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

M.B. Rudnick [a, d], J.A. van Veen [a, b], W. de Boer [a, c, *]

[a] Department of Microbial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, 6708 PB, The Netherlands
[b] Institute of Biology, Leiden University, Leiden, 2333 BE, The Netherlands
[c] Department of Soil Quality, Wageningen University, Wageningen, 6708 PB, The Netherlands
[d] Department of Plant Health, Institute for Vegetable and Ornamental Crops, Großbeeren/Erfurt e.V., Theodor Echtermeyer Weg 1, D-14979 Großbeeren, Germany

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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.

1. Introduction

The soil around plant roots (the rhizosphere) harbors an active and diverse community of microbiota. Plants release 20–50% 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 (Denef et al., 2007; Bueé et al., 2009). In a recent stable isotope labeling study, Hannula et al. (2012) followed a pulse of labeled $^{13}CO_2$ 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

* Corresponding author. Department of Microbial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, 6708 PB, The Netherlands. Tel.: +31 (0) 317 473 676.
E-mail addresses: m.rudnick@nioo.knaw.nl (M.B. Rudnick), h.vanveen@nioo.knaw.nl (J.A. van Veen), w.deboer@nioo.knaw.nl (W. de Boer).

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recently fixed plant carbon and proposed that this may be due to the penetration of plant roots by hyphae of saptrotrophic 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 (Waldrop and Firestone, 2004; Badri and Vivanco, 2009).

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 (Frey-Klett et al., 2007; Boersma et al., 2010). 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.

2. Material and methods

2.1. 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). *C. arenaria* L. (sand sedge) and *F. 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. *C. arenaria* is non-mycorrhizal whereas *F. rubra* associates with arbuscular mycorrhizal fungi. We defined rhizosphere soil as the soil that was still adhering to roots after vigorous shaking.

2.2. 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} KH_2PO_4 and 1 gL^{-1} $(NH_4)_2SO_4$ 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 (3000 rpm, 10 min) and re-suspended in 100 μL liquid M-Medium (see below) without glucose.

2.3. Fungal hosts

Two fungi, namely *T. harzianum* Rifai (Ascomycota) and *M. hiemalis* Wehmer (Zygomycota), were used as fungal host strains in the experiments. *T. harzianum* CECT 2413 was purchased from the Spanish type culture collection (CECT, University of Valencia, Spain) and *M. 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 Rooij-Van der Goes et al., 1995; de Boer et al., 2008). *T. 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). *M. 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).

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