Supplementary Information

Authors:

Martin Rozmoš[1] · Petra Bukovská[1] · Hana Hršelová[1] · Michala Kotianová[1] · Martin Dudáš[1] · Kateřina Gančarčíková[1] · Jan Jansa[1]

Affiliation (postal address):

[1] Laboratory of Fungal Biology, Institute of Microbiology, Czech Academy of Sciences, Vídeňská 1083, Prague 4, 14220, Czech Republic

Corresponding author:

Jan Jansa, email: jansa@biomed.cas.cz, phone: +420736404166

Supplementary Methods

Solidified growth medium from the labelling compartments recovered from Experiment 2 (*i.e.*, the modified MSR medium with added chitin, with or without bacteria or protists, and containing or not containing the arbuscular mycorrhizal [AM] fungal hyphae) were analyzed for abundance of bacteria, protists, and the AM fungus *Rhizophagus irregularis* upon harvest using quantitative real-time PCR (qPCR). The samples (15 ml of growth medium each) were first lyophilized and then DNA was extracted from the samples using a modified procedure described elsewhere [1] and while spiking each sample with a known amount (*i.e.*, 20 billion copies) of the internal DNA standard [2]. Briefly, the lyophilized samples were first ground in a ceramic

mortar with 2 ml of cetyltrimethyl ammonium bromide (CTAB) buffer (pH = 8.0) for 5 min and then transferred to a 50 ml centrifugation vial. The remainder of each sample was washed from the mortar using an additional 1 ml of the extraction buffer, which was then combined with the previous sample portion in the centrifugation vial. This mixture was incubated at 65 °C for 60 min, followed by centrifugation at 5,000 g for 10 min. The supernatant (1.2 ml) was transferred to a new 2 ml vial, 500 µl of chloroform was added, the mixture was vortexed for 10 s, and it was then centrifuged at 20,000 g for 10 min. The upper phase (not more than 200 µl) was then transferred to a new 1.5 ml vial, to which was added 1 ml of the Bind buffer (pH = 6.0) together with 10 µl of glassmilk (50%) as described elsewhere [3]. The suspension was then vortexed briefly and incubated for 5 min at ambient temperature with occasional stirring. Thereafter, the suspension was centrifuged at 20,000 g for 5 s and the supernatant discarded. The pellet was washed twice with 500 µl each of the NEET buffer (pH = 7.5), dried in a vacuum centrifuge for 3 min, then resuspended in 110 µl of the TE buffer (pH = 7.4), incubated at 65 °C for 5 min, centrifuged at 20,000 g for 30 s, and 100 µl of the supernatant was transferred to a new tube. This supernatant was used as a template in qPCR analyses using primers and hydrolysis (TaqMan) probes targeting specifically the internal DNA standard, *Rhizophagus irregularis* 28S rRNA gene, or the eubacterial 16S rRNA gene. Sequences of the primers and hydrolysis probes, together with the cycling conditions, were described earlier [4]. The abundance of *Polysphondylium pallidum* (targeting the 18S rRNA gene) was quantified using qPCR with the following primers (5′ to 3′ orientation): forward: GGCTACCACTTCTATGG, reverse: AATCTCAATCTCAACTACG, and a hydrolysis (TaqMan) probe, dually labelled with FAM and BHQ1 quencher: AAATTACTCAATCCCAATACGG. Cycling conditions for the *Polysphondylium* assay

were as follow: Initial denaturation at 95 °C for 3 min, followed by 55 PCR cycles (95 °C for 10 s, 47 °C for 30 s, and 72 °C for 20 s). Fluorescence of the qPCR reaction mixture was always measured after each amplification (72 °C) step. Calibration of the assay was made with amplicons generated by the primers described above specific for the protist from the *Polysphondylium*-containing samples from Experiment 2. For calibration of the qPCR assay, the amplicons were purified from a PCR mix, DNA concentration measured fluorometrically using Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), and serially (5-fold) diluted to cover a range of concentrations from hundreds of millions to less than 1 copy per µl.

The qPCR assays employed Luna® Universal qPCR Master Mix (New England Biolabs, Ipswich, MA, USA) for assessment of bacteria or Luna® Universal Probe qPCR Master Mix for assessment of internal DNA standard, *Rhizophagus irregularis*, and *P. pallidum*. The analyses were carried out in a LightCycler 480 II (Roche, Rotkreuz, Switzerland) equipped with 96-well plates.

Supplementary Results and their interpretation

Root dry biomass and the C, N, and P balances of the in vitro *microcosms*

Dry biomass of mycorrhizal roots per plate in Experiment 1 (263 mg, mean from 170 observations) was significantly lower ($p < 0.001$) than that of NM roots (345 mg, mean from 112 observations). The mean biomass of AM fungal hyphae in the mycorrhizal plates in Experiment 1 (recovered from the large plate volume) was 13.9 mg per plate. In the same experiment, significantly less C was also detected in the

mycorrhizal roots together with the AM fungal hyphae collected from the root-free compartment in the mycorrhizal plates than in the NM roots (27.3% vs. 32.9% of the added C as sucrose, respectively, $p < 0.001$). There also was less total N contained in the roots and their attached AM fungal hyphae than in the NM roots in Experiment 1 (75.7% vs. 81.5% of the N contained in the MSR medium, respectively, $p < 0.001$). No differences in the P content (*i.e.*, total amount of P in plant and fungal biomass per plate) were detected between the mycorrhizal and the NM microcosms in Experiment 1 ($p = 0.9$, although this analysis considered only six randomly chosen plates from each of the mycorrhizal and NM treatments in Experiment 1).

In Experiment 2, mean dry biomass of NM roots per plate was 317.5 mg, significantly (ANOVA $F_{1,180} = 134.2$, $p < 0.001$) greater than the biomass of mycorrhizal roots per plate, which reached a mean of 261.4 mg per plate. Mean biomass of the AM fungal hyphae extracted from mycorrhizal plates (*i.e.*, from the large Petri plate volume) was 7.5 mg. The N content (*i.e.*, total amount of N in plant and fungal biomass per plate) was greater (ANOVA $F_{1,180} = 67.7$, $p < 0.001$) in mycorrhizal microcosms in Experiment 2 (mean of 4.47 mg N per microcosm) than in the NM microcosms (mean of 3.72 mg N per microcosm).

The above analyses of the roots and AM fungal hyphae recovered from the *in vitro* plates indicated (although did not provide ultimate proof) that the mycorrhizal roots with their associated AM fungal hyphae were significantly more active in terms of respiration and thus also accumulated less biomass than did the NM roots in both of the experiments presented here. This effect was not necessarily accompanied by lower nutrient content in the root and fungal biomass per microcosm. This is consistent with the expected outcome of the interaction when energy is in limited supply [5]. Almost all (>80%) of N originally supplied with the MSR medium appeared

in the roots and/or AM fungal hyphae at the end of Experiment 1, thereby confirming that most of the N was freely available and actually used by the organisms (albeit slightly less efficiently in the mycorrhizal plates as compared to the NM treatment in Experiment 1, whereas higher N uptake into the roots and AM fungal hyphal biomass was encountered in Experiment 2). On the other hand, around 400% of the P supplied as orthophosphate in the MSR medium was actually detected in the roots and the mycorrhizal hyphae (the latter only in cases when mycorrhizal fungus was present, see Supplementary Data1), most of which obviously originated from the gelling agent. Importantly, the amounts of P in the root and/or AM fungal biomass per microcosm did not differ between the mycorrhizal and NM plates. These results indicate that both the P as well as the N supplied with the media in the main compartment were accessible by diffusion to both AM fungal hyphae and the roots (either mycorrhizal or the NM roots). The sharp contrast in C content of the plant and fungal biomass and inconsistent to no effect on mineral nutrient content between mycorrhizal and NM microcosms indicate that mineral nutrients were less likely to be limiting growth of the roots and AM fungal hyphae as compared to the carbon and/or energy supply.

Microbial abundances in the labelling compartment of Experiment 2

Large differences were detected in bacterial abundance within the labelling compartment in Experiment 2 among the different bacterial inoculation treatments. These differences were due to identities of the bacterial strains and to inoculation with the AM fungus and/or protist (Table S1). Significant values above the qPCR experimental noise detected in bacteria-free treatment were seen only for bacterial strains ID 2, 5, and 17 (*i.e., Janthinobacterium* sp. and *Paenibacillus* spp. only [Fig.

S5]). Furthermore, bacterial abundance in the labelling compartments was systematically suppressed by both the AM fungus and the protists across all bacterial inoculation treatments (Table S1).

Experimental noise for broadly specific bacterial primer assay could have resulted from co-detection of mitochondrial ribosomal rRNA genes in treatments lacking bacteria but with either the AM fungal hyphae or the *P. pallidum* present; from DNA impurities in recombinant Taq polymerase, most likely produced in *Escherichia coli*; or from random contamination of the qPCR water or chemicals, qPCR plates, or pipette tips in the lab. Although details of Taq polymerase production by the manufacturer of the qPCR master mixes used here are not publicly known, most DNA polymerases nowadays are routinely produced in transgenic bacteria and the master mixes often contain fragments of bacterial DNA unless subjected to a dedicated clean-up. Our experimental results (see Supplementary Data2 for details) indicate that the main source of experimental noise with bacterial qPCR quantification in bacteria-free treatments was the contamination of master mixes with bacterial DNA, possibly from the Taq polymerase production. Another issue in detection of the various bacteria was that we only detected very low values for some of the strains (e.g., ID 8, 12, 13, and 15) in Experiment 2 (Fig. S5), due either to poor growth of the specific bacteria on media where chitin was the only N source and also the source of most organic C in the labelling compartments (which is most likely, as these very strains were not capable of utilizing chitin, see Table 1), or (less likely) due to inefficient DNA extraction from cells of those strains.

Detection of *Rhizophagus irregularis* in non-mycorrhizal microcosms in Experiment 2 was very low and, because there were significant differences in abundance of the AM fungus as affected by the different bacteria in the mycorrhizal microcosms, also

the interaction between the two factors (*i.e.*, bacterial and AM fungal inoculation) turned significant (Table S1). Looking only at the mycorrhizal microcosms, development of the AM fungus in the labelling compartment was positively affected by three bacterial strains (ID 2, 5, and 12) – *Janthinobacterium* sp., *Paenibacillus chitinolyticus* CCM 4527, and *Bacillus* sp – as compared to the bacteria-free treatment (Fig. S5). None of the bacteria included in Experiment 2 showed antagonism against the AM fungus (Fig. S5).

Detection of the protist *P. pallidum* was very low in the protist-free microcosms and, because there obviously were significant effects of the different bacteria on the protist abundance in the protist-inoculated microcosms, the interaction between the protist and bacterial inoculation treatments turned significant (Table S1). In analyzing only data from protist-inoculated microcosms, it turned out that two of the seven bacterial isolates (*i.e.*, *Janthinobacterium* sp. and *Paenibacillus ehimensis* CCM 4526) included in Experiment 2 significantly stimulated the development of the protist (Fig. S5). None of the bacterial strains included in this study effectively inhibited development of the protist as compared to the bacteria-free treatment (Fig. S5).

Supplementary references:

1. Gardes M, Bruns TD. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. Mol Ecol. 1993;2:113–8.
2. Thonar C, Erb A, Jansa J. Real-time PCR to quantify composition of arbuscular mycorrhizal fungal communities – marker design, verification, calibration, and field validation. Mol Ecol Res. 2021;12:219–32.

3. Gryndler M, Trilčová J, Hršelová H, Streiblová E, Gryndlerová H, Jansa J. *Tuber aestivum* Vittad. mycelium quantified: Advantages and limitations of a qPCR approach. Mycorrhiza. 2013;23:341–8.

4. Bukovská P, Rozmoš M, Kotianová M, Gančarčíková K, Dudáš M, Hršelová H, et al. Arbuscular mycorrhiza mediates efficient recycling from soil to plants of nitrogen bound in chitin. Front Microbiol. 2021;12:574060.

5. Johnson NC, Wilson GWT, Wilson JA, Miller RM, Bowker MA. Mycorrhizal phenotypes and the law of the minimum. New Phytol. 2015;205:1473–84.

Supplementary Table S1

Results of three-way ANOVA for the abundance of bacteria, the arbuscular mycorrhizal (AM) fungus *Rhizophagus irregularis*, and the protist *Polysphondylium pallidum* in the labelling compartment of **Experiment 2**, detected by quantitative real-time PCR, as affected by identity of inoculated bacteria (factor A), presence or absence of the AM fungus (factor B), and presence or absence of the protist (factor C).

Source of variation (factors)	Degrees of freedom	Abundance of bacteria, *F*-ratio	Abundance of *Rhizophagus*, *F*-ratio	Abundance of *Polysphondylium*, *F*-ratio
A	7	268.2 ***	7.3 ***	20.2 ***
B	1	42.0 ***	348.8 ***	0.7 ns
C	1	22.4 ***	0.0 ns	105.5 ***
A×B	7	11.6 ***	7.3 ***	1 1 ns
A×C	7	25.1 ***	1.7 ns	20.2 ***
B×C	1	3.9 (*)	0.0 ns	0.7 ns
A×B×C	7	3.43 **	1.7 ns	1.1 ns
RESIDUAL	149			

ns $p \geq 0.1$, (*) $0.05 \leq p < 0.1$, ** $0.001 \leq p < 0.01$, *** $p < 0.001$

Supplementary Table S2

Results of three-way ANOVA for the fraction of ^{15}N transported out of the labelling compartment in **Experiment 1** to the roots (in the non-mycorrhizal control treatment) or to the roots together with the AM fungal hyphae extracted from the root-free zones of the large Petri dishes (in the mycorrhizal treatment), as affected by the presence or absence of the mycorrhizal fungus (factor A, 2 levels), nitrogen form supplied into the labelling compartment (factor B, mineral vs. organic, 2 levels), and identity of the bacterial isolate inoculated into the labelling compartment (factor C, none or one of five different isolates, 6 levels). The values were log (x+0.1) transformed prior to the analysis. Plates with no nitrogen supplied in the labelling compartment were not included into this analysis.

Source of variation (factors)	Sum of Squares	Degrees of freedom	Mean Square	*F*-ratio	*p*-value
A	16.1	1	16.1	1499.4	<0.001
B	34.8	1	34.8	3241.2	<0.001
C	0.9	5	0.2	15.8	<0.001
A×B	11.3	1	11.3	1049.3	<0.001
A×C	0.9	5	0.2	16.7	<0.001
B×C	0.9	5	0.2	17.2	<0.001
A×B×C	0.9	5	0.2	16.9	<0.001
RESIDUAL	1.3	120	<0.1		

Supplementary Table S3

Results of two-way ANOVA for the fraction of ^{15}N transported out of the labelling compartment amended with ^{15}N-labelled **NH_4Cl** in **Experiment 1** to the roots (in the non-mycorrhizal control treatment) or to the roots together with the AM fungal hyphae extracted from the root-free zones of the large Petri dishes (in the mycorrhizal treatment), as affected by the presence or absence of the mycorrhizal fungus (factor A, 2 levels) and the identity of bacterial isolate inoculated in the labelling compartment (factor B, none or one of five different isolates, 6 levels). The values were log (x+0.1) transformed prior to the analysis.

Source of variation (factors)	Sum of Squares	Degrees of freedom	Mean Square	*F*-ratio	*p*-value
A	27.9	1	27.9	1608.5	<0.001
B	<0.1	5	<0.1	0.2	0.95
A×B	<0.1	5	<0.1	0.5	0.81
RESIDUAL	1.1	62	<0.1		

Supplementary Table S4

Results of two-way ANOVA for the fraction of ^{15}N transported out of the labelling compartment amended with ^{15}N-labelled **chitin** in **Experiment 1** to the roots (in the non-mycorrhizal control treatment) or to the roots together with the AM fungal hyphae extracted from the root-free zones of the large Petri dishes (in the mycorrhizal treatment), as affected by the presence or absence of the mycorrhizal fungus (factor A, 2 levels) and the identity of bacterial isolate inoculated in the labelling compartment (factor B, none or one of five different isolates, 6 levels). The values were log (x+0.1) transformed prior to the analysis.

Source of variation (factors)	Sum of Squares	Degrees of freedom	Mean Square	*F*-ratio	*p*-value
A	0.21	1	0.21	57.1	<0.001
B	1.75	5	0.35	95.2	<0.001
A×B	1.76	5	0.35	96.0	<0.001
RESIDUAL	0.21	58	<0.1		

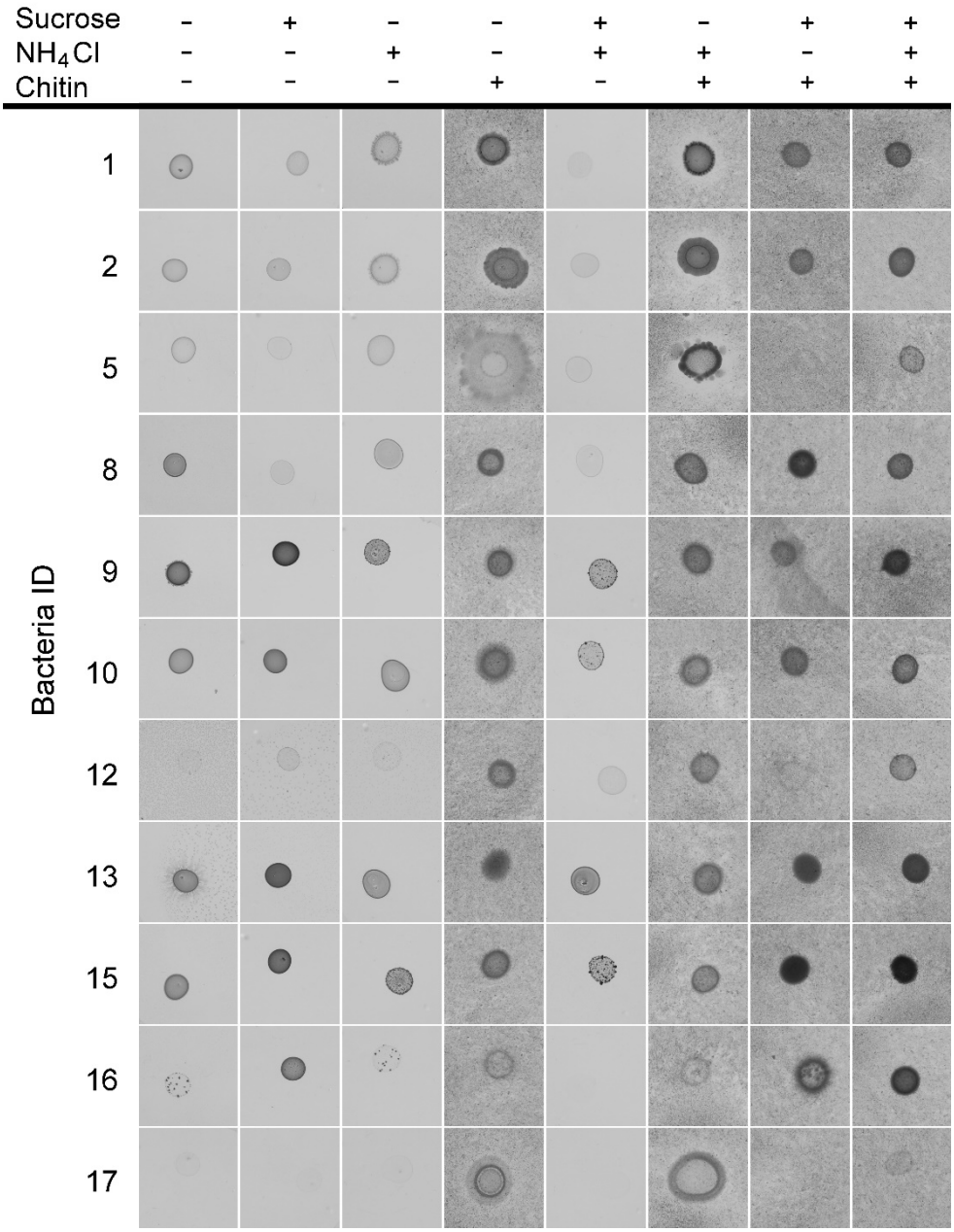
Supplementary Table S5

Results of three-way ANOVA for the fraction of ^{15}N transported out of the labelling compartment in **Experiment 2** to the roots (in the non-mycorrhizal control treatment) or to the roots together with the AM fungal hyphae extracted from the root-free zones of the main Petri dishes (in the mycorrhizal treatment), as affected by the presence or absence of the mycorrhizal fungus (factor A, 2 levels), identity of the bacterial isolate inoculated in the labelling compartment (factor B, none or one of seven different isolates, 8 levels), and inoculation of a protist *Polysphondylium pallidum* into the labelling compartment (factor C, yes or no, 2 levels). The values were log (x+0.1) transformed prior to the analysis.

Source of variation (factors)	Sum of Squares	Degrees of freedom	Mean Square	*F*-ratio	*p*-value
A	5.9	1	5.9	773.4	<0.001
B	14.2	7	2.0	267.6	<0.001
C	0.1	1	0.1	13.5	<0.001
A×B	8.8	7	1.3	165.2	<0.001
A×C	0.1	1	0.1	10.4	<0.01
B×C	0.3	7	<0.1	6.0	<0.001
A×B×C	0.2	7	<0.1	3.4	<0.01
RESIDUAL	1.1	149	<0.1		

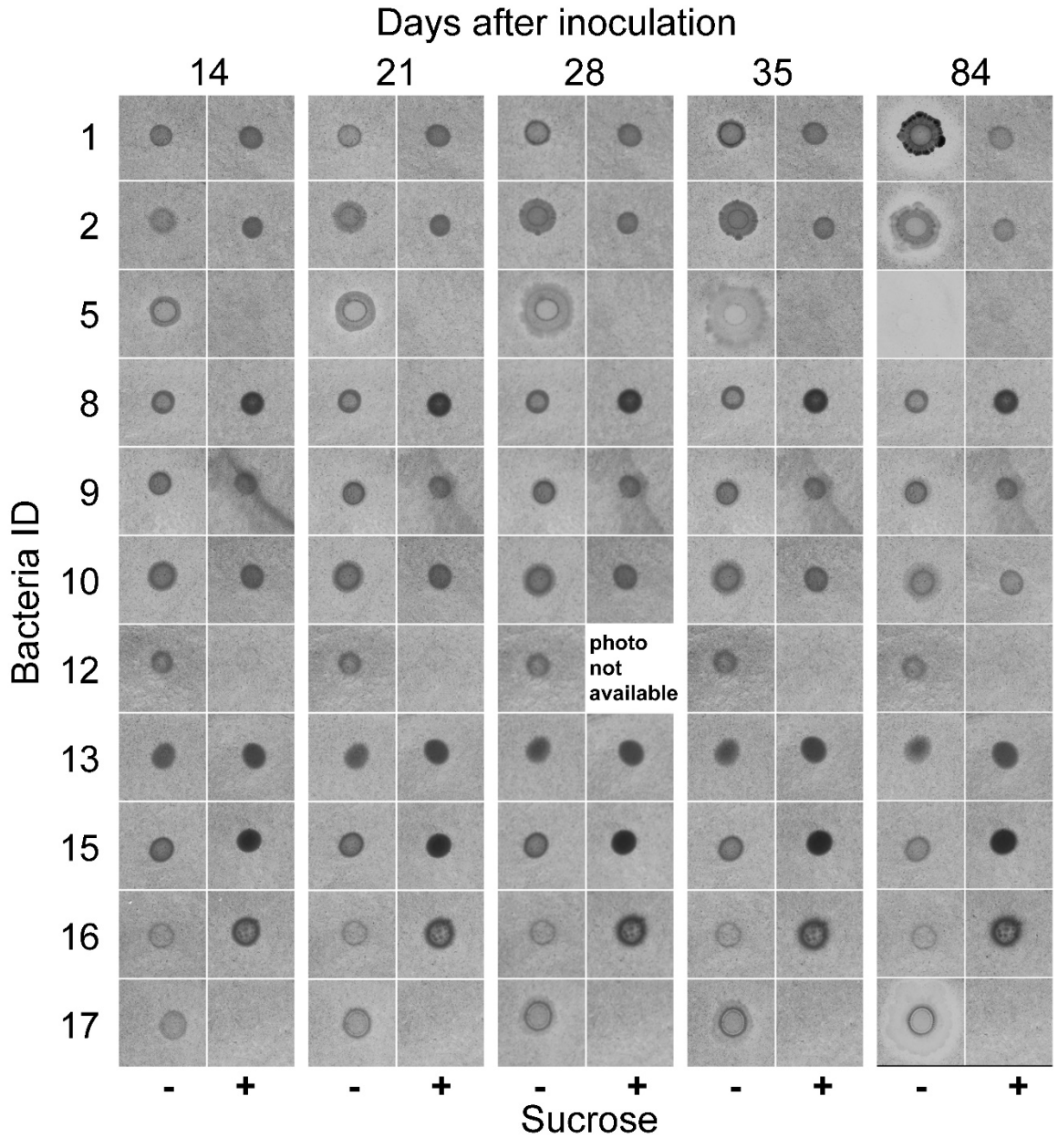
Supplementary Figure S1

Growth of different bacterial isolates included in this study, as affected by presence of sucrose (1% by volume added or not added), ammonium chloride, or crab-shell chitin additions at rates as in **Experiment 1** into the MSR medium. Chitinolytic activity is indicated by formation of clearing zones in the chitin-amended treatments around the bacterial colonies. Photographs were taken in a transmitted-light microscope at 35 days after inoculation, following incubation in darkness at 24 °C. Size of each photographic field is 2 × 2 cm.



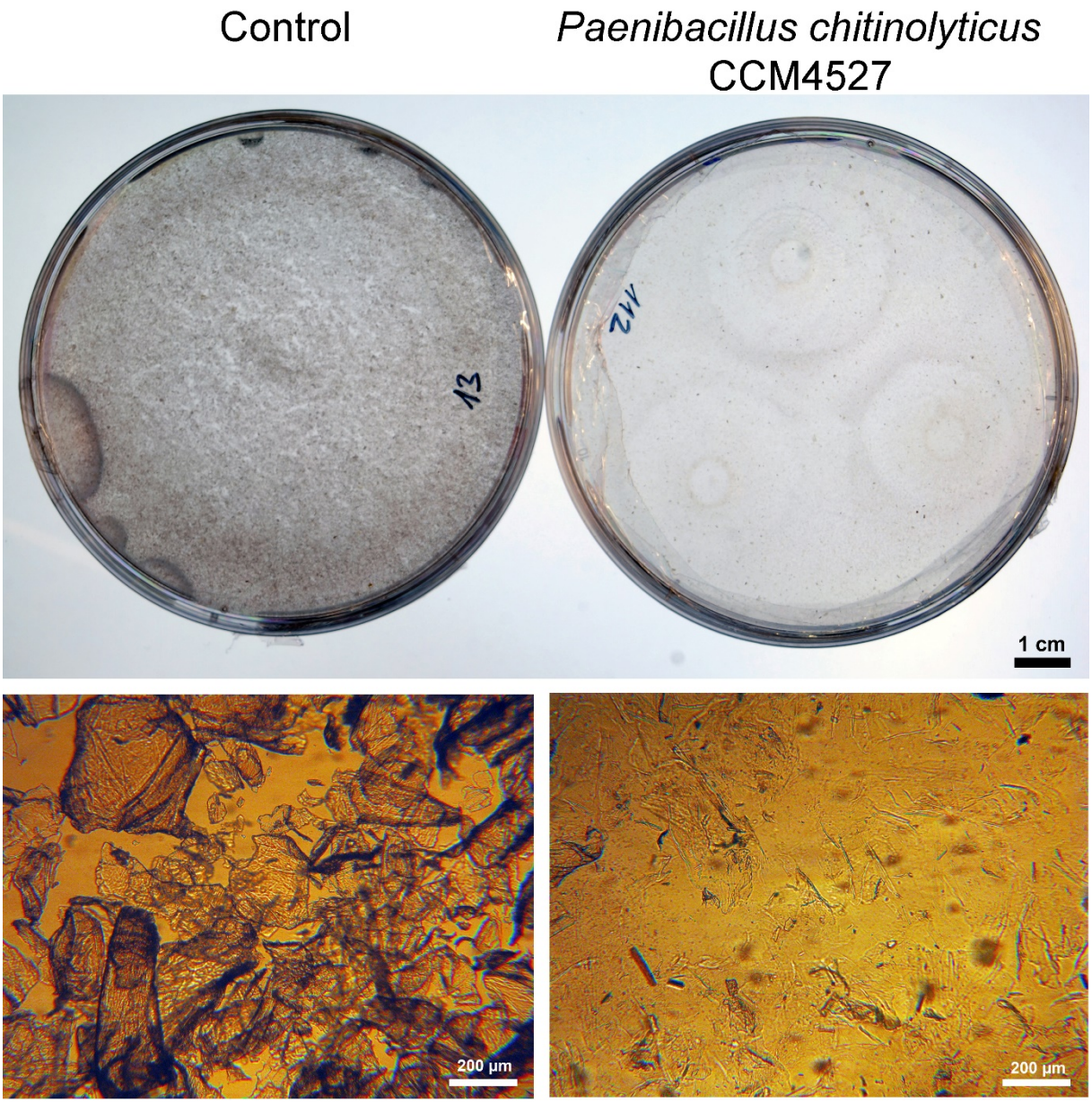
Supplementary Figure S2

Growth of and chitinolysis by the different bacterial isolates included in this study as affected by presence of sucrose (1% by volume added or not added) in the MSR medium supplemented with crab-shell chitin powder at five different time points. Chitinolytic activity is indicated by formation of clearing zones around the bacterial colonies. Photographs (of the same bacterial colony for each treatment) were taken in a transmitted-light microscope at different time points after inoculation, during incubation in darkness at 24 °C. Size of each photographic field is 2 × 2 cm.



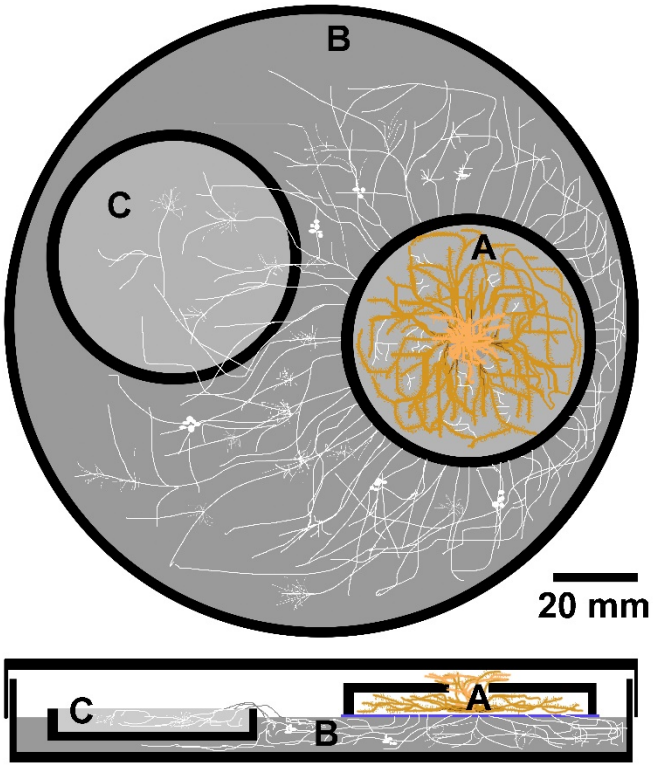
Supplementary Figure S3

Appearance of MSR medium in a Petri dish with added crab-shell chitin powder (fragments < 500 µm) as the only carbon and nitrogen source, after 99 days of incubation in darkness at 24 °C, either non-inoculated (Control) or inoculated with a chitinolytic bacterial strain *Paenibacillus chitinolyticus* CCM 4527 (*i.e.*, bacterial isolate ID 5). Clearance of the chitin in the cultivation media by the bacteria is illustrated at both macroscale (top) and microscale (bottom).



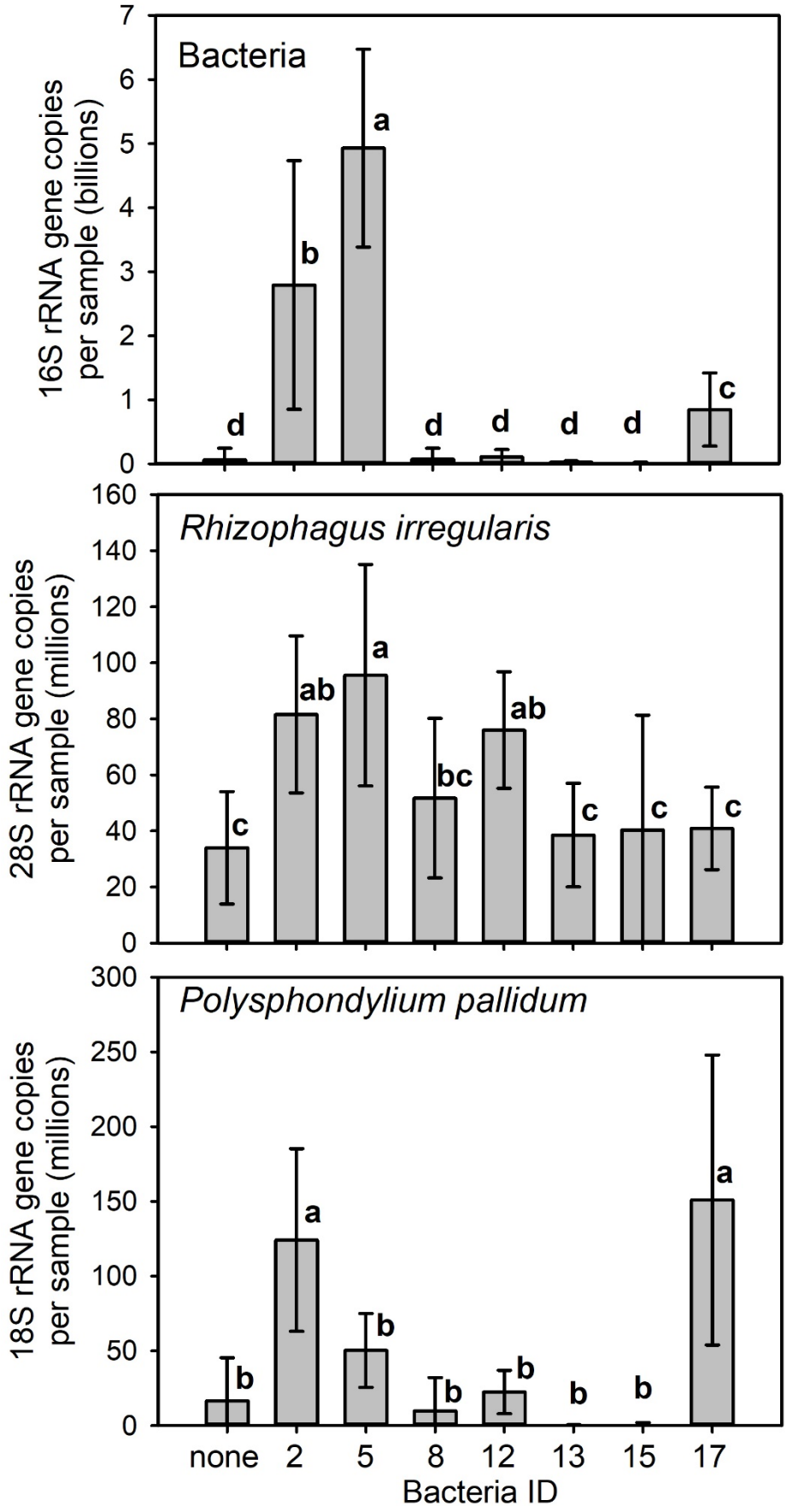
Supplementary Figure S4

The experimental system was constructed using a large Petri dish (150 mm diameter, 20 mm height). Inside that dish, a small rhizobox (Petri dish with 60 mm diameter, 8 mm height, and with a hole for root transfer delimited from the rest with a 42 µm nylon mesh (indicated by blue line) contained the Ri T-DNA transformed *Cichorium intybus* roots, either non-mycorrhizal or mycorrhizal (A). Mycorrhizal fungal hyphae growing out of the mycorrhizal roots through the mesh colonized the MSR medium filling the large Petri dish volume (B), and eventually reached the labelling compartment (C). The latter compartment was made of another small Petri dish (60 mm diameter, 8 mm height, the rim protruding above the surface of the medium in the large Petri dish by at least 1 mm, thereby constituting a diffusion barrier), and embedded in the MSR medium filling the large dish. The labelling compartment was filled with a modified (nitrogen-free) MSR medium, with or without various added ^{15}N-labelled nitrogen sources, bacteria, and/or protists.



Supplementary Figure S5

Abundance of bacteria, arbuscular mycorrhizal fungus *Rhizophagus irregularis*, and the protist *Polysphondylium pallidum* in the labelling compartment of **Experiment 2** as assessed by qPCR with taxon-specific primers and/or hydrolysis probes as described in the Supplementary Methods. Mean values of bacterial abundance and standard deviations of the means per bacterial inoculation treatment are shown for all samples (n = 22 or 23 per treatment). For *Rhizophagus* and *Polysphondylium* analyses, included were only samples where inoculation with the AM fungus or the protist had been carried out, respectively, resulting in n = 12 or 13 for the *Rhizophagus* analyses and n = 11 or 12 for the *Polysphondylium* analyses. Different letters indicate statistically significant differences between treatment means at $p < 0.05$ according to Duncan's post-hoc multiple range test following significant ANOVA. Results of preceding three-way ANOVA are shown in Supplementary Table S5.


Bacteria
16S rRNA gene copies per sample (billions)
7
6
5
4
3
2
1
0
d
b
a
d
d
d
d
c
Rhizophagus irregularis
28S rRNA gene copies per sample (millions)
160
140
120
100
80
60
40
20
0
c
ab
a
bc
ab
c
c
c
Polysphondylium pallidum
18S rRNA gene copies per sample (millions)
300
250
200
150
100
50
0
b
a
b
b
b
b
b
a
none
2
5
8
12
13
15
17
Bacteria ID

Supplementary Figure S6

Protist *Polysphondylium pallidum* at different stages of its life cycle. Amoebas feeding on a colony of *Paenibacillus ehimensis* CCM 4526 (*i.e.*, bacterial isolate ID 17), showing free amoebas and beginning of a slug formation 4 days after protist spore inoculation into a 3-week-old bacterial colony (A), amoebas aggregating on *Escherichia coli* culture 5 days after protist spore inoculation (B), and a mature fruiting body on the same culture 3 weeks after protist spore inoculation (C). Lysis of bacterial colonies and formation of *Polysphondylium* fruiting bodies on *Janthinobacterium* sp. (isolate ID 2) 4 days after introduction of protist spores (D), on *Paenibacillus ehimensis* (isolate ID 17) 5 days after protist inoculation (E), and on *Rhodococcus* sp. (isolate ID 15) 7 days after protist inoculation (F).