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FUNGAL MICROBIOLOGY

Interactions Between Hyphosphere-Associated Bacteria and the Fungus *Cladosporium herbarum* on Aquatic Leaf Litter

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Abstract We investigated microbial interactions of aquatic bacteria associated with hyphae (the hyphosphere) of freshwater fungi on leaf litter. Bacteria were isolated directly from the hyphae of fungi from sedimented leaves of a small stream in the National Park "Lower Oder," Germany. To investigate interactions, bacteria and fungi were pairwise co-cultivated on leaf-extract medium and in microcosms loaded with leaves. The performance of fungi and bacteria was monitored by measuring growth, enzyme production, and respiration of mono- and co-cultures. Growth inhibition of the fungus *Cladosporium herbarum* by *Ralstonia pickettii* was detected on leaf extract agar plates. In microcosms, the presence of *Chryseobacterium* sp. lowered the exocellulase, endocellulase, and cellobiase activity of the fungus. Additionally, the conversion of leaf material into microbial biomass was retarded in co-cultures. The respiration of the fungus was uninfluenced by the presence of the bacterium.

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Introduction

Plant litter decomposition in lentic and lotic systems is mediated by the activity of a large number of different organisms including fungi, bacteria, protozoa, and meiobenthic invertebrates. Leaf litter degrading microorganisms possess the enzymes necessary to digest the polymers of the leaf components and can turn refractory compounds into microbial biomass or other readily available nutrition sources for higher trophy levels [4, 36]. Fungi and bacteria live in close vicinity in the biofilm on submerged leaf litter and inside the leaf. Fungal hyphae can penetrate the leaf surface and may carry bacteria living directly at the hyphae or even inside the hyphae [9]. In lotic systems, the surface and direct surroundings of fungal hyphae, the "hyphosphere" [34], may provide a microenvironment in which enzymes, metabolites, and antibiotics secreted by the fungi are abundant in higher concentrations than in the surrounding water. Especially, the products of fungal exo-enzymatic activities could attract bacteria to the hyphosphere. Hence, the hyphosphere may be the "hotspot" of microbial interactions on and within submerged leaf litter. Interactions between fungi and bacteria are generally characterized by a range of mechanisms such as direct resource competition [22], commensalism, parasitism [33], mycophagy [11], bacteriophagy [6], and endosymbiosis [27]. In few laboratory studies, the effects of bacterial–fungal interactions associated with aquatic leaf litter have been studied and the alteration of biomass, enzyme activity, respiration patterns, and conidium production have been interpreted as the

effects of different modes of microbial interaction [e.g., 7, 16, 30]. Most studies of bacteria and freshwater fungi revealed antagonistic [16, 22, 24, 42] interactions. Synergistic interactions in the form of enhancement of fungal and/or bacterial growth were shown in two studies [7, 23]. Although both fungi and bacteria are relevant to leaf litter decomposition in streams [3, 5, 39], the relationships between these two groups of microorganisms on leaf litter are not fully understood yet. Specifically, the relations between hyphosphere associated bacteria and fungi in and on aquatic leaf litter are not clear.

The objective of this study was to clarify this important question by investigating the interactions between hyphosphere bacteria and fungi living on decomposing leaves. The main question of the study was: Does the presence of bacteria in the hyphosphere influence the fungal performance on solid media and on leaf litter? Specific objectives were (1) to isolate and determine bacteria from the hyphosphere of fungi originally isolated from submerged leaves and (2) to conduct co-cultivation experiments to detect potential interactions between bacteria and fungi/oomycetes. The study was conducted in two steps: (1) Initially, the spatial distribution of bacteria in the fungal mycelium and fungal growth was monitored on agar plates. (2) In order to measure the ecological implications of fungal–bacterial interactions, the activity of enzymes involved in the degradation of plant litter was measured in microcosms. Since plant leaves contain high cellulose content, both endo- and exocellulase as well as cellobiase activity were determined. Microbial cellulose degradation by extracellular enzymes [20] includes random cleaving of internal 1,4-glycosidic bonds by endocellulase, and the release of cellobiose by exocellulase activity and finally hydrolyzing cellobiose by cellobiases (beta-glucosidases). As an estimation of conversion of leaf material into microbial biomass, the ratio of carbon and nitrogen content was analyzed. To evaluate the metabolic potential of hyphosphere bacteria, BIOLOG was performed. Furthermore, CO_2 production in microcosms was measured in order to see if the presence of bacteria alters fungal respiration rates.

Materials and Methods

Study Site and Sampling

Sampling was performed in a nameless first order tributary of the Mummert stream which is running through the lower Oder floodplain in the northeast of Germany. The bank vegetation is dominated by Grey Sallow (*Salix cinerea*), White Elm (*Ulmus laevis*), and Purple Loosestrife (*Lythrum salicaria*). In autumn 2006, the water temperature was 12°C, and the O_2 concentration was 5.42 mg l^{-1} with a saturation of 44.85%. The conductivity was 776 µS cm^{-1} and the pH 7.46.

Willow leaves were taken from approximately 20 cm beneath the water surface. One leaf was cut into ten pieces of equal size (1 cm^2), and the pieces were placed on willow leaf extract agar (LEA: cloth filtered aqueous extract from 50 g willow leaves, 15 g agar, 1,000 ml pure water) plates. The biofilm of four other leaves was scratched onto a single LEA plate with a sterile spatula.

Isolation and Determination of Fungi and Hyphosphere-Associated Bacteria

Bacterial microcolonies were isolated directly from the fungal hyphosphere using a glass-capillary under a microscope (×100 magnification). Bacterial isolates were transferred to 10% tryptic soy agar (Difco, Augsburg, Germany) whereas fungal isolates and one oomycete were cultivated on 2% malt extract agar [14]. For fungal/bacterial isolates that could not be separated by this method, LEA plates with either 0.01% cycloheximide (for repression of eukaryotic growth, Sigma–Aldrich Germany), or 16 ml l^{-1} Penicillin–Streptomycin solution (for repression of prokaryotic growth, Sigma–Aldrich Germany) were used to obtain pure cultures. Genomic DNA from fungi and bacteria was isolated using the FastDNA®SPIN kit for soil in conjunction with the FastPrep® FP120 instrument (Qbiogene, Heidelberg, Germany). The fungal ITS region of ribosomal DNA was amplified with the primers SR6R (Vilgalys unpubl. [http://www.biology.duke.edu/fungi/mycolab/primers.htm]) and LR1 [38]. PCR mixtures contained a PCR buffer (Roboklon, Berlin, Germany), 200 µM of each deoxynucleotide, 1.5 mM magnesium chloride, 20 pM of each primer, 40–200 ng of genomic DNA, and 1 U of *Taq* polymerase (Roboklon, Berlin, Germany). The PCR was performed in a T Gradient Thermocycler (Biometra, Göttingen, Germany) with an initial denaturation step for 2 min at 94°C, followed by 35 cycles of denaturation for 1 min at 94°C, 45 s primer annealing at 46°C and elongation for 2 min at 72°C, final extension was for 5 min at 72°C. 16S rRNA genes of bacterial strains were sequenced by the company SMB Dr. Martin Meixner (Berlin, Germany) using the primers 27f, 699f, 1492r, and 699r [18]. The sequences were obtained from an ABI 373 sequencer (PE Applied Biosystems, Weiterstadt, Germany) and analyzed with Bionumerics (Applied Maths, Belgium). All sequences were used as queries in the GenBank sequence similarity search tool BLAST [2] with normal stringency. Two fungal strains were identified according to their morphological characters as *Alternaria alternata* (Fr.) Keissl. and *Cladosporium herbarum* (Pers.) Link.

Preparation of Suspensions for the Inoculation of Co-Cultures

Bacteria were grown overnight in 5 ml of 10% tryptic soy bouillon. The optical density of the suspensions was adjusted to an approximate value of 0.5 (*Curtobacter* sp.: 0.44, *R. pickettii* GR4: 0.664, *Chryseobacterium* sp.: 0.527). The optical density corresponded to cell counts of 2.6×10^8 ml^{-1} for *Chryseobacterium* sp., 3.2×10^8 ml^{-1} for *R. pickettii* GR4 and 1.8×10^8 ml^{-1} for *Curtobacter* sp. as determined with a counting chamber (0.02 mm depth).

Fungal strains were incubated on M20 agar for 14 days. Detachment of conidia from mycelia was achieved by panning and soft shaking with 5 ml of sterile deionized water. The conidia concentration of the suspensions was determined in a Thoma chamber (0.1 mm depth) and then adjusted to 10^6 conidia ml^{-1}. Mycelium of the oomycete *Pythium* was scratched from the surface of an M20 plate (which had been incubated for 14 days) with a sterile spatula. It was then hacked with the FastPrep FP120 instrument to yield smaller pieces of hyphae.

Co-Cultures on LEA Plates

In the first part of the study, LEA plates were inoculated with approximately 96×10^6 ml^{-1} bacteria and 0.7×10^6 ml^{-1}conidia/oomycete hyphae fragments by combining a suspension of one bacterium with one fungus/oomycete suspension to yield co-cultures. The plates were incubated at 18°C and were inspected daily to monitor fungal and bacterial growth using a stereomicroscope and a microscope (Zeiss, Jena, Germany). To test if *Ralstonia pickettii* produces allelochemicals a 0.2 µm bacterial lyophilized filtrate was inoculated into a *C. herbarum* culture as described by Anderson and coworkers [1]. Since the production of allelochemicals is likely to be induced in the presence of the fungus the filtrate of a co-culture of *R. pickettii* and *C. herbarum* was inoculated into a *C. herbarum* culture.

Co-Cultures in the Aquatic Microcosm

C. herbarum and *Chryseobacterium* sp. were chosen for the microcosm experiment since the bacterium constantly remained in the hyphosphere on LEA plates. Sterile Erlenmeyer flasks (V=100 ml) were filled with 25 ml of 0.2 µm filtered Mummert stream water and 5 ml of HEPES buffer to stabilize the pH [31] (Sigma–Aldrich, Munich, Germany). Willow leaf disks were each inoculated with15-µl suspensions of either 2.8×10^8 cells ml^{-1}of *Chryseobacterium* sp. or 15×10^6 conidia ml^{-1} of *C. herbarum* conidia, both suspensions, or none (control). Each microcosm was loaded with ten Willow leaf discs with diameters of 8 mm (for microscopy) and 17 mm (for all other analyses) and incubated on a laboratory shaker (138 rpm) at 18°C.

Spatial Distribution of Bacteria and Hyphae in Co-Cultures

The analysis of the spatial distribution bacteria was conducted by staining fungal chitin with 15 µl of 10% Fungalase™ (5,000 MWU/g, Anomeric Inc., Baton Rouge, LA, USA). Nucleic acids of fungi and bacteria were visualized with 15 µl of 1 µg ml^{-1} DAPI or SYTO 9 (Molecular Probes, Heidelberg, Germany) pipetted on each leaf disk. Epifluorescence microscopy was performed with a Zeiss Axioplan II (Oberkochen, Germany) fitted with a 100 W high-pressure bulb and Zeiss light filter sets no. 01 for DAPI (excitation 365 nm, dichroic mirror 395 nm, suppression 397 nm) and no. 09 for Oregon Green 488 (excitation 450–490 nm, dichroic mirror 510 nm, suppression 520 nm). Photographs were taken with a colorview 12, CY85 camera (Olympus, Hamburg, Germany). Leaf disks from microcosms were microscopically inspected on the 1st, 15th and 34th day of incubation.

C/N Ratio, Exoenzyme Activity, Respiration Rate, and Bacterial Substrate Utilization

Carbon/Nitrogen Ratio

To determine whether potential microbial interactions would alter the conversion of leaf material into microbial biomass, the C/N ratio was measured.

For the measurement of the C/N ratio during incubation time of inoculated and control leaf disks, the microcosm Erlenmeyer flasks (ten replicates for each measurement) were frozen at –20°C. Subsequently, all samples were thawed, dried at 105°C, minced in the AdW-IcT Vibrator (NARVA, Brand-Erbisdorf, GDR), and analyzed simultaneously in the gas chromatograph vario EL III CHNS (elementar, Hanau, Germany).

Exocellulase and Cellobiase Activity

The entire content of a microcosm Erlenmeyer flask was homogenized with an Ultraturrax T25 (Jahn & Kunkel, IKA-Labortechnik, Staufen i. Br., Germany). Carboxymethylcellulose was used as the substrate for cellulases present in the homogenate. One milliliter of homogenate was added to 4 ml of 5% CM-cellulose solution and incubated for 3 days at 31°C. The product glucose was then photometrically measured with the glucose liquicolor kit (Human GmbH, Wiesbaden, Germany) using the method of deproteinization, according to the manufacturer's specifications.

Endocellulase Activity

As a measure of long-chain cellulose still present after incubations, the change of the viscosity of the preparations was determined in the rotation viscosimeter RV3 (Haake, Berlin/Karlsruhe, Germany).

The dynamic viscosity η is calculated from the values (S) of the rotation viscosimeter using the following formula:

$$\eta = \frac{\tau}{D} = \frac{A \times S}{M \times n} = G \times \frac{S}{n}$$

where τ is the shear stress, D is the shear gradient, n is the rotational frequency, and A, M, and G are apparatus constants.

Viscosity is an indirect measure for endocellulase activity correlated with the decrease of long chain cellulose in the tested samples.

Erlenmeyer flasks (V=100 ml) were each filled with 40 ml of 10% (*w*/*w*) CM-cellulose solution and autoclaved. Ten milliliters of the homogenates from the 34th and 60th days of incubation, already used for exocellulase and cellobiase activity measurement, were pipetted into the flasks and incubated at room temperature for 3 days on a laboratory shaker (138 rpm) and then measured in the viscosimeter.

Respiration Rate Monitoring

Respiration rates were monitored by electric conductivity monitoring at a Respirocond system (Nordgren Innovations AB, Bygdeå, Sweden).

Vessels were each filled with 5 g of willow tree leaves, 25 ml of filtered water from the Mummert, and 5 ml of HEPES buffer (in order to stabilize the pH). The contents of the vessels were homogenized with an Ultraturrax T25 to a particle size of approximately 15 mm. The vessels were autoclaved, inoculated with 500 µl of either *C. herbarum* conidia solution (15×10^6 cells ml^{-1}), *Chryseobacterium* sp. solution (2.8×10^8 cells ml^{-1}), 500 µl of both solutions, or none (control) and placed into the Respirocond [26]. Three parallels of each preparation were analyzed. Incubation was performed over a period of 100 h. CO_2 production was measured twice per hour.

Substrate Utilization Analysis of Strain *Chryseobacterium* sp.

Chryseobacterium sp. was grown overnight on solid TSA medium. Bacterial colonies were collected and dissolved in a 0.9% NaCl solution to remove all medium components. The bacterial suspension was adjusted to an optical density corresponding to $3–5 \times 10^8$ cells/ml according to Mc Farland standard 1. One hundred fifty microliters of the suspension each was deposited into each of the 96 wells of a BIOLOG-GN-Plate (suitable for Gram negative bacteria, Oxoid, Wesel, Germany). The plates were incubated at 28°C in darkness for 1 day. Substrate utilization was checked by a purple formation (tetrazolium dye), which occurs when the microbe begins to respire due to utilization of the respective carbon source in the well. The test was performed in three replicates. Protease activity of *Chryseobacterium* sp. was determined by an agar plate assay. The test agar comprised the following ingredients per liter of distilled water: 4 g skim milk, 11.9 g HEPES, 0.5 g $CaCl_2$ (Sigma–Aldrich, Augsburg, Germany), 2 g $MgSO_4$ (Sigma–Aldrich, Augsburg, Germany), 1 ml trace element solution SL8 [10], and 15 g agar. *Chryseobacterium* sp. was grown on the agar plates for 5 days at 28°C.

Statistical Analyses

Normality testing of data from carbon–nitrogen ratio, exoenzyme activity, and respiration rates was performed using Kolmogorov–Smirnov and Shapiro–Wilk tests. Differences in C/N ratio, exoenzyme activity, and respiration rates among control, single, and dual cultures at different extraction times were tested using repeated measures ANOVA followed by Tukey's all pairs comparison. Data derived from C/N ratio were arcsine square root and respiration rates were $\ln(x+1)$ transformed before analyses. Additionally, all raw data were tested with a nonparametric test for significant differences using the Kruskal–Wallis test in conjunction with the Wilcoxon–Mann–Whitney Rank sum test. In graphs nontransformed data (mean ± standard deviation) were used. Data analyses were conducted using SigmaStat 2.0; SPSS 12.0 and Kaleidagraph 4.0.

Results

Fifteen different bacterial strains were isolated. Three strains, directly isolated from the fungal hyphosphere, were identified as *Curtobacter* sp., *R. pickettii* (GenBank accession number EU622981), and *Chryseobacterium* sp. (GenBank accession number EF640932). Two fungal strains, *C. herbarum* and *Alternaria alternata*, were isolated as well as three sterile mycelia, one of which was identified as the oomycete *Pythium*.

Interaction Experiments on Solid Nutrient Media

All LEA plates showed simultaneous growth of bacteria and fungi/oomycete. Growth of *C. herbarum*, however, was significantly less vigorous when plated with *R. pickettii* GR4 (Fig. 1a) than with the other bacterial strains. *R. pickettii* GR4 showed a high production of extracellular polymeric substances (EPS; not shown here) in single and co-cultures.

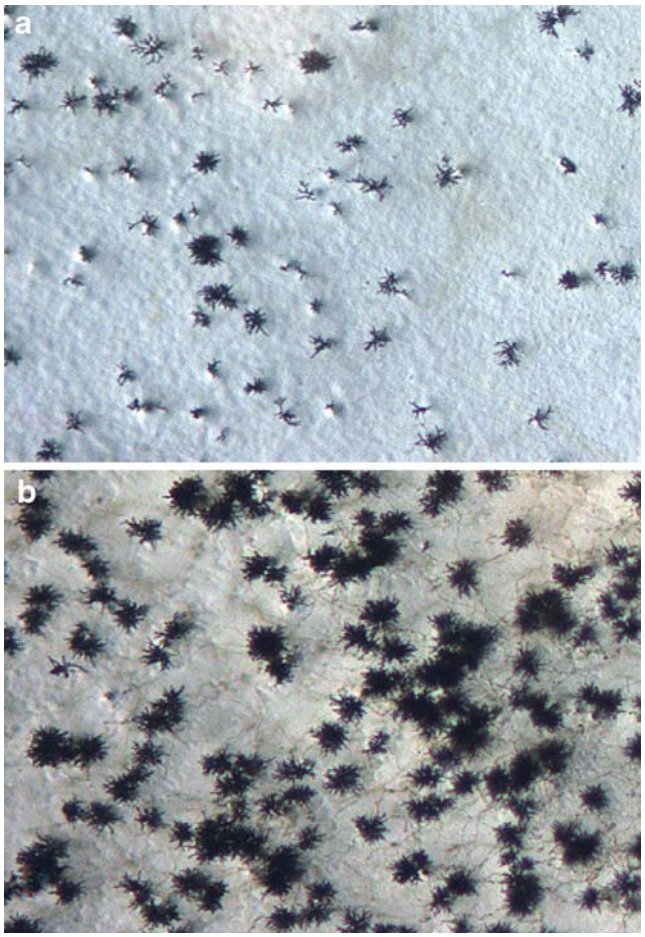

Figure 1 **a** Inhibited growth of *C. herbarum*: *C. herbarum* and *R. pickettii* GR4 on LEA, 8th day of incubation, 50×. **b** Uninhibited growth of *C. herbarum*: *C. herbarum* and *Chryseobacterium* sp. on LEA, 8th day of incubation, 50×

On LEA plates, *Chryseobacterium* sp. and *C. herbarum* grew together without apparent antagonism. (Fig. 1b). In bioassays, disks loaded with filtrate of *R. pickettii* or filtrate from a co-culture produced no inhibition of fungal growth.

Co-Incubation in the Aquatic Microcosm: C/N Ratio

In leaves inoculated with only *Chryseobacterium*, the C/N ratio did not change significantly compared to leaves without organisms (control) throughout incubation (Fig. 2). In microcosms inoculated with only the fungus, the C/N ratio decreased significantly during the incubation (days 1–34, $p=0.0058$). At day 15, the C/N ratio was significantly lower in fungus only treatments compared to two-membered microcosms ($p<0.0001$), bacteria-only ($p<0.0001$), and control ($p<0.0001$). The C/N ratio in the leaves inoculated with both organisms did not decrease until day 15. Between days 15 and 34, the C/N ratio decreased ($p=0.0013$) to a value similar to one of fungus-only treatments.

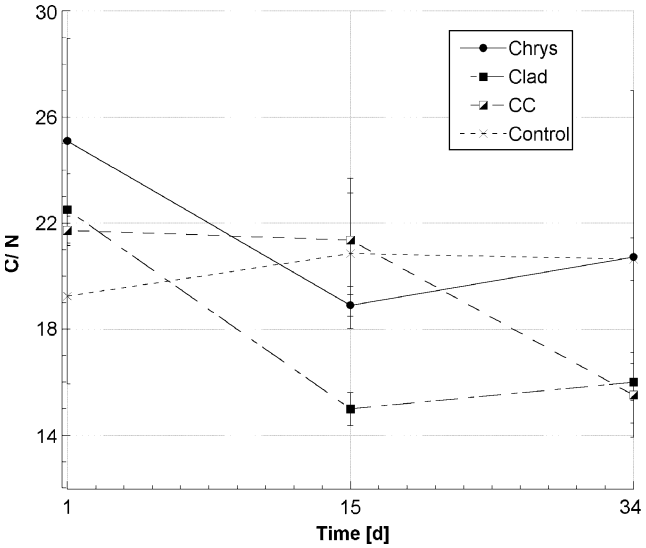

Figure 2 Carbon/nitrogen ratio of incubated leaves during the microcosm experiment between days 1 and 34. *Chrys Chryseobacterium* sp. only; *Clad C. herbarum* only, *CC* co-culture of *Chryseobacterium* sp. and *C. herbarum*. *Symbols* indicate means, *error bars* show standard deviation ($n=10$)

Exocellulase and Cellobiase Activity

The detection limit of the method was determined using a calibration curve to be 0.5 mmol glucose l^{-1}. Glucose, as product of exocellulase and cellobiase activity, was hardly detectable in bacteria-only microcosms at days 1 and 15 (Fig. 3). Also, on days 34 and 60, glucose levels of microcosms with only *Chryseobacterium* sp. were not significantly different than those of the control treatments.

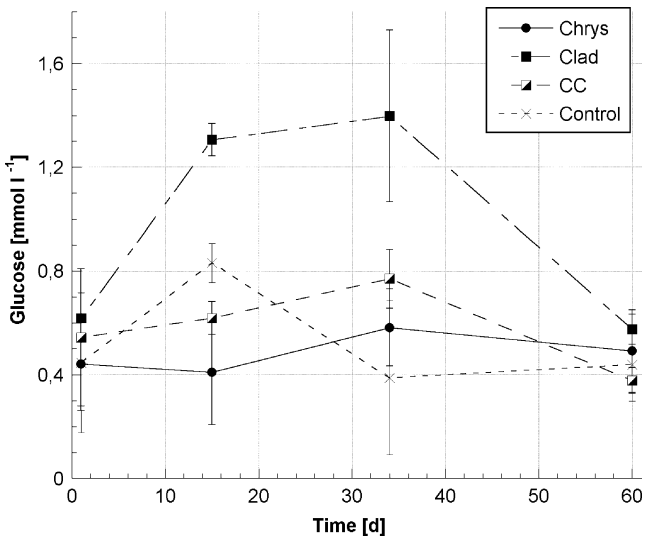

Figure 3 Exocellulase and cellobiase activity by glucose determination during the microcosm experiment between days 1 and 57. *Chrys Chryseobacterium* sp. only; *Clad C. herbarum* only, *CC* co-culture of *Chryseobacterium* sp. and *C. herbarum*. *Symbols* indicate means, *error bars* show standard deviation ($n=10$)

The highest glucose levels were measured when *C. herbarum* was inoculated singly in the microcosms on days 15 and 34. The enzymatic activity of fungal cellulases decreased significantly when *C. herbarum* and *Chryseobacterium* sp. were incubated together compared to days 15 ($p<0.0001$) and 34 ($p<0.0001$) of the fungus-only treatment. The glucose concentration in fungus only treatments increased significantly from the beginning of the incubation until day 15 ($p<0.0001$) and decreased between days 34 and 60 ($p<0.0001$). In contrast, the glucose amount did not increase in two-membered microcosms between the days 1, 25, and 34 but decreased between days 34 and 60 ($p=0.0009$). In bacteria-only treatments, the glucose concentration did not change over the incubation time.

Endocellulase Activity

The dynamic viscosity did not decrease in the control and bacteria-only microcosms (Fig. 4). In contrast, strong endocellulase activity, indicated by significantly decreased viscosity, was observed in microcosms inoculated with only *C. herbarum* on both sampling dates ($p=0.00015$, $p=0.00017$, $p=0.00015$, $p=0.00016$, *C. herbarum*, control, and *Chryseobacterium* sp. days 34 and 60, respectively). In two-membered microcosms, the fungal endocellulase activity was significantly lower (day 34, $p=0.0007$; day 60, $p<0.0001$) compared to that of the fungus-only treatment. Dynamic viscosity values of the respective microcosms did not differ significantly between days 34 and 60.

Respiration-Rate Monitoring

Respiration rates in two-membered microcosms and microcosms inoculated with only *C. herbarum* were consistently higher than those of the control and bacteria-only treatments (Fig. 5). Analyses by ANOVA and Kruskal–Wallis–Wilcoxon tests revealed no significant difference between the respiration rates of the control and *Chryseobacterium* sp. only treatments (ANOVA $p=0.3428$, KWW $p=0.025$). Similarly, there was no difference between the respiration rates of microcosms inoculated with only the fungus and microcosms inoculated with fungus and bacterium. Regarding the whole incubation period of 350 h, the carbon dioxide production escalated in fungus-only and co-culture treatments until 60 h and then decreased slowly until reaching constant values at 200 h. In microcosms inoculated with bacteria only, no significant change of respiration rates was observed over time.

BIOLOG Assay

Interestingly, the BIOLOG pattern revealed that the isolated *Chryseobacterium* sp. strain was unable to use cellulose as a carbon source. However, it used D-cellobiose as a carbon source. Furthermore, out of the 96 offered substrates, the strain could only catabolize the following: Tween 40 and 80, Gentobiose, D-raffinose, sucrose, alpha-keto butyric acid, alpha-keto glutaric acid, alpha-keto pentanoic acid,

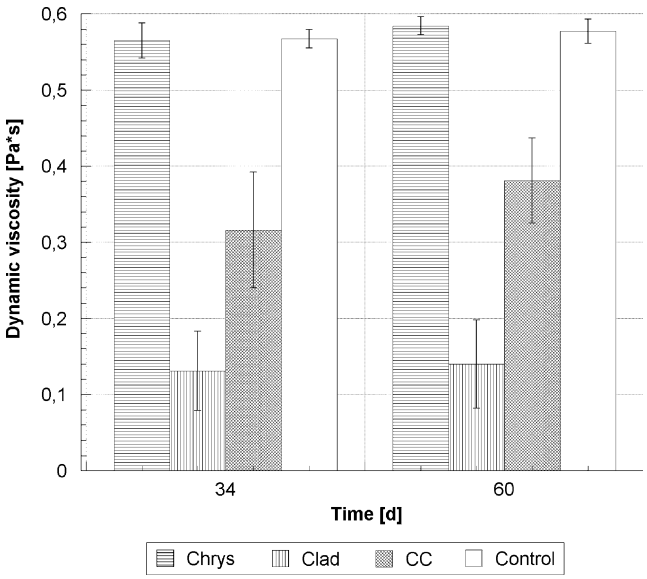

Figure 4 Viscosity values as an indirect measure of the endocellulase activity during the microcosm experiment at days 34 and 60. *Chrys Chryseobacterium* sp. only; *Clad C. herbarum* only, *CC* co-culture of *Chryseobacterium* sp. and *C. herbarum*. *Symbols* indicate means, *error bars* show standard deviation ($n=10$)

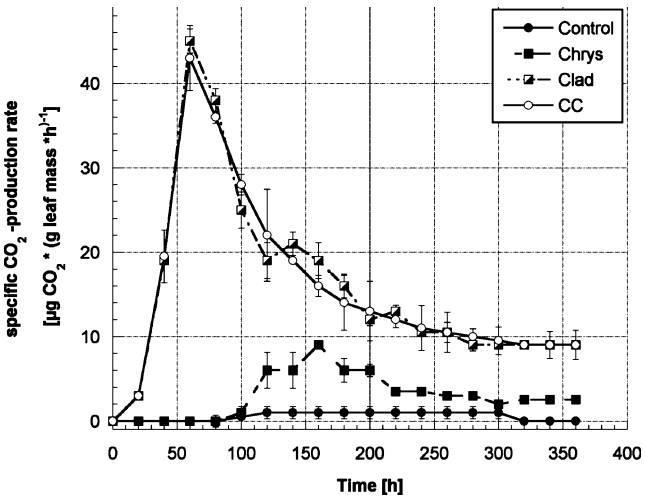

Figure 5 Respiration determined by measurement of CO_2 during the first 100 h of the microcosm experiment. *Chrys Chryseobacterium* sp. only; *Clad C. herbarum* only, *CC* co-culture of *Chryseobacterium* sp. and *C. herbarum*. *Symbols* indicate means, *error bars* show standard deviation ($n=10$)

and D,L lactic acid. *Chryseobacterium* sp. did not excrete a protease on skim milk medium.

Microscopy

On LEA plates and willow leaves from the microcosms, single bacteria and microcolonies of *Chryseobacterium* sp. were always localized in the hyphosphere of *C. herbarum* (Fig. 6). In microcosms, the fungus showed uninhibited growth and sporulation throughout the incubation period.

Discussion

Experiments on LEA revealed that *R. pickettii* GR4 significantly inhibited the growth of *C. herbarum*. The degree of inhibition was rather mild, however, as the fungus continued to grow and sporulate. *C. herbarum* (Teleomorph: *Davidiella tassiana* (De Not.) Crous & U. Braun) is one of the most common species isolated from dead organic matter in terrestrial and aquatic environments [13]. *R. pickettii* is a widely distributed Gram-negative rod that has been isolated from water, soil, and plants and is a member of the commensal oral microbial population of vertebrates [35]. Possible causes of the observed antagonism of bacterium toward fungus may be resource (exploitation) or interference competition. On LEA plates, the organisms may compete for the easily extractable carbon sources available from the extraction method. In vitro, many fungi and bacteria produce substances with antibiotic activity [15, 17, 25, 28, 40]. However, the production of interfering allelochemicals by our *R. pickettii* strain was not observed in bioassays. As suspected for fungal secretes, the substances of bacterial and chemical warfare on submerged leaves in a stream may be washed away [32]. On the other hand, the hyphosphere may provide a microenvironment where, fostered by bacterial EPS [19, 37] and fungal mucilage, microcompartiments of low velocity and drift may exist. The *R. pickettii* GR4 strain isolated in this study showed extensive production of exopolysaccharides on LEA plates (data not shown). It is also possible that a complex mechanism is responsible for the inhibition of fungal growth. Quorum sensing, a communication system between individuals of a bacteria population dependent upon its density, has been considered to be of significant importance in pathogenicity and virulence [41]. Raaijmakers et al. [29] showed that the production of the antifungal antibiotic 2,4-diacetylphloroglucinol (Ph1) was strongly related to the density of the bacterial population. Further research on bacterial–fungal interactions in the hyphosphere should include the elucidation of the biochemical nature of different modes of actions.

Interactions in the Microcosms

Co-incubation of *Chryseobacterium* sp. and *C. herbarum* on willow leaves in the aquatic microcosm showed antagonistic phenomena. The C/N ratio data suggest a delay in the conversion of leaf material into microbial biomass in dual culture relative to the *C. herbarum*-only culture. The measurement of exoenzymes showed that the exocellulase and cellobiase activity of the fungus decreased when it was cultured together with the bacterium. A source of error in respect to the exocellulase and cellobiase activity measurement may be the fact that all enzymes of the organisms in the samples were still present in the CM-cellulose preparation after addition of the digested sample. Thus, it is likely that a part of the formed glucose was metabolized and, therefore, not detectable. Therefore, the enzymatic activity might be underestimated, but this does not change the fact that the fungus has less glucose available in co-cultures than in single culture. Also, in fungus-only samples, the enzymatic activity might be underestimated due to glucose utilization by the fungus itself. In analogy with the exocellulase activity, the fungal endocellulase activity was less in dual cultures. In the microcosms, the respiration patterns of *C. herbarum* seemed to be uninfluenced by the presence of bacteria. The discrepancy between decreased enzyme activity and uninfluenced respiration might have been caused by a different allocation of the fungal energy usage for growth or production of conidia. On the other hand, it

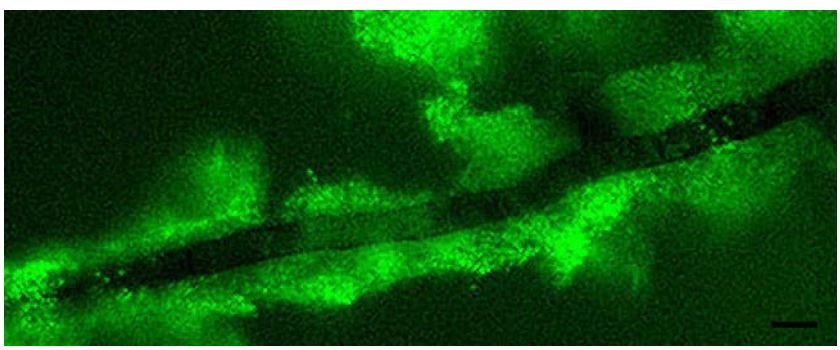

Figure 6 *Chryseobacterium* sp. in the hyphosphere of *C. herbarum* during co-incubation on willow leaf litter in the microcosm day 15. Stained with green fluorescent SYTO 9, *bar*=10 μm

is assumed that the Respirocond method is not sensitive enough to detect the respiration of *Chryseobacterium* sp. since large amounts of bacteria were microscopically observed in the samples. Two hypotheses may explain our results: either the presence of *Chryseobacterium* sp. causes a lower production of cellulases by *C. herbarum* or these enzymes are consumed by the bacteria. *Chryseobacterium* sp. did not excrete a protease, however, and, hence, is unable to consume polypeptides. The strain *Chryseobacterium* sp. is a member of the genus *Flavobacterium.* Members of the genus *Flavobacterium* are unable to decompose cellulose [8]. The BIOLOG pattern of *Chryseobacterium* sp. revealed that it is able to use cellobiose as a carbon source. The disaccharide cellobiose is a product of the consumption of cellulose by the exocellulase. In co-cultures *Chryseobacterium* sp. could, by the intake and catabolism of cellobiose, remove substrate from the fungus spreading its exocellulases. The commensalistic activity of *Chryseobacterium* sp. could then completely inhibit the fungus from utilizing its cellulase activity, resulting in a lower enzyme activity of the fungus overall. The decline of fungal enzyme activity in bacterial co-cultures is consistent with the results found by Mille-Lindblom and Tranvik [23]. They stated that reduced fungal growth in the presence of bacteria is the most probable cause for the lower enzymatic activity in co-cultures as compared to the fungi-only treatment. In a microcosm study of Gulis and Suberkropp [16], bacteria had a negative effect on fungal performance in terms of mycelial production and sporulation rates.

Microcosm conditions may favor bacteria in general. Cutting of leaves into small pieces may support the leaching of leaf substances and, thus, may ease degradation. Additionally, autoclaving leaves changes their chemical and physical properties [21], such as the leaching of condensed tannins and polyphenolic constituents as well as the protein precipitation capacity. Tannins and phenolics may reduce fungal cellulase activity. It seems unlikely, however, that the bacteria in the microcosms influenced the leaching of such compounds. Bacteria in microcosms surely benefit from the products of the extracellular enzyme activities of fungi. If the surrounding buffer is not exchanged, leaf leacheates and fungal decomposition products can accumulate in the microcosm. Accumulation of enzymes, decomposition products, and leaf leacheates may also occur in a natural hyphosphere-like microenvironment. The presence of *Chryseobacterium* sp. in the hyphosphere throughout the 60-day incubation indicates that the space near fungal hyphae may be a profitable location for freshwater bacteria. But the substances accumulating in the hyphosphere may not always be beneficial, as the bacteria may encounter antibiotics produced by the fungus. During the 60 days of incubation in the microcosm, neither the fungus nor the bacterium showed, by fluorescence microscopy, an obvious decline in biomass and/or sporulation activity. In contrast, other microbial interactions studies (e.g. [16, 22]) showed a decrease of fungal biomass measured by ergosterol analyses.

The effects of antagonistic interactions between fungi and hyphosphere-associated bacteria may appear in different ways [12]. In the case of *C. herbarum* and *R. pickettii* GR4, antagonism was visible as diminished fungus growth, as compared to growth with other bacterial species. The effects of antagonistic interactions between *C. herbarum* and *Chryseobacterium* sp. were not visible in co-cultures in Petri dishes but were apparent as lowered fungal enzymatic activity measured in microcosm experiments. Our results showed rather mild effects of antagonistic microbial relations. Nonetheless, even in aquatic habitats hyphosphere-associated bacteria seem to have the potential to affect the fungal performance significantly.

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