Fungal diversity in a transgenic poplar plantation and the role of ectomycorrhizal fungi for tree performance under field and controlled drought stress conditions

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Summary

The worldwide increasing energy demand comes along with diminishing fossil fuel resources. Thus, research on alternative bioenergy sources is urgently needed. Poplars with optimized properties for bioethanol production are available and have to be tested for sustainable usage in field experiments. It is an important issue to study possible environmental impacts of transgenic poplars on the biodiversity of associated organisms. Fungi play an important role in ecosystem functioning and information on their composition in the soil and on poplar roots of biomass plantations is rare. Poplars gain nutritional benefits from ectomycorrhizal (ECM) symbiosis and there is emerging evidence that ECM fungi could lead to enhanced water stress resistance in their host plants. The role of ECM symbiosis for poplar productivity and stress resistance is an important topic of research, especially in biomass plantations.

In this work, fungal biodiversity in soil and roots of a poplar plantation were analyzed. In addition the role of ECM fungal diversity for poplar productivity and the potential role of ECM in amelioration of drought resistance in poplar were investigated. The following research goals were pursued:

(I) The fungal communities in a short rotation plantation with *P.* × *canescens* wildtype (WT) and two transgenic lines with suppressed cinnamyl alcohol dehydrogenase (CAD) activity were investigated to elucidate (1) if the fungal composition in the soil served as a large species-rich reservoir for the establishment of the fungal composition in roots of WT and the two transgenic lines and (2) if the fungal community in soil and roots was affected by the modification of the two transgenic lines in comparison to the WT.

To investigate the soil/root fungal communities of WT and two transgenic CAD poplar lines, the pyrosequencing approach was used and to detect temporal dynamics of ECM communities on roots pyrosequencing was combined with the morphotyping/ Sanger-sequencing technique. Estimated species richness was highest in soil and decreased in the habitat order soil > root > root associated ECM. It was also shown that the soil serves as a fungal-rich reservoir for fungal species colonizing the roots. Analysis of the life style of the fungi in soil revealed dominance of saprophytic fungi followed by ECM, pathogenic and endophytic fungi, while in roots ECM fungi were the dominant group. Temporal dynamics of ECM fungi colonizing the poplar roots showed an increase in species richness after one year. Most species detected by morphotyping/ Sanger-sequencing in 2009 and 2010 were already detected by pyrosequencing in roots in 2009. The alteration of the CAD gene in poplars had no effect on the fungal community, neither in soil nor in roots.

(II) The biodiversity of ECM fungi in two short rotation plantations, one with commercial *P. deltoides* × *P. nigra* WT clones and the other with *P.* × *canescens* WT and seven transgenic lines with suppressed activities of CAD, caffeate/5-hydroxyferulate O-methyltransferase (COMT) or cinnamoyl-CoA reductase (CCR), were investigated to elucidate (1) if the ECM communities on the roots of poplars were affected by the gene modification of the transgenic lines compared to the WT and (2) if stem biomass and nutrient status in WT and transgenic lines were correlated with ECM colonization and community composition.

To investigate the ECM community on the roots of poplar and to investigate if ECM fungi are linked with stem biomass production and nutrition, roots of three clones of *P. deltoides* × *P. nigra* (WT) in 2010 and roots of *P.* × *canescens* (seven transgenic lines and the WT) in 2009 and 2010 were analyzed by morphotyping/ Sanger-sequencing approach. Stem material of *P.* × *canescens* lines was used for analyzing the nutritional status of the poplars. Non metric multidimensional scaling (NMDS) revealed a similar fungal community structure of the different genotypes in 2009, while in 2010 a clustering of fungal communities was detected. However, the variation was in the range of fungal community structures obtained in the commercial poplar field. Comparison of the fungal community structure of the WT from 2009 and 2010 revealed a highly dynamic succession. Fungal community structures of the transgenic lines were not affected by gene modifications of poplars. Furthermore, these results demonstrate that multiple poplar genotypes increase the ECM community composition in poplar plantations. Differences in growth and nutrient element concentrations in wood of transgenic poplars were found. A general mixed model revealed a link between the main factors for stem biomass prediction, ECM colonization and inverse wood N concentration.

(III) Drought stress responses of mycorrhizal and non-mycorrhizal *P.* × *canescens* plants were investigated in a controlled drought stress experiment to elucidate (1) if the ECM fungus *Paxillus involutus* improved the physiological responses of *P.* × *canescens* under water stress conditions and (2) if *P. involutus* enhanced the nutrition status of its host under drought stress conditions and (3) if the enhanced nutrition status was related to the extent of mycorrhization.

To elucidate the drought stress response of mycorrhizal and non-mycorrhizal drought stressed *P.* × *canescens* plants, the water supply was slowly decreased. The results showed that the gravimetric soil water content under mild and medium water stress was higher in mycorrhizal than non-mycorrhizal control and drought stressed plants. This effect was also reflected in a slower decreasing relative water content of leaves in mycorrhizal compared to

non-mycorrhizal drought stressed plants. The efficiency of photosystem II (PSII) was enhanced in mycorrhizal control and drought stressed plants and in case of drought treated plants the efficiency decreased only after severe water limitation. In contrast, the stomatal conductance was mainly affected by drought even under mild drought stress, while the effect of mycorrhiza was only apparent in combination with drought and time. Most of the stress related genes investigated were up- or down-regulated in non-mycorrhizal and mycorrhizal drought stressed plants compared to non-mycorrhizal control plants. The nutrient status in leaves of mycorrhizal plants was enhanced compared to non-mycorrhizal plants. ANCOVA results of leaves revealed a positive effect of mycorrhizal colonization on nutrient status in drought stressed plants.

In conclusion, the present study showed that soil serves as reservoir for ECM fungi establishing symbiotic interactions with poplar roots. Links between poplar productivity and nutrition status and ECM colonization were established and it was demonstrated that ECM fungi ameliorate the stress responses and nutrition status of poplars under drought stress conditions. Thus, the results of this work provide information which underpins the significant role of the ectomycorrhizal symbiosis in relation to nutrient status of the poplar under drought stress conditions, and in relation to stem biomass production in a poplar plantation. These informations could be of crusial importance in the establishment phase of a poplar plantation as well as in relation to predicted increasing extreme climate events which could have negative impacts on biomass production.

Zusammenfassung

Der weltweit steigende Energiebedarf geht einher mit abnehmenden Rohstoffquellen für fossile Brennstoffe. Die Forschung an alternativen Rohstoffen zur Bioenergie-Gewinnung ist daher ein wichtiges und notwendiges Thema. Pappeln mit optimierten Eigenschaften zur Bioethanol-Gewinnung sind verfügbar und müssen in Bezug auf nachhaltige Nutzung im Freiland getestet werden. Ein diesbezüglich sehr wichtiger Aspekt, ist die Erforschung negativer Einflüsse transgener Pappeln auf die Biodiversität der mit ihnen assoziierten Organismen. Pilze erfüllen eine wichtige Rolle im Ökosystem. Die Bedeutung von Pilzgemeinschaften im Boden und auf Pappelwurzeln in Biomasse-Plantagen ist bisher jedoch nur wenig untersucht. Durch Symbiosen mit Ektomykorrhizen (EM) besitzen Pappeln eine verbesserte Nährstoffversorgung und zunehmende Hinweise deuten darauf hin, dass EM-Pilze zu gesteigerter Trockenstress-Resistenz bei ihren Wirtspflanzen führen können. Den Einfluss der EM-Symbiosen auf die Produktivität von Pappeln und ihre Stressresistenz zu untersuchen, ist daher ein wichtiges Forschungsthema, besonders in Bezug auf Biomasse-Plantagen.

In dieser Arbeit wurde die Pilz-Biodiversität im Boden und an den Wurzeln der Bäume einer Pappel-Plantage untersucht. Zusätzlich wurde der Einfluss der EM-Biodiversität auf die Pappel-Produktivität sowie die potentielle Rolle der EM unter Trockenstress-Bedingungen in den Pappeln erforscht. Dabei wurden die folgenden Forschungsziele verfolgt:

(I) In einer Kurzumtriebsplantage mit *Populus* × *canescens* wurden die Pilzgemeinschaften im Boden und an den Wurzeln vom Wildtyp (WT) und zwei transgenen Linien mit unterdrückter Cinnamylalkoholdehydrogenase (CAD) Aktivität untersucht, um zu erforschen, (1) ob die Pilzgemeinschaft im Boden als ein großes, artenreiches Reservoir für die Besiedlung der Wurzeln von WT und den beiden transgenen Linien dient und (2) ob die Pilzgemeinschaft im Boden und in den Wurzeln durch die Genmodifikation der beiden transgenen Pappel-Linien, im Vergleich zur Kontrolle, beeinflusst wird.

Um die Pilzgemeinschaften im Boden und in den Wurzeln zu untersuchen, wurden die Pyrosequenzierung genutzt. Diese Methode wurde zur Ermittlung der temporären Dynamiken der EM-Gemeinschaften auf den Pappelwurzeln mit Morphotyping/ Sanger-Sequenzierung kombiniert. Die ermittelte Artenvielfalt der Pilze war im Boden am höchsten und nahm in der Reihenfolge Boden > Wurzel > Wurzel assoziierte EM ab. Die Ergebnisse zeigten auch, dass der Boden als pilzreiches Reservoir für Wurzeln besiedelnde Pilze dient. Die Analyse der Lebensweisen der Pilze zeigte eine klare Dominanz saprophytischer Pilze gefolgt von EM, pathogenen und endophytischen Pilzen, wohingegen in den Wurzeln die EM-Pilze dominierten. Die zeitliche Dynamik der EM-Pilze zeigte eine Zunahme der

Artenvielfalt nach einem Jahr. Die meisten durch Morphotyping/ Sanger-Sequenzierung detektierten Pilze in den Jahren 2009 und 2010 wurden mit der Pyrosequenzierung schon im Jahr 2009 gefunden. Die Genmanipulation am CAD Gen der Pappeln hatte keinen Effekt auf die Pilzgemeinschaft, weder im Boden, noch in den Wurzeln.

(II) Die Biodiversität von EM-Pilzen in zwei Pappel-Plantagen, eine bepflanzt mit kommerziellen *P. deltoides* × *P. nigra* WT Klonen, die andere mit *P.* × *canescens* WT sowie sieben transgenen Linien (unterdrückte CAD, Kaffeesäure-*O*-Methyltransferase (COMT) oder Cinnamoyl-CoA-Reduktase (CCR) Aktivität) wurde untersucht, um festzustellen, (1) ob die EM-Gemeinschaften in den Wurzeln durch die Genmodifikation der Pappeln im Vergleich zum WT beeinflusst werden und (2) ob die Stamm-Biomasse sowie der Nährstoffgehalt im WT und den transgenen Linien mit dem Grad der EM-Besiedlung und Artenzusammensetzung korrelieren.

Für diese Untersuchungen wurden 2010 Wurzeln von *P. deltoides* × *P. nigra* (WT) und 2009 und 2010 Wurzeln von *P.* × *canescens* (sieben transgene Linien und WT) mittels Morphotyping/ Sanger-Sequenzierung analysiert. Stamm-Material von den unterschiedlichen *P.* × *canescens* Linien wurden für die Analyse des Ernährungsstatus genutzt. Nicht Metrische Multidimensionale Skalierung (NMDS) ergab eine ähnliche Struktur der Pilzgemeinschaften der unterschiedlichen Pappel-Linien in 2009, während in 2010 eine Gruppierung der verschiedenen Linien gefunden wurde. Die beobachteten Unterschiede der Pilzgemeinschaften waren jedoch vergleichbar mit denen, die in der kommerziellen Pappel-Plantage beobachtet wurden. Der Vergleich der Pilzgemeinschaften der WT-Pappeln von 2009 und 2010 deutet auf eine hochdynamische Sukzession hin. Die gentechnische Veränderung der Pappeln hatte keinen Einfluss auf die Pilz-Gemeinschaften. Die Zusammensetzung der EM Gemeinschaften wurde jedoch durch den Einsatz unterschiedlicher Genotypen in der Pappel-Plantage beeinflusst. Es wurden Unterschiede in Wachstum und der Konzentration von Nähstoffen im Holz der transgenen Pappeln gefunden. Anhand eines general mixed models konnte der Zusammenhang zwischen EM Kolonisierung und inverser N Konzentration im Holz in Bezug auf die Stamm-Biomasse aufgezeigt werden.

(III) Die Trockenstress-Reaktion von mykorrhizierten und nicht-mykorrhizierten *P.* × *canescens* Pflanzen wurden in einem kontrollierten Trockenstress-Experiment untersucht, um zu ermitteln, (1) ob der EM-Pilz *Paxillus involutus* sich positive auf die physiologische Stressantwort von *P.* × *canescens* unter Trockenstress-Bedingungen auswirkt und (2) ob *P.*

involutus den Ernährungszustand der Pappeln unter Trockenstress-Bedingungen verbessert und (3) ob ein verbesserter Ernährungszustand durch die Mykorrhiza auch vom Grad der Mykorrhizierung abhängt.

Um die Trockenstress-Antwort von mykorrhizierten und nicht mykorrhizierten Pappeln zu untersuchen, wurde die Wasserzufuhr der Trockenstress-Pflanzen langsam reduziert. Die Ergebnisse des Versuchs zeigten, dass der gravimetrische Bodenwassergehalt der mykorrhizierten Pappeln bei leichtem und mittlerem Trockenstress höher war als der Bodenwassergehalt der nicht-mykorrhizierten Pappeln. Dieser Effekt wurde im Wassergehalt der Blätter widergespiegelt, nicht jedoch in den anderen Geweben. Die Effizienz des Photosystems II (PSII) war in den mykorrhizierten Kontroll- und Trockenstress-Pflanzen erhöht und sank in den Trockenstress-Pflanzen erst bei extremem Wassermangel. Im Gegensatz dazu verringerte sich die stomatäre Leitfähigkeit in mykorrhizierten und nicht mykorrhizierten Trockenstress-Pflanzen schon bei mildem Trockenstress. Die meisten der in diesem Versuch untersuchten, generell an Stressantworten beteiligten Gene, zeigten in mykorrhizierten und nicht mykorrhizierten Trockenstresspflanzen, im Vergleich zu nicht mykorrhizierten Kontrollpflanzen veränderte Expression. Der Ernährungszustand in Blättern von mykorrhizierten Pflanzen war erhöht verglichen mit nicht mykorrhizierten Pflanzen. Eine durchgeführte ANCOVA zeigte, dass der Grad der Mykorrhizierung ebenfalls einen positiven Effekt auf den Nährstoffstatus in Pflanzen unter Trockenstress hatte.

Die obigen Forschungsergebnisse konnten aufzeigen, dass der Boden als Reservoir für Ektomykorrhiza-Pilze fungiert, die symbiotische Interaktionen mit den Pappelwurzeln aufbauen. Weiterhin konnte der Zusammenhang zwischen Pappelproduktivität und Nährstoffstatus und EM-Kolonisierung demonstriert werden, was besonders in der Etablierungsphase eine Biomasse-Plantage von Bedeutung ist. Schließlich wurde noch die Signifikanz des EM-Pilzes *Paxillus involutus* auf die Stressreaktion und Ernährung der Pappeln unter Trockenstress-Einfluss gezeigt, was im Hinblick auf die Klimaerwärmung und damit einhergehenden häufigeren, extremen Wetterbedingungen von Bedeutung ist, da diese den Ertrag der Biomasseplantagen negativ beeinflussen können.

Chapter 1

Introduction

1 Introduction

1.1 *Populus* spp. as model organism

Poplars are economically important trees used in pulp and paper industry. The ecological relevance of these trees is reflected by their wide distribution, their ability to grow on marginal lands (Rooney *et al.*, 2009), their use for phytoremediation on heavy metal contaminated soils (Pulford & Watson, 2003) and their tolerance of waterlogging (Kreuzwieser *et al.*, 2002). Poplars have a rapid juvenile growth and are thus adequate for biomass production in short rotation coppice (Monclus *et al.*, 2006). Since global warming is accompanied by an increase in the greenhouse gas CO_2, research on fast growing trees as carbon sinks has gained importance (Gielen & Ceulemans, 2001). The use of plants as renewable energy from biomass reduces the fossil fuel combustion and contributes to a reduction in CO_2 emission (Lemus & Lal, 2005; Sims *et al.*, 2006).

The release of the *Populus trichocarpa* genome (Tuskan *et al.*, 2006) was a breakthrough in the poplar research. Ongoing research on the poplar pangenome drives the understanding of genetic diversity across populations and offers the possibility to create poplars with desirable traits for bioenergy production (Neale & Kremer, 2011). Biomass of woody plants mainly consists of hemicelluloses, cellulose, and lignin (Baucher *et al.*, 2003). This biomass has to be degraded by chemical and enzymatic processes which are hindered by recalcitrant components like lignin (Himmel *et al.*, 2007). To overcome this problem the lignin biosynthesis pathway could be modified and target genes for this purpose have been identified (Ehlting *et al.*, 2005). Genetically modified poplars with altered lignin composition or reduced lignin concentration are produced and have been investigated in greenhouse experiments (Baucher *et al.*, 1996; Leplé *et al.*, 2007; Van Doorsselaere *et al.*, 1995). However, to test these genetically modified trees in field experiments is often difficult due to high licensing requirements and sometimes encountered public resistance.

1.2 Biodiversity and fungal community structure

Since anthropogenic disturbance of ecosystems has driven the loss of biodiversity, the role of biodiversity for ecosystem functioning has gained much attention over the past decades (Chapin *et al.*, 2000; Mccann, 2000). Research was done to clarify the link between community structure and ecosystem productivity as well as the link between species diversity and ecosystem stability (Chapin *et al.*, 1997; Coleman & Whitman, 2005; Loreau *et al.*, 2001;

Naeem, 2002; Waide *et al.*, 1999). If genetically modified trees should be used for commercial biomass production, it is necessary to elucidate the possible risk of these modifications for the environment, in particular on biodiversity of organisms directly interacting with these trees.

Fungi play an important ecological role in ecosystems and can be classified according to their lifestyles into saprophytes, endophytes, pathogens and mycorrhizal fungi. Plants benefit from mycorrhizal fungi due to an enhanced nutrient supply in exchange for carbohydrates (Smith & Read, 2008). Over 90% of all land plants established mycorrhizal symbiosis (Cairney, 2000) and research of the evolution of mycorrhiza and plants indicating a strong correlation between the ability of plants to grow on land and the evolution of mycorrhizal symbiosis (Wang *et al.*, 2010). Fungal endophytes have been found in most plant species. They inhabit healthy tissue of plants and grow within roots, stems and leaves without damaging the inhabiting tissue (Stone *et al.*, 2000). Endophytes were shown to increase drought (Richardson *et al.*, 1992) or insect resistance (Cheplick & Clay, 1988). Pathogens on the other hand damage their host and may lead to severe reduction in health and growth. Thus, it is important to gain information on the abundance of each fungal group, and the possible alterations of these abundances due to tree modifications in biomass plantations. Furthermore, it is interesting to know in which way the soil fungal community serves as a species-rich reservoir for fungi colonizing the roots.

Due to the expenditure of time needed for studying these fungal lifestyles most studies focused only on one of these groups in the past. Molecular techniques such as cloning and Sanger sequencing and the use of public databases improved the accuracy of the identification of different fungal species (Horton & Bruns, 2001). Since White *et al*. (1990) designed the first ITS primers for amplification of the internal transcribed spacer (ITS) region of the ribosomal DNA it has become one of the most used target for fungal identification (Dahlberg, 2001; Ryberg *et al.*, 2009). Molecular techniques are, however, not unbiased. DNA extraction and amplification have been shown to affect the outcome of sequencing (Tedersoo *et al.*, 2010). In some fungal species the ITS region was shown to display a high intraspecific variability while other species showed high similarity (Glen *et al.*, 2001), together with sequencing mistakes and a lot of unidentified species an accurate identification of fungal species is difficult (Nilsson *et al.*, 2006). Thus a combination of different methods would be advisable. The development of new high throughput methods enables studying the whole fungal community with one molecular approach. Although these methods have a high sensitivity and could detect more species than other methods like cloning and Sanger sequencing they have their disadvantages. Methods like pyrosequencing are indeed able to detect for example the ectomycorrhizal fungi community on roots but lack information which

of the ECM species at least established the mycorrhizal symbiosis with the host plant. To answer questions regarding the degree of colonization or diversity of ECM fungi on roots a time intense method like morphotyping has to be used. Combining these two methods is a possibility to gain information of the potential pool of mycorrhizal fungi that inhabit the soil and/or root and those species which actually outcompete the others and establish the mycorrhizal symbiosis with the host plant.

1.3 Mycorrhizal fungi

Mycorrhizal fungi play important roles in soil ecosystems such as nutrient cycling and carbon sequestration (Smith & Read, 2008). In the symbiosis of mycorrhizal fungi with host plants the fungi provide nutrients to the host which would be otherwise inaccessible for them. The plants on the other hand provide the mycorrhizal fungi with energy in terms of carbohydrates (Smith & Read, 2008). Seven different types of mycorrhiza are described: ectomycorrhiza (ECM), arbuscular mycorrhiza (AM), ectendomycorrhiza, arbutoid mycorrhiza, ericoid mycorrhiza, monotropoid mycorrhiza and orchid mycorrhiza (Finlay, 2008; Smith & Read, 2008). Poplars are able to establish ECM and AM at the same time (Baum *et al.*, 2002b) and it was shown in several studies that ECM fungi are the dominate mycorrhiza in poplar plantations (Baum *et al.*, 2002a; Gehring *et al.*, 2006; Khasa *et al.*, 2002). Due to the focus on poplar in this thesis, further descriptions of mycorrhiza are limited to ECM fungi.

ECM fungi penetrate into the plant root where the hyphae grow between epidermis and cortex cells, building a network of hyphae called Hartig net. This network enlarges the surface area for nutrition exchange between fungi and host plant (Smith & Read, 2008). While the Hartig net is similar in all ECM species the hyphal mantle surrounding the root tips differ strongly in morphology. A.B. Frank (1885) was the first researcher describing ectomycorrhizal fungi. In Agerer (1987- 2006) a large collection of detailed descriptions of the morphology and anatomy of different ECM fungi on different host plants are found. ECM fungi can be distinguished by color, branching and surface texture of the mantle and by emanating mycelia. According to their emanating hyphae, ECM fungi were classified as different exploration types (Agerer, 2001). They are defined as contact type with few emanating hyphae, short-distance type with a lot of emanating hyphae, medium-distance type forming rhizomorphs (three subtypes distinguished by rhizomorphal structures), long-distance type with few highly differentiated rhizomorphs and pick-a-back type which can grow within mantles and/or rhizomorphs of other ectomycorrhizal fungi (Agerer, 2001). Different parts of the mantle and emanating hyphae could differ in their hydrophobic/hydrophilic

properties and thus influence nutrient and water uptake (Taylor & Alexander, 2005; Unestam & Sun, 1995).

1.4 Functional traits of ECM fungi

Mycorrhizae have been shown to improve nutrient acquisition, especially that of N and P for their host plants (Smith & Read, 2008). The symbiosis increases the absorptive surface area of the plant roots due to the extramatrical mycelium of the fungi (Harley, 1989; Rousseau *et al.*, 1994). The emanating hyphae can grow rapidly into soil areas behind the depletion zone of the plant roots (Bending & Read, 1995; Carleton & Read, 1991) and thus enhance the nutrient accessibility for the host. Enzyme activities (Courty *et al.*, 2005) and nutrient uptake rates and utilization of different nutrients vary among ECM fungi (Abuzinadah & Read, 1989; Finlay *et al.*, 1992; Wallander *et al.*, 2003) indicating an important role of mycorrhizal diversity for the host plant. The host plants benefit not only by improved nutrition from the mycorrhizal symbiosis, they were also shown to be better protected against heavy metals (Schützendübel & Polle, 2002), pathogens (Smith & Read, 2008) or drought stress (Morte *et al.*, 2000).

The IPCC report (2007) has forecast more extreme weather events due to global warming such as long drought periods which could negatively affect biomass production. It was shown that pines get access to water through the extraradical mycelia of the ECM fungus *Suillus bovines* (Duddridge *et al.*, 1980). The pathways in which water is transported from the external hyphae to the stele of the host plant are similar as in roots, namely, the apoplastic, symplastic or transmembrane pathway (Lehto & Zwiazek, 2011). Furthermore enhanced aquaporin expression in mycorrhizal seedlings compared to non-mycorrhizal seedlings was found by Marjanovic *et al.* (2005) indicating a direct benefit of mycorrhiza symbiosis under drought conditions due to enhanced water transport through this class of transmembrane proteins. When the accessibility of water becomes more and more impaired the plant is exposed to drought stress, leading to a reduced uptake of nutrients and a reduction of photosynthesis, which altogether hinders growth (Finlay, 2008; Smith & Read, 2008). However, the role of ECM symbiosis for plant nutrition under drought stress condition is less understood (Smith & Read, 2008). Alvarez et al. (2009) could demonstrate that ECM fungi improved nutrient N and P status in *Nothofagus dombeyi* under drought stress conditions. Thus, research on potential benefits of plants due to mycorrhizal symbiosis under drought stress conditions is an interesting research topic, especially in relation to biomass plantations.

1.5 Research topics addressed in this thesis:

In **Chapter 2**, the fungal community in soil and roots of a transgenic poplar plantation was examined in relation to fungal diversity and fungal lifestyles. Additionally, the succession dynamics of ECM fungi in two adjacent years were highlighted and the potential environmental risk of the gene modification was investigated. For this purpose the fungal communities of the different poplar lines were analyzed by a combined approach of pyrosequencing and morphotyping/ Sanger-sequencing.

In **Chapter 3**, the ECM fungal communities on roots of wildtype and transgenic poplars were examinded by morphotyping/ Sanger-sequencing to verify the potential effect of the transgenics on the ECM diversity and to elucidate the role of ECM fungi for biomass production and nutrition in a poplar plantation.

In **Chapter 4**, the physiological and nutrional responses of *P.× canescens* with and without mycorrhiza on slowly decreasing water availability were investigated in a controlled drought stress experiment. The goal was to elucidate if the ECM fungi *Paxillus involutus* ameliorate the drought stress response of the host.

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Chapter 2

Fungal soil communities in a young transgenic poplar plantation form a rich reservoir for fungal root communities

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Fungal soil communities in a young transgenic poplar plantation form a rich reservoir for fungal root communities

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Abstract

Fungal communities play a key role in ecosystem functioning. However, only little is known about their composition in plant roots and the soil of biomass plantations. The goal of this study was to analyze fungal biodiversity in their belowground habitats and to gain information on the strategies by which ectomycorrhizal (ECM) fungi form colonies. In a 2-year-old plantation, fungal communities in the soil and roots of three different poplar genotypes (*Populus* x *canescens*, wildtype and two transgenic lines with suppressed cinnamyl alcohol dehydrogenase activity) were analyzed by 454 pyrosequencing targeting the rDNA internal transcribed spacer 1 (ITS) region. The results were compared with the dynamics of the root-associated ECM community studied by morphotyping/Sanger sequencing in two subsequent years. Fungal species and family richness in the soil was surprisingly high in this simple plantation ecosystem, with 5944 operational taxonomic units (OTUs) and 186 described fungal families. These findings indicate the importance that fungal species are already available for colonization of plant roots (2399 OTUs and 115 families). The transgenic modification of poplar plants had no influence on fungal root or soil communities. Fungal families and OTUs were more evenly distributed in the soil than in roots, probably as a result of soil plowing before the establishment of the plantation. Saprophytic, pathogenic, and endophytic fungi were the dominating groups in soil, whereas ECMs were dominant in roots

(87%). Arbuscular mycorrhizal diversity was higher in soil than in roots. Species richness of the root-associated ECM community, which was low compared to ECM fungi detected by 454 analyses, increased after 1 year. This increase was mainly caused by ECM fungal species already traced in the preceding year in roots. This result supports the priority concept that ECMs present on roots have a competitive advantage over soil-localized ECM fungi

Keywords: Community Ecology, Fungi, Environmental DNA, Genetically Modified Organisms, Metagenomics, Microbial Biology

2.1 Introduction

Anthropogenic activities can cause dramatic changes of ecosystem structures and their ecological services (Dawson, 2011). Stability and maintenance of ecosystems rely on biodiversity and functional dynamics of organisms (Johnson *et al.,* 1996). The impact of organismal groups on ecosystem stability depends on several factors such as adaptation strategies, interaction with other organisms (Johnson et al, 1996) and manner of nutrient acquisition. Fungi are a group of central importance as they play key roles in the carbon and nitrogen cycle improving the availability of nutrients for other organisms. They are distributed across all climatic zones and colonize different habitats in ecosystems such as soil (Bridge & Spooner, 2001), plant tissues (Arnold *et al.,* 2000), water (Jones, 2011) or rocks (Gadd, 2007).

Fungi can be classified according to their life style and ecological function to be saprophytic, pathogenic, endophytic, and mycorrhizal. Traditionally, those different groups have been analyzed separately by targeted approaches. With the advent of deep sequencing techniques it is now possible to record these communities comprehensively as a precondition to understanding their interactions. For example, the analysis of rhizosphere and root endophyte communities in two natural poplar stands on contrasting soils revealed differentiation of the communities between roots and soil as habitats, but surprisingly no significant soil-related effects (Gottel *et al.,* 2011). Furthermore, in contrast to previous morphotyping/cloning studies in poplar plantations (Kaldorf *et al.,* 2004; Stefani *et al.,* 2009), deep sequencing suggested that mycorrhiza-forming fungal genera were underrepresented in roots (Gottel *et al.,* 2011). It has been speculated that genetic differences between poplar species affect mycorrhizal colonization (Tagu *et al.,* 2001; Karlinski *et al.,* 2010) and thus influence the composition of fungal communities in roots (Gottel *et al.,* 2011). Strong variation has been found among ectomycorrhizal fungi that colonize specific coniferous

species influenced by plant genotypes (Dučić *et al.,* 2009; Karlinski *et al.,* 2010). Many ECM fungi show strong host preferences (Lang *et al.,* 2011), but the whole root-inhabiting fungal community is composed of different ecological groups. It is unknown if fungal root communities as a whole can also be affected by the plant genotype.

Poplars are an important feedstock for biofuel production (Polle & Douglas, 2010). Agro-forest areas are currently being expanded to meet the demand for sustainable biomass production. Since soil-borne fungi have critical impact on plant health and productivity the conservation of healthy communities of soil biota and biological soil management are considered pivotal to ensure soil fertility and overall productive and sustainable agricultural systems (Matson *et al.,* 1997). However, knowledge on structure, function and ecology of soil microbial communities is still very limited, especially for managed agro-forest plantations. As there is increasing interest in the use of fast growing tree species for production of second generation biofuel, attempts are underway to increase pulping properties of the wood by transgenic modification of lignin content and composition (Baucher *et al.,* 1996; Pilate *et al.,* 2002). Previous studies showed faster decomposition of leaf litter of poplars with suppressed activity of cinnamyl alcohol dehydrogenase (antisense CAD) than that of wildtype leaves (Pilate *et al.,* 2002). It is currently unknown if changes in tissue composition of transgenic poplar also influence the assemblage of root-inhabiting fungi or if transgenic poplars affect the fungal community in the soil.

The main goal of the present study was a comprehensive analysis of fungal biota in soil and roots of wildtype and two antisense CAD poplar genotypes to test the hypothesis that the soil forms a large species-rich reservoir that leads to the differentiation of distinct fungal communities in wildtype and transgenic poplars. We conducted our study in a recently established experimental short rotation plantation of hybrid poplar (*Populus tremula* x *P. alba*, syn. *P.* x *canescens*) wildtype and transgenic lines. We applied 454 pyrosequencing analyses for in-depth characterization of fungal communities using the rDNA ITS1 region as marker gene.

The role of soil as reservoir for root colonization was investigated (i) on the base of taxa composition in fungal soil and root communities, (ii) with respect to clustering of functional fungal groups in roots of different genotypes and adjacent soil, and (iii) with regard to temporal dynamics of ECM communities identified by morphotyping/sequencing techniques compared to 454 pyrosequencing.

2.2 Materials and Methods

2.2.1 Plant materials and study site

Populus tremula x *Populus alba* (female clone INRA #717-1B4) wildtype and transgenic lines with a modified lignin metabolism were multiplied by micropropagation (Leplé *et al.,* 1992). In June 2008, rooted plantlets were planted outdoors in a field trial (47°83´N, 1°91´E) nearby the INRA in Orleans, France, on sandy soil with flint (Fig. 2.1). Climate is typical of the Loire Valley with oceanic tendencies, westerly dominant winds, average annual precipitation of 600 mm and a mean annual temperature of 10.4°C. Natural flora is acidophilic and characteristic of poor soils, with oak, birch, chestnut, pine and heather as prominent species belonging to the phytosociologic order *Quercetalia robori-petraeae*.

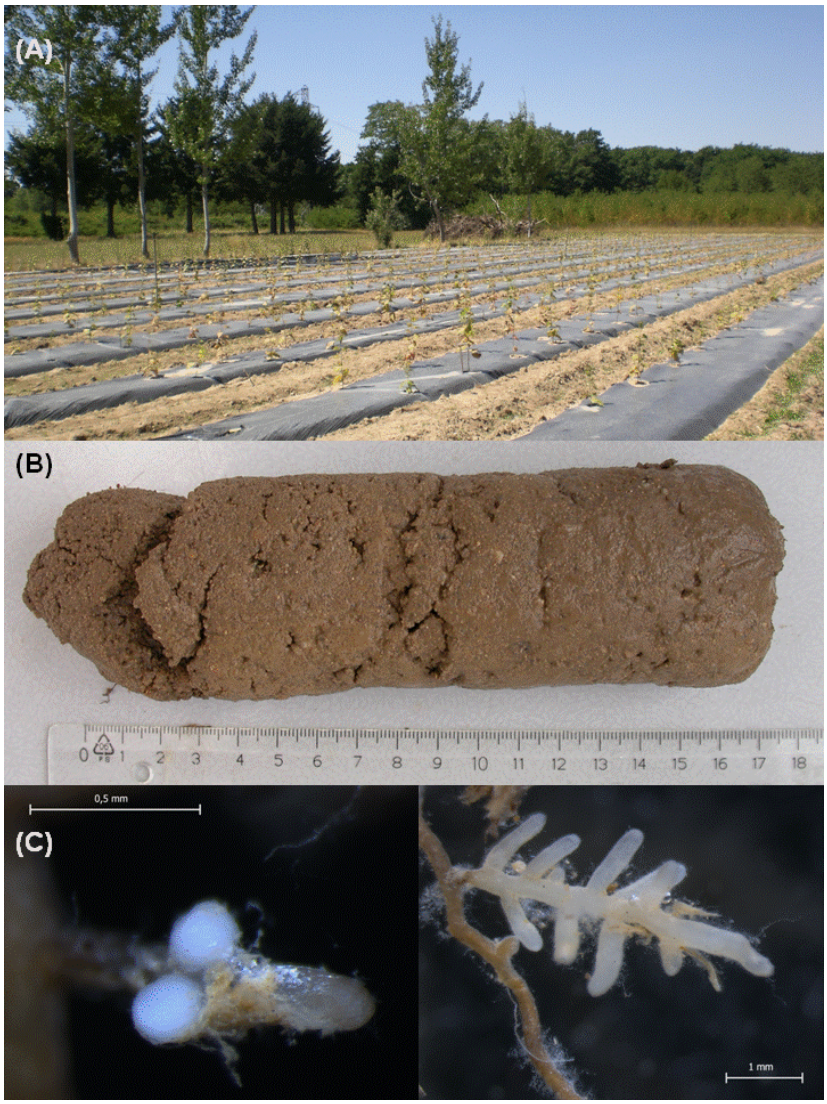

Fig. 2.1: Soil cores (B) were taken on a 2-year-old poplar plantation (A) and cut into two longitudinal sections. 454 Pyrosequencing was applied on one half to study fungal soil and root communities. Out of the second half, poplar roots were described by morphotyping (C) and ITS-sequencing (left picture: *Hebelome sacchariolens*; right: *Laccaria tortilis*).

The field trial was established in an area of 1365 m^2 with 120 plants per line (seven transgenic and one wildtype). The poplars were planted in randomized subplots, each consisting of 24 plants (four lines of six individual plants (Supporting Information Fig. S2.1). Plants were drip irrigated during the growing period. In March 2010, all trees were coppiced according to typical management practices in a short rotation plantation.

2.2.2 Sampling strategy

In October 2009 wildtype plants and two transgenic lines (ASCAD21 = L21, ASCAD52 =L18) with a decreased activity of CAD (Lapierre *et al.* 1999), were used for sampling. Three plots per line were chosen (Supporting information Fig. S2.1). In each plot nine soil cores (depth: 0.2 m, diameter: 0.05 m) (Fig. 2.1) were collected at a distance of 0.25 m between two neighboring poplar stems (for details, see Supporting Information Fig. S2.1). In total we collected 81 soil cores (27 per poplar line). In addition, leaves were collected. Soil cores and leaves were transported on ice and processed in the laboratory within 72 h after sampling.

The soil cores (Fig. 2.1) were cut longitudinally into two halves with a sterile scalpel (Supporting information Fig. S2.1). One half was used for analyses of ECM fungal community by morphotyping/ITS-sequencing and the other half for analyses of the overall fungal soil and root community by deep sequencing.

For ECM analyses three halves were pooled, resulting in three samples per plot. The samples were soaked in tap water and roots were removed by gentle washing. They were stored between wet filter papers at 4°C until further processing.

For analyses of 454 pyrosequencing, each sample was processed individually. Roots were cautiously removed from the soil, washed in autoclaved water, separated from roots of other plant species by shape and color under a stereomicroscope (Stemi SV 11, Zeiss, Jena, Germany) and frozen at -20°C. The soil was sieved, homogenized, subsampled in volumes of 2 ml, and stored by -20°C. Aliquots of the soil samples were used for nutrient element analyses.

2.2.3 Soil analyses

Soil pH was determined after extraction in water for 4h. Aliquots of the soil were weighed, dried for 4 days at 60°C, weighed again, and used to calculate the dry-to-fresh mass ratio. Carbon (C) and nitrogen (N) concentrations were determined by dry combustion using a C/N

analyser (Carlo Erbas Instruments, Italy). Mineral element concentrations of P, S, K, Ca, Mg, Mn, and Fe were determined using an Inductively Coupled Plasma – Atomic Emission Spectrometer (Spectro Flame, Spectro Analytic Instruments, Kleve, Germany) after pressure digestion of samples in 65% HNO_3 for 12 h (Heinrichs *et al.,* 1986). To determine the nitrate and ammonium concentrations, samples of 20 g soil were extracted in 40 mL 1 mmol/L $CaCl_2$, filtered, freeze-dried, and dissolved in 0.5 mL double deionized water. The aliquots were used for photospectrometric measurement of nitrate and ammonium using commercial kits (Spectroquant, Merck, Darmstadt, Germany).

2.2.4 DNA extraction and quality check

Eighty-one root samples and 10 leaf samples were freeze-dried and ground in a ball mill Type MM2 (Retsch, Haan, Germany). Hundred milligram root powder was suspended in 400 µL LSS-buffer of the "innuPREP Plant DNA kit" (analytikjena, Jena, Germany). Genomic DNA was extracted according to the manufacturer's instructions and eluted in 100 µL nuclease-free water (AppliChem, Darmstadt, Germany). Samples were checked for contamination by roots of other plant species by amplifying the trnL intron-region of the chloroplast DNA with the plant specific primer pair c (CGAAATCGGTAGACGCTACG) and d (GGGGATAGAGGGACTTGAAC) (Taberlet *et al.,* 1991). The polymerase chain reaction (PCR) reaction mix was composed of 2 µL template DNA (up to 15 ng), 2.5 µL 10x buffer (Fermentas, St. Leon-Rot, Germany), 2 µL of $MgCl_2$ (25mM, Fermentas), 1.25 µL of each primer (10 mmol/L) (Eurofins MWG Operon, Ebersberg, Germany), 0.5 µL dNTPs mix (10 mmol/L, Fermentas), 0.125 µL *Taq* polymerase (>10 U/µL, Fermentas) and 16.625 µL of nuclease-free water, resulting in a total volume of 25 µL. The PCR was performed in a Mastercycler Gradient (Eppendorf, Hamburg, Germany) starting with a hot-start at 95°C followed by 95°C for 1 min, 35 cycles of 30 s at 94°C (denaturation), 30 s at 53°C (annealing) and 1 min at 72°C (extension), and terminated with 72°C for 5 min. PCR products were subjected to electrophoresis in 2% agarose gels, ethidium bromide staining, and were scanned (Raytest scanner FLA 5100, Straubenhardt, Germany). PCR products on the DNA of leaves of the same poplar lines as for roots were used as positive control. In the few cases where contamination was detected, new samples were prepared.

Eighty-one soil samples were dried in a SpeedVac-Concentrator Savant SPD 11V230 (Thermo, Bonn, Germany) and ground in a ball-mill. Genomic DNA was extracted using the Soil kit (MoBio, Carlsbad, USA) following the manufacturer's instructions.

2.2.5 Amplicon generation and 454 pyrosequencing

All 162 DNA samples were amplified separately. Total extracted DNA was employed in the amplification at different concentrations (undiluted, 1:10, 1:50, 1:100). The Amplicon libraries were generated with primers including the Roche GS FLX Titanium Amplicon-Adaptor Sequences (A-Key, B-Key, Key: TCAG), a 10bp multiplex identifier (MID1-29, see Table 1, TCB No. 005-2009, Roche, Mannheim, Germany) in front of the B-Adaptor for multiplexing the PCR Products and the template-specific primers ITS1f (Gardes & Bruns, 1993) and ITS2 (White *et al.,* 1990), resulting in fusion primers A-ITS1F (5` CGTATCGCCTCCCTCGCGCCATCAG-CTTGGTCATTTAGAGGAAGTAA- 3`) and B-MID-ITS2 (5` CTATGCGCCTTGCCAGCCCGCTCAG-MID-GCTGCGTTCTTCATCGATGC). PCR reactions were performed as described above but 0.7 µL of 16 mg/mL bovine serum albumin (Merck, Darmstadt, Germany) was added to a total PCR mix volume of 25 µL. After amplification, the PCR products were purified using the "innuPREP PCRpure Kit" (analytikjena, Jena, Germany). Then, the PCR products from three cores of the same tree were pooled, resulting in 27 amplicon libraries for root and soil, respectively, with independent replicates for each line. Amplicon concentration was determined with the Qubit™ dsDNA HS Assay Kit in a Qubit fluorometer (Invitrogen GmbH, Karlsruhe, Germany). The 27 amplicon libraries of root- and soil-samples, respectively, were pooled in equal amounts for 454-pyrosequencing. Amplicon libraries were sequenced with the 454 Genome Sequencer FLX (Roche, Mannheim, Germany) using the amplicon sequencing protocol and Titanium chemistry (Roche, Mannheim, Germany). Sequencing was performed by the Göttingen Genomics laboratory (http://www.g2l.bio.uni-goettingen.de/). Three medium lanes of a Titanium picotiter plate were used for sequencing of the complete amplicon libraries. The entire quality data set as unprocessed data files were deposited into the sequence read archive (SRA). The study accession number is ERP001442 and can be accessed by following link: http://www.ebi.ac.uk/ena/data/view/ERP001442.

2.2.6 Bioinformatics and OTU clustering

After the removal of barcodes and tags, 454 pyrosequencing reads were processed with a perl script discarding all reads shorter than 150 bp and reads containing more than four ambiguity symbols. On average 74% of all reads passed these criteria. The individual sample FASTA files were subjected to cluster analysis for a tentative OTU count using the clustering function of USEARCH v5.3.23 (Edgar, 2010) with the following criteria: ≥ 97% similarity over ≥ 90% sequence length. Cluster analyses were carried out on individual and "combined" samples, the latter ones including sequence read information of all soil or all root samples.

All singletons were removed prior to further analyses. To identify OTUs at taxonomic level, a randomly selected sequence of an OTU cluster was compared to the nonredundant GenBank database (Benson *et al.,* 2008) and the custom-curated database RSyst (http://mycor.nancy.inra.fr/RSyst/) using BLASTn (Altschul *et al.,* 1997). A post-processing perl script stored the ten best BLASTn hits per cluster with an expectation value of $<10e^{-3}$ in a BLASTn-file. OTUs with a taxonomic assignment at the species level were classified with respect to their ecological lifestyle by literature research (Tab. S2.1, Supplemental information). Ecological groups were categorized as follows: AM, arbuscular mycorrhizal; ECM, ectomycorrhizal; lichenized; saprotrophic; endophytic or pathogenic.

2.2.7 Morphotyping on root tips

Grass roots were identified by differences in morphology and removed. Three-hundred living root tips were inspected per poplar tree. ECM fungi were morphotyped (Fig. 2.1) using a simplified method after Agerer (1987-2006) recording shape, color, texture of the mantle, and presence or absence of hyphae or rhizomorphes under a stereomicroscope (M205 FA, Leica, Wetzlar, Germany). ECM colonization (%) was calculated as: number of ECM root tips x 100/total number of root tips. Three to four ECM root tips of each morphotype were collected and stored at -20°C.

2.2.8 Cloning and sequencing of ectomycorrhizal species

Genomic DNA of the frozen ECM root tips was extracted using the "innuPREP Plant DNA kit" (analytikjena). The rDNA ITS-region was amplified by PCR with the primer pair ITS5/ITS4 (White *et al.,* 1990) as described above with the following modifications: 34 cycles and an annealing temperature of 55°C. Direct Sanger-sequencing or cloning/sequencing was carried out according to Lang *et al*. (2011). Sequences were blasted using the following databases: NCBI (nBLAST) (http://www.ncbi.nih.gov/), Fungal RSyst (http://mycor.nancy.inra.fr/RSyst/), and UNITE (http://unite.ut.ee/). Sequences are available at NCBI (accession JQ409279-JQ409296).

2.2.9 Data analyses

To test for possible variability of fungal communities of different samples, 454 pyrosequencing data were blasted against the RSyst database. A perl script stored the top

BLASTn hit (E-value < $10e^{-3}$) and the number of reads per species of each sample in a csv-file. Statistical analyses were performed on the basis of the number of reads per species and the relative abundance of reads. Samples were compared by a pairwise test based on the relative frequencies. The Wilcoxon rank-sum test was used to identify significant differences according to a *P*-value ≤ 0.05 after Bonferroni-correction. All our statistical analyses were carried out by using the software R-2.9.2 (R Developent Core Team, 2009). Additionally, nonmetric multidimensional scaling (NMDS) with the function metaMDS of the "vegan" package (Oksanen *et al.,* 2010) was applied. Before running the NMDS, data were square root transformed.

Statistical analyses of the fungal communities forming visible ECM with roots were based on the relative abundance of the morphotypes. The Kruskal-Wallis rank sum test (package "stats") was used to identify differences between poplar genotpyes ($P \leq 0.05$).

The Wilcoxon rank sum test with an additional Bonferroni correction was carried out to examine differences in biodiversity indices and the relative abundances of fungal families within different sample types, respectively.

The defined OTUs were used to calculate taxon accumulation curves with the freeware software Analytic Rarefaction version 1.3 (http://www.uga.edu/strata/software/Software.html). Biodiversity indices and species richness estimators were calculated using the software EstimateS version 8.0.0 (Collwell, 2006). Evenness was additionally determined by the formula (Shannon/LN [number of detected OTUs]).

Presence/absence data of fungal families in individual soil and root samples were subjected to hierarchical cluster analysis using EPCLUST (http://www.bioinf.ebc.ee/EP/EP/EPCLUST/index.cgi). Correlation-based distance measure was chosen as similarity metric and average distance as clustering method.

Differences in soil parameters were tested with one-way analysis of variance (ANOVA).

2.3 Results

2.3.1 Fungal species richness and diversity in soil and roots

In total, 811,900 sequence reads were generated by 454 pyrosequencing. Sequence reads that did not match our quality criteria were removed (see Material and Methods) resulting in 686,053 sequence reads for further analyses. In all, 4,706 - 17,994 sequences were obtained

per sample (Tab. 2.1). These sequences were clustered according to similarity and yielded 750 - 800 non-singleton OTUs per sample (Tab. 2.1). Forty-eight to 703 singletons per sample were obtained (Tab. 2.1).

Tab. 2.1: Summary of 454 pyrosequencing data. Samples are defined by sample type (soil or root samples) and poplar genotype (transgenic or wildtype). Twenty-seven samples were taken per sample type. Eighteen samples of transgenic and nine of wildtype plants, respectively. OTUs = operational taxonomic units

	Soil beneath		Roots from	
	Transgenic poplar	Wildtype poplar	Transgenic poplar	Wildtype poplars
Sequence reads	297,836	153,626	203,238	157,200
Sequence reads after quality control	251,883	129,962	166,556	137,652
Sequences per sample	11,631 - 15,965	9,524 - 17,994	6,568 - 10,835	4,706 - 9,620
Number of OTUs (non-Singletons per sample)	392 – 800	395 – 736	75 – 225	118 – 249
Number of Singletons per sample	326 – 675	307 – 703	48 – 143	2 – 112

Rarefaction curves based on 97% sequence identity leveled off between 398 and 817 OTUs for soil samples and between 91 to 249 OTUs for root samples (Supporting Information, Fig. S2.2A and B). Rarefaction analyses for complete fungal richness of the study site in soil and roots showed saturation at 5944 and 2399 OTUs, respectively (Supporting Information, Fig. S2.2D). Root samples exhibited higher variability in the shape of their species accumulation curves indicating strong scattering of species richness between different samples. Rarefaction analyses of ECM root communities revealed complete coverage (Supporting Information, Fig. S2.2C). Estimated species richness (H_{max}) showed a clear decrease in the order of the habitats soil > roots > root-associated ECM communities (Fig. 2.2). The decrease in species richness from soil to roots and ECM communities was also reflected by the Shannon indices (Fig. 2.2). Evenness was highest for EM communities and lowest for fungi in roots (Fig. 2.2).

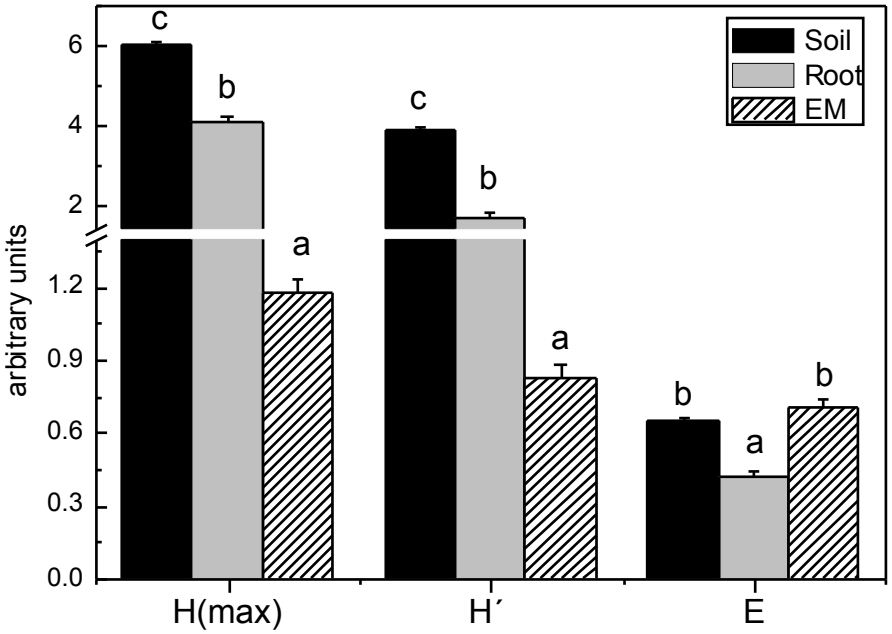

Fig. 2.2: Species richness (Hmax), Shannon index (H´) and Evenness (E) of fungi in soil, roots and of root-associated ECM communities. Diversity indices are means (n = 27 ± SE). Significant differences between bar heights (P ≤ 0.05) are indicated by different letters above bars. Hmax = ln (species number).

2.3.2 Fungal community structure in different habitats and poplar genotypes

To find out if the poplar genotype affected fungal abundance or community structures in roots or soil, Wilcoxon rank-sum tests with Bonferroni correction were conducted. However, no significant difference was detected between the three investigated genotypes (transgenic lines ASCAD52 [=L18], ASCAD21 [=L21] and wildtype; $P \leq 0.05$) with respect to the presence of fungal species or their abundance. These findings held true for soil and root samples as well as root-associated ECM fungi. Soil nutrients (per gram dry soil) did not differ between samples of different poplar genotypes (NO_3^-, 17.2 ± 2.04 µmol; NH_4^+, 15.3 ± 1.7 µmol; total N, 0.88 ± 0.08 mg; P, 0.22 ± 0.01 mg; S, 0.09 ± 0.01 mg; Ca, 0.93 ± 0.04 mg; Mg, 0.51 ± 0.02 mg; Mn, 0.17 ± 0.01 mg; Fe, 4.16 ± 0.20 mg; C, 15.5 ± 1.4 mg; pH 5.85 ± 0.03), with the exception of K (mean: 1.19 ± 0.04 mg/g dry soil), which was slightly higher (13% above the mean) in soil collected beneath poplar line 18 than in that beneath the wildtype ($P < 0.02$).

An NMDS plot calculated for OTUs revealed strong clustering of fungal communities for soil and roots, respectively (stress = 13.63, nonmetric fit R^2 = 0.98) (Fig. 2.3). Permutation test confirmed significant classification with $P < 0.001$ (R^2 = 0.6332). No separation of samples related to plant genotype or the position in the field was detected.

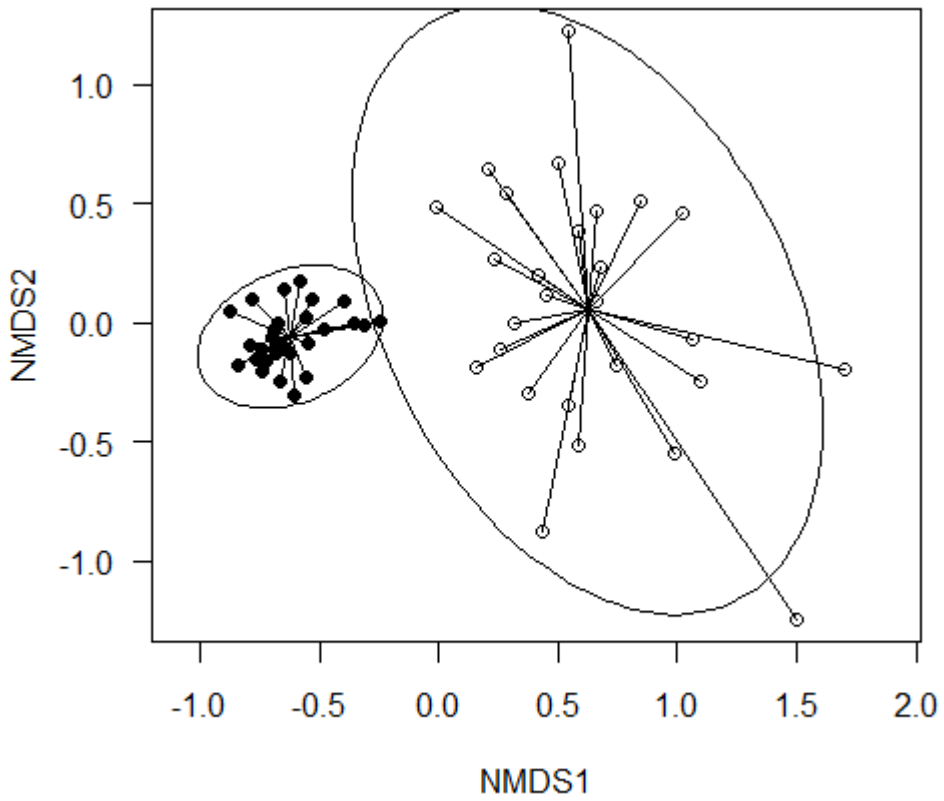

Fig. 2.3: NMDS plot of the fungal community structure using the Bray-Curtis dissimilarity measure. Each point represents the fungal community of a given sample. Permutation tests revealed a highly significant classification (P = 0.001). Samples were classified according to the plant genotype (wildtype; transgenic CAD lines L18 and L21), sampling point, and sample type (black circles, soil; open circles, root). Stress value = 13.63, R^2 = 0.98. Ellipses separate samples into two categories: left ellipse = soil samples, right one = root samples. Confidence area of ellipses = 0.95.

The significant differences between the fungal communities of soil and roots originated, therefore, from the lower species richness of roots compared with soil. Although soil contained higher species richness than roots, the scattering of data was lower (see ellipses in Fig. 2.3), indicating higher homogeneity of species distribution in soil than in roots.

2.3.3 Fungal family abundance and distribution across soil and root samples

OTUs were clustered according to their taxonomic affiliation into overall 196 fungal families. Soil (186) and root (115) samples differed in their fungal family composition. Eighty-one fungal families were solely found in soil samples and 10 only in root samples (Supporting Information, Tab. S2.2). Among the common families 59 were significantly more abundant in soil samples than in roots (Fig. 2.4).

Two families, Filobasidiaceae and Mortierellaceae, were dominant in soil, each comprising about 15% of all OTUs. The relative abundance of nine further families in soil ranged between 1 and 5%, whereas all other fungal families that differed significantly from roots were present only with low abundance (<1%) (Fig. 2.4).

In root samples six fungal families were significantly enriched in comparison to soil (Fig. 2.4). Pyronemataceae dominated the community (13.5%) in roots, while the relative abundances of Paxillaceae, Paraglomeraceae, Rhytismataceae, and Sporormiaceae ranged between 1.3% and 2%. Russulaceae were represented by 0.04% of the OTUs (Supporting Information, Tab. S.2.2).

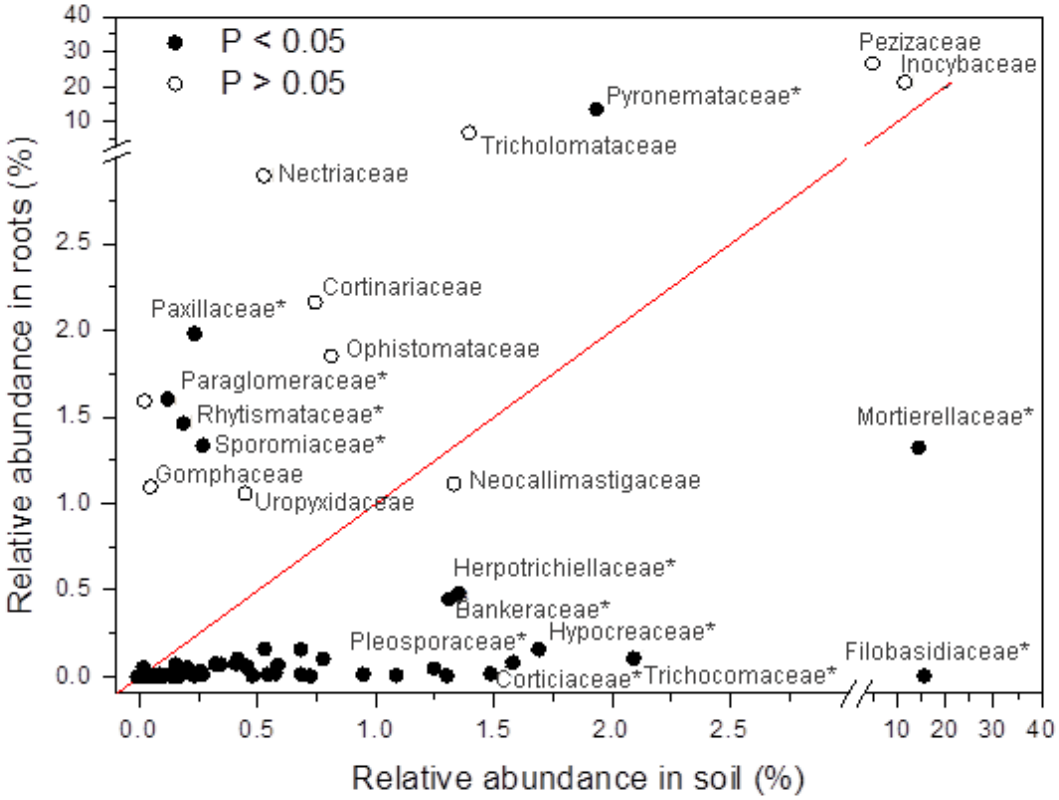

Fig. 2.4: Distribution of fungal families in soil and root samples according to their relative abundance. Significant differences (P < 0.05) between soil and roots are indicated by black circles; open circles indicate fungal families with similar abundance in soil and roots. Families with abundances above 1% were labeled with a star. Red line indicates equal abundances in both roots and soil. 100% is the total abundance of all fungal families.

Hierarchical cluster analyses demonstrated the distribution pattern of fungal families in individual samples (Fig. 2.5). In soil samples, about one quarter of all fungal families were present in >90% of the samples. Forty-six percent and 59% of fungal families were detected in at least >50% and >25% of all soil samples, respectively (Fig. 2.5A). In contrast, the clustering of fungal families in root samples differed (Fig. 2.7B). Only 8% of all fungal families in root samples were present in >90% of all samples. Twenty percent and 38% of fungal families were present in >50% and >25% of the samples, respectively (Fig. 2.5B).

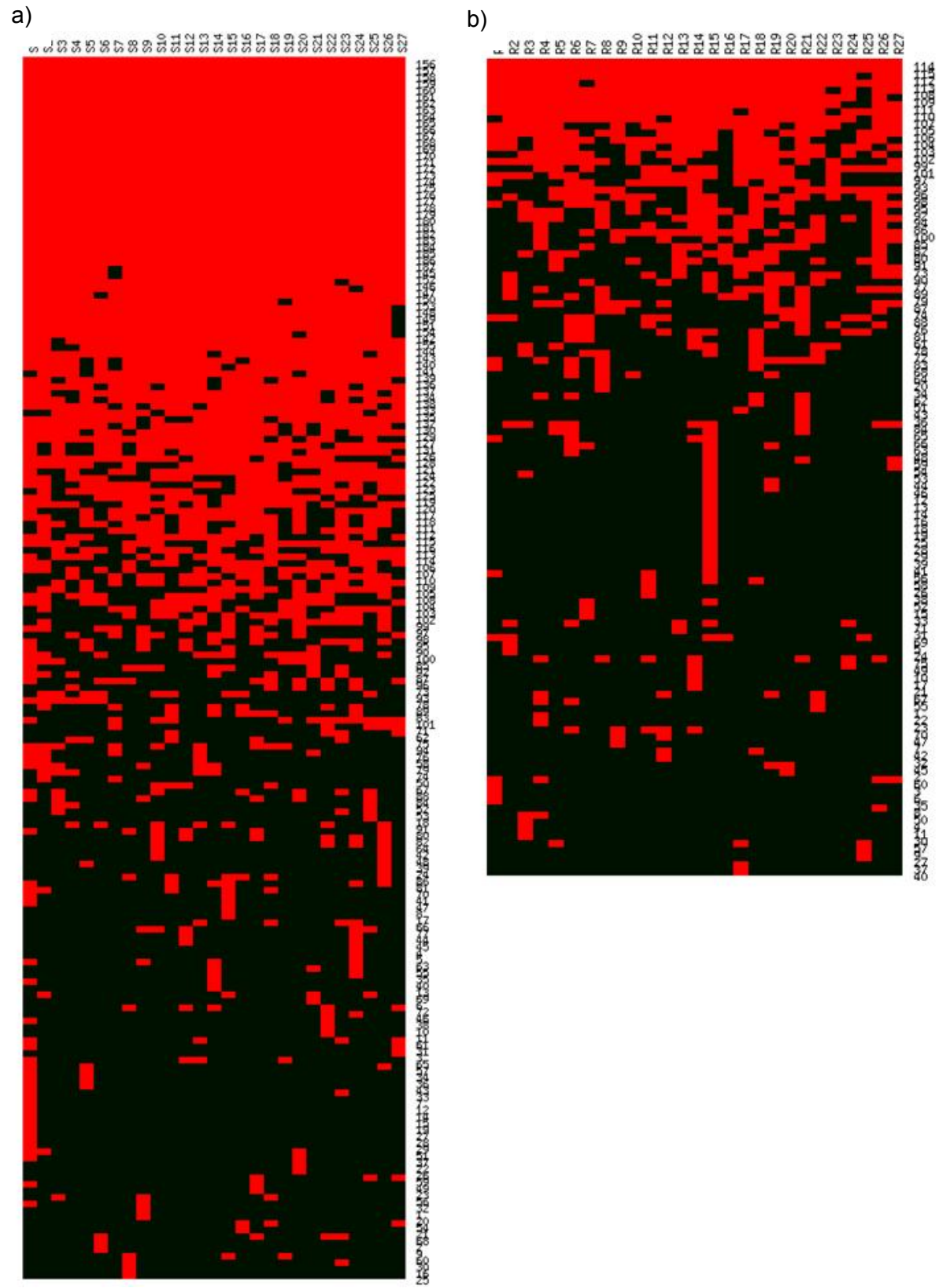

Fig. 2.5: Heat map showing clustering of fungal families in (a) soil and (b) root samples. The color code of the heat map indicates presence (red) or absence (black) of fungal families (in rows) in the individual samples (in columns).

2.3.4 Ecological groups in soil and root samples

To examine the distribution of ecological groups in soil and root samples 1272 and 463 OTUs, respectively, that could be assigned to species levels were selected and their abundances were set 100%. One hundred and fity-six and 27 of these species constituted 90% of the relative abundance in soil and roots, respectively, and were classified after literature research as ECM, AM, saprophytic, endophytic, pathogenic or lichenized fungi (Supporting Information, Tab. S2.1). In soil samples, saprophytic fungi (47%) formed the largest group, followed by 23% ECM, 19% pathogenic, and 8% endophytic fungi. Lichenized and AM fungi were present only in low abundances of 1.8% and 0.4%, respectively (Fig. 2.6).

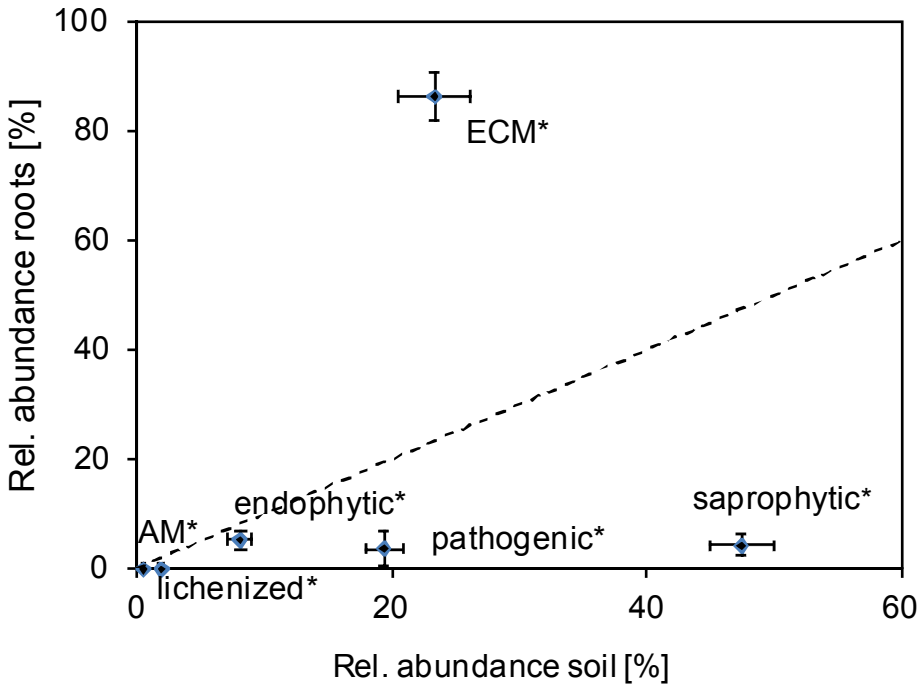

Fig. 2.6: Distribution of fungal species with different ecological lifestyle in soil and root samples. OTUs with >97% sequence identity to known species were classified based on their taxonomic affiliation to six different ecological lifestyles. Only OTUs accounting for 90% of overall relative abundance were included in the analysis. All ecological lifestyle groups were significantly different ($P < 0.05$) from the dashed line which indicates the same abundance in roots and soil. AM, arbuscular mycorrhizal; ECM, ectomycorrhizal fungal species; OUT, operational taxonomic unit.

In root samples, ECM fungi were the dominant group encompassing 87% of the total abundance. Endophytic, pathogenic and saprophytic fungi showed lower abundances of 5%, 4%, and 4%, respectively. On the species level, no AM or lichenized fungi were detected (Fig. 2.6).

2.3.5 Dynamic of the ectomycorrhizal community on poplar roots

With increasing age, poplar roots showed a typical increase in ECM fungal richness (Smith & Read, 2008). In October 2009, seven, and in October 2010, nineteen ECM fungal species were detected on roots, of which six (2009) and 16 (2010) were identified by rDNA ITS sequencing (Supplemental Information Tab. S2.4). With the exception of *Hebeloma* sp. and an uncultured Pezizales (JQ409284), the ECMs identified in 2009 were also present in 2010.

To understand dynamic processes within the ECM community and root colonization, morphotyping/ITS-sequencing and 454 pyrosequencing approaches were compared. All ECM species detected in 2009 were also detected by 454 pyrosequencing in both soil and root samples (Tab. 2.2). Furthermore, 13 of the 16 fungal species that colonized the roots in 2010 were already detected on poplar roots by 454 pyrosequencing in 2009.

Tab. 2.2: Fungal species detected by two approaches: morphotyping/ITS-sequencing and 454 pyrosequencing. In October 2009 and 2010, ectomycorrhizal poplar root tips were sampled, classified by morphotyping and analysed by ITS-sequencing (in total 27 samples). Additionally in 2009, poplar roots and soil samples were taken and subjected to 454 pyrosequencing analysis.

Fungal species	**ITS-Sequencing ECM root tips**		**454 pyrosequencing**	
			roots	**soil**
	2009	**2010**	**2009**	**2009**
Peziza ostracoderma	x	x	x	x
Paxillus involutus	x	x	x	x
Laccaria tortilis	x	x	x	x
Hebeloma sacchariolens	x	x	x	x
Tomentella ellisii		x	x	x
Scleroderma bovista		x		x
Cenococcum geophilum		x	x	x
Xerocomus ripariellus		x	x	x
Hebeloma sp.	x		x	x
Geopora sp.		x	x	x
Tuber sp.		x		x
uncultured Ascomycota JQ409293		x		
uncultured Ascomycota JQ409292		x	x	x
uncultured ectomycorrhizal fungi JQ409294		x	x	x
uncultured fungus JQ409288		x	x	x
uncultured fungus JQ409287		x	x	x
uncultured *Peziza* JQ409295		x		
uncultured Pezizales JQ409284	x		x	x

Two of the three missing species, *Scleroderma bovista*, and *Tuber* sp., were detected solely in soil samples. Only two species, an uncultured Ascomycota (JQ409293) and an uncultured Peziza (JQ409295) that formed ECM in 2010, were detected neither in soil nor root samples in 2009 by 454 pyrosequencing approach.

2.4 Discussion

2.4.1 Massive 454 pyrosequencing reveals surprisingly high fungal species richness in a young short rotation plantation

Rarefaction analyses indicated that we detected the majority of non-singleton OTUs present in soil (average 556 per sample, 5944 OTUs for the complete survey) and roots (145 per sample, 2399 OTUs) of the complete experimental site of a 2-year old poplar stand (Supporting Information, Fig. S2.2A, B). These numbers are high compared to other studies reporting deep sequencing of fungal communities in soil of mature forest stands (Buée *et al.,* 2009; Gottel *et al.,* 2011) and roots from mature oak trees (Jumpponen *et al.,* 2010). One reason may be a higher sampling density in our study compared to the previous ones. Nevertheless, it is remarkable that even in simple and young agro-ecosystems established on a tilled soil (Fig. 2.1) very high sequencing depth is needed for comprehensive characterization of fungal community composition.

The fungal family richness (186 in soil and 115 in poplar roots, Supporting Information Tab. S2.2) also exceeded values that have been previously reported for fungal soil communities (O'Brien *et al.,* 2005; Buee *et al.,* 2009), fungal phyllosphere (Jumpponen & Jones, 2009), and root communities of oak (Jumpponen *et al.,* 2010). As no adjacent forest or mature site existed that could cause “vicinal invasion” (Kaldorf *et al.,* 2004), our study shows that already very young stands own a rich and diverse reservoir of fungal propagules.

2.4.2 Roots and soil constitute distinct ecological fungal biomes

We observed a clear separation of soil and root fungal communities (Fig. 2.3). A clear separation of microbiomes has also been reported for the rhizosphere and endosphere of mature poplar sites (Gottel *et al*., 2011). Our study shows that the differentiation of these habitats occurs already in an early phase of stand development and is mainly the result of fungal families enriched in soil (about 1/3 of all soil families) compared to roots. This

observation points to high selectivity of interactions of roots with soil fungal genera (Fig. 2.3; Supporting Information, Tab. S2.2). The majority of significant fungal soil families shared saprophytic or pathogenic lifestyles (Fig. 2.6) including the two most abundant fungal soil families, the Filobasidiaceae and Mortierellaceae (Hibbett *et al*., 2007). Members of these families are widespread, occurred also with high abundance in soils of six different tree mono-plantations and have therefore been classified as generalistic families (Buée *et al.,* 2009).

Analysis of the lifestyle of the most abundant fungal species revealed significant enrichment of pathogenic, endophytic, lichenized fungi and AM fungi in soil compared to roots (Fig. 2.6). Some earlier studies demonstrated that pathogenic fungi are forming a large group within fungal communities in plant tissues (Bills & Polishook, 1994; Monk & Samuels, 1990) and that (bacterial) antagonists affect overall abundance of pathogenic fungi (Berg *et al.,* 2002). However, the analysis of fungal communities in plant tissue samples has been challenging in the past due to inadequate isolation techniques (Bayman, 2007). For example, in a deep sequencing study Jumpponen *et al.* (2010) reported 12.3% of all detected fungi in mycorrhizal oak roots to be pathogenic. Our study shows that fungi with this lifestyle were about five-times more abundant in soil than in roots (Fig. 2.6).

Interestingly, the abundance of endophytic fungi was also higher in soil than in roots (Fig. 2.6). The mechanism of endophytic transmission is very variable and depends on the endophytic class (Rodriguez *et al.,* 2009) ranging from spores dispersed by wind or rain to released hyphal fragments or infected (dead) plant tissue passively distributed by herbivores (Monk & Samuels, 1990) or physical disturbance. These pathways and the influence of abiotic factors such as land-use leads to sometimes unexpected abundance and diversity of endophytic fungi (Rodriguez *et al.,* 2009) found in different biomes such as agro-systems and terrestrial ecosystems (Arnold & Lutzoni, 2007). Additionally, the identification of fungi as endophytes is problematic as the classification is often based on the momentary status of detection without regarding the future status of interaction (Schulz & Boyle, 2005). Thus, fungi termed endophytic might be saprophytic or pathogenic in a certain part of their lifecycle.

Some distinct classes of mutualistic fungi including two families of mycorrhizal fungi (Archaeosporaceae (AM) and Bankeraceae (ECM), Fig. 2.4, Supporting Information, Tab. S.2.2) were significantly enriched in soil. The overabundance of AM fungi in soil is surprising since poplar trees are able to associate with both AM and ECM fungi at the same time (Molina *et al.,* 1992). However, here ECM fungi formed the largest ecological group in roots with almost 90% abundance (Fig. 2.6) more than previously reported by Jumpponen *et al.* (2010) for ECM-colonized oak roots (72%). The strong colonization with ECM was probably

caused by preceding long-term cultivation of poplars on the experimental sites and this may have suppressed AM proliferation (Dhillion, 1994; Chen *et al.*, 2000).

The ECM accumulation in roots was mainly due to OTUs assigned to four families: Inocybaceae, Pezizaceae, Paxillaceae and Pyronemataceae (Fig. 2.4). Whereas the former two were evenly distributed between soil and roots, the latter two were predominantly present in roots. Assignment of pezizalean Pyronemataceae taxa to specific ecological lifestyles remained problematic as they comprise a heterologous family. In fact, they are nowadays considered as paraphyletic (Perry *et al.,* 2007). In our study, Pyronemataceae showed significant presence in root samples (Fig. 2.4) and were one of the families with the highest genera richness (Supporting Information, Fig. S2.3). The different distribution of genera in soil and roots support previously assigned ecological lifestyles of some taxa of the Pyronemataceae: in roots solely, genera described as mycorrhiza forming fungi were detected, while in soil, additional taxa with other ecological lifestyles were found.

The distribution of fungal families in individual samples was more homogeneous in soil than in roots (Fig. 2.5). This was also supported by the narrow clustering of OTUs in the NMDS analysis (Fig. 2.3) and the larger calculated Evenness in soil than in roots (Fig. 2.2). Mycorrhizal fungi are known to cluster along the root system of their host plants forming a patchy distribution (Smith & Read, 2008). This may also be expected for fungal soil communities on early-successional sites, as soil factors can differ widely at one site (Reverchon *et al.,* 2010). In our study, the lack of significant differences in soil factors and soil plowing before the establishment of the plantation may have resulted in the relative homogeneous distribution of soil inhabiting fungi. The observation that a small number of ECM forming genera were dominant in roots and that roots contained a high number of rare OTUs at the same time suggests that roots were underlying high colonization pressure, but fungal proliferation was effectively suppressed with the exception of ECM. However, further studies are needed to shed light on the mechanisms influencing the composition of ecological groups in fungal communities in different habitats.

2.4.3 Deep sequencing reveals host effects on the priority of ECM root colonization

The application of a double approach, morphotyping/Sanger-sequencing and 454 pyrosequencing, allowed us to draw a picture of dynamic processes and cross-links of fungal soil and root communities in relation to ECM colonization. The ECM community on poplar roots showed the well-known increases in colonization rate and diversity with increasing tree age (Chen *et al.,* 2000; Dhillion, 1994; Egerton-Warburton & Allen, 2001). The fungal soil

community (2009) harbored already all but two of the fungal species that formed ECM with poplar roots in the following year (2010, Tab. 2.2). This finding indicates the strength of fungal soil communities as a source for plant root colonization, and suggests low invasion by soil fungi from outside the agro-system within one annual cycle. Furthermore, most fungal species with ECM development in 2010 were already traced on poplar roots in 2009 by 454 pyrosequencing (Tab. 2.2). The experimental site was underlying early successional dynamics with factors that influence fungal root colonization such as the pattern of C allocation (Druebert *et al.,* 2009), fungal competition (Kennedy *et al.,* 2009), or availability of nutrients (Peter *et al.,* 2001). While pronounced changes in soil nutrient availability appear unlikely, the growth of the poplars from about 0.2 to 1.9 m in the first year after planting (L. Danielsen, unpublished results) indicates a strong increment in carbon productivity, which is one of the main drivers of ECM diversity (Druebert *et al.,* 2009; Pena *et al.,* 2010). The priority concept for ECM colonization, which has experimental support under controlled conditions (e.g., Kennedy *et al.,* 2009), holds that the first mycorrhizal species to colonize a host´s roots subsequently is the stronger competitor, when other fungal species are added. Our results suggest that this concept needs to be expanded to account for the dynamics of the colonized habitat. Most changes in ECM root communities were caused by fungal species already present on roots, that is, prior to other ECM present in soil that became more competitive forming functional ECM in the second year. As there were no changes in climatic or edaphic factors, which could have resulted in changes in the ECM assemblages, plant-related factors such as changes in carbon availability must have been responsible for the shift in the dominance of fungal species in the ECM communities.

2.4.4 Transgenic poplars with suppressed CAD activity do not affect soil, root, or ECM communities

One important goal of this study was the assessment of the impact of transgenic versus wildtype poplar plants on fungal soil, root, and ECM communities; but no significant differences were observed (Fig. 2.5). Previous studies have already indicated no influence of transgenic poplar genotypes (rolC – a transformation causing stunting, nplI::GUS – a selection marker coupled with a reporter gene) on ECM community structures (Kaldorf *et al.,* 2004; Stefani *et al.,* 2009). Here, we show that this also holds true for transgenic poplars (antisense CAD) with improved pulping properties that were modified in their phenylpropanoid metabolism (Pilate *et al.,* 2002). This is an important result because other studies revealed significant correlations between phenolic concentrations and associated above-ground organismic interactions (Kleemann *et al.,* 2011). Earlier studies on genetically

modified poplars were limited because only ECM or cultivable soil fungi could be analyzed. Our data add important information with regard to the bio-safety discussion because we show that *in situ* fungal soil and root communities were unaffected by host modification of an important commercial trait. These results are especially interesting where fungi are concerned that depend on host plant features, such as endophytic or pathogenic fungi. In contrast to our working hypothesis, we did not detect any significant differences between the fungal communities of wildtype and antisense CAD poplars. Nevertheless, it is clear that in general, genotype x biotic environment interactions cannot be excluded because intra-specific variations of ECM colonization have been demonstrated in crossing pedigrees (Labbé *et al.,* 2011). Therefore, biotic interactions will have to be tested for each transgenic line that is planted in the field.

2.5 Conclusions

The results of our analyses indicate that fungal soil and root community interact by dynamic processes and that soil is playing an important role as a fungal reservoir. Poplar roots were dominated by ECM fungi. The down-regulation of an enzyme of lignin biosynthesis (antisenseCAD) did not affect ECM, root, or soil fungal assemblages. To our knowledge, we described for the first time the proportional composition of fungal ecological groups of two interacting fungal communities. Information on ecological groups and composition of fungal communities are urgently needed to understand the variable nature of fungal communities and underlying mechanisms of interaction. Additionally, the combination of two different detection techniques allowed us to draw a comprehensive picture of fungal soil and root communities of the experimental site.

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Supporting Information

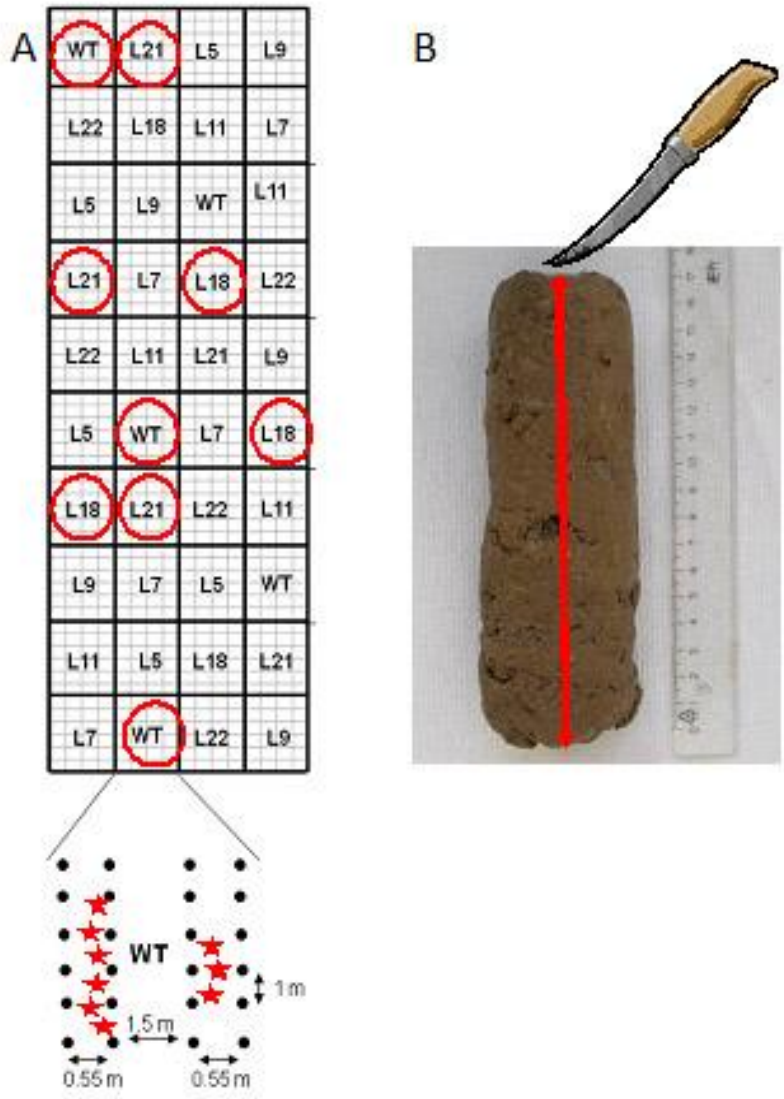

Fig. S2.1: Scheme of the poplar field trial (A) and picture of a soil core (B). Within an area of 1365 m^2, 120 plants of each *Populus* × *canescens* line, seven transgenic (L5, L7, L9, L11, L18, L21, L22) and one wildetype (WT) line, were planted in a randomized design with 5 replicates. Each replicate consisted of eight plots one plot (6 x 4 plant,black dots)for each line. Planting distance within one column was 1 m. The row distance within double rows was 0.55 m, whereas 1.5 m was kept between double rows. Sampling plots are indicated by red circles and location of soil cores by red stars. For further analyses, the soil cores were cut longitudinally and one half was used for classical EM analysis and the other for deep sequencing.

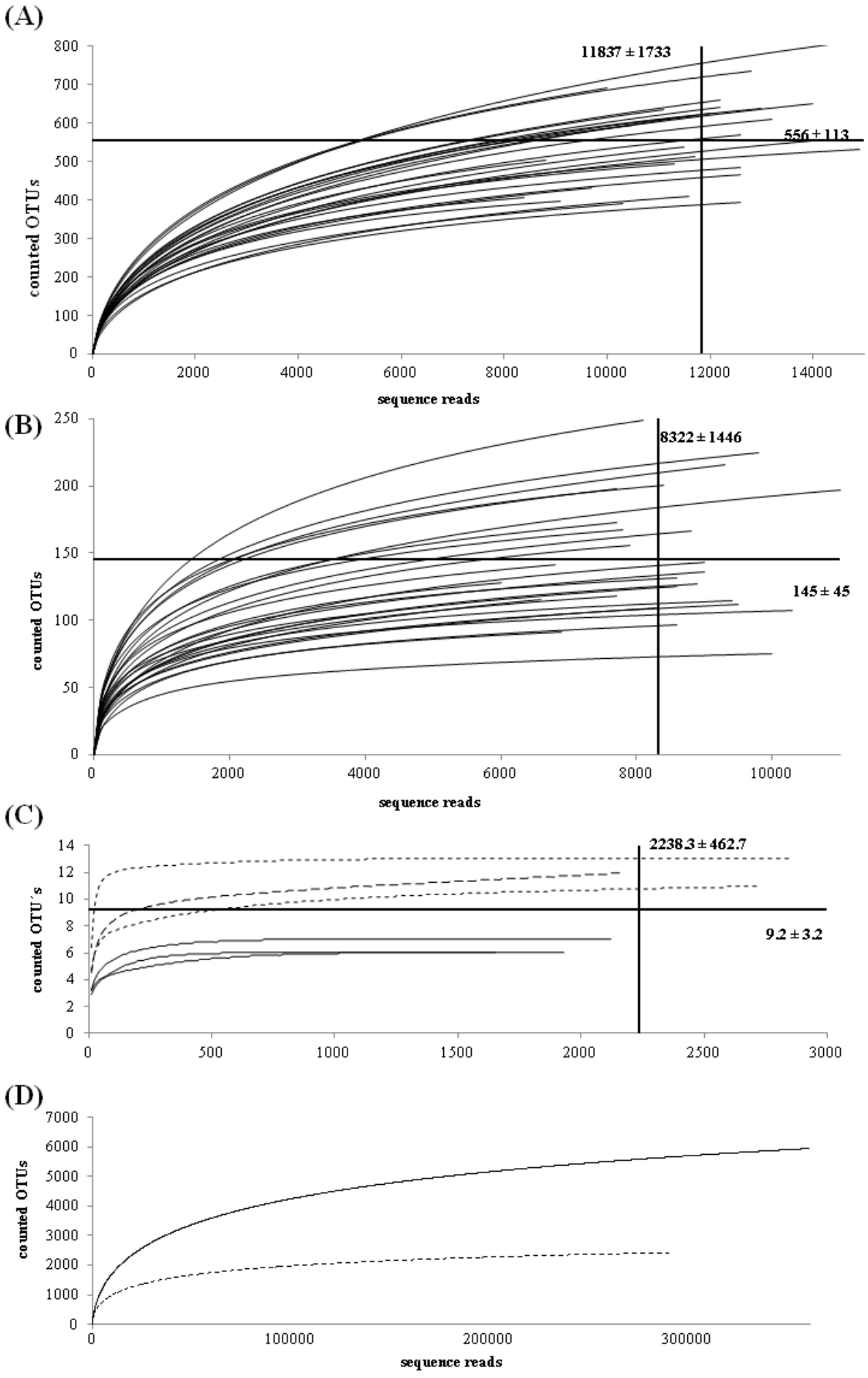
(A)
11837 ± 1733
556 ± 113
counted OTUs
sequence reads
(B)
8322 ± 1446
145 ± 45
counted OTUs
sequence reads
(C)
2238.3 ± 462.7
9.2 ± 3.2
counted OTU's
(D)
counted OTUs
sequence reads

Fig. S2.2: Rarefaction curves of (A) soil, (B) root, (C) ECM root tip and (D) combined samples. For each individual sample a separate rarefaction curve was calculated (27 samples for soil and root samples, three for ECM root tip samples). Solid vertical line, for (A) and (B) mean of sequence reads ± standard deviation (SD) or for (C) mean of counted ECM root tips. Solid horizontal line, mean of counted OTU´s ± SD. Solid curves in (C) samples from 2009; dashed curves, samples from 2010. For (D) data of all soil and root samples were combined prior to cluster analysis, respectively. Solid curve in (D) combined soil samples; dashed curve, combined root samples.

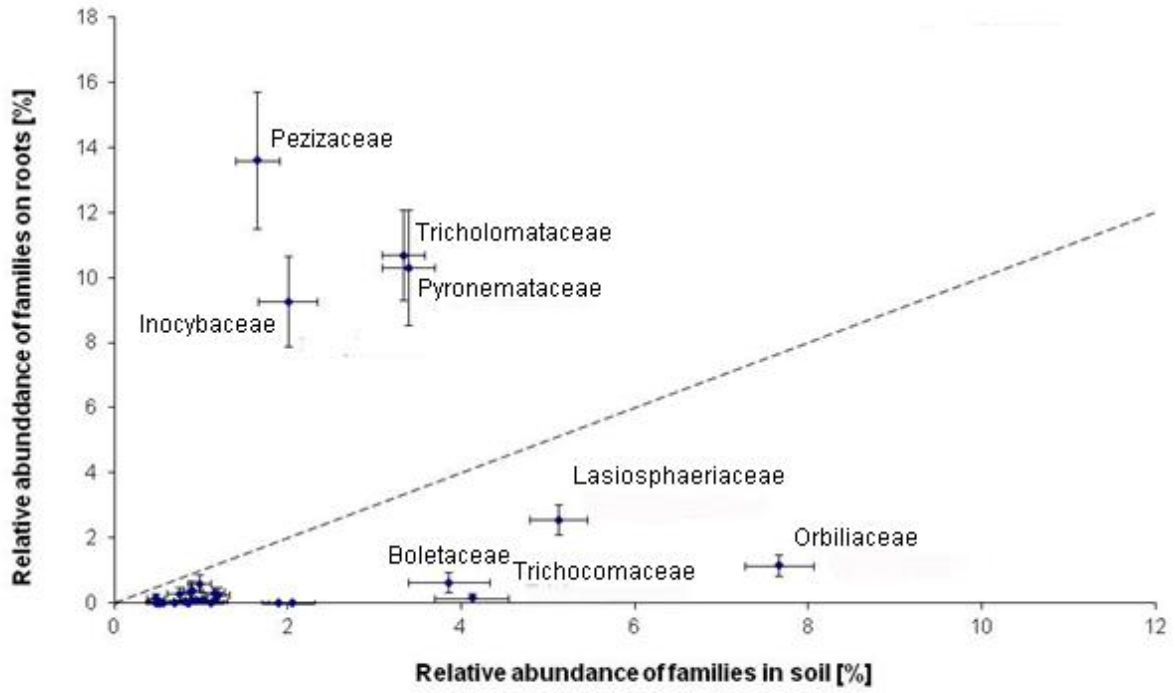

Fig. S2.3: Detected genera richness per fungal families clustered along their presence in soil and root samples. Dashed line, equal frequency in both sample types.

Tab. S2.1: Fungal species were classified into different groups according to their ecological lifestyles. Literature source is indicated. Only species accounting for 90% of overall relative abundance were included in the analysis. (A) Fungal species detected in soil samples. (B) Fungal species detected in root samples. (C) Reference list.

(A) Soil samples

Species	Functional Group	Source
Acremonium furcatum	endophyte	(Macia-Vicente *et al.,* 2008)
Aleuria aurentia	saprophyte	(Rahi *et al.*, 2009)
Alternaria citri	pathogen	(Isshiki *et al.,* 2001)
Alternaria longipes	pathogen	(Stavely & Main, 1970)
Alternaria macrospora	pathogen	(Bashi *et al.,* 1983)
Ampelomyces humuli	pathogen	(Kiss, 1997)
Apodus deciduus	saprophyte	(Malloch, 1971)
Apophysomyces elegans	pathogen	(Lakshmi *et al.,* 1993)
Arthrobotrys amerospora	pathogen	(Schenck *et al.,* 1980)
Arthrobotrys hertziana	pathogen	(Nordbring-Hertz, 2004)
Arthrographis cuboidea	pathogen	(Anagnost *et al.,* 1994)
Aspergillus versicolor	pathogen	(Jussila *et al.,* 2002)
Athelia bombacina	pathogen	www.mycobank.org
Basidiobolus ranarum	pathogen	(Zavasky *et al.,* 1999)
Bionectria ochroleuca	saprophyte	(Ravnskov *et al.,* 2006)
Blastobotrys proliferans	pathogen	(Quirin *et al.,* 2007)
Boletus dryophilus	ECM	(Egerton-Warburton *et al.,* 2007)
Cenococcum geophilum	ECM	(Jany *et al.,* 2002)
Cercophora sparsa	saprophyte	(Hilber & Hilber, 1979)
Chaetosphaeria chloroconia	saprophyte	(Midgley *et al.,* 2002)
Cheilymenia stercorea	saprophyte	(Denison, 1964)
Chroogomphus rutilus	ECM	www.deemy.de
Cladophialophora chaetospira	saprophyte	(Mouhamadou *et al.*, 2011)
Coniothyrium sporulosum	pathogen	(Montecchio *et al.,* 2004)
Coprinopsis latispora	saprophyte	(Prydiuk, 2010)
Cortinarius saturninus	ECM	(Clemmensen & Mechelsen, 2006)
Cryptococcus podzolicus	saprophyte	(Botes *et al.,* 2005)
Cryptococcus saitoi	saprophyte	(Passoth *et al.,* 2009)
Cryptococcus terricola	saprophyte	(Pedersen, 1958)
Cudoniella clavus	saprophyte	(Dennis, 1971)
Cylindrocarpon olidum	saprophyte	(Allegrucci *et al.,* 2009)
Discostroma tricellulare	endophyte	(Okane *et al.,* 1998)
Drechslera biseptata	pathogen	(Leach& Tulloch, 1972)
Entrophospora infrequens	AM	(Vogelsang *et al.,* 2006)
Fusarium lateritium	pathogen	(Hyun & Clark, 1998)
Fusarium oxysporum	pathogen	(Allegrucci *et al.,* 2009)
Fusarium solani	pathogen	(Woloshuk & Kolattukudy, 1986)
Fusarium solanifradicicola	pathogen	(Suga *et al.,* 2000)
Glomus aurantium	AM	(Blaszkowski *et al.,* 2004)
Handkea excipuliformis	saprophyte	www.mycobank.org

Hebeloma sacchariolens	ECM	(Leski *et al.,* 2010)
Hyalodendriella betulae	endophyte	(Crous *et al.,* 2007a)
Hypocrea viridescens	pathogen	(Blaszczyk *et al.,* 2011)
Inocybe curvipes	ECM	(Leski *et al.,* 2010)
Kabatiella zeae	pathogen	(Pronczuk *et al.,* 2004)
Laccaria tortilis	ECM	www.deemy.de
Leccinum lepidum	ECM	www.deemy.de
Leptodontidium elatius	pathogen	(Vasiliauskas *et al.,* 2005)
Leptodontidium orchidicola	endophyte	(Fernando & Currah, 1996)
Leptosphaeria dryadis	endophyte	(Promputtha *et al.,* 2007)
Leptosphaerulina chartarum	pathogen	(Toth *et al.,* 2007)
Lirula macrospora	pathogen	(Hennon, 1990)
Mariannaea elegans	saprophyte	(Vasiliauskas *et al.,* 2005)
Metarhizium anisopliae	pathogen	(Kershaw *et al.,* 1999)
Microbotryum stellariae	pathogen	(Lutz *et al.,* 2008)
Mollisia cinerea	endophyte	(Barklund & Kowalski, 1996)
Monacrosporium elegans	pathogen	(Hao *et al.,* 2004)
Monacrosporium lobatum	pathogen	(Li *et al.,* 2005)
Mortierella alpina	saprophyte	(Kwasna *et al.*, 2000)
Mortierella elongata	saprophyte	(Gams *et al.*, 1972)
Mortierella gamsii	saprophyte	(Vasiliauskas *et al.,* 2005)
Mortierella horticola	endophyte	(Holdenrieder & Sieber, 1992)
Mortierella humilis	saprophyte	(Varnaite & Raudoniene, 2005)
Mortierella hyalina	saprophyte	(Carreiro & Koske, 1992)
Mortierella verticillata	endophyte	(Summerbell, 2005)
Nectria lugdunensis	endophyte	(Seymour *et al.,* 2004)
Neofabraea alba	pathogen	(Henriquez, 2005)
Neofabraea malicorticis	pathogen	(de Jong *et al.,* 2001)
Neonectria ramulariae	endophyte	(Shiono *et al.*,2008)
Nolanea sericea	saprophyte	www.mycobank.org
Olpidium brassicae	pathogen	(Teakle, 1960)
Ophiostoma floccosum	pathogen	(Tanguay *et al.,* 2006)
Paxillus vernalis	ECM	(Cripps, 2003)
Penicillium chrysogenum	saprophyte	(Allegrucci *et al.,* 2009)
Penicillium concentricum	saprophyte	(Samson *et al.,* 1976)
Peziza ostracoderma	ECM	(Leski *et al.,* 2010)
Phaeosphaeria nodorum	pathogen	(Keller *et al.,* 1997)
Phellodon niger	ECM	www.deemy.de
Phialocephala fortinii	endophyte	(Grunig *et al.*, 2002)
Phialocephala xalapensis	endophyte	(Grunig *et al.,* 2002)
Phialophora finlandia	ECM	(Wilcox & Wang, 1987)
Phillipsia olivacea	saprophyte	(Hansen *et al.,* 1999)
Phoma multirostrata	endophyte	(Taylor *et al.,* 1999)
Phoma pomorum	pathogen	(Conner *et al.,* 2000)
Pleopsidium discurrens	lichen	(Obermayer, 1996)
Pleurotus cystidiosus	saprophyte	(Cohen *et al.,* 2002)
Plicaria endocarpoides	endophyte	(Hoffman *et al.,* 2008)
Podospora curvicolla	saprophyte	(Wicklow & Yocom, 1981)

Podospora ellisiana	saprophyte	(Angel & Wicklow, 1983)
Polyporus grammocephalus	saprophyte	(Huang *et al.,* 2011)
Preussia africana	endophyte	(Hoffman & Arnold, 2010)
Protoblastenia calva	lichen	(Türk & Breuss, 1994)
Protoblastenia lilacina	lichen	(Vezda, 2008)
Psathyrella hydrophila	saprophyte	(Dix, 1985)
Pseudeurotium bakeri	endophyte	(Tejesvi *et al.,* 2011)
Pseudeurotium ovale	pathogen	(Willcox & Tribe, 1974)
Psora testacea	lichen	(Papp *et al.,* 1999)
Pulvinula constellatio	ECM	(Amicucci *et al.,* 2001)
Rhizopogon subbadius	ECM	(Cripps & Grimme, 2001)
Rhizopus stolonifer	pathogen	(Tian *et al.,* 2002)
Rhodotorula glutinis	pathogen	(Davoli *et al.,* 2004)
Saccharicola bicolor	pathogen	(Eriksson & Hawksworth, 2003)
Sclerotinia homoeocarpa	pathogen	(Burpee, 1997)
Sistotrema sernanderi	saprophyte	(Vasiliauskas *et al.,* 2005)
Sphaerosporella brunnea	ECM	www.deemy.de
Sporopachydermia quercuum	endophyte	(Lachance *et al.,* 1982)
Stachybotrys echinata	pathogen	www.mycobank.org
Stachybotrys elegans	pathogen	(Archambault *et al.,* 1998)
Stilbella byssiseda	pathogen	www.mycobank.org
Talaromyces ocotl	saprophyte	(Heredia *et al.*, 2001)
Tetracladium maxilliforme	endophyte	(Tedersoo *et al.,* 2007)
Tomentella ellisii	ECM	(Cline *et al.,* 2005)
Tranzschelia fusca	pathogen	(Maier *et al.,* 2003)
Trichocladium opacum	saprophyte	(Allegrucci *et al.,* 2009)
Trichoderma aggressivum	pathogen	(Savoie & Mata, 2003)
Trichoderma hamatum	saprophyte	(Bae *et al.,* 2009)
Truncatella angustata	pathogen	(Eken *et al.,* 2009)
Umbelopsis autotrophica	saprophyte	(Renker *et al.*, 2005)
Umbelopsis isabellina	saprophyte	(Kwasna *et al.,* 2000)
Vascellum pratense	saprophyte	(Borovicka *et al.,* 2005)
Wilcoxina mikolae	ECM	(Kernaghan *et al.,* 2003)
Xenasmatella vaga	saprophyte	www.mycobank.org
Xerocomus ripariellus	ECM	(van der Heijden & Kuyper, 2003)
Zalerion varium	saprophyte	(Ananda & Sridhar, 2004)
Zeloasperisporium hyphopodioides	saprophyte	(Crous *et al,.* 2007b)

(B) Root samples

Species	Functional group	Source
Clavariadelphus ligula	ECM	(Smith *et al.,* 2002)
Dioszegia hungarica	saprophyte	(Gacser *et al.,* 2001)
Flagelloscypha minutissima	saprophyte	(Piatek & Cabala, 2004)
Hebeloma crustuliniforme	ECM	www.deemy.de

Hebeloma sacchariolens	ECM	(Fox, 1986)
Inocybe curvipes	ECM	(Leski *et al.,* 2010)
Laccaria tortilis	ECM	www.deemy.de
Lachnum pygmaeum	endophyte	(Marquez *et al.,* 2007)
Leptodontidium orchidicola	endophyte	(Wu & Guo, 2008)
Lirula macrospora	pathogen	(Hennon, 1990)
Mortierella elongata	saprophyte	(Gams *et al.,* 1972)
Nigrospora oryzae	pathogen	(Wicklow & Poling, 2009)
Paxillus vernalis	ECM	(Cripps, 2003)
Peziza ostracoderma	ECM	(Leski *et al.,* 2010)
Phialocephala fortinii	endophyte	(Grüning, 2003)
Phialophora finlandia	ECM	(Wilcox & Wang, 1987)
Sphaerosporella brunnea	ECM	www.deemy.de

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Tab. S2.2: Relative abundance (mean ± SD) of the 198 fungal families detected by 454 pyrosequencing analysis in soil and root samples. Fifty-nine and six fungal families showed significant rel. abundance in soil and root samples, respectively (*, $P \leq 0.05$).

	Rel. Abundance (mean±SD)	
Fungal Family	**Soil Samples**	**Root Samples**
Acaulosporaceae	0.01±0.04	0.04±0.10
Acarosporaceae*	0.19±0.31	0.02±0.06
Agaricaceae*	0.01±0.03	0.00±0.00
Agyriaceae	0.00±0.00	0.00±0.00
Albatrellaceae*	0.03±0.06	0.00±0.00
Amanitaceae*	0.02±0.04	0.00±0.00
Amphisphaeriaceae*	0.47±0.59	0.00±0.01
Annulatascaceae	0.00±0.00	0.00±0.00
Archaeosporaceae*	0.00±0.01	0.00±0.00
Arthrodermataceae	0.05±0.23	0.00±0.00
Ascobolaceae	0.00±0.02	0.00±0.00
Astraeaceae	0.00±0.00	0.00±0.00
Atheliaceae*	0.07±0.23	0.00±0.02
Auriculariaceae	0.00±0.00	0.01±0.04
Auriscalpiaceae	0.00±0.00	0.00±0.00
Bankeraceae*	1.30±2.75	0.44±2.23
Basidiobolaceae*	0.07±0.08	0.00±0.00
Bionectriaceae*	0.54±0.27	0.00±0.04
Blastocladiaceae	0.00±0.00	0.00±0.00

Bolbitiaceae	0.10±0.26	0.00±0.00
Boletaceae*	0.41±0.54	0.10±0.22
Bondarzewiaceae	0.00±0.00	0.00±0.00
Botryobasidiaceae	0.00±0.00	0.00±0.00
Botryosphaeriaceae	0.00±0.00	0.00±0.00
Caliciaceae	0.01±0.09	0.00±0.00
Calosphaeriaceae	0.00±0.01	0.00±0.00
Candelariaceae	0.00±0.02	0.00±0.00
Cantharellaceae	0.00±0.00	0.02±0.11
Ceratobasidiaceae	0.01±0.06	0.00±0.00
Ceratocystidaceae	0.00±0.00	0.00±0.00
Chaetomiaceae*	0.08±0.12	0.00±0.01
Chaetosphaeriaceae*	0.23±0.30	0.00±0.01
Chionosphaeraceae	0.00±0.00	0.00±0.00
Choanephoraceae	0.00±0.01	0.00±0.00
Chytridiaceae*	0.02±0.03	0.00±0.00
Cladoniaceae	0.00±0.00	0.00±0.00
Clavariaceae	0.00±0.00	0.00±0.00
Clavicipitaceae*	1.08±2.58	0.00±0.00
Clavulinaceae	0.00±0.00	0.00±0.00
Coccotremataceae	0.00±0.00	0.00±0.00
Coniochaetaceae	0.00±0.01	0.00±0.00
Cordycipitaceae*	0.09±0.15	0.00±0.00
Coriolaceae*	0.06±0.13	0.00±0.00
Corticiaceae*	1.48±2.10	0.01±0.05
Cortinariaceae	0.74±0.59	2.16±5.64
Cunninghamellaceae	0.00±0.00	0.00±0.00
Cyphellaceae*	0.05±0.20	0.00±0.00
Cystofilobasidiaceae	0.00±0.00	0.00±0.00
Dacrymycetaceae	0.00±0.00	0.00±0.00
Davidiellaceae*	0.34±0.26	0.06±0.26
Debaryomycetaceae	0.00±0.00	0.00±0.00
Dermateaceae	0.61±1.16	0.41±0.90
Diatrypaceae	0.00±0.00	0.00±0.03
Didymellaceae*	0.58±0.69	0.06±0.16
Didymosphaeriaceae	0.01±0.06	0.00±0.00
Dipodascaceae*	0.03±0.03	0.00±0.00
Discinaceae	0.00±0.00	0.00±0.00
Dissoconiaceae	0.00±0.00	0.00±0.00
Dothioraceae*	0.10±0.14	0.00±0.02
Entolomataceae*	0.13±0.27	0.00±0.00
Entylomataceae	0.00±0.00	0.00±0.00
Eremomycetaceae*	0.10±0.14	0.00±0.00
Erysiphaceae	0.00±0.00	0.01±0.08
Erythrobasidiaceae	0.00±0.00	0.01±0.06
Exidiaceae	0.03±0.03	0.35±0.85
Filobasidiaceae*	15.40±5.61	0.00±0.00
Fistulinaceae	0.02±0.08	0.00±0.00

Ganodermataceae*	0.01±0.01	0.00±0.04
Gautieriaceae	0.00±0.02	0.00±0.00
Geoglossaceae	0.00±0.01	0.00±0.00
Gloeophyllaceae	0.00±0.00	0.00±0.00
Glomeraceae*	0.31±0.20	0.07±0.13
Glomerellaceae	0.06±0.10	0.03±0.07
Gomphaceae	0.04±0.11	1.09±4.96
Gomphidiaceae*	0.57±0.56	0.01±0.05
Haematommataceae	0.01±0.06	0.00±0.00
Halosphaeriaceae	0.01±0.03	0.00±0.02
Helotiaceae*	0.45±0.84	0.05±0.11
Helvellaceae	0.00±0.00	0.00±0.00
Hemiphacidiaceae	0.07±0.16	0.00±0.00
Herpotrichiellaceae*	1.34±0.76	0.47±0.65
Heterogastridiaceae	0.00±0.01	0.00±0.00
Hyaloscyphaceae	0.01±0.02	1.59±3.33
Hygrophoraceae	0.00±0.02	0.00±0.00
Hymenochaetaceae	0.00±0.02	0.00±0.00
Hymenogastraceae	0.00±0.01	0.00±0.00
Hypocreaceae*	1.68±0.70	0.15±0.22
Hyponectriaceae	0.00±0.00	0.00±0.00
Inocybaceae	11.40±11.1	21.2±26.80
Kickxellaceae	0.00±0.00	0.00±0.00
Lasiosphaeriaceae*	0.77±0.55	0.09±0.17
Lecanoraceae	0.00±0.00	0.00±0.00
Lecideaceae	0.00±0.00	0.00±0.00
Legeriomycetaceae	0.00±0.00	0.00±0.00
Lentinaceae	0.00±0.00	0.00±0.00
Leotiaceae	0.04±0.07	0.06±0.22
Leptosphaeriaceae*	0.52±0.62	0.15±0.32
Leucosporidiales	0.09±0.14	0.00±0.00
Lipomycetaceae*	0.01±0.02	0.00±0.00
Lobariaceae	0.00±0.00	0.00±0.00
Lophiostomataceae	0.00±0.00	0.00±0.00
Lycoperdaceae*	0.72±2.09	0.00±0.00
Lyophyllaceae	0.00±0.00	0.00±0.00
Magnaporthaceae	0.11±0.44	0.73±1.45
Malasseziaceae	0.00±0.00	0.00±0.00
Marasmiaceae	0.00±0.00	0.00±0.00
Massarinaceae*	0.23±0.19	0.00±0.01
Melanommataceae	0.00±0.01	0.00±0.00
Melanotaeniaceae	0.00±0.00	0.03±0.14
Meruliaceae	0.00±0.00	0.00±0.00
Metschnikowiaceae	0.00±0.00	0.00±0.00
Microascaceae	0.00±0.00	0.00±0.00
Microbotryaceae*	0.15±0.18	0.06±0.35
Monoblepharidaceae	0.02±0.04	0.00±0.00
Mortierellaceae*	14.30±7.55	1.32±2.08

Mucoraceae*	0.16±0.15	0.00±0.00
Mycosphaerellaceae	0.07±0.07	0.30±0.67
Myxotrichaceae	0.03±0.15	0.00±0.00
Nectriaceae	0.52±0.57	2.89±3.23
Neocallimastigaceae	1.33±5.33	1.11±4.64
Olpidiaceae*	0.26±0.15	0.01±0.03
Onygenaceae	0.00±0.00	0.00±0.00
Ophiocordycipitaceae	0.10±0.19	0.03±0.14
Ophiostomataceae	0.81±0.57	1.85±3.15
Orbiliaceae*	1.24±1.15	0.04±0.07
Pannariaceae	0.00±0.01	0.00±0.00
Paraglomeraceae*	0.11±0.52	1.60±8.32
Parmeliaceae	0.00±0.00	0.00±0.00
Paxillaceae*	0.23±0.30	1.97±6.31
Peniophoraceae	0.00±0.01	0.01±0.07
Pezizaceae	4.76±6.00	26.50±27.2
Phaeosphaeriaceae*	0.68±0.71	0.15±0.42
Phallaceae	0.00±0.00	0.00±0.00
Physalacriaceae	0.03±0.15	0.00±0.01
Physciaceae	0.00±0.00	0.00±0.01
Physodermataceae	0.00±0.00	0.00±0.00
Pilobolaceae	0.00±0.00	0.00±0.00
Pisolithaceae	0.00±0.00	0.00±0.00
Plectosphaerellaceae*	0.25±0.26	0.03±0.09
Pleosporaceae*	1.57±1.20	0.07±0.17
Pleurotaceae*	0.68±0.64	0.00±0.03
Podoscyphaceae	0.01±0.03	0.00±0.00
Polyporaceae*	0.20±0.32	0.04±0.18
Protomycetaceae	0.00±0.00	0.00±0.00
Psathyrellaceae	0.27±0.53	0.17±0.39
Pseudeurotiaceae*	1.29±1.44	0.00±0.01
Psoraceae*	0.94±0.59	0.01±0.02
Pucciniaceae	0.00±0.00	0.00±0.00
Pyronemataceae*	1.93±2.07	13.50±13.5
Rhizocarpaceae	0.00±0.00	0.00±0.00
Rhizophydiaceae*	0.14±0.26	0.00±0.00
Rhizopogonaceae*	0.23±0.30	0.00±0.00
Rhytismataceae*	0.18±0.39	1.46±7.59
Roccellaceae	0.00±0.00	0.00±0.00
Russulaceae*	0.01±0.01	0.04±0.25
Saccharomycetaceae	0.04±0.17	0.00±0.00
Saccharomycodaceae	0.00±0.01	0.00±0.00
Saccharomycopsidaceae	0.00±0.00	0.00±0.00
Sarcoscyphaceae	0.15±0.72	0.00±0.03
Schizophyllaceae	0.00±0.00	0.00±0.00
Schizosaccharomycetaceae	0.00±0.00	0.00±0.00
Sclerodermataceae	0.01±0.06	0.00±0.00
Sclerotiniaceae*	0.40±0.54	0.07±0.30

Scutellosporaceae	0.00±0.01	0.00±0.00
Sebacinaceae	0.00±0.00	0.00±0.01
Septobasidiaceae	0.01±0.06	0.00±0.00
Sordariaceae	0.00±0.01	0.00±0.00
Sphaerobolaceae	0.00±0.00	0.00±0.02
Spizellomycetaceae*	0.15±0.25	0.00±0.00
Sporormiaceae*	0.26±0.33	1.33±6.91
Stereocaulaceae	0.00±0.00	0.00±0.00
Strophariaceae*	0.01±0.02	0.00±0.03
Suillaceae	0.02±0.13	0.00±0.00
Sympoventuriaceae	0.00±0.01	0.00±0.00
Taphrinaceae	0.03±0.07	0.00±0.00
Tapinellaceae*	0.02±0.02	0.00±0.00
Teloschistaceae	0.00±0.00	0.00±0.00
Teratosphaeriaceae	0.00±0.00	0.00±0.00
Thelebolaceae*	0.04±0.08	0.00±0.00
Thelephoraceae	0.21±0.28	0.75±2.07
Trechisporaceae	0.00±0.00	0.03±0.17
Tremellaceae*	0.02±0.02	0.00±0.03
Trichocomaceae*	2.09±1.76	0.10±0.32
Tricholomataceae	1.39±1.03	6.79±11.4
Trichomonascaceae	0.08±0.20	0.13±0.35
Tuberaceae	0.00±0.01	0.00±0.00
Tubeufiaceae	0.00±0.00	0.00±0.00
Typhulaceae	0.00±0.00	0.00±0.00
Umbilicariaceae*	0.08±0.10	0.01±0.03
Uropyxidaceae	0.44±1.34	1.05±2.90
Ustilaginaceae	0.00±0.00	0.00±0.00
Venturiaceae	0.00±0.00	0.00±0.00
Verrucariaceae	0.00±0.00	0.00±0.00
Vuilleminiaceae	0.00±0.00	0.00±0.00
Xenasmataceae	0.07±0.18	0.03±0.18
Xylariaceae	0.00±0.00	0.00±0.00

Tab. S2.3: List of fungal families used for cluster analyses (see Fig. 4). Listed in the descending order of appearance in heat map.

Clustering of Fungal Families in	
Soil Samples	**Root Samples**
Acarosporaceae	Pezizaceae
Bionectriaceae	Pyronemataceae
Boletaceae	Inocybaceae
Clavicipitaceae	Nectriaceae
Cortinariaceae	Herpotrichiellaceae
Davidiellaceae	Mortierellaceae

Dermateaceae	Ophiostomataceae
Didymellaceae	Mycosphaerellaceae
Filobasidiaceae	Tricholomataceae
Glomeraceae	Magnaporthaceae
Herpotrichiellaceae	Nephromataceae
Hypocreaceae	Dermateaceae
Inocybaceae	Cortinariaceae
Lasiosphaeriaceae	Lasiosphaeriaceae
Leptosphaeriaceae	Glomeraceae
Microbotryaceae	Orbiliaceae
Mortierellaceae	Leptosphaeriaceae
Nectriaceae	Didymellaceae
Nephromataceae	Hypocreaceae
Ophiostomataceae	Phaeosphaeriaceae
Orbiliaceae	Pleosporaceae
Paxillaceae	Psathyrellaceae
Pezizaceae	Exidiaceae
Pleosporaceae	Bankeraceae
Pleurotaceae	Hyaloscyphaceae
Psathyrellaceae	Trichomonascaceae
Pseudeurotiaceae	Glomerellaceae
Psoraceae	Boletaceae
Pyronemataceae	Uropyxidaceae
Sporormiaceae	Leotiaceae
Trichocomaceae	Thelephoraceae
Tricholomataceae	Neocallimastigaceae
Amphisphaeriaceae	Helotiaceae
Phaeosphaeriaceae	Sporormiaceae
Eremomycetaceae	Paxillaceae
Helotiaceae	Umbilicariaceae
Mucoraceae	Trichocomaceae
Plectosphaerellaceae	Chaetosphaeriaceae
Lycoperdaceae	Davidiellaceae
Massarinaceae	Bionectriaceae
Olpidiaceae	Ophiocordycipitaceae
Polyporaceae	Acarosporaceae
Basidiobolaceae	Polyporaceae
Thelephoraceae	Pseudeurotiaceae
Umbilicariaceae	Gomphaceae
Mycosphaerellaceae	Halosphaeriaceae
Chaetosphaeriaceae	Rhytismataceae
Rhizopogonaceae	Clavicipitaceae
Bankeraceae	Sebacinaceae
Neocallimastigaceae	Bolbitiaceae
Rhizophydiaceae	Sphaerobolaceae
Corticiaceae	Psoraceae
Sclerotiniaceae	Gomphidiaceae
Cordycipitaceae	Melanotaeniaceae

Dothioraceae	Dothioraceae
Chaetomiaceae	Massarinaceae
Gomphidiaceae	Taphrinaceae
Dipodascaceae	Xenasmataceae
Entolomataceae	Vuilleminiaceae
Spizellomycetaceae	Calosphaeriaceae
Tapinellaceae	Erysiphaceae
Ophiocordycipitaceae	Cyphellaceae
Amanitaceae	Diatrypaceae
Ramalinaceae	Discinaceae
Exidiaceae	Erythrobasidiaceae
Glomerellaceae	Ganodermataceae
Rhytismataceae	Gloeophyllaceae
Thelebolaceae	Meruliaceae
Tremellaceae	Parmeliaceae
Russulaceae	Peniophoraceae
Strophariaceae	Teratosphaeriaceae
Albatrellaceae	Tremellaceae
Coriolaceae	Russulaceae
Lipomycetaceae	Sclerotiniaceae
Magnaporthaceae	Myxotrichaceae
Cyphellaceae	Sympoventuriaceae
Leotiaceae	Thelebolaceae
Atheliaceae	Eremomycetaceae
Chytridiaceae	Rhizopogonaceae
Trichomonascaceae	Pleurotaceae
Taphrinaceae	Podoscyphaceae
Uropyxidaceae	Corticiaceae
Ganodermataceae	Ascobolaceae
Paraglomeraceae	Lycoperdaceae
Agaricaceae	Acaulosporaceae
Archaeosporaceae	Microbotryaceae
Ascobolaceae	Chaetomiaceae
Myxotrichaceae	Filobasidiaceae
Sympoventuriaceae	Hyponectriaceae
Bolbitiaceae	Physalacriaceae
Venturiaceae	Olpidiaceae
Halosphaeriaceae	Agaricaceae
Onygenaceae	Kickxellaceae
Saccharomycodaceae	Lecanoraceae
Podoscyphaceae	Plectosphaerellaceae
Fistulinaceae	Lyophyllaceae
Melanommataceae	Basidiobolaceae
Hemiphacidiaceae	Amphisphaeriaceae
Acaulosporaceae	Ramalinaceae
Sarcoscyphaceae	Cantharellaceae
Kickxellaceae	Albatrellaceae
Hyaloscyphaceae	Auriculariaceae

Coniochaetaceae
Cladoniaceae
Septobasidiaceae
Lyophyllaceae
Sordariaceae
Sclerodermataceae
Arthrodermataceae
Pisolithaceae
Trechisporaceae
Schizosaccharomycetaceae
Saccharomycetaceae
Monoblepharidaceae
Ceratocystidaceae
Cystofilobasidiaceae
Malasseziaceae
Xenasmataceae
Bondarzewiaceae
Gomphaceae
Lecideaceae
Parmeliaceae
Stereocaulaceae
Lentinaceae
Rhizocarpaceae
Physalacriaceae
Erysiphaceae
Auriculariaceae
Pannariaceae
Sebacinaceae
Calosphaeriaceae
Lophiostomataceae
Pucciniaceae
Verrucariaceae
Pilobolaceae
Roccellaceae
Blastocladiaceae
Botryobasidiaceae
Clavulinaceae
Hygrophoraceae
Helvellaceae
Lobariaceae
Debaryomycetaceae
Xylariaceae
Botryosphaeriaceae
Erythrobasidiaceae
Scutellosporaceae
Lecanoraceae
Choanephoraceae
Clavariaceae
Amanitaceae
Atheliaceae
Saccharomycetaceae
Botryobasidiaceae
Physciaceae
Annulatascaceae
Coccotremataceae
Pisolithaceae
Sarcoscyphaceae
Ceratocystidaceae
Paraglomeraceae
Strophariaceae
Trechisporaceae

Cantharellaceae
Dacrymycetaceae
Auriscalpiaceae
Microascaceae
Peniophoraceae
Geoglossaceae
Heterogastridiaceae
Physciaceae
Entylomataceae
Caliciaceae
Cunninghamellaceae
Didymosphaeriaceae
Gautieriaceae
Marasmiaceae
Sphaerobolaceae
Tuberaceae
Tubeufiaceae
Ceratobasidiaceae
Hymenogastraceae
Physodermataceae
Schizophyllaceae
Typhulaceae
Teloschistaceae
Protomycetaceae
Hymenochaetaceae
Dissoconiaceae
Agyriaceae
Metschnikowiaceae
Haematommataceae
Phallaceae
Suillaceae
Astraeaceae
Candelariaceae
Ustilaginaceae
Chionosphaeraceae
Legeriomycetaceae
Saccharomycopsidaceae

Tab. S2.4: Fungal species detected on ECM root tips of poplar plants by morphotyping/ITS-sequencing. Poplar roots were sampled in October 2009 and 2010.

Species	ACC	Best BLAST hit	Source database	Source ACC	Length of fragment	Homology [%]	Score
Hebeloma sacchariolens	JQ409280	*Hebeloma sacchariolens*	RSyst	AY312985	460	97	850
Hebeloma sp.	JQ409279	*Hebeloma* sp .	UNITE	UDB001188	605	96	957
Laccaria tortilis	JQ409281	*Laccaria tortilis*	UNITE	UDB001589	568	99	1126
MT5	no sequence available						
Paxillus involutus	JQ409282	*Paxillus involutus*	RSyst	EU078741	638	99	1203
Peziza ostracoderma	JQ409283	*Peziza ostracoderma*	NCBI	EU819461.1	657	99	1158
uncultured Pezizales	JQ409284	uncultured Pezizales	NCBI	DQ469743.1	669	98	1112
Cenococcum geophilum	JQ409285	*Cenococcum geophilum*	NCBI	HQ406817.1	857	96	1375
Geopora sp.	JQ409286	*Geopora* sp. TAA 192232	NCBI	FM206420.1	489	99	878
MT13	no sequence available						
Uncultured fungi (Ascomycota)	JQ409287	uncultured fungus	NCBI	EU555000.1	510	100	942
Uncultured fungi (Ascomycota)	JQ409288	uncultured fungus	NCBI	EU554730.1	539	100	996
MT30	no sequence available						
MT33	no sequence available						
Scleroderma bovista	JQ409289	*Scleroderma bovista*	UNITE	UDB002179	630	98	1205
Tomentella ellisii	JQ409290	*Tomentella ellisii*	NCBI	DQ068971.1	504	100	931
Tuber sp.	JQ409291	*Tuber* sp. GMB-2010b	NCBI	HM485376.1	473	100	874
uncultured Ascomycota	JQ409292	uncultured Ascomycota	NCBI	EU562601.1	522	97	883
uncultured Ascomycota	JQ409293	uncultured Ascomycota	NCBI	EU557319.1	544	99	992
uncultured ectomycorrhizal fungus	JQ409294	uncultured ectomycorrhizal fungus	NCBI	EF484931.1	571	97	965
uncultured *Peziza*	JQ409295	uncultured *Peziza*	NCBI	GU969261.1	539	99	979
Xerocomus ripariellus	JQ409296	*Xerocomus ripariellus*	UNITE	UDB000485	649	100	1287

Chapter 3

Ectomycorrhizal colonization and diversity in relation to tree biomass and nutrition in a plantation of transgenic poplars with modified lignin biosynthesis

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Ectomycorrhizal Colonization and Diversity in Relation to Tree Biomass and Nutrition in a Plantation of Transgenic Poplars with *M*odified Lignin Biosynthesis

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Abstract

Wood from biomass plantations with fast growing tree species such as poplars can be used as an alternative feedstock for production of biofuels. To facilitate utilization of lignocellulose for saccharification, transgenic poplars with modified or reduced lignin contents may be useful. However, the potential impact of poplars modified in the lignification pathway on ectomycorrhizal (EM) fungi, which play important roles for plant nutrition, is not known. The goal of this study was to investigate EM colonization and community composition in relation to biomass and nutrient status in wildtype (WT, *Populus tremula* × *Populus alba*) and transgenic poplar lines with suppressed activities of cinnamyl alcohol dehydrogenase, caffeate/5-hydroxyferulate O-methyltransferase, and cinnamoyl-CoA reductase in a biomass plantation. In different one-year-old poplar lines EM colonization varied from 58% to 86%, but the EM community composition of WT and transgenic poplars were indistinguishable. After two years, the colonization rate of all lines was increased to about 100%, but separation of EM communities between distinct transgenic poplar genotypes was observed. The differentiation of the EM assemblages was similar to that found between different genotypes of commercial clones of *Populus* × *euramericana*. The transgenic poplars exhibited significant growth and nutrient element differences in wood, with generally higher nutrient accumulation in stems of genotypes with lower than in those with higher biomass. A general linear mixed model simulated biomass of one-year-old poplar stems with high accuracy (adjusted R^2 = 97%) by two factors: EM colonization and inverse wood N concentration. These results imply a link between N allocation and EM colonization, which may be crucial for wood production in the establishment phase of poplar biomass plantations. Our data

further support that multiple poplar genotypes regardless whether generated by transgenic approaches or conventional breeding increase the variation in EM community composition in biomass plantations.

Keywords
Transgenic poplar; ectomycorrhizal colonization; diversity; biomass; lignin alteration; nitrogen; phosphorus

3.1 Introduction

The growing world population inevitably entails an increasing energy demand along with diminishing fossil fuel resources [1]. Renewable energies from biomass can be used as an alternative to partially replace conventional energy supplies. Trees, especially fast-growing species such as poplars, are an appealing feedstock for this purpose because they can be grown in dense short rotation plantations allowing several harvests without the need to re-plant [2]. Furthermore, poplars have a low nitrogen demand compared with other potential bioenergy crops [3]. Thus, their cultivation may contribute to the mitigation of nitrogen emissions from intensely used agricultural areas [4].

The conversion process of biomass to biofuels requires the breakdown of plant cell walls, which mainly consist of cellulose, hemicelluloses, and lignin [5]. Lignin is a recalcitrant polymer composed of phenylpropanoid units that hinder chemical and enzymatic cellulose degradation necessary for bioethanol production [6]. To amend wood utilization cell wall properties have been changed by targeted genetic approaches [7]. Genes of the biosynthetic pathway of lignin and cellulose have been isolated and characterized [8–10]. Suppression of cinnamyl alcohol dehydrogenase (CAD), an enzyme which converts cinnamyl aldehydes to the respective alcohols [5] and caffeate/5-hydroxyferulate O-methyltransferase (COMT), an enzyme involved in biosynthesis of syringyl lignin [5] result in altered lignin composition compared to wildtype (WT) poplars [11–13]. Overexpression of ferulate 5-hydroxylase (F5H), an enzyme that catalyzes an intermediate step in lignin biosynthesis, also results in compositional changes and less polymerization of monolignol units compared to the WT [14]. Suppression of cinnamoyl-CoA reductase (CCR) causes reduced lignin contents [15]. Transgenic poplars with alterations in lignin content and composition have been tested for industrial usage and display improved Kraft pulping [16]. The saccharification efficiency is also increased by genetic engineering of the lignin biosynthetic pathway [17].

If the use of genetically modified (GM) poplars with improved wood properties for bioenergy production was expanded, it will be necessary to know whether nutrient status and ecological

interactions of GM poplars are changed compared with the WT. In a preceding study we compared whole fungal communities in soil and roots of poplars with suppressed CAD activities and of the WT by pyrosequencing and found a strong dominance of ectomycorrhizal (EM) in roots, whereas saprophytes were prevalent in soil [18]; significant differences of these traits between the CAD lines and WT were not found [18]. The interaction of poplar roots with EM fungi is of particular importance for nutrient acquisition [19]. But other benefits have also been reported such as higher survival rates of EM-inoculated young poplar saplings [20–23] and increased resistance to drought stress [24–26], issues gaining importance with increasing poplar cultivation in a warming climate. Currently, it is still unclear if changes in the lignification pathway have significant ecological implication for interacting organisms. Lignin is the end product of the phenylpropanoid pathway, whose modification generally has consequences for the biosynthesis of other phenol-bearing compounds. For example, the suppression of CCR results in decreased lignin, but increased concentrations of phenolic compounds [15]. Phenolic compounds have been implicated in a wide range of ecological interactions. Greenhouse studies have shown that enzymatic activities of microbial communities are altered in soil of poplars with reduced lignin concentrations [27]. Field studies on the EM communities in relation to the performance of poplars with changes in the lignin composition and reduction of the lignin concentrations are lacking.

The aim of this study was to characterize the EM community composition and dynamics in the first cycle of a short rotation plantation with poplars modified in the lignification pathway. To assess the relationship between EM diversity, plant nutrient status and dendromass we analyzed height growth, biomass, and nutrient element composition in leaves, stem and roots of transgenic *Populus* × *canescens* with suppressed activities of COMT (L9 and L11), CCR (L5 and L7), or CAD (L18, L21 and L22) and the wildtype (WT). We further compared the EM assemblages in the GM plantation with those of commercial poplar clones (*P.* x *euramericana* , syn, *Populus deltoides* × *Populus nigra* c.v. Ghoy, I-214, and Soligo). Our study shows that in the first year after plantation establishment, EM fungal colonization and diversity were linked with tree productivity and low stem nitrogen concentrations. The variation of the EM fungal community composition found on roots of different transgenic poplar genotypes was similar to that found on different commercial poplar genotypes.

3.2 Material and Methods

3.2.1 Plant material and field site

One hybrid clone of *Populus tremula* × *Populus alba* (INRA #717-1B4, syn. *P.* × *canescens*) referred to as wild type (WT) and seven transgenic lines from this WT clone modified in key enzymes of the lignin biosynthetic pathway were used to establish a field trial. The transgenic lines were down regulated in one of the following enzymes of the lignin biosynthesis pathway: CCR (cinnamoyl coenzyme A reductase) with line FS3 = L5 and FAS13 = L7 [15], COMT (caffeic acid O-methyl transferase) with line ASOMTB2B = L9 and ASOMTB10B = L11 [11], and CAD (cinnamyl alcohol dehydrogenase) with line ASCAD21= L21, ASCAD52 = L18, and SCAD1= L22 [28]. After multiplying the clones by micropropagation [29] 120 plants of each of the 8 different poplar lines were planted in a plowed area of 1365 m^2 on sandy soil with flint in June 2008, next to INRA in Orléans, Sologne, France (47°83´N, 1°91´E). The field trial with GM poplars with modified lignin (application B/FR/07/06/01) has been approved by the "Bureau de la réglementation alimentaire et des Biotechnologies" from the "Direction Générale de l'Alimentation" from the French "Ministère de l'Agriculture et de la Pêche" (ministerial decision #07/015 on September 21, 2007 for a 5 year period). The land, where the field trial was conducted, is owned by INRA. Protected species were no sampled.

In this area the mean annual temperature is 10.4°C and precipitation 600 mm. The plant density was chosen according to short rotation coppice practice as follows: the space between trees of one double row was 0.55 m while the interspace between the two double rows was 1.5 m, and the planting distance within a line was 1 m (Fig. S3.1). The poplar lines were planted in a randomized block design with 5 blocks. Each block consisted of eight plots, one for each line. Each plot consisted of 24 trees (4 x 6) planted in two double rows. To prevent edge effects the experimental plantation was bordered with one row of WT clones (Fig. S3.1). During the growing season the poplars were drip irrigated.

A second plantation with 11 commercial clones of *Populus deltoides* × *P. nigra* including the cultivars Blanc de Poitou, Carpaccio, Dorskamp, Flevo, Ghoy, I-214, Koster, Lambro, Robusta, Soligo, and Triplo was established in May 2009 in the same area. The random block design consisted of three blocks. Each block consisted of 11 plots. Each plot consisted of 16 trees (4 x 4) of one commercial clone. The space between trees of one double row was 0.6 m while the interspace between the two double rows was 1.5 m, and planting distance within a line was 0.6 m (Fig. S3.2)

3.2.2 Sampling of soil cores for analyses of roots and soil

Soil cores were harvested immediately after planting (July 2008) to assess the heterogeneity of soil fungi and nitrogen at the beginning. After plantation establishment soil were collected for ECM fungal community analysis in October 2009 and October 2010. In July 2008, 25 soil

cores (diameter: 8 cm, depth: 20 cm) were taken randomly in the experimental field, the border area, and the area between the experimental field and a nearby poplar plantation.

In October 2009 and 2010 three plots per clone (i.e. 1 WT + 7 GM lines) were randomly chosen and soil cores (diameter: 5 cm, depth: 20 cm) were collected within these plots. Three trees per plot were chosen and three soil cores per tree were taken at a distance of 0.25 m from the trunk. In total 27 soil cores per line were collected. Soil cores were transported on ice and stored at 4°C until further processing.

Sampling in the *P. deltoides* × *P. nigra* plantation took place in October 2010, one year after planting. The same sampling strategy was used for the plantation with the commercial poplar clones as described above for the transgenic poplars. Three clones were selected for the analysis based on growth differences, which were mainly caused by differences in *Melampsora larici-populina* leaf rust infection: Soligo (high growth and high rust resistance), Ghoy (low growth and low rust resistance) and I-214 (intermediate growth and intermediate rust resistance).

3.2.3 Fungal soil communities analyzed by denaturing gradient gel electrophoresis (DGGE)

DGGE was performed for fungal soil communities at the time point of GM plantation establishment (June 2008). Twenty-five soil samples were sieved and 250 mg sieved soil was used for DNA isolation with the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc., Canada). The primer pair ITS1 and ITS4 [30] was used to amplify the rDNA ITS-region of fungi. A GC-clamp was added to the 5' end of the ITS4 primer to stabilize the melting behavior of the Polymerase Chain Reaction (PCR) products in the gel according to Muyzer *et al.* [31].

PCR was performed according to the following protocol: the total volume of the reaction mix was 25 µl, containing 2 µl template DNA, 2 µl of $MgCl_2$ (25 mM) (Fermentas, St. Leon-Rot, Germany), 2.5 µl 10x buffer (Fermentas, St. Leon-Rot, Germany), 1.25 µl of each primer (stock: 10 µM) (Eurofins MWG Operon, Ebersberg, Germany), 0.5 µl dNTPs mix (10 mM each, Fermentas, St. Leon-Rot, Germany), 15.375 µl of nuclease-free water, and 0.125 µl *Taq* polymerase (>10 U/µl, Fermentas, St. Leon-Rot, Germany). A Master Cycler (Eppendorf, Hamburg, Germany) was used to amplify the DNA with the following cycle steps: hot-start at 95°C for 15 min, followed by 95°C for 1 min, 34 cycles of 30 s at 94°C (denaturation), 30 s at 55°C (annealing) and 1 min at 72°C (extension), and termination at 72°C for 5 min.

The separation of the rDNA sequences was achieved in a 7.5% polyacrylamide (37.5 : 1= acrylamide : bis-acrylamide) gel with a linear denaturing gradient from 32-65% of denaturant (100% denaturant containing 40% (v/v) formamide and 7 M urea). After 2 h of polymerization 7.5 ml of 7.5% polyacrylamide gel without denaturant was added (stacking gel). After 20 min of polymerization the gel was loaded with 4 µl of PCR product per lane of each of the 25 samples. Running buffer contained 0.5 x TAE (20 mM tris(hydroxymethyl)-aminomethane, pH 7.4, 10 mM sodium acetate, 0.5 mM disodium ethylenedinitrilo-tetraacetic acid). An INGENYphorU-2 system (Ingeny International, Goes, The Netherlands) was used for the DGGE at a constant temperature of 58°C, 120 V and a running time of 16 h. DNA bands were visualized by silver staining following the "SILVER SEQUENCE™" protocol (Promega Corporation, Madison, USA). The stained gels were scanned on a flat-bed scanner. The band patterns were manually converted into a present/absent matrix, which was subjected to similarity analyses (Table S3.1).

3.2.4 Free amino acids, nitrate and ammonium in soil samples

At the time point of plantation establishment (June 2008), the concentrations of nitrogen compounds (nitrate, ammonium, amino acids) in the soil solution were determined. Soil samples were sieved (mesh width 5 mm) and 40 g of fresh soil were mixed with 40 ml 1 mM $CaCl_2$, incubated for 10 min and filtered through a Whatman® folded filter (Ø185 mm, Ref.No. 10314747, Whatmann, Dassel, Germany). After 1 h the resulting filtrate was passed through a glass fiber filter (pore size 1 µm, Pall Life Science, Port Washington, NY, USA) and subsequently through a sterilization filter (0.2 µm Sarstedt Filtropur S, Nümbrecht, Germany). After volume determination, the filtrate was freeze-dried and dissolved in 0.5 ml double deionized H_2O. Amino acids were analyzed by high-performance liquid chromatography (Pharmacia/LBK, Freiburg, Germany) according to Tilsner et al. [32]. Nitrate and ammonium were determined by photometric measurements (Shimadzu UV 1602, Hannover, Germany) using enzymatic ammonium and nitrate test kits (Merck 100683, Merck 109713, Merck, Darmstadt, Germany). The concentrations of inorganic nitrogen and amino acids are reported in Supplement Table S3.2.

3.2.5 Ectomycorrhizal colonization and morphotyping

For the investigation of the EM fungal community of roots, soil cores were divided longitudinally, and the three samples, which had been collected around the stem of one tree, were pooled resulting in nine samples per poplar line. Roots were carefully separated from

the soil by washing in a sieve under running tap water. The washed roots were inspected under a stereomicroscope (M205 FA, Leica, Wetzlar, Germany) and non-poplar roots were removed from the sample. The root samples were weighed and aliquots were removed, dried and used for nutrient element analyses.

Subsequently, living and dead root tips were counted until a total number of 300 living roots tips per sample was reached. The numbers of the different morphotypes and of the dead root tips were recorded applying a simplified method after Agerer [33]. Dead root tips exhibited a shrunken and dry appearance. EM morphotypes were distinguished by color, shape, texture of the mantle, and absence or presence of rhizomorphes and/or hyphae. Samples of each morphotype were collected and stored at -20°C for molecular analysis.

EM colonization (%) was calculated as: EM root tips x 100/ (EM root tips + vital non-mycorrhizal root tips).

The vitality index of root tips was determined as: number of living root tips x 100 / total number of counted root tips.

3.2.6 Sanger sequencing of the fungal ITS region

For the extraction of genomic DNA of frozen EM root tips the “innuPREP Plant DNA kit” (Analytik jena, Jena, Germany) was used following the instructions of the manufacturer. The primer pair ITS4 and ITS5 [30] was used to amplify the rDNA ITS-region by PCR with the PCR protocol described above for the DGGE. Cloning and sequencing or direct sequencing were conducted according to Druebert et al. [34]. The following databases were used for nucleotide BLAST searches: UNITE (http://unite.ut.ee/), Fungal RSyst (http://mycor.nancy.inra.fr/RSyst/), and NCBI BLASTn (http://www.ncbi.nih.gov/). Fungal sequences have been deposited at NCBI with the accession numbers JQ409279 to JQ409296 and JQ824878 to JQ824884, respectively.

3.2.7 Stem heights and biomass

Heights of trees chosen for EM fungal analysis were measured in October 2009 and 2010, respectively, when seasonal growth had stopped. In 2010 in addition to the height (h) of the leader shoot the number and lengths of side shoots, and stem diameters (d) of all shoots (15 cm above ground) were measured. Fully expanded top leaves were collected (Oct 2009) and dried for nutrient analyses.

Trees were coppiced in March 2010 and above ground stem biomass was determined after drying at 40° for two weeks to constant weight. Since there is no growth between October and March (fall/winter season), the stem biomass data measured in March 2010 represent that of the preceding year (2009).

Biomass in October 2010 was calculated as: $V \cdot \rho$ with $V = 1/3 \cdot r^2 \cdot \pi \cdot h$ where $r = d/2$ and $\rho = 0.50\ g \cdot cm^{-3}$ [35,36].

3.2.8 Nutrient element and $\delta^{13}C$ analyses

Dry stem wood (March 2010), roots (October 2010) and leaves (October 2010) were cut into small pieces, mixed and aliquots were removed and milled to a fine powder (MM2, Retsch, Hannover, Germany). Nutrient elements were pressure-extracted in HNO_3 and measured by inductively coupled plasma optical emission spectrometry (ICP-OES) after Heinrichs *et al.* [37]. For N and C analyses powdered dry tissues were weighed into tin cartridges (Hekatech, Wegberg, Germany) and measured with an element analyzer (Element Analyzer EA-1108, Carlo, Erba Instruments, Rodano, Italy). Leaf and wood samples for $\delta^{13}C$ analysis were weighed into tin cartridges (Hekatech, Wegberg, Germany) and analyzed with an isotope mass spectrometer (Delta plus XP, Finnigan MAT, Bremen, Germany) coupled with an element analyzer (EuroVektor, HEKAtech GmbH, Wegberg, Germany).

3.2.9 Statistical analyses

Statistical analyses were conducted using R statistics version 2.9.2 [38]. To identify potential clusters in the distribution of soil fungi (detected by DGGE) and soil nutrients (soluble amino acids, nitrate, and ammonium) across the plots non metric multidimensional scaling (NMDS) was conducted with package: “vegan” [39]. Input parameters were Jaccard distance for soil fungi and Euclidean distance for soil nutrients, respectively. To find out if the soil fungal assemblages were related to the composition of the soluble nitrogen compounds in soil, data were subjected to a Mantel test with the package ”vegan” [39].

Similarities of EM fungal community structures in 2009 and 2010 were analyzed by NMDS using Bray-Curtis distance as input parameter. In all cases a maximum of 100 starts were used to find a stable solution. The procedure was repeated with the best previous solution to prevent local optima. Function envfit() was used to fit grouping factors (different lines) onto the ordination. 95% confidence ellipses were drawn with function ordiellipse(), package:”vegan” [39].

Data for height, biomass, mycorrhizal colonization, vitality index, nutrient element concentrations and $\delta^{13}C$ signature are shown as means (±SE). Significant differences at $p \leq 0.05$ were detected by one-way ANOVA followed by multiple comparisons with TukeyHSD (package: "stats"). Residuals of the models were analyzed by Kolmogorov-Smirnov and Levene's test to check for normal distribution and homogeneity of variances, respectively. If one of the assumptions of the ANOVA had to be rejected, Kruskal-Wallis rank sum test followed by Mann Whitney U test was conducted.

Rarefied diversity indices (Shannon-Wiener Index (H'), species richness, and Pielou's Evenness) based on 850 root tips per sample were calculated using the EcoSim software version 7.72 [40]. Since cumulative rarefied diversity indices for the EM fungi community were calculated, only one value per line and year was obtained. Regression analysis and general mixed models (GLM) were calculated with Statgraphics Centurion (StatPoint Technologies, Inc., Warrenton, VA). Residuals of the regression models were tested by Shapiro Wilks normality test to check the assumption of normal distribution. If the assumption of normal distribution had to be rejected the Null Hypothesis that the slope is equal to zero was tested by Spearman's rank correlation. Before starting the analysis the data were checked graphically for outliers followed by Dixon test for outliers, package: "outliers" [41].

3.3 Results

3.3.1 Absence of fungal clusters and nutrient patches in the soil of a poplar plantation

When the poplar plantation was established in June 2008, nitrogen in the soil solution and fungal distribution were determined to detect potential patchy distribution of soil nutrients and fungi. NMDS did neither reveal any clustering for the patterns of soil fungi (Fig. 3.1a, permutation test, $R^2 = 0.30$, $p = 0.144$) nor for soluble nitrogen in the soil solution at different sampling spots in the plantation (Fig. 3.1b, $R^2 = 0.34$, $p = 0.101$). Other soil nutrient elements and soil pH neither showed positional effects [18]. The mean concentration of the sum amino acids was 415 ± 38 nmol kg^{-1} soil. Glycine, alanine, serine, phenylalanine and isoleucine were the most abundant amino acids in the soil (Fig. 3.1c). The mean soil concentrations of inorganic nitrogen were 82.6 ± 7.0 µmol kg^{-1} for nitrate and 16.6 ± 0.9 µmol kg^{-1} for ammonium. To test if the concentrations of the soluble nitrogen compounds in the soil were correlated with the fungal distribution a Mantel test was conducted. No correlation of those parameters was found ($r = -0.065$, $p = 0.634$). Since we did not detect clustering of soil fungi or nutrient patches when the plantation was established it is unlikely that further results were influenced by local variations of these environmental factors.

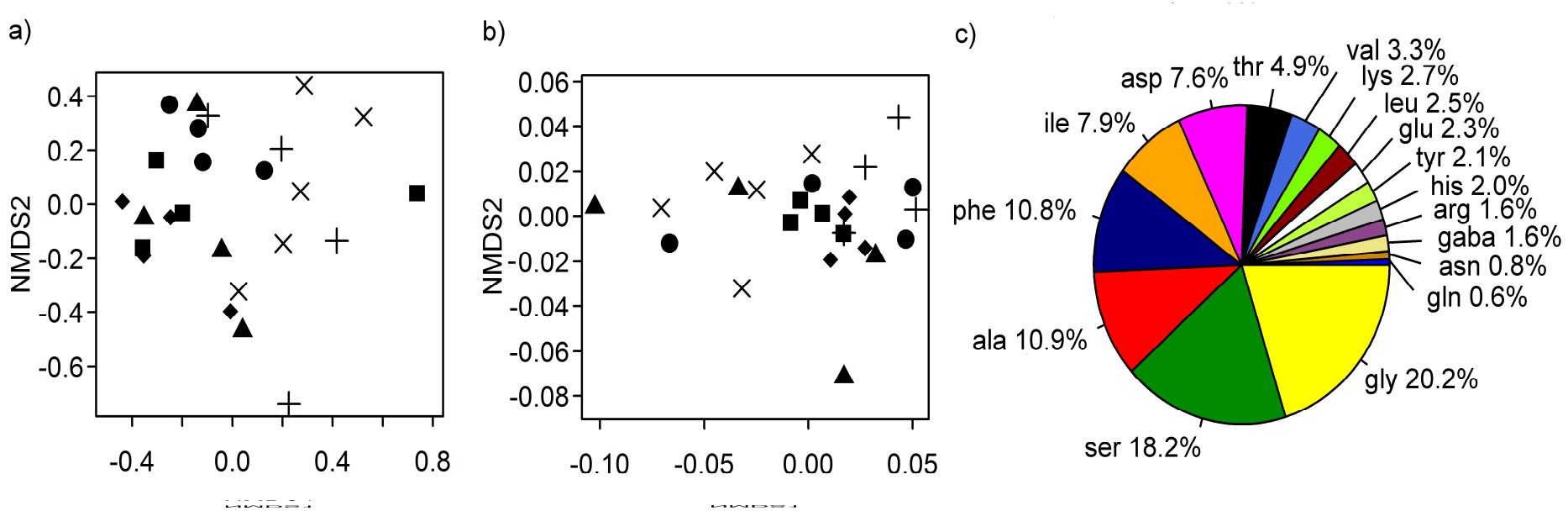

Fig 3.1: Non metric multidimensional scaling (NMDS) of soil fungal communities (a), soluble nitrogen compounds (b), and amino acid composition of the soil solution (c) in a poplar (*P.* x *canescens*) plantation. (a) The soil fungal pattern was determined by DGGE and similarities determined as Jaccard distances were used for the NMDS analysis (two of four dimension are shown, stress = 9.72). (b) NMDS of sum of free amino acids, nitrate, ammonium (two of three dimensions are shown, stress = 5.91). For the analysis 25 soil samples were used collected at the positions marked in supplemental Figure S1. The samples were annotated to their location in the plantation: upper part (filled diamond), upper-middle (filled square), middle-bottom (filled triangle), bottom (filled circle) and outside as border area (+) and distant area (X). (c) Mean percentage of soluble amino acids of all samples. Ser: serine, asn: asparagine, glu: glutamic acid, asp: aspartic acid, lys: lysine, leu: leucine, phe: phenylalanine, ile: isoleucine, val: valine, tyr: tyrosine, gaba: gamma-aminobutyric acid, ala: alanine, arg: arginine, thr: threonine, gly: glycine, gln: glutamine, his: histidine. Measurements were conducted when the plantation was installed (2008).

3.3.2 Ectomycorrhizal colonization show temporal dynamics and genotype- but not gene-specific effects in GM poplars

One year after planting (2009) the EM colonization varied between the different transgenic poplar lines and WT from 58% to 86% (Table 3.1). CAD line L22 showed the lowest and CAD line L18 the highest colonization (Table 3.1). At the end of the following growing season (2010) almost all vital root tips were colonized with EM (Table 3.1). There was only very little variation between the lines (Table 3.1).

The higher EM colonization of roots after two years than after one was also accompanied by higher EM species richness: only eight different EM species were detected after one, however, 30 after two years (Fig. 3.2, Table S3.3). Of the 30 EM species, six (*Paxillus involutus, Laccaria tortilis, Hebeloma sacchariolens, Hebeloma* sp.*, Cenococcum geophilum* and *Peziza ostracoderma*) had already been present in the preceding year (Table S3.3). The increases in total ECM species numbers were also reflected in the Shannon-Wiener Index, which increased from a mean across all poplar lines of 1.2 in 2009 to 2.1 in 2010 ($p < 0.001$), the Simpson Index, which increased from 0.65 to 0.83 ($p < 0.001$), and rarefied species richness, which increased from 5.5 to 13.6 ($p < 0.001$), whereas Evenness was unaffected (mean 2009: 0.72, mean 2010: 0.78, $p = 0.22$, Table S3.4). It was striking that CAD line L22 showed for all diversity indices one of the lowest and COMT line L9 generally the highest values, especially in the first year after plantation. CAD line 22 also displayed higher root tip mortality in 2009 than the other poplar genotypes, whereas its root density assumed an intermediate position between CCR line L5 (highest) and CCR line L 7 (lowest, Table 3.1).

Tab.3.1: Ectomycorrhizal (EM) colonization, vitality index and root density of *P. × canescens*. Root density was determined as root mass per liter of soil volume. Significant differences are indicated by different letters (ANOVA, followed by TukeyHSD, p≤ 0.05). Values indicate mean ± SE, (n =7-9). CCR, COMT and CAD refer to transgenic poplar lines with suppressed activities of cinnamoyl coenzyme A reductase, caffeic acid O-methyl transferase, and cinnamyl alcohol dehydrogenase, respectively.

	EM colonization [%]		**Vitality index [%]**		**Root density [g l $^{-1}$]**	
	2009	**2010**	**2009**	**2010**	**2009**	**2010**
	F= 2.1939	F= 1.1465	F=2.3565	F=1.9684	F=6.783	F=0.9578
	p = 0.04758*	p = 0.3462	p = 0.0342	p = 0.0735	p < 0.001	p = 0.4697
WT	71 ± 5.4 a	99 ± 0.4 a	85 ± 4.9 ab	96 ± 1.2 a	0.503 ± 0.168 bc	0.962 ± 0.321 a
CCR L5	64 ± 7.3 a	99 ± 0.6 a	89 ± 5.1 ab	98 ± 0.6 a	0.543 ± 0.205 c	0.896 ± 0.299 a
CCR L7	73 ±10.2 a	100± 0.0 a	79 ± 5.9 ab	92 ± 2.3 a	0.104 ± 0.039 a	0.739 ± 0.246 a
COMT L9	82 ± 4.8 a	99 ± 0.4 a	76 ± 5.2 ab	95 ± 1.5 a	0.133 ± 0.047 ab	0.652 ± 0.217 a
COMT L11	75 ± 4.1 a	100± 0.1 a	91 ± 2.5 a	94 ± 1.9 a	0.384 ± 0.128 c	0.862 ± 0.287 a
CAD L18	86 ± 1.7 a	99 ± 0.3 a	86 ± 5.0 ab	96 ± 1.3 a	0.497 ± 0.166 c	0.774 ± 0.258 a
CAD L21	64 ± 5.9 a	100± 0.2 a	91 ± 2.3 ab	97 ± 1.1 a	0.447 ± 0.149 c	1.146 ± 0.382 a
CAD L22	58 ± 8.2 a	99 ± 0.4 a	67 ± 8.8 b	91 ± 2.8 a	0.256 ± 0.090 abc	0.689 ± 0.230 a

*no significant differences were detected by TukeyHS

To investigate potential genotype-related effects on EM associations, we analyzed the EM community composition in greater detail. One year after plantation establishment, four of the total number of eight detected EM species were dominant colonizing >90% of the mycorrhizal root tips of all poplar lines; no significant differences between CAD, CCR, COMT and WT lines were found (Fig. 3.2). NMDS of the ECM fungal community on 1-year-old poplars neither revealed significant separation of different poplar lines (permutation test R^2 = 0.1649, p = 0.073, Fig. 3.3a).

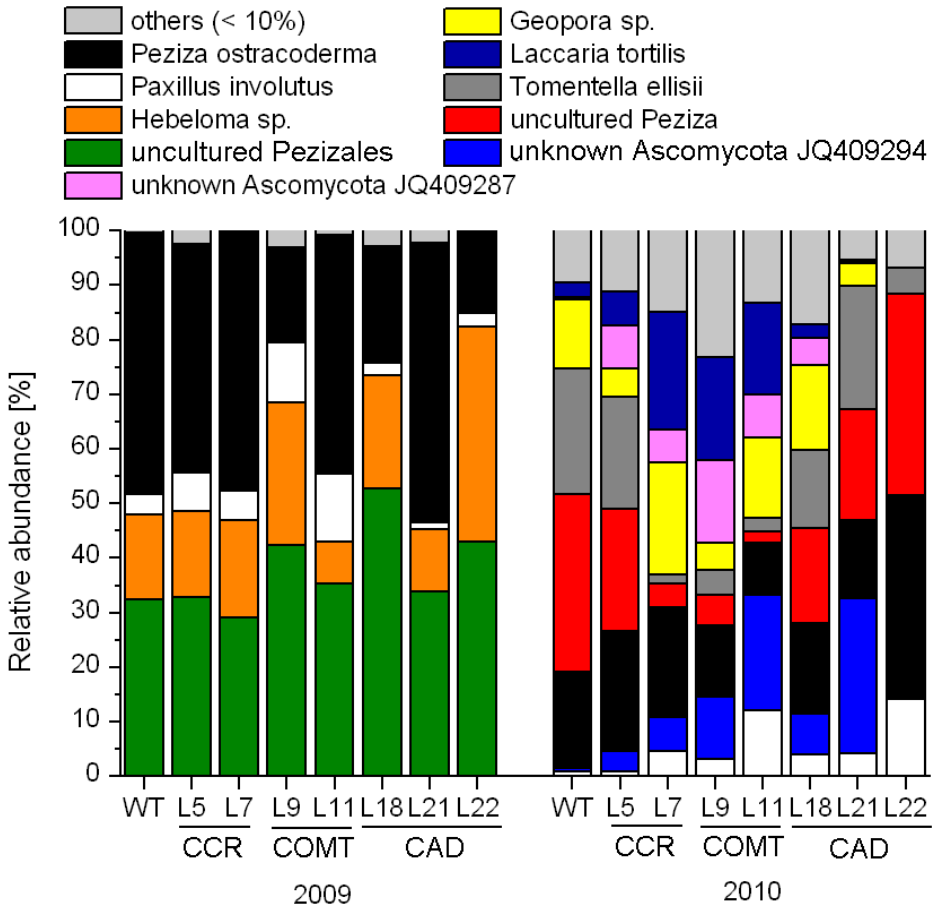

Fig. 3.2: Relative abundance of the most frequent ectomycorrhizal species on the roots of wildtype (WT) and transgenic *Populus* × *canescens* genotypes. The plantation was established in June 2008 and ectomycorrhizal (EM) colonization were determined in October 2009 and October 2010. Only those EM species are shown that exceed on average at least 10% colonization in one host line, other detected species are summarized as "others". Different colours represent different ECM species. The complete species list is found in Supplement Table S3. CCR, COMT and CAD refer to transgenic poplar lines with suppressed activities of cinnamoyl coenzyme A reductase, caffeic acid O-methyl transferase, and cinnamyl alcohol dehydrogenase, respectively.

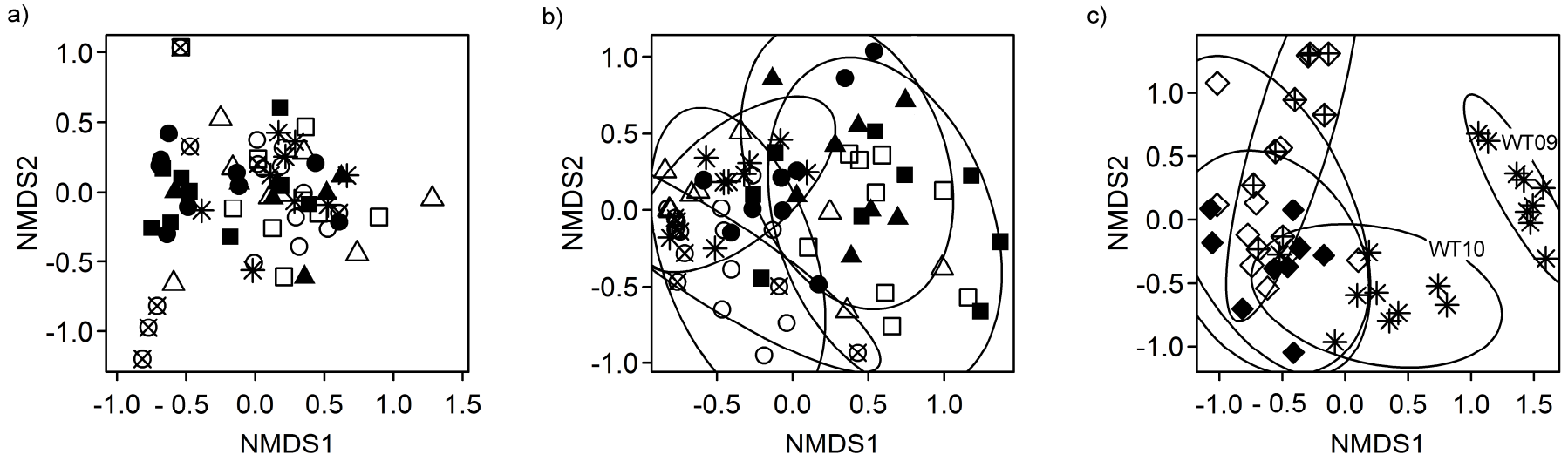

Fig 3.3: Non metric multidimensional scaling (NMDS) of the ectomycorrhizal communities associated with transgenic (a, b) and commercial (c) poplar genotypes. (a) NMDS of EM communities of wildtype and transgenic *P.* x *canescens* in 2009. Two of three dimensions are shown (stress = 10.20, permutation test for separation R^2= 0.49, p = 0.073). (b) NMDS of EM communities of wildtype and transgenic *P.* x *canescens* in 2010. Two of four dimensions are shown (stress = 11.70, permutation test for separation R^2 = 0.43, p = 0.001). (c) NMDS of EM communities of three *P. deltoides* × *P. nigra* clones (2010) and the wildtype of *P.* × *canescens* in 2009 and 2010. Two of four dimension are shown (stress = 7.80, permutation test for separation R^2 = 0.76, p = 0.001). Symbols correspond to different poplar lines. (a,b) COMT: open (L11) and filled squares (L9), CCR: open (L5) and filled triangles (L7), CAD: open (L21), filled (L18) and crossed circles (L22) and WT: star. (c) *P. deltoides* × *P. nigra* clones: open (Ghoy), filled (I-214) and crossed diamonds (Soligo), *P.* x *canescens*: stars.

At the end of the second year (2010), eight EM species were relatively frequent on the root tips (> 10% colonization per EM species) with some significant differences between the poplars lines (Fig. 3.2): an uncultured *Peziza* was more abundant on WT than on CCR line L7 and COMT line L11 roots (Mann-Whitney U-Test, p = 0.022 and p = 0.031, respectively). *Laccaria tortilis* was more abundant on COMT line L11 than on CAD lines L21 and CAD line L22 (p = 0.0077 and p = 0.0087, respectively) (Fig. 3.2). The changes in fungal abundance and composition resulted in genotype-related shifts in the EM communities as documented by NMDS (Fig. 3.3b, permutation test R^2 = 0.43, p = 0.001). The transgenic lines CCR L7 and CAD L22 showed a complete separation of their EM community structures (Fig. 3.3b). CAD line L18, CCR line L5 and COMT line L9 showed the strongest overlap (for clearness of display ellipses not drawn). The EM community structure of the WT was overlapping with all other lines indicating no significant separation (Fig. 3.3b).

To elucidate the ecological importance of these observations we also analyzed the EM species composition of three genotypes of high-yielding, commercial clones of *P.* x *euramericana* (Ghoy, I-214, and Soligo) in comparison with the WT of *P.* × *canescens*. The ordination shows a clear separation of the EM communities of one- and two-year-old *P.* × *canescens* (permutation test: R^2= 0.76, p= 0.001, Fig. 3.3c). Among the three *P.* x *euramericana* clones studied Ghoy and I-214 showed overlapping EM communities with *P.* × *canescens*, whereas Soligo was almost completely separated from *P.* × *canescens* and had less overlap with Ghoy and I-214 than those two genotypes among each other (Fig. 3.3c). These results support that the EM communities underlie temporal and genotype-specific differentiation. However, a separation of EM communities according to the modification of lignification genes was not found.

3.3 Early genotype-specific variation of growth is related to stem N concentrations and ectomycorrhizal root colonization

Since EM fungi can affect nutrient uptake and plant performance, we investigated growth and nutrient status of the poplars in the GM plantation. Significant differences were found for height growth and biomass among the poplar genotypes (Table 3.2). CAD line L22 generally exhibited the lowest performance and CAD line L18 the best (Table 3.2). CAD line L18 also produced more side shoots than the other poplar genotypes (Table 3.2). In comparison with the WT the lines CAD L22 and CCR L7 showed reduced biomass production, whereas biomass of the other genotypes was unaffected by the genetic modification (Table 3.2).

Table 3.2: Growth and biomass of wildtype (WT) and transgenic *P. × canescens* genotypes. CCR, COMT and CAD refer to transgenic poplar lines with suppressed activities of cinnamoyl coenzyme A reductase, caffeic acid O-methyl transferase, and cinnamyl alcohol dehydrogenase, respectively. The plantation was established in June 2008 and measurements were taken in October 2009 and October 2010. Data are means (± SE, n = 9). Cum Height: cumulated height of all stems of one plant was calculated as the sum of the length of the main stem and the side shoots. Biomass = dry mass of the main stem, RCD: root collar diameter. Significant differences are indicated by different letters (ANOVA, followed by TukeyHSD $p \leq 0.05$).

	Height (cm) 2009	Height (cm) 2010	Cum height (cm) 2010	Biomass (g) 2009	Biomass* (g) 2010	Shoots no. 2010	RCD (mm) 2010
	F = 5.349 p < 0.001***	F = 9.9638 p < 0.001***	F = 5.129 p < 0.001***	F = 3.291 p = 0.006**	F = 7.9226 p < 0.001***	F = 2.862 p = 0.012*	F = 5.101 p < 0.001
WT	205.1 ± 11.2 ac	322.7 ± 10.5 c	1100.6 ± 135.1 bc	132.6 ± 13.0 a	417.2 ± 32.9 c	5.6 ± 0.9 ab	23.8 ± 1.4 a
CCR L5	185.4 ± 18.8 abc	304.6 ± 12.1 ac	847.6 ± 103.8 abc	88.0 ± 18.3 ab	247.8 ± 51.3 ac	4.4 ± 0.6 ab	20.0 ± 1.4 ab
CCR L7	154.6 ± 15.5 ab	239.7 ± 19.3 ab	639.3 ± 117.8 ab	79.8 ± 18.1 ab	179.1 ± 46.9 ab	3.3 ± 0.3 a	16.7 ± 1.7 ab
COMT L9	203.6 ± 6.9 ac	309.9 ± 22.4 ac	786.7 ± 84.8 ab	139.1 ± 12.7 a	330.0 ± 58.6 ac	3.8 ± 0.5 ab	21.5 ± 1.7 a
COMT L11	216.6 ± 15.5 c	305.6 ± 14.0 ac	837.8 ± 121.0 abc	121.8 ± 16.5 ab	302.1 ± 43.8 ac	4.3 ± 0.8 ab	19.6 ± 2.2 ab
CAD L18	224.2 ± 13.9 c	328.3 ± 15.4 c	1310.0 ± 103.8 c	130.5 ± 14.7 a	466.9 ± 65.9 c	6.7 ± 0.6 b	24.1 ± 1.8 a
CAD L21	220.1 ± 9.8 c	343.1 ± 4.6 c	970.4 ± 102.5 abc	118.0 ± 18.8 ab	371.4 ± 42.8 c	4.4 ± 0.6 ab	22.0 ± 1.9 a
CAD L22	137.8 ± 7.4 b	194.2 ± 9.1 b	505.2 ± 63.2 a	33.4 ± 4.1 b	77.5 ± 11.7 b	3.7 ± 0.5 ab	11.9 ± 0.4 b

* = calculated with estimated stem volumes and wood density.

To find out whether the growth differences of the different poplar genotypes were the results of compromised nutrient supply, the nutrient element status was characterized for leaves, wood and stem, and carbon allocation was assessed by analyses of the $\delta^{13}C$ signatures in leaves and stem biomass (Supplement Table S3.5). The mean $\delta^{13}C$ value of leaves was -27.34 ± 0.11‰ and that of stems -24.92 ± 0.03‰ ($p < 0.001$). This indicates differences in carbon discrimination between leaves and stem; but no genotype-related effects within leaves or stems were found. We have, therefore, no evidence that the growth differences were caused by genotype-related differences in photosynthetic carbon allocation to wood.

The nutrient element concentrations did not reveal nutritional deficits in comparison with other poplars [42], but significant differences between the analyzed poplar genotypes were detected (Table 3.3, Supplemental Table S3.5). The highest number of differences in nutrient element concentrations among the genotypes was found in stems (P, N, K, Mg, Ca, Mn), an intermediate number in leaves (P, N, K, C, S) and the lowest number of differences were found in roots (P, K, Mn). These results indicate genotype-specific differences in internal nutrient element allocation. The macronutrients P and K showed genotype-related effects in all tissues and N in leaves and stems. The latter three nutrient elements were analyzed in greater detail since their uptake is known to be regulated by EM fungal associations [19].

Multiple variable analyses revealed no significant correlations of the P concentrations in any of the analyzed tissues with EM-related parameters such as root colonization, EM species richness, the Shannon Wiener index or root tip vitality (Table S3.6). To find out if the P concentrations were related to the abundance of specific EM fungi, i.e., related to fungal identity, multiple variable analyses were carried out for the dominant fungi with the tissue nutrient concentrations. None of the nutrient elements (stem concentrations of P, K, or N) showed significant correlations with the abundance of any of the major EM fungi in 2009. In 2010, the leaf P and K concentrations were negatively correlated with the relative abundance of *Peziza ostracoderma* (for P: $R = -0.808$, $p = 0.015$; for K: $R = -0.713$, $p = 0.047$) and the leaf P concentrations were positively correlated with the abundance of an unknown ascomycete JQ409294 ($R = 0.747$, $p = 0.033$). Although leaf P concentrations were correlated with height (Table S3.6), a link between height and the abundance of the ascomycete JQ409294 could not be established ($p = 0.19$). Therefore, we have no evidence for interactions between distinct EM fungal species, P concentrations and growth.

Tab.3.3: P, N and K concentrations in stems of wildtype and transgenic poplar (*P. × canescens*). CCR, COMT and CAD refer to transgenic poplar lines with suppressed activities of cinnamoyl coenzyme A reductase, caffeic acid O-methyl transferase, and cinnamyl alcohol dehydrogenase, respectively. F statistics and p-values are given for one-way ANOVA ($p \leq 0.05$). Significant differences between poplar lines are indicated by different letters. Data indicate means ± SE (L22: n = 4, all other n = 7-9)

Tissue	Genotype	P (mg/g)	N [mg/g]	K [mg/g]
Leaves	WT	2.832 ± 0.170 ab	25.479 ± 0.898 abc	11.544 ± 0.307 abc
Leaves	CCR L5	3.021 ± 0.092 ab	28.486 ± 0.700 a	11.901 ± 0.299 ab
Leaves	CCR L7	2.616 ± 0.124 ab	23.632 ± 0.783 b	10.615 ± 0.271 ac
Leaves	COMT L9	2.776 ± 0.179 ab	25.488 ± 0.389 abc	12.273 ± 0.471 ab
Leaves	COMT L11	3.184 ± 0.178 a	26.492 ± 0.561 abc	12.049 ± 0.532 ab
Leaves	CAD L18	2.749 ± 0.059 ab	25.053 ± 0.734 bc	12.613 ± 0.517 b
Leaves	CAD L21	3.169 ± 0.139 a	28.169 ± 1.150 ac	12.552 ± 0.442 b
Leaves	CAD L22	2.461 ± 0.063 b	23.890 ± 0.518 b	9.926 ± 0.339 c
Leaves	All	F= 3.72	F= 5.47	F= 5.54
Leaves	All	P= 0.002	p< 0.0001	p< 0.0001
Stem	WT	1.139 ± 0.021 b	8.226 ± 0.314 bd	2.653 ± 0.032 c
Stem	CCR L5	1.221 ± 0.052 ab	9.222 ± 0.315 ab	3.352 ± 0.152 a
Stem	CCR L7	1.318 ± 0.055 ab	9.881 ± 0.222 ac	3.422 ± 0.173 a
Stem	COMT L9	1.215 ± 0.053 ab	8.204 ± 0.229 bd	2.813 ± 0.122 bc
Stem	COMT L11	1.250 ± 0.029 ab	8.197 ± 0.197 bd	2.963 ± 0.078 abc
Stem	CAD L18	NA ± NA NA	NA ± NA NA	NA ± NA NA
Stem	CAD L21	1.330 ± 0.050 a	8.055 ± 0.210 d	2.772 ± 0.086 bc
Stem	CAD L22	1.370 ± 0.056 a	10.757 ± 0.263 c	3.245 ± 0.122 ab
Stem	All	F= 2.83	F= 13.62	F= 7.18
Stem	All	p= 0.019	p< 0.0001	p< 0.0001
Roots	WT	1.561 ± 0.071 ab	8.717 ± 1.104 a	5.369 ± 0.238 ab
Roots	CCR L5	1.825 ± 0.066 abc	9.444 ± 1.103 a	5.834 ± 0.249 ab
Roots	CCR L7	1.760 ± 0.094 ab	9.794 ± 0.618 a	6.036 ± 0.357 ab
Roots	COMT L9	1.618 ± 0.094 ab	10.185 ± 0.538 a	5.542 ± 0.306 ab
Roots	COMT L11	1.499 ± 0.030 a	10.283 ± 0.969 a	5.502 ± 0.286 ab
Roots	CAD L18	1.931 ± 0.111 bc	11.201 ± 0.949 a	6.054 ± 0.438 ab
Roots	CAD L21	2.156 ± 0.094 c	10.169 ± 1.106 a	6.672 ± 0.399 a
Roots	CAD L22	1.609 ± 0.088 ab	9.555 ± 1.070 a	5.063 ± 0.330 b
Roots	All	F= 6.87	F= 0.59	F= 2.23
Roots	All	p< 0.0001	p= 0.760	p= 0.043

NA = not available

To further evaluate the relationship between growth, tissue nutrient element concentrations and EM assemblages, we searched the correlation matrix for significant p values (Table S3.6). Stem biomass (2009) was significantly correlated with EM fungal species richness (2009), root tip colonization (2009), stem K and stem N concentrations. GLM analyses with

these parameters and stepwise removal of the factor with the least significant P-value revealed that stem biomass (2009) was modeled with high accuracy by only two factors: stem N concentrations and mycorrhizal root colonization (adjusted R^2 = 97%, $F_{(model)}$ = 108.4, $P_{(model)}$ = 0.0003, $F_{(N)}$ = 101.1, $P_{(N)}$ = 0.0006, $F_{(EM)}$ = 10.8, $P_{(EM)}$ = 0.03, Fig. 3.4). Stem biomass was negatively related to N concentrations and positively with the degree of EM root tip colonization (Fig. 3.4).

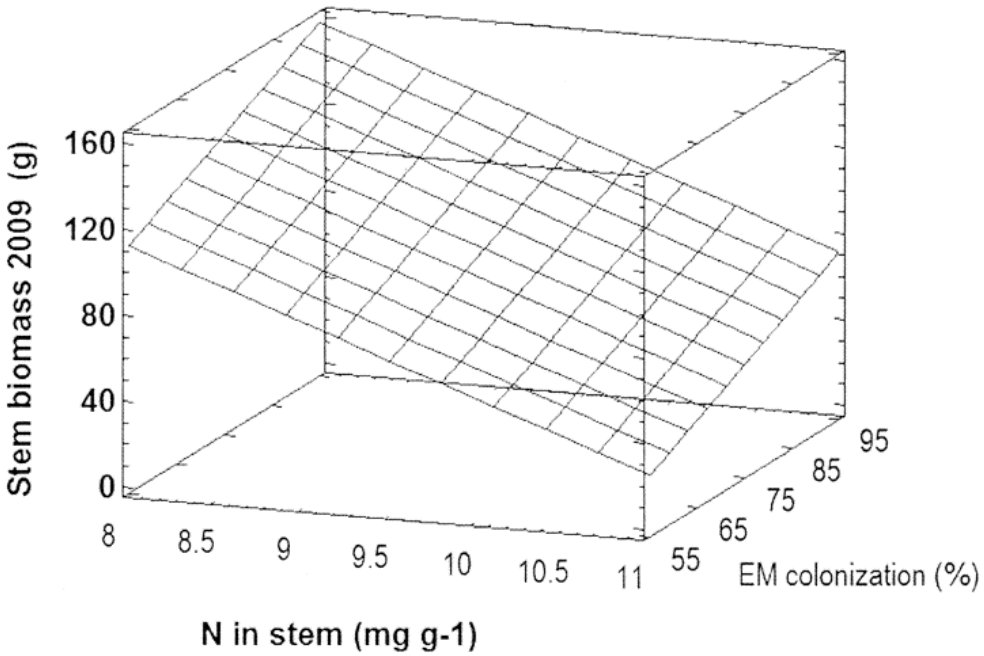

Fig. 3.4: A general linear mixed model for stem biomass with stem N concentrations and root ectomycorrhizal colonization as quantitative independent factors. The surface (hatched lines) shows the 3-dimensional relationship between biomass, N concentration and mycorrhizal colonization.

3.4. Discussion

3.4.1 Influence of gene modification on mycorrhizal colonization and community structure

Poplars can form mutualistic associations with both arbuscular mycorrhizal and EM fungi [19]. However, in poplar plantations associations with EM fungi are the dominant symbiotic form [18,21]. Age-related increases in root tip colonization and EM species diversity as observed here for GM and WT poplars are well known for non-transgenic as well as transgenic poplars (e.g., suppression of the *rolC* gene in *P.* x *canescens* [43], wildtype *P. tremuloides* [44]). Besides the dynamic fungal succession, we observed initially differences in root tip colonization, which vanished in the second year and a differentiation of distinct EM communities on different poplar genotypes.

A main question of the current study, therefore, was if the changes in EM colonization and fungal species composition were caused by the suppression of genes of the lignification pathway. Decreases in lignin as caused by CCR suppression or changes in the lignin composition as caused by CAD and COMT suppression interfere with secondary metabolism and entail changes in the profiles of phenolic compounds [45]. Since phenolic compounds belong to the defense arsenal of poplars [46–49], negative effects on biotic interactions with EM fungi may be anticipated in transgenic trees with changed lignin biosynthesis. Although we found differences in the EM community composition in the second year after planting, these differences could not be related to the suppression of CCR, CAD or COMT.

The composition of EM communities can be influenced by abiotic and biotic environmental factors such as fungal competition [50], soil nutrient and water availability [51–53] and the physiology and genetic constitution of the host [34,54,55]. Variations of abiotic factors and patchiness of soil fungi were not detected in our study plantation. Therefore, EM species composition and abundance might have been influenced by host factors. During transformation the positioning of the introduced DNA in the genome cannot be controlled. Thus, the insertion may have side-effects when the introduced DNA fragment unintentionally hits a functional plant gene locus. Therefore, each transformation event may cause intra-specific variation of traits, in addition to the target gene. Controlled experiments testing the colonization efficiency of the EM fungus *Laccaria bicolor* with the F1 progeny of an inter-specific poplar hybrid revealed that the ability to form mycorrhizas underlies natural intra-specific variation [55–57]. Different EM assemblages were also observed in the present study for different varieties of *P.* x *euramericana*, a poplar hybrid bred for biomass plantations [58,59]. The intra-specific and inter-specific variation in EM assemblages on the WT hybrids of *P.* x *euramericana* and *P.* x *canescens* was similar to that between CCR line L7 and CAD line 22, which exhibited the largest difference of EM species composition. Our study, therefore, supports that the host genotype can affect the colonization ability of distinct mycorrhizal fugal species. However, the intra-specific variation introduced by the transformation of poplars with the antisense constructs to suppress CCR, COMT or CAD activities did not result in larger differences in the EM community composition than those observed for different varieties of conventionally bred high-yielding poplar clones.

3.4.2 The link between EM colonization and diversity and poplar dendromass and nutrient status

The GM poplars with suppressed activities of enzymes of lignin biosynthesis showed strong (ca. 5-fold) differences in growth and biomass in the plantation. This was not surprising since

similar results had been obtained by others studying the performance of lignin-modified plants. For example, Leplé *et al.* [15] found reduced growth in two of five investigated CCR-suppressed poplar lines under field conditions. Voelker *et al*. [60] observed extensive variations in aboveground biomass of 14 different lines of *P.* × *canescens* down-regulated in 4-coumarate:coenzyme A ligase (4CL). Furthermore, greenhouse-grown transgenic poplars with suppressed coumaroyl 3'-hydrolase (C3'H) activity showed drastic growth reductions [61]. The suppression of C3'H activity also reduced the water use efficiency resulting in lower $\delta^{13}C$ signatures in the transgenic compared to WT poplars [61]. If the growth reductions found here were due to impairment of photosynthesis such as reduced stomatal conductance, we would have expected a shift in the $\delta^{13}C$ signature to higher values because of decreased carbon discrimination. However, this was not observed and, therefore, effects on water use and carbon allocation to wood are unlikely reasons for growth reductions in the GM poplars of our study.

Another possibility is that changes in EM colonization and changes in the EM communities had negative impact on tree nutrition leading to reduced growth. This option is not unlikely since the interactions of mycorrhizas with their hosts cover the whole range from beneficial to parasitic effects [62,63]. For example, colonization of *P.* x *euramericana* (cv Ghoy) with different arbuscular mycorrhizal fungal species caused reductions in plant biomass [64]. Although the P concentrations of the aboveground tissues increased, P content of the shoot was diminished because of overall biomass loss [64]. In our study, the abundance of the EM fungi *Peziza ostracoderma* and the ascomycete JQ JQ409294 on root tips of the transgenic poplar genotypes showed negative and positive correlations with foliar P concentrations, respectively. *Paxillus involutus*, which was present in our plantation, has been shown to increase K and P nutrition of poplars [20–23,65]. These observations might imply that distinct EM-poplar genotype associations contributed to facilitating or suppressing P or K transfer to their host trees. However, this suggestion is currently speculative since a full nutrient budget of the trees was not possible and the regulation of tree-fungal-environmental interaction is barely understood. Further functional analyses of EM fungi are, therefore, required.

N is one of the most important nutrient elements for plant growth [66]. In young strongly growing poplars N is mainly present in leaves, but a significant fraction is resorbed in fall, present in woody tissues during the dormant season and re-utilized for sprouting in spring [67,68]. Here, we observed a negative relationship between stem N concentrations and stem biomass indicating higher storage in the wood of smaller poplars than in those of taller plants. The biomass differences of stems were maintained in the following season, and could obviously not be compensated by increased internal N utilization of smaller trees for stem growth. Thus, poplars with low growth have the additional disadvantage of wasting N when

utilizing woody biomass. There is evidence that N allocation differs between fast and slow growing poplar species since trees with inherently higher biomass production exhibit lower N concentrations in the wood and higher nitrogen productivity [69–71]. Poplars grown on a previous agricultural field also showed increased biomass production, decreased N concentrations, and increased nitrogen use efficiency in response to long-term free air CO_2 enrichment [72,73]. Our present data support that, at least in the initial phase, EM colonization is linked with these traits. Positive relationships for growth, nitrogen utilization and EM colonization rates have also been found in Douglas fir [74]. Based on the current data it is not possible to distinguish if poplar growth was stimulated because of higher rates of EM colonization or if trees with higher growth were more amenable to EM colonization. However, the latter possibility is more likely since other studies have already shown that EM colonization and diversity were driven by carbon availability and productivity of the host tree and not vice versa [34,54,74]. Since the root tips of the GM poplars were almost completely colonized with EM at the end of the second growing season, it is clear that the GLM model developed for biomass, nitrogen and root colonization will not be applicable in older plantations. The establishment phase is, however, very important and biomass increments realized during this crucial period will result in further gains because of the exponential nature of growth.

3.5 Conclusion

Genetically modified poplars are a potential alternative for the production of renewable energy since their properties can be optimized to facilitate saccharification. The release of transgenic organisms into the field needs to be carefully controlled to avoid negative effects on environmental interactions, especially with potentially beneficial soil microbes. In this study we demonstrated that transgenic poplar lines modified in the lignin biosynthesis pathway show normal abilities to form ectomycorrhizas. Gene-specific effects of the transformed poplars on mycorrhizal community structure were not found. Variations in EM community structures found between different GM poplar genotypes were in a range similar to the intra-specific variation of commercial poplar clones. The transgenic lines displayed strong differences in stem biomass production. Wood production in the initial phase of plantation establishment was positively correlated with EM colonization rates and negatively with stem N concentrations. Growth advantages realized in the establishment phase were pertained in the following year. Our results suggest that initial differences in EM colonization may have consequences for long term biomass production.

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Author Contributions

Conceived and designed the experiments: LD CB GP AP, Performed the experiments: LD GL AS PK. Analyzed the data: LD GL AS PK CB GP AP. Contributed reagents/ materials/ analysis tools: LD GL AS PK CB GP AP. Wrote the paper: LD GL AS PK CB GP AP.

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3.8 Supplementary data

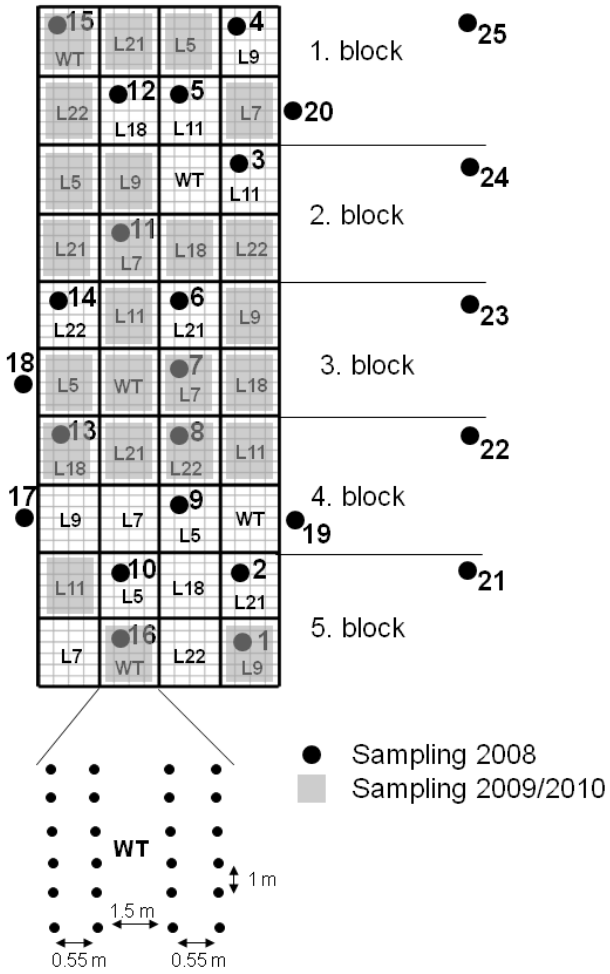


Fig. S3.1: Overview of the experimental plantation of *Populus* x *canescens*.
The experimental field covered an area of 1365 m^2. In total 120 plants per poplar line were planted in a randomized block design. Each block consist 8 subplots one for each poplar line. Each subplot consists of 24 trees planted in two double rows with 6 trees in each row. The space between trees of one double row was 0.55 m while the interspace between the two double rows was 1.5 m, planting distance within one row was 0.5 m. Different transgenic lines are labeled by different abbreviations (WT, CCR: L5 and L7, COMT: L9 and L11, CAD: L18, L21, and L22). To prevent an edge effect the field was bordered with one row of wildtype clones (not shown). Sampling location in 2008 (black pots), 2009 and 2010 (both grey square) are labeled in the figure.

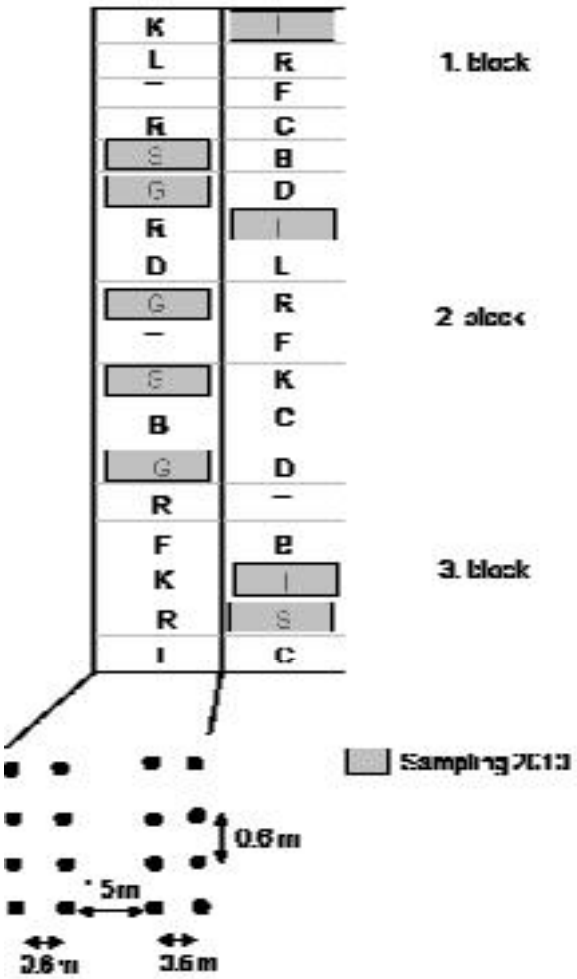


Fig. S3.2: Overview of the commercial plantation of *Populus deltoids* × *P. nigra*
Commercial clones were planted in a randomized block design. Each of the three blocks consist of 11 subplots one for each clone. Each subplot consists of 16 trees planted in four rows with 4 trees in each row. The space between trees of one double row was 0.6 m while the interspace between the two double rows was 1.5 m, planting distance within one row was 0.6 m. Different commercial clones are labeled by different abbreviations (S: Soligo, G: Ghoy, I: I-214, R: Robusta, L: Lambro, K: Koster, F: Flevo, T: Triplo, B: Blanc de Poitou, C: Carpaccio, D: Dorskamp). To prevent an edge effect the field was bordered with two rows of wildtype clones (not shown). Sampling location for in 2010 for Ghoy, Soligo and I-214 are indicated by a grey square.

Tab. S3.1: **Dissimilarity Matrix of fungal communities based on the DGGE band pattern.** 25 soil samples were collected in 2008. Jaccard distance was used for binary data as underlying distance for calculations.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
2	0.20																		
3	0.15	0.27																	
4	0.30	0.25	0.27																
5	0.23	0.27	0.07	0.27															
6	0.18	0.22	0.17	0.11	0.17														
7	0.17	0.20	0.08	0.20	0.08	0.09													
8	0.27	0.22	0.25	0.11	0.17	0.20	0.18												
9	0.17	0.20	0.23	0.30	0.23	0.27	0.25	0.18											
10	0.17	0.20	0.15	0.20	0.23	0.18	0.17	0.18	0.17										
11	0.22	0.16	0.20	0.16	0.12	0.14	0.13	0.05	0.13	0.13									
12	0.25	0.20	0.23	0.20	0.15	0.18	0.17	0.09	0.17	0.08	0.04								
13	0.33	0.29	0.40	0.14	0.40	0.25	0.33	0.25	0.33	0.33	0.29	0.33							
14	0.40	0.25	0.36	0.38	0.36	0.44	0.40	0.33	0.40	0.30	0.37	0.30	0.43						
15	0.18	0.22	0.17	0.22	0.17	0.10	0.09	0.20	0.27	0.18	0.14	0.18	0.25	0.44					
16	0.13	0.16	0.12	0.26	0.20	0.14	0.13	0.24	0.22	0.13	0.18	0.22	0.41	0.37	0.14				
17	0.47	0.47	0.43	0.33	0.43	0.41	0.37	0.29	0.47	0.37	0.33	0.37	0.38	0.47	0.29	0.44			
18	0.26	0.20	0.33	0.20	0.33	0.29	0.26	0.18	0.26	0.26	0.22	0.26	0.23	0.20	0.29	0.33	0.29		
19	0.27	0.22	0.17	0.22	0.25	0.30	0.27	0.20	0.27	0.18	0.24	0.27	0.38	0.22	0.30	0.14	0.41	0.29	
20	0.22	0.16	0.20	0.26	0.20	0.24	0.22	0.14	0.13	0.13	0.09	0.13	0.41	0.37	0.24	0.09	0.44	0.33	0.14
21	0.44	0.29	0.40	0.29	0.40	0.25	0.33	0.38	0.44	0.33	0.29	0.33	0.33	0.43	0.25	0.29	0.38	0.38	0.38
22	0.30	0.12	0.27	0.12	0.27	0.22	0.20	0.11	0.30	0.20	0.16	0.20	0.29	0.25	0.22	0.16	0.33	0.20	0.11
23	0.24	0.29	0.22	0.18	0.30	0.26	0.24	0.16	0.24	0.14	0.20	0.24	0.33	0.29	0.26	0.20	0.25	0.12	0.16
24	0.20	0.12	0.27	0.38	0.36	0.33	0.30	0.33	0.20	0.20	0.26	0.30	0.43	0.25	0.33	0.16	0.47	0.20	0.22
25	0.26	0.20	0.33	0.33	0.43	0.41	0.37	0.29	0.26	0.26	0.33	0.37	0.38	0.20	0.41	0.22	0.43	0.14	0.18

	20	21	22	23	24
21	0.29				
22	0.16	0.29			
23	0.20	0.33	0.18		
24	0.16	0.29	0.25	0.18	
25	0.22	0.38	0.20	0.12	0.07

Tab. S3.2: **Soluble amino acid, nitrate and ammonium concentration in soil samples collected in 2008.** 25 soil samples (ID) were analyzed. Amino acids are presented in nmol kg^{-1} soil and nitrate and ammonium are presented in µmol kg^{-1} soil.

ID	asp	glu	asn	ser	his	gln	gly	thr	arg	ala	gaba	tyr	val	ile	phe	leu	lys	NO_3	NH_4
1	18.5	5.3	1.6	47.9	4.9	2.7	51.8	8.7	4.0	24.0	5.1	4.0	7.8	15.0	52.5	5.5	8.1	55.5	15.6
2	88.6	27.0	7.0	184.0	24.0	4.0	210.8	64.0	13.0	123.4	6.0	28.0	30.0	38.1	31.0	37.0	33.0	127.2	17.8
3	13.8	4.9	1.8	33.1	4.8	2.5	37.1	8.9	8.0	21.4	5.9	4.9	9.8	21.4	39.2	2.9	4.5	81.6	13.8
4	22.1	4.3	2.0	46.6	8.3	1.2	45.8	9.4	4.1	16.6	5.9	5.1	11.1	31.3	43.3	4.1	11.1	100.2	17.9
5	15.3	4.9	1.9	28.7	1.6	0.7	31.8	6.1	2.7	14.9	6.0	5.1	7.9	20.9	36.8	5.8	5.1	94.3	15.3
6	19.4	5.6	1.2	51.9	2.1	1.3	44.4	10.8	4.6	17.6	6.5	5.6	9.8	29.6	35.9	4.3	5.1	96.5	15.2
7	121.9	36.0	17.0	372.0	54.0	10.0	534.4	94.0	37.0	262.1	7.0	25.0	59.0	44.5	31.0	47.0	46.0	70.6	15.3
8	64.6	20.5	2.2	109.0	12.1	3.1	131.1	39.3	7.8	83.8	5.0	16.0	16.4	47.2	64.6	16.3	22.3	42.2	20.5
9	16.6	3.9	1.5	49.2	2.8	0.6	48.9	9.2	0.5	19.2	3.3	4.0	5.3	27.7	41.5	5.1	6.7	108.6	13.1
10	9.4	2.3	0.9	17.0	0.7	0.5	26.4	3.8	1.7	13.4	3.4	6.0	5.4	23.0	37.1	4.1	4.9	74.9	13.1
11	56.4	11.9	2.2	103.0	1.8	1.3	112.9	26.1	1.1	77.8	6.7	13.0	15.6	50.5	65.3	15.9	15.2	51.3	23.9
12	16.3	4.2	1.1	35.5	1.0	0.8	42.0	8.1	2.4	24.2	4.1	5.9	13.7	32.8	41.1	6.4	6.1	47.9	13.2
13	10.5	2.7	1.2	15.5	1.2	0.4	25.0	5.5	2.1	11.3	3.8	4.2	5.2	4.1	9.1	5.2	4.7	90.3	13.8
14	36.3	9.3	3.2	52.0	4.1	1.0	85.0	19.5	2.8	41.4	4.7	11.7	10.5	36.1	45.9	12.6	14.7	67.7	16.5
15	30.1	10.6	3.1	80.6	3.6	1.5	72.8	17.7	5.5	37.2	6.6	8.1	12.5	41.3	57.3	9.9	11.7	193.5	18.1
16	8.9	3.4	1.6	22.0	6.6	0.6	23.1	4.5	1.8	13.9	7.1	6.3	8.6	31.6	47.3	3.6	4.3	65.5	21.5
17	14.8	4.4	1.7	31.3	9.8	0.7	31.5	6.2	2.1	11.6	4.5	3.5	17.4	38.8	58.8	3.1	6.7	31.2	22.8
18	17.1	3.6	1.2	42.7	1.3	1.4	41.9	11.1	2.3	33.3	4.9	5.7	12.2	49.3	72.2	6.4	4.7	119.9	28.4
19	15.4	3.6	1.1	28.4	11.7	2.5	29.0	5.4	3.0	19.1	4.6	6.5	8.6	31.4	49.3	2.9	3.8	65.4	15.3
20	22.5	5.3	2.0	61.0	5.0	2.4	54.2	11.3	1.7	27.5	7.3	8.3	6.9	32.8	46.8	4.9	6.6	117.8	17.5
21	25.7	8.6	4.6	89.5	3.0	3.1	64.7	17.4	5.6	35.5	16.4	7.4	17.2	46.6	65.0	10.1	8.4	49.6	22.1
22	25.9	6.4	3.7	97.3	11.1	3.5	71.7	22.6	6.0	33.8	3.6	5.7	9.4	32.4	37.3	7.5	7.8	61.7	13.7
23	25.7	9.5	5.2	95.7	11.2	4.0	63.6	22.3	31.3	37.0	5.2	4.3	9.4	30.4	40.8	8.6	9.2	73.8	11.8
24	52.5	26.7	8.3	107.0	10.3	5.9	124.6	50.8	13.1	74.3	6.9	13.6	18.9	32.4	32.1	18.3	14.8	60.9	8.0
25	43.7	13.4	6.6	85.0	10.1	2.2	92.5	30.9	5.4	54.1	25.7	9.6	14.6	35.2	36.8	12.4	10.0	115.7	10.7

Tab. S3.3: Relative abundance of fungal species detected on ectomycorrhizal root tips of *P. × canescens* and *P. deltoides × nigra* by morphotyping/ ITS- sequencing. Values indicate mean ± SE.

Species	WT	CCR L5	CCR L7	COMT L9	COMT L11	CAD L18	CAD L21	CAD L22
2009								
Cenococcum geophilum	0.00± 0.00	0.00± 0.00	0.00± 0.00	1.73± 0.23	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00
Hebeloma sp.	15.62± 0.52	15.88± 0.76	17.93± 0.51	26.10± 0.55	7.62± 0.33	20.68± 0.42	11.45± 0.36	39.47± 0.87
Hebeloma sacchariolens	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.78± 0.16	0.00± 0.00	0.55± 0.14	0.00± 0.00	0.00± 0.00
Laccaria tortilis	0.19± 0.08	2.44± 0.36	0.00± 0.00	0.58± 0.15	0.00± 0.00	2.03± 0.27	1.12± 0.17	0.00± 0.00
Paxillus involutus	3.55± 0.32	7.08± 0.52	5.49± 0.43	11.00± 0.57	12.42± 0.53	2.26± 0.28	1.14± 0.19	2.56± 0.32
Peziza ostracoderma	48.01± 0.58	41.85± 0.91	47.57± 0.77	17.46± 0.59	43.77± 0.63	21.39± 0.65	51.38± 0.49	15.04± 0.71
uncultured Pezizales	32.44± 0.49	32.76± 0.84	29.02± 0.66	42.34± 0.52	35.39± 0.64	52.77± 0.55	33.83± 0.54	42.93± 0.84
MT5	0.19± 0.08	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.80± 0.14	0.33± 0.11	1.08± 0.19	0.00± 0.00
2010								
Cenococcum geophilum	3.07± 0.33	0.11± 0.06	0.18± 0.08	0.41± 0.12	0.12± 0.07	0.00± 0.00	0.04± 0.04	0.00± 0.00
Geopora cervina	0.00± 0.00	0.00± 0.00	0.00± 0.00	9.14± 0.57	1.37± 0.23	0.00± 0.00	0.00± 0.00	0.00± 0.00
Geopora sp.	12.72± 0.38	5.14± 0.35	20.48± 0.46	4.87± 0.31	14.84± 0.44	15.56± 0.43	4.24± 0.32	0.00± 0.00
Hebeloma sp.	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	4.46± 0.44
Hebeloma sacchariolens	2.75± 0.28	0.00± 0.00	1.01± 0.17	0.07± 0.04	0.04± 0.04	0.22± 0.09	0.10± 0.05	0.00± 0.00
Inocybe cf. splendens	0.00± 0.00	0.00± 0.00	0.25± 0.10	0.00± 0.00	1.55± 0.20	0.00± 0.00	0.00± 0.00	0.00± 0.00
Inocybe curvipes	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.66± 0.16	0.00± 0.00	0.00± 0.00	0.00± 0.00
Laccaria laccata	0.00± 0.00	0.00± 0.00	0.00± 0.00	2.08± 0.28	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00
Laccaria tortilis	2.78± 0.32	6.32± 0.39	21.52± 0.51	18.79± 0.57	16.85± 0.39	2.56± 0.30	0.45± 0.11	0.00± 0.00
Paxillus involutus	0.80± 0.17	0.90± 0.18	4.64± 0.34	3.13± 0.21	12.02± 0.43	3.95± 0.28	4.16± 0.32	14.24± 0.73
Peziza ostracoderma	17.67± 0.59	21.90± 0.46	20.16± 0.47	13.09± 0.37	9.45± 0.32	16.68± 0.42	14.40± 0.35	36.99± 0.73
Scleroderma bovista	0.10± 0.06	2.85± 0.26	0.00± 0.00	0.00± 0.00	0.00± 0.00	1.68± 0.20	3.29± 0.31	0.00± 0.00
Tomentella ellisii	23.00± 0.54	20.55± 0.54	1.65± 0.20	4.65± 0.35	2.43± 0.23	14.41± 0.48	22.55± 0.54	4.69± 0.28
Tuber sp. JQ409291	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	3.52± 0.36	0.00± 0.00	0.00± 0.00	0.00± 0.00
Tuber sp. JQ824882	0.03± 0.03	0.00± 0.00	0.00± 0.00	1.63± 0.22	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00
Xerocomus ripariellus	3.07± 0.33	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00
uncultured ectomycorrhizal fungus JQ824883	0.00± 0.00	5.15±0.43	1.43± 0.23	0.00± 0.00	0.79± 0.17	0.00± 0.00	0.00± 0.00	0.00± 0.00
uncultured ectomycorrhizal fungus JQ824884	0.00± 0.00	0.00± 0.00	0.53± 0.14	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00
uncultured *Peziza*	32.46± 0.55	22.48± 0.44	4.46± 0.21	5.60± 0.36	2.20± 0.20	17.29± 0.43	20.42± 0.46	36.85± 0.69

Table continued

Species	WT	CCR L5	CCR L7	COMT L9	COMT L11	CAD L18	CAD L21	CAD L22
unknown Ascomycota JQ409287	0.44± 0.10	7.80± 0.54	5.96± 0.34	15.30± 0.51	7.84± 0.38	4.96± 0.27	0.18± 0.07	0.00± 0.00
unknown Ascomycota JQ409288	0.00± 0.00	0.00± 0.00	0.00± 0.00	1.73± 0.21	0.68± 0.16	6.67± 0.50	0.00± 0.00	0.00± 0.00
unknown Ascomycota JQ409292	0.19± 0.08	0.41± 0.12	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	2.39± 0.30
unknown Ascomycota JQ409293	0.00± 0.00	0.00± 0.00	3.61± 0.35	6.89± 0.51	1.98± 0.20	7.85± 0.54	0.00± 0.00	0.00± 0.00
unknown Ascomycota JQ409294	0.73± 0.16	3.80± 0.31	6.15± 0.44	11.40± 0.59	21.24± 0.61	7.47± 0.42	28.36± 0.56	0.37± 0.13
MT13	0.19± 0.08	0.00± 0.00	1.19± 0.20	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00
MT17	0.00± 0.00	0.11± 0.07	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00
MT30	0.00± 0.00	2.48± 0.30	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	1.81± 0.26	0.00± 0.00
MT33	0.00± 0.00	0.00± 0.00	0.48± 0.13	1.10± 0.17	0.18± 0.08	0.72± 0.16	0.00± 0.00	0.00± 0.00
MT38	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.25± 0.10	0.00± 0.00	0.00± 0.00	0.00± 0.00
MT42	0.00± 0.00	0.00± 0.00	6.31± 0.39	0.11± 0.06	1.99± 0.22	0.00± 0.00	0.00± 0.00	0.00± 0.00

Tab. S3.4: Fungal species detected on ECM root tips of *P. × canescens* and *P. deltoides × nigra* by morphotyping/ITS-sequencing In the transgenic poplar field sampling took place both years, in the commercial poplar field only in 2010. ACC: accession number. Field: 1 = transgenic field, 2 = commercial field. (-) indicates that this species was not found on roots of all lines within this year. Values indicate mean ± SE.

Species	ACC	Best BLAST hit	Source database	ACC	length of fragment	Homo-logy [%]	Score	Field
Cenococcum geophilum	JQ409285	*Cenococcum geophilum*	NCBI	HQ406817.1	857	96	1375	1+2
Geopora cervina	JQ824878	*Geopora cervina*	NCBI	FM206386.1	547	99	989	1+2
Geopora sp.	JQ409286	*Geopora* sp. TAA 192232	NCBI	FM206420.1	489	99	878	1+2
Hebeloma sp.	JQ409279	*Hebeloma* sp.	UNITE	UDB001188			1	
Hebeloma sacchariolens	JQ409280	*Hebeloma sacchariolens*	RSyst	UDB001188	605	96	957	1+2
Inocybe cf. splendens	JQ824880	*Inocybe cf. splendens* EL21906	NCBI	FN550912.1	514	99	944	1+2
Inocybe curvipes	JQ824879	*Inocybe curvipes*	UNITE	UDB000616	575	99	1104	1+2
Laccaria laccata	JQ824881	*Laccaria laccata* voucher	NCBI	JN021050.1	637	100	1107	1+2
Laccaria tortilis	JQ409281	*Laccaria tortilis* (Bolton) Cooke	UNITE	UDB001589	568	99	1126	1+2
Paxillus involutus	JQ409282	*Paxillus involutus*	RSyst	EU078741	638	99	1203	1+2
Peziza ostracoderma	JQ409283	*Peziza ostracoderma*	NCBI	EU819461.1	657	99	1158	1+2
Scleroderma bovista	JQ409289	*Scleroderma bovista* Fr.	UNITE	UDB002179	630	98	1205	1
Tomentella ellisii	JQ409290	*Tomentella ellisii*	NCBI	DQ068971.1	504	100	931	1+2
Tuber sp.	JQ824882	*Tuber* sp. NZT0251	NCBI	AM900418	562	100	1035	1+2
Tuber sp.	JQ409291	*Tuber* sp. GMB-2010b	NCBI	HM485376.1	473	100	874	1
Xerocomus ripariellus	JQ409296	*Xerocomus ripariellus* Redeuilh	UNITE	UDB000485	649	100	1287	1+2
uncultured ectomycorrhizal fungus	JQ824883	Uncultured fungus	NCBI	AJ875375.1	522	99	953	1+2
uncultured ectomycorrhizal fungus	JQ824884	Uncultured ectomycorrhizal fungus	NCBI	FJ013060.1	469	99	854	1
uncultured *Peziza*	JQ409295	uncultured *Peziza*	NCBI	GU969261.1	539	99	979	1+2
uncultured Pezizales	JQ409284	uncultured Pezizales	NCBI	DQ469743.1	669	98	1112	1
unknown Ascomycota	JQ409287	Uncultured fungus	NCBI	EU555000.1	510	100	942	1+2
unknown Ascomycota	JQ409288	uncultured fungus	NCBI	EU554730.1	539	100	996	1+2
unknown Ascomycota	JQ409292	uncultured Ascomycota	NCBI	EU562601.1	522	97	883	1+2
unknown Ascomycota	JQ409293	uncultured Ascomycota	NCBI	EU557319.1	544	99	992	1+2
unknown Ascomycota	JQ409294	uncultured ectomycorrhizal fungus	NCBI	EF484931.1|	571	97	965	1
MT5		only morphotyping						1
MT13		only morphotyping						1
MT17		only morphotyping						1
MT30		only morphotyping						1
MT33		only morphotyping						1+2
MT38		only morphotyping						1+2
MT42		only morphotyping						1+2

Tab. S3.5: **Diversity indices of ECM fungal community on the roots of *P. × canescens* in 2009 and 2010.** Rarefied diversity-Indices were calculated on the basis of 850 root tips per line. Cumulative diversity indices are presented.

	H'	Simpson	Species Richness	Pielou's Evenness
2009				
WT	1.18	0.65	6	0.66
L5	1.33	0.70	5	0.83
L7	1.17	0.66	4	0.84
L9	1.43	0.72	7	0.74
L11	1.24	0.67	5	0.77
L18	1.18	0.61	7	0.61
L21	1.06	0.60	6	0.59
L22	1.08	0.63	4	0.78
2010				
WT	1.82	0.79	13	0.70
L5	2.05	0.84	13	0.79
L7	2.18	0.85	17	0.77
L9	2.38	0.89	16	0.85
L11	2.30	0.87	19	0.78
L18	2.23	0.88	13	0.87
L21	1.79	0.80	11	0.75
L22	1.39	0.70	7	0.72

Tab. S3.6: **Mean nutrient element concentrations in leaf, stem, and roots of wildtype and transgenic poplar (*P. × canescens) genotypes*** Data indicate mean ± SE. F statistics and p-values are given for one-way ANOVA. Small letters indicated significant differences (TukeyHSD, $p \leq 0.05$). C concentration was analyzed by Kruskal-Wallis rank sum test and Mann-Whitney U-Test ($p \leq 0.05$). All element concentrations in mg g-1 dry mass. Leaves and roots were harvested in October 2009, and stems in March 2010.

Leaves	P [mg/g]	C [mg/g]	N [mg/g]	S [mg/g]	K [mg/g]
	F = 3.72	Chi2= 3.46	F= 5.47	F = 14.83	F = 5.54
	p= 0.002	p= 0.003	p< 0.0001	p= 0.038	p< 0.0001
WT	2.832 ±0.170 ab	496.148 ± 2.216 ab	25.479 ± 0.898 abc	1.875 ± 0.239 ab	11.544 ± 0.307 abc
CCR L5	3.021 ±0.092 ab	493.286 ± 3.076 a	28.486 ± 0.700 a	2.056 ± 0.216 a	11.901 ± 0.299 ab
CCR L7	2.616 ±0.124 ab	494.398 ± 1.296 a	23.632 ± 0.783 b	1.605 ± 0.031 ab	10.615 ± 0.271 ac
COMT L9	2.776 ±0.179 ab	499.395 ± 1.236 ab	25.488 ± 0.389 abc	1.607 ± 0.050 ab	12.273 ± 0.471 ab
COMT L11	3.184 ±0.178 a	497.447 ± 1.242 ab	26.492 ± 0.561 abc	1.647 ± 0.038 ab	12.049 ± 0.532 ab
CAD L18	12.749 ±0.059 ab	496.852 ± 1.120 ab	25.053 ± 0.734 bc	1.595 ± 0.031 ab	12.613 ± 0.517 b
CAD L21	3.169 ±0.139 a	500.149 ± 0.942 ab	28.169 ± 1.150 ac	1.706 ± 0.048 ab	12.552 ± 0.442 b
CAD L22	2.461 ±0.063 b	502.660 ± 0.901 b	23.890 ± 0.518 b	1.555 ± 0.018 b	9.926 ± 0.339 c
Stem	P [mg/g]	C [mg/g]	N [mg/g]	S [mg/g]	K [mg/g]
	F = 2.83	Chi2= 13.50	F = 13.62	F= 10.90	F= 7.18
	p= 0.019	p= 0.036	p< 0.0001	p< 0.0001	p< 0.0001
WT	1.139 ±0.021 b	462.720 ± 2.170 a	8.226 ± 0.314 bd	0.364 ± 0.010 c	2.653 ± 0.032 c
CCR L5	1.221 ±0.052 ab	459.039 ± 1.280 a	9.222 ± 0.315 ab	0.481 ± 0.028 bd	3.352 ± 0.152 a
CCR L7	1.318 ±0.055 ab	451.224 ± 3.222 a	9.881 ± 0.222 ac	0.573 ± 0.017 abd	3.422 ± 0.173 a
COMT L9	1.215 ±0.053 ab	459.153 ± 0.708 a	8.204 ± 0.229 bd	0.420 ± 0.026 cd	2.813 ± 0.122 bc
COMT L11	1.250 ±0.029 ab	456.294 ± 0.591 a	8.197 ± 0.197 bd	0.453 ± 0.017 cd	2.963 ± 0.078 abc
CAD L18	NA ± NA NA	NA ± NA NA	NA ± NA NA	NA ± NA NA	NA ± NA NA
CAD L21	1.330 ±0.050 a	458.457 ± 0.983 a	8.055 ± 0.210 d	0.444 ± 0.028 cd	2.772 ± 0.086 bc
CAD L22	1.370 ±0.056 a	459.383 ± 0.353 a	10.757 ± 0.263 c	0.647 ± 0.012 a	3.245 ± 0.122 ab
Roots	P [mg/g]	C [mg/g]	N [mg/g]	S [mg/g]	K [mg/g]
	F= 6.87	F= 1.32	F= 0.59	F = 2.06	F= 2.23
	p< 0.0001	p= 0.258	p= 0.760	p= 0.061	p= 0.043
WT	1.561 ± 0.071 ab	429.159 ± 3.765 a	8.717 ± 1.104 a	1.036 ± 0.102 a	5.369 ± 0.238 ab
CCR L5	1.825 ± 0.066 abc	418.172 ± 4.750 a	9.444 ± 1.103 a	1.171 ± 0.110 a	5.834 ± 0.249 ab
CCR L7	1.760 ± 0.094 ab	433.813 ± 6.373 a	9.794 ± 0.618 a	1.202 ± 0.072 a	6.036 ± 0.357 ab
COMT L9	1.618 ± 0.094 ab	417.123 ± 8.440 a	10.185 ± 0.538 a	1.205 ± 0.056 a	5.542 ± 0.306 ab
COMT L11	1.499 ± 0.030 a	429.196 ± 5.159 a	10.283 ± 0.969 a	1.009 ± 0.066 a	5.502 ± 0.286 ab
CAD L18	11.931 ±0.111 bc	438.245 ± 3.358 a	11.201 ± 0.949 a	1.363 ± 0.104 a	6.054 ± 0.438 ab
CAD L21	2.156 ±0.094 c	429.684 ± 7.183 a	10.169 ± 1.106 a	1.332 ± 0.113 a	6.672 ± 0.399 a
CAD L22	1.609 ±0.088 ab	430.494 ± 8.943 a	9.555 ± 1.070 a	1.089 ± 0.072 a	5.063 ± 0.330 b

Tab S3.6 continued

Leaves	Ca [mg/g]	Mg [mg/g]	Mn [mg/g]	Fe [mg/g]	δ C13
	F= 0.68	F= 1.91	F= 2.65	F= 0.73	F= 1.84
	p= 0.690	p= 0.082	p= 0.018	p= 0.645	p= 0.095
WT	7.220 ± 0.394 a	1.464 ± 0.025 a	0.069 ± 0.007 a	0.090 ± 0.005 a	-28.025 ± 0.195 a
CCR L5	7.177 ± 0.661 a	1.497 ± 0.064 a	0.090 ± 0.006 a	0.090 ± 0.006 a	-27.356 ± 0.320 a
CCR L7	7.898 ± 0.730 a	1.475 ± 0.039 a	0.081 ± 0.009 a	0.099 ± 0.010 a	-27.470 ± 0.258 a
COMT L9	7.541 ± 0.500 a	1.575 ± 0.064 a	0.079 ± 0.006 a	0.104 ± 0.012 a	-27.255 ± 0.212 a
COMT L11	7.466 ± 0.464 a	1.655 ± 0.061 a	0.106 ± 0.004 a	0.091 ± 0.008 a	-27.303 ± 0.267 a
CAD L18	7.244 ± 0.407 a	1.486 ± 0.047 a	0.076 ± 0.009 a	0.090 ± 0.005 a	-27.354 ± 0.296 a
CAD L21	7.618 ± 0.403 a	1.453 ± 0.051 a	0.100 ± 0.013 a	0.086 ± 0.007 a	-27.127 ± 0.214 a
CAD L22	8.387 ± 0.238 a	1.461 ± 0.041 a	0.071 ± 0.008 a	0.083 ± 0.003 a	-26.848 ± 0.084 a
Roots	Ca [mg/g]	Mg [mg/g]	Mn [mg/g]	Fe [mg/g]	δ C13
	F= 10.46	F= 3.61	F= 7.11	F= 2.15	F= 0.676
	p< 0.0001	p= 0.005	p< 0.0001	p= 0.064	p= 0.670
WT	6.246 ± 0.295 ab	0.700 ± 0.019 b	0.016 ± 0.001 b	0.047 ± 0.001 a	-24.743 ± 0.136 a
CCR L5	7.466 ± 0.266 ab	0.743 ± 0.029 ab	0.020 ± 0.002 ab	0.054 ± 0.010 a	-25.025 ± 0.129 a
CCR L7	9.623 ± 0.426 c	0.851 ± 0.046 a	0.027 ± 0.003 c	0.046 ± 0.004 a	-24.906 ± 0.077 a
COMT L9	6.106 ± 0.417 a	0.686 ± 0.036 b	0.019 ± 0.001 ab	0.057 ± 0.008 a	-24.990 ± 0.138 a
COMT L11	7.654 ± 0.213 b	0.792 ± 0.022 ab	0.024 ± 0.001 ac	0.044 ± 0.003 a	-24.964 ± 0.089 a
CAD L18	NA ± NA NA	NA ± NA NA	NA ± NA NA	NA ± NA NA	NA ± NA NA
CAD L21	6.638 ± 0.447 ab	0.788 ± 0.033 ab	0.017 ± 0.001 b	0.065 ± 0.009 a	-24.929 ± 0.098 a
CAD L22	7.591 ± 0.073 ab	0.802 ± 0.014 ab	0.016 ± 0.001 b	0.083 ± 0.018 a	-24.891 ± 0.071 a
Stem	Ca [mg/g]	Mg [mg/g]	Mn [mg/g]	Fe [mg/g]	
	F= 3.01	F= 1.86	F= 2.60	F= 1.18	
	p= 0.009	p= 0.091	p= 0.020	p= 0.326	
WT	7.783 ± 0.241 a	1.171 ± 0.118 a	0.114 ± 0.021 ab	2.372 ± 0.461 a	
CCR L5	8.565 ± 0.527 a	1.323 ± 0.106 a	0.121 ± 0.016 ab	2.476 ± 0.336 a	
CCR L7	9.329 ± 0.318 a	1.248 ± 0.093 a	0.107 ± 0.010 a	1.883 ± 0.201 a	
COMT L9	7.727 ± 0.292 a	1.277 ± 0.080 a	0.182 ± 0.019 ab	2.936 ± 0.337 a	
COMT L11	7.865 ± 0.345 a	1.113 ± 0.094 a	0.133 ± 0.023 ab	2.229 ± 0.446 a	
CAD L18	8.851 ± 0.479 a	1.540 ± 0.125 a	0.155 ± 0.022 ab	3.054 ± 0.392 a	
CAD L21	9.107 ± 0.171 a	1.506 ± 0.156 a	0.210 ± 0.038 b	3.006 ± 0.407 a	
CAD L22	8.912 ± 0.458 a	1.264 ± 0.084 a	0.131 ± 0.022 ab	2.899 ± 0.535 a	

Tab. S3.7: Pearson product moment correlations between biomass, ectomycorrhiza and nutrient related parameters. These correlation coefficients range between -1 and +1 and measure the strength of the linear relationship between the variables. Also shown in parentheses is the number of pairs of data values used to compute each coefficient. The third number in each location of the table is a P-value which tests the statistical significance of the estimated correlations. P-values below 0.05 indicate statistically significant non-zero correlations at the 95.0% confidence level. The following pairs of variables have P-values below 0.05:

		Biomass-related parameters						EM-related parameters						Nutrient-related parameters			
		Height 09	Height 10	Biomass 09	Biomass 10	Root_ density _09	Root_ density _10	SWI _09	SWI _10	Hmax _09	Hmax _10	RTC _09	VI	K_leaf	N_leaf	P_leaf	K_root
Correlation	Height_10	0.9517															
(Sample Size)		8															
P-Value		0.0003															
Correlation	Biomass_09	0.9151	0.8955														
(Sample Size)		8	8														
P-Value		0.0014	0.0026														
Correlation	Biomass_10	0.921	0.915	0.9118													
(Sample Size)		8	8	8													
P-Value		0.0012	0.0014	0.0016													
Correlation	Root_density_09	0.5222	0.5919	0.2783	0.5394												
(Sample Size)		8	8	8	8												
P-Value		0.1843	0.1222	0.5046	0.1676												
Correlation	Root_density_10	0.4828	0.6067	0.296	0.429	0.6621											
(Sample Size)		8	8	8	8	8											
P-Value		0.2256	0.1108	0.4766	0.2888	0.0737											
Correlation	SWI_09	0.2163	0.2483	0.4119	0.1357	-0.1675	-0.4126										
(Sample Size)		8	8	8	8	8	8										
P-Value		0.6069	0.5533	0.3106	0.7486	0.6918	0.3097										
Correlation	SWI_10	0.5022	0.4551	0.6496	0.4251	-0.1619	-0.2375	0.7023									
(Sample Size)		8	8	8	8	8	8	8									
P-Value		0.2047	0.2572	0.0813	0.2938	0.7017	0.5711	0.0521									
Correlation	Hmax_09	0.8196	0.7916	0.8494	0.8692	0.2883	0.1265	0.3634	0.4293								
(Sample Size)		8	8	8	8	8	8	8	8								
P-Value		0.0128	0.0192	0.0076	0.0051	0.4886	0.7653	0.3762	0.2885								
Correlation	Hmax_10	0.3523	0.3015	0.5303	0.2454	-0.244	-0.1274	0.5471	0.8824	0.1119							
(Sample Size)		8	8	8	8	8	8	8	8	8							
P-Value		0.3921	0.468	0.1764	0.5581	0.5603	0.7636	0.1605	0.0037	0.792							

Table S3.7 continued

		Biomass-related parameters						EM-related parameters						Nutrient-related parameters			
		Height_09	Height_10	Biomass_09	Biomass_10	Root_density_09	Root_density_10	SWI_09	SWI_10	Hmax_09	Hmax_10	RTC_09	VI	K_leaf	N_leaf	P_leaf	K_root
Correlation	RTC_09	0.5666	0.4482	0.7211	0.6322	-0.121	-0.3469	0.4782	0.8251	0.6741	0.6197						
(Sample Size)		8	8	8	8	8	8	8	8	8	8						
P-Value		0.1431	0.2654	0.0435	0.0926	0.7752	0.3999	0.2307	0.0117	0.0668	0.1012						
Correlation	VI	0.7499	0.8104	0.6004	0.6414	0.6827	0.7444	0.0426	0.3952	0.3118	0.4122	0.1821					
(Sample Size)		8	8	8	8	8	8	8	8	8	8	8					
P-Value		0.0322	0.0147	0.1155	0.0865	0.0621	0.0342	0.9203	0.3326	0.4522	0.3102	0.666					
Correlation	K_leaf	0.9511	0.9477	0.8599	0.8509	0.4795	0.4323	0.3441	0.5777	0.824	0.3394	0.5513	0.7433				
(Sample Size)		8	8	8	8	8	8	8	8	8	8	8	8				
P-Value		0.0003	0.0003	0.0062	0.0074	0.2292	0.2847	0.4039	0.1337	0.0119	0.4108	0.1567	0.0346				
Correlation	N_leaf	0.542	0.6682	0.3373	0.3405	0.6433	0.7208	0.1855	0.092	0.2581	0.0232	-0.2268	0.7492	0.6461			
(Sample Size)		8	8	8	8	8	8	8	8	8	8	8	8	8			
P-Value		0.1652	0.0701	0.4139	0.4092	0.0853	0.0437	0.6601	0.8285	0.5371	0.9566	0.5891	0.0324	0.0835			
Correlation	P_leaf	0.7142	0.7448	0.541	0.4696	0.5389	0.7346	0.1345	0.3158	0.2644	0.3762	0.0094	0.8933	0.7164	0.868		
(Sample Size)		8	8	8	8	8	8	8	8	8	8	8	8	8	8		
P-Value		0.0466	0.034	0.1661	0.2404	0.1681	0.0379	0.7508	0.4461	0.5268	0.3583	0.9824	0.0028	0.0456	0.0052		
Correlation	K_root	0.4225	0.5258	0.308	0.3995	0.2023	0.5777	-0.2341	0.2283	0.2623	0.0966	0.1112	0.5873	0.5505	0.4609	0.4626	
(Sample Size)		8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	
P-Value		0.2971	0.1808	0.458	0.3268	0.6309	0.1337	0.5768	0.5866	0.5303	0.82	0.7931	0.1258	0.1574	0.2504	0.2484	
Correlation	N_root	0.445	0.2667	0.3002	0.3371	-0.049	-0.1876	0.0394	0.4992	0.4316	0.2182	0.6123	0.1849	0.5194	-0.0131	0.1242	0.4331
(Sample Size)		8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
P-Value		0.2693	0.5232	0.47	0.4142	0.9083	0.6563	0.9261	0.2079	0.2857	0.6036	0.1066	0.6612	0.1871	0.9754	0.7696	0.2838
Correlation	P_root	0.2764	0.3896	0.0867	0.2971	0.3112	0.5312	-0.3702	-0.0838	0.2533	-0.3128	-0.0841	0.3844	0.4285	0.4373	0.2669	0.9047
(Sample Size)		8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
P-Value		0.5075	0.3401	0.8382	0.4749	0.4531	0.1755	0.3667	0.8436	0.545	0.4506	0.843	0.3472	0.2895	0.2786	0.5228	0.002
Correlation	K_stem	-0.7774	-0.6964	-0.7729	-0.8413	-0.2844	-0.4388	-0.0195	-0.0391	-0.8203	-0.0359	-0.3423	-0.2959	-0.6007	-0.2476	-0.4004	-0.0422
(Sample Size)		7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
P-Value		0.0397	0.0822	0.0416	0.0177	0.5365	0.3247	0.9669	0.9336	0.0238	0.9391	0.4524	0.5194	0.1537	0.5925	0.3734	0.9285
Correlation	N_stem	-0.9855	-0.9488	-0.9658	-0.9524	-0.3669	-0.5386	-0.3221	-0.51	-0.8276	-0.4716	-0.5596	-0.7103	-0.9195	-0.5742	-0.7729	-0.3508
(Sample Size)		7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
P-Value		0	0.0011	0.0004	0.0009	0.4182	0.2123	0.481	0.2423	0.0216	0.2854	0.1915	0.0737	0.0034	0.1776	0.0416	0.4405

Table S3.7 continued

		Biomass-related parameters						EM-related parameters						Nutrient-related parameters			
		Height_09	Height_10	Biomass_09	Biomass_10	Root_density_09	Root_density_10	SWI_09	SWI_10	Hmax_09	Hmax_10	RTC_09	VI	K_leaf	N_leaf	P_leaf	K_root
Correlation	P_stem	-0.54137	-0.60367	-0.70857	-0.70017	-0.40267	-0.11977	-0.60247	-0.4297	-0.58957	-0.4027	-0.52177	-0.38347	-0.46687	-0.25387	-0.29437	0.24157
(Sample Size)																	
P-Value		0.2095	0.1512	0.0748	0.0799	0.3706	0.7982	0.1523	0.3368	0.1636	0.3713	0.2298	0.3958	0.291	0.5829	0.5218	0.6019

Table S7 continued

		Nutrient-related parameters			
		N-root	P-root	K-stem	N-stem
Correlation	P_root	0.399			
(Sample Size)		8			
P-Value		0.3274			
Correlation	K_stem	0.02767	0.02447		
(Sample Size)					
P-Value		0.9532	0.9585		
Correlation	N_stem	-0.21377	-0.11877	0.80417	
(Sample Size)					
P-Value		0.6455	0.7999	0.0293	
Correlation	P_stem	0.45997	0.39647	0.48687	0.60917
(Sample Size)					
P-Value		0.2991	0.3786	0.268	0.1466

Chapter 4

Paxillus involutus improves poplar nutrition in *Populus* × *canescens* under drought conditions

Paxillus involutus improves poplar nutrition in *Populus* × *canescens* under drought conditions

4.1 Introduction

The predicted global warming will lead to an increase in extreme climatic events like regional heatwaves (IPCC, 2007; Saxe *et al.*, 2001). The accompanying drought is one of the main factors limiting plant growth and productivity (Ciais *et al.*, 2005). When soil water becomes limited, the water movement from soil through the plant into the air decreases, which leads to reduced photosynthesis, and eventually will negatively affect biomass production (Bréda *et al.*, 2006). Under drought conditions the plant has to compromise to prevent water loss and afford CO_2 uptake for photosynthesis through stomatal regulation. The balance between CO_2/H_2O has also been described as water use efficiency (Jones *et al.*, 2004). An increasing soil water deficit is one of the main factors expected to influence tree productivity (Loustau *et al.*, 2005).

Poplars are commercially important species used in paper and pulp industry as well as a renewable source for bioethanol production (Polle & Douglas, 2010; Yuan *et al.*, 2008). Most poplar species are fast growing drought sensitive trees with large water requirements (Monclus *et al.*, 2006; Tschaplinski & Tuskan, 1994). Since their productivity is closely related to water availability (Tschaplinski *et al.*, 1998; Zhang *et al.*, 2004) poplars used in short rotation coppice for biomass production could suffer severely from global warming. Research on drought stress responses of poplars has focused on physiological responses, like changes in photosynthesis (Monclus *et al.*, 2006; Ni & Pallardy, 1991; Silim *et al.*, 2009; Yin *et al.*, 2006), gene expression and enzyme activity (Bogeat-Triboulot *et al.*, 2007) as well as osmotic adjustment (Luo *et al.*, 2009b). However, less is known about the role of ectomycorrhizal symbiosis in relation to water uptake and results gained thus far are controversial (Lehto & Zwiazek, 2011).

Mycorrhiza symbiosis is characterized by the exchange of nutrients between the host plant and root-colonizing fungi. While the fungi supply nutrients like N and P, the tree delivers photosynthates to the symbiont (Smith & Read, 2008). The benefits of this mutualism for the host plant with regard to nutrient acquisition have been shown in many studies (Smith & Read, 2008) but the mechanisms for improved drought resistance found in some experiments remain unclear (Lehto & Zwiazek, 2011). Increased stomatal conductance and shoot water potential was accompanied by enhanced photosynthesis and growth in mycorrhizal plants compared to non-mycorrhizal plants in a drought stress experiment

conducted by Lehto *et al.* (1992). One important finding was the enhanced expression of plasmamembrane intrinsic proteins (PIP) of the main branches PIP1 and PIP2 in inoculated poplars compared to non-inoculated plants (Marjanovic *et al.*, 2005b; Marjanovic *et al.*, 2005a). When water moves along the apoplastic pathway, it has to overcome the Casparian strip in the roots and thus, is forced to find its way through the plasma membrane. Aquaporins are membrane intrinsic proteins that build water channels through the membrane (Maurel *et al.*, 2008) and hence could regulate water flow into the xylem (Hacke *et al.*, 2010).

The ectomycorrhizal fungus *Paxillus involutus* is well known to establish symbioses with poplar species (Baum *et al.*, 2000; Gafur *et al.*, 2004; Langenfeld-Heyser *et al.*, 2007). This fungus is classified according to its extraradical mycelium as a long distance exploration type (Agerer, 2001). This type of mycorrhiza builds highly differentiated rhizomorphs that grow into soil areas far behind the nutrient depletion zone of plant roots (Bending & Read, 1995; Carleton & Read, 1991) and thus, could transport water and nutrients over long distances to the host plants, which is advantageously when water becomes a limiting factor.

The objectives of this study were to elucidate (1) if the ECM fungi *P. involutus* on *Populus* × *canescens* roots ameliorated the physiological responses to water limitation in the host and (2) if the mycorrhiza improved the nutrition status of *P.* × *canescens* under this drought stress condition and (3) if nutrient x drought interactions were related to the extent of mycorrhization. To investigate these questions a controlled drought stress experiment with slowly decreasing water availability was conducted using non-mycorrhizal and mycorrhizal *P.* × *canescens* plants.

4.2 Material & Methods

4.2.1 Plant material and cultivation of fungi

Plantlets of *Populus* x *canescens* (*P. alba* x *P. tremula*, INRA clone 717-1B4) were multiplied by micropropagation according to Leplé *et al.* (1992). The plantlets were transferred to aerated hydroponic Long Ashton solution with low nitrogen (LN solution) [modified after Matzner *et al.* (1982): 300 μM NH_4NO_3, 200 μM K_2SO_4, 130 μM $CaSO_4$, 100 μM Na_2SO_4, 60 μM $MgSO_4$, 30 μM KH_2PO_4, 10 μM $MnSO_4$, 7.8 μM Fe-ethylene diamine-di(o-hydroxyphenylacetate), 5 μM H_3BO_3, 0.1 μM $NaMoO_4$, 0.1 μM $ZnSo_4$, 0.1 μM $CuSO_4$, pH 3.9] and acclimatized to ambient conditions. After acclimatization for 14 days in an air-conditioned growth room (16 h light / 8 h dark rhythm, 150 μmol photons $m^{-2}s^{-1}$ of photosynthetic active radiation, 50-60% relative air humidity, 21°C) the plants were planted into growth tubes with

and without fungal inoculum and transferred into a climate chamber with similar conditions as described above, except that the air humidity and temperature were kept constant at 60% and 20°C, respectively.

The ectomycorrhizal fungus *Paxillus involutus* (Bartsch.) (strain MAJ, stock collection Göttingen) was used as inoculum. The fungus was cultivated on a sand peat mixture on top of a cellophane layer covering a 2% modified Melin-Norkans medium (MMN) [10 g glucose, 0.5 g KH_2PO_4, 0.25 g $(NH_4)_2SO_4$, 0.15 g $MgSO_4$ x $7H_2O$, 0.05 $CaCl_2$, 0.025 g NaCl, 100 μg thiamine x HCL, 0.01 g $FeCl_3$, 5 g malt extract per 1L, pH 5.2] at 25°C in the dark. After two weeks of cultivation the temperature was decreased to 22°C to retard growth.

4.2.2 Mycorrhizal inoculation and plant growth conditions

The soil mixture consisted of a sand peat mixture with two parts peat, eight parts fine sand (grain size: 0.4-0.8 mm), and 10 parts coarse sand (grain size: 0.7-1.3 mm). Prior to use the sand was washed three times with demineralized water, dried, mixed with peat, and sterilized by autoclaving two times at 120°C for 20 min. *P.* × *canescens* clones were planted into growth tubes of 5 cm in diameter and 41 cm in length, containing a nylon mesh at the bottom to prevent leaking of soil. Six hundred sixty milliliters of inoculated or non-inoculated soil mixture was used per growth tube. The inoculated soil mixture was prepared by mixing the non-inoculated soil mixture with mycelia of *P. involutus.* In total 105 *P. involutus* culture plates were used. Mycelia of *P. involutus* cultivated plates were carefully scratched off the cellophane layer and transferred into a big Petri dish and mixed vigorously with a spoon. Each Petri dish contained mycelia of 15 fungal plates. The mixed mycelia of those Petri dishes were transferred into a bucket and again vigorously mixed with a spoon to gain a homogenous fungal inoculum. One hundred g fungal inoculum was used for inoculation and mixed with 660 mL of sterile soil mixture.

To record the volumetric soil water content five growth tubes of each treatment were equipped with soil moisture sensors (ECH_2O Check, Decagon Devices, USA). After the plantlets had been transferred into the soil mixture they were immediately watered to field capacity and placed under a transparent plastic bag for protection against evaporation. The plastic bag was lifted daily a few centimeters to allow a slow acclimatization. Until drought stress treatment the plants were automatically irrigated three times per day with 10 mL LN-nutrient solution per growth tube.

4.2.3 Drought stress and harvest time points

Half of the mycorrhizal and non-mycorrhizal plants were exposed to drought by stepwise reduction of LN-nutrient solution. Three reduction steps were used, first from 3 x 10 mL to 3 x 5 mL (start), second from 3 x 5 mL to 3 x 3.5 mL (day 9), and finally from 3 x 3.5 mL to 3 x 2.5 mL (day 18). Control plants were irrigated with 3 x 10 mL a day. Time points of reduction steps and harvests were determined by considering predawn leaf water potential and volumetric soil water content (SWC). The first harvest occurred at day 8 when SWC of the drought stressed plants was decreased by 50% of the SWC of control plants (mild drought stress). The second harvest was conducted at day 14 (medium drought stress), when additionally to the SWC also the predawn leaf water content was significantly decreased in drought stressed plants compared to control plants. Since the predawn leaf water potential of drought stressed plants recovered to the level of control plants (day 18), irrigation was further reduced to provoke severe drought stress. At day 20 the last harvest was carried out when predawn leaf water potential had strongly decreased.

4.2.4 Physiological measurements

Height was measured over the entire term of the drought stress treatment once a week.

The predawn leaf water potential was measured with a Scholander pressure chamber. Two hours before the lights were switched on leaves of five trees per treatment from overnight dark adapted plants were cut off at the petiole with a razor blade and clamped into the pressure chamber. The pressure was slowly elevated until water appeared on the cut surface of the leaf petiole and recorded as leaf water potential (Scholander *et al.*, 1965).

The photochemical quantum yield of photosystem II (PSII) was measured on illuminated plants in the climate chamber light environment using a portable chlorophyll fluorometer (MINI-PAM, Walz, Effeltrich, Germany) based on pulse-amplitude-modulation (PAM). Fluorescence was measured briefly before and during the saturation pulse. Thus, yield of fluorescence was calculated as: yield = (maximal chlorophyll fluorescence – steady state chlorophyll fluorescence) / maximal chlorophyll fluorescence (Maxwell & Johnson, 2000).

Stomatal conductance was measured on light adapted plants with a portable porometer (AP4 Porometer, Delta-T Devices Ltd, Cambridge, England) at ambient light, temperature and air humidity according to the manufacturers' instructions.

Measurements of predawn leaf water potential, chlorophyll fluorescence, and stomatal conductance were conducted every second day during the time course of the drought stress experiment.

4.2.5 Harvest

Plants were harvested after 8, 14, and 20 days of drought stress. The height of 12 trees per treatment was recorded before the plants were dissected into leaves, stem, and roots. For biochemical and molecular analysis subsamples of leaves and roots were immediately frozen in liquid nitrogen. Additional subsamples were collected for leaf area, biomass determination and element analysis and dried in an oven at 60°C for one week. For the investigation of mycorrhizal colonization subsamples of fine roots were taken from the upper part of the root system (upper 20 cm) and stored in wet tissues at 4°C.The frozen material was stored at -80°C until use. Relative water content (RWC) of tissues, here defined as the actual water content at harvest time, was calculated as follows: RWC = 100 - (dry weight * 100 / fresh weight). Relative growth rate was calculated as: rel. growth = ($height_{end}$ – $height_{start}$) / (time [d] * $height_{start}$). Start was the first day of the drought stress experiment and end was the harvest day of plants. Soil samples were taken from each plant to determine the gravimetric soil water content (SWC) which was calculated as: SWC = 100- (dry weight * 100 / fresh weight).

4.2.6 Determination of mycorrhizal colonization of root tips

Mycorrhizal root tips were determined on 12 samples per treatment. For this purpose 500 root tips from at least three different fine roots were counted under a stereo microscope (M205 FA, Leica, Wetzlar, Germany) and the number of mycorrhizal, non-mycorrhizal, and dead root tips were recorded. Mycorrhizal colonization was calculated as follows: number of mycorrhizal root tips x 100 / number of living root tips. Vitality index was calculated as: number of living root tips x 100 / total number of counted root rips per sample. On roots of six non-inoculated plants ectomycorrhizae establishment was detected. These plants were excluded from further analysis.

4.2.7 Quantitative Real Time PCR (qRT-PCR)

Leaf material of 10 plants per treatment was used for quantitative real time PCR. Tissues were ground in pre-cooled cups in a ball mill (Retsch, Haan, Germany). Five hundred mg of

frozen ground material was used for total RNA extraction according to Chang *et al.* (1993) with slight modifications: 2% β-mercaptoethanol was used in the extraction buffer and no spermidine was applied. To check the integrity of the RNA, electrophoresis was performed loading 0.5 – 1 µg RNA (depending on the RNA concentration) on an RNA denaturing agarose gel.

Before cDNA synthesis total RNA was DNase treated to remove remaining DNA. For this purpose the DNA-free Kit "Turbo DNA-freeTM" (Ambion Inc., Austin, Texas, USA) was used according to the manufacturer's instruction. The total RNA concentration and purity were measured spectrophotometrically (BioPhotometer, Eppendorf, Hamburg, Germany) at A_{260} and $A_{280.}$ The First Strand cDNA Synthesis Kit (Fermentas, St.-Leon-Rot, Deutschland) was used for cDNA synthesis. 1 µg of RNA was transcribed into cDNA according to the manufacturer's instruction.

For primer design RD26, Bet v I, and GLP3 gene sequences from *Arabidopsis* (AT4G27410, AT1G24020 and AT5G20630, respectively) were compared against the *P. trichocarpa* genome using BLAST search of Phytozome (http://www.phytozome.net/search.php). The best hit sequences were than compared against the NCBI (http://blast.ncbi.nlm.nih.gov) expressed sequence tags (EST) database for Poplar. In case of gene PIP2.5 poplar gene model was known (POPTR_0006s12980) and compared against NCBI EST database. If no hit was observed for *P.* x *canescens* several poplar species were selected and aligned with GeneDoc software Version 2.6.002 (http://www.psc.edu/biomed/genedoc) to look for conserved regions. Primer design was conducted with Oligo Explorer (Gene Link, Hawthorne, NY) followed by testing for primer dimers, primer loops and similar melting temperature (Tm) using Oligo Analyser (Gene Link, Hawthorne, NY). The primer sequences are shown in Table S4.1.

Primer efficiency was tested by running serial dilutions of template cDNA for each primer pair followed by a melting curve in a LightCycler® 480 (Roche, Grenzach-Whylen, Deutschland) with the same cycling conditions as in qRT-PCR (see below). Efficiencies were calculated with LightCycler® 480 Software release 1.15.0, version 1.5.0.39 (Roche, Grenzach-Whylen, Deutschland).

Before running the PCR the cDNA was diluted 1:10. Reaction volume consisted of 20 µL containing 5 µl cDNA, 10 µL 2x Mastermix (LightCycler® 480 SYBR Green I Mastermix, Roche, Grenzach-Whylen, Deutschland), 2 µL of each primer (10 µM), and 3 µL nuclease free water. The qRT-PCR was performed in a LightCycler® 480 (Roche, Grenzach-Whylen, Deutschland) with 10 samples per treatment and two technical replicates. The initial denaturing step of 95°C for 5 min was followed by 45 cycles with the following conditions:

95°C for 10 sec (denaturing), 58°C for 10 sec (annealing), 72°C for 20 sec (elongation). After amplification a melting curve was performed with a continuously increasing temperature (4.4 °C s^{-1}) from 65°C to 95°C. Changes in expression of actin9 and ß-Tubulin were tested prior the RT-PCR run of all samples with 3 samples of each treatment and two technical replicates. No changes were found for both housekeeping genes. Actin9 was used as housekeeping gene for further analysis. For relative expression of genes the following equations were used:

(1) relative expression of target gene of each sample compared to reference gene of the same sample:

$$\text{Relative expression} = E_{ref}^{(Cp\ \text{reference gene})} / E_{tag}^{(Cp\ \text{target gene})}$$

With E_{ref} representing the efficiency of the primer for the reference gene, E_{tag} is the efficiency of the primer for the target gene, and Cp is the threshold cycle.

(2) relative expression ratio of target gene

$$\text{ratio} = E_{tag}^{(\Delta Cp\ \text{taget (control} - \text{sample))}} / E_{ref}^{(\Delta Cp\ \text{reference (control} - \text{sample))}}$$

Significant differences between control and mycorrhizal/drought stressed samples were calculated with the Relative Expression Software Tool (REST)-384 beta (Pfaffl, 2001; Pfaffl *et al.*, 2002).

4.2.8 Element analysis

For element analysis dried leaf and root material was ground in a ball mill (Retsch, Haan, Germany). Fifty mg was extracted in HNO_3 according to Heinrichs *et al.* (1986) followed by element analysis via inductively coupled plasma-optical emission spectrometry (ICP-OES; Spectroflame, Spectro Analytical Instruments, Kleve, Germany). For determination of carbon and nitrogen concentrations 800 µg ground plant material was weighed into tin capsules and analyzed with an element analyzer (Elemental Analyzer EA-1108, Carlo, Erba Instruments, Rodano, Italy). Acetanilid (C_8H_9NO, HEKAtech GmbH, Wegberg, Germany) was used as standard.

4.2.9 Carbohydrate analysis

Glucose, fructose, sucrose, and starch concentrations were determined spectrophotometrically in leaf and root material after enzymatic conversion (Schopfer, 1989). Frozen material was ground in precooled cups of a ball mill (Retsch, Haan, Germany) and 75 mg tissue was used for analysis. Ten plants per treatment were analyzed. Carbohydrates were extracted in 1.5 mL DMSO/HCl (dimethylsulfoxide: 25% HCl = 80:20 (v:v)) at 60°C for 30 min. Samples were cooled on ice and centrifuged for 5 min at 4°C and 5000 rpm (Centrifuge 5417R, Eppendorf, Hamburg). The supernatant was used for determination of carbohydrates. In the first step the absorbance of NADPH is measured after adding the enzyme hexokinase which catalyses the conversion of glucose to gluconat-6-phosphate. The amount of NADPH generated in this step is equivalent to the amount of converted glucose. Hexokinase also catalyzes the conversion of fructose to fructose-6-phosphate. In the second step, to determine the content of fructose, the enzyme phosphoglucose isomerase was added which converts fructose-6-phosphate to glucose-6-phosphate, which is further converted to gluconat-6-phosphate. The generated NADPH is measured which is here equivalent to the amount of fructose in the supernatant. In the last step sucrose was determined by adding the enzyme β-fructosidase which hydrolyses the sucrose to glucose and fructose. Glucose and fructose are converted to gluconat-6-phosphate and the absorption of generated NADPH is measured. For determination of starch α-amyloglucosidase was added to the supernatant which catalyses the degradation of starch to glucose. NADPH was than measured as glucose equivalents. This method was conducted as described in detail in Luo *et al.* (2006). For correlations with osmolality the concentrations of sugars were transformed into mol as follows: sugar concentration [mg g^{-1} FW] / molecular weight 180.16 [mol g^{-1}].

4.2.10 Osmolyte content

Osmolytes in leaves and roots were analyzed in 10 biological replicates per treatment. Dried material was ground in a ball mill (Retsch, Haan, Germany). 250 µL H_2O was added to 25 mg of leaf and 500 µL to 100 mg of root sample, respectively and incubated at 55°C overnight. After centrifuging for 25 min at 1000 rpm, 50 µL of the supernatant was measured in a cryoscopic osmometer (Osmomat 030, Gonotec, Berlin, Germany). To determine the osmolyte concentration in fresh weight, values were back calculated using the RWC of tissues determined after harvest. The following equation was used: osmol kg^{-1} FW = osmol kg^{-1} DW * dilution / ((tissue FW * sample DW / tissue DW) – sample DW). Osmol kg^{-1} DW was obtained by measurements. Tissue FW and DW were determined after harvest and

sample DW was the weighted sample used for osmolyte determination. Dilution was the amount of H_2O added to the dried material.

4.2.11 Statistical analysis

Statistical analyses were conducted using R statistics software version R-2.9.2 (R Development Core Team, 2009). Unless otherwise stated the package: "stats" (R Development Core Team, 2009) was used for analysis. Differences between treatments in mycorrhizal colonization and vitality index were analyzed by non-parametric tests using Kruskal-Wallis rank sum test followed by Mann-Whitney U-test. Repeated measurement ANOVAs were conducted by function gls() in package: "nlme" (Pinheiro *et al.*, 2009). Linear models were fitted by generalized least squares accounting for correlated and/or unequal variances if necessary. Different models were tested and the best model was chosen according to the lowest value of Akaike's Information Criterion (AIC). Normal distributions and variance homogeneity were inspected visually. Multifactorial analysis of variance was conducted to test differences between treatments (mycorrhizal inoculation and drought) over time using function aov(). Two way analysis of variance (ANOVA) with interaction term was used to analyze the data sets with factors drought and mycorrhiza using function aov(). Normal distribution and homogeneity of variances of residuals were analyzed by Kolmogorov-Smirnov test and Levene's Test, functions ks.test() and leveneTest(), package: "car" (Fox & Weisberg, 2011). Analysis of covariance (ANCOVA) was conducted with the continuous variable mycorrhizal colonization and the factor drought and the interaction term of both. For visualizing the result of the ANCOVA, regression lines were fitted to the data based on the estimated coefficients of the ANCOVA. If the p-value of the interaction term was > 0.05 regression lines were calculated based on an additive model. Regression analyses of osmolality of cations and carbohydrates against total osmolality was conducted using a linear model with function lm(). $P \leq 0.05$ was considered to indicate significant effects of factors/covariable on the response variable.

4.3 Results

4.3.1 Effect of drought and mycorrhiza on soil water content and physiological parameters

After reducing the irrigation from 10 mL (control status) to 5 mL in the drought stress treatment, the volumetric soil water content (SWC) decreased within five days from about 6% (control status) to 1.5% and rose again to 3% after 8 days (Fig. 4.1). With decreasing water supply from 5 mL to 3.5 and finally 2.5 mL per day, the volumetric SWC decreased steadily until it was no longer detectable by the soil moisture sensors (Fig. 4.1).

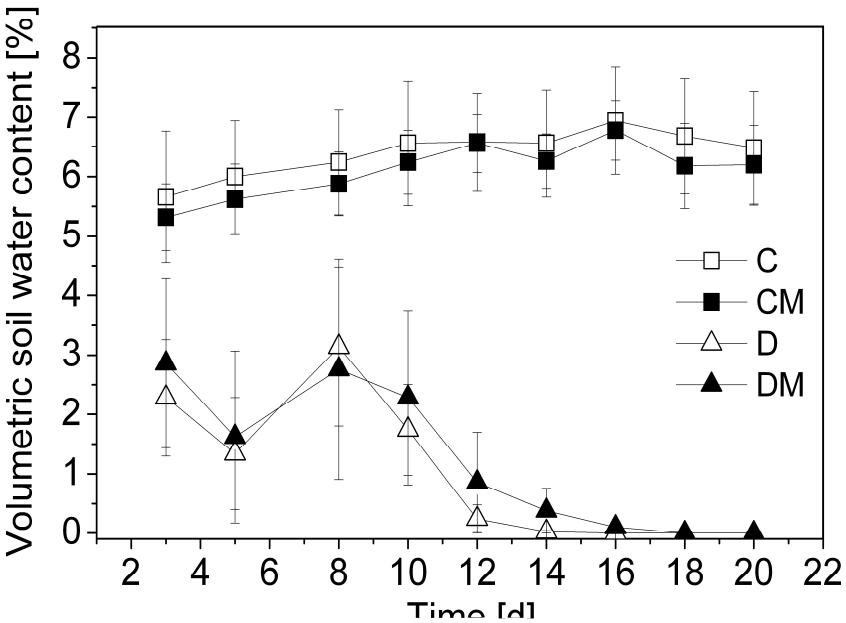


Fig. 4.1: Volumetric soil water content of different treatments measured continuously over 20 day of drought treatment. C: control plants (open square), CM: control plants with mycorrhiza (filled square), D: drought stressed plants (open triangle), DM: drought stressed plants with mycorrhiza (filled triangle). Values represent mean ± SE. (n = 5)

Repeated measures ANOVA revealed a significant effect of drought as single factor ($F = 74.41$, $p < 0.001$) and of the interaction term of drought x time ($F = 6.42$, $p < 0.001$) while no significant effect of mycorrhiza, neither alone nor in combination with drought or time, was found for volumetric SWC (Tab. 4.1).

Tab. 4.1: ANOVA table of probabilities for physiological variables and volumetric soil water content measured continuously over 20 day of drought treatment. Soil water content: SWC (n =5), predawn leaf water potential: Ψ_{pd} (n = 5), stomatal conductance: g_s (n = 6-8), quantum yield of photosystem II: Φ (n = 6-8). Factors used are D: drought, M: mycorrhiza, T: time. Interactions of these factors are indicated by "x". Significant factors are highlighted in bold lettering.

Factor	Response variable							
	SWC		Ψ_{pd}		g_s		Φ	
	F	p	F	p	F	p	F	p
D	74.41	**<0.001**	67.87	**<0.001**	94.00	**<0.001**	0.1	0.743
M	0.24	0.624	4.84	**0.030**	2.68	0.103	4.7	**0.032**
T	3.63	**<0.001**	23.31	**<0.001**	57.74	**<0.001**	2.7	**0.007**
D x M	1.66	0.684	0.48	0.488	2.27	0.133	1.3	0.264
D x T	6.42	**<0.001**	21.11	**<0.001**	11.24	**0.001**	2.6	**0.010**
M x T	0.84	0.569	3.03	**0.004**	1.16	0.323	3.8	**<0.001**
D x M x T	0.43	0.899	1.44	0.184	2.19	**0.029**	0.7	0.701

Predawn leaf water potentials (Ψ_{pd}) were measured to monitor the water status of the plants as an indicator of drought stress. The Ψ_{pd} of non-mycorrhizal drought stressed plants decreased immediately after the second reduction of irrigation at day nine while mycorrhizal drought stressed plants responded four days later. Both drought stressed treatments recovered to control levels after 18 days of water limitation (Fig. 4.2).

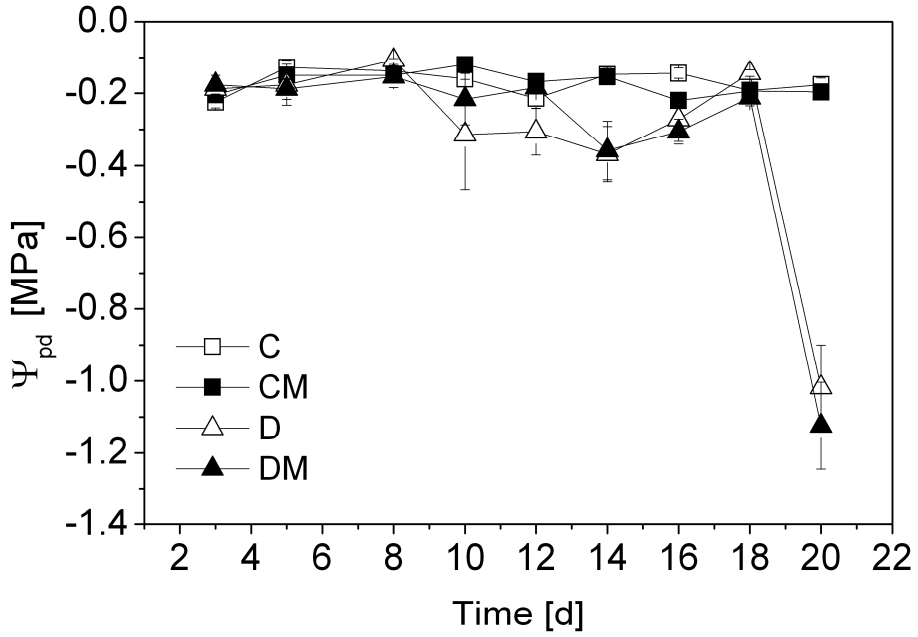


Fig. 4.2: Predawn leaf water potential of *P. × canescens* plants in response to increasing drought stress. C: control plants (open square), CM: control plants with mycorrhiza (filled square), D: drought stressed plants (open triangle), DM: drought stressed plants with mycorrhiza (filled triangle). Values represent mean ± SE. (n = 5)

After the third reduction of irrigation (day 18, 3 x 2.5 mL per day) the Ψ_{pd} of water limited plants strongly and rapidly decreased to -1.12 ± 0.12 MPa and -1.02 ± 0.12 MPa in mycorrhizal and non-mycorrhizal drought stress plants, respectively (Fig. 4.2). Statistical analysis showed no clear order of all treatments over 20 days of experimental treatment, indicated by significant results of all factors (drought, mycorrhiza and time) together with significant interaction terms for D x T and M x T (Tab. 4.1).

The response of the quantum yield of photosystem II efficiency to increasing drought stress was determined during the course of the experiment (Fig. 4.3). Most of the time mycorrhizal plants showed higher values of PSII efficiency compared to non-mycorrhizal plants ($F = 4.7$, $p = 0.032$). The effect of drought was apparent in combination with time ($F = 2.6$, $p = 0.010$), reflected in decreasing values in drought stressed plants at the end of the experiment (Fig. 4.3, Tab. 4.1).

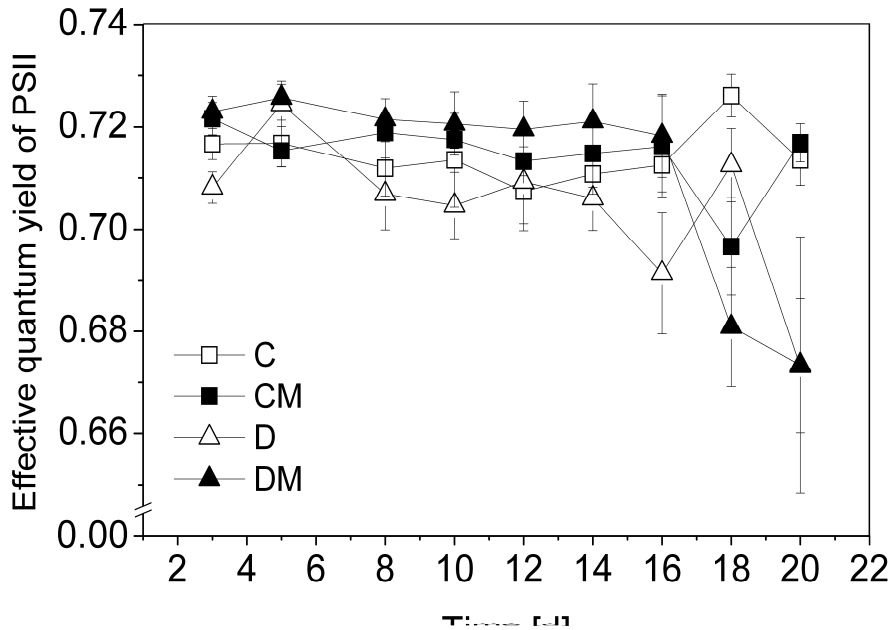


Fig. 4.3: Yield of photosystem II efficiency of *P. × canescens* plants in response to increasing drought stress. C: control plants (open square), CM: control plants with mycorrhiza (filled square), D: drought stressed plants (open triangle), DM: drought stressed plants with mycorrhiza (filled triangle). Values represent mean ± SE. (n = 6 -8)

Stomatal conductance of mycorrhizal and non-mycorrhizal control plants showed a similar decline over time ($F = 1.16$, $p = 0.323$, Tab. 4.1, Fig. 4.4). Drought strongly affected mycorrhizal and non-mycorrhizal drought stressed plants ($F = 11.24$, $p = 0.001$, Tab. 4.1), reflected in a strong reduction of stomatal conductance over time (Fig. 4.4). The effect of

mycorrhiza was apparent in combination with drought and time ($F = 2.19$, $p = 0.029$, Tab. 4.1) indicating no clear separation of mycorrhizal and non-mycorrhizal drought stressed plants over time.

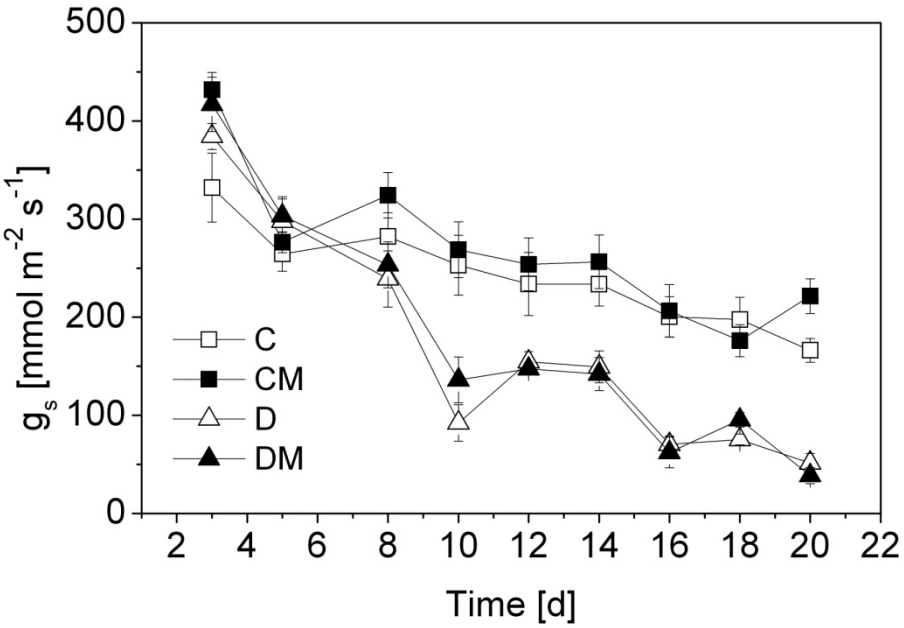


Fig. 4.4: Stomatal conductance of *P. × canescens* in response to increasing drought stress. C: control plants (open square), CM: control plants with mycorrhiza (filled square), D: drought stressed plants (open triangle), DM: drought stressed plants with mycorrhiza (filled triangle). Values represent mean ± SE. (n = 6-8)

4.3.2 Mycorrhizal colonization and vitality index

Mycorrhizal colonization of root tips of *P. × canescens* plants inoculated with *Paxillus involutus* was not affected by drought (Fig. 4.5a-c). Mean colonization of root tips was 47.7 ± 1.6% (Fig. 4.5a-c).

The vitality index of root tips was significantly reduced in response to drought (Fig. 4.5d-f). Differences between mycorrhizal and non-mycorrhizal drought stressed plants occurred after eight and 14 days of drought treatment, with non-mycorrhizal plants showing lower vitality of root tips (Fig. 4.5). Significant differences within treatments over time were only found for mycorrhizal drought stressed plants which showed lower vitality in the last harvest compared to the first and second harvest ($\chi^2 = 12.21$, $p = 0.002$).

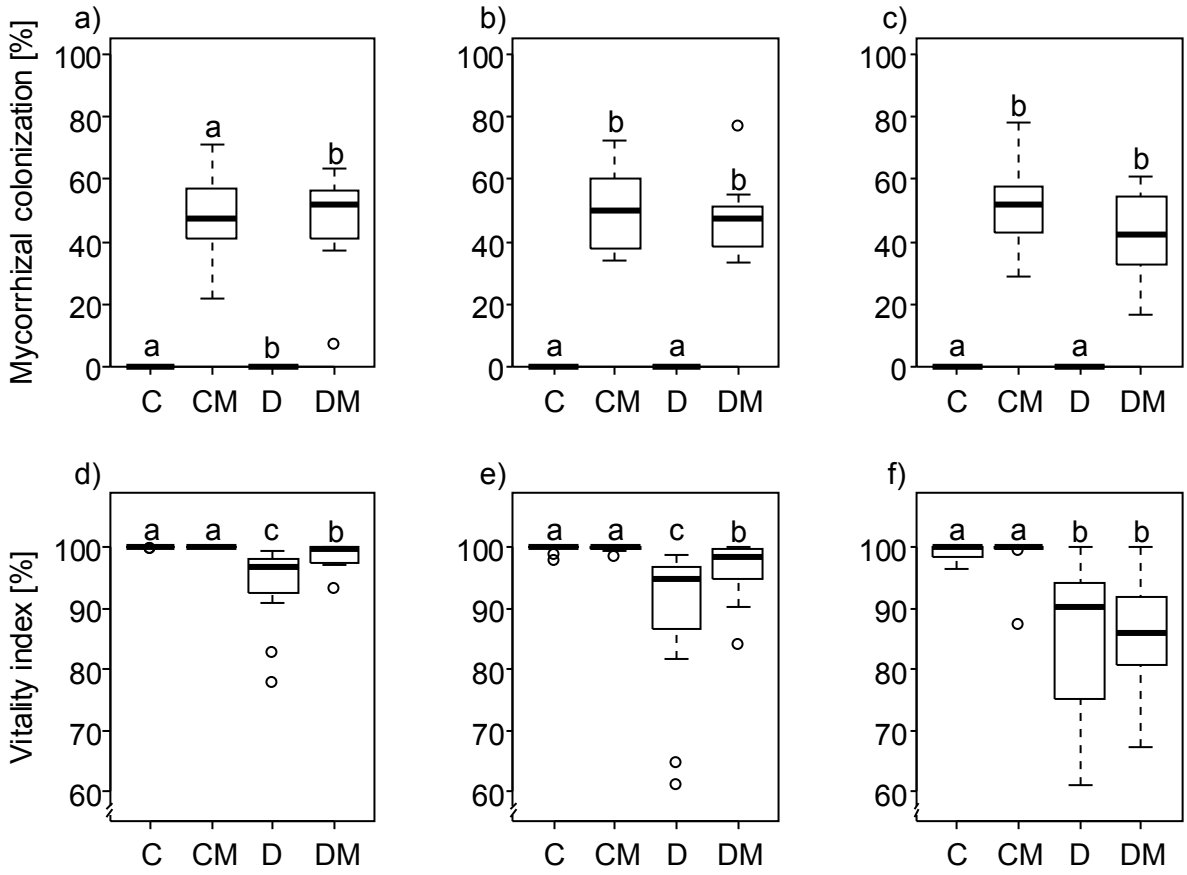


Fig. 4.5: Mycorrhizal colonization and vitality index of *P. × canescens* root tips after 8 (a, d), 14 (b, e) and 20 (c, e) days of drought treatment. C: control plants, CM: control plants with mycorrhiza, D: drought stressed plants, DM: drought stressed plants with mycorrhiza. Boxplots represent the median (black line) and interquartile length (box) of the data, upper and lower whiskers indicate maximum and minimum value and outliers are represented by single dots. Significant differences are indicated by small letters ($p \leq 0.05$). (n = 10-12)

4.3.3 Effect of drought and mycorrhiza on growth performance

After eight days of drought treatment mycorrhizal plants were smaller than non-mycorrhizal plants (Fig. 4.6a). While this effect of mycorrhiza was consistent over time for control plants the effect of mycorrhiza diminished in drought stress plants after 20 days of treatment (Fig. 4.6a). The response of poplars to mycorrhiza and drought over time was reflected in significant values of the interaction term of D x M and D x T ($F = 4.42$, $p = 0.037$ and $F = 6.01$, $p = 0.003$, respectively, Tab. 4.2).

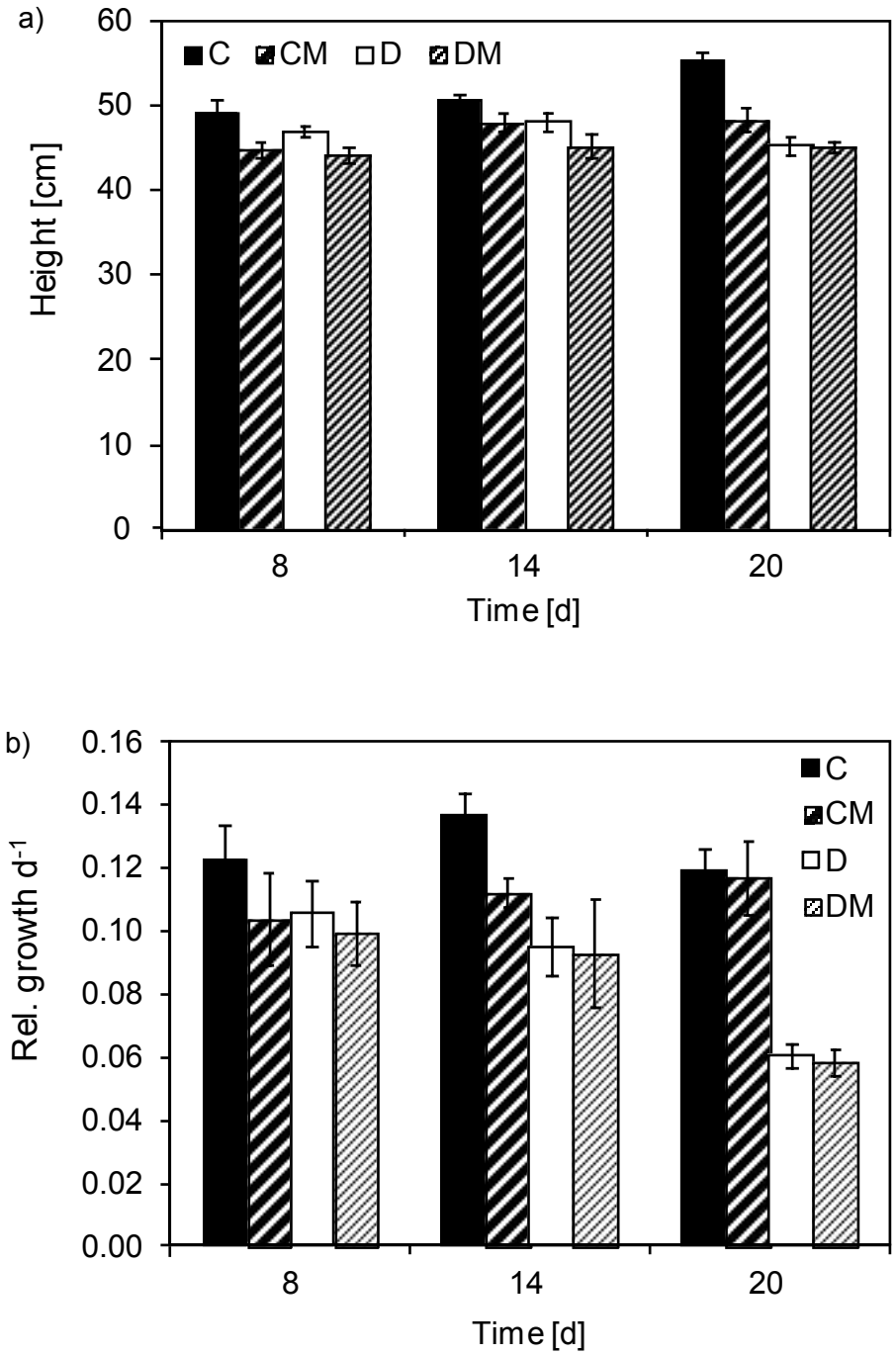


Fig. 4.6: Height and relative growth of *P. × canescens* determined after 8, 14 and 20 days of drought treatment. a) height, b) rel. growth. C: control plants (black), CM: control plants with mycorrhiza (hatched), D: drought stressed plants (white), DM: drought stressed plants with mycorrhiza (narrow hatched). Values represent mean ± SE. (n =10-12)

Relative growth of poplars was affected by drought, time and the interaction of drought x time with drought stressed plants showing continuously decreasing values of relative growth over time (Fig. 4.6b, Tab. 4.2).

Tab. 4.2: ANOVA Table of height and relative growth of *P. × canescens* measured at three harvest time points. D: drought, M: mycorrhiza, T: time. Interactions of factors are indicated by "x". F and p-values are given ($p \leq 0.05$). Significant factors are highlighted in bold lettering. (n = 10-12)

	Rel. growth d^{-1}		Height [cm]	
	F	p	F	p
D	29.67	**< 0.001**	28.60	**< 0.001**
M	2.39	0.125	26.27	**< 0.001**
T	4.76	**0.010**	4.40	**0.014**
D x M	0.92	0.340	4.42	**0.037**
D x T	5.49	**0.005**	6.01	**0.003**
M x T	0.34	0.713	0.18	0.837
D x M x T	0.26	0.771	2.91	0.058

The total biomass of mycorrhizal and non-mycorrhizal control plants was generally higher than that of mycorrhizal and non-mycorrhizal drought stressed plants ($F = 7.33$, $p = 0.007$, Tab. 4.3). Biomass of leaves was affected by drought ($F = 14.08$, $p < 0.001$) and mycorrhiza ($F = 37.37$, $p < 0.001$). No effect was found for time or interactions of factors (Tab. 4.3). The stem biomass at day eight was higher in non-mycorrhizal plants than mycorrhizal plants, but to a lesser extent in drought stressed plants reflected in a significant interaction term of D x M ($F = 4.01$, $p = 0.048$). After 14 days a contrasting result was found with a more pronounced reduction in stem biomass for mycorrhizal drought stressed plants compared to non-mycorrhizal drought stressed plants. After 20 days only the stem biomass of control plants differed significantly from all other treatments. These different effects of factors over time on the stem biomass data are reflected by the significant interaction term of D x M x T ($F = 3.62$, $p = 0.030$, Tab. 4.3). Results of root biomass showed a significant effect of drought and time ($F = 7.39$, $p = 0.008$ and $F = 3.83$, $p = 0.024$, Tab. 4.3) with higher values of control plants compared to drought stressed plants. Total biomass was affected by drought as single factor and showed significantly higher values in control treatments ($F = 7.33$, $p = 0.007$).

Tab. 4.3: Biomass data of plants harvested after eight, 14 and 20 days of drought treatment. C: Control plants, CM: control plants with mycorrhiza, D: drought stressed plants, DM: drought stressed plants with mycorrhiza. Table of 3-factorial ANOVA with interaction terms is given with F- and p-values ($p \leq 0.05$). Significant factors or interactions of factors are highlighted in bold lettering. Factors used are D: drought, M: mycorrhiza, T: time. Interactions of these factors are indicated by "x". (n=10-12)

	Biomass [g DW]			ANOVA Table			
	day 8	day 14	day 20	Factor	DF	F	p
Leaves							
C	2.14 ± 0.13	2.03 ± 0.08	2.35 ± 0.14	D	1	14.08	**<0.001**
CM	1.68 ± 0.13	1.82 ± 0.10	1.89 ± 0.13	M	1	37.37	**<0.001**
D	1.87 ± 0.06	2.07 ± 0.08	1.84 ± 0.10	T	2	1.07	0.346
DM	1.60 ± 0.07	1.61 ± 0.07	1.62 ± 0.05	D x M	1	0.34	0.562
				D x T	2	2.29	0.106
				M x T	2	0.02	0.980
				D x M x T	2	1.76	0.177
Stem							
C	1.10 ± 0.08	1.01 ± 0.04	1.38 ± 0.08	D	1	13.39	**<0.001**
CM	0.85 ± 0.07	0.94 ± 0.04	1.03 ± 0.08	M	1	14.70	**<0.001**
D	0.89 ± 0.03	1.08 ± 0.07	0.99 ± 0.06	T	2	10.77	**<0.001**
DM	0.77 ± 0.04	0.87 ± 0.05	1.01 ± 0.03	D x M	1	4.01	**0.048**
				D x T	2	1.27	0.286
				M x T	2	0.02	0.984
				D x M x T	2	3.62	**0.030**
Root							
C	10.01±1.82	6.59 ± 0.84	7.11 ± 0.95	D	1	7.39	**0.008**
CM	8.43 ± 1.19	8.73 ± 1.19	6.76 ± 0.96	M	1	0.00	0.983
D	7.11 ± 0.66	7.17 ± 0.98	5.18 ± 0.54	T	2	3.83	**0.024**
DM	6.68 ± 0.71	7.11 ± 0.62	6.15 ± 0.42	D x M	1	0.27	0.604
				D x T	2	0.34	0.714
				M x T	2	0.67	0.512
				D x M x T	2	0.30	0.740
Total							
C	13.25 ± 1.97	11.10 ± 1.73	10.71 ± 1.03	D	1	7.33	**0.007**
CM	10.95 ± 1.32	11.54 ± 1.26	9.67 ± 1.10	M	1	0.76	0.385
D	9.80 ± 0.70	10.31 ± 1.08	8.01 ± 0.61	T	2	1.85	0.160
DM	9.04 ± 0.74	9.60 ± 0.71	8.78 ± 0.43	D x M	1	0.31	0.577
				D x T	2	0.18	0.833
				M x T	2	0.48	0.620
				D x M x T	2	0.57	0.567

4.3.4 Effects of drought and mycorrhiza on water status

After eight and 14 days of drought treatment, a positive effect of mycorrhizal colonization on gravimetrically determined SWC in control and drought stressed plants was found by ANCOVA, in which drought treated plants showed significant lower values of SWC (Fig. 4.7a-b, Tab. S4.2). The effect of mycorrhizal colonization decreased after eight days and was no more detectable after 20 days of drought treatment (Fig. 4.7c, Tab. S4.2).

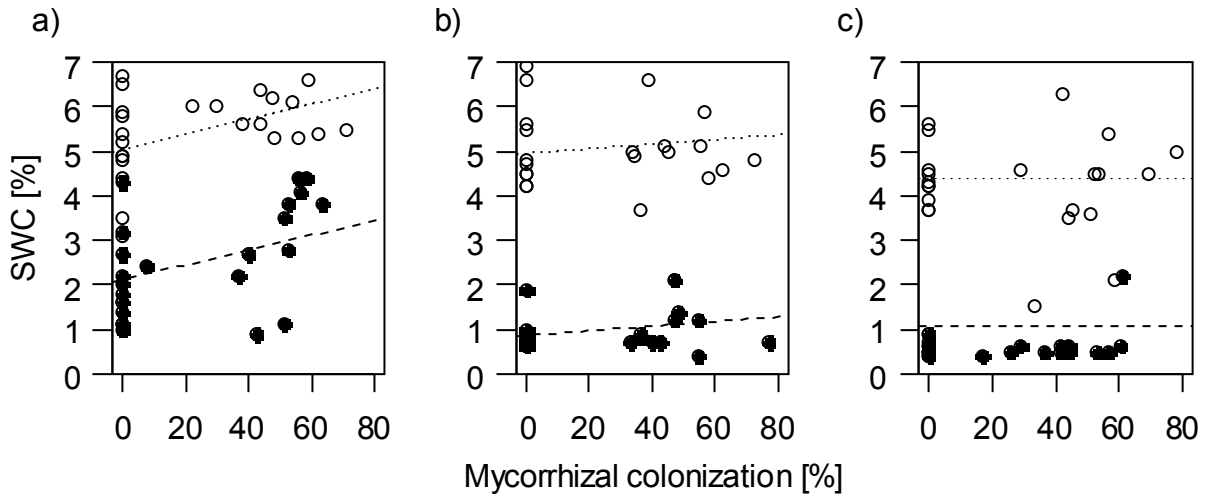


Fig. 4.7: Regression of gravimetric soil water content (SWC) against mycorrhizal colonization. SWC was determined after harvest of plants. Regression after a) 8 days b) 14 days c) 20 days of experimental treatment. Black dot: mycorrhizal and non-mycorrhizal drought stressed plants, white dots: mycorrhizal and non-mycorrhizal control plants. For statistics and coefficients (slope and intercept) see Tab. S4.2.

However, a significant effect of mycorrhiza on RWC of tissues was only detected in leaves in combination with drought and time, with lowest values in drought stressed plants at the third harvest ($F = 2.87$, $p = 0.016$, Tab. 4.4). RWC of stem was affected by the interaction of D x T, with a more pronounced effect of reduced RWC in drought stressed plants compared to control plants after 20 days of drought treatment ($F = 31.79$, $p < 0.001$). RWC of roots was neither affected by drought nor mycorrhiza (Tab. 4.4).

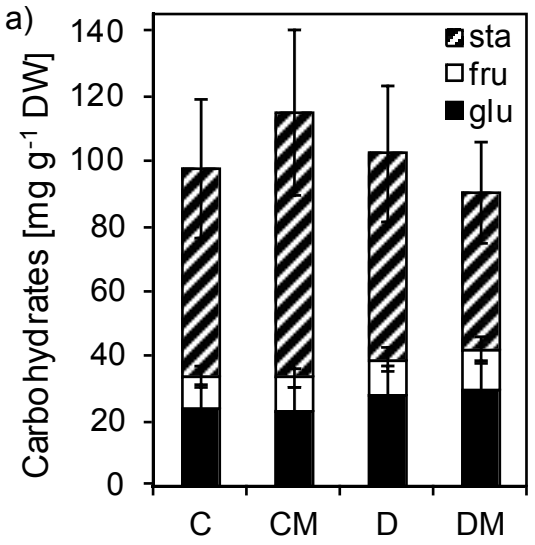
Tab. 4.4: Relative water content of leaves, stem and roots of *P. × canescens* after 8, 14 and 20 days of drought treatment. C: Control plants, CM: control plants with mycorrhiza, D: drought stressed plants, DM: drought stressed plants with mycorrhiza. Table of 3-factorial ANOVA with interaction terms is given with degrees of freedom (DF), F-value and p-values ($p \leq 0.05$). Significant factors or interactions of those are highlighted in bold lettering. Factors used are D: drought, M: mycorrhiza, T: time. Interactions of these factors are indicated by "x". Data represent mean ± SE (n = 10-12)

	Relative water content [%]			**ANOVA Table**			
	8 days	**14 days**	**20 days**	**Factor**	**DF**	**F**	**p**
Leaves							
C	69.1 ± 0.9	70.4 ± 0.9	69.4 ± 0.9	D	1	14.10	**0.006**
CM	70.5 ± 0.7	69.3 ± 0.7	68.8 ± 0.8	M	1	37.40	0.427
D	70.6 ± 0.9	68.1 ± 0.8	64.5 ± 1.0	T	2	7.20	……..**<0.001**
DM	69.2 ± 0.9	69.5 ± 0.9	67.3 ± 0.4	D x M	1	0.62	0.362
				D x T	2	2.27	**0.019**
				M x T	2	0.42	0.560
				D x M x T	2	2.87	**0.016**
Stem							
C	69.9 ± 0.5	69.7 ± 0.4	67.5 ± 0.5	D	1	86.21	**0.001**
CM	70.8 ± 0.4	68.5 ± 0.3	66.7 ± 0.6	M	1	0.01	0.922
D	69.9 ± 0.2	66.1 ± 0.5	61.6 ± 0.6	T	2	143.08	**<0.001**
DM	70.4 ± 0.4	67.9 ± 0.7	61.0 ± 0.5	D x M	1	1.64	0.202
				D x T	2	31.79	**<0.001**
				M x T	2	2.04	0.134
				D x M x T	2	3.01	0.053
Root							
C	71.3 ± 2.6	69.8 ± 2.2	69.1 ± 2.1	D	1	2.96	0.088
CM	71.5 ± 1.5	67.6 ± 1.8	69.1 ± 1.7	M	1	0.77	0.380
D	71.0 ± 1.7	66.8 ± 1.8	67.5 ± 2.7	T	2	4.79	**0.009**
DM	69.3 ± 1.1	67.8 ± 1.2	63.7 ± 0.9	D x M	1	0.06	0.810
				D x T	2	0.71	0.492
				M x T	2	0.25	0.778
				D x M x T	2	0.89	0.415

4.3.5 Effect of drought and mycorrhiza on carbohydrates and osmolality

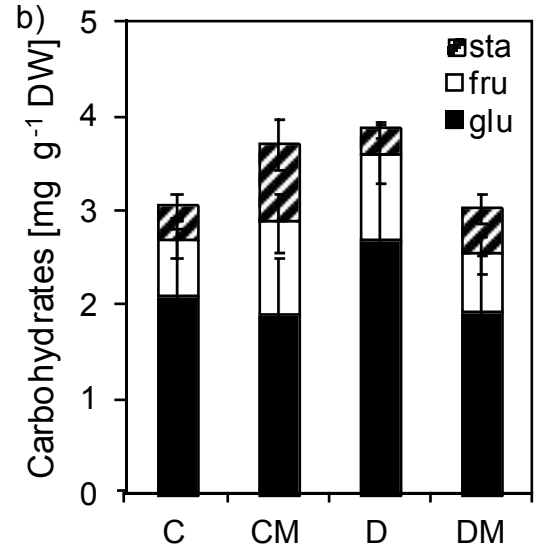
Sucrose was not detectable, neither in roots nor in leaves. In leaves starch was the dominant carbohydrate, while in roots glucose was most prominent (Fig. 4.8).

In leaves glucose and fructose showed increased concentrations in drought stressed plants ($F = 6.39$, $p = 0.016$ and $F = 8.44$, $p = 0.006$, respectively), while starch concentration was significantly lower in drought stressed plants ($F = 4.41$, $p = 0.043$, Fig. 4.8a). In roots an opposite pattern occurred with glucose and fructose being not significantly affected by drought or mycorrhiza and starch being affected by mycorrhiza as single factor, showing higher values in mycorrhizal plants ($F = 6.34$, $p = 0.015$, Fig. 4.8b).



ANOVA Table of leaf carbohydrates.

	Glucose		Fructose		Starch	
	F	p	F	p	F	p
D	6.39	**0.016**	8.44	**0.006**	4.41	**0.043**
M	0.11	0.743	0.47	0.499	0.02	0.874
D x M	0.29	0.595	1.86	0.181	3.63	0.065



ANOVA Table of root carbohydrates.

	Glucose		Fructose		Starch	
	F	p	F	p	F	p
D	0.66	0.422	0.03	0.872	3.12	0.086
M	1.89	0.178	0.06	0.815	6.34	**0.015**
D x M	0.69	0.410	1.87	0.180	1.01	0.322

Fig. 4.8: Carbohydrate concentrations of *P. × canescens* leaves (a) and roots (b) after 20 days of drought treatment. Sta: starch, fru: fructose, glu: glucose, C: control plants, CM: control plants with mycorrhiza, D: drought stressed plants, DM: drought stressed plants with mycorrhiza. Values represent mean ± SE. ANOVA tables of carbohydrates are shown next to the figures. Factors of the ANOVA are D: drought, M: mycorrhiza, D x M: interaction of drought and mycorrhiza. Significant effects are highlighted by bold lettering. (n = 9-10)

Under drought stress conditions changes in osmoregulation are expected. Thus, the osmolality was analyzed and the extent to which solutes such as ions and soluble carbohydrates contribute to osmoregulation was determined (Tab. 4.5). Osmolality in leaves was significantly affected by drought, with lower values of control plants compared to drought stressed plants ($F = 27.3$, $p < 0.001$). In roots no significant effect of drought or mycorrhiza on osmolality was detected (Tab. 4.5). The osmolality from cations in leaves was affected by drought and mycorrhiza, with higher values in drought stressed and mycorrhizal plants (F =

4.9, p = 0.035 and F = 4.4, p = 0.044, respectively). For roots the osmolality from cations was affected by drought with higher osmolality from cations in drought treated plants (F = 18.1, p < 0.001). In case of osmolality from soluble sugars, an effect of drought was found in leaves, with increased values in drought stressed plants (F = 21.4, p < 0.001). No effect was found for roots (Tab. 4.5).

Tab. 4.5: Total osmolality, and contribution of cations and soluble sugars in leaves and roots of *P. × canescens* after 20 days of drought treatment. C: control plants, CM: control with mycorrhiza, D: drought stressed plants, DM: drought stressed with mycorrhiza. Osmolality from soluble sugars represent the sum of glucose and fructose. Osmolality from cations represent the sum of K, Na, Mg, and Ca. Values represent mean ± SE. ANOVA tables with factors D: drought, M: mycorrhiza, and D x M: interaction of drought and mycorrhiza, F- and p-values are given (p ≤ 0.05). Significant effects are highlighted by bold lettering. (n = 9-10)

	Osmolality [mosmol kg^{-1}]	Cations [mmol kg^{-1}]	Soluble sugar [mmol kg^{-1}]
Leaves			
C	596.6 ± 17.3	191.1 ± 5.4	56.6 ± 3.1
CM	620.1 ± 24.2	195.3 ± 5.9	56.0 ± 2.2
D	732.0 ± 27.5	195.1 ± 3.9	74.3 ± 6.3
DM	742.8 ± 21.8	211.9 ± 3.6	76.5 ± 4.2
ANOVA Table			
	F p	F p	F p
D	27.3 **<0.001**	4.9 **0.035**	21.4 **<0.001**
M	0.9 0.347	4.4 **0.044**	0.0 0.846
D X M	0.0 0.982	1.6 0.212	0.1 0.737
Roots			
C	82.6 ± 8.1	110.1 ± 10.4	4.26 ± 0.23
CM	60.7 ± 7.4	96.5 ± 7.2	4.65 ± 0.98
D	64.8 ± 8.5	147.8 ± 15.8	5.59 ± 0.34
DM	70.3 ± 5.5	147.8 ± 6.9	4.97 ± 0.54
ANOVA Table			
	F p	F p	F p
D	0.2 0.703	18.1 **<0.001**	1.6 0.212
M	0.7 0.406	0.4 0.547	0.0 0.857
D x M	2.9 0.097	0.4 0.520	0.6 0.428

In roots, the contribution of cations to osmolality exceeds the measured total osmolality which is impossible (Tab. 4.5). No error was detected in case of the measurement procedure or the followed calculation of osmolality. Thus, the root osmolality was excluded from further analysis.

In leaves, regression analysis of osmolality from cations and total osmolality revealed a positive correlation (R^2 = 0.41, p < 0.001, Fig. 4.9a). Regression analysis of osmolality from soluble sugars and total osmolality in leaves revealed a weak positive correlation (R^2 = 0.25, p = 0.001, Fig. 4.9b).

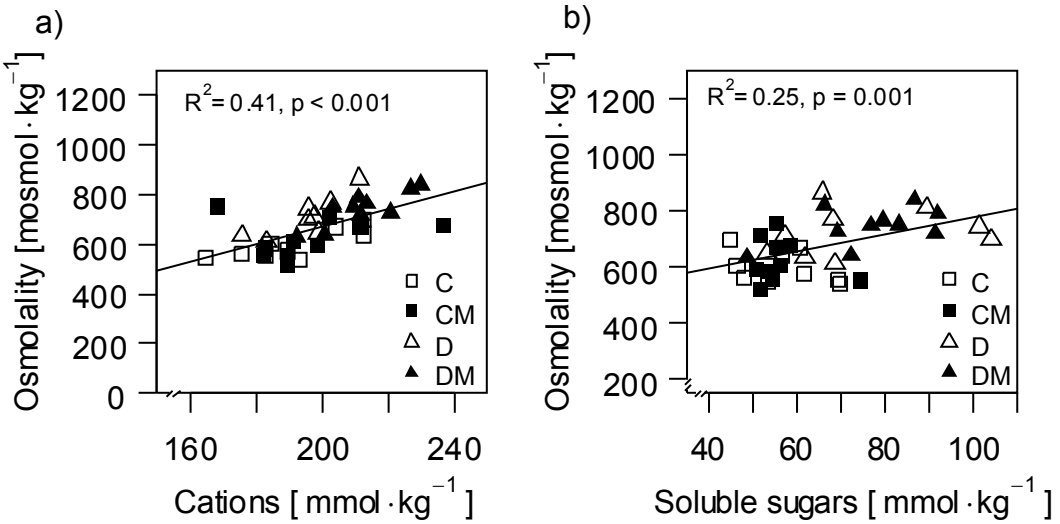
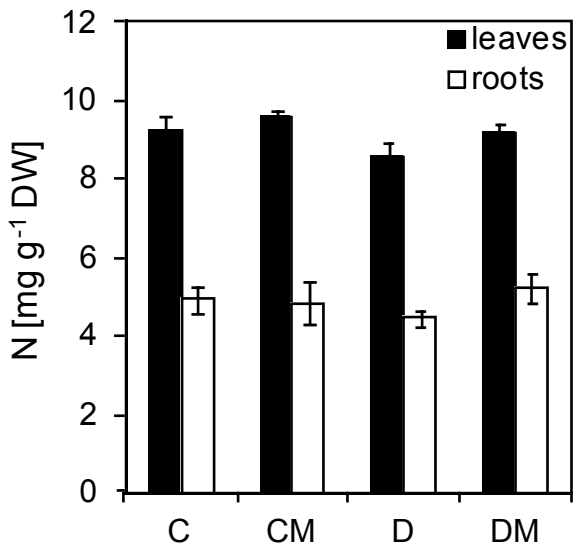


Fig. 4.9: Regression of total osmolality with osmolality from cations and from soluble sugars of leaves of *P. × canescens* after 20 days of drought treatment. a) Osmolality from cations represents the sum of K, Na, Mg, Ca. b) Osmolality from soluble sugars represents the sum of glucose and fructose. C: control plants (open square), CM: control plants with mycorrhiza (filled square), D: drought stressed plants (open triangle), DM: drought stressed plants with mycorrhiza (filled triangle). R-square and p values are given. (n = 9-10)

4.3.6 Effect of drought and mycorrhiza on nutrient status of plants

The main nutrient elements N, P and S in leaves and roots of *P. × canescens* are illustrated in Fig. 4.10. Surprisingly no effect of mycorrhiza was found for N, neither in leaves nor in roots. In contrast to this result, the P concentrations of leaves were significantly affected by drought and mycorrhiza as single factors, as well as by the interaction of both factors (Fig. 4.10b). Phosphorous concentrations were increased in mycorrhizal treatments, especially under drought stress conditions. For the S concentrations in leaves a similar pattern was found for mycorrhiza, but not for drought (F = 0.65, p = 0.427, Fig. 4.10c). P and S concentrations in roots were not affected by treatments (Fig. 4.10b-c).

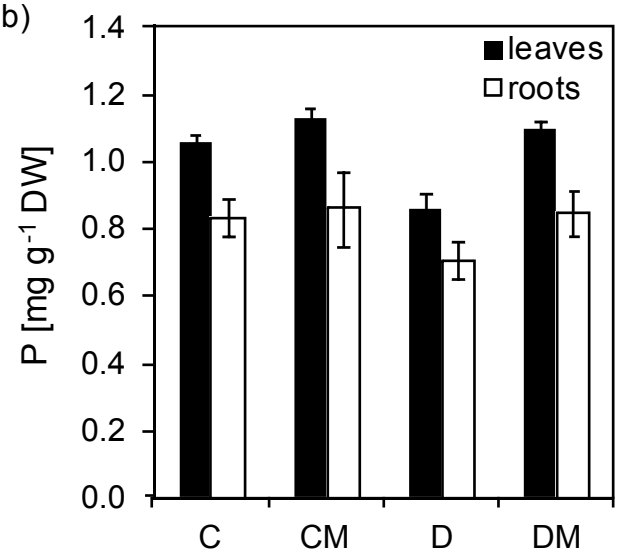
a)



ANOVA Table

	Leaves		Roots	
	F	p	F	p
D	3.02	0.092	0.01	0.943
M	2.53	0.121	0.74	0.396
D x M	0.21	0.647	1.096	0.303

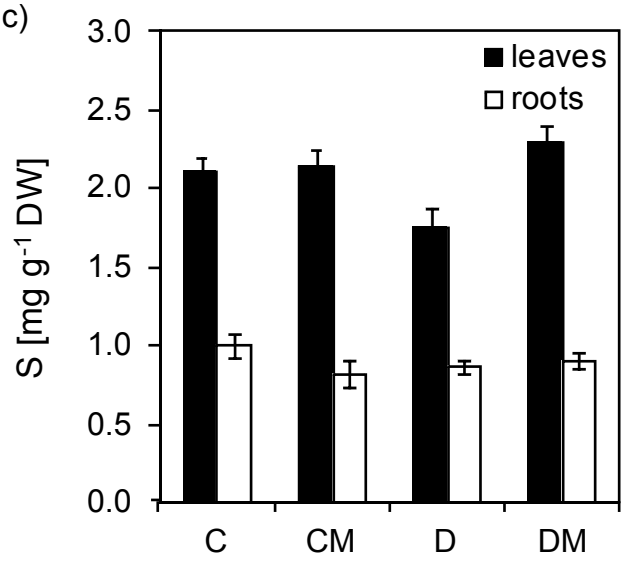
b)



ANOVA Table

	Leaves		Roots	
	F	p	F	p
D	13.66	**< 0.001**	0.78	0.383
M	29.20	**< 0.001**	1.32	0.260
D x M	7.682	**0.008**	0.57	0.454

c)



ANOVA Table

	Leaves		Roots	
	F	p	F	p
D	0.65	0.427	0.07	0.790
M	7.26	**0.011**	0.87	0.357
D x M	5.89	**0.021**	2.55	0.120

Fig. 4.10: Main nutrient elements concentrations in leaves and roots of *P. × canescens* after 20 days of drought treatment. C: control plants, CM: control plants with mycorrhiza, D: drought stressed plants, DM: drought stressed plants with mycorrhiza. a) nitrogen concentration, b) phosphorous

concentration and c) sulfur concentration. Black bars represent nutrient concentrations of leaves, white bars represent those of roots. Values indicate mean ± SE. ANOVA tables with F- and p-values are given. D: drought, M: mycorrhiza, D x M: interaction of drought and mycorrhiza. Significant effects ($p \leq 0.05$) are highlighted by bold lettering. (n = 9-10)

In leaves other macro- and microelements were mainly affected by mycorrhiza or the interaction of drought and mycorrhiza, showing higher values in mycorrhizal than non-mycorrhizal plants (Tab. 4.6). The K concentration, for example, was significantly higher in mycorrhizal plants compared to non-mycorrhizal plants ($F = 7.0$, $p = 0.012$). The concentrations of other nutrients like Ca, Mg, Mn, and Na were higher in mycorrhizal plants under drought stress conditions than mycorrhizal control plants indicated by a significant interaction term of drought and mycorrhiza (Tab. 4.6). In contrast to leaves, root elements were mainly affected by drought and only for Mg, Na, and Al also an effect of mycorrhiza was found, displayed by lower values in mycorrhizal than non-mycorrhizal plants (Tab. 4.6).

Aluminum is not considered to be a nutrient element but was listed in Tab. 4.6 due to its remarkable increase under drought stress conditions in roots, which was not only affected by drought ($F = 92.5$, $p < 0.001$) but also by mycorrhiza ($F = 8.9$, $p = 0.005$), with lower values in mycorrhizal plants. In case of leaves Al was affected by the interaction of drought and mycorrhiza ($F = 5.6$, $p = 0.024$) and showed higher values in mycorrhizal plants under drought stress conditions.

To elucidate if the predominant effects of mycorrhiza found for leaf nutrients was also dependent on the degree of mycorrhization an ANCOVA was conducted including the covariable mycorrhizal colonization and the factor drought stress. The result revealed that the extent of mycorrhizal colonization is an important factor in leaf nutrient concentrations (Tab. 4.7). Whereas the ANOVA detected mostly significant effects of mycorrhiza and the interaction of mycorrhiza x drought, the ANCOVA revealed predominantly a significant interaction term. In case of P, S, Mg, Ca, K and Na the significant interaction revealed a positive effect of mycorrhizal colonization in drought stress plants leading to higher concentrations at higher colonization rates. In contrast to the ANOVA Mn was not affected by mycorrhizal colonization or the interaction of mycorrhizal colonization x drought (Tab. 4.7), and Fe was significantly affected by mycorrhizal colonization ($F = 7.2$, $p = 0.011$). In case of N no significant effect was found neither of mycorrhizal colonization nor of drought stress (Tab. 4.7). For the main nutrients P, N, and S the results of the ANCOVA are visualized in Fig. S4.1.

Tab. 4.6: Macro- and micronutrient and aluminum concentrations in leaves and roots of *P. × canescens* after 20 days of drought treatment. C: control plants, CM: control plants with mycorrhiza, D: drought stressed plants, DM: drought stressed plants with mycorrhiza. Macronutrients (K: potassium, Ca: calcium, Mg: magnesium), micronutrients (Na: sodium, Mn: manganese, Fe: iron), Al: aluminum. ANOVA tables with factors D: drought, M: mycorrhiza and D x M: interaction of D and M, F and p-values are given. Significant effects are highlighted with bold lettering ($p \leq 0.05$). (n = 9 -10)

Leaves	Response variable [mg g^{-1} DW]						
	K	Ca	Mg	Na	Mn	Fe	Al
C	11.8 ± 0.6	9.0 ± 0.3	2.23 ± 0.06	0.093 ± 0.028	0.314 ± 0.009	0.074 ± 0.003	0.259 ± 0.070
CM	12.5 ± 0.4	8.7 ± 0.3	2.15 ± 0.07	0.051 ± 0.001	0.309 ± 0.008	0.092 ± 0.008	0.188 ± 0.030
D	10.6 ± 0.5	7.6 ± 0.4	1.92 ± 0.09	0.060 ± 0.009	0.276 ± 0.015	0.079 ± 0.012	0.139 ± 0.017
DM	12.4 ± 0.3	9.2 ± 0.2	2.35 ± 0.06	0.170 ± 0.047	0.338 ± 0.011	0.092 ± 0.006	0.308 ± 0.064
ANOVA table							
	F p	F p	F p	F p	F p	F p	F P
D	1.9 0.173	1.7 0.203	0.3 0.590	2.7 0.111	0.1 0.775	0.1 0.754	0.0 0.896
M	7.0 **0.012**	4.2 **0.048**	5.9 **0.021**	1.4 0.243	6.6 **0.015**	3.6 0.066	1.0 0.337
D x M	1.7 0.208	9.5 **0.004**	13.2 <**0.001**	7.1 **0.012**	9.0 **0.005**	0.1 0.760	5.6 **0.024**
Roots	Response variable [mg g^{-1} DW]						
	K	Ca	Mg	Na	Mn	Fe	Al
C	4.0 ± 0.3	7.1 ± 0.5	1.29 ± 0.07	0.833 ± 0.066	0.159 ± 0.010	11.5 ± 1.3	5.4 ± 0.5
CM	3.5 ± 0.4	7.1 ± 0.8	1.10 ± 0.12	0.541 ± 0.075	0.149 ± 0.016	12.3 ± 2.0	4.5 ± 0.6
D	5.1 ± 0.3	8.6 ± 0.2	1.92 ± 0.10	0.671 ± 0.051	0.187 ± 0.010	19.7 ± 2.0	14.4 ± 1.0
DM	4.4 ± 0.3	8.7 ± 0.5	1.50 ± 0.06	0.532 ± 0.033	0.164 ± 0.012	13.5 ± 1.4	10.7 ± 0.9
ANOVA table							
	F p	F p	F p	F p	F p	F p	F p
D	10.1 **0.003**	8.5 **0.006**	31.7 <**0.001**	2.0 0.167	3.0 0.094	7.1 **0.012**	92.5 <**0.001**
M	4.1 0.051	0.0 0.978	11.4 **0.002**	13.6 <**0.001**	1.9 0.183	2.9 0.100	8.9 **0.005**
D x M	0.2 0.705	0.0 0.847	1.6 0.222	1.8 0.188	0.3 0.588	4.2 **0.048**	3.3 0.079

Tab. 4.7: ANCOVA Table of leaf elements after 20 days of drought treatment. D: drought, myc.col: mycorrhizal colonization, D x myc.col: interaction of mycorrhizal colonization and drought. F and p-values are given. (n = 10-12). Significant effects ($p \leq 0.05$) are highlighted by bold lettering.

	N		P		S		K		Ca	
	F	p	F	p	F	p	F	p	F	p
D	2.5	0.121	9.2	**0.005**	0.4	0.531	1.3	0.269	1.4	0.238
myc.col	1.7	0.208	17.0	**0.002**	1.9	0.177	10.4	**0.003**	0.0	0.903
D x myc.col	0.0	0.911	6.4	**0.016**	4.8	**0.036**	3.0	0.091	6.7	**0.014**
	Mg		Na		Mn		Fe		Al	
	F	p	F	p	F	p	F	p	F	p
D	0.1	0.756	2.3	0.139	0.1	0.793	0.3	0.594	0.0	0.847
myc.col	0.2	0.636	0.6	0.453	0.0	0.959	7.2	**0.011**	0.5	0.491
D x myc.col	10.0	**0.003**	4.2	**0.050**	3.7	0.061	0.3	0.567	4.7	**0.038**

Although no effect of mycorrhiza and drought on the N concentration in leaves was detected (Fig. 4.10a, Tab. 4.7), gene expression of the ammonium transporter AMT3.1 was significantly upregulated in mycorrhizal drought stressed plants after 20 days of drought treatment compared to control plants (Fig. 4.11).

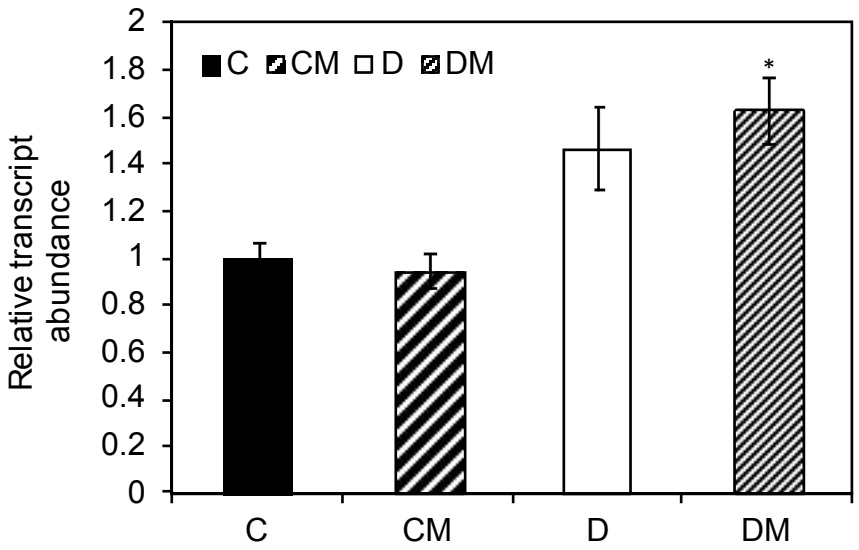


Fig. 4.11: Relative transcript abundance of gene AMT3.1 of leaf material of *P. × canescens* after 20 days of drought stress treatment. Values are normalized against actin. Relative transcript abundance is expressed as treatment/control with treatments C: control plants, CM: control plants with mycorrhiza, D: drought stressed plants, DM: drought stressed plants with mycorrhiza. Significant differences to control plants are indicated by (*) ($p \leq 0.05$). (n = 9-10).

4.3.7 Effect of drought and mycorrhiza on expression of stress related genes

The transcript abundance of the following prominent stress related genes was determined: PIP2.5, aquaporin (drought stress), GLP3, RD26 and Bet v I. Except aquaporin PIP2.5, all these genes showed significant changes in drought stressed plants compared to control plants (Fig. 4.12). GLP3 was significantly downregulated in mycorrhizal and non-mycorrhizal drought stressed plants, while RD26 was significantly upregulated in both of these treatments (Fig. 4.12). Bet v I was only significantly affected in mycorrhizal drought stressed plants, showing a reduced expression (Fig. 4.12).

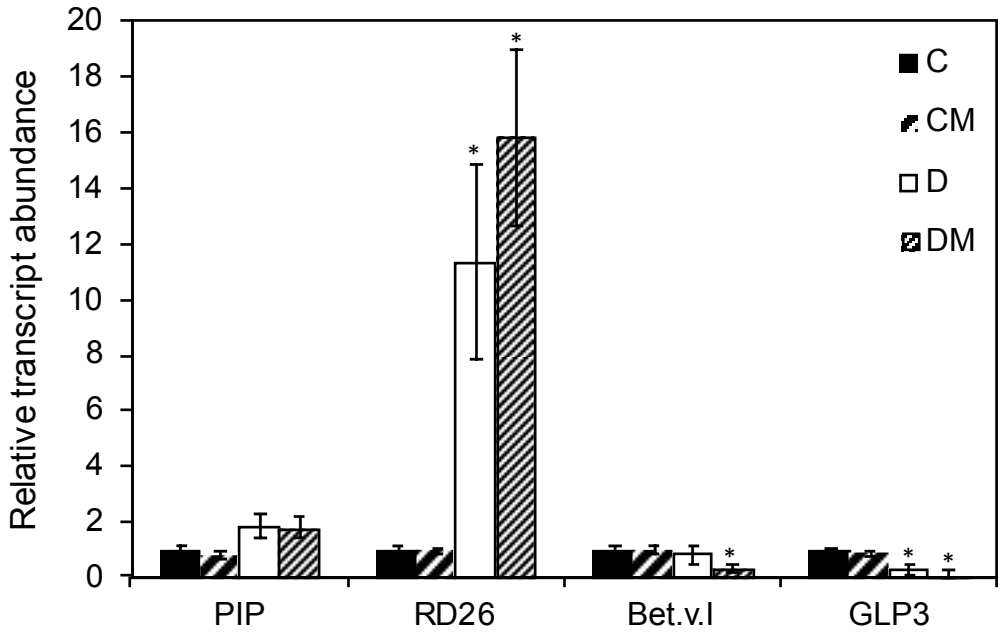


Fig. 4.12: Relative transcript abundance of stress-related genes in leaves of *P. × canescens* after 20 days of drought stress treatment. Values are normalized against actin. Relative transcript abundance is expressed as treatment/control, with treatments C: control plants, CM: control with mycorrhiza, D: drought stressed plants, DM: drought stressed with mycorrhiza. Significant differences ($p \leq 0.05$) to control plants are indicated by (*). (n = 9-10).

4.4 Discussion

4.4.1 *Paxillus involutus affects the water status of P. × canescens under drought stress conditions*

The volumetric SWC of about 6% measured over the duration of drought treatment in control plants appeared relatively low, but physiological measurements like pre-dawn leaf water potential, stomatal conductance or efficiency of PSII did not indicate drought stress in these plants. Bogeat-Triboulot *et al.* (2004) demonstrated constant predawn shoot water potential

over a wide range of volumetric SWC (27%-2%) in *Pinus pinaster* seedlings grown in a sandy soil. The SWC for drought treatment was kept between 1-3% in this study (Bogeat-Triboulot et al., 2004). These data support that control plants kept at 6% SWC in our study were not suffering from drought. Since the ECM fungus *P. involutus* is able to explore with its rhizomorphs a large soil volume, a positive effect on the water status of mycorrhizal drought stressed plants was expected. The colonization of *P.* × *canescens* roots with *P. involutus* or maintenance of mycorrhiza was not affected by drought stress. However, the vitality index of root tips was higher in mycorrhizal compared to non-mycorrhizal drought stressed plants indicating a positive effect of the fungus on survival of root tips. This effect might have been the result of higher SCW found in mycorrhizal compared to non-mycorrhizal plants (Fig. 3a, Tab S4.3). Studies on arbuscular mycorrhiza have shown that they affect soil moisture retention curves (Augé, 2004). Such measurements are currently still leaking for ECM. Our data suggest that *P. involutus* also improves the SW retention capacity. *P. involutus* is known to build large rhizomorphs (Agerer, 1987-2006) and thus might affect at least partly the soil water status. SWC decreased with increasing drought stress, which lead to an increased number of dead root tips in mycorrhizal drought stressed plants to the level of non-mycorrhizal drought stressed plants at the end of the experiment (Fig. 4.5c). However, the effect of *P. involutus* on the SWC was not reflected in the RWC of stem and roots of poplar (Tab 4.4). In case of RWC of leaves an effect of mycorrhiza was apparent in combination with drought and time, reflected in a slower decrease of the RWC over time in mycorrhizal than non mycorrhizal drought stressed plants (Tab. 4.4). In contrast, Luo *et al.* (2009b) found no effect of inoculation in RWC of leaves of *P. euphratica* after drought treatment, while RWC of stem and roots was significantly affected by drought and inoculation with higher RWC in inoculated plants. Thus *P. involutus* in our study seem not or in case of leaves only slightly enhance the water status of drought stressed plants.

Predawn leaf water potential was significantly affected by mycorrhiza over time, reflected in delayed response of mycorrhizal plants to increasing drought stress compared with non-mycorrhizal drought stressed plants (Fig. 4.4). A positive effect of inoculation with ECM fungi in relation to predawn leaf water potential under drought conditions was also found by Dosskey *et al*. (1991) investigating different ECM species associated with Douglas fir. However, the result was dependent on host-species combination. A positive effect on predawn leaf water potential was found for the ECM fungus *Rhizopogon vinicolor,* while no effect was found for *Laccaria lacata* or *Hebeloma crustuliniforme (*Dosskey *et al*., 1991).

The stomatal conductance in mycorrhizal and non-mycorrhizal drought stressed plants decreased rapidly over time. Stomatal closure is an effective mechanism of plants to prevent severe water loss through transpiration and associated cavitation. An effect of mycorrhiza on

stomatal conductance was only apparent in combination with drought and time, indicating that there is no clear separation of mycorrhizal and non-mycorrhizal drought stress treatment over time. In contrast to our result Lehto *et al.* (1992) could demonstrate a higher stomatal conductance of *Picea sitchensis* inoculated with *P. involutus* under well watered and drought stressed conditions compared to non-inoculated plants. In an experiment without mycorrhiza Almeida-Rodriguez *et al.* (2010) investigated two poplar genotypes and found contrasting results. While stomatal conductance of *P. simonii* × *balsamifera* was already reduced under mild drought stress, it was not affected in *P. balsamifera* until severe drought stress. In addition to these results Almeida-Rodriguez *et al.* (2010) found an upregulation of the aquaporin PIP2.5 in leaves of *P. balsamifera,* while expression levels in *P. simonii* × *balsamifera* showed no significant changes. Due to the function of this aquaporin as water channel (Secchi *et al.*, 2009) they concluded that PIP2.5 may support the plant water flow, which is driven by transpiration (Almeida-Rodriguez *et al.*, 2010). Although the stomatal conductance in mycorrhizal and non-mycorrhizal drought stress plants in our study showed a rapid decrease over time, the PIP2.5 expression in leaves was not significantly altered in both treatments compared to control plants, indicating no effect of drought on the expression level of this aquaporin in leaves under strong stress conditions after 20 days of treatment. Marjanovic *et al.* (2005a) could demonstrate that the ECM fungus *Amanita muscaria* increased the expression of PIP2.5 in mycorrhizal fine roots of *P. tremula* × *tremuloides* compared to fine roots of non-inoculated poplar plants. In our study, no effect of mycorrhiza on the expression level of PIP2.5 in leaves was found.

Transcripts of Bet v I allergen were often found to be induced upon colonization by mycorrhiza (Duplessis *et al.*, 2005; Johansson *et al.*, 2004; Le Quéré *et al.*, 2005). In our study Bet v I was downregulated in mycorrhizal drought stressed plants but not in non-mycorrhizal drought stress plants or mycorrhizal control plants. In contrast to our results Bet v I gene expression was up-regulated in mycorrhizal compared to non-mycorrhizal roots of *P.* × *canescens* associated with *P. involutus* in control as well as in salt stressed treatments (Luo *et al.*, 2009a). Since genes with high homology to the Bet v I gene family are involved in triggering ABA response (Ma *et al.*, 2009) it is possible that mycorrhizal drought stressed plants react faster to environmental stress on a molecular level.

RD26 is a dehydration-induced NAC protein shown to be involved in ABA-dependent stress response (Fujita *et al.*, 2004). RD26 was shown to be up-regulated by drought, salt or ABA treatment in *Arabidopsis,* suggesting a key role of this gene in stress-signaling pathways (Fujita *et al.*, 2004). Thus, we expected the expression level of this gene to be up-regulated in non-mycorrhizal and mycorrhizal drought stressed plants compared to non-mycorrhizal control plants in our study, which was confirmed by qRT-PCR. Germin-like genes have been

implicated in responses to external stimuli as flower inductive-darkness (Ono *et al.*, 1996), drought (Bray, 2004) and salt stress (Nakata *et al.*, 2002). Previous studies showed that GLP3 was expressed in response to pathogens in *Arabidopsis* (Floerl *et al.*, 2012) and *MtGlp*1 was found to be induced in response to arbuscular mycorrhiza in the legume *Medicago truncatula* (Doll *et al.*, 2003). Thus, we expected altered expression in mycorrhizal control and drought stressed plants in our experiment. However, the expression was down-regulated in mycorrhizal and non-mycorrhizal drought stressed plants and no changes in the expression level of mycorrhizal control plants compared to non-mycorrhizal control plants were detected, indicating a role of this gene in drought stress response of *P. × canescens*. Down-regulation of GLP3 under drought stress conditions is in line with Bray (2004) who analyzed three independent water-deficit experiments and showed that two germin-like proteins (AtGER1 and AtGER3) were down-regulated in *Arabidopsis* under drought conditions.

4.4.2 *Paxillus involutus affects physiology and nutrition of P. × canescens under drought stress conditions*

The quantum yield of PSII efficiency was higher in mycorrhizal than in non-mycorrhizal plants, indicating an enhanced photosynthesis activity even under drought stress conditions in mycorrhizal plants. Similar results were also found by Fini *et al*. (2011) in mycorrhizal linden and maple trees compared to control plants. Mycorrhizae were often found to increase photosynthetic activity in host plants (Nehls, 2008). In contrast to these results Luo *et al.* (2009b) found lower values in efficiency of PSII in *P. euphratica* inoculated with *P. involutus*. Improved nutrition is known to be positively correlated with increased photosynthesis (Lehto & Zwiazek, 2011). Thus, the enhanced nutrition status in leaves of mycorrhizal drought stressed and mycorrhizal control plants could be the reason for the higher efficiency of PSII. Although in our study the stomatal conductance in mycorrhizal and non-mycorrhizal plants decreased early in response to drought stress, no changes in efficiency of the PS II of those plants were detected before drought stress reached a severe level. Mycorrhizal and non-mycorrhizal drought stressed plants had a decreased CO_2 supply through stomatal closure but seemed to maintain photochemistry until suffering from severe drought stress.

Mycorrhizae are a strong sink of photosynthates and favor glucose over fructose (Nehls, 2008). Thus it was expected that changes in soluble sugar concentration would be detected between mycorrhizal and non-mycorrhizal plants. Furthermore, drought stress leads to lower C assimilation when stomatal closure occurs and thus could also lead to changes in carbohydrate concentrations. Retention of soluble sugar concentrations in leaves was often

observed together with a strong decrease in starch (Chaves, 1991). Carbohydrate concentrations in our study were not significantly affected by mycorrhiza in leaves but drought stress led to an increase in glucose and fructose concentrations. The decrease in starch concentration may indicate the necessity to utilize this carbohydrate resource for production of osmolytes. A high amount of starch in leaves was also found in *P. nigra* clone 58-861 growing at two different water regimes, with starch concentrations in well watered plants ranging from 84.3 to 134 mg/g DW in young and old leaves, respectively (Regier *et al.*, 2009). An increase of glucose and fructose content in poplar in response to drought stress was also found by other groups (Bogeat-Triboulot *et al.*, 2007; Xiao *et al.*, 2008). Carbohydrate concentrations of roots were not affected by drought while mycorrhiza increased starch concentration under control and drought stress conditions. An increase of starch concentration in roots was also found in *Betula pendula* inoculated with *P. involutus* compared to non-mycorrhizal plants (Wright *et al.*, 2000). In contrast to our findings Luo *et al.* (2009b) found markedly higher glucose and fructose concentration in roots of *P. euphratica* inoculated with *P. involutus* compared to non-mycorrhizal plants. The accumulation of starch in roots of mycorrhizal control and drought stressed plants in our study could be interpreted as an indication for the higher sink of photosynthates due to the mycorrhizal symbiosis.

Sugars have multiple roles in plants such as sugar-mediated alteration in gene expression in response to environmental stress, protein stability and activity, energy storage, and osmotic potential (Koch, 1996; Rolland *et al.*, 2006; Smeekens, 2000). Thus, we looked for the contribution of soluble sugars to osmolality. Soluble sugars in leaves showed a very week positive correlation with osmolality, while the correlation of cations with osmolality was stronger. In total the contribution of cations and sugars to leaf osmolality was 41-42% in mycorrhizal and non-mycorrhizal control plants, and in mycorrhizal and non-mycorrhizal drought stressed plants the contribution was even lower (37-39%). Although the cation and sugar concentration increased under drought stress conditions in mycorrhizal and non-mycorrhizal plants the proportion of the contribution of these compounds to the total osmolality decreased. Thus, other osmotic compounds have been accumulated or synthesized for osmotic adjustment.

Although mycorrhizal plants showed an improved leaf nutrition status (Fig. 4.12, Tab. 4.6), the total biomass production as well as relative growth rates were not positively affected by mycorrhiza, which might have been expected due to a better nutrition status. Biomass of *P. euphratica* was shown to decrease in response to *P. involutus,* although the mycorrhiza was not established (Luo *et al.*, 2009b). In contrast, inoculation of *P. involutus* increased root and shoot biomass as well as N and P concentration of *Picea abies* (Brandes *et al.*, 1998). Javelle *et al.* (1999) demonstrated in an ^{15}N labeling approach that *P. involutus* enhance

nitrogen nutrition in *Betula pendula*. In contrast to these results we could not detect enhanced N concentrations in mycorrhizal plants compared to non-mycorrhizal plants neither in control nor in drought stress treatments. However, our results are in line with Langenfeld-Heyser *et al.* (2007) investigating the salt stress response of non-mycorrhizal and mycorrhizal *P.* × *canescens* plants inoculated with *P. involutus.* They found no effect of treatment on N concentrations, whereas P concentrations were increased in mycorrhizal plants (Langenfeld-Heyser *et al.*, 2007).

Although the N concentration in leaves in our study seemed to be quite low it was still in the range found by Jug *et al.* (1999). In contrast Rivest *et al.* (2009) found N concentration ranging from 16.0 to 68.83 mg/g in leaf tissue. The low N concentration in our study was not surprising because the N content in the nutrient solution was kept low to foster ectomycorrhizal establishment. The P concentrations in leaves were in the range found by other researchers (Jug *et al.*, 1999; Rivest *et al.*, 2009). Since the improved nutrition status found for mycorrhizal plants did not affect biomass production but might have increased the efficiency of PSII, it is possible that *P. involutus* was a sink for carbohydrates which were therefore not available for growth. The carbohydrates might have been used to increase the biomass of the external mycelia indicating that the positive nutrition effect is not sufficient for increasing biomass of both symbiosis partners.

4.5 Conclusion

P. involutus seems to ameliorate the soil water status of the plant which was also reflected in a slower decrease over time of the RWC of leaves in mycorrhizal drought stressed plants. The ectomycorrhizal fungus positively affected the nutrient status of the control and drought stressed plants. This enhanced nutrient status could be responsible for a higher efficiency of PSII of the mycorrhizal treatments. Our analyses furthermore revealed that the nutrient status was linked to the extent of mycorrhizal colonization. The improved nutrient supply in plants, especially when water becomes limited, underpins the importance of this symbiosis for the host trees.

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Supplemental Data

Tab. S4.1: Oligonucleotid used for RT-PCR

Poplar gene model	AGI	Gene	Primer-Forward Name	Primer-Reverse	Primer Efficiency
POPTR_0011s12400	AT4G27410	rd26	5´-GTAATCACGACAGAGGGACG-3´	5´-TACTTCCACTTTTGCGAGAGG-3´	2.0
POPTR_0010s10600	AT1G24020	Bet v I	5´-ACTGTAGACGAAGCAAAGAAGG-3´	5´-TCACACATCCATTTCACCAAGC-3´	1.977
a		AMT3	5´-GGTTGCTATGGATGGGATGG-3´	5´-CAGATGTTAGTGTTAAGGACAGC-3´	1.920
POPTR_0006s12980		PIP2.5	5´-CATTGATTGATGCCGAGGAGC-3´	5´-CACATACATCGCCGTTCTTGG-3´	1.994
POPTR_0006s14510	AT5G20630	GLP3	5´-CACCCAGGAGGTTCAGAGG-3´	5´-AGGGAAAACCATTATGTCTCCC-3´	2.0
b		ACT9	5´-TGGTGGTTCCACTATGTTCC-3´	5´-TGGAAATCCACATCTGCTGG-3´	1.950
c		β-TUB	5´-GATTTATCCCTCGCGCTGT-3'	5´-TCGGTATAATGACCCTTGGCC-3´	1.916

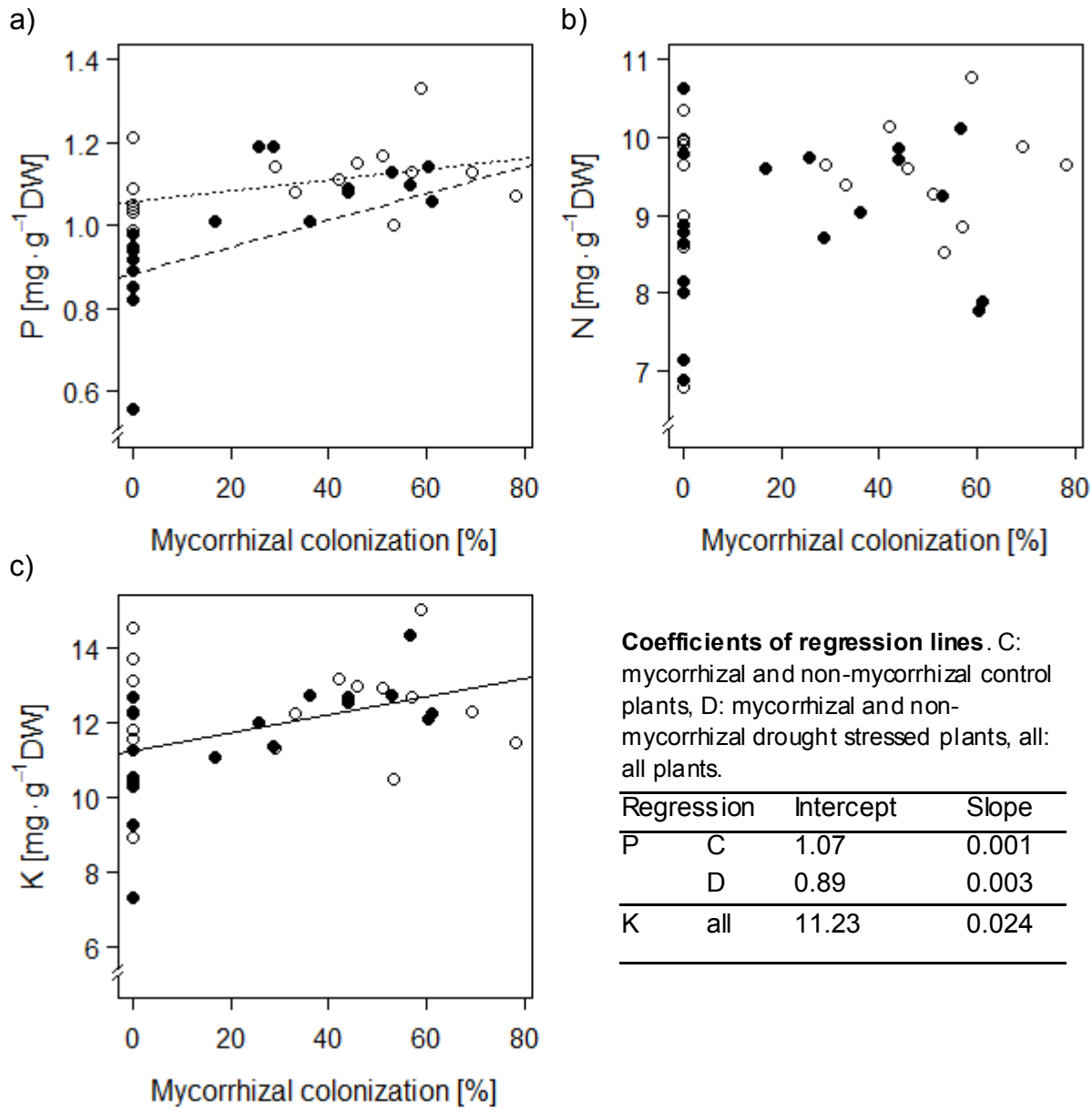
c:**Escalamte-Pérez M, Lautner S, Nehls U, Selle A, Teuber M, Schnitzler JP,Teichmann T, Fayyaz P, Hartung W, Polle A, Fromm J, Hedrich R, Ache P. 2009.** Salt stress affects xylem differentiation of grey poplar (*Populus x canescens*). *Planta* **229** (2): 229-309

a: **Lou ZB, Janz D, Jiang X, Göbel C, Wildhagen H, Tan Y, Rennenberg H, Feussner I, Polle A. 2009.** Upgrading root physiology for stress tolerance by ectomycorrhizas: Insights from metabolite and transcriptional profiling into reprogramming for stress anticipation. *Plant Physiology* **151**:1902-1917

b: **Janz D, Behnke K, Schnitzler JP, Kanawati B, Schmitt-Kopplin P, Polle A. 2010.** Pathway analysis of the transcriptome and metabolome of salt sensitive and tolerant poplar species reveals evolutionary adaption of stress tolerance mechanisms. *BMC Plant Biology* **10**: 150

Tab. S4.2: Summary of the ANCOVA of soil water content (SWC) after 8, 14 and 20 days of drought stress treatment. Additive models were used due to the insignificant interaction term with the covariable mycorrhizal colonization (Myc.col) and the factor drought stress (D). F and p-values of the ANOVA and coefficients (a: intercept, b: slope) for the non-mycorrhizal and mycorrhizal control plants (Control) and for non-mycorrhizal and mycorrhizal drought stress plants are given. (n = 10-12)

	d8		**d14**		**d20**	
ANOVA table	F	p	F	p	F	p
Myc.col	11.16	**0.002**	4.13	**0.049**	0.15	0.693
D	116.68	**<0.002**	365.91	**<0.001**	68.92	**<0.001**
Coefficients	a	b	a	b	a	b
Control	5.05	0.017	4.96	0.005	4.42	-0.005
Drought	2.10	0.017	0.86	0.005	1.06	-0.005



Coefficients of regression lines. C: mycorrhizal and non-mycorrhizal control plants, D: mycorrhizal and non-mycorrhizal drought stressed plants, all: all plants.

Regression		Intercept	Slope
P	C	1.07	0.001
	D	0.89	0.003
K	all	11.23	0.024

Fig. S4.1: Correlation of nutrient element concentrations and mycorrhizal colonization in leaves of *P.* × *canescens* after 20 day of drought treatment. White circles: control plants, black circles: drought stressed plants. Dotted line: regression line of control plants, broken line: regression line of drought stressed plants, solid line: regression line of all plants. For F and p-values of the ANCOVA see Tab. 4.7.

Chapter 5

Overall conclusion

5 Overall conclusion

Renewable energy from biomass, especially from second generation biofuels, is an appealing alternative to reduce the consumption of fossil fuels. However, little information is available of these transgenic poplar lines with respect to biological interactions with soil inhabiting biota, especially with ECM fungi. This work is therefore an important gain of information.

The results obtained in this thesis display for the first time a comparison of fungal communities in soil and roots of transgenic CAD poplar lines and WT poplar. Neither for soil nor for root fungal communities an effect of gene modification was detected. Saprophytes were the dominating fungal group in soil followed by similar proportions of pathogens and ECM fungi. In roots the ECM fungi were most abundant, which underlines the importance of this fungal group in a small scale ecosystem like this poplar plantation (Fig. 5.1). Furthermore, the ECM fungal diversity in the soil was also high which was unexpected due to the long use of the respective plantation for poplar cultivation and thus recurrent disturbances of the soil. Therefore it was concluded, that the soil served as a species rich reservoir for the root colonization of ECM fungi. Furthermore it could be shown that the ECM families Paxillaceae and Pyronemataceae were enriched in roots, while the fungal families Archaeosporaceae (arbuscular mycorrhizae) and Bankeraceae (ECM) were significantly enriched in soil. However, the two most abundant fungal families in soil Fliobasidiaceae and Mortierellacea shared saprophytic or pathogenic lifestyles. There is emerging evidence that ECM fungi could also positively affect the host response to above-ground pathogenic rust fungi (Pfabel *et al.*, 2012). Thus, a high degree of ECM colonization is a desirable aspect in poplar plantations and was already achieved after the second vegetation period in our field experiment. The correlation of mycorrhizal colonization and plant height and biomass underpins the importance of this fungal symbiosis for poplar performance (Fig. 5.1).

The experimental poplar plantation was irrigated over the whole vegetation period and ^{13}C analysis revealed no evidence for water limitation. However, under natural conditions fluctuations in water and nutrient availability could be expected due to predicted extreme weather scenarios caused by global warming (IPCC, 2007). Drought periods could lead to severe loss of biomass yield. The ECM symbiosis on the other hand could partly counteract drought effects through enhanced nutrient and water availability. However, less information is available in this respect, especially for biomass plantations. Thus, a controlled drought stress experiment was conducted to elucidate the role of ECM fungi in the plant stress response to water limitation. The results showed a positive effect of mycorrhizal colonization on the

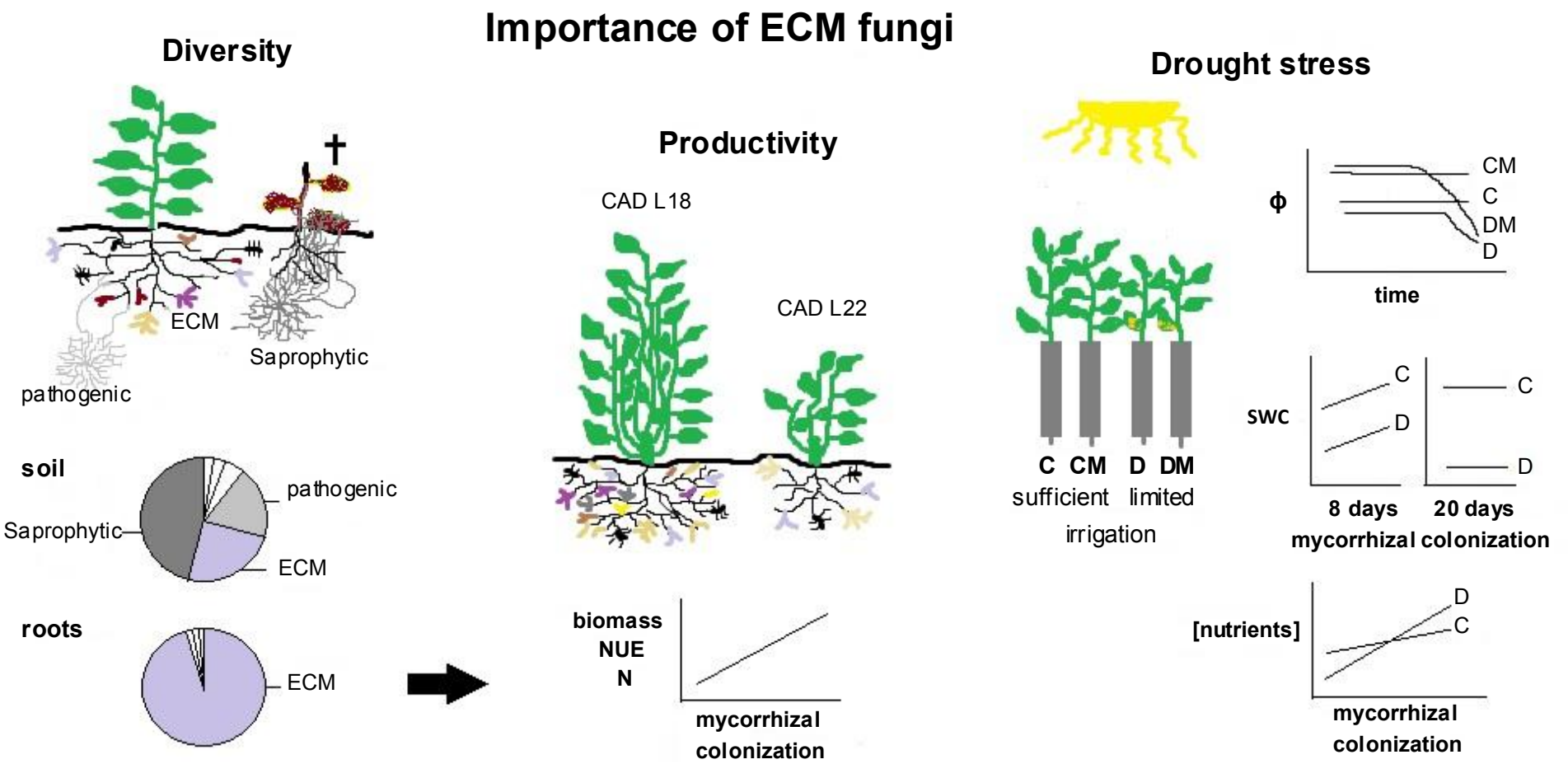


Fig. 5.1: Important findings of this thesis demonstrating the relevance of ECM fungi in soil and root fungal diversity, plant productivity and in plant stress responses. The pie charts display the relative abundance of different fungal lifestyles in soil and roots of the biomass plantation with WT and transgenic poplar. The dominance of ECM fungi in roots display their important role for poplar productivity, which is demonstrated in a positive correlation of biomass, nitrogen use efficiency (NUE) and nitrogen concentration (N) with mycorrhizal colonization. Differences in biomass were especially found for CAD line L22

compared to other lines, which was discussed as transformation rather gene effect due to the "normal" growth of other CAD lines like L18. The potential importance of ECM fungi with respect to global warming, which might come along with drought periods, was shown in a drought stress experiment. Enhanced nutritional status of e.g. phosphorous (P) and potassium (K) concentration in mycorrhizal plants under water limitation were found, as well as earlier closure of stomatal conductance. Mycorrhizal plants had higher efficiency of quantum yield of PSII than non-mycorrhizal plants and gravimetric soil water content (SWC) was positively affected by mycorrhiza under mild and medium drought stress. C: control plants, CM: control mycorrhiza, D: drought stressed plants, DM: drought mycorrhiza.

gravimetric SWC in well watered as well as in drought stress plants. This effect might be higher under natural conditions, when the fungal mycelium is not restricted to the small volume of the growth tube used in this experiment. If the mycelium is able to grow beyond the depletion zone of the roots the ECM fungi might not only be able to provide the tree with water from more distant regions but also with nutrients. The main finding of this experiment was that *P. involutus* enhanced the nutrient status of the poplar under control and particular under drought stress conditions. These results also demonstrate the importance of symbiotic interaction with ECM fungi for poplar performance (Fig. 5.1).

Although the important role of ECM fungi for poplar performance could be demonstrated in this thesis there are still open questions remaining. We found changes in community structure in two adjacent years but it remains still unclear what the driving forces for these changes were. Thus, it will be interesting and essential to monitor successional dynamics in the fungal community structure of this poplar plantation over a longer period. In addition, further field experiments are needed to confirm whether the results obtained in this controlled drought stress experiment can be confirmed under field conditions. For this purpose it will be necessary to produce poplars modified in their ability to establish mycorrhizal symbiosis to be able to differentiate in a field experiment between mycorrhizal and random effects.

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