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Fungal Community Associated with Genetically Modified Poplar During Metal Phytoremediation[§]

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Due to the increasing demand for phytoremediation, many transgenic poplars have been developed to enhance the bioremediation of heavy metals. However, structural changes to indigenous fungal communities by genetically modified organisms (GMO) presents a major ecological issue, due to the important role of fungi for plant growth in natural environments. To evaluate the effect of GM plant use on environmental fungal soil communities, extensive sequencing-based community analysis was conducted, while controlling the influence of plant clonality, plant age, soil condition, and harvesting season. The rhizosphere soils of GM and wild type (WT) poplars at a range of growth stages were sampled together with unplanted, contaminated soil, and the fungal community structures were investigated by pyrosequencing the D1/D2 region of the 28S rRNA gene. The results show that the overall structure of the rhizosphere fungal community was not significantly influenced by GM poplars. However, the presence of GM specific taxa, and faster rate of community change during poplar growth, appeared to be characteristic of the GM plant-induced effects on soil-born fungal communities. The results of this study provide additional information about the potential effects of GM poplar trees aged 1.5–3 years, on the soil fungal community.

Keywords: fungal community, GMO, phytoremediation, metal contamination, NGS

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Introduction

Recently, there has been increasing demand for phytoremediation, which is the use of plants to clean up environmental pollution, for example, by organic compound degradation or heavy metal removal (Cherian and Oliveira, 2005). To increase performance, faster growing and more stress-tolerant plants have been developed through genetic engineering (Cherian and Oliveira, 2005). However, the effect of introducing genetically modified organisms (GMO) into the environment has long been a controversial issue (Wolfenbarger and Phifer, 2000). The possible structural change of indigenous microbial communities by GMO is a major ecological concern, because of the important role that microorganisms have in regulating soil conditions (Conrad, 1996). For example, fungal-plant interactions are very important for plant function, because mycorrhizal fungi are crucial for plant nutrition and tolerance to various abiotic and biotic stresses (Lodge, 1989; Raven *et al*., 1992; Gehring *et al*., 2006). This type of natural symbiotic relationship between plant and fungi exists for over 98% of vascular plants (Raven *et al*., 1992). Thus, understanding how GM plants might alter the soil fungal community is of great interest.

Poplar trees are good model organisms for phytoremediation, and many transgenic poplars have been developed to enhance the phytoremediation of heavy metals (Rugh *et al*., 1998; Cherian and Oliveira, 2005). Because poplars are among the few tree genera that develop both ectomycorrhizal and arbuscular mycorrhizal associations (Karlinski *et al*., 2010), intimate interactions between the fungi and plant during phytoremediation are expected. According to previous reports about the variable colonization ratios of fungi on different poplar species and hybrids, the influence of poplar genotype was found to be minor compared to the influence of environmental factors (Karlinski *et al*., 2010). In 18S rRNA gene or ITS sequencing-based fungal community studies, only a minor difference was found between GM and WT poplar plantations (Oliver *et al*., 2008; Stefani *et al*., 2009). On the other hand, a recent study using the D1/D2 region of the 28S rRNA gene revealed marked fungal community differences between the rhizosphere and endophytes of poplar roots (Gottel *et al*., 2011). The results also showed that rhizosphere fungal communities are quite consistent, regardless of the physicochemical characteristics in host trees and soil, whereas endophyte communities are variable, depending on conditions (Gottel *et al*., 2011).

The majority of recent fungal community studies indicate that genetic modification has no significant impact on rhizosphere microbial communities (Becker *et al*., 2008; Oliver *et al*., 2008; Stefani *et al*., 2009; Weinert *et al*., 2009; Karlinski

et al., 2010; Lottmann *et al*., 2010). However, the majority of studies conducted so far used general molecular techniques, such as T-RFLP, DGGE, ARDRA, and clone-library sequencing. These techniques are useful for evaluating differences in overall community structure, but are not sensitive enough to detect minor changes. Thus, the potential effects of GM plants on microbial communities require evaluation by using extensive sequencing-based experiments. Our recent sequencing-based study showed that the rate of prokaryotic community change was faster in the GM than wild-type (WT) rhizosphere during the phytoremediation of metals by poplars (Hur *et al*., 2011). Thus, further investigation of the fungal community under such environmental conditions would enhance our understanding of the relationship between GMO and indigenous microbes.

The objective of this study was to evaluate the effect of GM plant use on natural fungal soil communities. Hence, the rhizosphere soils of 1.5-, 2.5-, and 3-year-old GM and WT poplars were sampled, together with unplanted, contaminated soil. The fungal community composition was investigated by pyrosequencing the D1/D2 region of the 28S rRNA gene.

Materials and Methods

Soil samples

A schematic diagram of the sampling site is given in Fig. 1. Seven composite soil samples (named CS, WT1.5, WT2.5, WT3.0, GM1.5, GM2.5, and GM3.0), each of which was a mixture of 5 individual soil samples taken from sites with corresponding conditions, were collected in early July 2010. The study site is a trial station belonging to the Korea Forest Research Institute, Korea Forest Service, and is located at Socheon-myeon, Bonghwa-gun, Gyeongsangbuk-do Province, Korea (129° 3′ 17.343″ E, 36° 51′ 46.201″ N, 614 m a.s.l.). Ten years ago, the study site had been filled with zinc mine tailings, after which it was covered with a top layer of uncontaminated soils. Wild-type (WT) poplars and genetically modified (GM) poplars of the non-flowering mutant hybrid (*P. alba* × *P. tremular* var. *glandulosa*) (Choi *et al*., 2007) were planted 1.5, 2.5, and 3.0 years ago. Within the study site, a composite of contaminated soil (CS) samples was obtained from 5 locations without poplars in the metal-contaminated area. Three composite samples (named WT1.5, WT2.5, and WT3.0) comprised rhizosphere soils of WT poplars aged 1.5, 2.5, and 3.0 years, respectively. Three other composite samples (named GM1.5, GM2.5, and GM3) comprised rhizosphere soils of GM poplars aged 1.5, 2.5, and 3.0 years, respectively. Each composite soil was prepared by mixing 5 individual soil samples. The soil within 30 cm of a main root in the comtaminated soil layer was regarded as the rhizosphere, because rootlets measured in this study extend up to 30 cm from the main root. The soil samples were kept on ice until sieving for subsequent analysis, and stored at -80°C. Soil pH, electrical conductivity (EC), total organic carbon content, total nitrogen content, and total Zn concentration were analyzed using 8 composite soils as described previously (Hur *et al*., 2011).

DNA extraction, PCR, and pyrosequencing

The seven composite soil samples were subjected to pyrosequencing. DNA was extracted from 1 g of soil sample with a commercial soil DNA isolation kit (MoBio). The extracted DNA was amplified using primers that targeted the D1 to D2 regions of the eukaryotic 28S rRNA gene (Van der Auwera

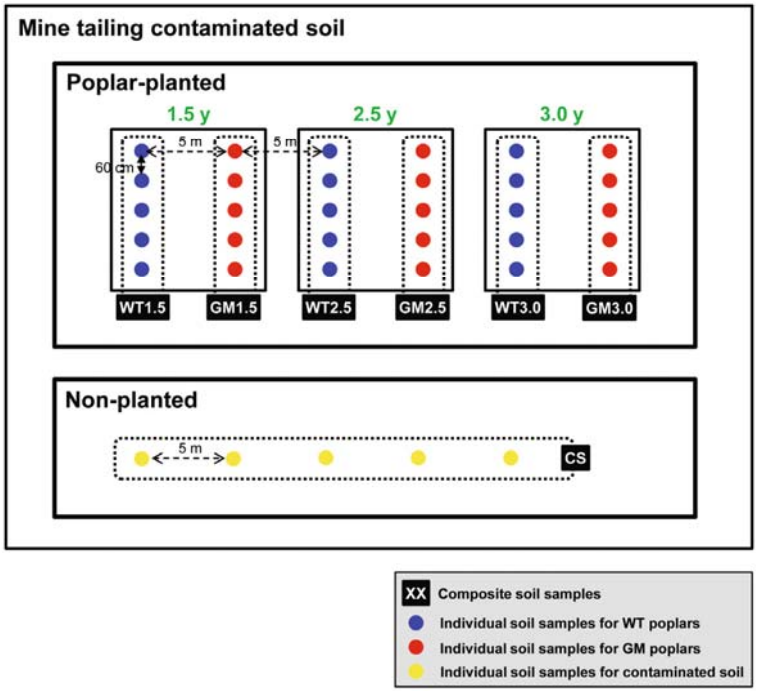

Fig. 1. Schematic diagram of the sampling scheme. Each composite soil sample consisted of five individual soil sub-samples.

Table 1. Summary of sequencing data and diversity indices

	No. of sequencing reads			No. of MOTUs[b]		Pielou's evenness	pH
	Total[a]	Fungal	Sub-sample	Observed	Chao1		
CS	2,163	2,085	556	63	80	0.66	2.95
WT 1.5y	585	556	556	72	89	0.68	2.87
WT 2.5y	7,984	7,575	556	89	122	0.77	4.43
WT 3.0y	5,589	4,909	556	142	256	0.84	5.52
GM 1.5y	623	538	538	109	193	0.73	4.01
GM 2.5y	560	525	525	117	235	0.73	5.34
GM 3.0y	632	441	441	126	242	0.80	6.77

[a] Excluding chimeric and low quality (< 300 bp and Ns ≥ 2) sequences.
[b] MOTU was a group of sequences showing less than 1% sequence differences.

et al., 1994). The primers used were LSU-26F (CCTATCC CCTGTGTGCCTTGGCAGTC-TCAG-TG-ACCCGCTG AAYTTAAGCATAT, with the underlined section indicating the gene specific section) and LSU-657R (CCATCTCATC CCTGCGTGTCTCCGAC-TCAG-*X*-GA-CTTGGTCCGT GTTTCAAGAC, with an *X* barcode being uniquely designed for each subject). PCR reactions were carried out under the following conditions: initial denaturation at 94℃ for 5 min, followed by 10 cycles of denaturation at 94℃ for 30 sec, annealing at 60℃ to 55℃ with a touchdown program for 45 sec, and elongation at 72℃ for 90 sec. This was followed by an additional 20 cycles of denaturation at 94℃ for 30 sec, annealing at 55℃ for 45 sec, and elongation at 72℃ for 90 sec. The amplified products were purified using resin columns (Qiagen) and subjected to DNA pyrosequencing. Seven sequencing libraries were mixed equally (1 μg of PCR product for each subject) and sequenced simultaneously without replication. DNA sequencing was performed by Chunlab Inc. (Seoul, Korea) with a Roche/454 GS FLX Titanium platform, according to the manufacturer's instructions.

Processing of sequencing data

The sequencing reads were sorted by the unique barcodes. Then, the sequences of the barcode, linker, and PCR primers were removed from both sides of the original sequencing reads. The resultant sequences were subjected to a filtering process where only reads containing 0–1 ambiguous base calls (Ns) and 300 or more base pairs were selected for the final bioinformatic analyses. Chimeric sequences and non-specific PCR amplicons, which showed no match in the NCBI nt database, were removed from the analyses. Random subsampling was conducted to equilibrate fungal sequence coverage and to facilitate abundance-based community comparison. All of the sequence analyses were performed using this subset. For the molecular operational taxonomic unit (MOTU) diversity analyses, the pre-processed subset sequences were further clustered as phylotypes at a 99% similarity threshold using CD-Hit (Li and Godzik, 2006). Diversity and richness indices were based on the clustering results, and were calculated using the PRIMER v6 program (PRIMER-E Ltd.). For the taxonomic assignment of the 28S rRNA gene sequences, each of the sub-sampled fungal sequences was searched against an in-house 28S rRNA gene database using the BLASTN program (expectation value of $>e^{-5}$). The in-house database was constructed by collecting fungal 28S rRNA gene sequences from the NCBI nt database, while excluding sequences defined as environmental, uncultured, or unidentified fungi. The result of the BLAST search was visualized using MEGAN software (Huson *et al.*, 2007), and was based on the taxonomic hierarchy of the NCBI taxonomy. The distribution of the sequences was represented by trees and pie diagrams provided in MEGAN. A phylogenetic tree was constructed using the neighbor-joining (Saitou and Nei, 1987) method, which was implemented in the MEGA program (Kumar *et al.*, 2008) after the alignment of sequences using CLUSTALW (Thompson *et al.*, 2002) and jPHYDIT (Jeon *et al.*, 2005) programs. The overall phylogenetic distance between each pair of communities was estimated using the Fast UniFrac web interface (Hamady *et al.*, 2010). The correlation between community structure and soil physicochemical features was analyzed using the R program.

Results and Discussion

The total number of reads that remained after pre-processing varied according to the samples. Thus, the sequence coverage equilibrated subsets (n=556 sequences per sample) were used

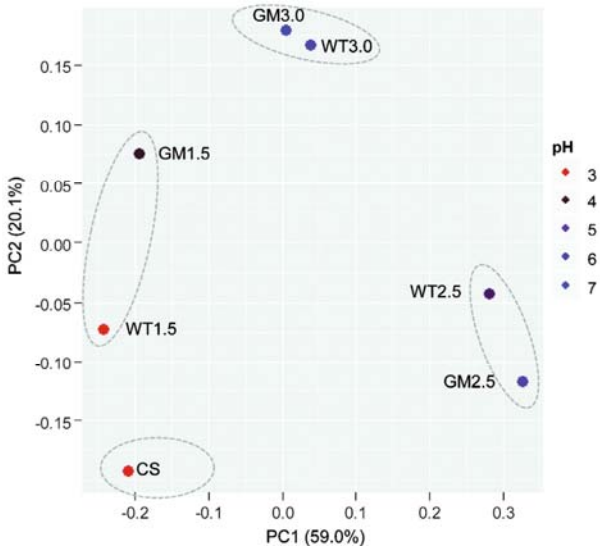

Fig. 2. Principal coordinate analysis (PCoA) of the fungal community. The weighted pairwise UniFrac distances were used for the distance matrix. Samples are color-coded according to the soil pH gradient.

for the analyses. Rarefaction analyses were performed to evaluate the depth of sequencing and to compare the relative fungal community diversity in different samples (data not shown). When a species-level MOTU was defined using a threshold of 99% nucleotide sequence similarity, 63 to 142 phylotypes were found, depending on samples (Table 1). Although the gradients of collector's curves decreased as the number of sequences increased, the number of phylotypes continued to increase even for the highest numbers of sampled sequences. This result indicates that the underlying sequence diversity of the actual fungal population was not fully encompassed by the experiment. The Chao1 estimator of species richness (Colwell and Coddington, 1994) ranged from 80 to 256, indicating that about 1.6-fold more fungal species might be present in the tested soils compared to those observed in the present study. Overall, slightly greater fungal MOTU richness was observed for GM compared to WT poplars. The greater richness of phylotypes for GM compared to WT poplars is congruent with a previous report that investigated the rhizosphere of 5-month-old aspen (Oliver *et al.*, 2008). In particular, the number of estimated phylotypes for 1.5- and 2.5-year poplars was remarkable (Table 1), indicating higher diversity in GM than WT poplars during the early growth stage. In comparison, the Pielou's evenness index suggests an unclear differentiation in the level of dominance by some fungal species for GM and WT poplars.

The UniFrac-based PCoA showed that variation in the fungal community was strongly associated with poplar age, but not poplar type (Fig. 2). The 7 samples were separated into 4 groups; the first contained contaminated soil; the second contained 1.5-year soils; the third contained 2.5-year soils; and the fourth contained 3-year soils. In addition to poplar age, soil pH strongly influenced variability in fungal community structure. The contaminated soil was acidic, and gradually became neutral as poplars aged (Fig. 2). Among the soil physicochemical properties analyzed in this study, only pH showed a linear correlation with the ordination graph groupings (data not shown). Thus, the pH gradient generated by the neutralization of soil during phytoremediation appears to be a fundamental means of controlling fungal community structure. The results of this study were congruent with previous reports, in which GM poplar plantations only had a minor effect on fungal community structure compared to other environmental factors (Oliver *et al.*, 2008; Stefani *et al.*, 2009; Karlinski *et al.*, 2010). However, the positioning of GM1.5 in the ordination was noteworthy. GM1.5 was positioned at a greater distance to CS than WT1.5. In a related study using prokaryotes, a faster rate in community change was observed for GM poplars (Hur *et al.*, 2011). This phenomenon is possibly explained by a higher rate and greater extent of metal accumulation in GM compared to naturally occurring plants. This, in turn, would result in a greater change to the soil environment, and hence the microbial habitat. Thus, the positioning of GM1.5 might indicate a faster rate of fungal community change during the early growth stage of GM poplars (Table 1). However, the number of soil samples used in this study was insufficient to validate this theory. Further study using experimental replicates, in addition to taking regional and individual variability into consideration, would be required.

Overall, the phyla Dikarya (Ascomycota and Basidiomycota) dominated (98.1%, average) the fungal community of tested soils, followed by Chytridiomycota (0.9%), Glomeromycota (0.2%), and Neocallimastigomycota (0.2%) (Fig. 3A). The composition of fungal phyla varied depending on the growth stage of poplars. In CS and rhizospheres of 1.5-year-old poplars, the proportion of Basidiomycota (5.5–6.2%) was remarkably lower than Ascomycota (92.0–93.0%). However, the proportion of Basidiomycota explosively increased at 2.5 years for both WT (43.2%) and GM (49.0%) poplars, and then decreased at 3 years (24.6% for WT; 19.7% for GM). The fungal class responsible for this phenomenon was the Agaricomycetes, which showed the same pattern of change as the Basidiomycota (Fig. 3B). Aside from Agaricomycetes,

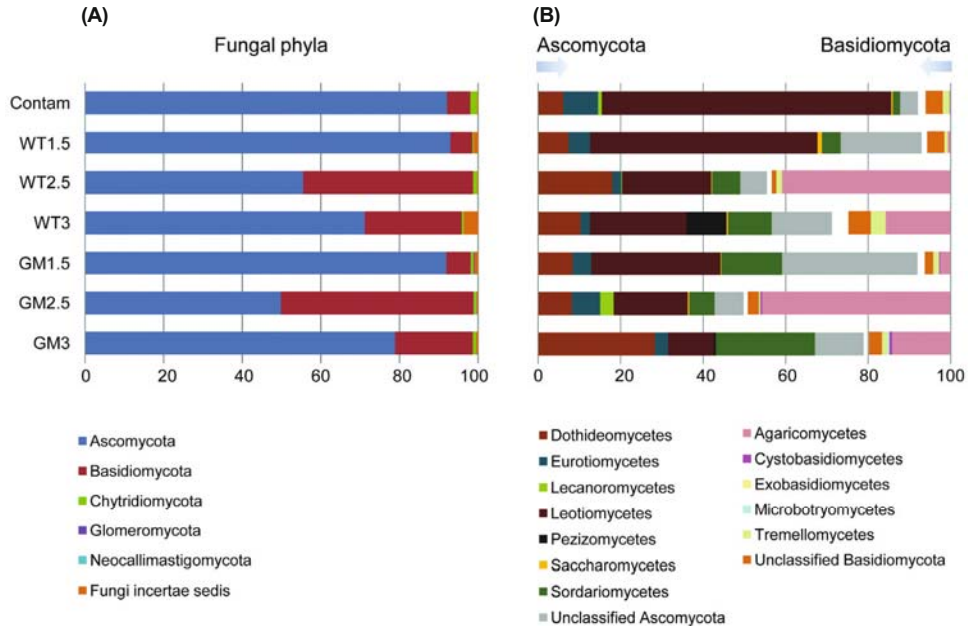

Fig. 3. Relative abundance of fungal taxa identified in contaminated unplanted soil (CS), wild type (WT), and genetically modified (GM) poplar soils: (A) Fungal phyla composition, (B) Composition of fungal classes belonging to Ascomycota and Basidiomycota.

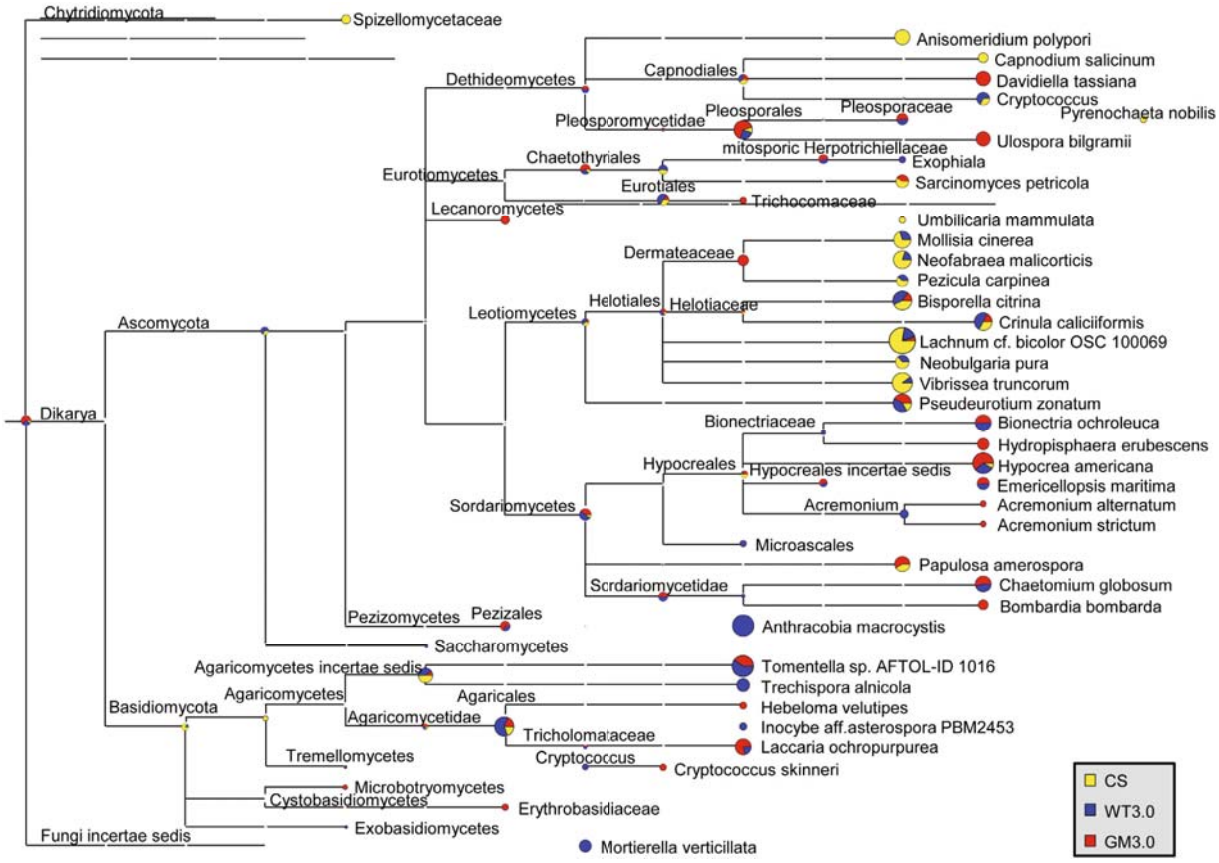

Fig. 4. Schematic phylogenetic tree of pyrosequencing reads based on a BLASTN search against the NCBI nt database. The tree was constructed using MEGAN software. The size of the pie chart at each taxonomic node is proportional to the number of assigned reads. CS (yellow), WT3.0 (blue), and GM3 (red) samples were displayed as representatives of the seven samples investigated in this study.

small numbers of Cystobasidiomycetes (0.0–0.7%), Exobasidiomycetes (0.0–0.2%), Microbotryomycetes (0–0.5%), and Tremellomycetes (0.4–3.4%) were also detected.

The association of Ascomycota abundance with poplar tree age was from changes in the abundance of 3 dominant fungal classes, namely Leotiomycetes (11.1–70.1%), Dothideomycetes (6.1–28.3%), and Sordariomycetes (1.8–24.0%) (Fig. 3B). For example, Leotiomycetes was the most dominant fungal class in CS (70.1%), with its abundance gradually decreasing as poplars grew. In comparison, the abundance of Dothideomycetes and Sordariomycetes increased as the poplars grew. Other minor classes that showed no apparent change in abundance were also detected, including Eurotiomycetes (2.1–8.5%), Lecanoromycetes (0.0–0.9%), Pezizomycetes (0.0–9.7%), and Saccharomycetes (0.0–0.4%).

The full taxonomic contents of the sequencing reads were visualized by the schematic phylogenetic tree drawn by MEGAN (Fig. 4 and Supplementary data Fig. S1). The fungal taxa that were exclusively detected in CS were Spizellomycetaceae and *Pyrenochaeta nobilis*. Many fungal species are known to tolerate and detoxify metals (Ashida, 1965; Gadd, 1993) and/or live in extreme acidic habitats, with pH values <3 (Gross and Robbins, 2000). Thus, while no study has been reported for the metal or acid resistance of these 2 taxa, our study results indicate that they might be metal resistant and/or acidophilic fungi.

Interestingly, the class Cystobasidiomycetes was only detected in the rhizosphere of GM poplars, exhibiting a gradual increase in abundance as poplars grew (0.2, 0.4, and 0.7% in GM1.5, 2.5, and 3 poplars, respectively; Figs. 3B and 4). Thus, the presence of a GM-specific taxon might indicate GM plant-induced effects on soil-born fungal communities. In addition, several fungal species were detected exclusively in GM poplars, whereas only 1 taxon was exclusively present in WT poplars. *Ulospora bilgramii*, *Hydropisphaera erubescens*, *Acremonium strictum*, *Bombardia bombarda*, and *Cryptococcus skinneri* were also only detected in the rhizospheres of GM poplars. The taxon that was exclusive to WT poplar rhizospheres was *Trechispora alnicola*. However, the biological replicates or sequencing coverage obtained in this study was insufficient to confirm that the presence/absence of those taxa was specifically related to poplar genotype.

The majority of recent fungal community studies suggest that the influence of poplar genotype is minor compared to the influence of environmental factors, and that the observed differences do not exceed natural variability (Becker *et al.*, 2008; Oliver *et al.*, 2008; Stefani *et al.*, 2009; Weinert *et al.*, 2009; Karlinski *et al.*, 2010; Lottmann *et al.*, 2010). The effects

detected to date are known to have originated from agricultural practices, season, plant developmental stage, soil type, or plant genotype rather than actual genetic modification (Lottmann *et al*., 2010). Thus, in this study, the influence of environmental factors was restricted by controlling plant clonality, plant age, soil condition, and harvesting season. Furthermore, highly sensitive extensive-sequencing was employed as a detection technique. The minor effects discovered in this study were meaningful in this context; however, sample size was insufficient to obtain statistical significance. More studies using biological duplicates would be required to provide convincing conclusions with respect to potential fungal community changes from the effects of genetic modification.

In conclusion, the overall structure of the rhizosphere fungal community was not significantly influenced by GM poplars. However, minor changes in fungal diversity were detected in association with the genetic modification of trees, such as the presence of GM-specific taxa and the faster rate of community change during poplar growth. Although further systematic investigation is required to determine the biological consequences for these minor changes, the results of this study provide new insights about the potential effects of GM plants on soil fungal communities.

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