultivar	Least Sq Mean	Std Error	Student's T-test	
SX64	290.61458	8.3822240	SX64	B
SX61	307.94792	8.3822240	SX61	A

Note: different letters indicate better than 0.05 p-value difference between means. Data transformed for the analysis but not before generating this table, to allow comparison with other agricultural and forestry work.

Table SII.2.V: 2012 Height ANOVA results (LOG transformed)

Source	Nparm	DF	DFDen	F Ratio	Prob > F
field	2	2	29	69.0521	<.0001*
inoc	1	1	29	0.0321	0.8590
field*inoc	2	2	29	1.7677	0.1886
fert	1	1	29	34.4261	<.0001*
field*fert	2	2	29	0.3813	0.6863
inoc*fert	1	1	29	1.7255	0.1993
field*inoc*fert	2	2	29	0.5438	0.5863
cultivar	1	1	29	1.3861	0.2486
field*cultivar	2	2	29	1.9688	0.1578
inoc*cultivar	1	1	29	0.1091	0.7436
field*inoc*cultivar	2	2	29	0.3310	0.7209
fert*cultivar	1	1	29	3.9326	0.0569
field*fert*cultivar	2	2	29	1.9303	0.1633
inoc*fert*cultivar	1	1	29	0.6383	0.4308
field*inoc*fert*cultivar	2	2	29	1.2489	0.3018

Note: All combinations of the block treatment, by itself and with the other treatment variables were part of the model, but block was treated differently since it was nested in the field variable and labeled as a random attribute. It therefore does not appear in this table.

Table SII.2.VI: 2012 Height cm ANOVA predicted values and test results

field	Least Sq Mean	Std Error	Tukey's test	
Dry	355.32292	4.6853161	Dry	A
Rocky	287.23958	4.6853161	Rocky	A
Sandy	358.76563	5.7383168	Sandy	A

inoculation	Least Sq Mean	Std Error	Student's T-test	
no	333.22917	4.5282070	not inoculated	A
yes	334.32292	4.5282070	inoculated	A

nitrogen	Least Sq Mean	Std Error	Student's T-test		
fertilized	346.47917	3.6574027	fertilized	A	
unfertilized	321.07292	3.6574027	unfertilized		B

cultivar	Least Sq Mean	Std Error	Student's T-test	
SX64	330.07639	4.2739418	SX64	A
SX61	337.47569	4.2739418	SX61	A

Note: different letters indicate better than 0.05 p-value difference between means. Data transformed for the analysis but not before generating this table, to allow comparison with other agricultural and forestry work.

SII.3: Additional mass analyses

Table SII.3.I: 2011 Oven dry tons /ha ANOVA results (LOG transformed)

Source	Nparm	DF	DFDen	F Ratio	Prob > F
field	2	2	33	18.6140	<.0001*
inoc	1	1	33	0.9277	0.3425
field*inoc	2	2	33	0.0934	0.9110
fert	1	1	33	58.7500	<.0001*
field*fert	2	2	33	4.3614	0.0208*
inoc*fert	1	1	33	0.0399	0.8429
field*inoc*fert	2	2	33	0.2666	0.7676
cultivar	1	1	33	1.8588	0.1820
field*cultivar	2	2	33	0.5180	0.6005
inoc*cultivar	1	1	33	1.9361	0.1734
field*inoc*cultivar	2	2	33	0.2127	0.8095
fert*cultivar	1	1	33	1.0404	0.3153
field*fert*cultivar	2	2	33	0.2383	0.7893
inoc*fert*cultivar	1	1	33	3.8483	0.0582
field*inoc*fert*cultivar	2	2	33	0.5025	0.6096

Note: All combinations of the block treatment, by itself and with the other treatment variables were part of the model, but block was treated differently since it was nested in the field variable and labeled as a random attribute. It therefore does not appear in this table.

Table SII.3.II: 2011 Oven dry tons /ha ANOVA predicted values and test results

inoculation	Least Sq Mean	Std Error	Student's T-test	
no	3.2559861	0.18766585	not inoculated	A
yes	3.3419896	0.18759477	inoculated	A

cultivar	Least Sq Mean	Std Error	Student's T-test	
SX64	3.3655104	0.19033297	SX64	A
SX61	3.2324653	0.19026289	SX61	A

By fertilized

field	Least Sq Mean	Std Error	Tukey's test		
Dry	2.9263348	0.34074839	Dry		B
Rocky	2.8243854	0.34030058	Rocky		B
Sandy	5.7627500	0.34030058	Sandy	A	

By unfertilized

field	Least Sq Mean	Std Error	Tukey's test		
Dry	2.5881667	0.25714174	Dry		B
Rocky	1.8655000	0.25714174	Rocky		B
Sandy	3.8195625	0.25714174	Sandy	A	

By Dry

nitrogen	Least Sq Mean	Std Error	Student's T-test	
fertilized	2.9287531	0.27895837	fertilized	A
unfertilized	2.5881667	0.27869378	unfertilized	A

Table SII.3.II: 2011 Oven dry tons /ha ANOVA predicted values and test results (continued)

By Rocky

nitrogen	Least Sq Mean	Std Error	Student's T-test		
fertilized	2.8243854	0.17892886	fertilized	A	
unfertilized	1.8655000	0.17892886	unfertilized		B

Sandy

nitrogen	Least Sq Mean	Std Error	Student's T-test		
fertilized	5.7627500	0.40362255	fertilized	A	
unfertilized	3.8195625	0.40362255	unfertilized		B

Note: different letters indicate better than 0.05 p-value difference between means. Data transformed for the analysis but not before generating this table, to allow comparison with other agricultural and forestry work.

Table SII.3.III: 2012 Oven dry tons /ha ANOVA results (LOG transformed)

Source	Nparm	DF	DFDen	F Ratio	Prob > F
field	2	2	29	18.7248	<.0001*
cultivar	1	1	29	4.5113	0.0423*
field*cultivar	2	2	29	3.1593	0.0574
fert	1	1	29	24.2716	<.0001*
field*fert	2	2	29	0.3974	0.6757
cultivar*fert	1	1	29	0.0001	0.9921
field*cultivar*fert	2	2	29	1.1389	0.3341
inoc	1	1	29	0.1710	0.6823
field*inoc	2	2	29	0.9470	0.3996
cultivar*inoc	1	1	29	0.0881	0.7688
field*cultivar*inoc	2	2	29	0.1770	0.8387
fert*inoc	1	1	29	0.0401	0.8426
field*fert*inoc	2	2	29	0.7959	0.4608
cultivar*fert*inoc	1	1	29	0.2776	0.6023
field*cultivar*fert*inoc	2	2	29	0.0050	0.9950

Note: All combinations of the block treatment, by itself and with the other treatment variables were part of the model, but block was treated differently since it was nested in the field variable and labeled as a random attribute. It therefore does not appear in this table.

Table SII.3.IV: 2012 Oven dry tons /ha ANOVA predicted values and test results

field	Least Sq Mean	Std Error	Tukey's test		
Dry	9.146802	0.42706946	Dry	A	
Rocky	6.755115	0.42706946	Rocky		B
Sandy	10.650906	0.52305114	Sandy	A	

inoculation	Least Sq Mean	Std Error	Student's T-test	
no	9.1420208	0.43277771	not inoculated	A
yes	8.5598611	0.43277771	inoculated	A

nitrogen	Least Sq Mean	Std Error	Student's T-test		
fertilized	9.8563368	0.34441827	fertilized	A	
unfertilized	7.8455451	0.34441827	unfertilized		B

cultivar	Least Sq Mean	Std Error	Student's T-test	
SX64	9.3320451	0.37632919	SX64	A
SX61	8.3698368	0.37632919	SX61	A

Note: different letters indicate better than 0.05 p-value difference between means. These values represent two seasons of growth. Data transformed for the analysis but not before generating this table, to allow comparison with other agricultural and forestry work.

Chapter III: Exploration of willow-associated fungal communities in short-rotation coppice fields

(A preliminary draft for the introduction and a portion of the materials and methods sections were contributed by K Abram with the assistance of T Bell, during an initial period of planned coauthorship, but all subsequent work and writing were done by TJ Pray. K Abram was also the lab technician who did the preparatory work for Illumina MiSeq sequencing as well as the initial sequence data processing under the direction of T Bell and TJ Pray. After moving, K Abram decided not to continue as a coauthor. The framework for the project was proposed by M St-Arnaud and M Labrecque, in the initial proposal to the *Programme de soutien à l'innovation en agroalimentaire* of the *Ministère de l'agriculture, des pêcheries et de l'alimentation du Québec*, but final design and implementation were done by TJ Pray. As thesis advisors, M St-Arnaud and M Labrecque gave advice and input throughout the project, as well as suggested edits and proof-reading of the document, but the final product represents the contributions and decisions made by TJ Pray.)

Abstract

Mycorrhizal fungi, the symbiotic fungi living in and attached to plants' roots, are key ecosystem components. They are an important bridge between below ground nutrient resources and above ground biomass. Understanding what determines the particular mix of mycorrhizal species in an area is one key to making new breakthroughs in any field that manages areas of land, such as habitat restoration and conservation, or agriculture. This study examined the mycorrhizal population inhabiting the rhizospheres of short-rotation coppice shrub willows—two cultivars of *Salix miyabeana*—in three different fields at a farm in southern Quebec, Canada. Our study used MiSeq Illumina sequencing of the ITS region, on DNA extracted from 96 soil rhizospheric samples and identified 702 unique fungal operational taxonomic units (OTUs). The majority of these OTUs did not match known species in the NCBI database, indicating they are yet unstudied, but of those that were identifiable almost half were ectomycorrhiza. Our findings are further evidence that the lists of species commercial mycorrhizal inocula are drawn from are not those species dominant under field conditions. Also, the fact that not one arbuscular mycorrhizal OTU was found suggests shrub willows

under many conditions tend to preferentially associate with ectomycorrhiza. Finally, the soil characteristics of our fields appeared to be the biggest determinant of mycorrhizal species composition. A marked shift in fungal population was seen that correlated with soil texture differences between the field we labeled "Sandy" and the other two. Plant host did not control fungal identity, as different species of fungi were found on the many-planted clones of the same two cultivars. Neither pH nor nitrogen fertilization correlated with any marked shifts in mycorrhizal association.

III.1. Introduction

The soil microbiome is central to soil fertility, and affects both crop productivity and cropping security (Rooney *et al*. 2009). Mycorrhizal fungi are a key component of the soil microbiome (van der Heijden *et al.* 2015), colonizing the roots of their host plant, and exchanging soil-derived nutrients for carbohydrates provided by the plant host (Smith and Read 1997). *Salix* spp. are able to host both arbuscular mycorrhizal (AM) and ectomycorrhizal (EM) fungi (Khan 1993). AM fungi form tree-like hyphal structures (arbuscules) within root cells, while EM fungi are characterized by extensive hyphal growth between cortical root cells and a sheath that envelops the root tip (Smith and Read 1997).

Soil texture and moisture availability appear to be major factors that drive differences in AM fungal communities (Moebius-Clune *et al*. 2013). Different soil types, ranging from fine sands to compact clay soils, can be used in coppicing systems (Crow and Houston 2004). Therefore, exploration of fungal communities in different soil types in our experimental fields is a first step towards separating the effects of host plant communities on fungal distribution from the influence of soil characteristics. We used three different fields in this experiment. In all three, weeds were controlled and the same two willow cultivars planted, allowing us to focus on the relative influence of soil parameters and nitrogen fertilization on willows treated with mycorrhizal inocula.

Fertilization studies have shown that nitrogen enrichment can have strong effects on AM fungal community dynamics (Egerton-Warburton and Allen 2000). Empirical field and greenhouse research indicates that nitrogen fertilization can be associated with an increase (Heijne *et al*. 1992, 1994) or decrease (Hayman 1982) in root infection, a reduction in AM spore abundance and species diversity (Hayman 1970, Johnson *et al*. 1991), and selection for aggressive, possibly less effective,

mutualist fungi (Johnson 1993). Nitrogen enrichment is also associated with a decrease in EM root infection (Dighton and Jansen 1991).

Using Illumina MiSeq sequencing of ITS amplicons and measurements of plant and soil characteristics, we studied the composition of native fungal communities under different willow coppicing treatments in the field. More specifically, we aimed to (1) identify native mycorrhizal fungi (both AM and EM) supported by willows planted on different marginal agricultural soils, and (2) investigate the effect of fertilization and soil type on fungal community composition directly linked to plant productivity.

III.2. Materials and methods

III.2.1. Experimental design

Three experimental fields were set up in June of 2010 in Saint-Roch-de-l'Achigan, QC, Canada (N45.848783, W73.674546) as described in chapter II. Briefly, the experiment used a hierarchical design with inoculation treatments randomized first, cultivars randomized second, and fertilization treatments randomized third, that was repeated across three fields (given the descriptive names in our experiment of Sandy, Rocky, and Dry and described in table III.I). The inoculum species were *Rhizoglomus irregulare* and *Hebeloma longicaudum*, an AM and an EM fungi respectively, and the shrub willow cultivars used were *Salix miyabeana,* Seeman (SX61 and SX64). During the second growing season, in May, half of the trees receive nitrogen fertilization (75 kg/ha N, as pelleted chicken feather compost—Fertilec's 12-0-0 "Farine de Plume"—scattered by hand).

III.2.2. Harvest of soil and plant materials

Ninety-six root samples were harvested in October 2011, after the second growing season (four replicates of the 24 treatment combinations, selected from four randomly chosen blocks over the twelve full blocks repeated in each field). Because root systems were too large to dig up entirely, several sections of the last several cm of root ends, each with their many branching off root tips, were dug up from around each selected willow (taking care to ensure that the roots could be followed back to below the main stems). These root samples were shaken free of bulk soil and stored with desiccant at 3 °C for a few weeks (for later microbial culturing to be done in parallel with the DNA sequencing), and then frozen until further processing and DNA extraction.

III.2.3. Soil DNA extraction, ITS amplification, and Illumina MiSeq sequencing

Dried unwashed roots were thawed, and the soil worked free by hand and separated from the roots. One gram of rhizospheric soil was weighed from each sample for extraction. DNA was extracted using MoBio Laboratories PowerSoil Extraction kit according to the manufacturer's instructions with one modification. Instead of the standard homogenization and lysis with a vortexer in the first step, an MP Biomedicals FastPrep machine at setting 4 was used for 25s and six repetitions. Amplification of DNA extracts and subsequent processing for sequencing was performed following primarily the Illumina 16S Metagenomic Sequencing Library Preparation guide. We performed initial amplifications of soil DNA extracts using the primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and 58A2R (5'-CTGCGTTCTTCATCGAT-3') (Gardes and Bruns, 1993; Martin and Rygiewicz, 2005), containing the required Illumina adaptors at the 5' end of the primer sequences (5' - TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG - 3' for the forward primer and 5' - GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG - 3' for the reverse primer).

PCR products were cleaned using NucleoMag NGS Clean-Up and Size Select beads (Macherey-Nagel, Bethlehem, PA). We transferred 2.5 µl of this product to a new 96-well plate, and added 5 ul each of 5'- and 3'-targeted Index Primers (a unique combination for each sample to allow *in silico* differentiation), as well as 12.5 µl of 2x KAPA HiFi HotStart ReadyMix, and 5 µl of water (total volume of 25 µl per sample). We performed PCR amplifications using the following conditions: 3 min at 95 °C, 8 cycles of 30 s at 95 °C, 30 s at 55 °C, and 45 s at 72 °C, and a final elongation step of 5 min at 72 °C. PCR products were again cleaned with NucleoMag beads, quantified using a Qubit Fluorometer (Life Technologies, Burlington, ON, Canada), and combined in an equimolar ratio. This final product was run out on a 1.2% agarose gel, cut to isolate only the prominent band at the expected size, and purified using the PureLink Quick Gel Extraction Kit (Life Technologies). This final pool was sequenced on an Illumina MiSeq using one 600-cycle MiSeq Reagent Kit v.3, following the manufacturer's recommendations.

III.2.4. Sequence processing

Processing of ITS sequences followed the Brazilian Microbiome Project (BMP) ITS pipeline (Pylro *et al.* 2014), after the initial processing steps suggested in Mothur v.1.32.1 (Schloss *et al.*

2011). Following Mothur, we merged paired-end reads with 'make.contigs'. Primers were trimmed using 'trim.seqs' (pdiffs=2, maxambig=0), and the group sequences matching the trimmed fasta were obtained with 'list.seqs' followed by 'get.seqs'. We removed singletons using 'unique.seqs' followed by 'split.abund' (cutoff=1), and we repopulated the fasta with all of the original sequences, minus those identified as singletons, using 'deunique.seqs'. We split the single fasta into separate files for each sample to facilitate naming in QIIME (Caporaso *et al.* 2010; MacQIIME v.1.8.0), and the command 'add_qiime_labels.py' was used to name and merge individual files. After this, we followed the steps described in the BMP pipeline (starting at step 3; http://www.brmicrobiome.org/#!its-profiling-illumina/c22js; accessed March 2015). The resulting OTU table was uploaded to Excel v.12.3.6 and R v.3.0.2 (2013) for further analyses.

III.2.5. Graphical and statistical analysis

Of the 96 samples, 13 did not develop usable sequences. Several of these were from Sandy field, but enough samples from each treatment combination did work, to allow a complete analysis. Principal coordinates analyses (PCoA) of sequence matrices were performed using the statistical language R (v 3.0.2, The R Foundation for Statistical Computing), and were performed on Bray–Curtis distances using the function 'cmdscale' in the 'stats' package.

The number of sequence reads for each identified OTU was converted in Excel to a percentage of total usable reads in each sample, to give an estimate of relative abundance. These relative abundance percentages were averaged together for each OTU to get an idea of total diversity, and tables were made to display this information. To make the tables more readable, Table III.II employed a cutoff of 0.40%. Those OTUs that represented less than that percentage of the community as a function of total sequence reads were left out. Supplementary Table SIII.1.II used a cutoff of 0.01% making it more complete, but harder to use. Other tables showing total diversity were made listing only those OTUs that could be identified to the genus and species level. Table III.III was made using a cutoff of 0.002% to show the most interesting of these rarer, but information rich OTUs.

The same relative abundance percentages for each OTU in each sample were also averaged with all the other samples by field. This allowed combined relative abundances to be compared between the three fields. In order to be easily read in 100% stacked column chart form, Figure III.1 shows totals that added all the OTUs in a known fungal Order together. For some of the OTUs that

were not known to the Order level, they were added together into a column with others in their Phylum. Figure SIII.1.1 displays another attempt at constructing a 100% stacked column of our data by field, showing the most information possible without overwhelming the image. Only the dominant 50 OTUs were used to create the figure, and their values were added together by Family and Genus when possible.

III.3. Results

Even after rigorous screening for quality and elimination of singleton sequences, 1,482 different operational taxonomic units (OTUs) were found in rhizospheric soil samples from the willow field experiment (containing ~4,230,000 sequences). However, only 702 OTUs could be assigned a fungal identity by the pipeline database (these did contain almost 94% of the sequences, or ~3,970,000). Furthermore, 264 OTUs (containing ~15% of the total sequences) of the 702 OTUs that could be identified as belonging to the fungal kingdom remained unclassified at the phylum level. This fraction of unknown fungi was not concentrated in a few bizzare or unusual samples. When rhizospheric samples were looked at on an individual basis, close to ~15% of the fungal sequences in each samples could not be identified at the phylum level.

Table III.II lists the OTUs that dominate the communities in the experiment's fields. By far the most prevalent organism with ~26% of the sequences on average is an unclassified fungus in the Pyronemataceae family. It is in the Pezizales order, and is in the Ascomycota phylum, but cannot be identified as ectomycorrhizal (EM) without at least genus identification. It is however very closely related to *Geopora sepulta*, which makes up ~2% of our sample's sequences, and that is a known EM genus. Many other unclassified OTUs (see Table III.II) are not in the same Pyronemataceae family, but are in the same Pezizales order as this known EM. It is also in the same family as *Sphaerosporella brunnea*, found among the dominant OTUs associated with willows roots at both Varennes and Valcartier (Bell et al 2014, 2015).

Similarly, Table III.II lists an unclassified fungus in the Cortinariaceae family that makes up a little under ~11% of our samples. This fungus in the Agaricale order and the Basidiomycota phylum is closely related to *Inocybe curvipes* and *Inocybe lacera* var. *lacera*. Both are present in our samples at ~4% and ~2% respectively, and both are in a known EM genus. Also of interest are the several OTUs listed in Table III.II in the same Cortinariaceae family that were assigned uncultured *Hebeloma*

labels by the pipeline database. This can only be considered speculative, based on the morphology of the reference's mushroom or mycorrhizal root tips, but *Hebeloma* is a known EM and the label should be noted.

Table III.III lists the 19 OTUs that were identifiable to species level, and were present in our samples at least at 0.002% or greater on average. This list actually consists of 16 unique species, as three OTUs are duplicates of other reference species in the list, despite the greater than 3% difference in their ITS sequences. Only 6 out of the 19 OTUs are of known EM genus, but a further 2 OTUs are of genus that have been tentatively identified as having EM members in the past.

Figure III.1 shows the proportion of sequences in each Order by field (using the average of the many samples from each). The fraction of unclassified fungi can be seen at the bottom of each stacked column. Two other small fractions (less than 5%) show those that cannot be identified within an Order but were identified at the Phylum level, as well as numerous OTUs that were identified at the Order level, but were combined with the unclassified as "other" due to their very small number of sequences (generally around $1/10^{th}$ of a percent of the samples on average, and no more than 1 percent of the samples on average).

Figure III.1 shows a marked shift between Dry and Rocky fields, which have very similarly proportioned rhizospheric fungal communities, and Sandy field, which has quite a different one. Two fungal Orders dominate the willow fields in our experiment, Agaricales in the Basidiomycota phylum, and Pezizales in the Ascomycota phylum. However, in Dry and Rocky fields, Pezizales outnumbers Agaricales by almost 7:1. However, in Sandy field this proportion is reversed, and Agaricales outnumbers Pezizales by a similar factor.

This shift between fields can also be seen in Figure III.2, a principal coordinates analysis (PCoA), which mathematically displays the statistical location of each sample on a two-axis graph based on the identity and proportion of sequences in each sample. Samples from the sandy field cluster somewhat linearly in the upper left, while Dry and Rocky field samples cluster together diagonally from the top right down to the center.

Figure III.3 is the same PCoA, but it labels the samples by fertilization treatment instead of by field. It is evident that the two treatments are scattered equally throughout the graph, and that fertilization did not have an effect on the overall fungal community composition in our experiment willow's rhizospheres. Similarly, willow rhizosphere fungal communities in our experiment were not

clearly shifted by either inoculation or willow cultivar. Since those PCoAs look almost identical to the one showing fertilization, the figures can be found in the supplementary data section as Figure SIII.1.2 and SIII.1.3.

III.4. Discussion

The most dominant fungal sequences were unclassifiable at the genus and species level. Many others were unclassifiable at the family, order, class, and even phylum level. This is noteworthy because it means the fungi recruited by the willows and physiogically linked to them, those in the rhizospheric soil, have probably never been studied closely or cultured. Other experiments have shown that the most frequent organisms isolated with traditional techniques are not those dominating in the soil, as found by sequencing soil DNA using next generation sequencing (Stefani *et al*. 2015, Bell *et al*. 2015). It would follow that commercially available inocula, as species that are easier to isolate and grow in the lab, have neither the same composition nor the same diversity as the communities that dominate healthy soils full of native organisms.

No AM fungi were found in our samples. This was surprising, given that we did inoculate an AM species. Also, cloning-sequencing with AM specific primers during the first year had found a diversity of AM fungi (Pray *et al*. 2016). Perhaps AM found the first year were merely spores left from historical land use and neighboring forest land (nested AM primers as described in Pray *et al.* can find rare sequences). Another reason could be that many more samples were dug up and extracted the second year and MiSeq data of unnested ITS amplicons should be more reliable. The lack of AM fungi in these second year samples does reinforce the perception in scientific literature that willows predominantly associate with EM fungi (Hashimoto and Higuchi 2003, Puettsepp *et al.* 2004, Milne *et al.* 2006, Paradi and Baar 2006, Ryberg *et al*. 2011).

Because the ITS gene does not differentiate AM fungi very well, there are probably fewer AM (Glomeromycota) sequences represented in the database. This raises the possibility that some of the completely unclassifiable fungi we found are in fact Glomerales or less well known Diversisporales or Archeosporales.

It is tempting to believe that the majority of the rhizospheric soil fungi we found are mycorrhizal. Doubtless some are endophytes that inhabit the rhizosphere without benefit or harm to the plants. Saprobes and disease fungi cannot be ruled out. Almost 100 individual plants were

sampled, though. Furthermore, the roots and the stems of the willows were visibly healthy. It is unlikely then, that the majority of our sequences would be disease fungi. The results suggestively agree with this logic, as seen in Table III.II the majority of our sequences were in the same Family as known mycorrhizal genera. Focusing on known species, as in Table III.III, demonstrates a similar trend even if those of known species were minor components scattered throughout the community. Despite the strong circumstantial evidence, the mycorrhizal identity of many of the fungi cannot be stated with certainty, and this is an area in need of further investigation.

Unexpectedly, as seen in Figure III.3 nitrogen fertilization did not have an effect on the rhizospheric soil fungal community. Under certain conditions, researchers report strong effects from fertilization on mycorrhizal communities (Beauregard *et al*. 2010). Perhaps those species adapted or drawn to actively farmed fields, and regularly exposed to nutrient additions, are generally unaffected by differences in fertilization. In the first year of the project, Pray *et al*. (2016) reported some effect from the inoculum on the community makeup of rhizospheric fungi. This effect was not very strong, however, and the inoculum itself did not appear to take hold. There were some closely related sequences found that first year, though, and it was not absolutely certain that the inoculum was not present but misidentified. The lack of the inoculum in any measurable form by the second year, nor even some impact left on the makeup of the rhizospheric fungal community, is clearly demonstrated by the second year data. Both the passage of time—allowing indigenous fungal populations to dominate—and the more thorough methods applied in year two may be responsible for this clearer "negative" result. There was no evidence either in Pray *et al*. (2016) that cultivar had any effect on the rhizospheric fungal community, and so it was not surprising no effect was found in this second year data.

Field type was the greatest determinant of fungal community structure in the experiment. This agrees with other research that pinpointed soil texture as the determining factor (Moebius-Clune *et al*. 2013, Jansa *et al.* 2014), potentially because of the unique mycorrhizal communities supported in different soil types. Variations in other factors, such as local water table levels, may also contribute. Soil moisture probes were installed at the beginning of the experiment, but only at a depth of 30 cm. It became apparent by the end of the first year (and even more so by the beginning of the second) that the reach of the willow roots extended well past this mark. Also, it was suspected that the water table fluctuated in meters rather than centimeters, as periodically evidenced by standing water at the bottom

of a large ditch alongside Dry field, and standing pools of water alongside Sandy field (for these reasons, the inconclusive soil moisture data is not reported). Sandy field was also proximate to a forested lot along one edge. Potentially, wind and animal movements of soil could transport fungi from the forest to the field, but such movement is limited (Dickie and Reich 2005). Although there is no reason to believe such transported fungi would take hold in the very different habitat of a cultivated field, such effects undoubtedly influence the natural mycorrhizal communities as well. Among these qualitative variables, as well as quantitative variables such as total phosphorus and pH (all listed in Table III.1), the only variable that correlated unequivocally with the community shift seen in Figures III.1 and III.2 was soil texture. Dry and Rocky fields were quite different in total phosphorus, and they still had almost identical communities of rhizospheric fungi. The three fields were very different in pH as well, but a shift in fungal population did not occur between Dry and Rocky fields, even with their ten-fold difference in proton concentration.

In conclusion, these results highlight the pressing need to complement laboratory and greenhouse-based knowledge of mycorrhiza with field-based findings. The sheer number of native rhizospheric fungi this study turned up in farm soil, that were previously unknown and currently unculturable, is exciting. The evidence for a very strong soil texture influence on mycorrhiza, while tentative, is promising enough to demand immediate further research. If it holds up, any future efforts at mycorrhizal inoculation or manipulation in agriculture must take it into account. The same would be true for any other soil ecology-based breakthroughs in land management.

(to be published with co-authors Michel Labrecque[1], Marc St-Arnaud[1])

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Table III.I: Characterization of the three field sites, including a soil analysis at two depths

Name (depth)	pH	Nitrate	Total Phosphorus	Organic matter	Clay	Silt	Sand	Soil Type
field Dry (N45.825276, E-73.624675)								
(0-20 cm)	7.1	5.77 ppm	130 kg/ha	4.0%	21.0%	40.9%	38.1%	Medium loam
(20-40 cm)	7.3	7.35 ppm	81 kg/ha	3.9%	19.9%	32.7%	47.4%	(edging towards sandy loam)
Principal qualitative features: heavily drained with a 0.5m deep ditch along its west side and a 2m deep channel along its south side.								
field Rocky (N45.827205, E-73.626789)								
(0-20 cm)	7.9	6.87 ppm	63 kg/ha	3.5%	29.5%	39.2%	31.3%	Medium loam
(20-40 cm)	7.9	5.77 ppm	39 kg/ha	2.8%	24.1%	43.5%	32.4%	(edging towards clay loam)
Principal qualitative features: ~30% of its surface covered with small and medium sized rocks (~1-5 cm).								
field Sandy (N45.825629, E-73.617733)								
(0-20 cm)	6.0	5.35 ppm	256 kg/ha	2.1%	2.5%	10.4%	87.1%	Loamy sand
(20-40 cm)	6.1	5.91 ppm	192 kg/ha	2.0%	3.4%	6.8%	89.8%	(very close to pure sand)
Principal qualitative features: far removed from any drainage, it often had standing water for a week at a time after any rainfall, between the field and the forest boardering its south side and the southern half of its east side.								

Note: all samples tested <0.2% total nitrogen—below detection level. Table modified and expanded from Table II.I in chapter II.

Table III.II: Identity of main fungal OTUs (comprising ~94% of the community) using the ITS gene, found in willow rhizosphere samples

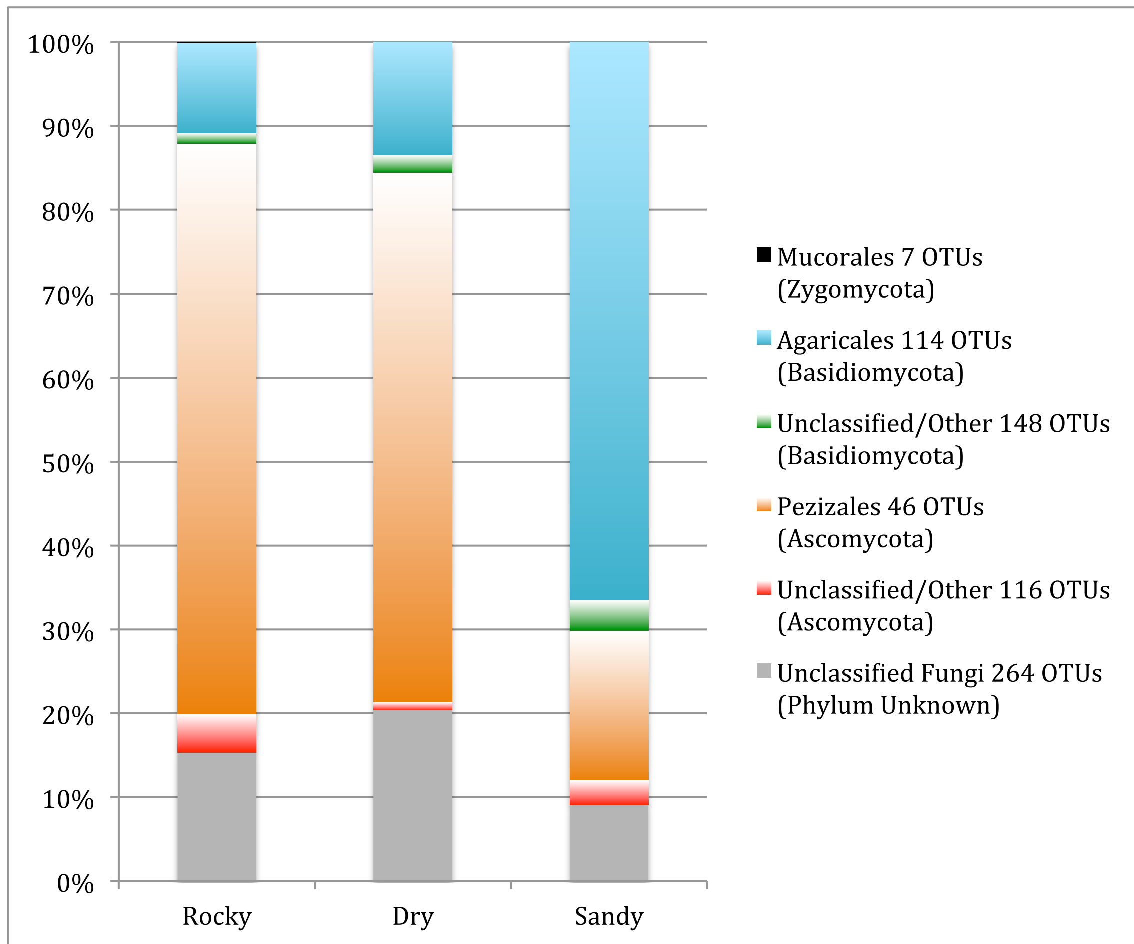
OTU Designation	Phylum	Class	Order	Family	Genus and Species/Label	Average
OTU879013980	Ascomycota	Leotiomycetes	Helotiales	Incertae_sedis	*Cadophora* unclassified	0.48688%
OTU568789266	Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	*Geopora sepulta*	1.67795%
OTU819080307	Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	Unclassified	26.34384%
OTU253682147	Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	Unclassified uncultured *Geopora*	0.87242%
OTU238480107	Ascomycota	Pezizomycetes	Pezizales	Unclassified		9.96437%
OTU50310567	Ascomycota	Pezizomycetes	Pezizales	Unclassified		6.57832%
OTU40225823	Ascomycota	Pezizomycetes	Pezizales	Unclassified		2.30437%
OTU446082359	Ascomycota	Pezizomycetes	Pezizales	Unclassified		1.59378%
OTU250957214	Ascomycota	Pezizomycetes	Pezizales	Unclassified		0.73486%
(20 OTUs)	Ascomycota	Pezizomycetes	Pezizales	Unclassified		0.49225%
OTU531110773	Ascomycota	Pezizomycetes	Pezizales	Unclassified		0.43645%
OTU692506221	Ascomycota	Sordariomycetes	Xylariales	Amphisphaeriaceae	*Truncatella angustata*	0.50808%
(26 OTUs)	Ascomycota	Unclassified				0.57585%
OTU86348303	Basidiomycota	Agaricomycetes	Agaricales	Cortinariaceae	*Inocybe curvipes*	3.74216%
OTU694722950	Basidiomycota	Agaricomycetes	Agaricales	Cortinariaceae	*Inocybe lacera* var. *lacera*	2.02034%
OTU652930571	Basidiomycota	Agaricomycetes	Agaricales	Cortinariaceae	Unclassified	10.51350%
OTU896147191	Basidiomycota	Agaricomycetes	Agaricales	Cortinariaceae	Unclassified uncultured *Hebeloma*	3.63312%
OTU237953421	Basidiomycota	Agaricomycetes	Agaricales	Cortinariaceae	Unclassified uncultured *Hebeloma*	1.48969%
OTU984249233	Basidiomycota	Agaricomycetes	Agaricales	Cortinariaceae	Unclassified uncultured *Hebeloma*	0.95373%
OTU364029024	Basidiomycota	Agaricomycetes	Agaricales	Cortinariaceae	Unclassified uncultured *Hebeloma*	0.69246%
OTU578686797	Basidiomycota	Agaricomycetes	Agaricales	Unclassified		1.15163%
(56 OTUs)	Basidiomycota	Unclassified				0.40042%
OTU925616399	Basidiomycota	Unclassified				0.71206%
(264 OTUs)	Unclassified					15.87007%

Table III.III: Identity of dominant fungal OTUs recognized with species names, using the ITS gene, found in willow rhizosphere samples.

OTU Designation	Phylum	Class	Order	Family	Genus and Species	Average
OTU195883303	Basidiomycota	Agaricomycetes	Agaricales	Bolbitiaceae	**Alnicola tantilla*	0.01103%
OTU248475881	Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	*Cladophialophora chaetospira*	0.01754%
OTU522652877	Basidiomycota	Agaricomycetes	Agaricales	Nidulariaceae	*Cyathus stercoreus*	0.05555%
OTU100449810	Ascomycota	Sordariomycetes	Diaporthales	Valsaceae	*Cytospora chrysosperma*	0.06720%
OTU568789266	Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	**Geopora sepulta*	1.67795%
(OTU436068696)	(Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae)	(**Geopora sepulta*)	(0.18389%)
OTU153963325	Basidiomycota	Agaricomycetes	Boletales	Hymenogasteraceae	*[?] *Hymenogaster vulgaris*	0.01236%
OTU86348303	Basidiomycota	Agaricomycetes	Agaricales	Cortinariaceae	**Inocybe curvipes*	3.74216%
OTU694722950	Basidiomycota	Agaricomycetes	Agaricales	Cortinariaceae	**Inocybe lacera* var. *lacera*	2.02034%
(OTU209944304)	(Basidiomycota	Agaricomycetes	Agaricales	Cortinariaceae)	(**Inocybe lacera* var. *lacera*)	(0.16927%)
OTU327480802	Basidiomycota	Agaricomycetes	Agaricales	Bolbitiaceae	*Naucoria salicis*	0.03915%
OTU743654468	Ascomycota	Dothideomycetes	Pleosporales	Montagnulaceae	*Paraconiothyrium sporulosum*	0.31531%
(OTU863735734)	(Ascomycota	Dothideomycetes	Pleosporales	Montagnulaceae)	(*Paraconiothyrium sporulosum*)	(0.09374%)
OTU773824548	Ascomycota	Pezizomycetes	Pezizales	Pezizaceae	*[?] *Peziza subcitrina*	0.13015%
OTU903697817	Basidiomycota	Agaricomycetes	Polyporales	Fomitopsidaceae	*Piptoporus betulinus*	0.00235%
OTU810640323	Basidiomycota	Ustilaginomycetes	Ustilaginales	Ustilaginaceae	*Pseudozyma prolifica*	0.06461%
OTU27178405	Zygomycota	Incertae sedis	Mucorales	Mucoraceae	*Rhizopus oryzae*	0.00271%
OTU362952107	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Talaromyces luteus*	0.00203%
OTU692506221	Ascomycota	Sordariomycetes	Xylariales	Amphisphaeriaceae	*Truncatella angustata*	0.50808%

Note: Even though this table represents relatively minor members compared to some of the unknowns from the previous table, it only contains those that were at least 0.002% of the total. Known mycorrhizal genera marked with an asterisk (*). Suspected because of mycorrhizal members within the genera marked with a question (*[?]). Duplicated species in parenthesis.

Figure III.1: ITS, rhizospheric soil, 2011, ~96 willows, showing proportion of sequences in each Order (and Phylum), all fungal sequences normalized by sample and averaged by field



Note: Plus four OTUs in the Chytridiomycota phylum, that are less than $1/1000^{th}$ of a percent of the sequences, and three other insignificant Zygomycota OTUs that are not shown.

Figure III.2: Principal coordinate analysis by field

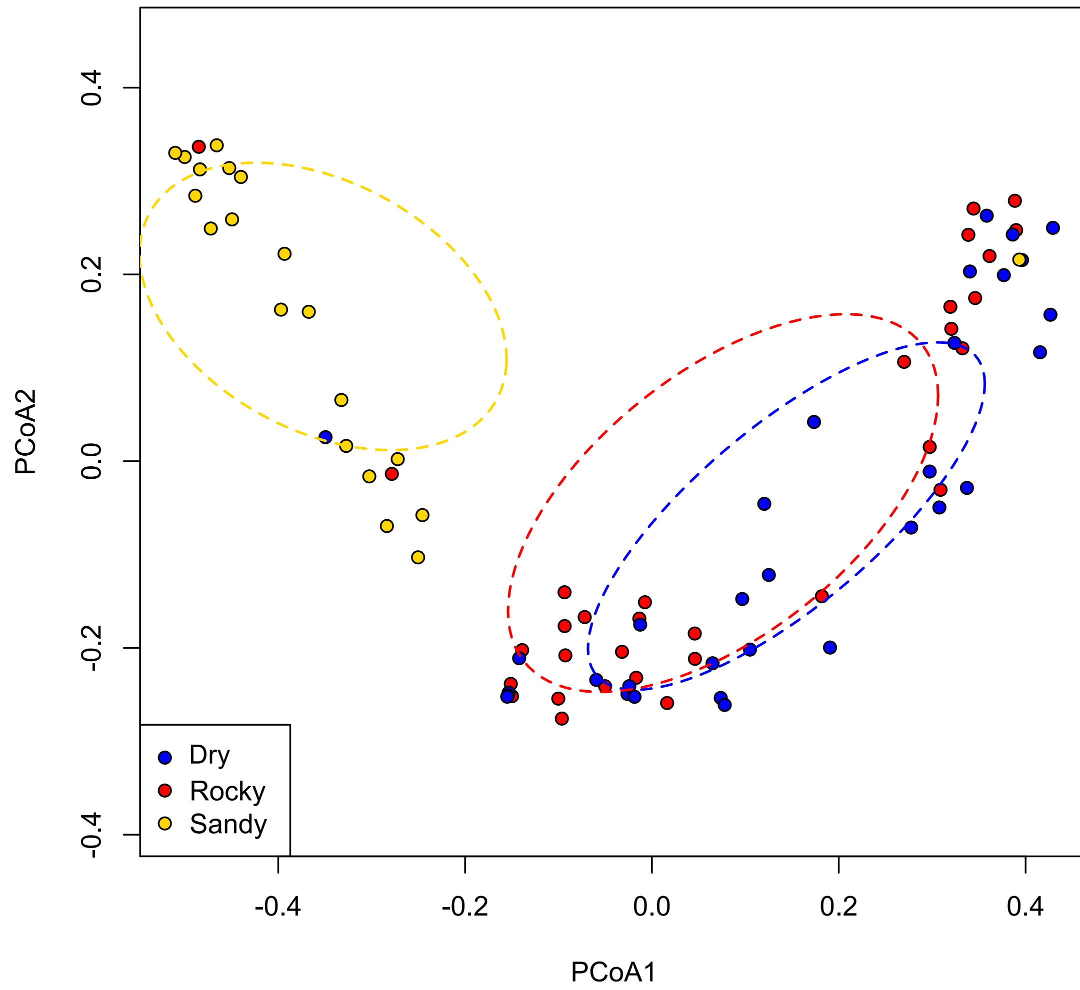
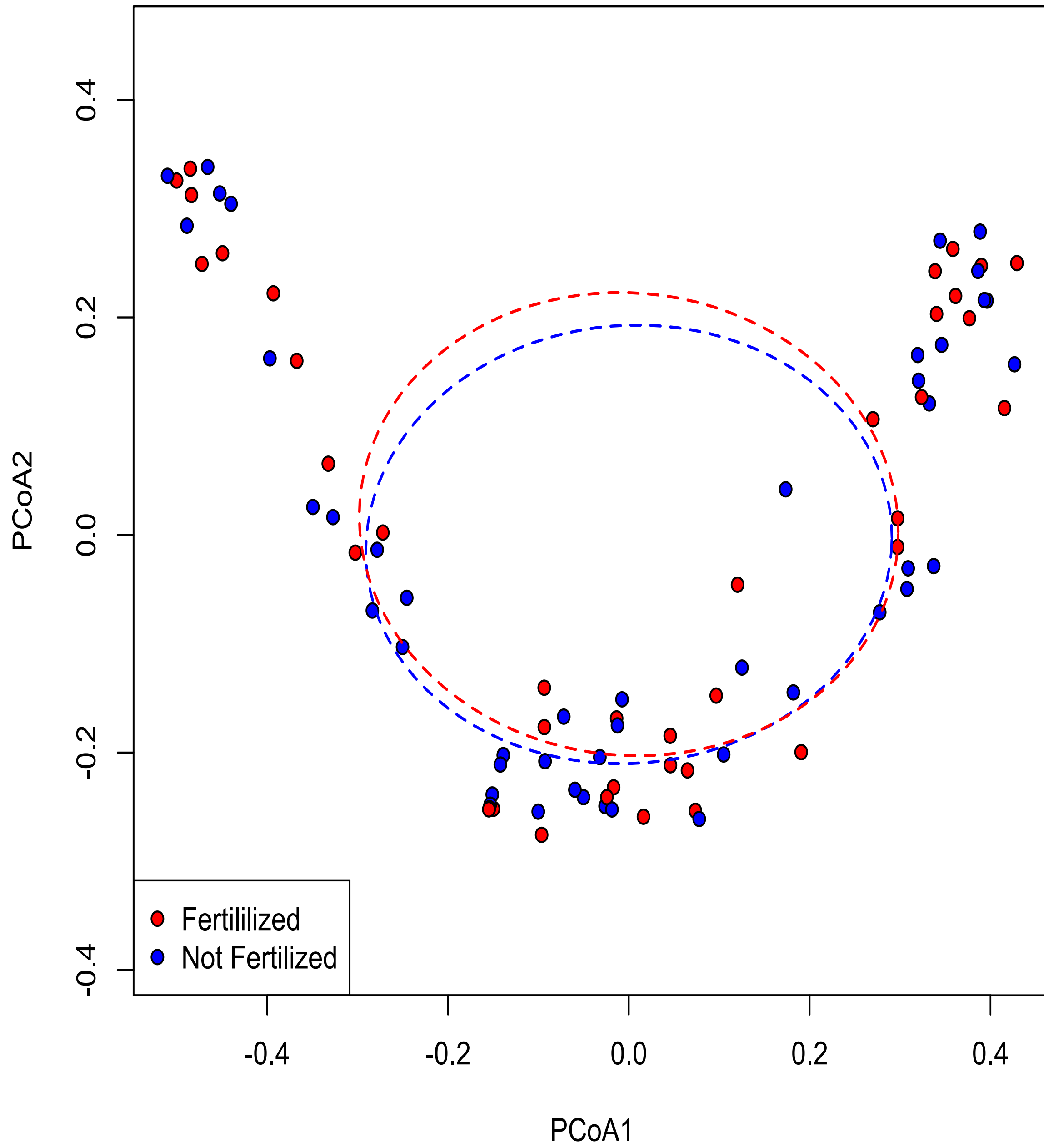


Figure III.3: Principal coordinate analysis by fertilization treatment, ITS gene with MiSeq sequencing, rhizospheric soil, 2011



Supplementary material to Chapter III

SIII.1: Additional tables and figures

Table SIII.1.I: Identity of fungal OTUs recognized with species names, using the ITS gene with MiSeq sequencing, found in willow rhizosphere samples

	Phylum	Class	Order	Family	Genus and Species	Average
OTU242438885	Basidiomycota	Agaricomycetes	Agaricales	Bolbitiaceae	*Agrocybe pusiola*	0.00067%
OTU711207212	Basidiomycota	Agaricomycetes	Russulales	Stereaceae	*Aleurodiscus aurantius*	0.00009%
OTU195883303	**Basidiomycota**	**Agaricomycetes**	**Agaricales**	**Bolbitiaceae**	***Alnicola tantilla***	**0.01103%**
OTU675118613	Ascomycota	Orbiliomycetes	Orbiliales	Orbiliaceae	*Arthrobotrys conoides*	0.00083%
OTU785190115	Ascomycota	Orbiliomycetes	Orbiliales	Orbiliaceae	*Arthrobotrys flagrans*	0.00026%
OTU442424634	Ascomycota	Orbiliomycetes	Orbiliales	Orbiliaceae	*Arthrobotrys oligospora*	0.00042%
OTU163212841	Ascomycota	Orbiliomycetes	Orbiliales	Orbiliaceae	*Arthrobotrys superba*	0.00012%
OTU863080015	Ascomycota	Eurotiomycetes	Onygenales	Arthrodermataceae	*Arthroderma uncinatum*	0.00010%
OTU125101945	Ascomycota	Sordariomycetes	Xylariales	Xylariaceae	*Biscogniauxia bartholomaei*	0.00004%
OTU984046671	Ascomycota	Eurotiomycetes	Onygenales	Onygenaceae	*Chrysosporium evolceanui*	0.00003%
OTU248475881	**Ascomycota**	**Eurotiomycetes**	**Chaetothyriales**	**Herpotrichiellaceae**	***Cladophialophora chaetospira***	**0.01754%**
OTU998936320	Zygomycota	Incertae_sedis	Kickxellales	Kickxellaceae	*Coemansia pectinata*	0.00004%
OTU740924459	Ascomycota	Dothideomycetes	Pleosporales	Leptosphaeriaceae	*Coniothyrium fuckelii*	0.00039%
OTU801630869	Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	*Coprinellus eurysporus*	0.00005%
OTU903946873	Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	*Coprinellus micaceus*	0.00026%
OTU167941583	Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	*Coprinopsis atramentaria*	0.00030%
OTU550379725	Basidiomycota	Agaricomycetes	Agaricales	Coprinaceae	*Coprinus bellulus*	0.00128%
OTU826828736	Basidiomycota	Agaricomycetes	Agaricales	Cortinariaceae	*Cortinarius anomalus*	0.00041%
OTU522652877	**Basidiomycota**	**Agaricomycetes**	**Agaricales**	**Nidulariaceae**	***Cyathus stercoreus***	**0.05555%**
OTU100449810	**Ascomycota**	**Sordariomycetes**	**Diaporthales**	**Valsaceae**	***Cytospora chrysosperma***	**0.06720%**
OTU839250985	Basidiomycota	Agaricomycetes	Agaricales	Entolomataceae	*Entoloma clandestinum*	0.00004%
OTU971515936	Basidiomycota	Agaricomycetes	Agaricales	Strophariaceae	*Flammula alnicola*	0.00104%
OTU800483531	Basidiomycota	Agaricomycetes	Agaricales	Cortinariaceae	*Galerina stylifera*	0.00006%
OTU568789266	**Ascomycota**	**Pezizomycetes**	**Pezizales**	**Pyronemataceae**	***Geopora sepulta***	**1.67795%**
OTU436068696	**Ascomycota**	**Pezizomycetes**	**Pezizales**	**Pyronemataceae**	***Geopora sepulta***	**0.18389%**
OTU546454278	Ascomycota	Leotiomycetes	Erysiphales	Erysiphaceae	*Golovinomyces cichoracearum*	0.00003%
OTU346178412	Basidiomycota	Agaricomycetes	Polyporales	Meripilaceae	*Grifola frondosa*	0.00041%
OTU870932573	Basidiomycota	Agaricomycetes	Agaricales	Lycoperdaceae	*Handkea utriformis*	0.00081%
OTU970697223	Basidiomycota	Agaricomycetes	Russulales	Hericiaceae	*Hericium coralloides*	0.00063%
OTU153963325	Basidiomycota	Agaricomycetes	Boletales	Hymenogasteraceae	*Hymenogaster vulgaris*	0.01236%
OTU387862542	Basidiomycota	Agaricomycetes	Polyporales	Hyphodermataceae	*Hyphoderma obtusiforme*	0.00010%
OTU17307192	Basidiomycota	Agaricomycetes	Polyporales	Hyphodermataceae	*Hypochnicium* sp.	0.00008%
OTU944504830	Ascomycota	Sordariomycetes	Xylariales	Xylariaceae	*Hypoxylon fragiforme*	0.00022%
OTU86348303	**Basidiomycota**	**Agaricomycetes**	**Agaricales**	**Cortinariaceae**	***Inocybe curvipes***	**3.74216%**
OTU694722950	**Basidiomycota**	**Agaricomycetes**	**Agaricales**	**Cortinariaceae**	***Inocybe lacera* var. *lacera***	**2.02034%**
OTU209944304	**Basidiomycota**	**Agaricomycetes**	**Agaricales**	**Cortinariaceae**	***Inocybe lacera* var. *lacera***	**0.16927%**
OTU775930526	Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	*Inonotus glomeratus*	0.00004%
OTU511819475	Basidiomycota	Agaricomycetes	Polyporales	Hapalopilaceae	*Ischnoderma benzoinum*	0.00138%
OTU343789308	Ascomycota	Dothideomycetes	Pleosporales	Leptosphaeriaceae	*Leptosphaeria doliolum*	0.00036%
OTU52444823	Ascomycota	Sordariomycetes	Diaporthales	Valsaceae	*Leucostoma persoonii*	0.00195%
OTU334299142	Zygomycota	Incertae_sedis	Kickxellales	Kickxellaceae	*Linderina macrospora*	0.00040%
OTU120026670	Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	*Mensularia radiata*	0.00084%
OTU327480802	**Basidiomycota**	**Agaricomycetes**	**Agaricales**	**Bolbitiaceae**	***Naucoria salicis***	**0.03915%**
OTU857636164	Ascomycota	Leotiomycetes	Erysiphales	Erysiphaceae	*Oidium mutisiae*	0.00046%
OTU743654468	**Ascomycota**	**Dothideomycetes**	**Pleosporales**	**Montagnulaceae**	***Paraconiothyrium sporulosum***	**0.31531%**
OTU863735734	**Ascomycota**	**Dothideomycetes**	**Pleosporales**	**Montagnulaceae**	***Paraconiothyrium sporulosum***	**0.09374%**
OTU357339150	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Penicillium purpurogenum*	0.00009%
OTU773824548	**Ascomycota**	**Pezizomycetes**	**Pezizales**	**Pezizaceae**	***Peziza subcitrina***	**0.13015%**
OTU150150053	Basidiomycota	Agaricomycetes	Polyporales	Phanerochaetaceae	*Phanerochaete chrysosporium*	0.00004%
OTU903697817	**Basidiomycota**	**Agaricomycetes**	**Polyporales**	**Fomitopsidaceae**	***Piptoporus betulinus***	**0.00235%**
OTU809295602	Basidiomycota	Agaricomycetes	Polyporales	Polyporaceae	*Polyporus squamosus*	0.00002%
OTU195558662	Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	*Pseudaleuria quinaultiana*	0.00028%
OTU810640323	**Basidiomycota**	**Ustilaginomycetes**	**Ustilaginales**	**Ustilaginaceae**	***Pseudozyma prolifica***	**0.06461%**
OTU390756117	Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	*Rhizoctonia solani*	0.00074%
OTU27178405	**Zygomycota**	**Incertae_sedis**	**Mucorales**	**Mucoraceae**	***Rhizopus oryzae***	**0.00271%**
OTU504408516	Basidiomycota	Microbotryomycetes	Sporidiobolales	Incertae_sedis	*Rhodotorula acheniorum*	0.00012%
OTU746656896	Basidiomycota	Agaricomycetes	Agaricales	Entolomataceae	*Richoniella asterospora*	0.00004%
OTU977575360	Ascomycota	Dothideomycetes	Unclassified	Unclassified	*Scleroramularia abundans*	0.00003%
OTU100250967	Chytridiomycota	Chytridiomycetes	Spizellomycetales	Spizellomycetaceae	*Spizellomyces pseudodichotomus*	0.00036%
OTU884302782	Basidiomycota	Microbotryomycetes	Microbotryales	Microbotryaceae	*Sporisorium destruens*	0.00062%
OTU966456327	Basidiomycota	Microbotryomycetes	Microbotryales	Microbotryaceae	*Sporisorium reilianum*	0.00093%
OTU211674455	Basidiomycota	Microbotryomycetes	Sporidiobolales	Incertae_sedis	*Sporobolomyces griseoflavus*	0.00097%
OTU325227420	Ascomycota	Sordariomycetes	Hypocreales	Incertae_sedis	*Stachybotrys bisbyi*	0.00119%
OTU533258817	Basidiomycota	Agaricomycetes	Agaricales	Strophariaceae	*Stropharia ambigua*	0.00049%
OTU267871275	Basidiomycota	Agaricomycetes	Agaricales	Strophariaceae	*Stropharia rugosoannulata*	0.00048%
OTU362952107	**Ascomycota**	**Eurotiomycetes**	**Eurotiales**	**Trichocomaceae**	***Talaromyces luteus***	**0.00203%**
OTU859836155	Basidiomycota	Agaricomycetes	Boletales	Hygrophoropsidaceae	*Tapinella atrotomentosa*	0.00010%
OTU687749927	Basidiomycota	Agaricomycetes	Thelephorales	Thelephoraceae	*Thelephora terrestris*	0.00017%

OTU43902637	Basidiomycota	Agaricomycetes	Polyporales	Polyporaceae	*Trichaptum biforme*	0.00006%
OTU692506221	**Ascomycota**	**Sordariomycetes**	**Xylariales**	**Amphisphaeriaceae**	***Truncatella angustata***	**0.50808%**
OTU425504232	Basidiomycota	Agaricomycetes	Boletales	Boletaceae	*Tylopilus felleus*	0.00006%

Note: Those in bold were used for Table III.III and represent the dominant ones (those above 0.002% of the total). Those in parentheses were given the same species and genus designation as the one above it, but were assigned different OTUs due to a difference in sequence.

Table SIII.1.II: Identity of fungal OTUs comprising ~97% of the community, using the ITS gene with MiSeq sequencing, found in willow rhizosphere samples

	Phylum	Class	Order	Family	Genus and Species	Average
OTU743654468	Ascomycota	Dothideomycetes	Pleosporales	Montagnulaceae	*Paraconiothyrium sporulosum*	0.31531%
OTU863735734	Ascomycota	Dothideomycetes	Pleosporales	Montagnulaceae	*Paraconiothyrium sporulosum*	0.09374%
OTU599341566	Ascomycota	Dothideomycetes	Pleosporales	Unclassified	Unclassified	0.01912%
OTU248475881	Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	*Cladophialophora chaetospira*	0.01754%
OTU879013980	**Ascomycota**	**Leotiomycetes**	**Helotiales**	**Incertae_sedis**	**Unclassified _Cadophora_**	**0.48688%**
OTU530559237	Ascomycota	Leotiomycetes	Helotiales	Incertae_sedis	Unclassified *Cadophora*	0.01580%
OTU695562143	Ascomycota	Leotiomycetes	Helotiales	Unclassified	Unclassified	0.02145%
OTU877102909	Ascomycota	Leotiomycetes	Leotiales	Leotiaceae	*Neobulgaria* sp.	0.10819%
OTU568789266	**Ascomycota**	**Pezizomycetes**	**Pezizales**	**Pyronemataceae**	***Geopora sepulta***	**1.67795%**
OTU436068696	Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	*Geopora sepulta*	0.18389%
OTU773824548	Ascomycota	Pezizomycetes	Pezizales	Pezizaceae	*Peziza subcitrina*	0.13015%
OTU819080307	**Ascomycota**	**Pezizomycetes**	**Pezizales**	**Pyronemataceae**	**Unclassified**	**26.34384%**
OTU238480107	**Ascomycota**	**Pezizomycetes**	**Pezizales**	**Unclassified**	**Unclassified**	**9.96437%**
OTU50310567	**Ascomycota**	**Pezizomycetes**	**Pezizales**	**Unclassified**	**Unclassified**	**6.57832%**
OTU40225823	**Ascomycota**	**Pezizomycetes**	**Pezizales**	**Unclassified**	**Unclassified**	**2.30437%**
OTU446082359	**Ascomycota**	**Pezizomycetes**	**Pezizales**	**Unclassified**	**Unclassified**	**1.59378%**
OTU250957214	Ascomycota	Pezizomycetes	Pezizales	Unclassified	Unclassified	0.73486%
(20 OTUs)	**Ascomycota**	**Pezizomycetes**	**Pezizales**	**Unclassified**	**Unclassified**	**0.49225%**
OTU531110773	**Ascomycota**	**Pezizomycetes**	**Pezizales**	**Unclassified**	**Unclassified**	**0.43645%**
OTU772196488	Ascomycota	Pezizomycetes	Pezizales	Unclassified	Unclassified	0.26245%
OTU111795707	Ascomycota	Pezizomycetes	Pezizales	Pezizaceae	Unclassified	0.15477%
OTU851546115	Ascomycota	Pezizomycetes	Pezizales	Unclassified	Unclassified	0.12622%
OTU313534712	Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	Unclassified	0.08926%
OTU253682147	Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	Unclassified uncultured *Geopora*	0.87242%
OTU655633880	Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	Unclassified uncultured *Geopora*	0.39477%
OTU470947479	Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	Unclassified uncultured *Geopora*	0.25917%
OTU100449810	Ascomycota	Sordariomycetes	Diaporthales	Valsaceae	*Cytospora chrysosperma*	0.06720%
OTU280089542	Ascomycota	Sordariomycetes	Diaporthales	Valsaceae	Unclassified *Valsa*	0.07373%
OTU975065746	Ascomycota	Sordariomycetes	Hypocreales	Incertae_sedis	Unclassified *Cephalosporium*	0.01359%
OTU369735641	Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	Unclassified *Trichoderma*	0.02936%
OTU205745926	Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	Unclassified *Trichoderma*	0.01628%
OTU126931823	Ascomycota	Sordariomycetes	Hypocreales	Unclassified	Unclassified	0.09951%
OTU857689461	Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	Unclassified	0.01565%
OTU828776762	Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	Unclassified uncultured *Hypocrea*	0.03090%
OTU692506221	Ascomycota	Sordariomycetes	Xylariales	Amphisphaeriaceae	*Truncatella angustata*	0.50808%
OTU124770399	**Ascomycota**	**Unclassified**	**Unclassified**	**Unclassified**	**Unclassified**	**0.36815%**
(25 OTUs)	**Ascomycota**	**Unclassified**	**Unclassified**	**Unclassified**	**Unclassified**	**0.20770%**
OTU939896726	Ascomycota	Unclassified	Unclassified	Unclassified	Unclassified	0.19396%
OTU43973557	Ascomycota	Unclassified	Unclassified	Unclassified	Unclassified	0.10740%
OTU890682727	Ascomycota	Unclassified	Unclassified	Unclassified	Unclassified uncultured Ascomycota	0.10240%
OTU195883303	Basidiomycota	Agaricomycetes	Agaricales	Bolbitiaceae	*Alnicola tantilla*	0.01103%
OTU522652877	Basidiomycota	Agaricomycetes	Agaricales	Nidulariaceae	*Cyathus stercoreus*	0.05555%
OTU86348303	**Basidiomycota**	**Agaricomycetes**	**Agaricales**	**Cortinariaceae**	***Inocybe curvipes***	**3.74216%**
OTU694722950	**Basidiomycota**	**Agaricomycetes**	**Agaricales**	**Cortinariaceae**	**_Inocybe lacera_ var. _lacera_**	**2.02034%**
OTU209944304	Basidiomycota	Agaricomycetes	Agaricales	Cortinariaceae	*Inocybe lacera* var. *lacera*	0.16927%
OTU115384453	Basidiomycota	Agaricomycetes	Agaricales	Cortinariaceae	*Inocybe lacera* var. *lacera*	0.04434%
OTU652930571	**Basidiomycota**	**Agaricomycetes**	**Agaricales**	**Cortinariaceae**	**Unclassified _Inocybe_**	**10.51350%**
OTU493811366	Basidiomycota	Agaricomycetes	Agaricales	Cortinariaceae	Unclassified *Inocybe*	0.26982%
OTU327480802	Basidiomycota	Agaricomycetes	Agaricales	Bolbitiaceae	*Naucoria salicis*	0.03915%
OTU667206613	Basidiomycota	Agaricomycetes	Agaricales	Bolbitiaceae	*Naucoria salicis*	0.01031%
OTU989439865	Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	Unclassified *Psathyrella*	0.01641%
OTU735317	Basidiomycota	Agaricomycetes	Agaricales	Strophariaceae	Unclassified *Stropharia*	0.14992%
OTU578686797	**Basidiomycota**	**Agaricomycetes**	**Agaricales**	**Unclassified**	**Unclassified**	**1.15163%**
OTU518257773	Basidiomycota	Agaricomycetes	Agaricales	Unclassified	Unclassified	0.10021%
OTU373163642	Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	Uncultured *Coprinellus*	0.12216%
OTU896147191	**Basidiomycota**	**Agaricomycetes**	**Agaricales**	**Cortinariaceae**	**Unclassified uncultured _Hebeloma_**	**3.63312%**
OTU237953421	**Basidiomycota**	**Agaricomycetes**	**Agaricales**	**Cortinariaceae**	**Unclassified uncultured _Hebeloma_**	**1.48969%**
OTU984249233	**Basidiomycota**	**Agaricomycetes**	**Agaricales**	**Cortinariaceae**	**Unclassified uncultured _Hebeloma_**	**0.95373%**
OTU364029024	**Basidiomycota**	**Agaricomycetes**	**Agaricales**	**Cortinariaceae**	**Unclassified uncultured _Hebeloma_**	**0.69246%**
OTU646369203	Basidiomycota	Agaricomycetes	Agaricales	Cortinariaceae	**Unclassified** uncultured *Hebeloma*	0.06001%
OTU153963325	Basidiomycota	Agaricomycetes	Boletales	Hymenogasteraceae	*Hymenogaster vulgaris*	0.01236%
OTU279526272	Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	Unclassified *Ceratobasidium*	0.02420%
OTU654018642	Basidiomycota	Agaricomycetes	Corticiales	Corticiaceae	Unclassified *Rhizoctonia*	0.32679%
OTU88269714	Basidiomycota	Agaricomycetes	Corticiales	Corticiaceae	Unclassified *Rhizoctonia*	0.08940%
OTU757113337	Basidiomycota	Agaricomycetes	Corticiales	Corticiaceae	Unclassified *Rhizoctonia*	0.06278%
OTU285576110	Basidiomycota	Agaricomycetes	Sebacinales	Sebacinaceae	Unclassified	0.09070%
OTU355069902	Basidiomycota	Agaricomycetes	Sebacinales	Sebacinaceae	Unclassified	0.06097%
OTU385801689	Basidiomycota	Agaricomycetes	Sebacinales	Sebacinaceae	Uncultured *Sebacina*	0.01818%
OTU498616323	Basidiomycota	Agaricomycetes	Thelephorales	Thelephoraceae	Uncultured *Thelephoraceae*	0.06145%
OTU487162098	Basidiomycota	Agaricomycetes	Unclassified	Unclassified	Unclassified sp.	0.09021%
OTU61070214	**Basidiomycota**	**Unclassified**	**Unclassified**	**Unclassified**	**Unclassified**	**0.20217%**
(55 OTUs)	**Basidiomycota**	**Unclassified**	**Unclassified**	**Unclassified**	**Unclassified**	**0.19826%**
OTU925616399	**Basidiomycota**	**Unclassified**	**Unclassified**	**Unclassified**	**Unclassified uncultured Basidiomycota**	**0.71206%**
OTU810640323	Basidiomycota	Ustilaginomycetes	Ustilaginales	Ustilaginaceae	*Pseudozyma prolifica*	0.06461%
(263 OTUs)	**Unclassified**	**Unclassified**	**Unclassified**	**Unclassified**	**Unclassified uncultured fungus**	**15.87007%**
OTU589950850	Zygomycota	Incertae_sedis	Mucorales	Mucoraceae	Unclassified	0.03971%

Figure SIII.1.1: ITS gene with MiSeq sequencing, rhizospheric soil, 2011, ~96 willows, 50 OTUs that made up the bulk of the sequence reads

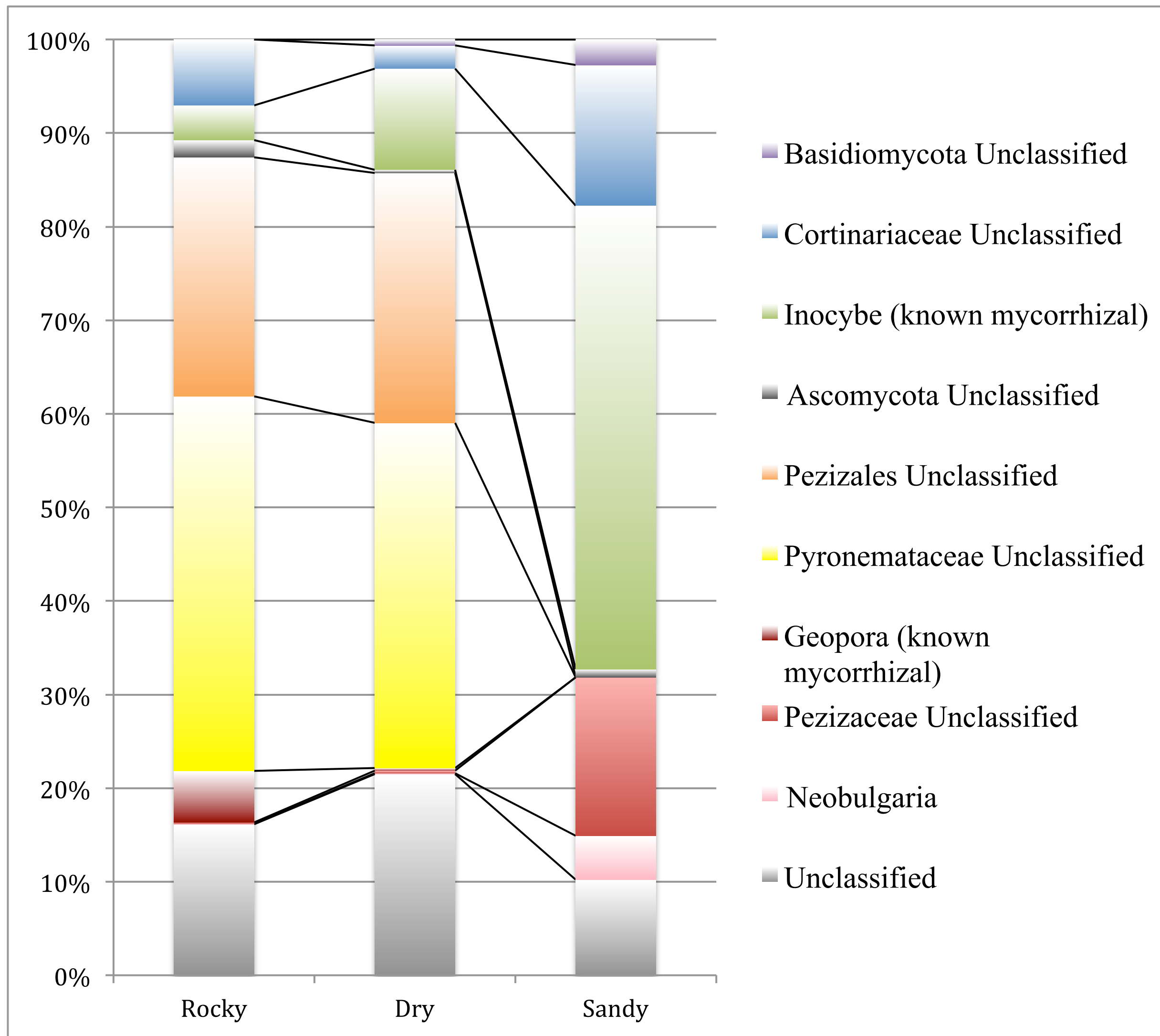


Figure SIII.1.2: Principal coordinate analysis by inoculation treatment

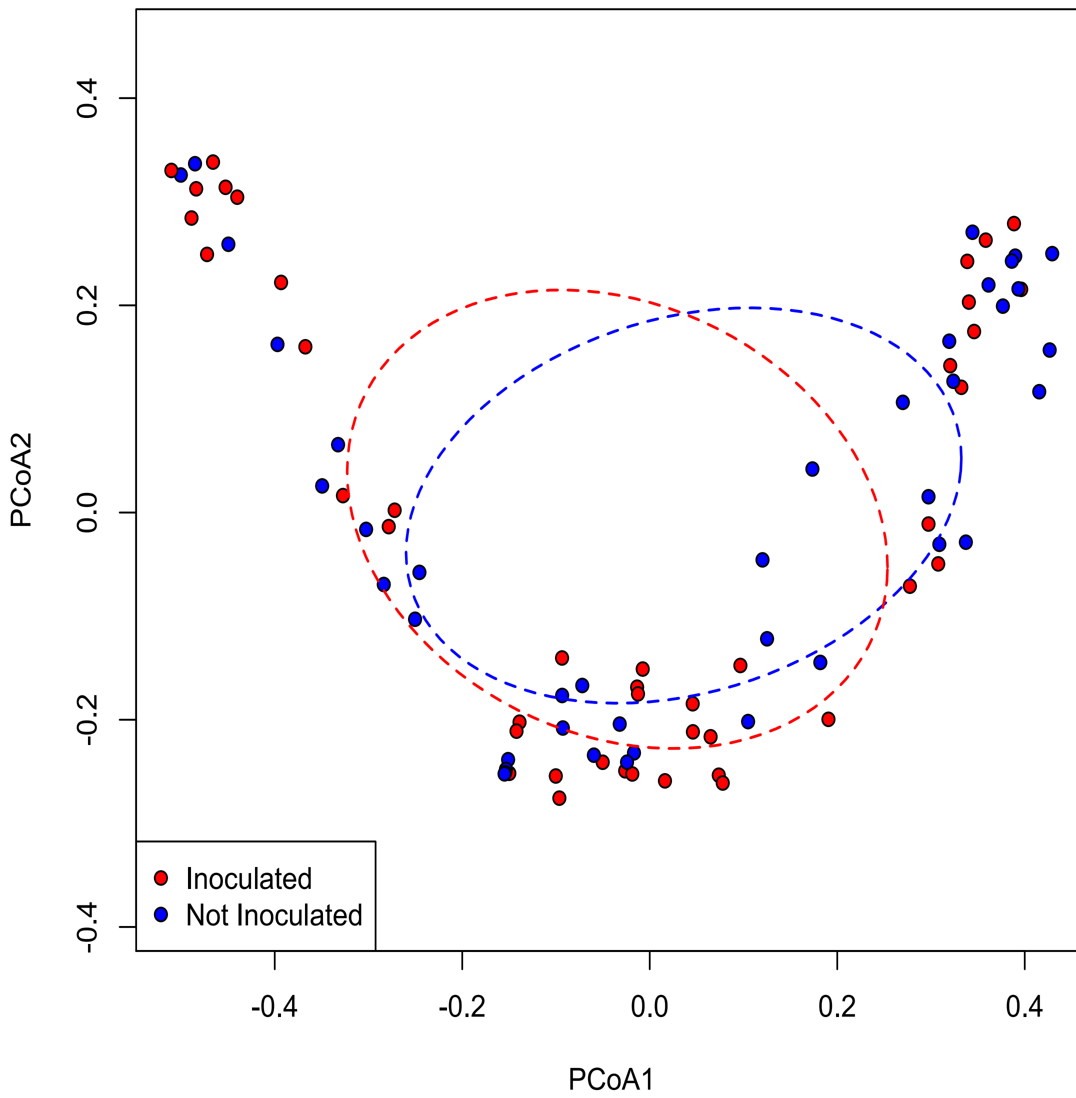
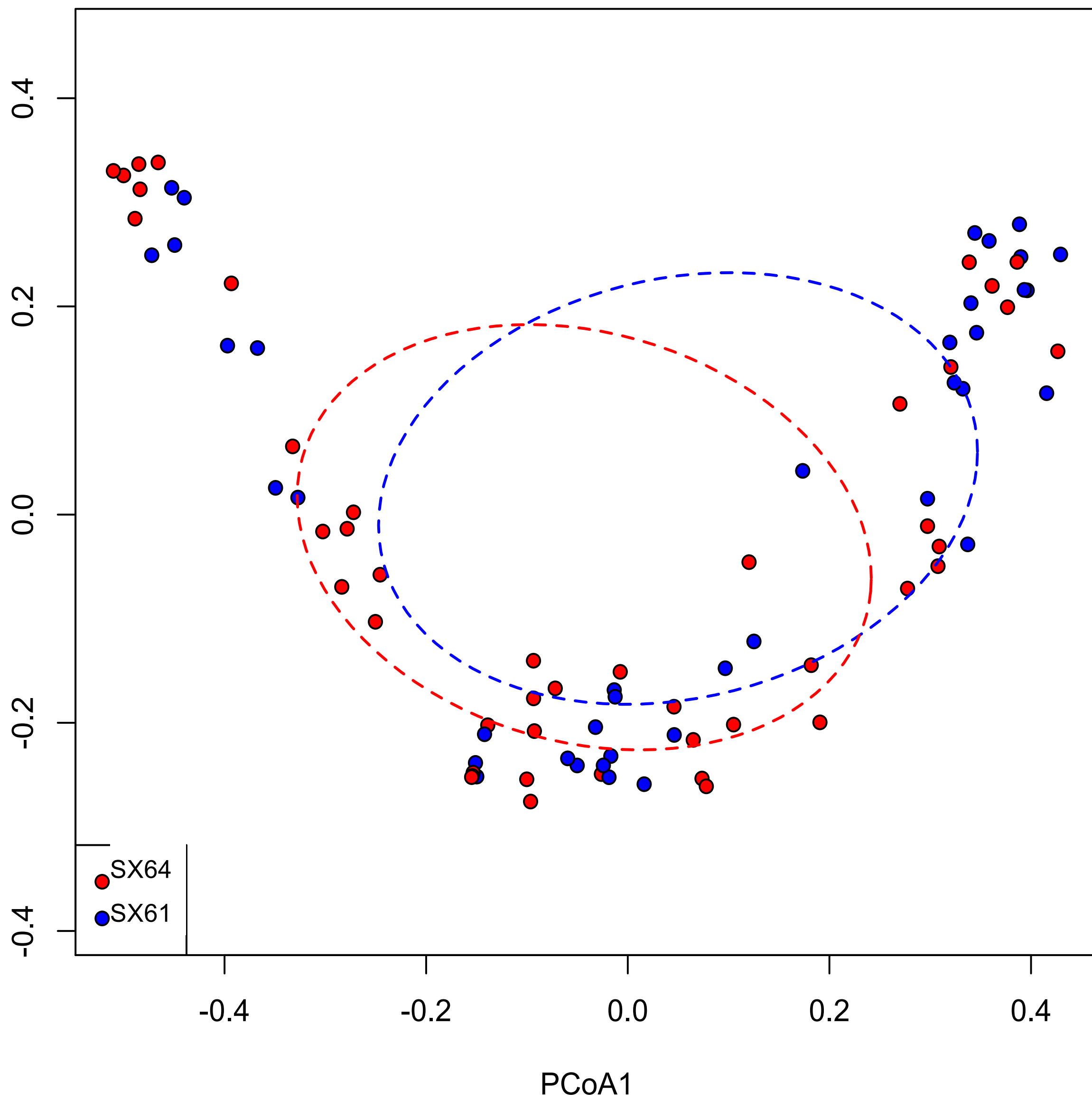


Figure SIII.1.3: Principal coordinate analysis by willow cultivar, ITS gene with MiSeq sequencing, rhizospheric soil, 2011



IV. Conclusion

IV.1. Findings from field experiments

The practical objective of our study was to improve the growth of SRC willows on marginal land by inoculating with mycorrhizal fungi. Chapter II reported the outcome of the study: that we found no productivity difference between inoculated and uninoculated *Salix miyabeana* Seeman (SX61 and SX64), despite measuring significant effects from nitrogen fertilization and field differences. The experiment was designed to be of practical interest to farmers and agricultural scientists, conducted at farm scale and with typical farm equipment, as well as being set up in unsterile farm soil. The wild fungal organisms present naturally, provided a truer control than a laboratory or greenhouse experiment could. The results should also be fairly robust, as we tested the inoculum's effect across three different marginal soil types and with and without nitrogen fertilization, as well as using the two different willow cultivars. The inoculum species, *Rhizoglomus irregulare* and *Hebeloma longicaudum*, likewise represented both arbuscular and ectomycorrhizal inoculum types. Furthermore, *R. irregulare* is the most widely available and popular commercial mycorrhizal inoculant. Preliminary molecular data presented in Chapter II suggested a diverse community of native fungi in the field soils could have either outcompeted or masked the effect of the introduced species.

Chapter III presented a more comprehensive look into the native community of mycorrhizal fungi associated with the shrub willows in our three fields. We did this to better understand the results of our experiment, as well as to further our long-term objective: to better inform agriculture through the lens of soil microbial ecology, focusing on mycorrhizal fungi. Sequencing DNA extracted from 96 soil rhizospheric samples, 702 unique fungal operational taxonomic units (OTUs) were identified, the majority previously unknown and unstudied. The high percentage of OTUs new to science reinforces the fact that the culturing methods used to study fungi, as well as to select and make commercial inoculum are simply not able to grow most species thriving in actual farm soils. Of relevance to shrub willow cultivation, the lack of AM fungi in such a complete survey of fungal sequences strongly

suggests that mature shrub willows preferentially interact with EM, even under agricultural field conditions. Finally, a marked shift in fungal population was seen between one field, labeled Sandy, and the other two, labeled Dry and Rocky. This suggests a stronger correlation between mycorrhizal fungi and soil texture, than between plant host and nitrogen fertilization, or probably soil pH and total soil phosphorus.

IV.2. Hypotheses revisited

The results we present in chapter II clearly show that the two mycorrhizal species we tested do not benefit the growth or survival of our shrub willows under realistic farm conditions in marginal fields. These results necessitate rejecting our original hypotheses, that we would see a benefit. Our results do not have to contradict the idea that mycorrhizal fungi aid plants. This is well established in laboratory experiments. Our results do, however, contradict the idea that farm soil is functionally deficient in wild mycorrhizal fungi. The most likely hypothesis for our results, given that we had evidence the inoculum was viable when we applied it, is that populations of wild mycorrhizal fungi in our control and treatment plots equaled or overwhelmed our inoculation (both in numbers and in benefits to the willows). As we did not see any benefit from inoculation, we could not test our hypothesis that the more marginal plots would demonstrate even greater benefit. Neither could we confirm or describe hypothetical interactions of fertilization and inoculation. Though the fact that our experiment tested different soil types (as well as willow cultivars, and fertilization regimes) does strengthen the conclusion that wild mycorrhizal fungi are functionally present for crops in many, or even most, situations.

In the third chapter our objective was to use the inoculation experiment as an opportunity to better understand rhizospheric fungal community dynamics. The chapter presented data from rhizospheric soil sampled the second year, and causes us to completely revise our hypotheses from the introduction. No obvious sign of our inoculated species could be found, certainly not in the dominant members of the fungal community. Instead, a robust native community of fungi appears in our data, or more accurately, distinct communities determined by specific environmental parameters. These communities do not seem to be determined chiefly by their plant host, as some hypotheses would suggest, but instead by soil

characteristics. Unexpectedly, by the second year EM appear to dominate our willow rhizospheres with almost no AM found using universal fungal primers. Our hypothesis that both AM and EM species would thrive in our SRC willow's rhizospheres must also be thrown out. It is possible that a small but physiologically significant percentage of AM is still present, but our sampling numbers and data are unable to show that.

IV.3. Discussion

Our biggest finding is that successful inoculation—the introduction of selected mycorrhizal fungi, populations of which go on to thrive—is extremely hard to do in non-sterile field soils. This is most likely because agricultural soils are healthier than suspected with an intact, diverse suit of rhizospheric fungi. Also, the fact that our inoculated species did not take hold in any of our fields or even have any impact on the fungal community by the second year, as well as the fact that so many unknown species were found, suggests that current culturing techniques simply do not select for species and strains that are competitive enough in wild environments to be used as inocula. Before experiments can truly test for growth and survival benefits to their host plants in field conditions, inoculum species must be screened for their ability to take hold and thrive in field conditions. Conversely, the fact that nitrogen fertilization had a significant effect in our experiment does hold out hope for researchers one day finding a beneficial mix of soil organisms that can fulfill such a nitrogen need.

The other important understanding that came from this experiment was an appreciation for the fundamental discoveries possible in soil ecology, through the use of a simplified ecosystem in a natural setting, as well as targeting a critical fungal functional group. In a way, our inoculation experiment became the background to a targeted ecological survey, with host plants all clones of two similar willows of the same species, and since they were perennials, multiple years for the community to develop undisrupted. Focusing on fungi in the soil attached to living roots allowed us to begin to find patterns governing the distribution of different mycorrhizal fungi, the key organism linking soil to plants.

To best continue this research, I believe we must first test inoculation delivery techniques and survival in willows in unsterile field conditions. Our results suggested that soil

structure is likely a key determinant of mycorrhizal species success and suitability. The artificial soils that are usually used to keep plants healthy in containers could be strongly biasing efforts to date. Realistic soil tests, on the other hand, might even find completely different EM and AM species than commonly used are necessary to compete in such an environment. I suspect that researchers will need to revolutionize their inoculum culturing techniques to grow these field-dominant mycorrhizal species.

IV.4. New ideas, and support from the scientific literature

One difficulty might lie in culturing candidate mycorrhizal species. Those that grow quickly and are easier to propagate might not be those that compete well in natural fields (Stefani *et al*. 2015). Large volumes of propagate for slower-growing, more competitive, and more beneficial mycorrhizal species might take months to develop. Perhaps culturing conditions would even have to mimic multiple summer-winter seasonal transitions to encourage sporulation and growth. If the candidate species grow best with helper bacteria (as in Taktek *et al.* 2016) or other fungi, entirely new culturing techniques and screening/propagating procedures would have to be worked out. Nutrients might also be applied at lower total concentrations, similar to levels seen in unfertilized farm soil, rather than the higher ones typical of plate culturing. Perhaps dead fungal and bacterial cells can be used as a nutrient source, instead of the more readily available chemical nutrients currently added to plates. Soil often has lower temperatures and slower oxygen diffusion than is typical of culture plates, and these differences might have to be taken into account. One very different idea, but along the lines of mixed-species cultures, would be to include nematodes that target "weed" species of fungi. In a sense, you would be culturing simple ecosystems.

Another idea is to devote time and energy to better DNA extraction and sequencing of AM species from field root samples. It is highly suspicious to me that we and so many others have to use nested PCR to find AM sequences. It can't be just that root or soil bacterial DNA get in the way, or the same problem would be seen searching for EM sequences. One possibility is to extract and sequence from root pieces that have been gently cleared and colonization verified (from Pitet *et al.* 2009), similar to extracting and sequencing only from EM root tips seen under a microscope. Sampling timing (as in Beauregard *et al.* 2010) and

technique should also be investigated. Perhaps sampling soil adhering to roots late in the season is a mistake. If at that time AM have retreated into structures either inside roots, or spores a small distance away from roots and lightly or not at all attached, sampling adhering soil could miss the majority of sequenceable AM genetic material.

Once multiple species of EM and AM candidates that are proven competitors in field soils and with shrub willow hosts are found, then field tests can be conducted to screen them for willow growth or willow survival benefits. These field inoculation tests could use minimum numbers of willows but test many more inoculation species. The dilemma is scale. Our results suggest that natural variation in fields make large trials necessary. A valuable calculation would be to determine how many trees would have to be tested (and therefore how big a field planted) to see a minimum 2% positive effect size for growth at a maximum p-value of 0.05. Perhaps fields with less natural heterogeneity would allow smaller totals, but soil assessment is not easy. Measuring growth simply with diameter (rather than height or weight) would save time and resources, and allow a larger overall experiment. Similarly, not worrying about fertilization or cultivar type (besides paying attention that tests were consistent) would allow the use of already planned commercial biomass plantings.

If inoculation in healthy fields does not make sense until these kinds of breakthroughs are made, I urge farmers to use strategies that encourage a healthy mycorrhizal fungal community made up of species already present in and around their field. I believe that keeping host plants on the fields with cover crops, is probably of even more value than limiting soil disruption, but both would help. Potentially, extra fertilization along with organic carbon sources at the beginning or towards the end of the regular growing season could encourage soil bacteria and fungi (Kirkby *et al.* 2014). Prevailing thought would anticipate soil organic carbon build up via saprotrophic bacteria and fungi with such “soil feeding” strategies, but I suspect that in fields that have kept cover crops on them—or perennial crops such as our biomass willows—significant growth could be seen with mycorrhizal fungi. Farmers might also explore the potential for unconventional fertilization sources (phosphorus mineral gravels, etc.) with strong mycorrhizal fungal communities.

Finally, if a farmer or landscaper is dealing with almost-sterile soil (after heavy fungicide use, or deposited subsoil fill, for example) and wanted to inoculate, our findings

would suggest they could consider local wild sources instead of, or in addition to, commercial inoculant. I would suggest they search for a healthy local wild plant in soil of a similar texture and make inoculum from shaken but not washed root pieces.

IV.5. Summary

Our project used biomass shrub willows to investigate the agricultural use of mycorrhizal inoculation. We first found unequivocally that the mycorrhizal fungal inoculums we used did not increase crop productivity, despite measuring strong productivity effects from nitrogen fertilization and different field conditions. Our next findings were that our agricultural fields contained a diverse suite of wild mycorrhizal species, and that current inoculum species were not competitive among them. The second of these findings should be applicable to many types of agriculture as well as willow plantations. Future inoculation attempts might need to wait for culturing techniques to progress. Until such breakthroughs, our work suggests farmers and researchers that support them should concentrate on better understanding the wild soil communities already present, and improving the health and positive interaction of that soil community with their crops.

Farmers and researchers working with willows on marginal lands can also benefit from looking over our growth data, a rather unique data set with qualitative lessons to be learned from farming willows in different field soil conditions, as well as different fertilization levels.

Much of the sequence data for this project came from rhizospheric soil. It is an investment of time and resources to dig up roots and separate the adhering soil from them. The value though is that it focuses on those organisms that were recruited by the willows and in fact aid the willows' growth (many are mycorrhizal). Our efforts represent early stage exploration, but the structure of our experiment allowed unique insight to the very local nature of mycorrhizal communities, with soil texture emerging as the key determinant.

Selective breeding and the improvement of plant strains is part of what defines agriculture. It seems inevitable that mycorrhizal fungal will also be selected and improved, and that inoculation will become an important part of agriculture, but for now this is still several breakthroughs away.

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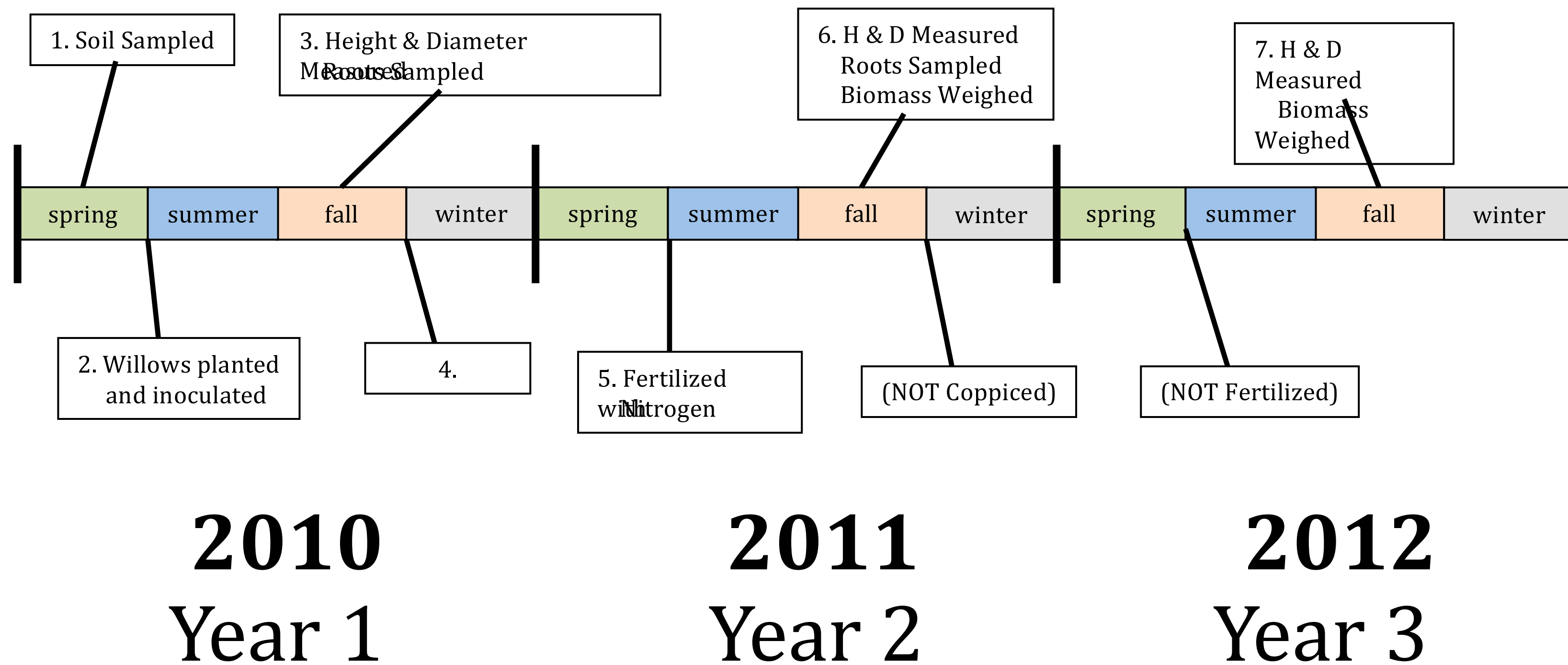
Appendix A1: Field experiment timeline

Figure A1.1: Field Experiment Timeline



Appendix A2: Experimental plan

Number of sites: three marginal fields (Sandy, Dry, and Rocky)

Area: 4665.6 m² per field (43.2 X 108), 12 blocks, 96 treatment subplots 48.6 m² each (9 X 5.4), 13996.8 m² in total (144 subplots), which equals 0.648 m² per tree (or 0.0000648 ha per tree)

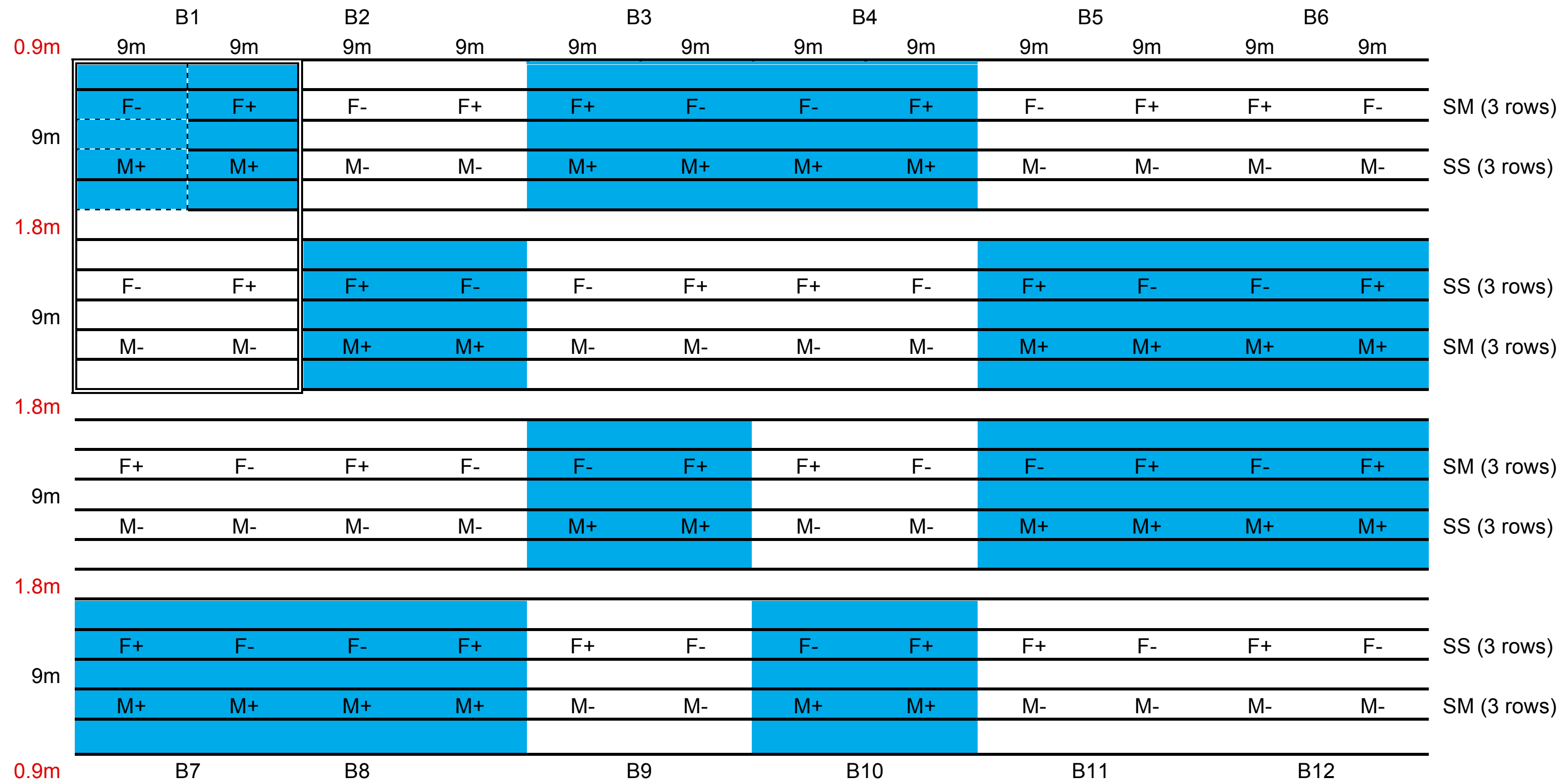
Number of total trees: 7200 willows in each field, planted in 24 rows (shown by the lines—not between them) spaced 1.8 m apart, trees every ~36 cm

Planting density: ~15,432 trees /ha, ~90,123 stems /ha (5.84 stems per tree, calculated from 2011 counts)

Treatments: two clone cultivars, *Salix miyabeana* SX61 (SS) and SX64 (SM) (laid out using strip randomization in strips three rows wide), mycorrhizally inoculated (M+), not inoculated (M-), fertilized with 75 kg N /ha (F+), not fertilized (F-)

Randomization: as shown by this plan, but each field randomized again for a different pattern (inoculated shaded blue for further clarity), 1st and 2nd subplot receive the same inoculation and fertilization treatment and are outlined with a dashed line, 1st block (B1) outlined with a double line

Figure A2.1: Experimental design (rocky field shown, used as example)



Appendix A3: Selected pictures

Tractor with modified 3-row cabbage planter, angled view and while planting. The tank of water to gravity feed the suspended wet inoculum can be seen on the fork in front of the tractor (kept mixed with a submerged circulating pump).

On left, boxes with willow cuttings in front of farmers seated on the planter. Just visible at the bottom of the photo are the cups that hold individual cuttings before they are dropped by the planter into the trench. On right are the cuttings in the ground (the closest pulled out slightly to be better visible).

On left, digging up whole root samples the first Fall. On right, an angled view of the Dry field the second Fall to see general growth and weed control. Also in the image on the right, a fertilization treatment can just be seen in the distance with its increased growth.

View of the ground around a willow tree the second Fall in the Rocky field. A 50 mL graduated tube can be seen for scale, as well as in preparation for digging up and sampling roots. The inset photo is a closer view of the lighter colored rock nearest the base of the willow, turned over to expose lignified but living root tips.

Appendix A4: Moisture probe data

Table A5.I: Moisture probe data 2010 (in fields at least three months at 30 cm depth)

Field and meter #	Avg. (Volts)	Range (Volts)
Dry 1	0.533	0.472-0.674
Dry 2	0.558	0.526-0.614
Rocky 1	0.556	0.505-0.662
Rocky 2	0.576	0.521-0.630
Sandy 1	0.460	0.360-0.642
Sandy 2	0.526	0.428-0.665

Appendix A5: Root staining and microscopy

For this project we spent several weeks working out a staining technique for the willow roots for visualization of mycorrhizal infection under a microscope. We were not able to use a protocol from others' work in the lab, because willow roots were far more lignified than the weedy grass and forbs plants worked with previously. We also spent a couple of those weeks working on a staining protocol that would allow extraction and PCR on the stained roots post-microscope viewing. This was interesting for the certainty it would provide, since amplifying DNA from roots with all of the natural PCR inhibitors is notoriously difficult. In the end, however, a modification of a protocol from the citation below proved more effective for visualization without the possibility to amplify DNA afterwards.

(modified from Veirheilig H, Coughlan AP, Wyss U and Piché Y (1998) Ink and vinegar, a simple staining technique for arbuscular-mycorrhizal fungi. Applied and Environmental Microbiology 64: 5004–5007)

-15-20min autoclave in 10% KOH

-thorough rinse (some vinegar sometimes to help neutralize KOH)

-1hr room temperature with 30% H_2O_2 (adding a few mL NH_4OH after ~10 minutes)

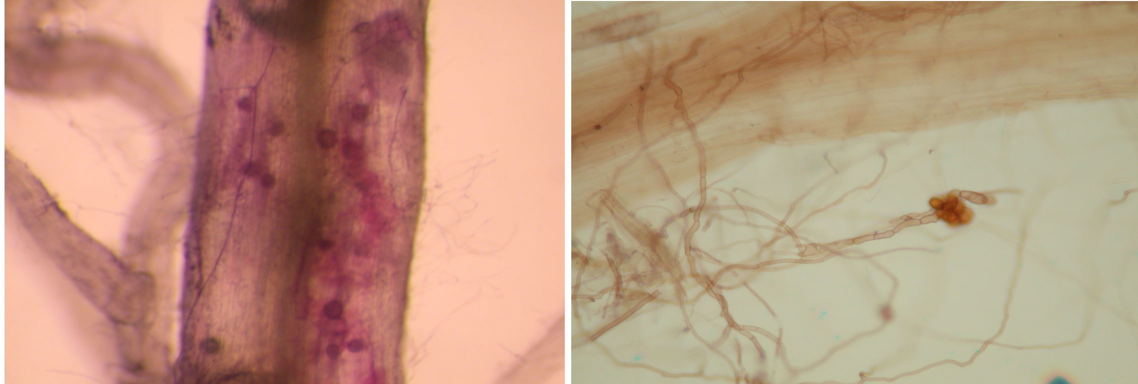
-thorough rinse

-10-20 min 1% HCl for acidification

-thorough rinse

-overnight (or over weekend) room temperature ink and vinegar (5% acetic acid), or trypan blue and vinegar

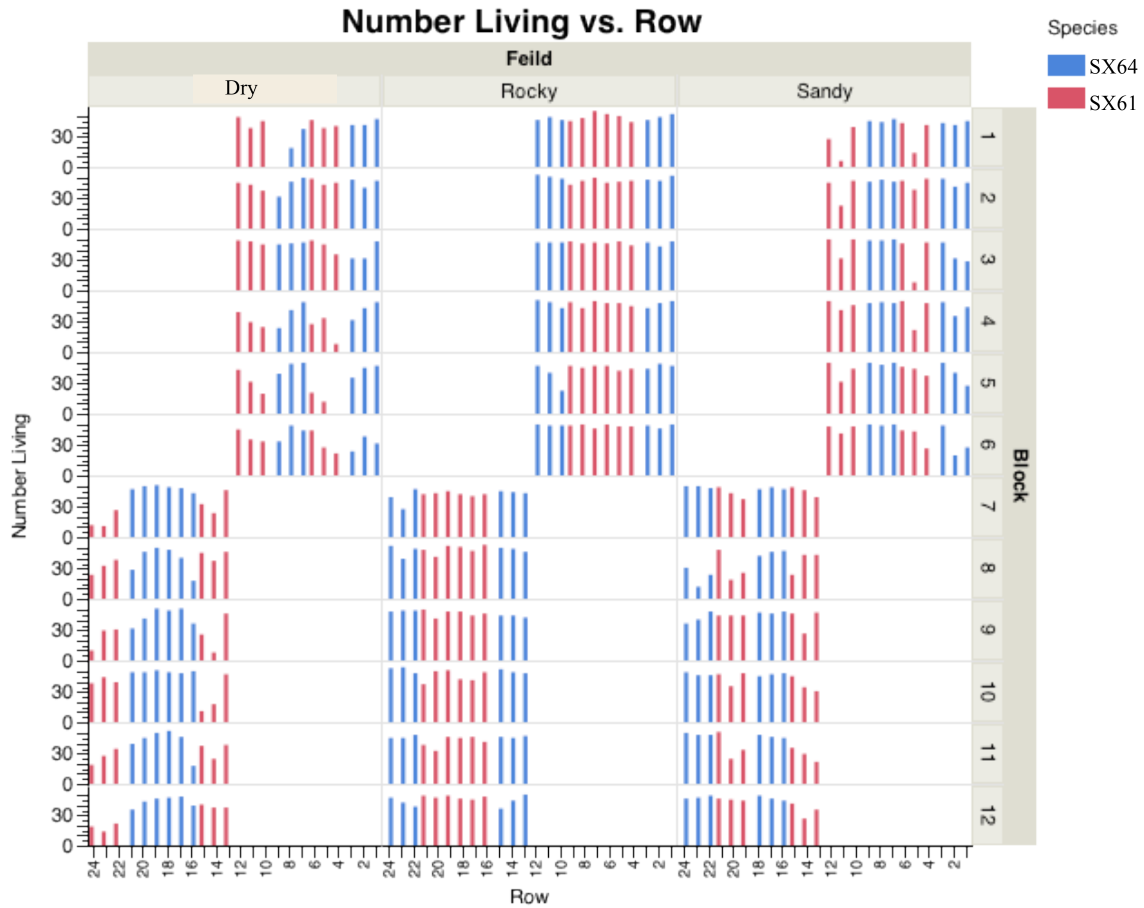
We did not choose to try to quantify colonization rates with a staining-transact protocol, but instead devoted our time to trouble-shooting molecular protocols for surveying the diversity of AM and EM fungi in our root and rhizospheric soil samples. Though we did confirm the presence of an AM fungi, and a putative EM fungi in samples of roots from our field experiment willows. This was to be sure mycorrhizal fungi were present since, again, amplifying DNA proved extremely difficult.



The left image is of a willow root sample taken from Block 5 of the Rocky field in October, 2011. It shows classic AM vesicles and hyphae within the roots, stained with Trypan blue. The right is of a different sample, stained with ink. The label for the right image was forgotten, but the root sample most likely came from a potted greenhouse willow inoculated with *Hebeloma longicaudum*.

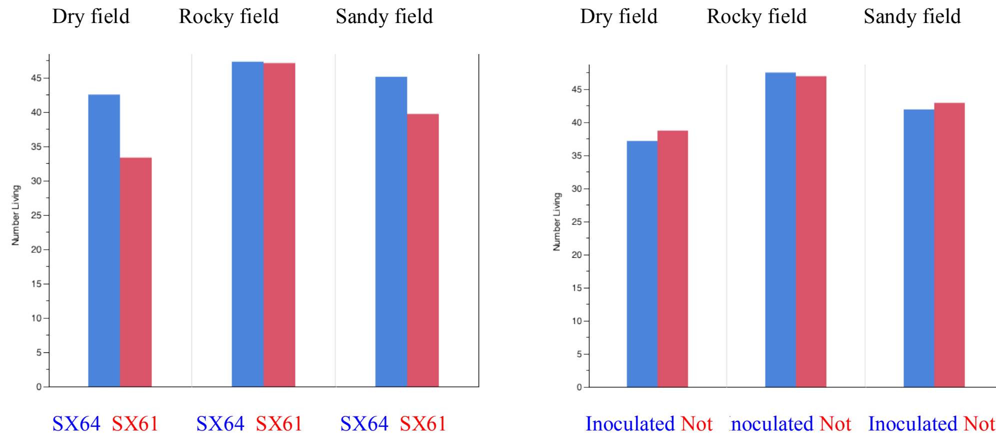
Appendix A6 Mortality charts

Figure A6.1: Willow Mortality



Note: This figure shows how mortality, while patchy, often spared a row just next to a severely affected row. This allowed us to measure growth parameters and sample roots, by shifting over a row in the few instances where there were not enough living trees to do so normally (done in JMP, a clickable interface program for SAS).

Figure A6.2: Number living on average per block-row



Note: Average number living per block-row on the y-axis, by type indicated on x-axis

Appendix A7: Primer investigation

While the NS31/AM1 primer pair (along with mixes of complements to AM1: AM2, 3, etc., to pick up missing groups of AMF) has a relatively long and successful history it has gradually been found to have serious shortcomings. Researchers working with cleaned root samples might not have as great a problem, but those trying to use the pair for rhizosphere or especially general soil analysis have found it not specific enough. In fact, in a recent pyrosequencing analysis of Mediterranean soil less than 40 percent of the amplified sequences by this pair were AM fungi (Lumini *et al.* 2010). Alternative primer pairs focusing on the same gene have been developed, and a map of the primer sites in relation to previous pairs was included with the AML1/AML2 pair (Lee *et al.* 2008).

For AML1/AML2 (a bit confusing after the AM mixes, as AML2 is the reverse—not an alternative or complement to AML1, as AM2 is to AM1), the sequence it targets is at the longer range of what is possible to use with DGGE. Another alternative primer pair, AMV4.5VF/AMDGR, was developed specifically for DGGE (Sato *et al.* 2005) and showed higher than 75 percent specificity for AM fungi even when pyrosequencing (Lumini *et al.* 2010). The map for this pair was not published with it, though, so I searched through a *Glomus* reference sequence in the NCBI database to find where it targeted in relation to the others we are trying, and highlighted the sequences when I found them to have a reference.

Figure A7.1: *Glomus vesiculiferum* 18S rRNA gene, isolate Att14-8, clone pWD193-2-3 (Schüßler group)

GenBank: FR750374.1

CCA**GTAGTCATATGCTTGTCTC**AAAGATTAAGCCATGCATGTCTAAGTATAAATCGTTTATACAGGTGAA
ACTGCGAATGGCTCATTAAATCAGTTATAATTTATTTGATAGTACCTTACTACTTGGATAACCGTGGTAA
TTCTAGAGCTAATACATGCTAAAACCTCCGACTTCTGGAAGAGGGTGTATTTATTAGATAAAAAACCAAT
ATCGGGCAACCGATTCCCTTGGTGATTCATAATAACTTTTCGAATCGTACGACTTTACGTTGACGATGAA
TCATTCAAATTTCTGCCCTATCAACTTTCGATGGTAGGATAGAGGCCTACCATGGCGGTAACGGGTAACG
GGGTGTTAGGGCACGACACCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGATGGCAGCAGGCG
CGCAAATTACCCAATCCCGACACGGGGAGGTAGTGACAATAAATAACAATACGGGGTTCTTTAGGATCTC
GTAATTGGAATGAGTACAATTTAAATCTCTTAACGAGGAACAATTGGAGGGCAAGTCTGGTGCCAGCAGC
CGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAA**AAGCTCGTAGTTGAATTTCG**G
GGTTAGTAGGTTGGTCATGCCTCTGGTATGTACTGGTCTCACTGATTCCTCCTTCCTGATGAATCTTAAT
GCCATTAATTTGGTGTTTTGGGGAATTTGGACTGTTACTTTGAAAAAATTAGAGTGTTTAAAGCAAGCTA
ACGCTTGAATACATTAGCATGGAATAATGAAATAGGACGTTCGATCCTATTTTGTTGGTTTCTAGGATTG
ACGTA**ATGATTAATAGGGATAGTTGGG**GGCATTAGTATTCAATTGTCAGAGGTGAAATTCTTGGATTTAT
TGAAGACTAACTACTGCGAAAGCATTTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATC
GAAGACGATCAGATACCGTvCGTAGTCTTAACCATAAACTATGCCGACTAGGGATCGGATGATGTTAATTT
TTTTATGACTCATTCGGCGCCTTACGGGAAACCAAAGTGTTTGGGTTCCGGGGGGAGTATGGTCGCAAGG
CTGAAA**CTTAAAGGAATTGACGGAAG**GGCACCACCAGGGGTGGAGCCTGCGGCT**TAATTTGACTCAACAC**
GGGGGAACTCACCAGGTCCAGACATAGTAAGGATTGACAGATTGAGAGCTCTTTCTTGATTCTATGGGTG
GTGGTGCATGGCCGGTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGTTACCGAACGAGACCTTAA
CCTGCTAAATAGCTAGGCCTAACATTGTTAGGTCGCCAGCTTCTTAGAGGGACTATCGGTGTTTAACCGA
TGGAAGTTTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCCACACTGAT
GAAGTCATCGAGTTCATTTCCTTTATCGGAAGATATGGGTAATCTTTTGAAACTTCATCGTGCTGGGGAT
AGAGTATTGCAACTATTGCTCTTGAACGAGGAATCCCTAGTAAGTACAAGTCACTAGCTTGTGCTGATTA
CGTCCCTGCCCTTTGTACACACCGCCCGTCGCTACTACCGGTTGAATGGCTTAGTGAGGCCCTCGGATTG
AGGCTCGGAGACTGGCAACAGACTCTCGGTTTTGAAGAGTTGGTCAAACTTGGTCATTTGGAGGAAGTAA
AAGTCGTAACAAGGT

(supposed to be AMF specific:)
fwd_name: GeoA2, fwd_seq: ccagtagtcatatgcttgtctc, (possible alternative to NS1?)
rev_name: Geo11, rev_seq: accttgttacgacttttacttcc"
(1765 long)

(**Sato *et al.* 2005 AMV4.5VF/AMDGR primer ((**AAG CTC GTA GTT GAA TTT CG ; CCC AAC TAT CCC TAT TAA TCA T)) amplified 259-long sequence within Lee *et al.* 2008 AML1/AML2 primer amplified 795–long sequence(ATC AAC TTT CGA TGG TAG GAT AGA, GAA CCC AAA CAC TTT GGT TTC C))

(also Santos-González *et al.* 2007 NS31/AM123 primer mix amplified 550-long sequence–based on Helgason *et al.* 1998 AM1/NS31)

(also **May LA *et al.* 2001 NS1/fung primer mix** amplified ~350-long sequence?)

NS1 and NS41 (1190 long)
NS4 (alternative to NS41, but same Tm so not much help)

Appendix A8: PCR-cloning-sequencing results

Table A8.I: Arbuscular mycorrhizal (AM) species found in rhizospheric soil (and one root sample from a same treatment) of *Salix* grown for biofuel

	Dry Field	**Rocky Field**	**Sandy Field**	**Name and GI of closest match in NCBI database**
18S_OTU-10	**1**	**-**	**-**	*Diversispora celata*: 224586636
18S_OTU-11	**32**	**-**	**-**	*Diversispora* sp. W4538: 342298391
18S_OTU-12	**2**	**-**	**-**	Uncultured *Diversispora*: 398649715
18S_OTU-13	**3**	**16**	**-**	*Glomus* sp. MC27: 334683211
18S_OTU-14	**1**	**30**	**-**	Uncultured *Glomus*: 401664149
18S_OTU-15	**-**	**-**	**10**	Uncultured *Ambispora*: 308084344
18S_OTU-17	**-**	**-**	**9**	*Glomeromycota* sp. MIB 8442: 328541374

Note: ~190 (47-48 each treatment) clones total were sequenced and analyzed, 4 cloning reactions (one for each treatment) were carried out, and each treatment's combined DNA extract was amplified with the 18S primers AML1/AML2 (nested in a previous reaction targeting NS1/NS41). The combined DNA extracts for each treatment represent 6 separate DNA extractions (18 individual trees total; *Salix* species was not considered for this investigation). OTU numbers not shown were from non-specific, non-fungal eukaryotic organisms, and were less than 15% of the cultivars.

Figure A8.1: 18S for AM PCR-cloning-sequencing, 2010, rhizospheric soil (and root for Sandy)

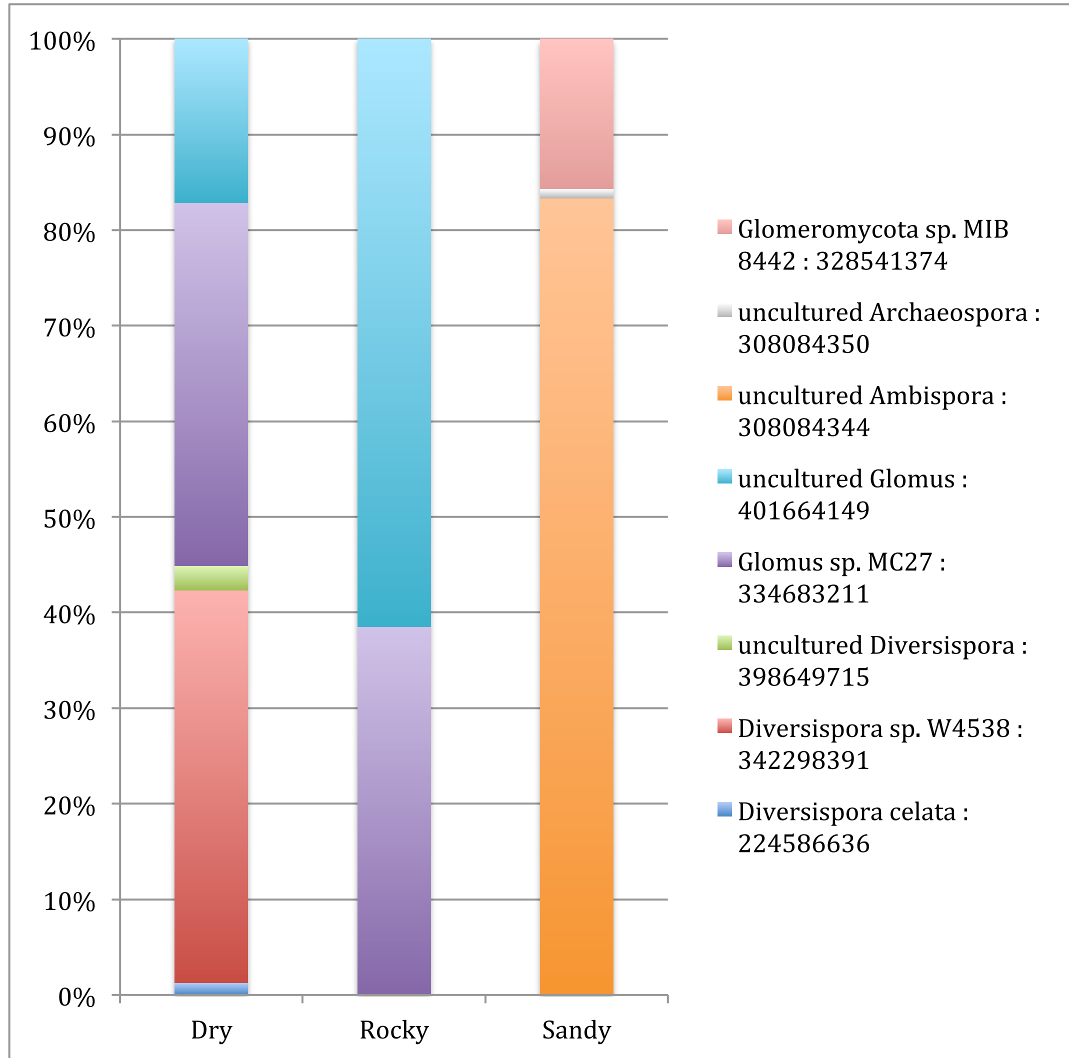
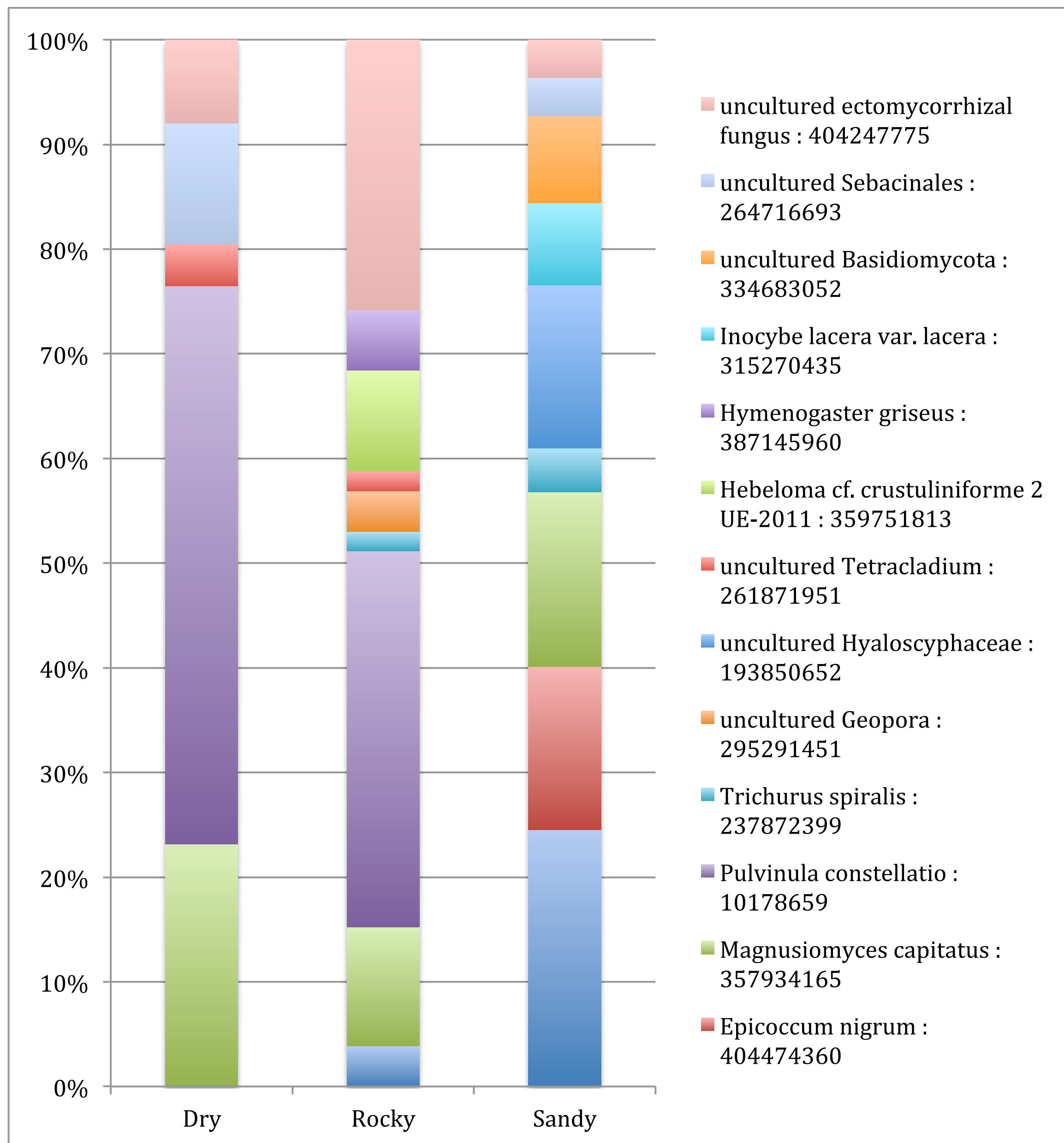


Table A8.II: Fungal species found in the roots of short-rotation coppice *Salix* Fall 2010 (planted early Summer 2010), ITS PCR-cloning-sequencing

	Dry Field	Rocky Field	Sandy Field	Name and GI of closest match in NCBI database	
ITS_OTU-1	1	-	-	*Chloridium* sp. GHJ-3: 254797324	Ascomycota
ITS_OTU-2	**-**	**-**	**5**	***Cladosporium cladosporioides*: 356484684**	**Ascomycota**
ITS_OTU-3	-	-	2	*Epicoccum nigrum*: 404474360	Ascomycota
ITS_OTU-6	**2**	**2**	**4**	***Magnusiomyces capitatus*: 357934165**	**Ascomycota**
ITS_OTU-8	-	-	1	*Pezizella discreta*: 344333528	Ascomycota
ITS_OTU-9	**1**	**8**	**-**	***Pulvinula constellatio*: 10178659**	**Ascomycota**
ITS_OTU-11	-	1	1	*Trichurus spiralis*: 237872399	Ascomycota
ITS_OTU-15	-	-	1	Uncultured *Apodus*: 261871958	Ascomycota
ITS_OTU-16	-	-	1	Uncultured Ascomycota: 299810505	Ascomycota
ITS_OTU-18	-	-	2	Uncultured Hyaloscyphaceae: 193850652	Ascomycota
ITS_OTU-19	-	-	1	Uncultured Pezizales: 54695082	Ascomycota
ITS_OTU-20	1	-	-	Uncultured *Tetracladium*: 261871951	Ascomycota
ITS_OTU-23	-	-	1	*Hymenogaster* sp. 4 SGT-2012: 399572828	Basidiomycota
ITS_OTU-24	-	-	1	*Inocybe lacera* var. *lacera*: 315270435	Basidiomycota
ITS_OTU-25	-	1	-	*Mrakiella aquatica*: 16209525	Basidiomycota
ITS_OTU-26	-	1	-	*Thanatephorus cucumeris*: 7415966	Basidiomycota
ITS_OTU-27	-	-	1	Uncultured *Auriculariales*: 401466721	Basidiomycota
ITS_OTU-28	-	-	2	Uncultured Basidiomycota: 334683052	Basidiomycota
ITS_OTU-30	1	-	-	*Uncultured Sebacinales: 264716693	Basidiomycota
ITS_OTU-31	-	-	1	*Nowakowskiella elegans*: 38146198	Chytridiomycota
ITS_OTU-33	**2**	**14**	**-**	***Uncultured ectomycorrhizal fungus: 404247775**	**environmental samples**
ITS_OTU-34	**33**	**20**	**18**	***Uncultured fungus (from *Salix* rhiz.): 402535072**	**environmental samples**
ITS_OTU-35	**2**	**-**	**2**	**Uncultured soil fungus: 195964332**	**environmental samples**
ITS_OTU-36	-	1	-	*Olpidium brassicae*: 87159723	Fungi incertae sedis
ITS_OTU-38	-	-	1	*Calluna vulgaris* root associated fungus: 283482652	Fungi Ukn
ITS_OTU-39	-	-	1	fungal sp. 5DI8-1PL2: 146218697	Fungi Ukn
ITS_OTU-40	-	-	2	**Entrophospora* sp. JJ38: 15809596	Glomeromycota

Note: 143 (47-48 each treatment) clones total were sequenced and analyzed, 3 cloning reactions (one for each treatment) were carried out, and each treatment's combined DNA extract was amplified with the primers ITS1F and ITS2 (for all fungi). The combined DNA extracts for each treatment represent 6 separate DNA extractions pooled by field (36 individual trees total, both *Salix miyabeana* SX61 and SX64). Operational taxonomic units, or OTUs, with more than 3 clones are in **bold**; ~90% of the clones are within these OTUs; *mycorrhizal species according to NCBI entry notes.

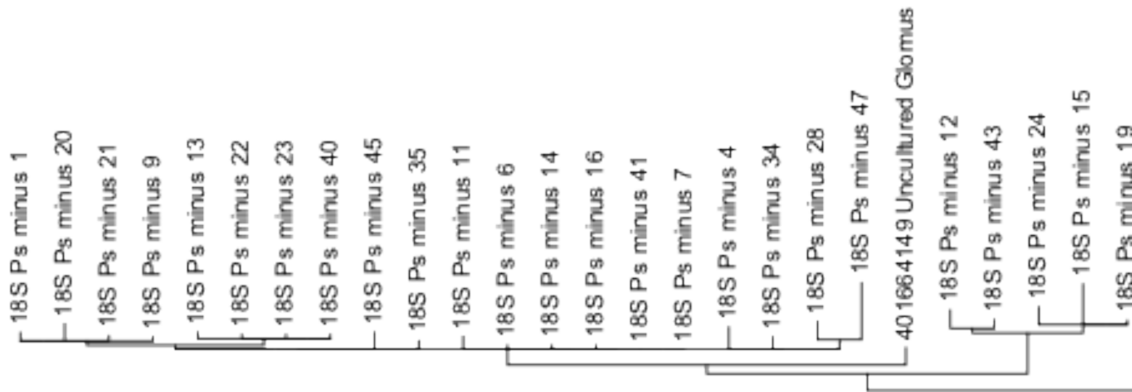
Figure A8.2: ITS PCR-cloning-sequencing, rinsed roots, 2010



Note: The three columns represent samples from our Dry, Rocky, and Sandy fields. See table A8.II for the key to further treatment label details.

Appendix A9: Phylogenetic trees

Figure A9.1: Arbuscular mycorrhiza maximum likelihood tree created using Mega5



Note: Clones are labeled with the primer used (18S), field initial (N, P, or S), from rhizospheric soil or root DNA extract (s or r), and uninoculated (minus), as well as with a number unique to the clone sequence. Database organisms are given by their GI number, followed by genus and species or other identifier as necessary. (page 1)

Figure A9.1: Arbuscular mycorrhiza maximum likelihood tree created using Mega5 (page 2)

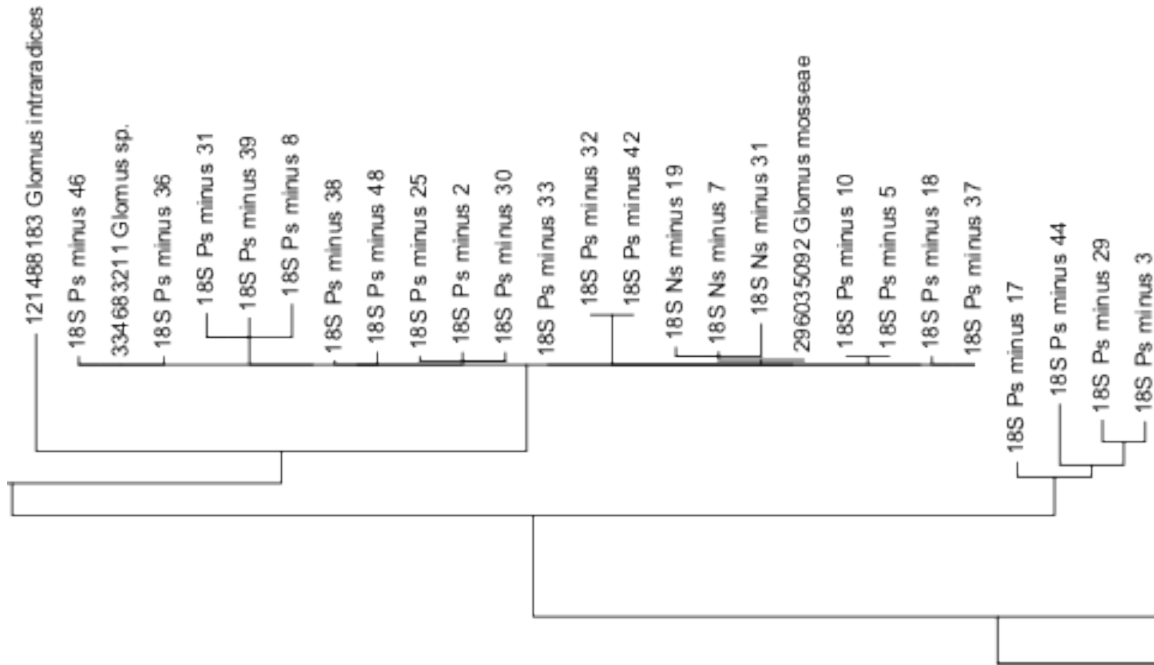


Figure A9.1: Arbuscular mycorrhiza maximum likelihood tree created using Mega5 (page 3)

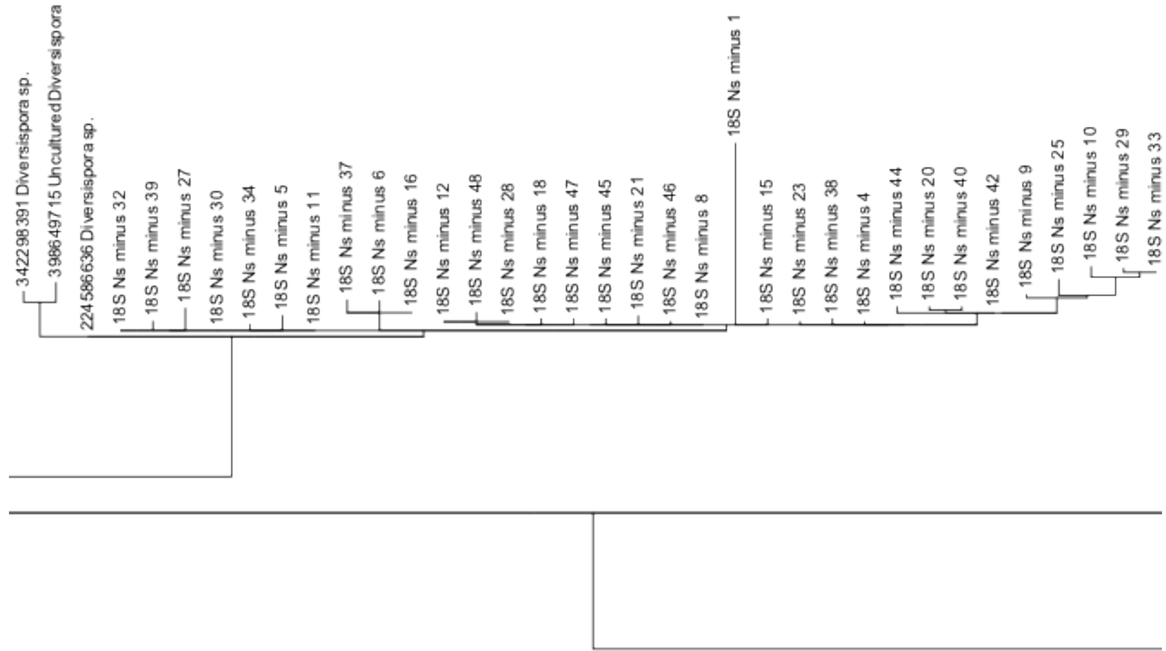


Figure A9.1: Arbuscular mycorrhiza maximum likelihood tree created using Mega5 (page 4)

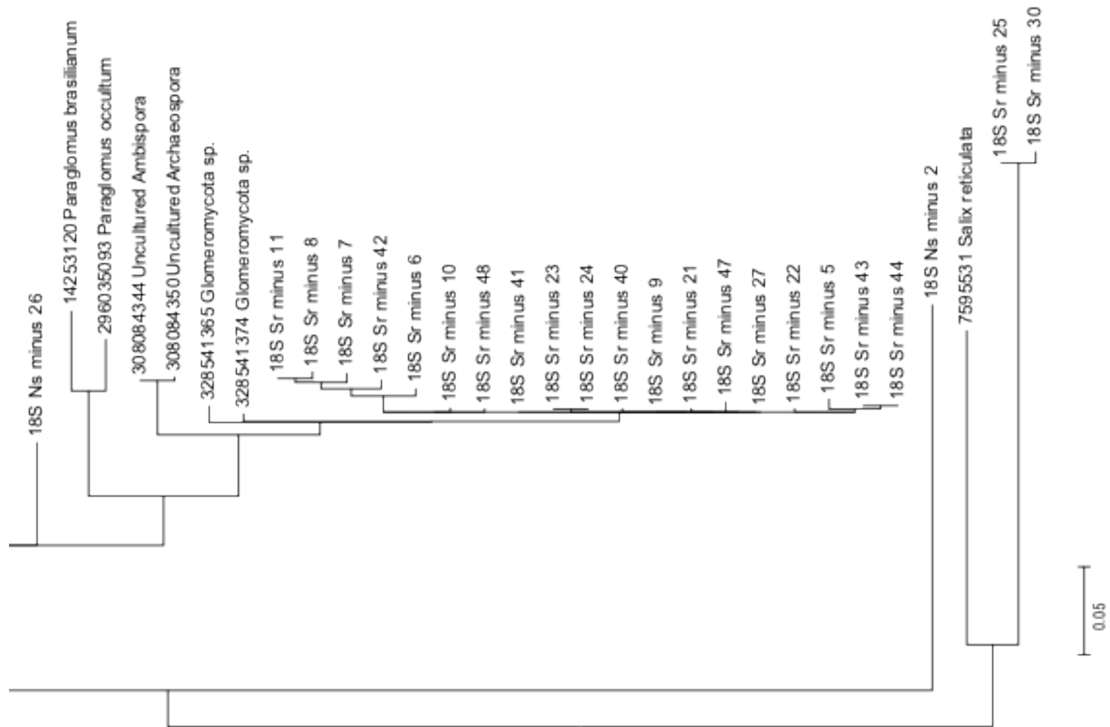
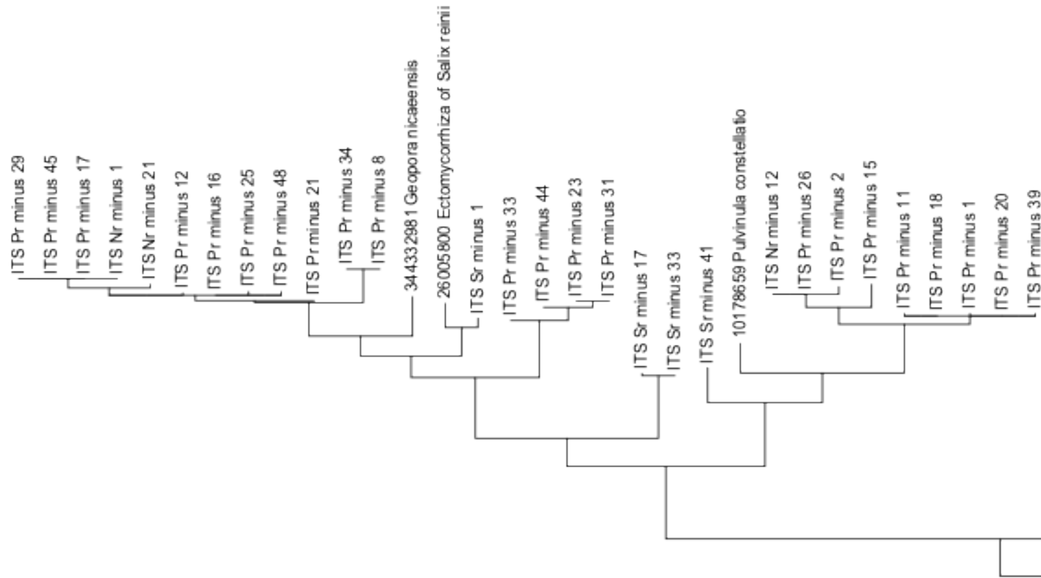


Figure A9.2: Total root fungi maximum likelihood tree created using Mega5



Note: Clones are labeled with the primer used (ITS), field initial (N, P, or S), from root DNA extract (r), and uninoculated (minus), as well as with a number unique to the clone sequence. Database organisms are given by their GI number, followed by genus and species or other identifier as necessary. (page 1)

Figure A9.2: Total root fungi maximum likelihood tree created using Mega5 (page 2)

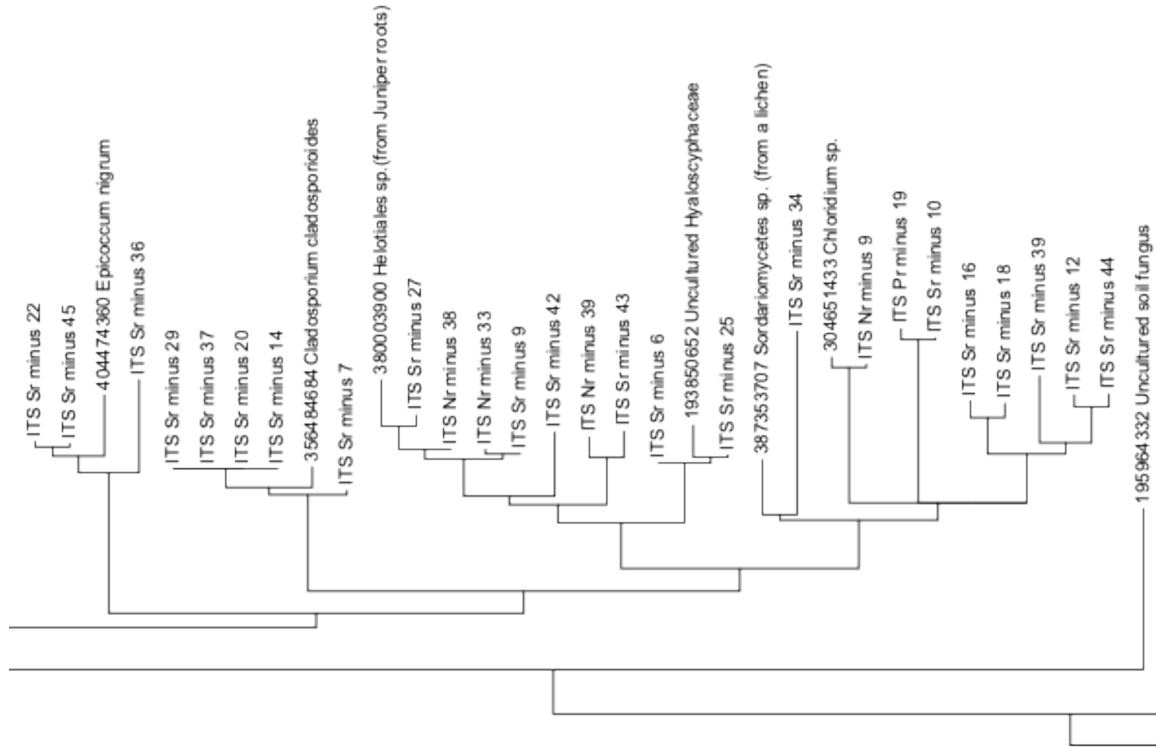


Figure A9.2: Total root fungi maximum likelihood tree created using Mega5 (page 3)

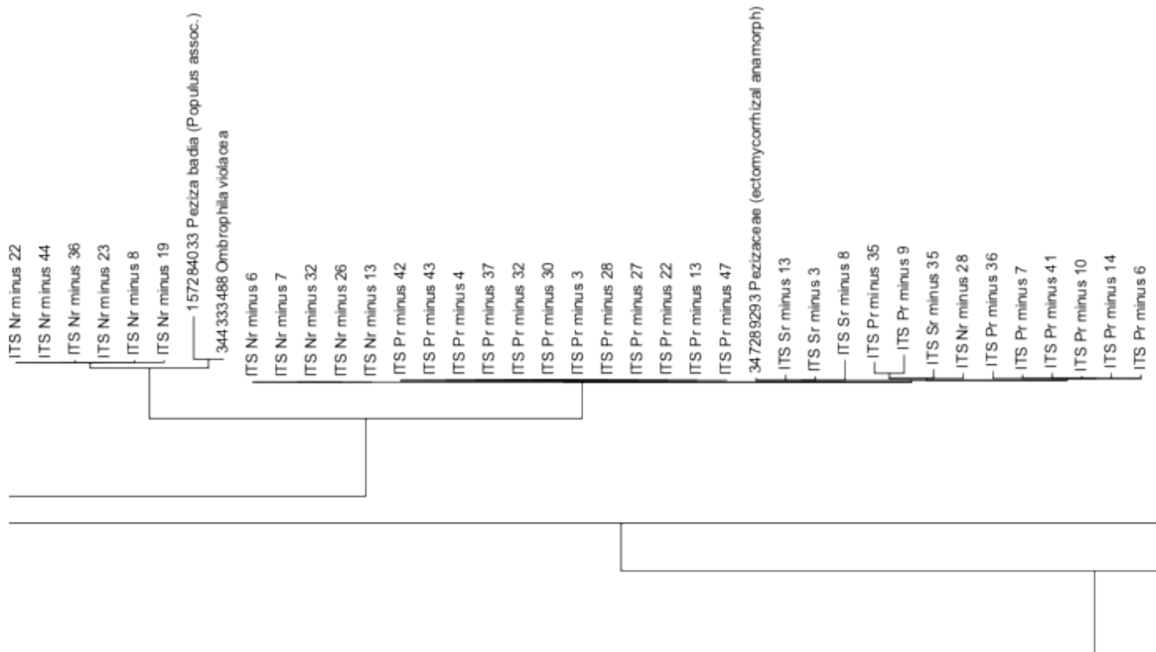


Figure A9.2: Total root fungi maximum likelihood tree created using Mega5 (page 4)

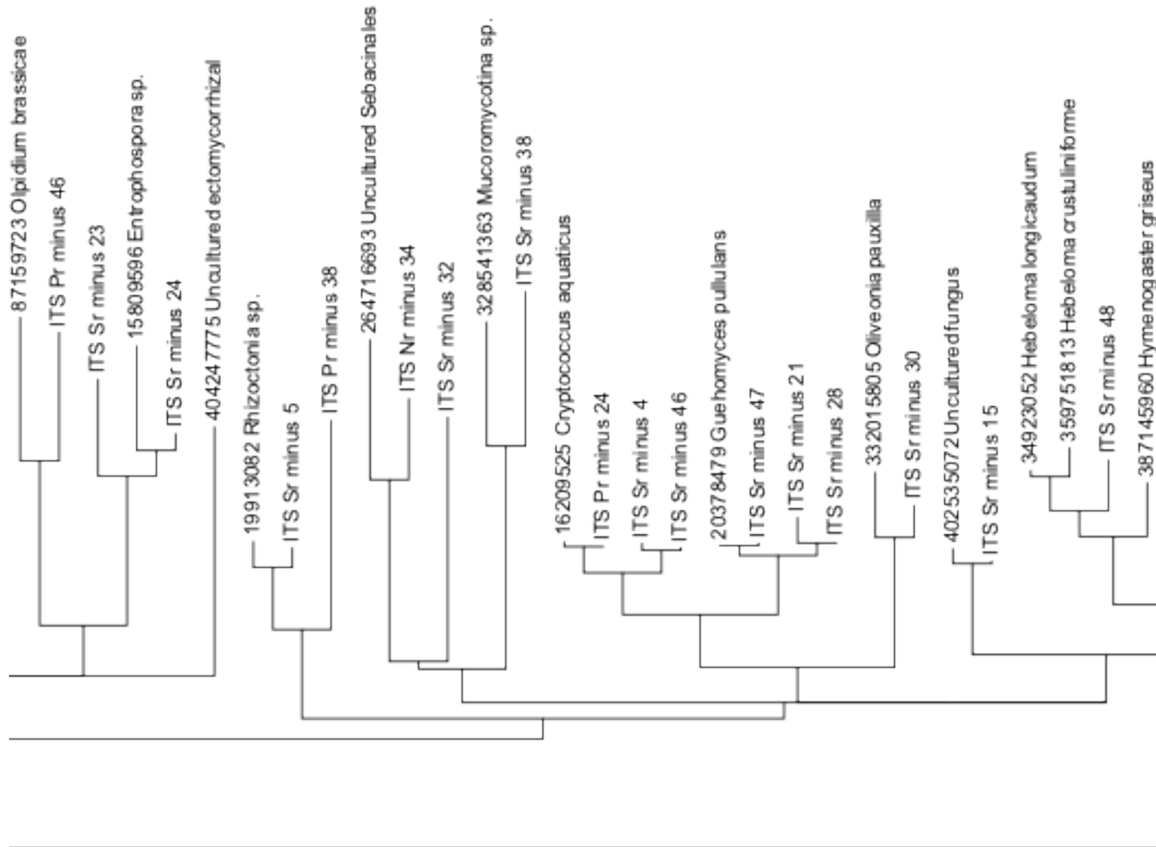


Figure A9.2: Total root fungi maximum likelihood tree created using Mega5 (page 5)