Title: Baiting of bacteria with hyphae of common soil fungi revealed a diverse group of potentially mycophagous secondary consumers in the rhizosphere

Running title: Saprotrophic fungi as food source for rhizobacteria

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Abstract:

Fungi and bacteria are primary consumers of plant-derived organic compounds and therefore considered as basal members of soil food webs. Trophic interactions among these microorganisms could, however, induce shifts in food web energy flows. Given increasing evidence for a prominent role of saprotrophic fungi as primary consumers of root-derived carbon, we propose that fungus-derived carbon may be an important resource for rhizosphere bacteria. To test this assumption, two common saprotrophic, rhizosphere-inhabiting fungi, *Trichoderma harzianum* and *Mucor hiemalis*, were confronted in a microcosm system with bacterial communities extracted from the rhizospheres of a grass and sedge species, *Carex arenaria* and *Festuca rubra*. This showed a widespread ability of rhizosphere bacteria to attach to and feed on living hyphae of saprotrophic fungi. The identity of the fungi had a strong effect on the composition of these potentially mycophagous bacteria, whereas plant species identity was less important. Based on our results, we suggest that food web models should account for bacterial secondary consumption since this has important consequences for carbon fluxes with more carbon dioxide released by microbes and less microbial carbon available for the soil animal food web.

Keywords: Fungal-bacterial interactions, Mycophagy, Trophic interactions, Mycorrhizosphere, Root exudates, Fungal inhibition, Fungal suppression, Rhizosphere

Introduction:

The soil around plant roots (the rhizosphere) harbors an active and diverse community of microbiota. Plants release 20 to 50% of their photosynthetically obtained carbon into the soil via their roots either directly or via associations with mycorrhizal fungi (mycorrhizosphere) (Kuzyakov and Domanski, 2000). Rhizodeposits are composed of passively sloughed off root cells and actively released substances such as mucilage, volatiles, and exudates (Dennis *et al.*, 2010). Rhizosphere microbial communities are structured by the amount and composition of rhizodeposits, which differ among plant species and age, but are also determined by biotic and abiotic factors (Berg and Smalla, 2009). Rhizodeposits are degraded by the saprobic rhizosphere microbial community. Whereas, bacteria are thought to be the main decomposers of simple soluble compounds, like root exudates, fungi are assumed to be mainly degrading solid recalcitrant polymers (de Boer *et al.*, 2006). However, it is becoming increasingly recognized that the role of fungi is not restricted to this “recalcitrant carbon” niche (van der Wal *et al.*, 2013). Several studies indicate that saprotrophic fungi can form an abundant fraction of the active microorganisms in the rhizosphere and that they metabolize root exudates (Bueé *et al.*, 2009; Denef *et al.*, 2007). In a recent stable isotope labeling study, Hannula *et al.* (2012) followed a pulse of labeled $^{13}\text{CO}_2$ through potato plants (*Solanum tuberosum*) and from the plant roots into the root associated microbiota. They showed that rhizosphere fungi belonging to the phylum *Ascomycota* were rapidly incorporating recently fixed plant carbon and proposed that this may be due to the penetration of plant roots by hyphae of saprotrophic rhizosphere fungi. Indeed, it has been shown that not only the obligatory symbiotic arbuscular mycorrhizal fungi and plant-pathogenic fungi but also

saprotrophic fungi such as *Trichoderma harzianum* are able to enter plant roots (Harman *et al.*, 2004). Hence, for such fungi, the hyphae in the rhizosphere (external phase) can be supplied with organic nutrients transported via hyphae that have entered the plant root (internal phase). Another explanation for rapid uptake of recently fixed plant carbon by saprotrophic fungi is that they could be advantageous in uptake competition with soil bacteria under specific conditions. This is not immediately expected for the most common root exudates, namely sugars, organic and amino acids (Paterson *et al.*, 2007). However, small aromatic compounds known to trigger fungal activity are increasingly released by older roots (Badri and Vivanco, 2009; Waldrop and Firestone, 2004).

Taken together, these studies suggest that there are incidences in which saprotrophic fungi could dominate the degradation of carbon compounds in the rhizosphere. In such situations, rhizosphere bacteria may exploit other niches. A niche for bacteria that cannot directly access root exudates would be to feed on fungal resources (tissue or fungal exudates) and thereby act as secondary consumers.. The existence of bacteria that are able to feed on living fungal tissue or fungal exudates has been described (Leveau and Preston, 2008). This so-called mycophagous nutrition has been extensively studied for soil bacteria of the genus *Collimonas* which use a combination of antibiotics and enzymes to get access to organic nutrients present in living fungal hyphae (Leveau *et al.*, 2010). According to the definition proposed by Leveau and Preston (2010) only bacteria that are actively involved in getting access to fungal nutrients, e.g. by causing leakage of fungal membranes, are considered mycophagous. So far, the documentation on the occurrence of mycophagous nutrition in other soil bacteria is limited. Growth on fungal exudates of bacteria that are associated with ectomycorrhizal fungi has been indicated (Boersma *et al.*, 2010; Frey-Klett *et al.*, 2007). However, since it is not clear if these bacteria have an effect on the exudate efflux they can only be considered as potentially mycophagous. The quantitative extent of mycophagy among rhizosphere bacteria is not known.

Given the aforementioned ability of saprotrophic fungi to rapidly incorporate root exudates we hypothesize that mycophagy is common among members of taxa that are known to be dominant in the rhizosphere.

Saprotrophic fungi inhabiting the rhizosphere are known for the three major phyla/divisions of terrestrial fungi, namely, the *Zygomycota*, the *Ascomycota* and the *Basidiomycota*. Fungi differ in cell wall composition (Bartnick, 1968), and since physical attachment is needed for hyphal colonization, this may select for different bacteria. We, therefore, further hypothesize that phylogenetically different fungi are colonized by different bacterial communities.

We tested our hypotheses in microcosms in which we confronted the fungi *Trichoderma harzanium* (*Ascomycota*) and *Mucor hiemalis* (*Zygomycota*) with bacterial inocula extracted from the rhizosphere of two wild plants, namely the sedge *Carex arenaria* and the grass *Festuca rubra*. Both fungal species have been found to be dominant among saprotrophic fungi colonizing the rhizosphere of these two plants (de Rooij-Van der Goes *et al.*, 1995). Bacterial community DNA was isolated from fungal hyphae and subjected to 454 pyrosequencing.

Simultaneously, hyphae-adhering bacteria were isolated into culture and further screened for their physiological ability to obtain nutrients from fungal hyphae. We found that a substantial number of rhizosphere bacteria were able to feed on fungus derived nutrients as their only source of energy and carbon.

Material and methods:

Plant species and soils:

Samples were taken at an inland river dune in Bergharen, The Netherlands (51°10'N, 05°40'E). The sampling area is characterized by slightly acidic sandy soils (pH 5.5) that are low in organic matter and colonized by early successional plant species. More detailed information on the location and soil characteristics is given in de Boer *et al.* (2008). *Carex*

arenaria L. (sand sedge) and *Festuca rubra* L. (red fescue) plants were dug up to collect rhizosphere soil samples in December 2011. Both plant species are dominant early colonizers of the sand dunes. *Carex arenaria* is non-mycorrhizal whereas *Festuca. rubra* associates with arbuscular mycorrhizal fungi. We defined rhizosphere soil as the soil that was still adhering to roots after vigorous shaking.

Preparation of bacterial rhizosphere inocula:

Bacterial inocula from both rhizosphere soils were prepared using the following protocol: 1 g of soil was added to 10 ml of 10 mM Morpholineethanesulfonic acid (MES) buffer (pH 5.5) containing 1 gL⁻¹ KH₂PO₄ and 1 gL⁻¹ (NH₄)₂SO₄ and was shaken for 90 min (200 rev min⁻¹, 20 °C). The soil suspensions were then subjected to sonification for 2 min at 47 kHz followed by another 30 min of shaking. Finally, the suspensions were passed through several mesh size filters down to a 3 µm cellulose-acetate filter (Whatman Netherlands BV, Den Bosch, Netherlands), to obtain inocula mainly consisting of bacteria (and possibly smaller fungal spores). The absence of fungi in these inocula was confirmed by plating 50 µl of the inoculum on Potato Dextrose Agar (PDA), (9.75 gL⁻¹ ; 3.75 gL⁻¹ agar) containing the bacterial antibiotics oxy-tetracycline (50 mgL⁻¹) and streptomycine (100 mgL⁻¹). The cells in 4 ml of the filtered microbial suspensions of *C. arenaria* or *F. rubra* rhizosphere were spun down (3000rpm, 10min) and re-suspended in 100 µL liquid M-Medium (see below) without glucose.

Fungal hosts:

Two fungi, namely *Trichoderma harzianum* Rifai (Ascomycota) and *Mucor hiemalis* Wehmer (Zygomycota), were used as fungal host strains in the experiments. *Trichoderma harzianum* CECT 2413 was purchased from the Spanish type culture collection (CECT, University of

Valencia, Spain) and *Mucor hiemalis* was originally isolated by de Rooij-Van der Goes *et al.* (1995) from coastal foredunes of the island Terschelling, the Netherlands.

The two fungal species are dominant members of the rhizosphere fungal communities of both plants (de Boer *et al.*, 2008; de Rooij-Van der Goes *et al.*, 1995). *Trichoderma harzianum* (phylum *Ascomycota*) is a saprotrophic fungus that is also able to feed on other fungi, a mode of feeding known as mycoparasitism or mycophagy, but it has also been reported to be able to penetrate the outer cell wall of living plant roots (Harman *et al.*, 2004). *Mucor hiemalis* is a saprotrophic soil fungus of the phylum *Zygomycota*, common in the rhizosphere of plants growing in sandy dune soils (de Rooij-Van der Goes *et al.*, 1995). In order to make sure that no bacterial contamination was introduced together with the fungi, both fungi were pre-cultured on PDA containing oxy-tetracycline (50 mgL⁻¹) and streptomycine (100 mgL⁻¹).

Fungi were verified to be devoid of endophytic bacteria by PCR, using the primers 27f and 1492r (see below).

Microcosm system with host fungi:

Microcosms were set up in two compartmented Petri dishes (Fig.1), similar to the Petri dish system established by Scheublin *et al.* (2010). Fungi were inoculated in the first compartment on autoclaved, modified M-Medium (Bécard and Fortin, 1988) amended with 1% glucose and 0.5% phytigel (Sigma-Aldrich, St. Louis, MO, USA.). Phytigel is a very pure agar and resistant to enzymatic breakdown by many digestive enzymes (Sutherland and Kennedy, 1996). Compared to the original medium, we replaced sucrose by glucose and the pH was adjusted to 5.5 before autoclaving. The medium in the second compartment was free of glucose. After the fungus crossed the barrier to the second compartment and colonized it halfway, a 10 cm² plug of phytigel was cut out near the growth zone of the fungus. This “incubation-slot” was filled with 4 ml liquid M-Medium without glucose and the liquid was left to be colonized by the fungus. The colonization of the Petri dish, including the

“incubation-slot”, took 20 to 22 days. Next, the “incubation-slots” were inoculated with concentrated rhizosphere bacterial suspensions (100 µl/slot). Three replicate microcosms were incubated at 20°C for 24 hours.

Bacterial sampling:

After 24 hours of incubation, the liquid in the “incubation-slots” and the hyphae that colonized the “incubation-slots” were sampled. A pipette was used to carefully collect 1 ml liquid from the “incubation-slot”, avoiding collection of hyphal material. Samples were centrifuged 5 min at 10.000 rpm (Sartorius Sigma Microcentrifuge 1-15P, Nieuwegein, the Netherlands) and pellets frozen at -20°C. Hyphae colonizing the “incubation-slot” were harvested with sterile, disposable inoculation loops and washed in 1 ml 10 mM MES buffer (pH 5.5) by vortexing for 5 sec before transfer with sterile, disposable inoculation loops to new, sterile Eppendorf tubes. Hyphal fragments were washed to remove bacteria not firmly attached to the hyphae. We thus selected for bacteria that rapidly attach to the fungal hyphae, which, we considered as an important strategy for feeding on fungal carbon. Hyphal samples were either frozen at -20 °C for later DNA extraction, or directly used for isolating attached bacteria.

DNA extraction:

DNA from frozen samples (hyphae with attached bacteria and bacterial pellets obtained by centrifugation) was extracted following a protocol based on Griffiths *et al.* (2000) with the following modifications: After CTAB addition, samples were incubated at 65 °C for 5 min and cell lysis was performed with bead beating, using 200 mg of 0.17 - 0.18 mm diameter glass beads for 15 sec at a speed of 4 m s⁻¹ in a MP FastPrep-24 machine (M.P. Biomedicals, Noordwijkerhout, the Netherlands).

454 Pyrosequencing:

The V4 region of the bacterial ribosomal small subunit gene (16S) was amplified with the forward primer 515f (5-CGTATCGCCTCCCTCGCGCCATCAG(10 base barcode)GTGTGCCAGCMGCCGCGGTAA-3) and the reverse primer 806r (5-CTATGCGCCTTGCCAGCCCGCTCAG(10 base barcode)GGGACTACVSGGGTATCTAAT-3)(Vos *et al.*, 2012). The forward primer consisted of primer A from 454 Life Sciences followed by a 10 base sample specific barcode, the 2-base linker sequence *GT*, and the conserved bacterial primer sequence 515F. The reverse primer consisted of 454 Life Sciences primer B, a 10 base long sample specific barcode, the 2-base linker sequence *GG*, and the conserved bacterial primer 806R. Each of the 26 bacterial community samples (2 rhizosphere bacterial origins (*C. arenaria*, *F. rubra*), 2 host fungi (*T. harzanium*, *M. hiemalis*), 2 bacterial community subsets (attached / suspended)), 3 replicates plus the two inocula prior to inoculation, received a unique barcode sequence.

PCRs were carried out in 4 separate 25 µL reactions. Detailed description of cycling parameters and PCR reagents can be found in Vos *et al.* (2012). PCRs were finally pooled and cleaned with the Qiaquick PCR purification kit (Qiagen, Venlo, the Netherlands). PCR amplicons were finally sent to Macrogen (Amsterdam) for equimolar pooling and sequencing, using GS-FLX Titanium chemistry.

Sequence analysis:

In a first step, the sequences and the corresponding quality information was extracted from the Standard Flowgram Format (SFF) files. This was done using the SFF converter tool in the Galaxy interface (Goecks *et al.*, 2010). Sequences were de-multiplexed and the quality was controlled using several scripts of the QIIME pipeline version 1.6. First, sequences that had a maximum of 6 ambiguous bases, 6 homopolymer runs, zero mismatches with the primer,

233 passed a quality score window of 50 and had a minimum average quality of 28, had a
234 minimum length of 200bp, a maximum length of 1000bp and a maximum of 1.5 errors in the
235 barcode sequence were binned according to sample identifier. Barcode sequences were
236 trimmed off. In a second step, the sequence data were corrected for sequencing errors by
237 using the DENOISER algorithm version 1.6.0 and chimeras were removed from the dataset
238 using USEARCH (Edgar, 2010). The sequences that passed the quality filtering were aligned
239 using PyNAST (Caporaso *et al.*, 2010) and UCLUST (Edgar, 2010) and assigned to
240 Operational Taxonomical Units (OTUs), using a minimum sequence identity cutoff of 97%.
241 From all sequences that clustered together as one OTU the most abundant was selected as
242 representative for taxonomy assignment. Identification was done by comparing those OTU
243 representatives with sequences in the SILVA database (release 108 SSU) with a minimum
244 identity cutoff of 75%. Finally, OTU tables were produced and an archeal outgroup sequence
245 was added to the alignment of all OTUs to construct a Lowest Common Ancestor (LCA)-
246 rooted tree by using the FastTree program (Price *et al.*, 2009). Statistical and graphical
247 analyses were done using the package phyloseq (McMurdie and Holmes, 2011) in the
248 program R (R Development Core Team, 2013). OTU abundance plots were created with the
249 program SigmaPlot (v.12.5). The composition of the bacterial communities was analyzed
250 using non-metric MultiDimensional Scaling (nMDS) of Bray-Curtis distances. The program
251 “PAST” (Hammer *et al.*, 2001) was used to test for differences in bacterial community
252 composition by non-parametric multivariate analysis of variance (NPMANOVA), using 9999
253 permutations (Anderson, 2001). Absolute abundance data were transformed into relative
254 abundance data for those analyses. An exception was made for the calculation of the richness.
255 Here, non-transformed, absolute abundance data were used since the Chao richness estimator
256 has been shown to be sensitive to data transformations (Chao and Shen, 2003). Since some of
257 the ten most abundant OTUs could not be identified to the genus level using the

aforementioned workflow, we further matched those against the rdp database (see below) in order to find the most closely related strain.

Bacterial isolation, culturing and Sanger sequencing:

Hyphal fragments collected from the “incubation-slots” were plated on 10% Tryptic Soy Agar (TSA) (de Boer *et al.* 1998) containing fungal inhibitors (100 mgL⁻¹ cycloheximide & 50 mgL⁻¹ delvocide). Growing colonies were picked randomly and dilution streaked until contamination-free. Since it is known that mycophagous *Collimonas* strains inhibit the growth of several fungi on nutrient-poor agar (Leveau *et al.* 2010), we conducted a growth inhibition assay on water-yeast agar as a first indication of mycophagy. For the fungus-inhibitory bacteria, colony-PCR with the primers 27f and 1492r (Weissburg et al 1991) were run with the reagents: 18.14 µl H₂O, 2.5 µl 10x PCR-buffer containing 2 mM MgCl₂ (Roche Scientific, Woerden, the Netherlands), 0.2 mM of each dNTP (Roche Scientific, Woerden, the Netherlands) and 0.4 µM of each primer, 1 U Fast Start High Fidelity Polymerase (Roche Scientific, Woerden, the Netherlands) and 1 µl DNA template. Thermal cycling conditions consisted of a pre-denaturation step of 10 min at 95°C to break the cells open, an initial denaturation of 94°C for 2 min, followed by 34 cycles of 94°C for 30 sec, 55°C for 1 min and 72°C for 90 sec with a 1 sec increment per cycle and a final elongation step at 72°C for 10 min. PCR products were examined by standard electrophoresis through a 1.5% agarose gel and cleaned with 20 % PEG8000 (Sigma-Aldrich). Sanger sequences were generated by unidirectional sequencing from the primer 1492r at MacroGen (Amsterdam, the Netherlands), yielding sequences ≥700bp after quality filtering and trimming (DNA BASER, Heracle BioSoft). Although such shorter fragments of the 16S rDNA do not suffice to distinguish bacterial strains, we successfully assigned bacterial genera to the fragments. Preliminary taxonomic assignments were made via BLASTn, which were also used to decide which isolates to test on mycophagy. Afterwards, distance trees were calculated using Neighbor-

Joining in the software MEGA version 5.2.1. Taxonomic assignments were made using Seqmatch (rdp classifier) (Wang *et al.*, 2007) and alignment with the ClustalW algorithm and finally tested with 100 bootstraps and exported using the software MEGA 5.2.1. (Tamura *et al.*, 2011). The results of the rdp assignment represent the closest matches in the database. Partial 16S sequences of the isolated bacteria were deposited in GenBank under the accession numbers KJ396109 - KJ396179.

Assay on putative mycophagy:

The ability of the bacterial isolates inhibiting fungal growth to live on fungal hyphae as the only organic energy source was tested in a Phytigel-based microcosm system. This system was developed and tested using the known mycophagous bacterium *Collimonas fungivorans* Ter331 (see Fig. S1 in Supporting Information). Bacteria were grown overnight in liquid 10 % TSB at 20 °C, washed twice in 10 mM MES buffer, and 100 µL of cell suspension (10^4 cells/ml) was spread on Petri dishes containing Phytigel. Subsequently plugs from the colony margin of *T. harzanium* and *M. hiemalis*, growing on PDA were introduced to the middle of Petri dishes on sterilized plastic- and metal discs, respectively, in order to prevent nutrient leakage from the PDA plugs into the Phytigel. During the following 14 days of incubation at 20 °C the growing fungal hyphae encountered the bacterial isolates. Living fungal tissue or compounds exuded by the fungus represented the only source of carbon for bacterial growth. Microcosms without fungi were used to control for possible traces of metabolizable carbon, introduced with the phytigel. To harvest bacterial cells, the fungal plug and plastic/metal disc were removed; 2 ml 10 mM MES buffer was added and uniformly spread over the Petri dish. The microcosms were incubated for 30 min at room temperature without shaking. Next, the buffer was swirled, collected, and the OD of the suspension measured at 600 nm. Mycophagy was quantified by dividing the average OD₆₀₀ of each treatment by the average of the highest OD₆₀₀ of the controls (either bacteria only or fungus only) to account for OD₆₀₀ increases by

hyphal fragments or bacterial growth on the Phytigel medium. Experiments were done in triplicates. Data of isolates taxonomically classified as identical were merged. Treatment and control OD were compared by a one-tailed t-test, with either homogeneously or heterogeneously distributed variance (F-test) in Excel (Microsoft Corp.). Mycophagy ratios were plotted in Excel, imported in the program Inkscape (open source, <http://www.inkscape.org>) and mapped on the phylogenetic trees.

Linking bacterial isolate identities to community members:

The sequences of the collection of bacterial isolates were searched by similarity with the 454 sequence reads to determine correspondences between the cultivated and uncultivated bacteria. The 16SrRNA data from the isolates (Sanger sequencing) was uploaded to the galaxy platform and treated as a database against which the obtained 454 reads were compared (blastn algorithm). Matches between Sanger sequencing reads and OTUs could then be compared by similarity values.

Results:

Fungus-associated bacterial communities:

Pyrosequencing of the V4 region of the bacterial 16S ribosomal genes of the 26 bacterial community samples of “hypha-adhering” bacteria, “liquid-phase” bacteria and inocula yielded 156.666 high-quality sequence reads (minimum sequence length 231bp, maximum sequence length 441bp, average read length 335bp) that could be classified to the kingdom level of bacteria. Those sequences could be assigned to 425 OTUs at a cutoff of 97%. The number of sequences varied between samples, with on average less sequences being generated from the hyphal than “incubation-slot” and inoculum samples (Table S3 in Supporting Information).

The pyrosequencing data sets have been deposited in the European Nucleotide Archive under the accession number PRJEB5862.

OTU Richness:

OTU richness was highest in the rhizosphere inocula with on average 343 taxa, followed by 125 taxa in the non-attached bacterial community sampled from the “incubations-slots” and 73 taxa in the bacterial communities attached to hyphae. The rhizosphere bacterial communities originating from *C. arenaria* and *F. rubra* showed similar trends for both fungi with the “incubation-slot” communities always having a higher number of taxa than the respective attached communities (Fig. S2 in Supporting Information). Rarefaction analyses confirmed adequate sampling if more than 2000 sequences were obtained (Fig. S4).

Bacterial community structure:

Ordination analysis of the Bray-Curtis distances among the bacterial communities revealed several clusters (Fig. 2). The clustering of the communities was determined by the bait fungi *M. hiemalis* or *T. harzianum*. Those clusters were significantly different from each other ($p < 0.0001$) and from the bacterial communities of the rhizosphere inocula ($p = 0.0121$ and $p = 0.009$, respectively), which formed a third group. For *T. harzianum* associated bacteria, there were two different subgroups ($p = 0.002$), namely bacteria attached to hyphae and bacteria present in the liquid medium of the “incubation-slot”. Such a difference was not found for the other host fungus, *M. hiemalis*. The grouping according to attachment or “incubation-slot” was not affected by the origin of the bacterial inocula communities (Fig.2).

In general, “the liquid phase” as well as hyphal surfaces were colonized by a complex microbial community with a few dominant OTUs and many low abundance OTUs (Fig.3).

The bacterial communities adhering to hyphae of *T. harzianum* were dominated (> 90 % of total OTUs) by four OTUs that had relatively high similarities with the following taxa: OTU 1 (class: β -Proteobacteria), 8 (family: Oxalobacteraceae), 7 (genus: *Pseudomonas*), and 9 (class: γ -Proteobacteria). The hyphae of *M. hiemalis* were dominated by the OTUs 3 (class: β -Proteobacteria), 1 (class: β -Proteobacteria), 22 (class: Actinobacteria), 23 (family: Spingomonadaceae) which accounted for about 61% of all adhering OTUs (Table 1). Several OTUs significantly increased in relative abundance on fungal hyphae as compared to the liquid phase in. In the case of *M. hiemalis*, these were OTU 22 (Actinobacteria), 23 (α -Proteobacteria), and 26 (β -Proteobacteria). For *T. harzianum* these were OTU 8 (β -Proteobacteria), 7 and 9 (both γ -Proteobacteria). These taxa seem to be very efficient hyphal colonizers. Although many were rare, these hypha-associated bacteria clearly grouped according to fungal identity (Fig. 2).

Phylogenetic affiliation and potential mycophagy of isolated bacteria:

In total, 390 isolates (cultivable on TSA) were retrieved from hypha-adhering communities. More than 50% (203) of these isolates inhibited fungal growth (slowdown or complete stop of hyphal growth) or altered fungal hyphal morphology on nutrient-poor agar. About 65% (n=132) of the fungus-inhibiting bacteria originated from *M. hiemalis*, 35% (n=71) from *T. harzianum*. Taxonomic classification revealed that several isolates belonged to the same strain, reducing the number of dissimilar mycophagous bacterial taxa to 71 (Table S4). Bacteria with antagonistic effects on fungal growth of both fungal species were well represented among the γ - and β -Proteobacteria (Fig. 4). However, fungus-antagonistic bacterial isolates belonging to the phylum Actinobacteria were only obtained from hyphae of *M. hiemalis*. Differences in abundance and taxonomic affiliation of cultivable antagonists clearly separated the two bacterial communities of the bait fungi (Fig.4).

Most of the antagonistic isolates (59% for *M. hiemalis* and 91% for *T. harzianum*) had representatives among the 10 most abundant OTUs in the pyrosequencing dataset with high sequence similarities. Those were slightly higher for isolates associating with *Mucor hiemalis* as compared to *T. harzianum* (Fig.S3, Table S1 and S2 in Supporting Information). Because Sanger sequences were longer (> 700 bp) and thus provided higher resolution than 454 pyrosequencing reads (~300 bp), several OTUs grouped with more than one bacterial isolate (Table S1 & S2).

The percentage of hypha-adhering-, antagonistic bacteria that were able to grow with living hyphae as the only source of nutrients was higher for *M. hiemalis* (80 %) than for *T. harzianum* (35%). The relative increase in bacterial biomass (average “mycophagy ratio”) was also considerably higher for *M. hiemalis* (9.1) than for *T. harzianum* (5.5) (Fig.4). For *M. hiemalis* the most efficient potentially mycophagous bacteria belonged to the genus *Burkholderia* (average “mycophagy ratio” 10) and the genera *Curtobacterium* and *Leifsonia* (class *Actinobacteria*) (average “mycophagy” ratio 3.4). In the case of *T. harzianum*, we found that the genus *Burkholderia* was also an important representative of potentially mycophagous bacteria, but we discovered the same number of potentially mycophagous isolates for the class γ -*Proteobacteria* (genera *Luteibacter* and *Pseudomonas*). Isolate names, accession numbers and taxonomic classification are listed in Table S4.

Discussion:

Studies tracking the fate of fixed $^{13}\text{CO}_2$ in plant-soil-systems have indicated that saprotrophic fungi can be the dominant primary consumers of root derived carbon (Bueé *et al.*, 2009; Deneff *et al.*, 2007; Hannula *et al.*, 2012). Therefore, the flow of energy from roots via saprotrophic fungi to fungal-feeding bacteria may be an important process. Indeed, Hannula *et*

al (2012) and also Drigo *et al.* (2010) showed an increase in ^{13}C -labeled bacteria after an initial ^{13}C enrichment of saprotrophic or mycorrhizal fungi in the rhizosphere. In a follow-up study, it was shown that the bacteria labeled in the experiment of Hannula *et al.* (2012) consisted of genera such as *Burkholderia* and *Pseudomonas* (Dias *et al.*, 2013) which harbor potentially mycophagous bacteria as shown in our research. Yet, they could not prove that these bacteria were feeding on fungi, as ^{13}C -labeled plant resources were also present. We want to highlight that differentiation between bacterial-, fungal- and plant derived carbon in such a system is extremely difficult. Therefore, we chose a growth assay-based approach to find support for the existence of bacteria acting as secondary consumers of fungus-derived carbon.

In the current study we revealed that part of the rhizosphere bacteria of a grass and sedge has the ability to rapidly form tight associations with hyphae of saprotrophic fungi and are able to grow with no other energy sources than the hyphae. Therefore, we propose that for those bacteria, fungal hyphae might be an important source of organic nutrients in the rhizosphere.

Since we tested hypha-adhering bacteria that showed *in vitro* antifungal activity, antifungal secondary metabolites may have had a role in getting access to fungal nutrients e.g. by degrading the exterior of fungal hyphae or by stimulating hyphal exudation. Such a feeding habit where bacteria are actively involved in obtaining fungal nutrients has been termed bacterial mycophagy (Leveau and Preston, 2008). However, since we did not study the interactions between bacteria and fungi in detail, we cannot exclude that bacteria may only have been feeding on fungal exudates without being actively involved in getting access to those nutrients. Therefore, we refer to the bacteria showing a positive growth response when encountering a host fungus as potentially mycophagous bacteria. Our results indicate that many rhizosphere bacteria were able to colonize fungal hyphae and to convert fungal nutrients

into bacterial biomass. Bacteria belonging to the order *Burkholderiales*, which are common members of rhizosphere bacterial communities, were overall well represented on fungal hyphae and also efficient feeders on *M. hiemalis* as evident from a high mycophagy ratio. Most of the mycophagous bacteria that we isolated belonged to *Burkholderia*, a genus of the order *Burkholderiales* that is known to colonize hyphae of a variety of fungi (Frey-Klett *et al.*, 2011). Despite the known association with fungi, there is only little knowledge about the actual mycophagy of *Burkholderia* (Cuong *et al.*, 2011). However, it has been indicated that a *Burkholderia terrae* strain can stimulate fungal exudation of glycerol, a compound that is efficiently used by this strain (Nazir *et al.*, 2013). Scheublin *et al.* (2010) showed that the family *Oxalobacteraceae* which also belongs to the order *Burkholderiales* was an abundant colonizer of hyphae of arbuscular mycorrhizal fungi. There are indications for *Oxalobacteraceae* possibly being involved in fungal pathogen inhibition (Cretoiu *et al.*, 2013). We showed that members of *Oxalobacteraceae* were able to colonize and feed on *T. harzianum*.

Bacteria of the genera *Burkholderia*, *Pseudomonas*, *Sphingomonas* and *Dyella* have been shown to migrate or co-migrate along fungal hyphae (Kohlmeier *et al.*, 2005; Warmink *et al.*, 2011). In our study, these genera were well represented among the potentially mycophagous colonizers of fungal hyphae as well. It has already been hypothesized that motility could be an important trait for mycophagous bacteria to find sites along the fungal mycelium where energy resources are most accessible (Leveau and Preston, 2008). For instance, it has been shown that the hyphal tips, the actively growing parts of mycelia, are most vulnerable to attacks by mycophagous bacteria of the genus *Collimonas* (Leveau *et al.*, 2010).

The ability of bacteria to colonize hyphae of arbuscular and ecto-mycorrhizal fungi is well known (Scheublin *et al.*, 2010; Voronina *et al.*, 2011). However, the mycophagy of those

bacteria has largely not been quantitatively assessed or its evaluation is based on indirect evidence, i.e. by showing the potential to grow on substrates that are mainly of fungal origin such as trehalose (Frey-Klett *et al.*, 2007). Based on the taxonomic similarity of mycophagous rhizosphere bacteria found in this study with those of fungal-adhering bacteria in other studies, it seems likely that many of the latter do also have the potential to obtain resources from hyphae.

The fungus associated bacteria in our study represent a subset of the diverse rhizosphere bacterial community. Hence, a part of the rhizosphere bacteria is able to attach, colonize and feed on saprotrophic fungal hyphae. This is in line with research by Frey-Klett *et al.* (2007), showing that ecto-mycorrhizal fungi exert a selective effect on the associated bacteria. On top of that, in our study, rhizosphere fungi of different phyla were colonized by distinct bacterial species. Differences in the mycophagy ratio of hyphal colonizers but also the percentage of potentially mycophagous bacteria recovered from hyphae showed that *M. hiemalis* was colonized by a higher number and more aggressive bacteria than *T. harzianum*.

Differences in the composition of the hypha-associated bacterial communities of *M. hiemalis* and *T. harzianum* could reflect selection for specific bacteria by cell wall/hyphal surface properties, composition of fungal exudates and production of bactericidal compounds (Bartnick, 1968; Keller *et al.*, 2005). Here, another notable result was the exclusive association of *Actinobacteria* with hyphae of *M. hiemalis* (Fig.4). Presence of *Actinobacteria* in the hyphosphere and their ability to feed on arbuscular mycorrhizal fungi was already previously reported. (Lecomte *et al.*, 2011; Schrey *et al.*, 2012). *Actinobacteria* are known to be potent producers of antimicrobial substances. The mycophagous bacteria of the genus *Collimonas* were also shown to produce a variety of metabolites which have antifungal properties (Fritsche *et al.*, 2014; Leveau *et al.*, 2010). Since the production of antimicrobials by mycophagous bacteria plays an important role in the interaction with the fungus, we

suggest that mycophagous bacteria could be a promising source for the discovery of novel antibiotic compounds. The selective association of *Actinobacteria* with *M.hiimalis* suggests that antibiotic mining of specific bacterial groups could be realized by use of different fungal “baits”.

Interactions between host-associated microbiota can range from mutualistic to antagonistic, including pathogenicity, and only small changes in the genome or the uptake of mobile genetic elements can make the difference (Freeman and Rodriguez, 1993; Zhang *et al.*, 2014). Several of the bacteria isolated from fungal hyphae are putative plant pathogens, such as *Curtobacterium flaccumfaciens*, *Enterobacter aerogenes*, *Leifsonia xyli* and *Burkholderia cepacia*, but also potential plant beneficial bacteria such as *Pseudomonas protegens* or *Luteibacter rhizovicinus*. It has already been shown that bacteria from those genera associate with fungal or other eukaryotic hosts (Aylward *et al.*, 2012; Cuong *et al.*, 2011; Hoffman *et al.*, 2013; Kamei *et al.*, 2012; Warmink and van Elsas, 2009). Both bacterial plant mutualists and antagonists could heavily depend on mycophagy to survive and during dispersal in the soil environment.

We observed attachment to fungal hyphae within 24 hours after inoculating the bacterial communities. Such quick attachment suggests that for mycophagous bacteria the association with the fungal hypha is of importance. The fact that we retrieved fungus-specific OTUs with low abundance in rhizosphere inocula (Fig.3), further underlines this. Fungi probably stimulate colonization by mycophagous bacteria via exudates, guiding mycophagous bacteria to the hyphae by secreting exudates or possibly quorum sensing molecules (Frey-Klett *et al.*, 2011) that might serve as signal molecules for directed bacterial movement towards the fungal host.

Our study indicates that many rhizosphere bacteria can act as fungus-feeding secondary consumers. Given the prominent role of saprotrophic fungi as primary decomposers of root-derived organic carbon that has been indicated by literature references, the flow of carbon from plants via fungi to fungal-feeding bacteria may be substantial. This may have major implications for the functioning of rhizosphere food webs. For instance, the partitioning of root-derived C into carbon dioxide and soil microbial biomass is primarily based on microbial consumers and their growth efficiencies (Farrar *et al.*, 2003). Secondary microbial consumption reduces the amount of energy flowing into the animal food web due to respiratory losses. Soil animal food webs rely strongly on root-derived carbon (Bonkowski *et al.*, 2009). Hence, to have a better understanding of the functioning of the soil animal food webs, it is necessary to understand how carbon and inorganic nutrients are cycled in rhizosphere microbial communities before they actually enter the soil animal food chain.

Conclusions:

Our results show that saprotrophic rhizosphere fungi may form a major source of energy for many members of the rhizosphere bacterial community. This could indicate that secondary consumption of saprotrophic fungi by bacteria may represent an important process in the rhizosphere. So far, the flow of root-derived organic nutrients via saprotrophic fungi to bacteria has not been considered, but it may be substantial, given the accumulating evidence that saprotrophic fungi can rapidly assimilate root exudates. This would, however, change our view on the functioning of rhizosphere bacterial communities and their relationship with the functioning of plants. Therefore, we encourage further studies using ^{13}C isotope tracing to elucidate the trophic relationship between plants, rhizosphere inhabiting saprotrophic fungi and bacteria.

Acknowledgements:

We thank Marlies van den Berg and Maria Hundscheid for technical assistance. We are grateful to Almudena Gonzales-Mula for technical assistance in conducting pilot studies. We thank two anonymous reviewers for their helpful comments and suggestions for improvement of previous manuscript versions. This research was supported by a grant from the Netherlands Organization for Scientific Research, division Earth and Life Sciences (NWO-ALW), grant number 819.01.016. This is publication XXX of the Netherlands Institute of Ecology.

Conflict of Interest Statement:

All authors declare no conflict of interest.

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Table legends:

Table 1 Taxonomic classification of the most abundant bacterial operational taxonomic units (OTUs) present in the “incubation slots” as hypha-attached (A), liquid-phase (L), and inoculum (I) bacteria. OTUs are ordered based on their relative abundance in the hyphal-attached fraction for *Mucor hiemalis* (MH) and *Trichoderma harzianum* (TH), respectively. P-values according to t-tests on the relative abundances in the two fractions are shown. Bold values indicate significance at $p \leq 0.05$. Stdev indicates the standard deviation of the mean. Closest matches of OTUs with the Rdp database are given, including the similarity score and accession number.

Figure legends:

Fig. 1. Two-compartment Petri dish microcosm for testing interactions among bacteria and fungi. While growing on glucose in one compartment, the fungus colonizes the other compartment with a liquid “incubation-slot” for bacterial inoculation. The two compartments are separated by a plastic barrier which prevents glucose diffusion.

Fig. 2. nMDS ordination of Bray-Curtis similarities between bacterial communities. Crosses = inoculum communities, open symbols = hypha-attached communities, filled symbols =

“incubation-slot” communities, triangles = host fungus *Trichoderma harzianum* with *Festuca rubra* inoculum, diamonds = host fungus *T. harzianum* with *Carex arenaria* inoculum, squares = host fungus *Mucor hiemalis* with inoculum of *F. rubra*, circles = host fungus *M. hiemalis* with inoculum of *C. arenaria*.

Fig. 3. Relative abundance of the 10 most abundant operational taxonomic units (OTUs) in each microcosm. (INO = Inoculum, C = *Carex arenaria*, F = *Festuca rubra*, A = hypha-attached, S = liquid-phase, M = *Mucor hiemalis*, T = *Trichoderma harzianum*)

Fig. 4. Neighbor joining distance trees of hypha-associated bacterial taxa of a) *Trichoderma harzianum* and b) *Mucor hiemalis* and their degree of mycophagy (growth on living fungal hyphae). Black bars show mycophagy ratios (mean treatment OD₆₀₀/mean control OD₆₀₀) > 1.0, grey bars show mycophagy ratios < 1.0. Significant differences ($p \leq 0.05$) between bacterial growth in the presence of fungal hyphae and in controls (without fungi) are marked with an asterisk. The trees were inferred from Sanger sequences > 700bp in length. Branch support values are based on a bootstrap analysis of 100 pseudoreplicated datasets. The scale bars indicate uncorrected p-distances (nucleotide substitutions per site).

ed fraction for *Mucor hiemalis* (MH) and *Trichoderma harzianum* (TH), respectively. P-values as a result of a t-test between relative abundances of fractions are shown. Bold values indicate significance at the standard deviation of the mean. Closest matches of OTUs with the Rdp database are given, including similarity score and accession number. Since Sanger sequences were longer (> 700 bp) and thus provided more sequencing reads (~300 bp), the same "closest matches" can appear several times.

λ	Liquid-phase (L)		Inocula (I)		Significance			Rdp database						
	Rel. ab	Stdev	Rel. ab	Stdev	A vs L	A vs I	L vs I	Phylum	Class	Order	Family	Genus	Closest match	Sim
71	47.57	5.89	7.92	6.42	0.20	0.00	0.00	Proteobacteria	β-Proteobacteria	Unknown	Unknown	Unknown	<i>Burkholderia phenazinium (T)</i>	0.98
49	23.82	7.54	1.87	2.04	0.09	0.06	0.00	Proteobacteria	β-Proteobacteria	Unknown	Unknown	Unknown	<i>Janthinobacterium sp.</i>	0.98
05	0.12	0.07	0.33	0.32	0.02	0.08	0.27	Actinobacteria	Actinobacteria	Unknown	Unknown	Unknown	<i>Sreptacidiphilus neutrinimicus</i>	0.98
16	0.38	0.12	0.21	0.03	0.02	0.02	0.05	Proteobacteria	α-Proteobacteria	Sphingomonadales	Sphingomonadaceae	Unknown	<i>Sphingomonas sp.</i>	0.98
13	5.44	3.05	0.85	0.75	0.17	0.05	0.05	Proteobacteria	γ-Proteobacteria	Xanthomonadales	Xanthomonadaceae	<i>Dyella</i>	<i>Dyella sp.</i>	0.98
32	0.54	0.62	1.14	0.24	0.00	0.08	0.12	Proteobacteria	β-Proteobacteria	Burkholderiales	Unknown	Unknown	<i>Leptothrix sp.</i>	0.98
50	3.32	2.51	1.71	1.76	0.19	0.36	0.22	Proteobacteria	β-Proteobacteria	Burkholderiales	Burkholderiaceae	<i>Burkholderia</i>	<i>Burkholderia glathei</i>	0.98
00	0.07	0.06	0.18	0.19	0.19	0.32	0.10	Actinobacteria	Actinobacteria	Streptomycetales	Streptomycetaceae	Unknown	<i>Sreptomyces aureofaciens</i>	0.98
34	0.08	0.09	0.16	0.18	0.08	0.22	0.19	Proteobacteria	α-Proteobacteria	Sphingomonadales	Sphingomonadaceae	<i>Novosphingobium</i>	<i>Novosphingobium sp.</i>	0.98
11	0.77	1.03	0.62	0.15	0.38	0.35	0.43	Proteobacteria	γ-Proteobacteria	Xanthomonadales	Xanthomonadaceae	<i>Luteibacter</i>	<i>Luteibacter rhizovicius</i>	0.98
15	80.74	6.52	1.87	2.04	0.00	0.01	0.00	Proteobacteria	β-Proteobacteria	Unknown	Unknown	Unknown	<i>Janthinobacterium sp.</i>	0.98
72	2.64	2.17	0.19	0.13	0.00	0.00	0.09	Proteobacteria	β-Proteobacteria	Burkholderiales	Oxalobacteraceae	Unknown	<i>Zoogloea ramigera</i>	0.98
40	2.28	1.98	3.10	0.47	0.00	0.03	0.30	Proteobacteria	γ-Proteobacteria	Pseudomonadales	Pseudomonadaceae	<i>Pseudomonas</i>	<i>Pseudomonas sp.</i>	0.98
69	1.31	0.84	3.27	2.00	0.00	0.07	0.04	Proteobacteria	γ-Proteobacteria	Unknown	Unknown	Unknown	<i>Pseudomonas meliae</i>	0.98
01	0.24	0.15	0.05	0.05	0.07	0.05	0.07	Proteobacteria	β-Proteobacteria	Burkholderiales	Oxalobacteraceae	Unknown	<i>Telluria mixta</i>	0.98
60	2.43	1.61	7.92	6.42	0.05	0.19	0.22	Proteobacteria	β-Proteobacteria	Unknown	Unknown	Unknown	<i>Burkholderia phenazinium (T)</i>	0.98
01	1.50	1.85	0.43	0.28	0.22	0.32	0.23	Proteobacteria	Unknown	Unknown	Unknown	Unknown	<i>Butiauxella sp.</i>	0.98
55	0.05	0.02	0.21	0.03	0.09	0.34	0.00	Proteobacteria	α-Proteobacteria	Sphingomonadales	Sphingomonadaceae	Unknown	<i>Sphingomonas sp.</i>	0.98
45	0.33	0.16	0.62	0.15	0.44	0.23	0.03	Proteobacteria	γ-Proteobacteria	Xanthomonadales	Xanthomonadaceae	<i>Luteibacter</i>	<i>Luteibacter rhizovicius</i>	0.98
23	3.13	2.68	0.85	0.75	0.02	0.20	0.15	Proteobacteria	γ-Proteobacteria	Xanthomonadales	Xanthomonadaceae	<i>Dyella</i>	<i>Dyella sp.</i>	0.98

Figure 2

Figure 3

Figure 4

Figure S1

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Figure S2

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