RESEARCH ARTICLE

Structural and functional variation in soil fungal communities associated with litter bags containing maize leaf

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Abstract

Soil fungi are key players in the degradation of recalcitrant organic matter in terrestrial ecosystems. To examine the organisms and genes responsible for complex organic matter degradation in soil, we tracked changes in fungal community composition and expressed genes in soil adjacent to mesh bags containing maize leaves undergoing decomposition. Using high-throughput sequencing approaches, changes in fungal community composition were determined by targeting 18S rRNA gene sequences, whereas community gene expression was examined via a metatranscriptomic approach. The majority of the 93 000 partial 18S rRNA gene sequences generated, were affiliated with the *Ascomycota* and *Basidiomycota*. Fungal diversity was at least 224 operational taxonomic units at the 97% similarity cutoff level. During litter degradation, the relative proportion of *Basidiomycota* increased, with a decrease in *Ascomycota* : *Basidiomycota* ratios over time. The most commonly detected decomposition-associated fungi included *Agaricomycetes* and *Tremellales* as well as unclassified *Mucoromycotina*. The majority of protein families found in the metatranscriptomic data were affiliated to fungal groups described to degrade plant-derived cellulose, such as *Mucoraceae*, *Chaetomiaceae*, *Sordariaceae*, *Sebacinaceae*, *Tremellaceae*, *Psathyrellaceae* and *Schizophyllaceae*. The combination of high-throughput rRNA gene-based and metatranscriptomic approaches provided perspectives into the organisms and genes involved in complex organic matter in soil.

Introduction

Soil fungi play several critical roles in terrestrial ecosystems, influencing important ecosystem processes such as nutrient acquisition, nitrogen and carbon cycling, and soil structure formation (van der Heijden *et al.*, 2008). They represent the majority of microbial biomass in many soils, decompose organic material, provide nutrients to plants and are indicators of ecosystem health (Verbruggen *et al.*, 2010). Litter-decomposing fungi are the primary players of organic matter decomposition in the upper layers of soils, thereby converting it into fungal biomass, organic acids, carbon dioxide and nutrients. Fungi belonging to the *Basidiomycota* are especially important for decomposition as they are among the few organisms that possess the ligninocellulolytic enzymes necessary to degrade residual plant biopolymers such as lignin (Baldrian, 2006; Lundell *et al.*, 2010). White rot fungi are especially well known for their secretion of oxidoreductases, such as lignin peroxidase and manganese peroxidase. The examination of enzymes from fungal isolates active in litter degradation (Mahajan & Master, 2010) and cultivation- or molecular-based analyses of fungal communities in soils and during organic matter degradation (Valaskova *et al.*, 2007; Hannula *et al.*, 2010) have provided insight into some of the players and activities involved. However, direct, in-depth analyses of these organisms and their activities in agriculture soils are scarce, except for studies

in forest soils on expressed fungal genes under *N* input (Edwards *et al.*, 2011), expressed cellobiohydrolases (Baldrian *et al.*, 2012) and forest transcriptomes (Damon *et al.*, 2012). It is anticipated that such knowledge of fungal decomposition will aid in our understanding of the impact of land management on nutrient cycling and in the development of biological indicators for these important ecosystem functions.

Despite the recent advance in the understanding of fungal-mediated organic matter decomposition, the majority of studies to date have generally lacked the depth or breadth of focus necessary to understand these complex activities. So far phylogenetic analyses of fungal communities via PCR-based techniques targeting ITS and 18S rRNA gene sequences have helped to provide a cultivation-independent perspective to the analysis of soil fungal communities (Buee *et al.*, 2009, 2011; Lim *et al.*, 2010; Jumpponen, 2011). However, such strategies provide little functional insight and are subject to PCR and cloning biases. To provide a more functional perspective, enzyme assays have been used to examine the development of fungal activities during organic matter degradation, with the focus primarily on peroxidases (i.e. lignin, manganese) and laccases (Pelaez *et al.*, 1995; Steffen *et al.*, 2000; Snajdr *et al.*, 2008; Sinsabaugh, 2010). However, such approaches are restricted to a rather narrow range of enzymes that are already suspected of being important in organic matter degradation, and it is not known how well assay conditions and resulting *in vitro* measurements relate to actual activities in the soil. The recent introduction of functional gene-based molecular assays for the interrogation of laccase and peroxidase genes (Kellner *et al.*, 2009) represents an important step forward in the examination of soil-borne fungal degraders, but even the most involved studies (Kellner & Vandenbol, 2010) are limited to the PCR-based examination of a limited suite of known genes.

Pyrosequencing-based methods for phylogenetic and functional analysis of microbial communities offer a number of advantages over previously used methods and open up the possibility for more comprehensive examinations of soil-borne microbial communities and their activities (Urich *et al.*, 2008; Bates *et al.*, 2011; Dumbrell *et al.*, 2011; Nacke *et al.*, 2011). Using such methods, much greater depth of coverage can be achieved in rRNA gene-based surveys (Roesch *et al.*, 2007; Lauber *et al.*, 2009). The application of high-throughput sequencing strategies has recently greatly expanded our appreciation of fungal diversity in soil habitats (Buee *et al.*, 2009; Lim *et al.*, 2010; Tian *et al.*, 2010; Dumbrell *et al.*, 2011; Verbruggen *et al.*, 2012). Furthermore, the development of shot-gun metagenomic and metatranscriptomic approaches has afforded a new perspective into the functional capacities and expressed activities, respectively, in complex microbial communities (Falkowski *et al.*, 2008; DeLong, 2009). These technical advances offer unprecedented opportunities to examine the activities of the fungal communities that drive critical steps in the degradation of complex organic compounds in soil.

To gain information on the organisms and genes involved in the degradation of complex organic matter in soil, the present study combines 18S rRNA gene fragment sequencing and eukaryote-targeted metatranscriptomic approaches. We hypothesized that soil communities adjacent to litter-containing mesh bags would be enriched over time in fungal taxa involved in litter degradation, especially specific *Basiodiomycetes*, and that community mRNA pools would be enriched for transcripts encoding enzymes involved in organic matter degradation. We firstly used a PCR-based pyrosequencing approach, using fungal-specific 18S rRNA gene primers, to gain phylogenetic coverage of the fungal communities responding to litter degradation. Secondly, we applied a eukaryote-specific metatranscriptomic approach in the same soils to provide detailed information about the protein families associated with maize litter undergoing decomposition.

Material and methods

Plant material

Maize DKC3420 variety was grown in the greenhouse (day/night: 16 h/8 h, 21 °C/16 °C). Maize seeds were sown in December 2007, and leaves were harvested in May 2008 (plants started to senesce) by cutting the stems at ± 10 cm above the soil. The leaves were separated from the stems, cut into pieces of about 1 cm and dried in a paper bag at 40 °C for 3 days. After drying, leaves were stored in a dry air controlled room until being used in the experiment.

Mesh bag incubation experiment

Soil used in this study (100 kg) was collected in December 2008 from an organic farm field used for agricultural purposes located in Helvoirt, Brabant, the Netherlands (51°38′0″N, 5°14′0″E). The soil chemical properties are as follows: total N = 1470 mg kg^{-1}, C : N ratio = 16, P = 80 mg P_2O_5 l^{-1}, K = 146 mg kg^{-1}, Mg = 114 mg kg^{-1}, Na = 15 mg kg^{-1}, pH = 5.9. Soil was taken from the upper 10-cm layer of the field. Dried cut leaves (3.50 g) were placed into 25 × 12.5 cm litterbags with a mesh size of 45 μm. Five litter bags with maize leaves were buried (8 cm depth) in each of five replicate boxes (50 × 40 cm) in soil in greenhouse (day/night: 16 h/8 h,

21 °C/16 °C). The controls were soils in boxes without mesh bags. Five soil samples (50 mL each) adjacent (attached) to litterbags were collected at 0, 21 and 70 days. A second set of soil samples was collected after 70 days of incubation: with no leaves ('bulk soil') and soils adjacent to bags containing maize leaves ('adjacent'). Samples were transferred into 50-mL tubes and immediately frozen at −80 °C.

Three replicates of 'bulk soil' and three replicates of 'adjacent' samples at 70 days were measured for laccase activity according to Bourbonnais & Paice (1990) and for manganese peroxidase activity according to Ngo & Lenhoff (1980).

DNA isolation and real-time PCR

DNA was extracted from a 2-g subsample from each of the five replicates, using the PowerSoil total DNA isolation kit (Mo Bio Laboratories, Inc.). DNA quality was checked by agarose gel (2%) electrophoresis and quantified on a ND-1000 spectrophotometer (Nanodrop Technology, Wilmington, DE). The resulting DNA extracts (20 ng μL^{-1}) were used as template for PCR using the EF4 and EF3 primers (Smit *et al.*, 1999) for partial 18S rRNA gene amplification. In this study, we used the EF4/EF3 primer set because it has been shown to be less biased than other 18S rRNA primer sets (Hagn *et al.*, 2003; Anderson & Cairney, 2004). Another reason to target a portion of the nuclear 18S rRNA (SSU) gene region was because of the availability of a well-curated and annotated database for this gene: the SILVA database (www.arb-silva.de) facilitates downstream phylogenetic and comparative analyses. PCR amplifications were carried out with 10 mM dNTP, 0.125 units of Hot Start *Taq* Polymerase (Roche), and 1.0 mM of each primer. PCR amplifications were performed via the following thermocycling scheme: an initial denaturation at 95 °C (5 min), 35 amplification cycles of denaturation 95 °C (30 s), annealing at 56.7 °C (30 s), and extension at 72 °C (60 s), followed by a final extension at 72 °C (10 min). Negative controls were carried out with water instead of DNA.

PCR products were purified using the MiniElute Reaction Cleanup Kit (Qiagen 28204) and pooled to a total of 5 μg per sample. Because the primers EF4 and EF3 amplify approximately 900-bp fragments of the 18S rRNA gene, the PCR products were fragmented and sequenced using a Roche 454 GS FLX system using titanium chemistry (454 Life Sciences, Branford, CT; Macrogen Inc. Company, South Korea). Each sample was run in one lane on a 454 pyrosequencing plate. Real-time PCR quantifications for total bacteria and total fungi were performed as previously described (Kuramae *et al.*, 2012).

RNA isolation, mRNA amplification and cDNA synthesis

Soil samples from each of the five replicates per time point from the second set of samples (bulk soil and soil with leaves at 70 days) were extracted separately. Two grams of soil from each replicate were subjected to RNA extraction using the RNA PowerSoil total RNA isolation kit (Mo Bio Laboratories, Inc.). DNA of each sample was removed by *DNase* I treatment (Qiagen) according to the manufacturer's guidelines. Total RNA was quantified on an ND-1000 spectrophotometer (Nanodrop Technology) and quality checked using the Experion system (BioRad).

The total RNA of five replicates per treatment was pooled and a 1-μg aliquot of total RNA was used for mRNA amplification using the MessageAmp™ II aRNA Amplification Kit (Ambion, Inc.). As the amplified mRNA quantity was not sufficient for pyrosequencing, a second round of amplification was carried out using 2 μg of amplified mRNA. A total of 2 μg of amplified mRNA was used for cDNA synthesis. The polyA cDNA synthesis was carried out according to the Joint Genome Institute guidelines for cDNA library construction adapted for the 454 sequencing protocol (http://my.jgi.doe.gov/general/): 2 μg polyA RNA was first-strand synthesized by adding 1 μL $dT_{15}VN_2$ primer (50 μM) and 1 μL 10 mM dNTP, and incubated at 65 °C, for 5 min and 4 °C for 2 min. Then, 4 μL of 5 × First-Strand buffer, 1 μL 0.1 M dithiothreitol (DTT), 1 μL RNaseOUT (40 U μL^{-1}), 2 μL SuperScript III RT (200 U μL^{-1}; Invitrogen) were added and incubated at 50 °C for 1 h and 70 °C for 15 min. Second strand synthesis was carried out in 150 μL total volume by adding to the previous volume, 30 μL 5 × Second-Strand buffer, 3 μL 10 mM dNTP, 1 μL *Escherichia coli* DNA ligase (10 U μL^{-1}), 4 μL *E. coli* DNA polymerase (10 U μL^{-1}), 1 μL *E. coli RNase*H (2 U μL^{-1}; Invitrogen) and incubated at 16 °C for 2 h. Then, 2 μL T4 DNA polymerase (5 U μL^{-1}; Invitrogen) was added and incubated at 16 °C for 5 min. The cDNA was purified by using a MiniElute Reaction Cleanup Kit (Qiagen) and sequenced on a Roche 454 GS FLX system using titanium chemistry (454 Life Sciences; Macrogen Inc. Company) as for 18S rRNA amplicons.

Data analysis

Overview of OTUs using 18S rRNA gene sequences

The 18S rRNA gene reads were filtered and analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) software package (Caporaso *et al.*, 2010). First, signal noise in the 18S rRNA gene sequences arising from

PCR and pyrosequencing was removed using the PYRONOISE software program (Quince *et al.*, 2009). Secondly, the sequences were checked for PCR chimeras using UCHIME (Edgar *et al.*, 2011). All reads starting with one of the primer sequences were subjected to flowgram clustering to group highly identical reads only differentiating in stretches of homopolymers or single nucleotide insertions or deletions. The longest read in each cluster was used to represent that cluster when constructing operational taxonomical units (OTUs).

Similar sequences were clustered into OTUs using a 97% identity threshold with UCLUST (Edgar, 2010). The most abundant sequence from each OTU was selected as a representative sequence. Taxonomy was assigned to each OTU using the Basic Local Alignment Search Tool (BLAST; Altschul *et al.*, 1990) for each representative sequence against a database of 18S rRNA gene sequences from the SILVA database (Pruesse *et al.*, 2007). OTUs were grouped at different taxonomic levels and visualized using the QIIME software. Statistical differences in 18S rRNA gene profiles of pairwise comparisons of samples were calculated using the Fisher Exact test with false discovery rate (FDR) correction implemented in the Statistical Analysis of Metagenomic Profiles (STAMP) software tool (Parks & Beiko, 2010). The diversity within each sample (alpha diversity) was evaluated using the observed species metric (count of unique OTUs in each sample), richness (Chao1), Shannon (diversity) and evenness were calculated in QIIME. The percentage of coverage was calculated by Good's method (Good, 1953).

Metatranscriptome analysis

Open reading frames (ORF) were predicted from metatranscriptomic sequences by six-frame translation in a similar way as the Global Ocean Survey (GOS) study (Rusch *et al.*, 2007). An ORF is commonly defined as a translated DNA sequence that begins with a start codon and ends with a stop codon. However, because pyrosequencing reads typically do not yield complete ORFs, we mostly detected only partial ORFs. These were defined as beginning with either a start codon or the beginning of a read and ending with a stop codon or the end of the read.

Sequences were annotated by comparison with protein families (Pfam) using HMMSEARCH and with the Kyoto Encyclopedia of Genes and Genomes (KEGG) using BLAST. A sequence was annotated if it matched with an expected value $< 10^{-4}$. Statistical differences in annotation profiles of pairwise comparisons of samples were calculated using the FDR correction implemented in the STAMP software tool (Parks & Beiko, 2010).

Searches for glycoside hydrolases (GHs) and carbohydrate-binding modules (CBMs) were performed as described before (Warnecke *et al.*, 2007). Briefly, database searches were performed using HMMER hmmsearch with Pfam HMMs (full-length models) to identify complete matches to the family, which were named in accordance with the CAZy nomenclature scheme (Cantarel *et al.*, 2009). All hits with E-values $< 10^{-4}$ were counted and their sequences further analyzed. For those GH and CBM families for which there is currently no Pfam HMM, the representative sequences selected from the CAZy web site and described by Warnecke *et al.* (2007) were used in BLAST searches of the metatranscriptomic data to identify these GH and CBM families. An E-value cutoff of 10^{-4} was used in these searches.

The reads of the most dominant Pfam and KEGG categories were translated in all reading frames and compared against the NCBI nr database using BLASTX with an E-value cutoff $< 10^{-8}$ in order to bin the proteins into taxonomical groups using MEGAN software (Huson *et al.*, 2007).

Results

DNA-based assessment of fungal community structure

The fungal : bacterial ratio, as determined by real-time PCR, increased across the time course of the litter incubation (Supporting Information Table S1). At day zero, 49% of all 18S rRNA genes sequences were affiliated with the *Ascomycota*. The second most abundant phylum was *Basidiomycota,* representing 28% of the sequences. At day 21 of maize litter decomposition, there was a decrease in the relative representation of *Ascomycota* sequences and an increase in sequences affiliated with the *Basidiomycota*. *Ascomycota* represented 38% and *Basidiomycota* 35% of the fungal sequences on day 21. At day 70 of decomposition, the percentage of *Ascomycota* sequences was similar (36%) to day 21, whereas the percentage of *Basidiomycota* was higher (46%) than day 21 (35%) and day zero (28%). The ratios of *Ascomycota* : *Basidiomycota* were 1.77, 1.09 and 0.79 for days zero, 21 and 70, respectively. The percentages of OTUs at finer taxonomic levels are illustrated in Fig. 1.

OTU richness and diversity

A total of 130 489, 111 243 and 120 430 18S rRNA gene fragment reads were generated from samples taken at days 0, 21 and 70, respectively. The average read length was 251 bp. There was a slight bias for reads containing the EF3 primer sequence as compared with the EF4 primer sequence (Table S2), and, in total, around 30% of all reads started with one of the two primer sequences.

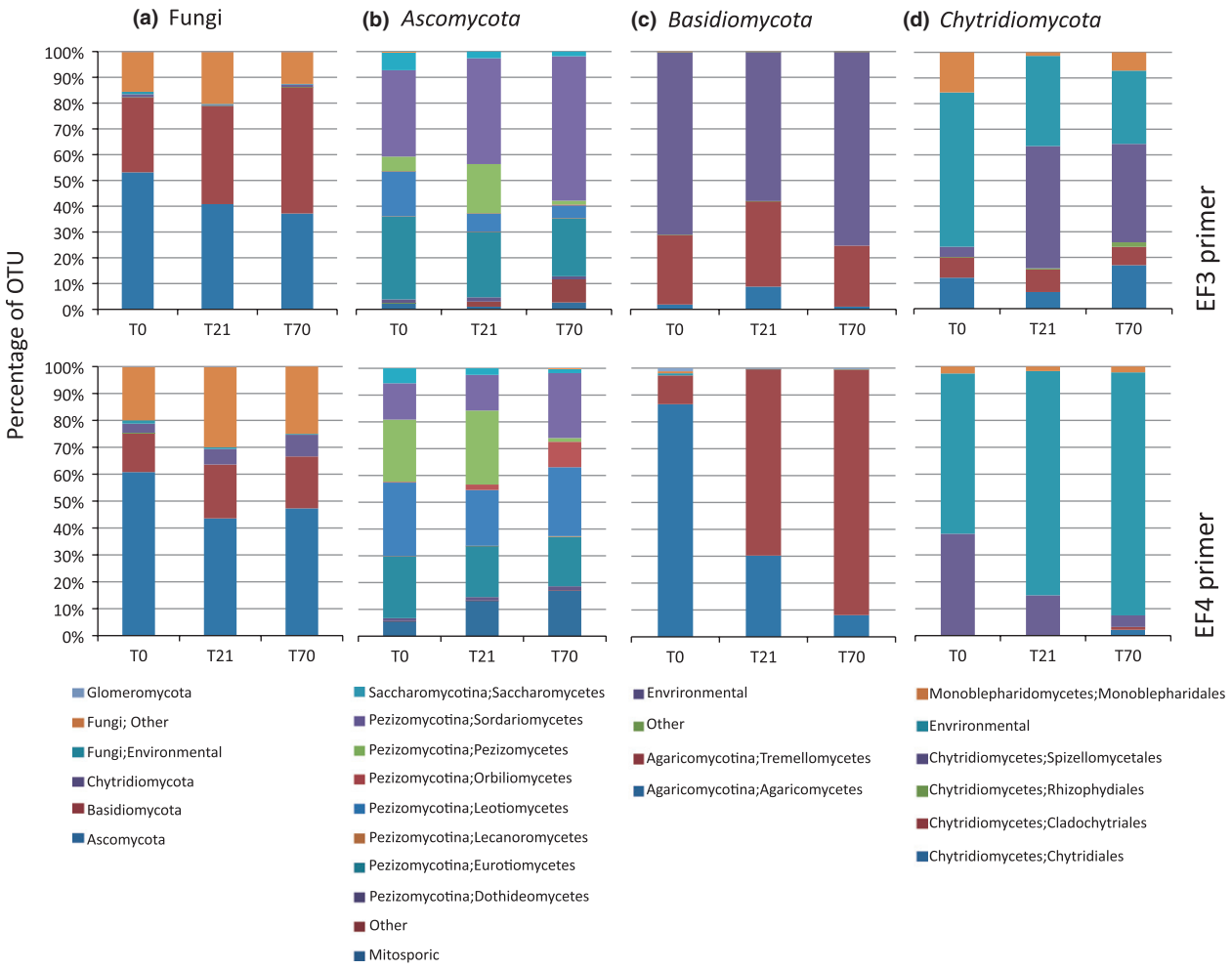

Fig. 1. Percentage of OTUs assigned to fungi (a), *Ascomycota* (b), *Basidiomycota* (c), *Chytridiomycota* (d) with primers EF4 and EF3 in DNA extracted from soil adjacent to mesh bags containing maize leaves collected at day zero (T0), day 21 (T21) and day 70 (T70). Categories with fewer than 2% of the total sequences are not shown.

To minimize effects of analytical errors, we excluded the reads that did not contain one of the primers. From all sequence reads starting with one of the primer sequences (EF4 or EF3), noise that arose from PCR (chimeras) and pyrosequencing techniques were removed by pre-clustering the patterns of light intensities (flowgrams) emitted during the incorporation of complementary bases. The clustering of flowgrams helps to prevent an overestimation of OTU numbers related to sequence error. The EF3 primer targets more OTUs of *Basidiomycota* than does the EF4 primer, and the EF4 primer targets more OTUs belonging to *Ascomycota*, *Chytridiomycota* and other fungi (not classified according to SILVA database) than *Basidiomycota* (Fig. 1).

There were some systematic differences in the range of sequences obtained via the two primers. These included a greater representation of environmental *Basidiomycota* among the *Basidiomycota* sequences recovered with the EF3 primer (Fig. 1c). In addition, *Ascomycota* sequences recovered with the EF4 primer were mainly affiliated with the *Leotiomycetes, Orbiliomycetes* (nematophagous fungi) and *Pezizomycetes* (Fig. 1b), and *Chytridiomycota* sequences with this primer mainly represented by environmental *Chytridiomycota* (Fig. 1d). The term environmental *Basidiomycota* and *Chytridiomycota* has been assigned to environmental sequences added to the database that have not yet received a taxonomic affiliation within the SILVA database.

Using a threshold of 97% sequence identity, the denoised sequence reads from the three samples yielded different numbers of OTUs (Table 1). *Ascomycota* and *Basidiomycota* groups represented the largest numbers of OTUs, with 225 and 72 OTUs, respectively. Trends with respect to the Shannon estimator of fungal diversity

Table 1. Estimators of sequences diversity, evenness and coverage given by different primers EF3 and EF4 at day zero (T0), day 21 (T21) and day 70 (T70) of incubation with decomposing maize leaves

	EF3_T0	EF4_T0	EF3_T21	EF4_T21	EF3_T70	EF4_T70
Number of reads	18 542	16 370	17 836	12 607	17 714	10 099
Number of singletons	1093	1111	1086	1113	1090	1093
Number of OTUs	306	389	272	362	224	348
Chao1 (richness)	395.62	530.28	371.81	520.66	291.24	591.67
Shannon (diversity)	3.97	4.45	4.06	4.90	3.55	4.94
Evenness	0.50	0.53	0.52	0.58	0.47	0.60
Good's coverage estimator	94.11	93.21	93.91	91.17	93.85	89.18

OTU, operational taxonomic unit.

depended on the primer sequence. The EF3 primer yielded the highest diversity for the day 21 sample, whereas EF4 primer gave the highest diversity estimates for the day 21 and 70 samples. Good's estimator suggested > 90% coverage for each of the samples, irrespective of the primer sequence (Table 1).

Soil metatranscriptome

A total of 81 258 and 89 926 transcript reads were obtained from bulk soil and soil adjacent to the mesh bags-containing maize leaves, respectively, with average read lengths of 421 and 423 bp, respectively. All reads were translated in all six reading frames into peptide sequences with a minimum cutoff of at least 30 amino acids. Approximately 30% of all reads of each sample could be assigned to Pfam categories (29 533 reads from bulk soil, 30 941 reads from soil adjacent to mesh bags) and to KEGG orthology groups (29 653 – bulk soil; 33 199 – soil adjacent to mesh bags). A total of 3301 Pfam categories and 3908 KEGG orthology groups were assigned. The functional profiles of bulk soil and soil adjacent to mesh bags were compared to detect statistical differences in the relative abundance of particular protein family transcripts. In all, 169 Pfam categories and 236 KEGGs were significantly different between the two communities (Tables S3 and S4). Pfams that were more expressed in soil adjacent to mesh bags than in bulk soil included (1) genes in biological categories of protein transport, primary metabolic processes (cellular amino acid, carbohydrates, nucleobase, nucleoside and nucleic acid), translation, organelle organization, and response to stress; (2) genes in molecular function categories of catalytic activity (transferases, hydrolases), binding (RNA, DNA, actin, nucleic acid), translation factor, signal transducer (receptor), ion channel transporter, structural molecule and transcription regulator; and (3) genes in cellular component categories of ribosomal genes. The trend in gene ontology for the 169 Pfams was also observed for the top 10 most significantly different transcripts of Pfam and KEGG categories (Table 2, Fig. 2). The shift of the top 10 Pfam transcripts was from general eukaryotes in bulk soil to a more specific group of eukaryotes, namely fungal-binned transcripts in soil

Table 2. Number of transcripts of the top 10 significantly different protein family domains (Pfam) and Kyoto Encyclopedia of Genes and Genomes (KEGG) categories from soil samples incubated for 70 days with no maize leaves (bulk soil) and soil adjacent to mesh bags containing maize leaves (soil leave)

Pfam/KEGG Name	Pfam KEGG	Bulk soil	Adjacent	*P*-values*
Protein kinase domain	PF00069	848	435	1.85e–31
Protein tyrosine kinase	PF07714	628	291	5.20e–29
Thiolase, C-terminal domain	PF02803	37	**197**	1.41e–23
Sugar (and other) transporter	PF00083	84	**276**	5.64e–21
Cell wall-associated hydrolase	PF10695	182	**437**	6.91e–21
Ribosomal protein family	PF01248	8	**93**	3.01e–18
Elongation factor domain	PF03143	215	**461**	1.87e–17
Endomembrane protein 70	PF02990	239	84	4.68e–17
Amino acid permease	PF00324	37	**150**	3.27e–14
Ubiquitin-conjugating enzyme	PF00179	33	**129**	1.42e–11
Acetyl-CoA acyltransferase	K00632	8	**184**	2.72e–37
MFS transporter, SP family, sugar:H+ Symporter	K08139	10	**192**	2.72e–37
Amino acid transporter, AAT family	K03293	24	**192**	5.72e–27
Elongation factor EF-1 alpha subunit	K03231	346	**742**	2.70e–22
S-adenosylmethionine synthetase	K00789	167	58	1.23e–13
Glutamine synthetase	K01915	21	**120**	3.67e–13
Adenosylhomocysteinase	K01251	207	88	7.39e–13
GINS complex subunit 2	K10733	36	0	7.39e–10
Unclassified	K07119	42	**143**	2.73e–09
1-Phosphatidylinositol-4-phosphate 5-kinase	K00889	176	82	3.85e–09

Bold = Pfam and KEGGs more present in soil adjacent to mesh bags containing maize leaves than bulk soil.
**P*-value with false discovery rate (FDR) correction.

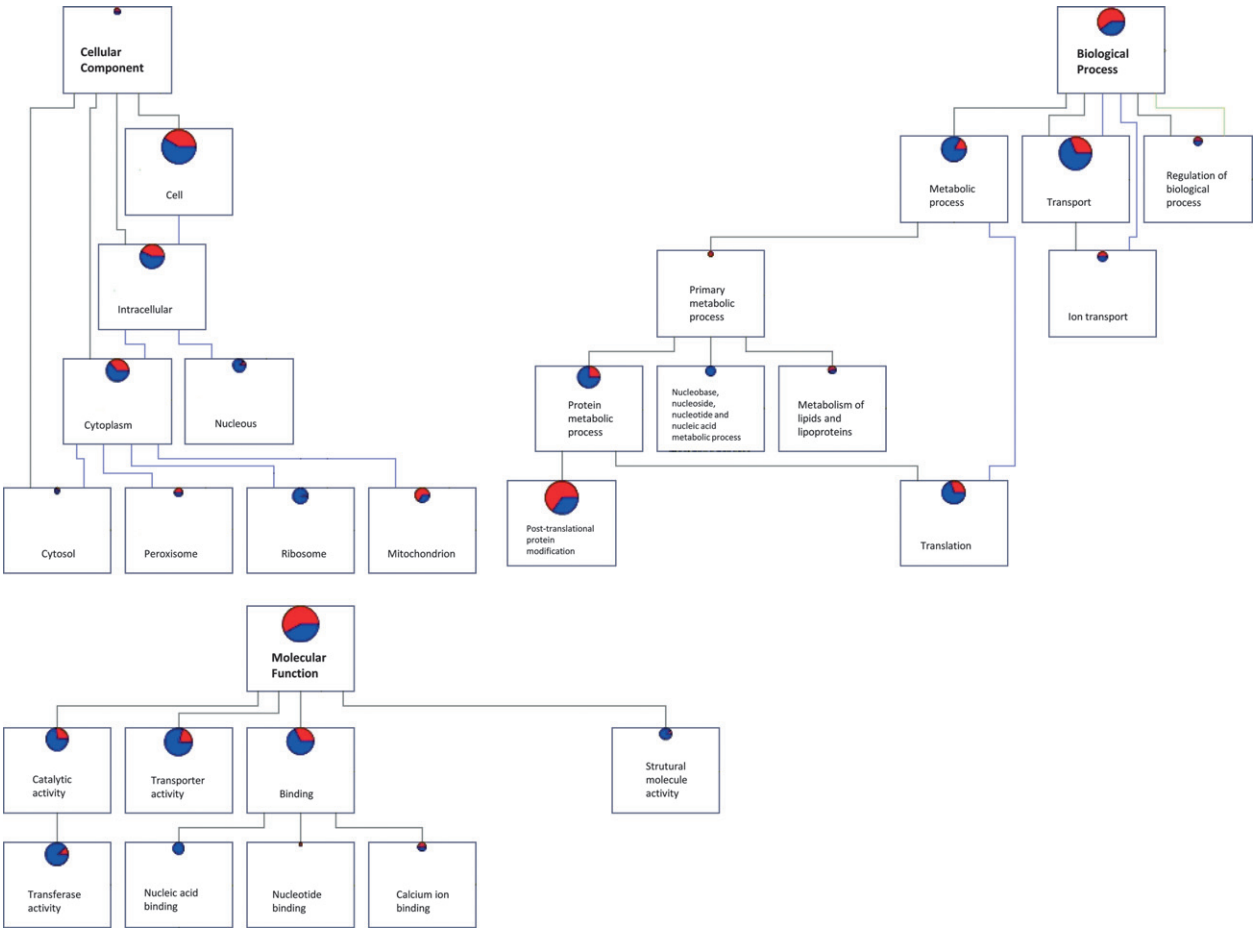

Fig. 2. Gene ontology of the top 10 Pfam proteins (listed in Table 2) with significantly different abundance between bulk soil and soil adjacent to mesh bags containing maize leaves at day 70 of incubation distributed according to biological process, molecular function and cellular component. Red = bulk soil; Blue = soil adjacent to mesh bags.

adjacent to mesh bags containing leaves (Fig. 3). These included the PF00083 and KO8139 ontologies, which correspond to major facilitator superfamily (MFS) transporters and sugar transporters family, respectively. Most of the reads assigned to PF00083 and KO8139 were fungal transporter-like genes, i.e. 90% in bulk soil and 100% in soil adjacent to mesh bag leaves. These transporters are mainly related to carboxylic acid, hexose, monosaccharide and phosphate transport. In soil adjacent to the mesh bags, the sugar transporters (Table S3 for PFAM abundances) appear to be binned mostly with species that have previously been implicated in the degradation of plant-derived cellulose, such as *Chaetomiaceae* (*Ascomycota*), and *Tremellaceae*, *Psathyrellaceae*, *Schizophyllaceae* (*Basidiomycota*) (Supporting Information Fig. S1A). The ribosomal protein family PF01248 (96 reads in total – see Table S3) in bulk soil was assigned to general eukaryotes, whereas in soil adjacent to mesh bags containing maize leaves, most ribosomal protein sequences were assigned to fungi, mainly to *Ascomycota* and *Basidiomycota* (Fig. S1B). The transcripts corresponding to elongation factor Tu C-terminal domain (PF03143) in bulk soil were assigned to a variety of organisms, whereas in soil adjacent to mesh bags containing leaves, the transcripts of fungal elongation factors were overrepresented (Fig. S1C). In bulk soil, amino acid permeases (PF 00324), integral membrane proteins involved in the transport of amino acids into the cell, were mostly binned to *Dictyostelium*. However, when mesh bags containing leaves were incubated for a period of 70 days in the same soil, most of the amino acid permeases were binned to fungal permeases (*Ascomycota Nectriacea*; *Basidiomycota Tremellaceae* and *Agaricales*) and a minor portion to unknown permeases (Fig. S1D).

The top two Pfam categories that were more abundant in bulk soil than in soils adjacent to mesh bags containing maize leaves were protein kinases (PFA00069; PF07714) and endomembrane protein 70 (PF02990). In

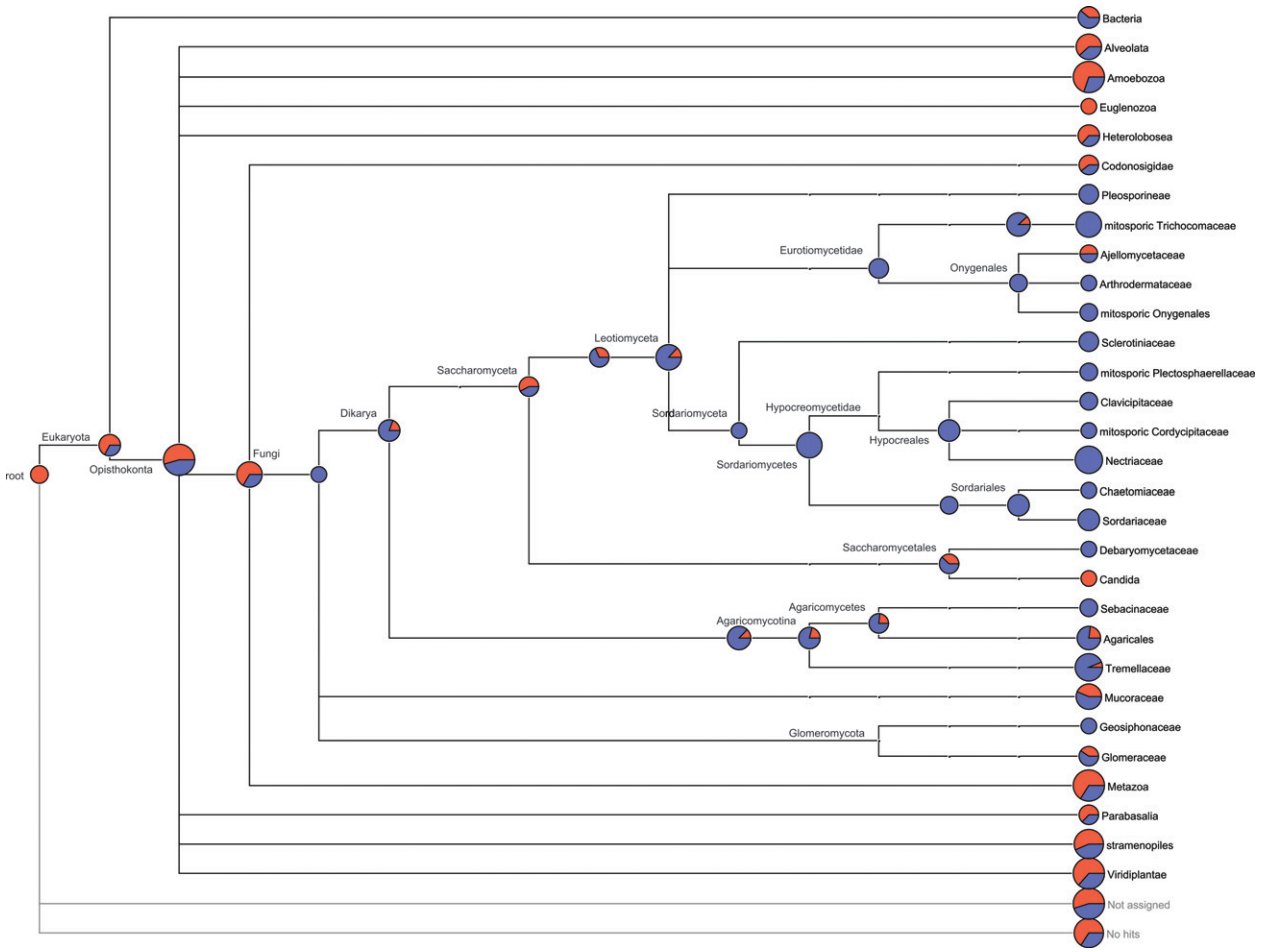

Fig. 3. Metagenomic analysis by MEtaGenome ANalyzer (Megan) of the top 10 PFAM listed in Table 2 binned to different taxonomical groups. Red = bulk soil; Blue = soil adjacent to mesh bags.

bulk soil, these protein kinase domains were binned to different eukaryotes whereas in soils adjacent to mesh bags with the decomposition of maize leaves, most of the transcripts in this protein family were of fungal origin.

The Foly database lists all the enzymes potentially involved in the breakdown of lignin (Levasseur *et al.*, 2008). Members of the Lignin Oxidase Families (LO1–LO3) were linked to Pfam categories to detect changes in the number of transcripts of enzymes potentially degrading lignin. In the sample containing soils adjacent to the mesh bags, 23 transcripts were annotated to the LO2 family, which contains a peroxidase domain (PF00141) [manganese peroxidase MnP (EC1.11.1.13), lignin peroxidase (EC1.11.1.14), versatile peroxidase (EC1.11.1.16)], whereas only 14 sequences were annotated with this term from the bulk soil ($P < 0.05$). The laccase and manganese peroxidase enzymes activities increased at day 70 in soil adjacent to mesh bags containing maize leaves as compared with bulk soil in the same time point (Fig. S2). The other families in the Foly database did not show any significant increase in relative transcript numbers.

The carbohydrate-active enzymes database (CAZy) (www.cazy.org) was used to assign sequences and link them to Pfam categories. A total of 27 glycoside hydrolases (GH) were present in two samples (Table S5); however, 10 glycosyl hydrolase families (GH 2 – TIM barrel domain, GH 6, GH 14, GH 20 – domain 2, GH 25, GH 29-sialyltransferase, GH 39, GH 59, GH 79 N-terminal domain, GH 92) were only detected in bulk soil and nine glycosyl hydrolase families (GH 10, GH 11, GH 17, GH 25 LPS biosynthesis protein, GH 32 N-terminal domain, GH 35, GH 53, GH 64, GH 67 C-terminus) were only detected in soil adjacent to mesh bags (Table S5). Although these glycoside hydrolases have been described to be important in the carbohydrate decomposition, none of them was statistically different between the two samples.

Discussion

PCR-based 18S rRNA gene pyrosequencing

In this study, our goal was to provide the first detailed functional and phylogenic analysis of fungi possibly involved in organic matter degradation in agricultural soil. The recent availability of pyrosequencing-based methods and metatranscriptomic technologies were critical to the ability to address this goal. The relative quantification of fungi : bacteria ratio in soil adjacent to the mesh bags at different time points showed the relative importance of fungi, since fungal 18S rRNA gene copies increased with time of decomposition. It has previously been proposed that agricultural practices reduce soil fungal diversity (Manici & Caputo, 2009; Verbruggen *et al.*, 2010), but sampling depth and primer limitations have hampered estimations of fungal diversity prior to the introduction of high throughput sequencing approaches (Baldrian *et al.*, 2012). Our Good's estimator of coverage showed that the current study sampled a large portion of the total fungal diversity in the soils examined.

With ongoing incubation, the proportion of *Basidiomycota* increased and the proportion of *Ascomycota* decreased, suggesting an enrichment of organisms involved in plant litter degradation. Members of the *Chytridiomycota* are common in soil environments (Lozupone & Klein, 1999, 2002). However, little is known about their ecology or abundance in nature. We have found an increase of environmental *Chytridiomycota* over time in the litter bag experiment, suggesting that these fungi may also contribute to decomposition.

Metatranscriptomic analysis

By comparing the genes expressed in bulk soil versus those expressed in soil adjacent to mesh bags with decomposing leaf litter, we were able to infer several soil functions associated with maize leaf decomposition. For transcripts associated with several protein families, we observed a range of eukaryotic sequences in bulk soil, yet an overrepresentation of specific fungal transcripts at day 70 of maize leaf decomposition. After incubation with decomposing maize leaves, the recovery of several gene families was significantly enhanced, including genes involved in sugar uptake, carboxylic acid and phosphate transporters, drug efflux systems, Krebs cycle metabolites, organophosphate: phosphate exchangers and oligosaccharide: H1 symport permease. The protein family PF00083 (MFS transporters) was one of the top 10 protein families in the metatranscriptomic data, which was not surprising since MFS transporters account for nearly half of the solute transporters encoded within the genomes of microorganisms. What was striking was that 100% of this protein family in soil adjacent to mesh bags containing maize leaves was binned to fungal proteins.

Members of the *Mucorales* (*Mucoromycotina*) are fast-growing organisms, supposedly ubiquitous in nature and widely found on organic substrates, including vegetable matter, crop debris and compost piles (Richardson, 2009). *Mucorales* depend on soluble products provided by lignocellulose-degrading *Ascomycota* and *Basidiomycota,* and they have been called sugar fungi because of their efficient utilization of simple carbohydrates (Thorn & Lynch, 2007). Our results also suggest that members of the *Mucoromycotina* may play a role in the decomposition of plant materials in agricultural soils given the recovery of multiple sugar and amino acid transporter sequences binned to the order *Mucoraceae*. In addition to sugar transport by fungal transporters into the fungal cells, amino acids may also be transported, as evidenced by the high read numbers assigned to amino acid permease protein family (PF00324), which contains integral membrane proteins involved in the transport of amino acids into the cell. Amino acid permease genes found in bulk soil were mostly binned to *Dictyostelium*. These organisms are cellular slime molds and have been found in soils all over the world in environments that vary from cold temperate to tropical (Swanson *et al.*, 1999). However, when maize leaves were incubated for a period of 70 days in the same soil, most of the amino acid permeases were binned to *Ascomycota* and *Basidiomycota* (*Agaricales* and *Tremellales*) fungal permeases and a minor portion to unknown permeases, suggesting that fungal activities in particular are stimulated by the presence of maize leaf litter.

The two Pfam categories are present more in bulk soil than in soils adjacent to mesh bags containing maize leaves, included protein kinases (PFA00069; PF07714) and endomembrane protein 70 (PF02990). Protein kinases transfer the gamma phosphate from nucleotide triphosphates (often ATP) to one or more amino acid residues in a protein substrate side chain, resulting in a conformational change affecting protein function. Protein kinases play a role in a multitude of cellular processes, including division, proliferation, apoptosis and differentiation (Manning *et al.*, 2002). In bulk soil, these protein kinase domains were from a wide range of different eukaryotes, but in soils adjacent to decomposing maize leaves, most of the transcripts of this protein family were of fungal origin, again indicating that fungi are of pivotal importance for litter degradation.

The most common *Basidiomycota* binned transcripts associated with litter decomposition belonged to the order *Agaricales* (families *Psathyrellaceae and Schizophylla-*

ceae), but there were also other groups of *Basidiomycota*, e.g. *Tremellales*, *Boletales, Sebacinales* and *Poriales*, with transcripts that were over-represented in soil adjacent to mesh bags with leaves. We should, however, emphasize that the composition of available databases may bias such analyses. The *Agaricales* have been characterized as being terrestrial, lignicolous, saprobic or mycorrhizal and associated with decomposing litter (Osono, 2007), and many *Tremellales* species have been found as saprobes associated with wood, plants and soil (Zugmaier *et al.*, 1994), which is in line with our findings. The presence of these two groups by 18S rRNA gene sequences and binned-transcripts in soil with maize leaves indicates their possible association with the breakdown of complex organic compounds such as lignin, waxes and tannins.

It was somewhat surprising that we have not detected significantly different transcript numbers for enzymes known to be involved in degrading lignin and cellulose, such as lignin peroxidase, manganese peroxidase and laccase, as well as transcripts of carbohydrate-active enzymes. Some of the glycosyl hydrolase families were, however, found in soil adjacent to mesh bags but not in bulk soil, albeit at low frequencies. These included proteins involved in the microbial degradation of cellulose and xylans, including the following: glycoside hydrolase family 10, which comprises enzymes with a number of known activities: xylanase (EC 3.2.1.8); endo-1,3-beta-xylanase (EC 3.2.1.32); cellobiohydrolase (EC 3.2.1.91); glycoside hydrolase family 53 (β-1,4-galactanase), whose members degrade a structural component of the primary plant cell wall; glycoside hydrolase 67 family, which contains enzymes involved in the hydrolysis of glucuronosyl links in the main chain of hardwood xylans; and glycosyl hydrolase family 53, which contains the plant cell wall-degrading enzyme β-1,4-galactanase. Although these glycoside hydrolases have been described to be important in carbohydrate decomposition, none of them was statistically different between the samples analyzed in our study. The low transcript numbers related to lignin degradation and carbohydrate-active enzymes may stem from the fact that these genes might be expressed only when the fungus is in direct contact with the substrate. The small mesh size of litter bags used in our experiment may have limited the amount of direct contact with the substrate, potentially explaining these low transcript numbers. Alternatively, these activities may represent only a relatively minor component of the degradation apparatus, at least at the time of sampling. One must also keep in mind that, despite the use of pyrosequencing approach, the limited numbers of replicates and sequence reads is not large enough to assess the entire functional diversity of the agricultural soil under study. These limitations should be overcome in the near future as high-throughput sequencing and metatranscriptomic approaches become more reliable and less expensive, allowing for the coverage necessary to compare levels of relatively rare transcripts.

Technical aspects

In this study, we targeted a 900-bp fragment of the nuclear 18S rRNA gene. However, this target size is too large to be fully sequenced using currently available 454-based pyrosequencing technology. Thus, to facilitate sequencing, sequences were fragmented and a separate analysis was performed for sequences generated with the forward (EF3) and reverse (EF4) primers. This analysis showed a slight bias in the sequences generated by the two primers with relatively more *Basidiomycota* sequences generated by EF3 and relatively more *Ascomycota* and *Chytridiomycota* sequences using EF4. This is explained by the bias of the sequencing approach, since different numbers of reads were obtained from different primers. It is also worth noting that, despite the fungal specificity of the primer set, we also recovered a number of non-fungal sequences, including *Rhizaria* and other eukaryotes. Moreover, it has to be kept in mind that the primer sets used in the different studies may have differential coverage of the total fungal communities, and it is known that the primers used in our study may bias against the recovery of particular fungal lineages. The deep sampling afforded by pyrosequencing, diversity analysis and Good's estimator coverage clearly show that we have sampled a significant part of the fungal species within the soils examined.

Soil eukaryotic metatranscriptomics (as opposed to metagenomics) still presents technical challenges. Although there have been other studies targeting mRNA isolated from environmental microbial communities (Bailly *et al.*, 2007; McGrath *et al.*, 2008; Damon *et al.*, 2012) for cDNA library construction and Sanger sequencing, this approach offers only limited coverage of fungal transcripts. The method applied in the current study also circumvents the need to isolate mRNA specifically, as cDNA generation via the polyA of mRNAs specifically enriches this pool of RNA. However, even with such an mRNA-targeted approach, it was necessary to perform rounds of mRNA amplification to obtain enough material for pyrosequencing, and potential biases associated with such amplification steps need to be kept in mind.

Accession numbers

Sequences were deposited in MG-RAST metagenome ID: 4460440.3.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Megan metatranscriptome comparison analysis of (A) MFS transporters and sugar transporters family (PF 00083), (B) ribosomal protein family (PF 01248), (C) elongation factor Tu C-terminal domain (Pfam 03143), (D) amino acid permeases (PF 00324) from samples at day 70.

Fig. S2. Manganese peroxidase and laccase enzymes activity of samples incubated for 70 days with no maize leaves (bulk soil) and soil adjacent to mesh bags containing maize leaves (adjacent).

Table S1. Bacterial 16S rRNA and fungal 18S rRNA gene copies and fungal: bacterial ratio in soils adjacent to mesh bags containing maize leaves collected at day zero (T0), day 21 (T21) and day 70 (T70).

Table S2. Number of de-noised sequences per taxonomic category. Only taxa containing > 30 sequences are presented (EF4/EF3 = primer pair).

Table S3. Significantly different protein families (PFAM) between bulk soil and soil adjacent to mesh bags containing maize leaves at day 70 of incubation.

Table S4. Significantly different Kyoto Encyclopedia of Genes and Genomes (KEGG) between bulk soil and soil adjacent to mesh bags containing maize leaves at day 70 of incubation.

Table S5. Numbers of glycosyl hydrolase families present in soil samples at day 70 with no mesh bags (Bulk soil) and adjacent to mesh bags containing maize leaves (Adjacent).