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Methods

Inverted bioassay
For the fungal inhibition test we used an inverted assay as described in Garbeva et al. (2014a), with bacteria growing on the bottom on a Petri-dish and fungi growing on the lids (Figure A1)

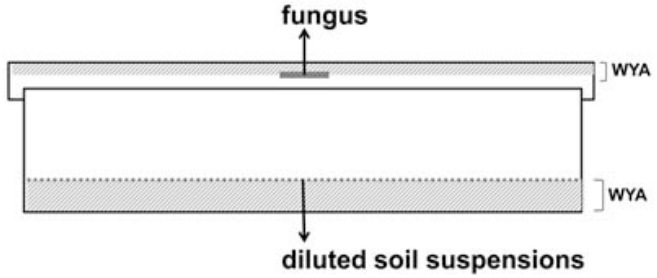


Figure A1. Petri-dish set-up used to assess areal extension of fungi growing in the presence of bacterial volatiles. Solid dark grey= fungal plug of potato dextrose agar serving as inoculum. Hatched grey = carbon-limited, water yeast agar (WYA), for fungus on the lid and for bacteria at the bottom of the petri-dish. Dashed line represents the bacterial colonies growing from the diluted soil suspensions

Fungi were pre-cultured on 0.5 potato dextrose agar (PDA Oxoid 19.5 g L^{-1} + 7.5 g L^{-1} CMN agar Boom). A plug of this culture was used as inoculum and placed on the inside of the lids of Petri dishes that contained water yeast agar (same composition as for the bacteria but without fungal inhibitors). The fungi were left overnight at 20˚C in the dark to grow attached to the lid. Thereafter, the lids were placed on top of the bacterial plates, closed with a double layer of parafilm and incubated at 20˚C. The placing of the lids on the bacterial plates was done after the two weeks incubation period, to expose the fungi to volatiles from a similar amount of bacteria. The edge of hyphal growth was indicated with a marker on the plate, after 3, 7 and 10 days. At the end of the experiment the areal extension of the colonies was determined.
Inverted bioassay with soil
In October 2011 *Fusarium oxysporum* colonies were also grown in the presence of soils rather than the cultivated bacterial communities. The inverted bioassay was used, where the water yeast agar was replaced by soil from the seven origins mentioned in Table A1. The amount of soil was equivalent to 20 g dry weight. Inhibition of fungal growth is calculated as percentage of extension of hyphae on control plates in absence of soil as $100*(1-(G_s/G_a))$ with G_s growth in the presence of soil and G_a growth in the absence of soil. Fungal inhibition in the presence of soil was tested with a rank ANOVA, with soil origin as explanatory variable. Posthoc comparisons between soil origins were done with Tukey HSD tests.

Isolation of DNA, amplification and pyrosequencing of 16S rRNA genes
The bacterial suspension obtained washing the agar plates was centrifuged for 10 min at 13000 rpm after which the pellet was used for DNA extraction with the Mobio Power Soil DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA), according to the manufacturer's instructions. Amplicons for barcoded pyrosequencing were generated by PCR using the primer set 515F and 806R (Caporaso et al. 2011), which primers target the V4 region of the bacterial 16S rRNA gene. The 515F primer included the Roche 454-B pyrosequencing adapter and GT linker, while 806R included the Roche 454-A sequencing adapter and GG linker. A 12-bp barcode (unique to each sample) was included in both primers. Bacterial DNA from the dilution plates was

used as template in PCR reactions. Five PCR reactions were performed for most DNA samples. For a few samples ten PCR reactions were performed to obtain sufficient amounts of purified product. The reactions contained 2.5 × reaction buffer, 0.2 mM of each dNTP, 0.5 µM of each primer (*Alpha DNA*, Montreal, Canada), 13 ng of template DNA, 0.056 U of FastStart High-Fidelity PCR System enzyme blend (Roche Applied Sciences, Indianapolis, IN, USA) and sterile water to 50 µL final volume. The thermocycling conditions were: initial denaturation for 5 min at 95°C; 25 cycles of 30 s at 95°C, 1 min at 53°C, and 1 min at 72°C; and a final extension for 10 min at 72°C. After product integrity confirmation by agarose gel electrophoresis, amplicons from 5 reactions were combined and cleaned with the Qiagen PCR purification kit (Qiagen, Valencia, CA, USA). After purification samples were eluted with 55 µl resulting in a concentration of ~180 ng μl^{-1}. The samples were sequenced on a Roche 454 automated sequencer and GS FLX system using titanium chemistry (454 Life Sciences, Branford, CT, USA) (Macrogen Inc. Company, South Korea).

Pyrosequencing data preprocessing

DNA sequences and quality information were extracted from the Standard Flowgram Format (SFF) files using the SFF converter tool in the Galaxy interface (Goecks et al. 2010). The data were processed using the QIIME pipeline (Caporaso et al. 2010). In brief, low-quality sequences (<150 bp in length with an average quality score of <25) were removed. After denoising the sequences and checking for chimeras using USEARCH (Edgar et al. 2011), OTUs (operational taxonomy units) were identified using UCLUST (Edgar 2010) with a phylotype being defined at the 97% sequence similarity level. Taxonomy was assigned to the most dominant OTU within in cluster using the Ribosomal Database Project (RDP) 2 classifier (release 10.4), with a minimum support threshold of 60%.

Volatile analysis

Volatiles were desorbed from the traps using an automated thermodesorption unit (model Unity, Markes International Ltd., Llantrisant, UK) at 200ºC for 12 min (He flow 30 $ml \cdot min^{-1}$). The trapped volatiles were introduced into the GC-MS (model Trace, ThermoFinnigan, Austin, TX, USA) by heating the cold trap for 3 min to 270°C. Split ratio was set to 1:4, and the column used was a 30 m × 0.32 mm ID RTX-5 Silms, film thickness 0.33 µm (Restek, Bellefonte, PA, USA). Temperature program used was as follows: from 40°C to 95°C at $3°C \cdot min^{-1}$, then to 165°C at $2°C \cdot min^{-1}$, and finally to 250°C at $15°C \cdot min^{-1}$. The VOCs were detected by the MS operating at 70 eV in EI mode. Mass spectra were acquired in full scan mode (33–300 AMU, 0.4 $scan \cdot sec^{-1}$). Compounds were identified by their mass spectra using deconvolution software (AMDIS) in combination with NIST 2008 (National Institute of Standards and Technology, USA, http://www.nist.gov) and Wiley 7th edition spectral libraries and by their linear retention indexes (lri).

Statistical analysis

In the experiment testing the effect of dilution treatment on the growth of *Fusarium oxysporum* for seven soil origins there is one missing value for soil C 10^{-1}. In our statistical analysis we compared the complete dataset with a dataset where either soil C or the whole first dilution treatment was omitted, in order to obtain a balanced dataset, and this showed that the unbalance in the data due to the missing value had no effect on the consistent significant effect of dilution on fungal inhibition.

Discussion

Based on the results of the first dilution experiment with multiple species and one soil, one fungal species (*F. oxysporum*) was chosen for further tests. All fungi showed the same trend of decreased inhibition in the presence of the most diluted bacterial communities, but the effect was less clear for the three fast-growing species, the oomycete *P. intermedium* and the fungi *F. culmorum* and *R. solani*. This is most likely due to the time it takes before an inhibiting level of volatiles has been re-established after opening the plates (when the original lid is replaced by the lid containing the fungus). Therefore, we have chosen the slightly slower growing *F. oxysporum* for further tests.

Remarkably, genera usually associated with fungal suppression (*Pseudomonas* and *Burkholderia*) were abundant in low diverse communities with both high and low production of fungal suppressing volatiles. It is quite likely that volatile production in these genera may vary among species and that the composition of antifungal volatile-producing species of these genera varies between soils (Zou et al. 2007). Similarly, comparison of soils that were conducive or suppressive for disease caused by the root-pathogenic fungus *Rhizoctonia solani* revealed that the suppression had to be ascribed to differences within dominant phyla (*Proteobacteria, Actinobacteria*) at lower taxonomic levels (Mendes et al. 2011).

Dilution series of microbial suspensions followed by regrowth in a sterile environment have been used successfully to manipulate species composition (Salonius 1981, Franklin and Mills 2006). One major issue with the dilution-to-extinction method is the possible confounding effect of biomass or density. Differences in density of bacteria could point at differences in the amount of available substrates that has been used for growth and can, therefore, also affect the quantity and quality of volatile production. Hence, this should be ruled out as confounding factor. Dilution does immediately affect the number of cells and this can be addressed by an incubation period to reach similar biomass in all treatments. Such an approach has been used in other media, such as soil (Matos et al. 2005), sewage (Franklin et al. 2001) and water (Szabo et al. 2007). This approach can only be successful when the whole studied “habitat” can be recolonized by microbes. This is not the case for agar-media as many bacterial colonies have a limited ability to expand. Therefore, we used glass beads to spread bacterial colonies after one week of growth and let that follow by another week of growth, and this resulted in full-grown plates with similar biomass for most bacterial communities. Biomass is an important factor in dilution experiments which should be handled by trying to avoid or reduce differences in biomass and by testing whether dilution effects remain after controlling for them, as was the case for our dataset.

This study is based on cultivable bacteria and thus one may question the relevance for actual soil microbial communities which are often dominated by non-cultivable bacteria species (Smit et al. 2001). Although we fully acknowledge that our approach is somewhat artificial, efforts have been made to keep bacterial communities on the media as close as possible to bacterial communities in soil. The bacterial communities develop directly from bacteria present in soil; cultivation time is as short as possible to reduce the risk of domestication (Eydallin et al. 2014), and the growth of these bacteria is on a carbon-limited medium resembling the prevailing condition in soils (Demoling et al. 2007). Therefore, processes driven by such communities, in our case volatile suppression of fungal pathogens, can give an important indication on the relationship between microbial community parameters (e.g., diversity) and ecosystem processes in real soils. The levels of volatile-induced pathogen inhibition observed with the developing bacterial communities on C-limited agar were similar to those observed with real soil. The major finding in our study is that the presence of low abundant species in the bacterial community is associated with the production of antifungal volatiles.

Appendix Table A1. Description of the seven sampling sites. A-G refer to different soil origins, for which coordinates, chemical characteristics and management are shown (*n*=1).

Tabel S1. Description of the sampling sites. Coordinates, chemical characteristics and management.

Code	A	B	C	D	E	F	G
Coordinates (degrees, min, sec)	52˚ 3' 38" N 5˚ 45' 5" E	52˚ 0' 5" N 5˚ 45' 15" E	51˚ 57' 26" N 5˚ 44' 33" E	51˚ 57' 10" N 5˚ 44' 36" E	51˚ 57' 10" N 5˚ 44' 36" E	51˚ 59' 8" N 5˚ 46' 42" E	51˚ 59' 8" N 5˚ 46' 42" E
Sand (% >63 µm)	73	75	57	55	31	80	71
Silt/Clay (% <63 µm)	27	25	43	45	69	20	29
Management	Ex-arable, Unfertilized	Ex-arable, Unfertilized	Ex-arable, Unfertilized	Permanent grassland	Fertilized maize	Permanent grassland	Fertilized maize
pH H_20	5.8	5.5	5.2	5.9	7	6.4	6.4
Olsen P (mg kg^{-1})	86	112	17	60	49	110	111
Total nitrogen (%)	0.25	0.25	0.35	0.83	0.35	0.31	0.18
Total carbon (%)	3.75	4.79	3.73	8.23	3.18	3.71	2.93

Appendix Table A2. Origin of the plant-pathogen strains used for inhibition assays

Name	Source*	Hosts
Fusarium culmorum	NIOO	*Ammophila arenaria*
Fusarium oxysporum TuA	PPO1	*Tulipa* sp.
Rhizoctonia solani AG2-2IIIB	IRS	*Beta vulgaris*
Pythium intermedium P52	PPO2	*Hyacinth* sp.

*NIOO: Netherlands Institute for Ecological Research, Department of Microbial Ecology, Paulien Klein Gunnewiek (Wageningen, The Netherlands). PPO1: Applied Plant Research, section Integrated Crop Protection, Marjan de Boer (Lisse, The Netherlands). PPO2: Applied Plant Research, section Flower Bulbs, Gera van Os (Lisse, The Netherlands). IRS: Institute for Sugar Beet Research (Bergen op Zoom, The Netherlands).

Appendix Table A3. Relative abundance of bacterial families across dilution treatments, and correlation between fungal inhibition and relative abundance of those families. Given the variation between soil origins, the median relative abundance is shown for each dilution treatment. n= number of samples in which the family was found out of a total of 71 samples. Rho and P are based on Spearman rank correlations between relative abundances and fungal inhibition data. Please note: based on non-rarefied data, only families that occurred in at least 5 samples are shown. OTUs which were frequently found but could not be identified at family level are shown in italics with the prefix "uk" for unknown.

	10^{-1}	10^{-2}	10^{-3}	10^{-4}	n	rho	*P*
Actinomycetales							
Microbacteriaceae	0.0027	0.0043	0.0077	0.0001	57	-0.09	0.469
Micrococcaceae	0.0072	0.0228	0.1081	0.2642	70	-0.39	0.001
Mycobacteriaceae	-	0.0001	-	-	28	0.18	0.143
Nocardiaceae	-	0.0002	0.0004	-	26	-0.15	0.227
Streptomycetaceae	0.0015	0.0028	0.0007	0.0003	64	0.14	0.229
ukBacteroidetes	0.0001	-	-	-	14	0.29	0.015
Sphingobacteriales							
Chitinophagaceae	0.0013	-	-	-	24	0.41	<0.000
Sphingobacteriaceae	0.0694	0.0395	-	-	45	0.42	<0.000
Bacillales							
Bacillaceae	0.0024	0.0018	0.0056	-	57	-0.06	0.613
Paenibacillaceae	-	0.0001	-	-	24	0.16	0.189
ukBacillus	0.0002	0.0001	0.0007	-	35	0.02	0.850
ukProteobacteria	0.0083	0.0005	-	-	38	0.28	0.018
ukAlphaproteobacteria	0.0075	0.0011	-	-	25	0.37	0.002
Rhizobiales							
Bradyrhizobiaceae	0.0007	0.0004	-	-	36	0.31	0.010
Phyllobacteriaceae	0.0005	0.0003	-	-	38	0.44	<0.000
Rhizobiaceae	0.0029	0.0064	0.0002	-	49	0.31	0.009
Xanthobacteraceae					30	0.42	<0.000

	10^{-1}	10^{-2}	10^{-3}	10^{-4}	n	rho	*P*
	0.0005	0.0008	-	-			
Sphingomonadales							
Sphingomonadaceae	0.0188	0.0031	-	-	46	0.45	<0.000
Burkholderiales							
Alcaligenaceae	0.0053	-	-	-	23	0.26	0.031
Burkholderiaceae	0.0806	0.0667	0.0767	0.0014	66	0.31	0.009
Comamonadaceae	0.0029	0.0057	0.0034	0.0004	61	0.19	0.113
Oxalobacteraceae	0.0060	0.0053	0.0011	-	51	0.38	0.001
ukGammaproteobacteria	0.0005	0.0008	-	-	42	0.21	0.079
Enterobacteriales							
Enterobacteriaceae	0.0442	0.0002	-	-	35	0.46	<0.000
Pseudomonadales							
Pseudomonadaceae	0.2448	0.2129	0.1962	0.0021	63	0.09	0.461
Xanthomonadales							
Xanthomonadaceae	0.2258	0.2101	0.2918	0.0032	68	0.14	0.249

Appendix Table A4. List of volatiles produced by two soil bacterial communities (soil D and F) on nutrient-poor agar. Shown here are only those compounds which were detected more frequently in the plates inoculated with 10^{-1} diluted soil suspensions (showing the highest fungal inhibition) than in those inoculated with 10^{-3} dilutes soil suspensions. Volatiles were measured for 5 replicates of dilutions 10^{-1} and 10^{-3}. Compounds known from the literature as antifungal are listed on top, with the references as footnotes. Compounds were identified on basis of retention index and mass spectra. In addition to the compounds listed below, 17 unidentified (unknown or mixed) compounds differed between the dilutions.

Compounds name (molecular formula)	LRI	CAS	Soil	Reference
Antifungal compounds				
2-methylfuran (C_5H_6O)	<650	533-22-5	F	Gao *et al*. 2010
2-furaldehyde ($C_5H_4O_2$)	831	98-01-1	F	De Corato *et al*. 2014
2-(methylthio) benzothiazole ($C_8H_7NS_2$)	1585	615-22-5	F	Zhao *et al*. 2011
muurolol isomer ($C_{15}H_{26}O$)	1637		D	Chang 2008
Activity unknown				
methyl ethyl disulphide ($C_3H_8S_2$)	828	20333-39-5	D	
4-heptanone ($C_7H_{14}O$)	871	123-19-3	D	
anisol (C_7H_8O)	913	100-66-3	F	
cadinene isomer ($C_{15}H_{24}$)	1519		F	

LRI- linear retention index Rtx-5ms column

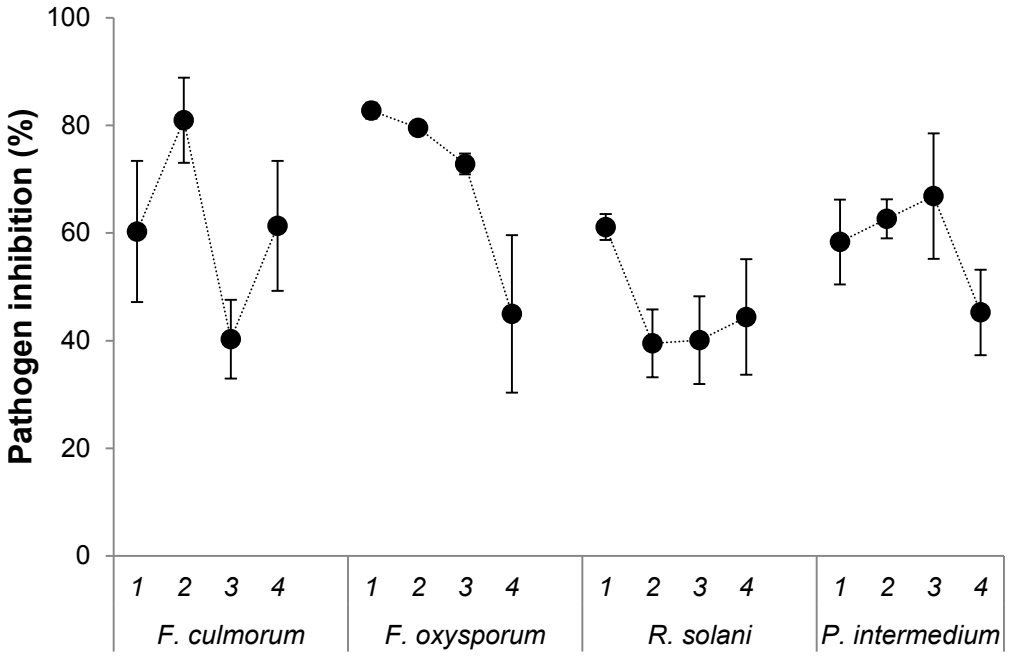
Chang H. (2008). Antifungal activity of essential oil and its constituents from *Calocedrus macrolepis* var. *formosana* Florin leaf against plant pathogenic fungi. *Biores Tech* **99**: 6266-6270.

De Corato U, Viola E, Arcieri G, Valerio V, Cancellara FA, Zimbardi F. (2014). Antifungal activity of liquid waste obtained from the detoxification of steam-exploded plant biomass against plant pathogenic fungi. *Crop Prot* **44**: 109-118.

Gao F, Dai C, Liu X. (2010). Mechanisms of fungal endophytes in plant protection against pathogens. *Afr J Microbiol Res* **4**: 1346-1351.

Zhao L, Yang X, Li X, My W, Liu F. (2011). Antifungal, insecticidal and herbicidal properties of volatile components from *Paenibacillus polymyxa* Strain BMP-11. *Agr Sci China* **10**: 728-736.

Appendix Figure A2



Appendix Fig. A2. Inhibition of four root pathogens by volatiles from cultivated soil bacterial communities. Average fungal inhibition of four root-rot pathogens (three fungi and one oomycete) exposed to volatiles produced by the dilutions of cultivated bacterial communities extracted from soil A. (Means ± s.e. given, $n = 3$). All species were significantly inhibited compared to the control plates (control = no bacteria present) and both species and dilution were significantly affecting pathogen growth with no significant interaction between species and dilution (rank ANOVA Species $Df = 3$, $F = 5.055$, $P = 0.005$, Dilution $Df = 1$, $F = 8.459$, $P = 0.006$, Species*Dilution $Df = 3$, $F = 0.985$, $P = 0.409$).

Appendix Figure A3

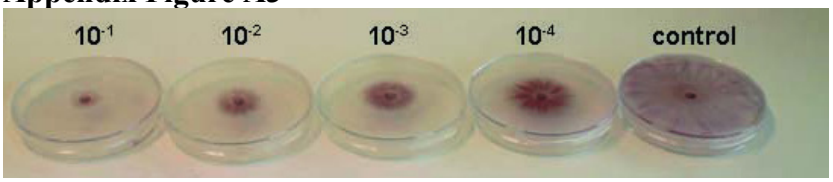
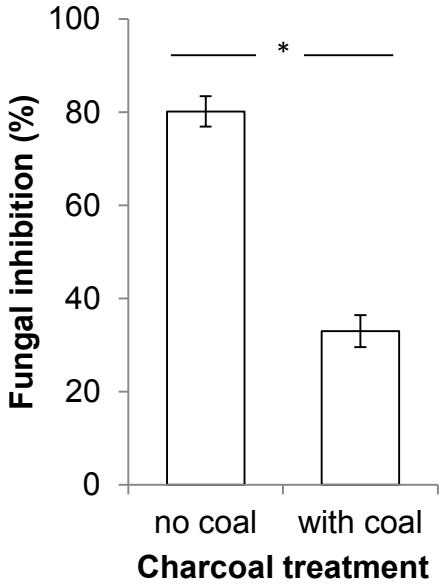


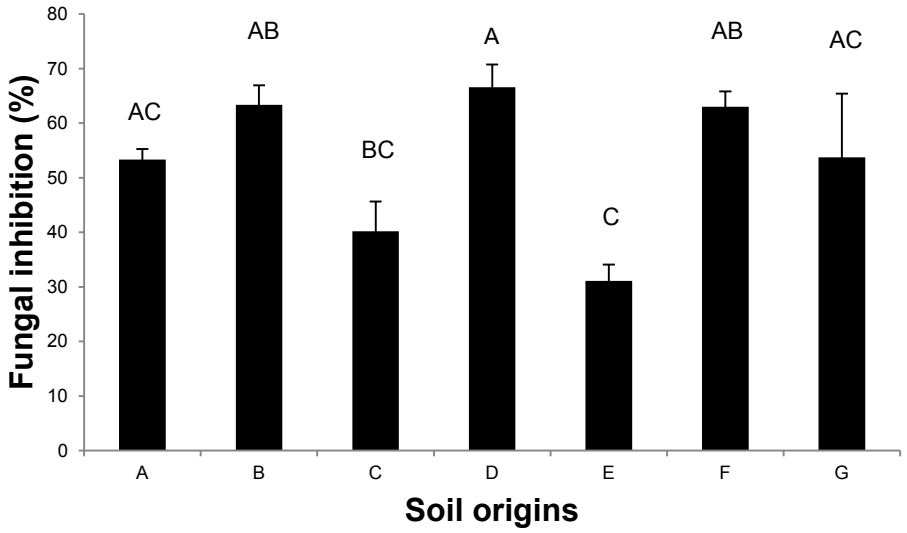
Fig. A3. Photograph illustrating the hyphal growth of the fungus *F. oxysporum* in response to volatiles produced by dilutions of soil bacterial suspensions, compared to a control without bacteria.

Appendix Figure A4



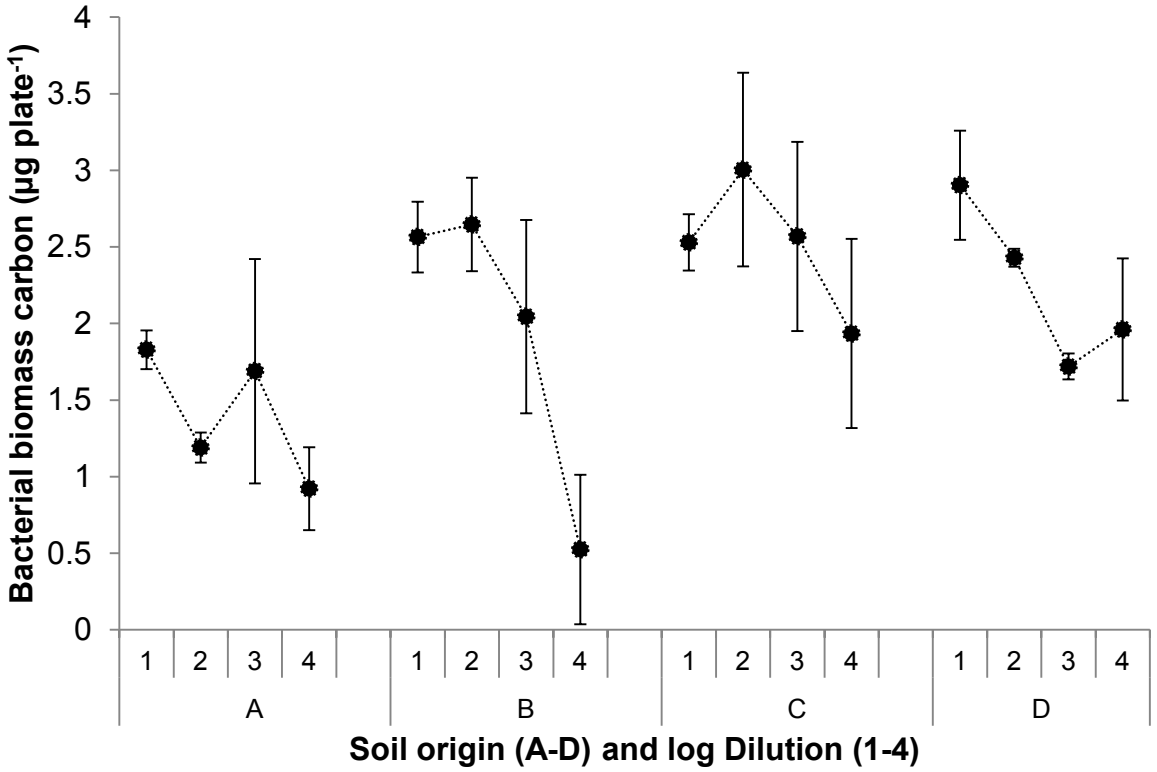
Appendix Fig. A4. Charcoal diminishes inhibition of *Fusarium oxysporum* by bacterial volatiles. This was tested for the 10^{-1} dilutions of all soil origins (Means ± s.e given, n = 7, paired t-test $P < 0.001$).

Appendix Figure A5



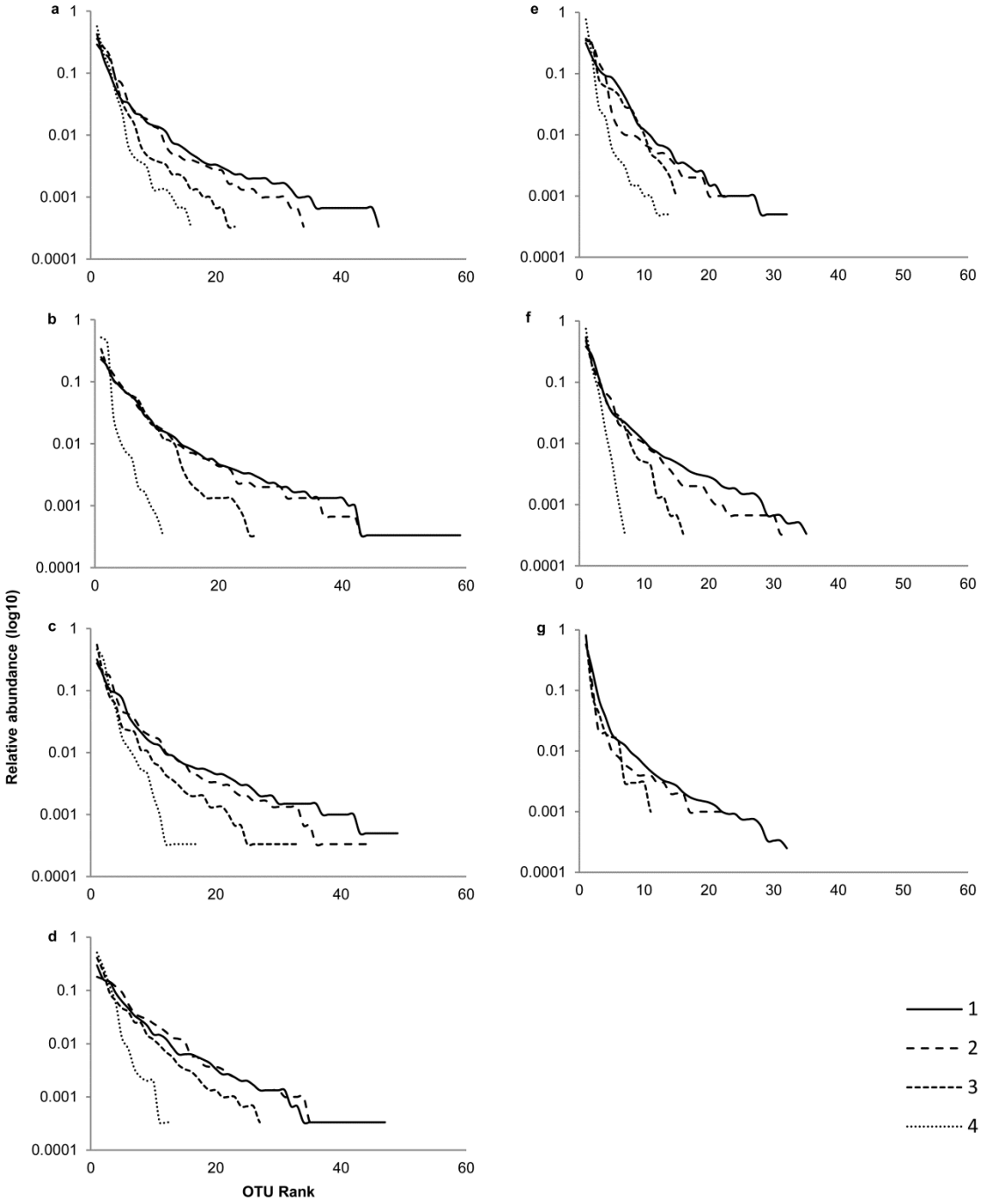
Appendix Fig. A5. The fungus *Fusarium oxysporum* is inhibited when exposed to volatiles from soils from different origins using an inverted bioassay. See Appendix Materials and Methods for details. Mean ± s.e. (n=3) are shown. Soil origins are described in Appendix Table A1. Data were analysed with an ANOVA on ranked data, followed by posthoc comparisons with Tukey HSD. Soil origins with different letters differ significantly in fungal inhibition.

Appendix Figure A6



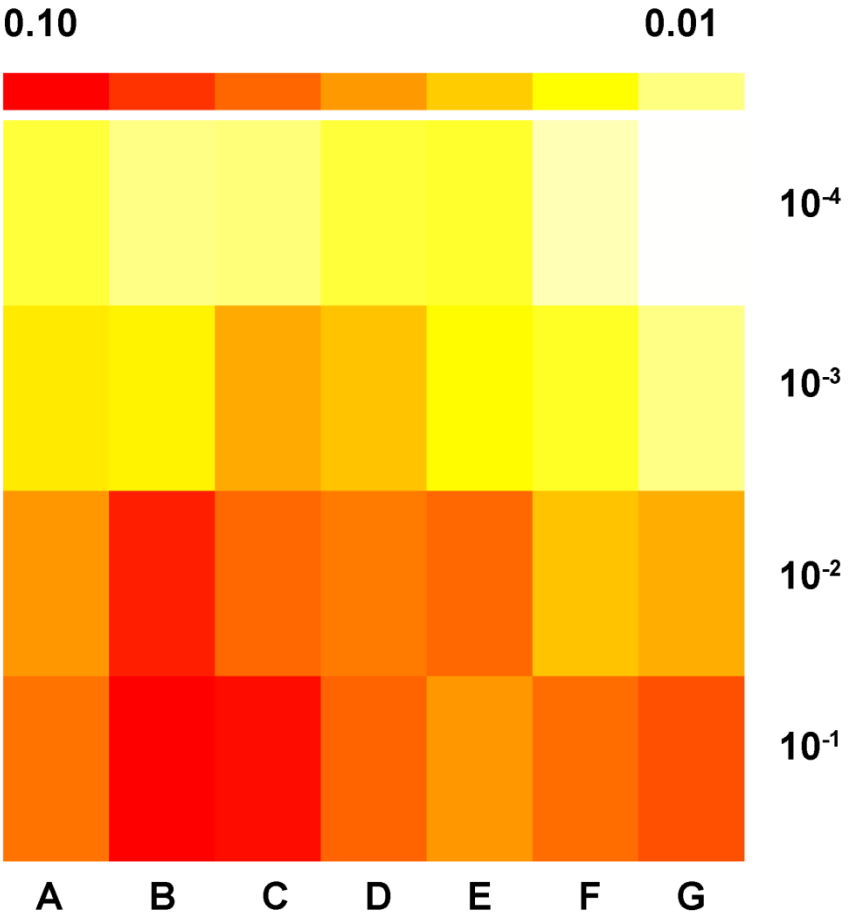
Appendix Fig. A6. Biomass of bacteria from diluted soil suspensions growing on nutrient poor agar. Means ± s.e. shown, $n = 3$. Bacterial biomass was collected by washing the plates and scraping the bacteria from the agar surface. Part of the collected suspension was freeze dried, weighed and the percentage carbon was determined on an elemental auto analyzer. Total carbon was calculated and used as estimate of bacterial biomass per dilution. Linear regression of biomass in relation to soil origin and dilution revealed no significant effect of dilution ($P = 0.36$), while data analysis with an ANOVA on ranked data (Dilution $Df = 1$, $F = 9.849$, $P = 0.003$), followed by posthoc comparisons with Tukey HSD, showed the last dilution (10^{-4}) to be significantly lower than the first dilution treatment ($P = 0.028$).

Appendix Figure A7



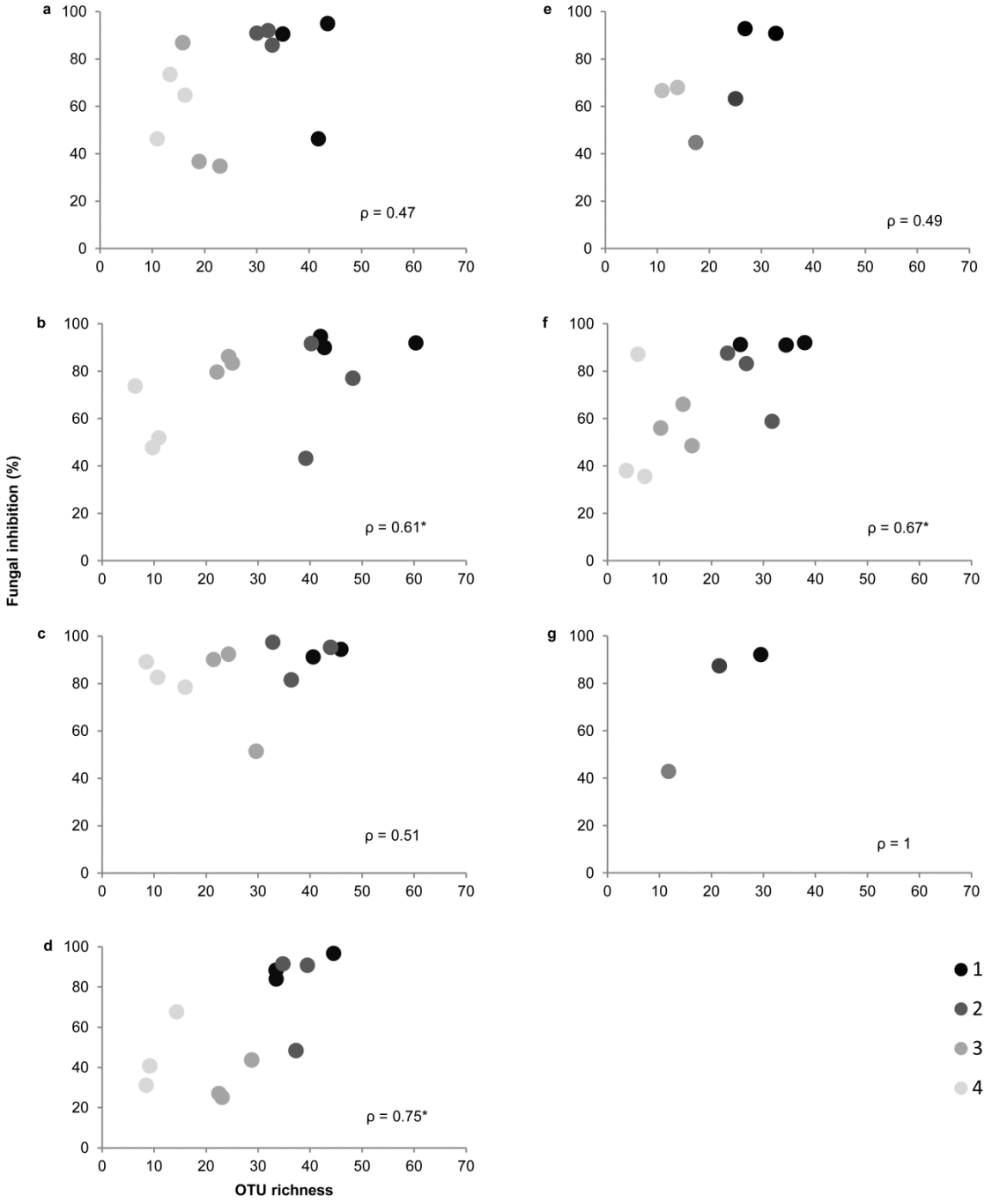
Appendix Fig. A7. Rank abundance curves for cultivated bacteria from dilution series of seven different soil origins. 1-4 indicates level of 10-fold dilution, with 1 least diluted and 4 most diluted. a-g refer to soil origins A-G in Appendix Table A1.

Appendix Figure A8



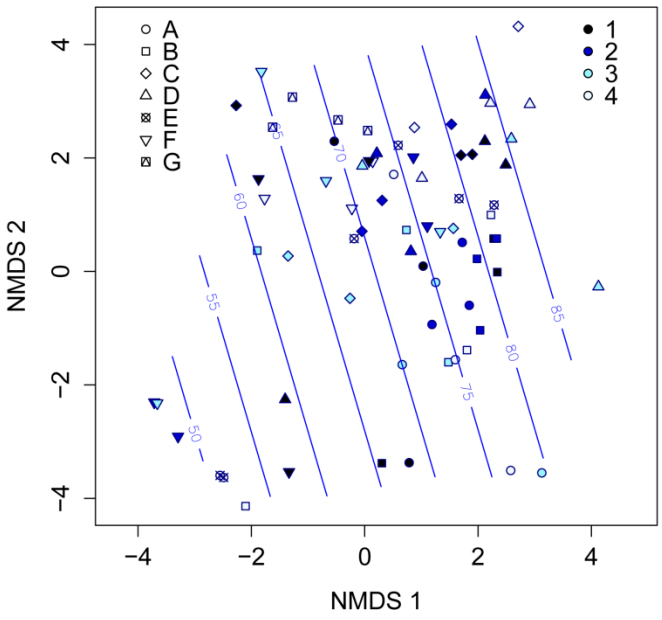
Appendix Fig. A8. Heatmap showing the cumulative abundance of all rare OTUs (<0.01 of total number of reads) per dilution treatment for all soil origins. In the weakest dilution the cumulative abundance of all rare OTUs is around 10% while in the strongest dilution treatment it is reduced to ~1%. Based on non-rarefied pyrosequencing data of the bacterial community with an average sampling depth of 6000.

Appendix Figure A9



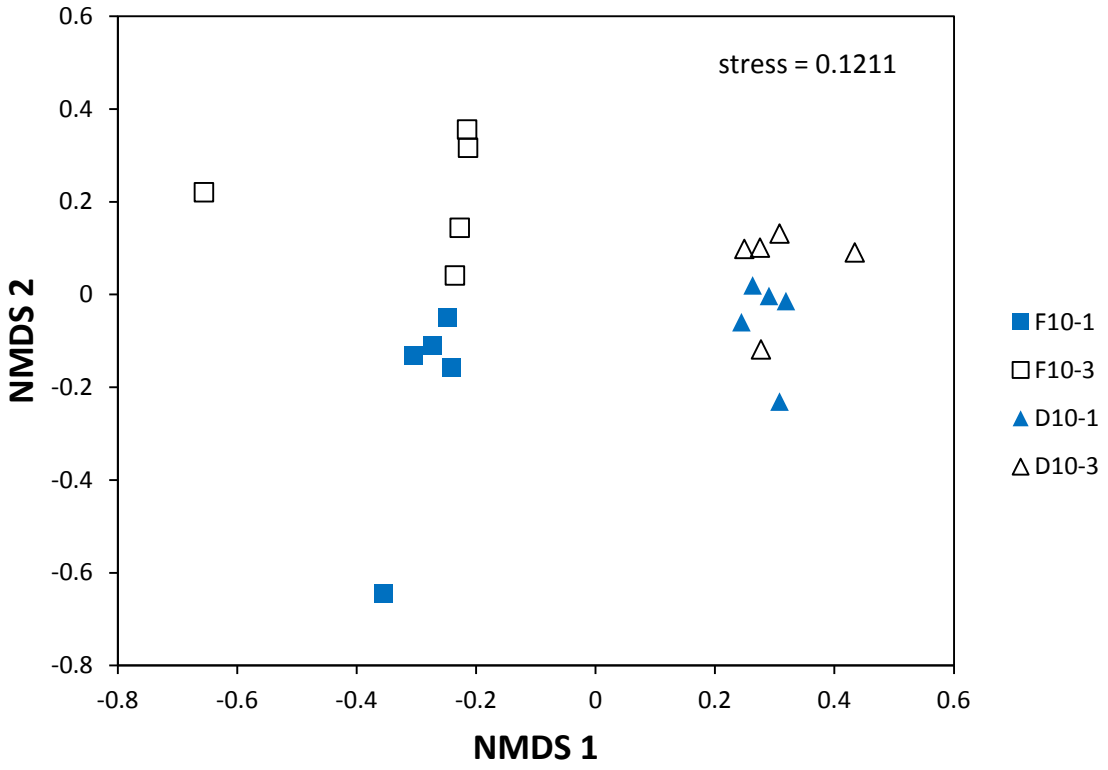
Appendix Fig. A9. Inhibition of the fungus *Fusarium oxysporum* by volatiles produced by dilution series of soil bacterial communities on nutrient-poor agar decreases with OTU loss. A-G = bacterial communities from soils A-G as described in Appendix Table A1; 1-4 indicates level of dilution, with 1 least diluted and 4 most diluted. ρ = Spearman rank correlation coefficient; asterisks indicate a significant positive correlation between OTU richness and fungal inhibition (n=3-12, P<0.05).

Appendix Figure A10



Appendix Fig. A10. NMDS plot of bacterial communities from different soil origins and dilution treatments. Soil origins A-G correspond to soil origins in Table A1 and are indicated by symbol. Dilution treatments are indicated by filling of the points, with black the weakest dilution and light blue the strongest. NMDS plot is based on Bray-Curtis dissimilarity matrix calculated based on relative abundance of bacterial families. The level of fungal inhibition is added as overlay on the figure, with the 'surf' function from the R package 'labdsv'. A Spearman rank correlation test between fungal inhibition and the position of points on the x-axis showed a weak but significant relation between NMDS axis 1 and fungal inhibition (rho = 0.26, P = 0.025).

Appendix Figure A11



Appendix Fig. A11.
NMDS plot of volatile profiles produced by bacteria from two different soil origins (D and F) and two dilution treatments (10^{-1} and 10^{-3}). NMDS is based on Bray-Curtis dissimilarity calculated over incidence data of the volatiles. Dissimilarity values correlated well with NMDS values (r = 0.939).

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