Systemic enrichment of antifungal traits in the rhizosphere microbiome after pathogen attack

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Running headline: *Recruitment of beneficial microbes*

Summary

1. Plant-associated microbial communities are crucial for plant growth and play an important role in disease suppression. Community composition and function change upon pathogen attack, yet to date we do not know if these changes are a side effect of the infection or actively driven by the plant.

2. Here we used a split-root approach to test whether barley plants recruit bacteria carrying antifungal traits upon infestation with *Fusarium graminearum*. Split-root systems allow disentangling local infection effects, such as root damage, from systemic, plant-driven effects on microbiome functionality. We assessed the recruitment of fluorescent pseudomonads, a taxon correlated with disease suppression, and of two well-described antifungal genes (*phlD* coding for 2,4-DAPG and *hcnAB* coding for HCN).

3. We show an enrichment of fluorescent pseudomonads, *phlD* and *hcnAB* upon pathogen infection. This effect was only measurable in the uninfected root compartment. We link these effects to an increased chemotaxis of pseudomonads towards exudates of infected plants.

4. *Synthesis.* We conclude that barley plants selectively recruited bacteria carrying antifungal traits upon pathogen attack and that the pathogen application locally interfered with this process. By disentangling these two effects we set the base for enhancing strategies unravelling how pathogens and plant hosts jointly shape microbiome functionality.

Key words:

Barley, *Fusarium graminearum*, Plant-microbe interactions, *Pseudomonas*, Recruitment, Split-root

Introduction

Plant pathogens cause significant loss of agricultural yield worldwide (Strange & Scott 2005) and are an important determinant of plant community structure and productivity (Packer & Clay 2000; Klironomos 2002; Van der Putten 2003; Petermann *et al*. 2008). Plant-associated microbes form a first line of defense that may complement plant innate immunity. In particular the rhizosphere microbiome, the microbial community associated with plant roots, is increasingly recognized to shape disease suppression (Berendsen, Pieterse & Bakker 2012; Mendes *et al.* 2013).

The rhizosphere is a place of complex interactions between plants and microbes. Plants invest important resources to secrete root exudates, a set of labile organic compounds able to recruit, feed and manipulate the physiology of a subset of microbes present in the surrounding soil (Bais *et al*. 2006). In return several of the associated microbes will protect plants against pathogens by producing antibiotics and stimulating plant immunity (Bakker *et al.* 2013).

Plant microbiome composition varies as a function of plant identity and soil type (Costa *et al.* 2006; Aira *et al.* 2010; Peiffer *et al.* 2013) and even with plant growth stage (Chaparro *et al.* 2013; Yuan *et al.* 2015), indicating that the host plant exerts a structuring influence on the selection of microbial communities from the available species pool. Also plant diseases play an important role and pathogen attack can be associated with alterations in microbiome structure, functionality and activity (Trivedi *et al.* 2012).

These different effects interactively shape microbiome functionality in response to diseases, making it hard to distinguish which changes are actively driven by the host plant (and thus indicate that a plant has the ability to structure its microbiome) and which changes are self-assembly processes driven by changes in the environmental conditions around the roots. Such passive changes encompass root damage by pathogens, causing leaking of compounds, or an altered exudation in pathogen-challenged plants as a result of a lower photosynthetic activity or a shunting of the nutrient flows toward healthy parts of the root system (Henkes *et al*. 2011). However, there are several cues indicating that plants react to pathogen infection by producing compounds that may attract and stimulate beneficial microbes (Rudrappa *et al*.

2008; Jousset *et al*. 2011). Such plant mediated processes might even favor specific functions if feedback loops allow plants to reward microbes performing certain tasks, as reported for different plant-microbe symbioses (Phillips *et al.* 2004; Kiers & Denison 2008).

In the present study we aim to disentangle pathogen- and plant-mediated effects on microbiome functionality. To separate these effects we set up a split-root system with barley plants growing in a natural soil. Split-root systems involve the separation of the root system into two hermetic compartments, allowing plant-microbe interactions to be tracked independently. In the present case we added a fungal pathogen to one of the compartments and measured alterations in functional traits of the root-associated communities. At the treatment compartment, the pathogen may alter plant-microbe interactions by preventing exudation or secreting mycotoxins (Henkes *et al.* 2011, Notz *et al*. 2002). In contrast, effects observed on the systemic, non-infected compartments, can be safely attributed to plant-mediated effects (Henkes *et al.* 2011; Jousset *et al.* 2011).

We specifically address whether 1) barley recruits different microbes upon pathogen infection, 2) whether microbial recruitment is related to an enrichment of anti-fungal traits (functional genes) and 3) whether the recruitment process contributes to the structuring of rhizosphere microbial communities. By combining these aspects we aim to prove the concept that a host plant is able to manipulate its rhizosphere microbiome structure to fit its functionality to the challenges, such as pathogen infection, that the plant is facing.

Methods

Study system

We used a model system with barley (*Hordeum vulgare* L.) as host plant, *Fusarium graminearum* (Schwabe) as fungal pathogen and fluorescent pseudomonads as potential pathogen antagonists. Barley is the fourth most important cereal crop worldwide. It serves as food for animals and humans as well for

malt production (Newton *et al.* 2011) and its general root microbiome structure was recently examined (Bulgarelli *et al.* 2015). One of its major pathogens, the soil-borne fungus *F. graminearum*, is responsible for blight in cereals and a growing concern in agriculture (Goswami & Kistler 2004; Kazan, Gardiner & Manners 2012). Especially its production of mycotoxins poses a serious threat to animal and human health and makes contaminated products unusable (D'Mello, Placinta & Macdonald 1999).

In an earlier study Henkes *et al.* (2011) showed that the local infection of barley roots with *F. graminearum* resulted in a reduction of carbon delivery to the infected root parts. An inoculation with fluorescent pseudomonads (*P. protegens* CHA0) counteracted this effect and successfully prevented barley biomass reduction due to the infection. In a further study Lanoue *et al.* (2010) showed that barley plants changed their exudation profiles after root infection with *F. graminearum*. The plants responded with the synthesis of organic acids exhibiting antifungal activity. Some of these compounds are also known to trigger the production of antifungal metabolites by fluorescent pseudomonads (Jousset *et al.* 2011) and act as chemo-attractants for this bacterial taxon (Oku *et al.* 2014). Based on these evidences we used this biological system as a model to test our concept that plants may actively manipulate their rhizosphere microbiome in response to an external stressor.

Therefore, we set up two experiments using a split-root approach to disentangle plant- and pathogen mediated effects on microbial communities. The two split-root systems consisted of two separated compartments. One compartment was infected with the pathogen (hereafter the 'treatment compartment') while the other compartment remained pathogen-free (hereafter the 'systemic compartment').

Seedling preparation

We used barley plants (*Hordeum vulgare* L. cv. 'Barke') grown from commercial seed material (Irnich Inc., Frechen, Germany). Barley seeds were surface-sterilized as described previously (Henkes *et al.* 2011). Briefly, seeds were soaked in H_2SO_4 (62% v/v) for 1 h to remove glumes, washed two times in sterile water and were surface-sterilized in $AgNO_3$ solution (2% w/v) for 20 min. $AgNO_3$ was removed by

five cycles of precipitation in 1% NaCl solution and washing in sterile water. Seeds were germinated on 3% water agar at 24 °C in darkness to check for contamination and to obtain roots big enough to be transferred into the split-root systems. One three day old seedling was transferred into each split-root microcosm and its roots were evenly separated to each of the compartments.

Fungal inoculum

We used *Fusarium graminearum* (Schwabe) strain FG17 as model pathogen. The fungal cultures were maintained on potato dextrose agar (PDA, Carl Roth GmbH, Essen, Germany) in darkness. Prior to the experimental application, we incubated a cube of actively growing culture of *F. graminearum* in sterile sucrose solution (40g l^{-1}) for 7 days at room temperature on an orbital shaker at 40 rpm. The resulting mycelium suspension was homogenized by placing it through a syringe and then repeatedly centrifuged at 2000 rpm for 2 min, rinsed five times with 0.1 x phosphate buffered solution (PBS) to remove nutrients and finally adjusted to OD_{600}= 0.4 (optical density at 600 nm) in 0.1 x PBS. For the chemotaxis experiment we used a spore suspension of the fungus. Therefore, an actively growing culture was transferred to autoclaved mung bean medium (40 g of mung beans boiled in 1 l water for 20 min) and incubated for 5 days at room temperature on an orbital shaker at 40 rpm. The resulting spore suspension was repeatedly centrifuged at 3000 rpm for 5 min, rinsed five times with sterile water and finally adjusted to OD_{600}= 0.1 in sterile water.

Split root set up

First, we assessed bacterial recruitment in a natural soil (hereafter ‘soil experiment’): We grew barley plants in split-root microcosms made of a polypropylene base part, containing the compartment chambers, (width: 78 mm, depth: 6 mm, height: 128 mm) and a polycarbonate cover plate. Before setting up, the microcosms were autoclaved at 105 °C for 60 min. The compartment chambers were filled with non-

sterile soil from the field site of the Jena Experiment (see Müller, Scheu & Jousset 2013) and were supplemented with 20% (w/w) sand to avoid soil clumping. Both compartments were irrigated separately with sterile water via fiber-glass capillaries and had apertures on the top to allow for gas exchange (see Fig. S1 in Supporting Information). After ten days growth at 20 °C and 12 h photoperiod under artificial light (120 µmol m^{-2} s^{-1}), the treatment compartment of the split-root microcosms were inoculated with 4 ml of the mycelium suspension, spread evenly on the whole root system. The control plants received the same amount of 0.1 x PBS.

Ten days after pathogen addition, the split-root systems were destructively sampled. Rhizosphere soil samples were collected by gently removing the roots with adherent soil and suspended in 30 ml of 0.1 x PBS on a horizontal shaker at 60 rpm for 30 min.

We chose the comparatively short growth period of ten days to allow the challenged plants to respond to the pathogen while preventing too much infection damage which may have caused biases in the interpretation.

Microbial communities

We followed changes in rhizosphere microbial communities at three levels: We assessed total bacterial density as a marker for unspecific changes coming along with the infection, such as nutrient leaking, affecting indiscriminately all microbes. Further we enumerated the density of fluorescent pseudomonads as model group, which is consistently associated with disease suppression (Mendes *et al.* 2013) and the specific antifungal genes *phlD* and *hcnAB* (as indicators for specific functional gene enrichment). Further, we monitored the diversity of pseudomonads and their community structure to test for selective processes structuring the rhizosphere.

In the second experiment (hereafter 'the chemotaxis experiment') we tested if exudates from infected plants are chemo-attractant for bacteria with potential antifungal activity. Therefore, we used a gnotobiotic system where the plants were confronted with the pathogen in otherwise sterile conditions. We built split-

root microcosms out of two sterilized 15 ml Falcon tubes (BD Biosciences, Heidelberg, Germany) filled with sterile demineralized water.

Plants were grown for ten days at 20 °C and 12 h photoperiod after which we inoculated the treatment compartment of half of the plants with 0.5 ml of fungal spore suspension or sterile water respectively.

After ten days, the water samples from the root compartments containing the root exudates, were filtered through a 0.22 µm filter (Millipore, Merck, Darmstadt, Germany) to remove remaining fungal or root particles and were stored at 4 °C until processing the chemotaxis assay.

Culture based enumeration of bacteria – plate counts

For the culture based enumeration of fluorescent pseudomonads and total heterotrophic bacteria, we spread-plated the rhizosphere soil suspensions on Petri dishes with Gould`s S1 medium (Gould *et al.* 1985) and R2A medium (Reasoner & Geldreich 1985) respectively. For plating on Gould`s S1 medium we used 50 µl of the 20 fold diluted suspensions and for R2A medium 50 µl of the 100 fold diluted suspensions. Petri dishes were incubated at 28 °C for 48 h in darkness before colonies were counted.

DNA extraction

DNA extraction was performed in triplicates directly from the soil suspensions, following the protocol of Lueders, Manefield & Friedrich. (2004) with slight modifications. Briefly, 600 µl of the soil suspensions were mixed with 150 µl $NaPO_4$ buffer (600 mM), 250 µl TNS solution and 0.7 g of 0.1 mm silica beads. Cells were lysed by beat-beating at 6.5 m s^{-1} for 30 s. The subsequent extraction steps were done according to the protocol. Finally, we eluted the DNA in 30 µl EB buffer. The quality of DNA extracts were checked by electrophoresis on 1.5 % agarose gels at 120 V for 30 min. For all further analyses triplicate samples were pooled.

Quantitative real-time PCR

We used quantitative real time PCR (qPCR) to enumerate total bacteria and specific disease-suppressive bacteria, producing the antifungal metabolites 2,4-diacetylphloroglucinol (2,4-DAPG) and hydrogen cyanide (HCN), which contribute to the suppression of fungal diseases in soil (Voisard *et al.* 1989; Weller *at al.* 2002). All reactions were performed in a final reaction volume of 20 µl containing 2 µl of the template DNA, 10 µl KAPA SYBR FAST qPCR MasterMix Universal (Kapa Biosystems, Wilmington, MA, USA), 24 µg bovine serum albumin (BSA) and 0.24 µM of each of the respective primers. All reactions were run in a Stratagene Mx3005P instrument (Agilent Technologies, Waldbronn, Germany). To enumerate total bacteria, we quantified the copy numbers of 16s rDNA using the primer pair EUB338 and EUB518 (Fierer *et al.* 2005). To enumerate HCN producing bacteria we quantified the copy numbers of *hcnAB* using the primer pair PM2 and PM-26R (Svercel, Duffy & Défago 2007) and for 2,4-DAPG producing bacteria we quantified the copy numbers of *phlD* using the primer pair BPF2 and BPR4 (McSpadden Gardener *et al.* 2001). As quantification standards we used a dilution series of already quantified amplification products of the targeted fragments. For reactions targeting 16s and *hcnAB* the standards were obtained from *P. protegens* strain CHA0 and for *phlD* from *P. fluorescens* strain Q2-87. Additionally, we performed qPCR targeting *F. graminearum* DNA using the primer pair FG16NF and FG16NR (Nicholson *et al.* 1998) to validate the success of pathogen inoculation and to check for potential cross-contamination. Details of oligonucleotide primers and the cycling conditions are listed in Supplementary Information Tables S1 and S2. Microcosms contaminated with *F. graminearum* (presence of the fungus in one of the compartments of control plants or in the systemic compartment of the infected plants respectively) were omitted from further analyses. This resulted in total of 10 replicated split-root microcosms for the control treatment and 16 replicated microcosms for the infection treatment.

Assessment of Pseudomonas community structure

Pseudomonas community structure was investigated using an established denaturing gel gradient electrophoresis (DGGE) based on a nested PCR amplification of the *Pseudomonas*-specific *gacA* gene (Costa *et al*. 2007). We performed the first PCR using the primer pair gacA-1F (Costa *et al.* 2007) and gacA2 (de Souza, Mazzola & Raaijmakers 2003). PCR was performed in a total reaction volume of 25 µl containing 1 µl of the template DNA, 5 µl KAPA2G Buffer A (Kapa Biosystems, Wilmington, MA, USA), 1.25 U KAPA2G Robust DNA polymerase (Kapa Biosystems, Wilmington, MA, USA), 100 µM dNTPs and 0.2 µM of each of the according primer. After purification, the products were subjected to a second PCR using the primers gacA-1FGC and gacA-2R (Costa *et al.* 2007) in a final reaction volume of 25 µl containing 1 µl of the first PCR product as template, 5 µl KAPA2G Buffer A, 1.25 U KAPA2G Robust DNA polymerase, 100 µM dNTPs and 0.2 µM of each of the according primer. Both PCR were performed in a TProffesional Thermal cycler (Analytik Jena, Jena, Germany). Details of oligonucleotide primers and the cycling conditions are listed in the Supplementary Information (Table S1 and Table S2). Electrophoresis was performed on 4 µl of the second PCR product in an 8% acrylamide gel with a denaturing gradient ranging from 20% to 80% denaturants (100% denaturants: 7M urea and 40% formamide) at 60 °C for 18 h and 140 V in 1 x Tris-acetate-EDTA buffer using a Dcode Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA). The gels were stained with 200x SYBR Green (Sigma-Aldrich, Hamburg, Germany). The DGGE fingerprints were digitized to greyscale images and analyzed using the Quantity One software (Bio-Rad, Hercules, CA, USA).

Bacterial chemotaxis towards infected and uninfected plants

We assessed the chemotactic activity of a GFP-tagged *Pseudomonas protegens* CHA0 (Jousset *et al.* 2006) towards the exudates of infected and uninfected plants. We chose this strain as reference fluorescent pseudomonad carrying the functional genes *phlD* and *hcnAB* also targeted in the natural soil experiment. Bacteria were pre-grown in 3 g l^{-1} tryptic soy broth (TSB) at 20 °C for 24 h, centrifuged (5 min at 10,000 rpm) and washed 5 times in 0.1 x PBS to remove remaining nutrients. Bacterial density was adjusted to

243 OD_{600}=0.1 in 0.1 x PBS. We used 200 µl pipette tips filled with 50 µl of the exudate samples, placed on a
244 multichannel pipette, as chemotaxis capillaries. The filled tips remained on the pipette and were carefully
245 placed into wells of a microtiter plate containing 100 µl of the bacterial suspension. The whole system was
246 incubated for 30 min at 20 °C in darkness to allow the bacteria migrating into the pipette tips. Bacterial
247 concentration in the pipette tips was measured by flow-cytometric enumeration using a BD Accuri C6
248 flow cytometer (Accuri, Ann Harbor, MI, USA) following the manufacturer's instructions. For both
249 treatments we set up 10 split-root microcosms as replicates.
250
251 *Statistical analyses*
252
253 All analyses were performed in R version 3.1.1 (R Core Team 2014). In the soil experiment, we tested for
254 the effect of the infection treatment on dry shoot biomass using a linear model (LM) with the infection
255 treatment (control vs. pathogen addition) as categorical predictor. To test for differences in root dry
256 biomass we used a linear model with the infection treatment, the compartment side (treatment
257 compartment vs. systemic compartment) and their interaction as categorical predictors.
258 In the soil experiment, bacterial and gene abundances were calculated as numbers of gene copies and
259 colony forming units (CFU) per gram soil dry weight respectively. We calculated *gacA* allelic richness as
260 the number of bands within each gel lane.
261 Because soil water is essential for bacterial motility, we included the soil water content (percentage soil
262 moisture) as covariate in the models targeting bacterial abundances, gene abundances and *gacA* allelic
263 richness in the soil experiment. The soil water content was not affected by the infection treatment
264 ($F_{1,48}$=0.51, p=0.48), the compartment side ($F_{1,48}$=0.06, p=0.81) nor their interaction ($F_{1,48}$=0.60, p=0.44),
265 indicating that there was no systematic bias.
266 Bacterial and gene abundances in the soil experiment exhibited a positive, right-skewed distribution with
267 an over proportional increase in variance. Thus, we used generalized linear models (GLM) with gamma
268 distributed errors and a log-link function (Faraway 2006; Crawley 2007), testing for the effects of the soil

water content (linear covariate), the infection treatment (factor with two levels), the compartment side (factor with two levels) and the treatment-compartment side interaction as categorical predictors.

Regarding the analysis of *gacA* allelic richness in the soil experiment, it is noteworthy, that the treatments were represented in equal proportions within a single gel, while the compartment sides were represented on separate gels. Therefore, we tested for the effect of the infection treatment on *gacA* allelic richness separately for the compartment sides using linear models and fitted the gel identity as a block effect before the soil water content and infection treatment. To adjust for the covariate soil water content, we calculated for all above mentioned response variables their least-square means and standard errors (r-package: lsmeans; Lenth 2015) based on the respective model (GLMs targeting bacterial and gene abundances and LMs targeting *gacA* allelic richness).

For the chemotaxis experiment, we defined chemo-attractant function of root exudates as the density of *P. protegens* CHA0 (cells μl^{-1} exudate) in capillaries filled with the root exudates. We tested for effects of the infection treatment, the compartment side and their interaction as categorical predictors on the chemo-attractant function of root exudates using a GLM with gamma distributed errors and a log-link function. If the models indicated differing effects of the infection treatment between the compartment sides (infection treatment x compartment side interaction at $p<0.1$), we additionally tested the pairwise contrasts of the infection treatments within both compartments sides (r-package: multcomp; Hothorn, Bretz & Westfall 2008).

The *gacA* community composition was analyzed using non-metric multidimensional scaling (NMDS) based on pairwise Bray-Curtis dissimilarities of the Hellinger-transformed (Legendre & Legendre 1998; Legendre & Gallagher 2001; Ramette 2007) greyscale band intensities. First, ordination was performed separately for each single gel to account for inter-gel variations resulting from potential differences of the denaturing gradient due to the gel casting procedure. To check for the comparability of the ordination results, we tested for the concordance of the single-gel ordinations within each compartment side using a procrustes rotation test based on 999 permutations. Within the compartment sides we tested for the significance of the infection treatment on the grouping of the *gacA* ordinations using permutational

295 multivariate analysis of variance (PERMANOVA) based on 999 permutations. Therefore, we constrained
296 the permutations within the single gels to account for gel identity in sense of a statistical block effect (r-
297 package: vegan; Oksanen *et al.* 2013).
298
299 **Results**
300
301 Dry shoot biomass of barley plants was not affected by the infection treatment ($F_{1,24}$=0.01, p=0.91). Also
302 dry root biomass was not affected by infection treatment ($F_{1,48}$=0.43, p=0.51), the compartment side
303 ($F_{1,48}$=1.78, p=0.19) nor their interaction ($F_{1,48}$=0.04, p=0.84), indicating that *F. graminearum* did not
304 generate measurable damage during the short incubation time.
305 The abundance of total heterotrophic bacteria (CFU on R2A medium) was not affected by any predictor
306 (Table 1, Fig. 1a). In contrast, the abundance of fluorescent pseudomonads (CFU on Gould's S1 medium)
307 was higher in the rhizosphere of infected plants than of uninfected plants at the systemic compartment
308 (Table 1, Fig. 1b). Further, the abundance of fluorescent pseudomonads was positively correlated with the
309 soil water content (Table 1, Fig. 2a).
310 Similar to the abundance of total heterotrophic bacteria, there were no effects of any predictor on the
311 abundance of 16s rDNA gene copy numbers (Table 1, Fig. 3a). However, the abundances of the two
312 assessed antifungal genes (*phlD* and *hcnAB*) were positively correlated with the soil water content (Table
313 1, Fig. 2b,c). The effect of the infection treatment on antifungal gene abundance depended on the
314 compartment side (Table 1) and their abundances were higher in the rhizosphere of infected plants than of
315 uninfected plants at the systemic compartments (Fig. 3a,b). In the treatment compartment, *gacA* allelic
316 richness was not significantly affected by the infection treatment (Table 2). However, in the systemic
317 compartment, *gacA* allelic richness was higher in the rhizosphere of infected plants than of uninfected
318 plants (Table 2, Fig. 4a).
319 Procrustes rotation test revealed that the single gel ordinations of the *gacA* DGGE profiles were congruent
320 in the systemic compartments (p=0.017), indicating a coherent structuring force on the communities.

Further, community composition was well discriminated by the infection treatment in the systemic compartment (Table 2, Fig. 4b). The single gel ordinations in the treatment compartment were not congruent (p=0.15) and community composition was not separated regarding the infection treatment (Table 2, Fig. 4b).

In the chemotaxis experiment, *P. protegens* CHA0 was more attracted by the root exudates of infected plants than by the root exudates of the control plants. This increase was independent of the compartment side (Table 1, Fig 5).

Discussion

The results of our study show an enrichment of the rhizosphere microbiome with potentially antifungal microbes for barley plants challenged with *F. graminearum*. By combining a split-root approach with different levels of resolution (zooming from all bacteria into one representative taxonomic group and two representative functional genes), we could disentangle the effects of plant mediated microbiome structuring from perturbations of the infection treatment.

Our results provide evidence for the concept that plants are able to actively recruit specific bacterial groups upon pathogen infection. Fluorescent pseudomonad abundance increased with infection. These bacteria are good colonizers of plant roots (Loper *et al.* 2012) and we propose that their enrichment in the rhizosphere of infected plants may be explained by conserved characteristics such as chemotactic activity toward root exudates. The fact that fluorescent pseudomonads are repeatedly associated with disease suppression (Weller 2007; Mendes *et al.* 2011) makes it plausible that a co-evolutionary process between plants and this bacterial group occurred, in which bacteria evolved to respond to plant alarm cues.

However, we can only speculate about the underlying mechanism. In contrast to symbioses with rhizobia or mycorrhiza, plants have very few possibilities to select for partners providing a particular function such as disease protection (Kiers & Denison 2008). We propose that feedback loops, such as the enhanced

346 exudation in presence of 2,4-DAPG (Philips *et al*. 2004), may provide a reward mechanism that may have
347 promoted the recruitment of bacteria harboring antifungal traits.
348 Bacterial recruitment can be the result of a specific attraction by exudates or by a higher growth on roots.
349 Chemotaxis appears here as a significant driver. Exudates from infected plants were more attractive than
350 exudates of uninfected plants for *P. protegens* CHA0, a model strain harboring the genes needed for the
351 production of both 2,4-DAPG and HCN, the two genes that were systemically enriched in the rhizosphere
352 of infected plants. These results are in line with a past study on *Bacillus*, showing that plants challenged
353 by the foliar pathogen *Pseudomonas syringae* recruited one specific *Bacillus* strain by secreting more
354 malic acid, to which the studied *Bacillus* strain showed a strong chemotactic response (Rudrappa *et al.*
355 2008). We propose that a similar effect occurred in the soil experiment with fluorescent pseudomonads.
356 The chemotaxis hypothesis is further backed by the importance of the soil water content for bacterial
357 recruitment. High soil moisture promotes the motility of free living bacteria (Wong & Griffin 1976) as
358 well as the diffusion of soluble nutrients and chemo-attractant compounds in the soil matrix (Raynaud
359 2010). The positive effect of the soil water content on the abundance of fluorescent pseudomonads and
360 antifungal genes indicates that the recruitment process is driven by increased migration of motile bacteria
361 towards the rhizosphere of the plants. Fluorescent pseudomonads are highly motile bacteria, which is an
362 important trait related to their rhizosphere competence (Turnbull *et al.* 2001; de Weert *et al.* 2002;
363 Martínez-Granero, Rivilla & Martín 2006). The hypothesis of plant triggered migration of disease-
364 suppressive bacteria is also supported by the chemotaxis experiment, documenting that sterile root
365 exudates from infected plants were more attractive to *P. protegens* than those from control plants. Root
366 exudates serve the plant to communicate with surrounding microbes and their composition can shift in
367 presence of pathogens. For instance, organic acids exert an attracting cue for the chemotactic activity of
368 fluorescent pseudomonads (Oku *et al.* 2014). Plants can rapidly change their exudation profile in response
369 to pathogen infection and initiate the synthesis of defensive organic acids to suppress pathogens (Lanoue
370 *et al.* 2010). Some of these compounds also stimulate the expression of antifungal genes (Jousset *et al.*
371 2011).

Alternatively, an increased water potential can rapidly lead to an increased bacterial biomass (Lund & Goksøyr 1980; Kieft, Soroker & Firestone 1987). In both scenarios we propose that sufficiently high soil moisture is essential for the plant to recruit beneficial microbes out of the existing microbial species pool. Pseudomonad richness, measured as *gacA* allelic richness, was higher in the rhizosphere of infected plants than of uninfected plants. This fits with an enhanced chemo-attractant function of the root exudates of infected plants. For instance, infected plants may have recruited bacteria from more distant soil regions, which would allow accessing a broader microbial species pool. This higher diversity may be crucial for efficient disease suppression as it may enhance the production of antifungal metabolites (Jousset *et al.* 2014). The distinctiveness of the bacterial community compositions between the rhizosphere of infected plants and uninfected plants at the systemic compartments further suggests that this selective plant driven recruitment functions as a structuring force for the assembly of rhizosphere bacteria.

Caveats

We observed enrichments of specific bacteria due to the infection treatment in the systemic compartment only. This leads us to propose that the inoculum application may locally interfere with plant driven effects as observed at the systemic compartments. The addition of PBS to the treatment compartment may have affected microbial communities via an addition of phosphorus or a mechanical perturbation of the root system. Even if we kept perturbation as low as possible by using highly diluted PBS and dripping it slowly onto the roots, it may account for some effects such as the higher bacterial abundances in the treatment compartment than the systemic compartment. Also effects of the fungal inoculum itself, e.g. an antagonistic (Notz *et al.* 2002) or attracting potential of fungal metabolites (de Weert *et al.* 2004), may mask plant driven processes. Such concomitant effects illustrate how important it is to physically separate the site of experimental manipulation from the site of measurements especially in the context of systemic plant driven processes.

This study focused on one specific plant-pathogen system and on three very simplified bacterial groups (all bacteria, one specific taxon and two functional genes). Also the plants were grown for a short time in small microcosms under the controlled conditions of a climate chamber. The potential differences to other plant-pathogen systems and the impact of environmental conditions or plant age remains a subject for further investigation, which would give valuable insights into the generality and limitations of the process observed in this study. Nevertheless, this simplification allowed us to conceptually demonstrate a new dimension in plant-microbiome interactions, disentangling effects of a host plant and a pathogen on the composition and functionality of microbial communities. However, this approach also brings a few caveats we would like to address. First, we would like to emphasize that the tested groups are examples. Microbial communities are very diverse, both phylogenetically and functionally. Further, we did not attempt to analyze exudate composition, as they would have in the present context added few to the observed net effects on bacteria. Rather, we see our approach as a conceptual base that can be combined with metabolomics or metagenomics to disentangle the roles of plant and pathogen on the assembly of the rhizosphere microbiome. Further, we did not assess the antifungal potential of root-associated bacteria. We propose that future studies assessing soil suppressiveness in the systemic compartment after several growth cycles may greatly improve our understanding of the role of the plants in building up soil suppressiveness as observed in take-all decline (Weller *et al.* 2002).

Conclusion

We demonstrated that barley plants actively manipulate their rhizosphere community to favor a specific function, such as here antifungal traits, in response to pathogen attack. This extends the finding from Jousset *et al.* (2011) showing a stimulation of antifungal gene expression in bacteria living on pathogen infested plants to the recruitment and enrichment of disease-suppressive bacteria in a non-sterile soil system. The specificity of the recruitment indicates an active selection process by the plant and its restriction to non-infected parts of the root systems suggests that pathogen presence or infection-linked

damages to the plant may reduce the ability of plants to select for beneficial microbes. The identification of the ability of plants to recruit beneficial microbes to counteract pathogens remains a challenge for plant breeding (Haney *et al.* 2015). Our study provides a new strategy to assess a plant`s potential to recruit disease-suppressive bacteria by removing the noise caused by pathogen infection.

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Data accessibility

Data associated with this study are deposited in the Dryad repository: doi:10.5061/dryad.83np7.

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Table 1 Results of the generalized linear models (with gamma distributed errors and a log-link function) targeting the abundances of total heterotrophic bacteria (CFU on R2A medium), fluorescent pseudomonads (CFU on Gould's S1 medium), 16s rDNA, the antifungal genes *hcnAB* and *phlD* in the rhizosphere of barley plants (soil experiment) and the chemo-attractant function of root exudates (chemotaxis experiment). In all models of the soil experiment the soil water content was fitted as linear covariate before the fixed effects of the infection treatment and the compartment side. Significant effects are highlighted in bold.

Response	Predictor	DF	Deviance	F	P
Total heterotrophic bacteria (# CFU $g_{soil\ dry}^{-1}$)	Soil water content	1	0.049	0.538	0.467
	Infection treatment (IT)	1	0.086	0.937	0.338
	Compartment side (CS)	1	0.156	1.698	0.199
	IT x CS	1	0.007	0.072	0.790
	Residuals	47	4.202		
Fluorescent pseudomonads (# CFU $g_{soil\ dry}^{-1}$)	Soil water content	1	5.970	26.818	**<0.001**
	Infection treatment (IT)	1	1.455	6.538	**<0.05**
	Compartment side (CS)	1	0.002	0.010	0.920
	IT x CS	1	0.722	3.243	0.078
	Residuals	47	10.226		
16s (# copies $g_{soil\ dry}^{-1}$)	Soil water content	1	0.180	3.389	0.072
	Infection treatment (IT)	1	0.104	1.969	0.167
	Compartment side (CS)	1	0.001	0.017	0.897
	IT x CS	1	0.002	0.044	0.836
	Residuals	47	2.471		
phlD (# copies $g_{soil\ dry}^{-1}$)	Soil water content	1	9.503	14.205	**<0.001**
	Infection treatment	1	0.891	1.332	0.254
	Compartment side	1	0.949	1.418	0.240
	IT x CS	1	3.459	5.171	**<0.05**
	Residuals	47	32.337		
hcnAB (# copies $g_{soil\ dry}^{-1}$)	Soil water content	1	6.264	18.002	**<0.001**
	Infection treatment (IT)	1	0.273	0.783	0.381
	Compartment side (CS)	1	0.073	0.210	0.649
	IT x CS	1	2.522	7.247	**<0.01**
	Residuals	47	18.352		
Chemo-attractant function (cells μl^{-1} exudate)	Infection treatment (IT)	1	1.529	4.457	**<0.05**
	Compartment side (CS)	1	0.260	0.759	0.389
	IT x CS	1	0.109	0.317	0.577
	Residuals	36	12.350		

Table 2 Results of the linear models (LM) targeting *gacA* allelic richness and the permutational multivariate analysis of variance (PERMANOVA) targeting the differences in the *gacA* community compositions. All analyses were performed separately for each compartment side. The gel identity was fitted before the soil water content and the infection treatment in the LMs. Permutations in the PERMANOVAs were constrained within the single gels.

Response	Compartment side	Predictor	DF	SS	F	P
gacA richness (# bands)	Treatment	Gel identity	1	30.154	4.560	**<0.05**
		Soil water content	1	0.529	0.080	0.778
		Infection treatment	1	4.307	0.651	0.428
		Residuals	22	145.472		
	Systemic	Gel identity	1	5.538	2.632	0.119
		Soil water content	1	6.135	2.916	0.102
		Infection treatment	1	42.034	19.976	**<0.001**
		Residuals	22	46.293		
gacA composition (NMDS)	Treatment	Infection treatment	1	0.023	0.377	0.767
		Residuals	24	1.472		
	Systemic	Infection treatment	1	0.185	2.018	**<0.001**
		Residuals	24	2.197		

Fig 1 Abundance (least-square means and standard errors) of (a) total heterotrophic bacteria (CFU on R2A medium) and (b) fluorescent pseudomonads (CFU on Gould's S1 medium) in the rhizosphere of uninfected plants (white circles, left) and infected plants (grey circles, right) of the soil experiment. Treatment compartments are paired to the left and the systemic compartments are paired to the right. For significant infection treatment x compartment side interactions ($p<0.1$) additional p-values are given for the pairwise contrasts of the infection treatment within each compartment side.

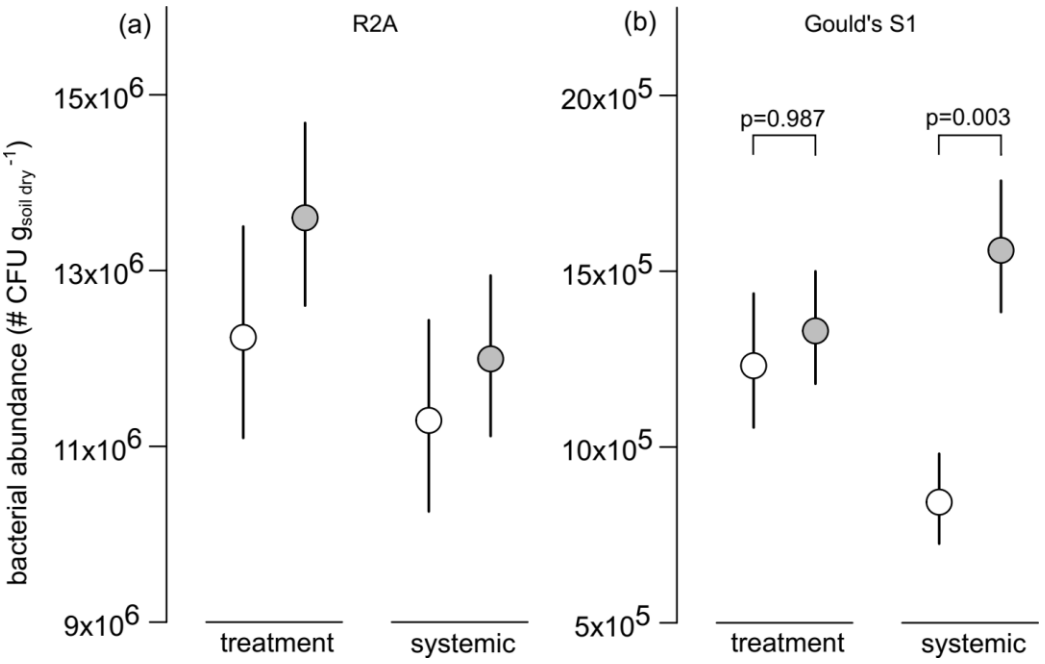

Fig 2 Effect of the soil water content on the abundances of (a) fluorescent pseudomonads (CFU on Gould's S1 medium) and the functional genes (b) *phlD* and (c) *hcnAB* in the rhizosphere of uninfected (white) and infected (grey) plants at the treatment compartments (triangles) and the systemic compartments (squares) of the soil experiment. Regression fits refer to the respective generalized linear model (with gamma distributed errors and a log-link function).

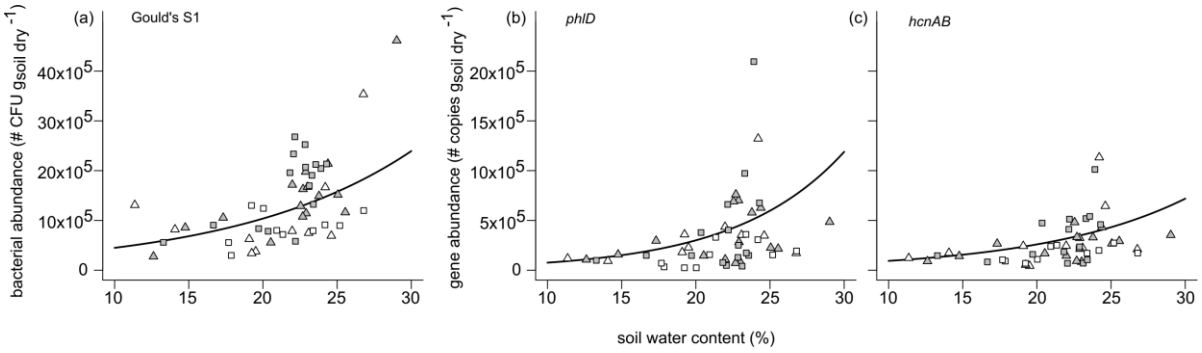

Fig 3 Abundance (least-square means and standard errors) of (a) 16s rDNA and the functional genes (b) *phlD* and (c) *hcnAB* in the rhizosphere of uninfected plants (white circles, left) and infected plants (grey circles, right) of the soil experiment. Treatment compartments are paired to the left and the systemic compartments are paired to the right. For significant infection treatment x compartment side interactions ($p<0.1$) additional p-values are given for the pairwise contrasts of the infection treatment within each compartment side.

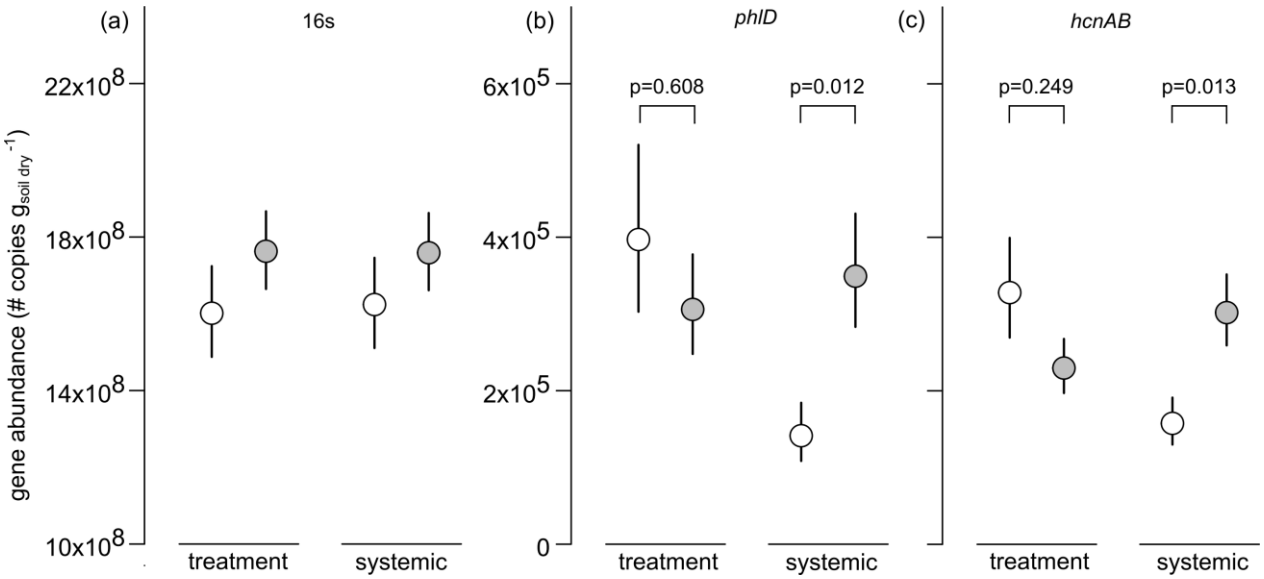

Fig 4 *gacA* allelic richness (a) (least-square means and standard errors) and *gacA* community composition (based on NMDS ordination) (b) in the rhizosphere of uninfected plants (white circles, left) and infected plants (grey circles, right) of the soil experiment. In panel (a) treatment compartments are paired to the left and the systemic compartments are paired to the right. In panel (b) each point represents one single plant. For both compartment sides, the results from the single gel ordinations are superimposed to aid visualization.

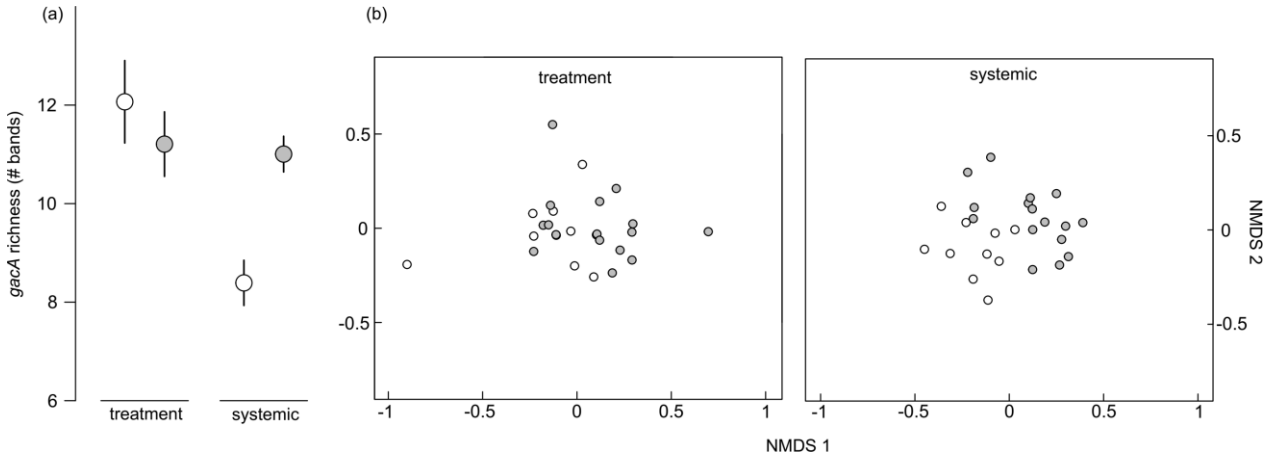

Fig 5 Chemo-attractant function (least-square means and standard errors) of *P. protegens* towards the root exudates of uninfected plants (white circles, left) and infected plants (grey circles, right) of the chemotaxis experiment. Treatment compartments are paired to the left and the systemic compartments are paired to the right.