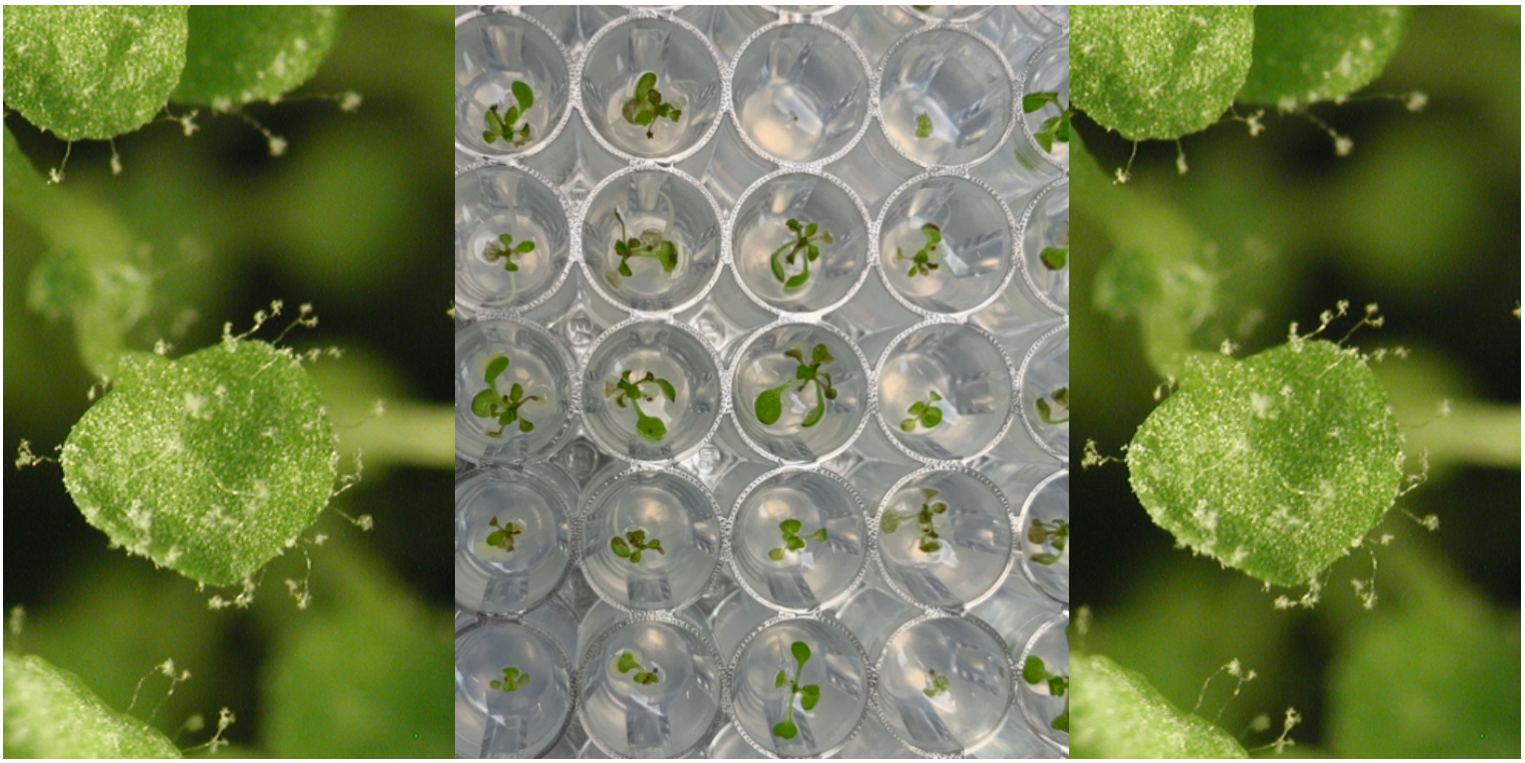
Downy mildew-Associated Bacteria: Their growth and influence on reproduction of *Hpa* spores in *Arabidopsis thaliana*



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February 2021 – December 2021

Abstract
Downy mildews are host specific, obligate biotrophic oomycetes that cause disease in a plant's foliage and lead to catastrophic agricultural crop loss annually. The role of phyllosphere microbes to protect a plant against Downy mildew infection is largely unknown, understood even though the phyllosphere is pivotal for the production of biocontrol agents that can prevent disease outbreaks in agriculture. A previous study has shown that leaf infection of the Downy mildew of *Arabidopsis thaliana* called *Hyaloperonospora arabidopsidis* (*Hpa*) strongly influenced the phyllosphere microbial community of a plant whereby specific bacteria (*Hpa*-associated bacteria) were enriched in multiple *Hpa* infected cultures. In this study, we looked at the influence a few selected *Hpa*-associated bacteria have on *Hpa* sporulation as well as how *Hpa* influences the growth of these single bacterial isolates. We did this in an axenic system using gnotobiotic *Hpa (*gno*Hpa)*. Our results validated that the previously identified *Hpa*-associated bacteria also benefited from the presence of *Hpa* in a gnotobiotic growth system. Next to that, we observed that two *Hpa*-associated bacterial isolates from the genera *Microbacterium sp.* and *Aeromicrobium sp.* significantly reduced gno*Hpa* sporulation. The reduction in sporulation seen by *Microbacterium sp.* is likely by directly antagonizing Downy mildew. Together our results opened an interesting pathway where further research can be conducted to understand the fundamental interactions occurring in the phyllosphere of plants infected with *Hpa*. Understanding these interactions is of key importance to uncover novel mechanisms to deal with pathogen attacks through stimulation of the leaf microbiota.

Keywords: Downy Mildew, Plant-Microbe Interactions, Gnotobiotic system, *Arabidopsis thaliana*, Biocontrol agents

Layman's summary
Downy mildews are a group of fungus-like oomycetes that are plant pathogenic and cause major agricultural crop losses annually. Downy mildews are host-specific, meaning that each species of Downy mildew organism is only able to cause disease in a specific type of plant species. Next to that they are obligate biotrophic and thereby require their host plant to be alive for their own survival. They reproduce by spreading spores from one plant leaf to the other. Current practices in agriculture to evade Downy mildew infections is limited to the use of fungicides and the breading for resistance traits. However, the pathogen evolves rapidly and is able to quickly by-pass fungicides and resistance traits making agricultural practices not sufficient to control disease. The aboveground part of plants, consisting of the stem, leaves, flower and fruits, contain a dynamic microbial community that is able to stimulate growth and development and are able to induce disease resistance to pathogens that try to invade and replicate inside a plant. The specific downy mildew of the plant *Arabidopsis thaliana* (Hereafter: Arabidopsis) is called *Hyaloperonospora arabidopsidis (*Hereafter*: Hpa).* Previous experiments have isolated and characterized microbes that were abundant in the leaves of plants that were infected with *Hpa*. Possible reasons for the abundance of these characterized microbes could be because they defend a plant against the *Hpa* pathogen. If that is the case, we can continue to research the ways the microbe helps a plant and ultimately optimize it as a tool to prevent downy mildew disease in agricultural fields. In this research, we investigated the influence microbes that were associated with downy mildew infection have on reproduction of downy mildew. We also

simultaneously looked at the influence downy mildew has on growth of the associated bacterial isolates. We did this by creating a system that is completely microbe-free. This allows to look at interactions of only three factors: A host plant, a pathogen and an individual microbe. Our results gave interesting insights in the way microbes react to the presence of a pathogen and paves way to further research how we can leverage from the leaf microbiome to enhance plant defense against pathogens.

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Introduction

Downy Mildew is an umbrella name for a large range of obligate biotrophic plant-pathogenic oomycetes that are host specific and cause devastating damage to commercial and natural ecosystem plants around the world (Correll et al., 2011). Downy mildew outbreaks have previously been so severe, that a complete shift in historical crop production has occurred and currently it is estimated that these oomycetes cause 30% of crop loss annually (Chang et al., 2013; Holmes et al., 2015). The downy mildew in Arabidopsis thaliana (Hereafter Arabidopsis) is called *Hyaloperonospora arabidopsidis* (Hereafter *Hpa*) and is a useful model for research on plant-pathogen interactions. Being obligate biotrophic, *Hpa* is unable to survive without Arabidopsis, causing a coevolving pathosystem that contains a high level of diversity in host resistance and pathogen avirulance proteins(Coates & Beynon, 2010)

Agricultural applications to deal with these pathogens and ensure sufficient and consistent yield heavily rely on the use of chemical protection products such as pesticides or herbicides and cross-breeding for dominant resistant genes (Cohen et al., 2019; Shaw et al., 2021). Many chemical products pose a threat to human health and the environment (Nishimoto, 2019). Additionally, downy mildew is able to rapidly evolve new virulence factors and bypass pathogen resistance genes and chemical protectants (Asai et al., 2018; Chen et al., 2007). To ensure food security for the growing world population, agricultural companies are continuously seeking novel mechanisms to enhance plant resistance against downy mildew infections (Michelmore et al, 2013).

Plants contain an intricate immune system that is fundamental for their growth & development and survival against pathogens. This immune system relies on fast recognition of microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs) by pattern recognition receptors (PRRs) and/or immune sensors termed nucleotide-binding, leucine-rich repeat receptors (NLRs), that recognize pathogen effectors (Cui et al., 2015; Zipfel, 2014). Within plant immunity there are multiple biotic stress response regulators including the Enhanced Disease Susceptibility1 (eds1) gene which is an essential component of basal immunity to biotrophic pathogens (such as Downy Mildews) as well as effector-triggered immunity (ETI) (Table S1, Falk et al., 1999; Parker et al., 1996). It has been previously shown that eds1 mutated plants exhibited high susceptibility to downy mildew (Parker et al., 1996). Next to that, there are also many signaling molecules such as the hormone salicylic acid (SA) that is essential for defense against biotrophic pathogens. Genes that influence SA levels in the cytoplasm are thereby extremely important in regulating defense. Notably, the Salicylic Acid Induction-Deficient 2(Sid2-1) that influences biosynthesis of SA and the Nonexpressor of PR genes1-1 (Npr1-1), a key gene that mediates crosstalk between SA and Jasmonic acid (JA) and if mutated causes decreased accumulation of SA(Table S1, Cao et al., 1994; Nawrath & Métraux, 1999).

A plant also contains an extremely diverse microbiome that is a major biotic driver of plant health. Particularly, the rhizosphere microbiome has been intensively studied for its functions in protection of a host plant against pathogenic attacks (Berendsen et al., 2012). One mechanism of protection is seen by a *Pseudomonas sp.* that suppresses the growth of *G. graminis* var. *tritici*

by producing the antifungal compound 2,4-diacetylphloroglucinol (DAPG) that directly interacts with the pathogen(Weller et al., 2002).
Next to that, *Pseudomonas sp.* can also protect plants by other more indirect mechanisms including competition with other soil microbes for nutrients and inducing systemic resistance (ISR), a disease resistance mechanism where prior pathogen infection causes a plants immune system to be "primed" and leads to an enhanced resistance for future pathogen attacks (Berendsen et al., 2015; Pieterse et al., 2014).
Other researchers have also revealed that plants themselves can recruit specific beneficial microbes or microbial functions to provide protection against an invading pathogen (Bakker et al., 2018; Berendsen et al., 2018; Carrión et al., 2019; Yuan et al., 2018). An example of such recruitment is seen in an experiment where infection of *Hpa* in the leaves leads to an enrichment of a *Microbacterium sp*., a *Stenotrophomonas sp*., and a *Xanthomonas sp.* in the rhizosphere microbial community that was able to induce ISR (Berendsen et al., 2018).
One aim of research focused on finding microbes that have plant beneficial properties is to create new biocontrol agents. These biocontrol agents use natural strains of microorganisms that have been previously identified to be able to suppress populations of plant pathogens through different modes of action(Legein et al., 2020). Biocontrol agents can directly interact with a pathogen by hyperparasitism, antibiosis or production of secondary metabolites and from there suppress disease. But they can also interfere with a pathogen's lifecycle without direct interaction, namely, through competition for nutrients, inducing resistance or priming for enhanced resistance (Pieterse et al., 2014; Spadaro & Droby, 2016). In some cases, there are multiple modes of action taking place simultaneously (Pieterse et al., 2014). Elucidating which mechanism microorganisms influences pathogen development is essential for an optimal disease control.

The aboveground part or "phyllosphere" of plants is a complex system where microbial communities on the outside (epiphytic) and inside (endophytic) surfaces interact with a host plant and play an important role in protecting a plant against disease (Legein et al., 2020). Pathogens must first adapt to the epiphytic surfaces of plants in order for them to initiate an infection in the intercellular space of plants (Pfeilmeier et al., 2016). Despite the importance of the phyllosphere, not much is known of how plant-microbe interactions in this area influence a pathogens attack. It is thereby of significant relevance to accelerate more understanding in the responsiveness of the phyllosphere microbiome towards pathogens since this could pave way to new applications in agriculture that prevent pathogen outbreaks.

Previous experiments by Goossens et al, unpublished data at Utrecht University (UU) using 16s rRNa amplicon sequencing showed that infection of *Hpa* on Arabidopsis leaves strongly affected the phyllosphere bacterial communities. Namely, infection of *Hpa* caused a shift in which amplicon sequence variants (ASVs) were abundant in the phyllosphere. This exact experiment was also performed in a laboratory at the Max Planck Institute for Plant Breeding research (MPIPZ), whereby they also saw that the previously abundant ASVs of UU were identical to the ASVs abundant at MPIPZ. Distinct locations caused abundance of identical ASVs. Additionally, another experiment termed the "9-passage" experiment showed that removal of *Hpa* via successive passaging of the *Hpa* microbiome resulted in a shift in the microbial community, giving

a stronger conviction that the bacterial isolates abundant in presence of *Hpa*, are indeed associated with *Hpa*. These results raised the question whether these *Hpa*-associated isolates are abundant because they have an impact in plant defense or they just benefit from the presence of the pathogen.

The bacterial isolates in the experiments mentioned above have been isolated and stored and offer a unique opportunity to further test their association with *Hpa*. Additionally, researchers at UU were successfully able to subsequent passage Noco2 in an axenic system, creating a gnotobiotic *Hpa* (Hereafter gno*Hpa)* culture. This gno*Hpa* culture allows for microbe-free bioassays to be performed, making it possible to look at interactions between only three components: the host plant (Arabidopsis), an individual *Hpa*-associated bacterial isolate and gno*Hpa*.

In this research, we investigated the effects of a few selected *Hpa*-associated isolates on sporulation of gno*Hpa* as well as how they influence plant growth in presence and absence of gno*Hpa*. Subsequently, we also investigated what influence gno*Hpa* has on the growth of the selected bacterial isolates. We hypothesized that if a single *Hpa*-associated isolate influence gno*Hpa* sporulation, this is mediated by the plants immune system.
Interestingly, our results showed that all *Hpa*-associated isolates were enriched in presence of gno*Hpa* in an axenic system. From the selected *Hpa*-associated isolates there were two isolates that continuously showed a significant inhibition of gno*Hpa* sporulation. Together our results highlights the importance of the phyllosphere microbiome during pathogen infection and paves way to further research phyllosphere associated microbes that could be used as defense mechanisms for diseases.

Material & Methods

Cultivation of plant material

Arabidopsis Col-0, *eds1-1, sid2-1, npr1-1* and *coi1-16* -seeds at Utrecht University (UU) were surface sterilized using vapor-phase sterilization (mixing 100 ml household bleach with 3,2 ml fuming HCl (37%)) in a desiccator for 3,5h. Sterilized seeds were left to aerate in a sterile environment to evaporate residual chlorine gas for at least 30 minutes before sowing seeds on 48well-plates. Each well contained 1,5mL of full-strength Hoagland medium. A plant agar percentage of 60% was used in order for roots to effectively penetrate in the medium. Plates containing sterilized seeds were left to stratify at 4°C for 3 days. After 3 days of stratification, the seeds were allowed to germinate in a growth chamber (21 °C, 70% relative humidity, 10 h light/14 h dark, light intensity 100 µmol m^{-2} s^{-1}).

Cultivation of bacterial isolates

A total of 88 bacterial isolates were previously isolated from *Hpa* infected leaves and placed in the -80 C freezer of PMI. These isolates were streaked on 9cm petri dishes with LB medium until single colonies appeared. Single colonies were picked with sterile p200 pipet points and placed in 5mL liquid LB to grow in a 28°C shaker in order for the bacteria to grow. After three days 1mL of bacterial suspension was mixed with 1mL of 50% glycerol in a cryotube and placed in the -20°C freezer. When doing experiments with bacterial isolates, isolates were taken from the -20°C storage.

Gnotobiotic assay to test bacterial growth and *Hpa* sporulation

Leaves of 10-day-old Arabidopsis seedlings were droplet-inoculated with a gno*Hpa* spore suspension, a single bacterial suspension or a co-inoculation of gno*Hpa* and a single bacterial isolate.

Preparation of gno*Hpa* spore suspension

A gno*Hpa* spore suspension was created by cutting approximately 15 gno*Hpa* leaves, placing it in a sterile 2mL tube and vigorously mixing it in 1,5mL $MgSO_4$ with a vortex for 20 seconds. To maximize the amounts of spores released, we performed this step an additional time in a new 2mL tube with the previously used leaves. The two spore suspensions were added together and the amounts of spores per µL was calculated under a binocular. Approximately 150 spores per µL is a decent amount for the inoculation. The final inoculant has a 1:9 ($MgSO_4$: *Hpa*) ratio by mixing 20µL of $MgSO_4$ with 180µL of the gno*Hpa* suspension. Additionally, Silwett L-77 is added to the mixture in order to obtain a total concentration of 0,02%.

Preparation of bacterial suspensions

An isolate is streaked on LB medium to obtain fresh single colonies. One single colony is picked with a sterile p200 pipette point, mixed with $MgSO_4$ and glass-beaded on a new petri dish in order to have a pure and uniform bacterial culture. On the day of inoculation, the bacteria are removed from the petri dish using $MgSO_4$ and placed in a 15mL tube. To wash the bacterial cells, we place the 15mL tubes in a centrifuge at 3500g for 5min. The supernatant is removed and fresh $MgSO_4$ is mixed with the bacterial pellet. This washing step is repeated a second time

before measuring the optical density (OD) with a spectrophotometer. All suspensions are diluted with $MgSO_4$ to obtain an OD_{600} = 0,2 and OD_{600} = 0,002. The final inoculant needs a 1:9 (Bacteria: $MgSO_4$) ratio by mixing 20µL of the bacterial suspension with 180µL $MgSO_4$.

Co-inoculation of gno*Hpa* and a single bacterial isolate

To make the co-inoculant, bacteria and gno*Hpa* were mixed at a 1:9 (bacteria: gno*Hpa*) ratio. Additionally, Silwett L-77 is added to the mixture to obtain a total concentration of 0,02%.
A repeater pipette is used to apply droplets of 0,3µL on the cotyledons and first two true leaves. After inoculation, the plates are placed in a climate chamber (16 °C, 10 h light/14 h dark, light intensity 343 100 µmol m−2 s−1).

Gno*Hpa* sporulation quantification assay

At seven days after inoculation, plant leaves were harvested in tubes and the fresh weight was determined using a theoretical scale. Afterwards, the leaves are thoroughly mixed with 400µL $MgSO_4$ using a vortex and three individual 1µL droplets are used to determine the average spores per mg fresh weight.

Bacterial growth quantification assay

At seven days after inoculation, plant leaves inoculated either with a single bacterial isolate or co-inoculated with a single bacterial isolate and gno*Hpa* were harvested in 2mL sterile tubes in a flow cabinet. Fresh weight was determined using a theoretical scale followed by the addition of 400µL $MgSO_4$ + 0,02% Silwett L-7 in each tube, in a flow cabinet. The tube racks were placed on a plate shaker at max. velocity for 1 hour. After one hour, tubes are immediately put on ice in order to continue with serial dilution. PCR plates are used to perform the serial dilution, meaning that 12 samples can be serial diluted in one dilution streak. Each bioassay contained 48 samples to be serial diluted, namely, 24 samples at OD600= 0,02 +/- gno*Hpa* and 24 samples at OD600 = 0,0002 +/- gno*Hpa*. To ensure waiting time for each serial dilution did not influence the bacterial growth that is seen, each dilution PCR plate contained 3 samples of each treatment. Before performing the serial dilution, 72µL of $MgSO_4$ is pipetted in all PCR plates with a multichannel pipette, excluding the first row of each plate. The first row will contain undiluted sample. Twelve samples are removed from ice and thoroughly vortexed for two rounds and 80µL of the liquid is pipetted in the first row of the PCR plate. From there, 8uL of the undiluted suspension is passed to the next row, making sure to pipette up and down to ensure a homogenous mixture is created. This step is continuously done until the lowest dilution of 10^7 times less the undiluted suspension is achieved. Five µL of each serial dilution suspension is plated on square TSA plates. This step is performed twice, to ensure two replicas are taken in account in the bacterial quantification. The TSA plates are covered with parafilm and placed in a 28°C stove. At two days after serial plating, colony counting can take place (This may vary considering different growth times of each bacterium).

Confirmation of bacterial isolates

Single colonies of bacterial isolates were grown in liquid LB medium for two days and the gDNA was extracted using the GenElute Bacterial Genomic DNA Kit according to instructions of the manufacturer, followed by a PCR reaction containing 10 µM of the forward primer 16S-27F

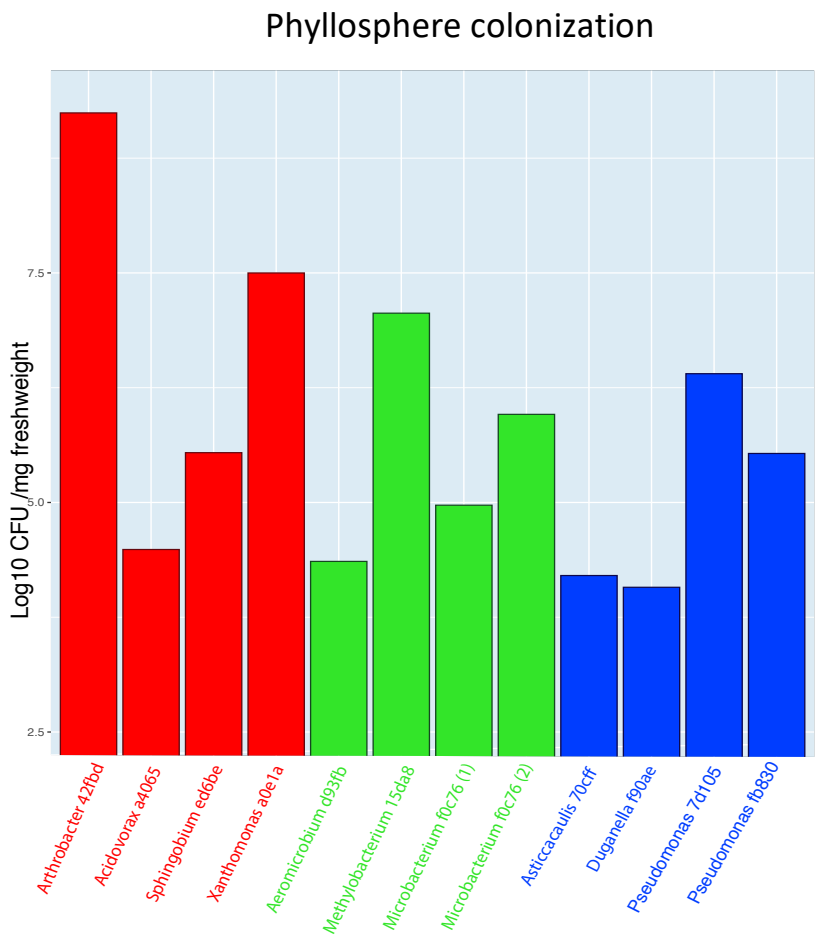
(5'AGAGTTTGATCMTGGCTCAG), 10 µM of the reverse primer 16S-1492R (5'CGGTTACCTTGTTACGACTT), 1.0 units/50µL reaction of Phusion DNA polymerase, 10 mM dNTPs and 10X Standard *Taq* Reaction Buffer. PCR cycling was performed as follows: 10 minutes at 95 °C followed by 30 cycles of; 30 seconds at 95 °C, 30 seconds at 55 °C, 30 seconds at 72 °C; and a final elongation step for 5 minutes at 72°C. ThPCR products were purified using AMPure XP beads with 9 µl of bead solution per 15 µl PCR mixture and washing with 80% ethanol as described in the standard protocol for Illumina16S rDNA amplicon sequencing. PCR products were quantified using Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific) and are sent for sequencing by MacroGen. Using the application GenElute, sequences are matched with the previously known genome sequence.

Results

Arthrobacter sp. reaches higher densities in the phyllosphere than all other bacteria

All microorganisms (whether pathogenic or beneficial) who establish in a plant are initially recognized as a potential invader, which triggers a MAMP-triggered immunity (MTI) (Zamioudis & Pieterse, 2012). In order for a *Hpa*-associated microbe to be able to establish a mutualistic interaction with Arabidopsis, it needs to be able to cope with this MTI. Knowing more of how a microbe establishes in the phyllosphere, can give relevant insights to how resilient it is against MTI as well as how well it adapts to this discrete environment. Previous literature suggest that bacterial community densities reach up to 10^5 cells/mg in the phyllosphere (Lindow & Brandl, 2003). We set out to test how a few selected *Hpa*-associated microbes colonize the phyllosphere of a plant in mono-association. We selected bacterial isolates that were isolated from previous experiments done by Goossens et al; unpublished data. We chose four bacterial isolates that were consistently present in two *Hpa* cultures from UU + MPIPZ (*Hpa*-Core bacteria; red color), four bacterial isolates that benefited from the presence of *Hpa* in the 9-passage experiment (*Hpa*-associated bacteria; green color) and four bacterial isolates that were depleted or unaffected by the presence of *Hpa* (blue color). All bacterial isolates, with their given association are illustrated in supplemental table 2.

We inoculated Col-0 seedlings at 10 DAG with a single bacterial isolate at an OD_{600}=0,02. At seven DPI we harvested the leaves and calculated the CFU per mg fresh weight. Our results showed that the *Hpa*-Core bacteria, *Arthrobacter 42fbd*. showed the highest phyllosphere colonization, reaching above 10^6 cells/g (Figure 1A). This is not surprising as bacteria from the *Arthrobacter* genus are commonly found in the phyllosphere due to their fast ability to adapt and multiply in this environment(Scheublin & Leveau, 2013). The high colonization of *Arthrobacter sp*. resulted in Arabidopsis leaves covered in visible bacterial residues, suggesting that this isolate is pathogenic (Figure 1B). *Xanthomonas sp.* and *Methylobacterium sp*. showed a remarkably high colonization, whilst the other *Hpa*-associated isolates had a colonization below 10^3 CFU/mg (Figure 1A). From the unaffected or depleted isolates we see that *Asticcacaulis sp.* and *Duganella sp.* had the lowest observed phyllosphere colonization, suggesting that these microbes are the least competent in the phyllosphere environment (Figure 1A). When looking at the phenotype of leaves inoculated with *Xanthomonas sp.,* pathogenic traits were observed including, leaf discoloration and visible bacterial growth on plant medium (Figure 1B).



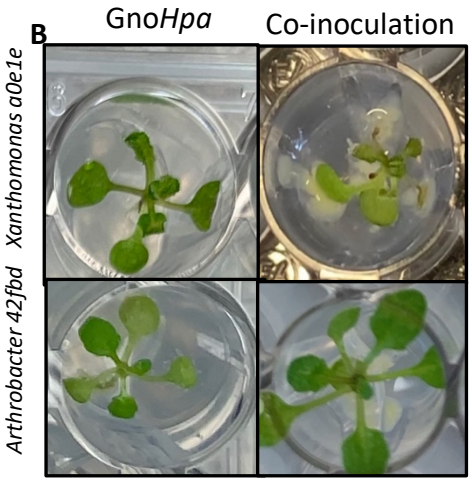


Figure 1 *Arthrobacter sp*. reaches higher densities in the phyllosphere than all other bacteria
(A) Barplot showing phyllosphere colonization in *Hpa*-Core bacteria (red), *Hpa*-associated bacteria (green) and bacteria who were unaffected or depleted in presence of *Hpa* (blue). The phyllosphere colonization is determined by the Log10 CFU/mg fresh weight. **B** Pictures showing the phenotype of plants at 7 DPI with gno*Hpa* or co-inoculated with gno*Hpa* and *Xanthomonas sp. (*above*) or* gno*Hpa* and *Arthrobacter sp. (*below*)*.

Xanthomonas sp.* reduces plant growth in presence of gno*Hpa

Extensive communication occurs between a plant, microorganisms and pathogens where signaling molecules play an important role to activate plant immunity or plant growth (Berg, 2009; Huot et al., 2014). Given this knowledge, we wanted to see if the interactions occurring between the twelve previously mentioned bacterial isolates and gno*Hpa* are reflected in the plant weight since these results can give more insights whether some bacterial isolates have plant beneficial properties. To test this, we measured the fresh weight of plants inoculated with only a bacterial isolate, only gno*Hpa* and co-inoculated with a single bacterial isolate and gno*Hpa*. When comparing the difference in fresh weight of plants inoculated with only gno*Hpa* vs those co-inoculated with a single bacterial isolate, we observed that plants co-inoculated with *Xanthomonas sp*. showed significantly lower fresh weight in both a high and low inoculation density (Figure 2A). These results give a stronger confirmation that the pathogenicity that was observed in Figure 1B, is also associated with a reduced plant weight. The presence of two *Hpa*-associated isolates also significantly influenced plant growth solely at a low inoculation density (Figure S1E) and none of the unaffected or depleted bacterial isolates showed a significant change in plant growth (Figure S1F). Further research should look more into what effects addition of these bacterial isolates have on plant weight by comparing them with plants that were treated with only $MgSO_4$.

We also compared the difference in fresh weight of plants inoculated with a single bacterial isolate vs plants co-inoculated with gno*Hpa*. Here, we observed that the presence of gno*Hpa* significantly reduced the fresh weight of plants (Figure 2B). Additionally, we observed that gno*Hpa* also significantly reduced plant growth in two other *Hpa*-associated isolates at a high inoculation density and none of the unaffected or depleted isolates showed any statistical difference (Figure S1B-C).

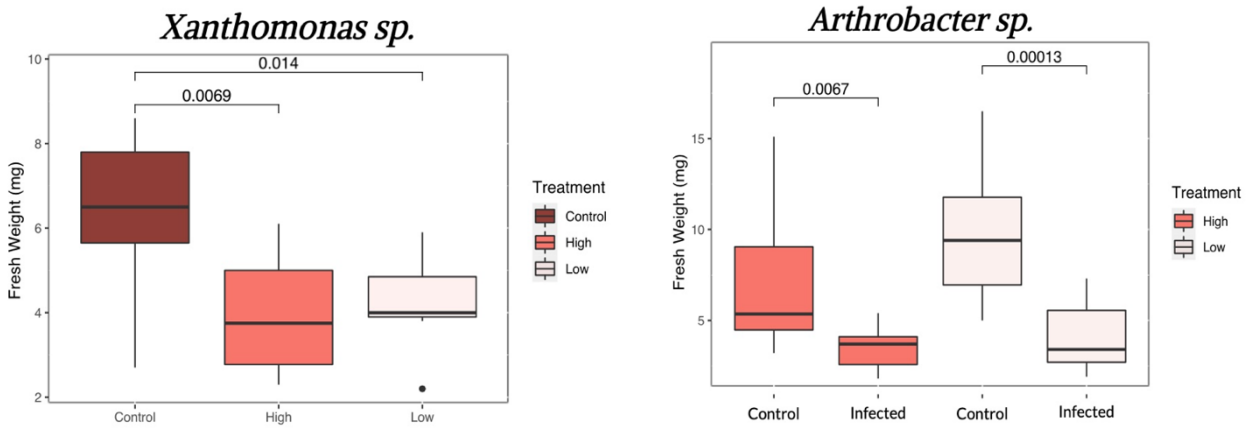


Figure 2 *Xanthomonas sp.* reduces plant growth in presence of gno*Hpa*

(A) Boxplot of the average fresh weight (mg) of Arabidopsis plants at 17 DAG that were inoculated at 10DAG with gno*Hpa* (Control) or co-inoculated with *Xanthomonas a0e1a* at a high (pink) or low (light pink) bacterial density. (B) Boxplot of the average fresh weight (mg) of Arabidopsis plants at 17 DAG that were inoculated at 10DAG with only Arthrobacter 42fbd (Control) or co-inoculated with Gno*Hpa* at a high (pink) or low (light pink) bacterial density.

All *Hpa*-associated bacteria benefit from the presence of gno*Hpa*

After looking at how well our twelve selected isolates colonize the phyllosphere of Arabidopsis plants, we wanted to see if in a gnotobiotic system, the presence of *Hpa* also increases growth of our selected *Hpa*-associated bacteria. To test this, Arabidopsis seedlings were inoculated at 10 DAG with an individual bacterial isolate (control) or co-inoculated with gno*Hpa*. The experiments were performed at an od_{600}= 0,02 and od_{600} = 0,0002. At seven DPI, all *Hpa*-Core (red) and *Hpa*-associated (green) bacterial isolates were significantly enriched in presence of gno*Hpa* at an od_{600}= 0,02, suggesting that these bacteria indeed benefit from the presence of gno*Hpa* (Wilcoxon signed-rank Test p-value < 0,05)(Figure 3A). Contrastingly, the depleted or unaffected bacteria, *Asticcacaulis 70cff, Duganella f90ae* and *Pseudomonas 7d105* were unaffected by the presenceof gno*Hpa*. Interestingly, *Pseudomonas fb830*, was significantly depleted in one experiment and unaffected in a second experiment. The depletion of *pseudomonas fb830* in presence of gno*Hpa* could be due to a plants pathogens ability to produce compounds to decrease the growth and development of bacteria. The unaffected or depleted bacterial isolates were not tested with an OD_{600} = 0,0002. When looking at the average fold change we can further see the contrast between the *Hpa*-associated and depleted/unaffected bacteria, whereby the *Hpa*-associated bacteria all have higher growth rates than the unaffected or depleted bacteria (Figure 3B). Particularly *Arthrobacter sp*. and *Sphinogobium sp* show more than 100x higher growth rates in presence of gno*Hpa* than all other bacteria (Figure 3B).

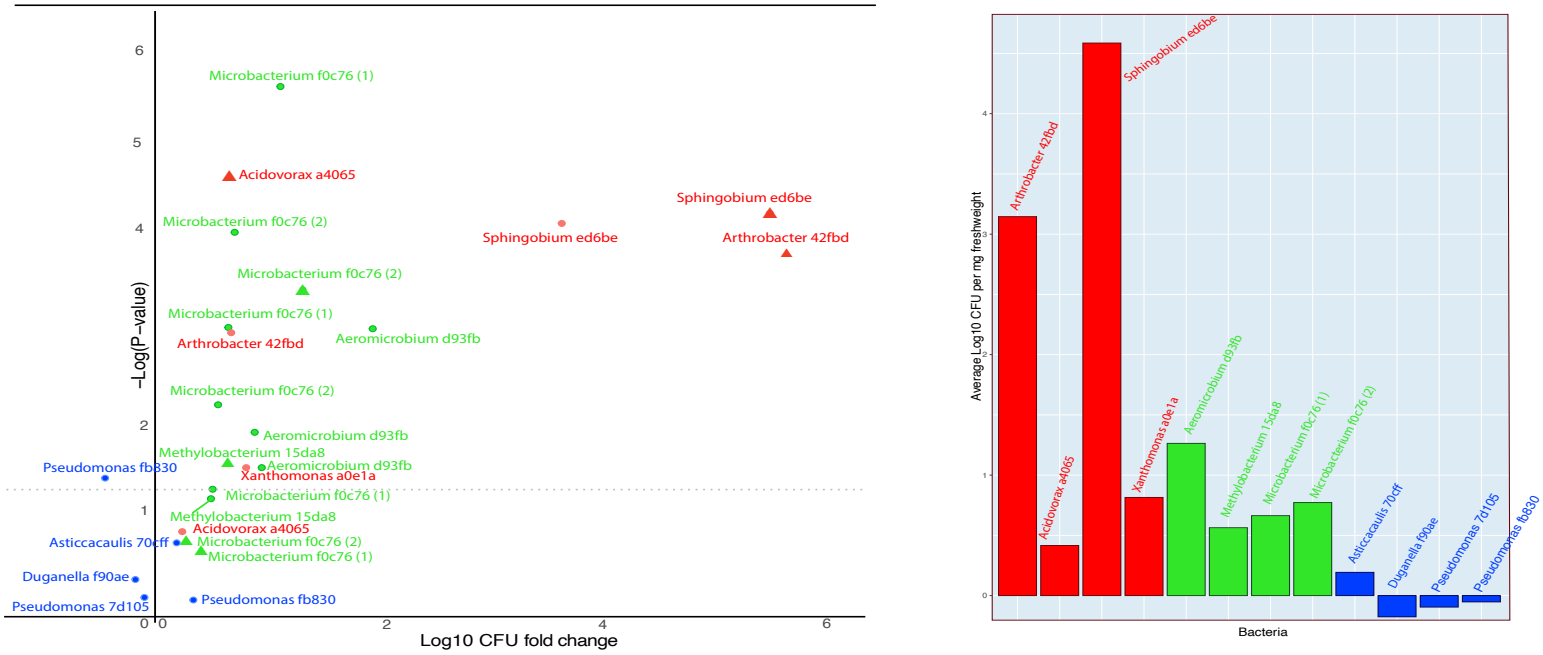


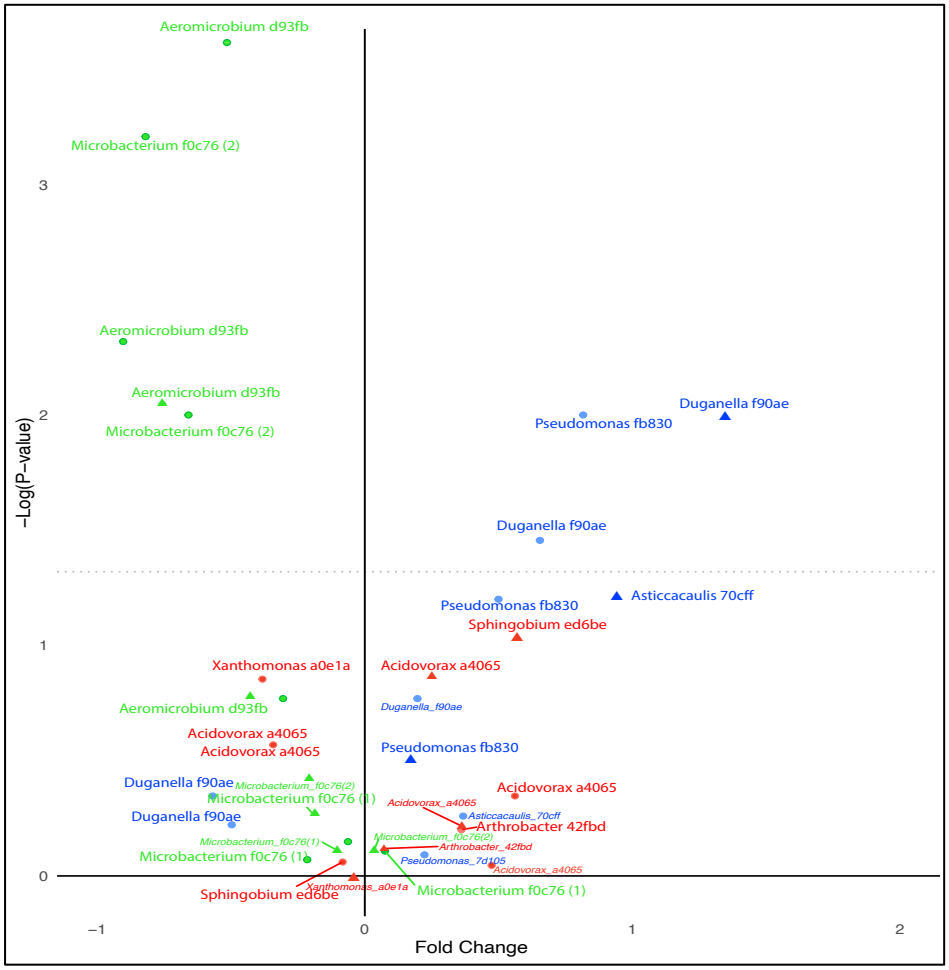
Figure 3 All *Hpa*-associated bacteria benefit from the presence of gno*Hpa*

(A) Scatterplot illustrating the log10 fold change of Arabidopsis seedlings inoculated with a single bacterial isolate vs a single bacterial isolate and gno*Hpa.* Y-axis indicates the $-^{10}$log-transformed *P*-value of the comparison (Wilcoxon test). The scattered line in the graph represents a *P*-value = 0,05, all data-points above this line are statistically significant. • Indicates a bacterial density of OD_{600} = 0.02, • Indicates an inoculation density of OD_{600} = 0.02, ▲ indicates an inoculation density of OD_{600} = 0.0002. All labels contain the isolate genus and *asv*. (B) Boxplot illustrating the average Log10 fold / mg fresh weight of the 4 *Hpa*-Core bacterial isolates (red), 4 *Hpa*-associated bacteria (green) and 4 unaffected or depleted bacterial isolates (blue).

Microbacterium sp.* and *Aeromicrobium sp.* inhibit sporulation of gno*Hpa

We sought to investigate whether the enrichment of *Hpa*-associated bacteria in presence of gno*Hpa* could be because these bacteria impact reproduction of gno*Hpa*. To test this, we compared gno*Hpa* sporulation between plants inoculated at 10 DAG with only *gnoHpa* or Co-inoculated with a single bacterial isolate. Interestingly, we observed that two *Hpa*-associated bacteria, namely *Microbacterium f0c76(2)* and *Aeromicrobium d93fb* significantly inhibited gno*Hpa* sporulation at a high inoculation density in two separate experiments (Figure 4A-E). At a low inoculation rate however, no effects were observed in presence of *microbacterium f0c76* (2), whilst with *Aeromicrobium* d93fb there was also a significant decrease in one of the two experiments (Figure 4D). The other two *Hpa*-associated isolates, *Methylobacterium 15da8* and *Microbacterium f0c76(1*) did not affect gno*Hpa* sporulation. All *Hpa*-Core bacteria did not affect gno*Hpa* reproduction at none of the bacterial inoculation densities (Figure 4a, Figure S2).

When looking at the influence of non *Hpa*-associated isolates, we saw that *Duganella f90ae* significantly increased sporulation of gno*Hpa* at a high and low inoculation density, whilst *Pseudomonas fb830* also increased sporulation in one experiment (Figure 4A, figure S2). When repeating this experiment however, our results showed a small and insignificant increase in sporulation. Nonetheless, these results suggest that some *Hpa*-associated bacteria suppress sporulation of gno*Hpa*, whilst the unaffected/depleted bacteria do not affect or promote sporulatin of gno*Hpa.*



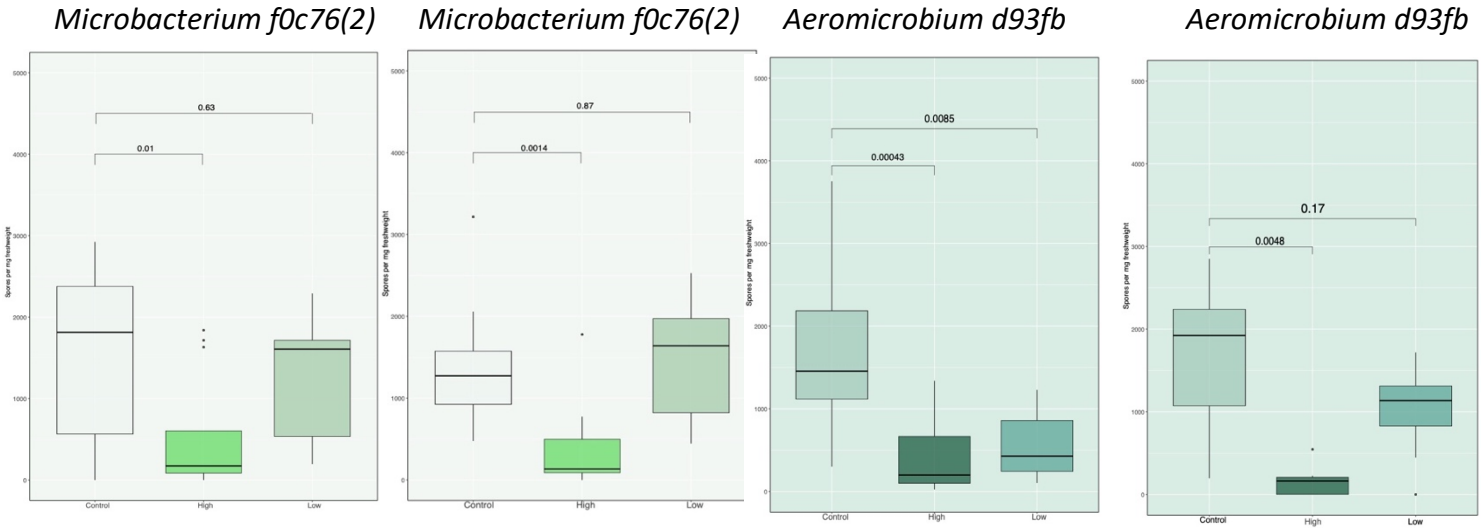


Figure 4 *Microbacterium sp.* and *Aeromicrobium sp.* inhibit sporulation of gno*Hpa*

(A) Scatter plot showing the log10 foldchange of gno*Hpa* spore production in seedlings in absence and presence of a single bacterial isolate (horizontal axis). Y-axis indicates the $-^{10}$log-transformed *P*-value of the comparison (Wilcoxon test). The dashed line in the graph represents *P*-value = 0,05, all data-point above this line are statistically significant. • Indicates a bacterial density of OD_{600} = 0.02, ▲ indicates a bacterial density of OD_{600} = 0.0002. All labels contain the isolate genus and *asv*. (B-E) Boxplot of four experiments performed separately showing average spores per mg fresh weight at seven DPI in plants inoculated with *Microbacterium f0c76 (2)*(Figure 4B+C) or *Aeromicrobium d93fb*(Figure 4D+E) . *P*-values are shown in the graphs using a Wilcoxan signed-ranked test.

***Microbacterium f0c76(2)* inhibits gno*Hpa* sporulation in immune system compromised mutants**

We previously hypothesized that if a single bacterial isolate influences gno*Hpa* sporulation, this is mediated through the plant immune system. To test this hypothesis, we performed two separate experiments where we inoculated Col-0 and four different plant mutants with *Microbacterium f0c76(2)*. The plant mutants (*eds1, sid2-1, npr1 and coi1-16*) that were used, were compromised in specific areas of the plant immune system that have previously shown to be key regulators of defense towards biotrophic pathogens (Backer et al., 2019; Nawrath & Métraux, 1999; Parker et al., 1996). An overview of the mutants is illustrated in supplemental table 1.

In the first experiment, we observed that *Microbacterium sp.* inhibited gno*Hpa* sporulation in *eds1* and *sid2-1* seeds but not in Col-0 (Figure 5A). Next to that we also observed that *eds1* and *sid2-1* seedlings inoculated with only gno*Hpa*, showed a higher sporulation in comparison to Col-0 seedlings (Figure 5A). Notably, only *sid2-1* seedlings results were significantly higher (Wilcoxan signed-rank test p-value = 0,038). An increased sporulation in *eds1* and *sid2-1* seedlings compared to Col-0 was expected since these plant mutants are associated with an increased susceptibility to pathogen infections (Falk et al., 1999; Nawrath & Métraux, 1999; Wiermer et al., 2005).

In the second experiment, we observed that *Microbacterium sp.* inhibited gno*Hpa* sporulation in Col-0 and *npr1* but not in *coi1-16* seedlings (Figure 5C). *Npr1* seedlings had a similar sporulation compared to Col-0. We expected that *Npr1* seedlings are more susceptible to *Hpa* since SA mediates resistance against biotrophic pathogens and *npr1* mutants cannot accumulate SA for enhanced protection. On the other hand, *coi1-16* showed a significantly lower sporulation compared to Col-0. This is not surprising as it could be that *coi1-16* plants direct more energy in the SA biosynthesis pathway since there is no crosstalk between JA and SA taking place. All experiments had the same result whereby *Microbacterium sp*. was significantly enriched in presence of gno*Hpa* (Figure 5B+D*)*. These results suggest that the inhibition that was seen by the presence of *Microbacterium sp.* may be caused by a direct interaction (hyperparasitism, antibiosis or production of secondary metabolites) with gno*Hpa*.

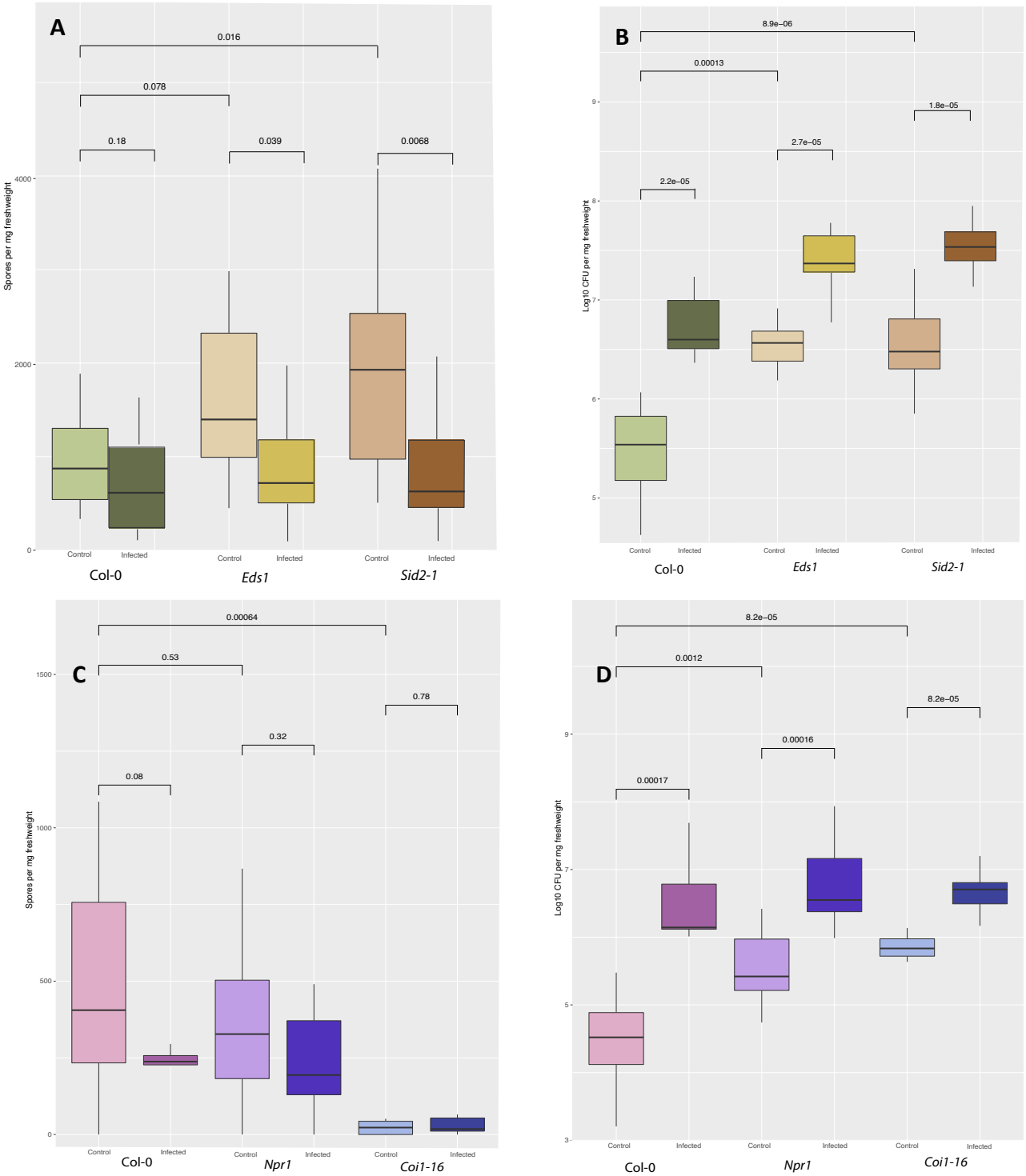


Figure 5 *Microbacterium sp.* inhibits *gnoHpa* sporulation independent from SA signaling and effector triggered immunity in the plant

(**A+C)** Boxplots showing the average spore production in spores per mg fresh weight of Arabidopsis seedlings at 17 DAG inoculated with only gno*Hpa* (control)) or co-inoculated with *microbacterium f0c76(2).* (B+D) Boxplots showing the Log10 CFU per mg fresh weight of Arabidopsis seedlings at 17 DAG inoculated with only gno*Hpa* (control)) or co-inoculated with *microbacterium f0c76(2).* The titles below each graph indicates the plant mutant. *P*-values are shown in the graphs using a Wilcoxan signed-ranked test. Bars indicate minimum and maximum numbers in a set.

Consortium of *Hpa*-Core bacterial isolates inhibited gno*Hpa* sporulation

Since the *Hpa*-core bacterial isolates were abundantly present in 4 of the 4 *Hpa* cultures we expected that these isolates might have interesting interactions with *Hpa* in mono-association. However, our results showed that none of these *Hpa*-Core bacteria influence gno*Hpa* sporulation (Figure 4A). There is also emerging evidence that microbial consortia as oppose to single inoculants might be better biocontrol agents since a combination of strains can cover a wider range of target organisms and conditions and induce systemic resistance (Minchev et al., 2021). We thereby questioned whether inoculating plants with a consortium of the *Hpa*-Core isolates would affect plant growth and gno*Hpa* sporulation. Subsequently, we also questioned whether a *Hpa*-Core consortium impacts the pathogenic traits previously caused by *Xanthomonas sp.* and *Arthrobacter sp.* (Figure 1B), since consortiums may lead to competition between microbes that ultimately prevents dysbiosis. To test this, we inoculated Col-0 seedlings with $MgSO_4$ (Mock), gno*Hpa,* the *Hpa*-Core consortium (AASX) or a co-inoculation of gno*Hpa* (gno*Hpa*) and *Hpa*-Core consortium (AASX+gno*Hpa*). At seven DPI, we observed that the *Hpa*-Core consortium significantly reduced plant growth in presence and absence of gno*Hpa*, suggesting that this

Hpa-Core consortium

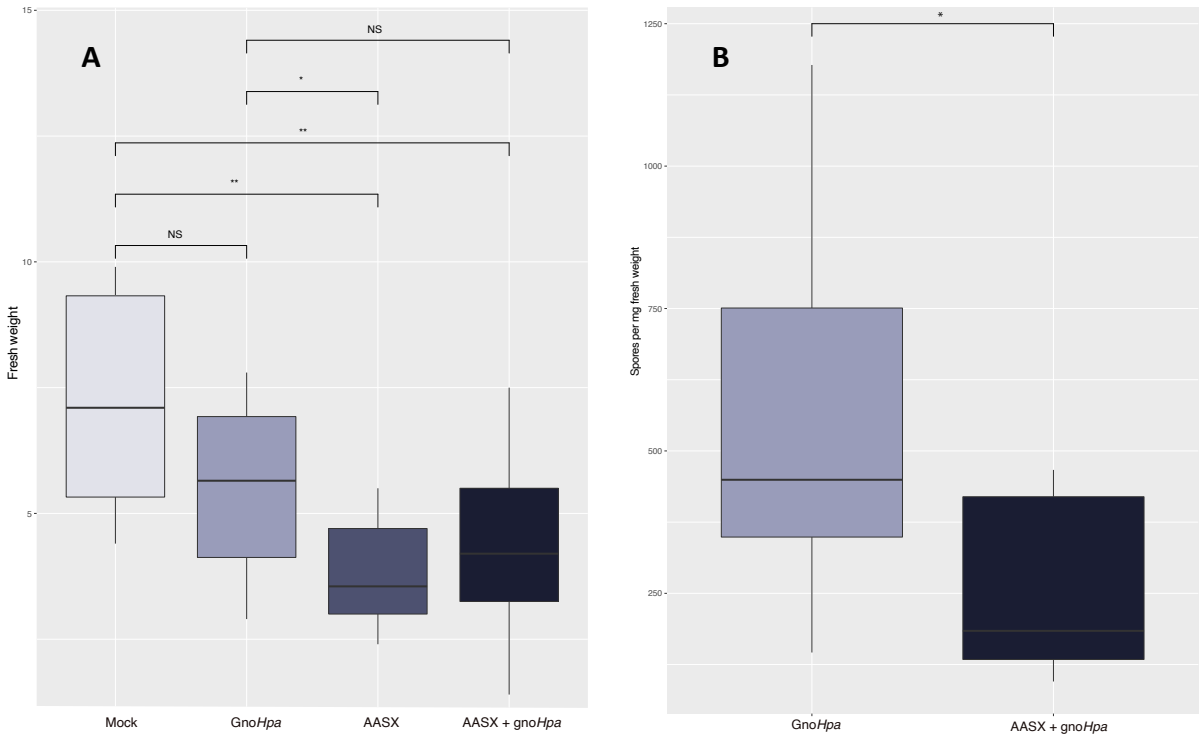


Figure 6 Consortium of *Hpa*-Core bacterial isolates inhibited gno*Hpa* sporulation
(A) Boxplot showing fresh weight of plants inoculated at 7 DPI with mgso4 (mock), gnoHpa (GnoHpa), *Hpa*- core consortium (AASX) or co-inoculated with *Hpa* core- consortium and gno*Hpa*. (B) Boxplots showing the average spore production in spores per mg fresh weight inoculated with gno*Hpa* only (purple) or co-inoculated with gno*Hpa* + the core consortium (dark purple). Asterisk* depict statistical significance (*P*=0.016) using a Wilcoxan signed-ranked test. Bars indicate minimum and maximum numbers in a set.

consortium causes stress to the plants (Figure 6A). In contrast, we observed that plants inoculated with gno*Hpa* did not effect plant growth (Figure 6A). Interestingly, we observed that the *Hpa*-Core consortium significantly reduced sporulation (Figure 6B). The pathogenic traits that were previously observed in *Xanthomonas sp.* or *Arthrobacter sp.* inoculated plants, were not observed in this experiment, suggesting that this consortium may regulate microbial homeostasis. This experiment was only performed once and thereby require additional experiments that focus on the reproducibility of this result.

Discussion

Previous experiments have shown that specific bacteria have been consistently enriched in the phyllosphere of cultures infected with *Hpa*. Subsequently, it was also shown that removal of *Hpa* via successive passaging diminishes the presence of these specific disease associated bacteria, suggesting that these bacteria benefit from the presence of *Hpa*. In this study, we investigated if the presence of gno*Hpa* enhances growth of a few selected *Hpa*-associated bacteria. Next to that, we also investigated if these *Hpa*-associated bacteria influence sporulation of gno*Hpa*.

Our experiments showed that in a gnotobiotic system, all single *Hpa*-associated bacteria grew better in presence of gno*Hpa*, whilst unaffected/depleted bacteria were unaffected in presence of gno*Hpa* (Figure 3*)*. These results further validate that the previously seen *Hpa*-associated isolates indeed benefit from the presence of *Hpa* and that Hpa influences the phyllosphere microbiome colonization. It remains however a mystery what plant or microbe mechanisms drives this significant enrichment. Further research should look more into how these *Hpa*-associated bacteria modulate a plant's physiological processes such as production of phytohormones, since these processes have shown to intervene with plant growth and induce ISR (Bakker et al., 2014).

Interestingly, no effect was seen on spore formation by all four *Hpa*-Core bacteria when bioassays were performed in mono-association, suggesting that these isolates are not strong enough to have an effect to combat disease individually. Further research should be done by firstly inoculating plants with bacterial isolates and afterwards with gno*Hpa*. This would give the individual isolates more time to colonize the phyllosphere, prime the plants and induce ISR (Pieterse et al., 1998). This approach better reflects the way biocontrol agents are used in agriculture. We identified two Hp-associated bacteria, namely *Aeromicrobium sp*. and *Microbacterium sp* that reduced sporulation of gno*Hpa* significantly. Using mutants, we observed that the reduced sporulation in presence of *microbacterium sp.* was still observed when plants were compromised in multiple important SA regulating genes as well as an important regulator of basal and effector triggered immunity. These results suggest that *microbacterium sp*. directly interacts with downy mildew through antibiosis .(Legein et al., 2020). To further investigate the exact mode of action of direct inhibition, including acidification of growth substrates, hyper-parasitism or presence of secondary metabolites, further direct interaction screenings should be performed (Anith et al., 2003; Köhl et al., 2019).

Arthrobacter 42fbd showed high colonization capabilities both in presence and absence of gno*Hpa.* Next to that, we also observed that plants inoculated with this isolate were also significantly bigger compared to when they are co-inoculated with gno*Hpa*. Previous research in

the rhizosphere microbiome has shown that *Arthrobacter spp*. secreted the enzyme aminocyclopropane-1-carboxylate (ACC) deaminase (Gaiero et al., 2013), an enzyme that indirectly promoted plant growth. Given that the phyllosphere might have other not yet known, growth related enzymes, It might be interesting to further look into the gene expression of key plant immunity genes in presence of *Arthrobacter 42fbd* since this could give more directions in the mechanisms this microbe possibly stimulates plant growth in the phyllosphere.

The *Xanthomonas a0e1a* was one of the four *Hpa*-Core associated isolates. Inoculation with *Xanthomonas a0e1a* in the phyllsphere led to diseased plants both in presence and absence of gno*Hpa (Figure 1B)*, suggesting that this isolate is a conditional/opportunistic pathogen. This conditional pathogenicity has also been observed by Pfeilmeier et al., 2021 whereby a similar *Xanthomonas* strain was only pathogenic when the RBOHD gene, a gene that is important for activation of immune responses against pathogen infection, was compromised. On the other hand, *Xanthomonas a0e1a* was shown to stimulate plant growth and induce disease resistance in consortium with other bacteria(Berendsen et al., 2018).

Preliminary bioassays focused on inoculating all four *Hpa*-Core bacterial isolates on Arabidopsis leaves resulted in a loss of pathogenicity of *Xanthomonas a0e1a*. These results give further conviction that *Xanthomonas* a0e1a is an opportunistic pathogen and that phyllosphere microbial communities members could prevent dysbiosis. To further understand the mechanisms by which a microbiota reduces the effects of opportunistic pathogens, bipartite interactions screening should be performed to look if this reduction in pathogenicity could be explained due to direct interactions with other bacteria or by the stimulation of the plant immunity (Helfrich et al., 2018). We also observed that inoculation of all four Core-*Hpa* bacteria significantly reduced plant growth both in presence and absence of gno*Hpa* (Figure 6A*)*. A possible explanation for this observation could be that plants grown in gnotobiotic systems might give a hypersensitive response when exposed to microbial consortiums. Lastly, we observed that the *Hpa*-Core consortium significantly reduced sporulation of gno*Hpa*. To validate if this *Hpa*-Core consortium is indeed specific to Downy Mildew, a follow up experiment should aim at testing the effects of this consortium using other plant pathogens.

In summary, we validated that all *Hpa* associated bacteria from previous screening benefit from the presence of gno*Hpa* in the phyllosphere of the plant whereby two bacterial strains significantly reduced sporulation of gno*Hpa*. Our findings emphasize the role of plant microbe interaction in the *Hpa* pathobiome and further research is required to find out how we can benefit from the enrichment of these bacterial isolates in a way that plants are systemically protected against infection of downy mildew.

Acknowledgments

I would like to thank my daily supervisor Pim Goossens for his guidance during my major internship. Pim was always willing to help me with questions and doubts I had when doing experiments in the lab as well as teaching me many tricks in the laboratory. Furthermore, he gave me fantastic constructive feedback in the way I performed, organized, gave presentations and wrote my report. I would also like to thank Roeland Berendsen for his involvement in my research. Additionally, I thank Guido van den Ackerveken for giving me the opportunity to do an internship in his research group. The members of the Plant-Microbe interactions department

have offered me an inspiring and warm environment, particularly the members of the Guido Lab. I also would like to thank Annemiek Andel for her help in maintaining the Gno*Hpa* cultures and Marrit Alderkamp for always giving me a help in my Bioassays.

References

Anith, K. N., Radhakrishnan, N. v., & Manomohandas, T. P. (2003). Screening of antagonistic bacteria for biological control of nursery wilt of black pepper (Piper nigrum). *Microbiological Research*, *158*(2). https://doi.org/10.1078/0944-5013-00179

Asai, S., Furzer, O. J., Cevik, V., Kim, D. S., Ishaque, N., Goritschnig, S., Staskawicz, B. J., Shirasu, K., & Jones, J. D. G. (2018). A downy mildew effector evades recognition by polymorphism of expression and subcellular localization. *Nature Communications*, *9*(1). https://doi.org/10.1038/s41467-018-07469-3

Backer, R., Naidoo, S., & van den Berg, N. (2019). The NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1) and related family: Mechanistic insights in plant disease resistance. In *Frontiers in Plant Science* (Vol. 10). https://doi.org/10.3389/fpls.2019.00102

Bakker, P. A. H. M., Pieterse, C. M. J., de Jonge, R., & Berendsen, R. L. (2018). The Soil-Borne Legacy. In *Cell* (Vol. 172, Issue 6). https://doi.org/10.1016/j.cell.2018.02.024

Bakker, P. A. H. M., Ran, L. X., & Mercado-Blanco, J. (2014). Rhizobacterial salicylate production provokes headaches! In *Plant and Soil* (Vol. 382, Issues 1–2). https://doi.org/10.1007/s11104-014-2102-0

Berendsen, R. L., Pieterse, C. M. J., & Bakker, P. A. H. M. (2012). The rhizosphere microbiome and plant health. In *Trends in Plant Science* (Vol. 17, Issue 8). https://doi.org/10.1016/j.tplants.2012.04.001

Berendsen, R. L., Vismans, G., Yu, K., Song, Y., de Jonge, R., Burgman, W. P., Burmølle, M., Herschend, J., Bakker, P. A. H. M., & Pieterse, C. M. J. (2018). Disease-induced assemblage of a plant-beneficial bacterial consortium. *ISME Journal*, *12*(6). https://doi.org/10.1038/s41396-018-0093-1

Berg, G. (2009). Plant-microbe interactions promoting plant growth and health: Perspectives for controlled use of microorganisms in agriculture. In *Applied Microbiology and Biotechnology* (Vol. 84, Issue 1). https://doi.org/10.1007/s00253-009-2092-7

Cao, H., Bowling, S. A., Gordon, A. S., & Dong, X. (1994). Characterization of an Arabidopsis mutant that is nonresponsive to inducers of systemic acquired resistance. *Plant Cell*, *6*(11). https://doi.org/10.2307/3869945

Carrión, V. J., Perez-Jaramillo, J., Cordovez, V., Tracanna, V., de Hollander, M., Ruiz-Buck, D., Mendes, L. W., van Ijcken, W. F. J., Gomez-Exposito, R., Elsayed, S. S., Mohanraju, P., Arifah, A., van der Oost, J., Paulson, J. N., Mendes, R., van Wezel, G. P., Medema, M. H., & Raaijmakers, J. M. (2019). Pathogen-induced activation of disease-suppressive functions in the endophytic root microbiome. *Science*, *366*(6465). https://doi.org/10.1126/science.aaw9285

Chang, K. F., Hwang, S. F., Ahmed, H. U., Strelkov, S. E., Conner, R. L., Gossen, B. D., Bing, D. J., & Turnbull, G. D. (2013). Yield loss and management of downy mildew on field pea in Alberta, Canada. *Crop Protection*, *46*. https://doi.org/10.1016/j.cropro.2012.12.001

Chen, W. J., Delmotte, F., Richard-Cervera, S., Douence, L., Greif, C., & Corio-Costet, M. F. (2007). At least two origins of fungicide resistance in grapevine downy mildew populations. *Applied and Environmental Microbiology*, *73*(16). https://doi.org/10.1128/AEM.00507-07

Coates, M. E., & Beynon, J. L. (2010). Hyaloperonospora arabidopsidis as a pathogen model. *Annual Review of Phytopathology*, *48*. https://doi.org/10.1146/annurev-phyto-080508-094422

Cohen, Y., Rubin, A. E., & Galperin, M. (2019). Novel synergistic fungicidal mixtures of oxathiapiprolin protect sunflower seeds from downy mildew caused by Plasmopara halstedii. *PLoS ONE*, *14*(9). https://doi.org/10.1371/journal.pone.0222827

Correll, J. C., Bluhm, B. H., Feng, C., Lamour, K., du Toit, L. J., & Koike, S. T. (2011). Spinach: Better management of downy mildew and white rust through genomics. *European Journal of Plant Pathology*, *129*(2). https://doi.org/10.1007/s10658-010-9713-y

Cui, H., Tsuda, K., & Parker, J. E. (2015). Effector-triggered immunity: From pathogen perception to robust defense. *Annual Review of Plant Biology*, *66*. https://doi.org/10.1146/annurev-arplant-050213-040012

Falk, A., Feys, B. J., Frost, L. N., Jones, J. D. G., Daniels, M. J., & Parker, J. E. (1999). EDS1, an essential component of R gene-mediated disease resistance in Arabidopsis has homology to eukaryotic lipases. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(6). https://doi.org/10.1073/pnas.96.6.3292

Gaiero, J. R., McCall, C. A., Thompson, K. A., Day, N. J., Best, A. S., & Dunfield, K. E. (2013). Inside the root microbiome: Bacterial root endophytes and plant growth promotion. *American Journal of Botany*, *100*(9). https://doi.org/10.3732/ajb.1200572

Helfrich, E. J. N., Vogel, C. M., Ueoka, R., Schäfer, M., Ryffel, F., Müller, D. B., Probst, S., Kreuzer, M., Piel, J., & Vorholt, J. A. (2018). Bipartite interactions, antibiotic production and biosynthetic potential of the Arabidopsis leaf microbiome. *Nature Microbiology*, *3*(8). https://doi.org/10.1038/s41564-018-0200-0

Holmes, G. J., Ojiambo, P. S., Hausbeck, M. K., Quesada-Ocampo, L., & Keinath, A. P. (2015). Resurgence of cucurbit downy mildew in the United States: A watershed event for research and extension. *Plant Disease*, *99*(4). https://doi.org/10.1094/PDIS-09-14-0990-FE

Huot, B., Yao, J., Montgomery, B. L., & He, S. Y. (2014). Growth-defense tradeoffs in plants: A balancing act to optimize fitness. In *Molecular Plant* (Vol. 7, Issue 8). https://doi.org/10.1093/mp/ssu049

Köhl, J., Kolnaar, R., & Ravensberg, W. J. (2019). Mode of action of microbial biological control agents against plant diseases: Relevance beyond efficacy. In *Frontiers in Plant Science* (Vol. 10). https://doi.org/10.3389/fpls.2019.00845

Legein, M., Smets, W., Vandenheuvel, D., Eilers, T., Muyshondt, B., Prinsen, E., Samson, R., & Lebeer, S. (2020). Modes of Action of Microbial Biocontrol in the Phyllosphere. In *Frontiers in Microbiology* (Vol. 11). https://doi.org/10.3389/fmicb.2020.01619

Lindow, S. E., & Brandl, M. T. (2003). Microbiology of the phyllosphere. In *Applied and Environmental Microbiology* (Vol. 69, Issue 4). https://doi.org/10.1128/AEM.69.4.1875-1883.2003

Minchev, Z., Kostenko, O., Soler, R., & Pozo, M. J. (2021). Microbial Consortia for Effective Biocontrol of Root and Foliar Diseases in Tomato. *Frontiers in Plant Science*, *12*. https://doi.org/10.3389/fpls.2021.756368

Nawrath, C., & Métraux, J. P. (1999). Salicylic acid induction-deficient mutants of Arabidopsis express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation. *Plant Cell*, *11*(8). https://doi.org/10.1105/tpc.11.8.1393

Nishimoto, R. (2019). Global trends in the crop protection industry. In *Journal of Pesticide Science* (Vol. 44, Issue 3). https://doi.org/10.1584/jpestics.D19-101

Parker, J. E., Holub, E. B., Frost, L. N., Falk, A., Gunn, N. D., & Daniels, M. J. (1996). Characterization of eds1, a mutation in Arabidopsis suppressing resistance to Peronospora parasitica specified by several different RPP genes. *Plant Cell*, *8*(11). https://doi.org/10.1105/tpc.8.11.2033

Pfeilmeier, S., Caly, D. L., & Malone, J. G. (2016). Bacterial pathogenesis of plants: future challenges from a microbial perspective: Challenges in Bacterial Molecular Plant Pathology. In *Molecular plant pathology* (Vol. 17, Issue 8). https://doi.org/10.1111/mpp.12427

Pfeilmeier, S., Petti, G. C., Bortfeld-Miller, M., Daniel, B., Field, C. M., Sunagawa, S., & Vorholt, J. A. (2021). The plant NADPH oxidase RBOHD is required for microbiota homeostasis in leaves. *Nature Microbiology*, *6*(7). https://doi.org/10.1038/s41564-021-00929-5

Pieterse, C. M. J., van Wees, S. C. M., van Pelt, J. A., Knoester, M., Laan, R., Gerrits, H., Weisbeek, P. J., & van Loon, L. C. (1998). A novel signaling pathway controlling induced systemic resistance in arabidopsis. *Plant Cell*, *10*(9). https://doi.org/10.1105/tpc.10.9.1571

Pieterse, C. M. J., Zamioudis, C., Berendsen, R. L., Weller, D. M., van Wees, S. C. M., & Bakker, P. A. H. M. (2014). Induced systemic resistance by beneficial microbes. *Annual Review of Phytopathology*, *52*. https://doi.org/10.1146/annurev-phyto-082712-102340

Scheublin, T. R., & Leveau, J. H. J. (2013). Isolation of Arthrobacter species from the phyllosphere and demonstration of their epiphytic fitness. *MicrobiologyOpen*, *2*(1). https://doi.org/10.1002/mbo3.59

Shaw, R. K., Shen, Y., Zhao, Z., Sheng, X., Wang, J., Yu, H., & Gu, H. (2021). Molecular Breeding Strategy and Challenges Towards Improvement of Downy Mildew Resistance in Cauliflower (Brassica oleracea var. botrytis L.). In *Frontiers in Plant Science* (Vol. 12). https://doi.org/10.3389/fpls.2021.667757

Spadaro, D., & Droby, S. (2016). Development of biocontrol products for postharvest diseases of fruit: The importance of elucidating the mechanisms of action of yeast antagonists. In *Trends in Food Science and Technology* (Vol. 47). https://doi.org/10.1016/j.tifs.2015.11.003

Weller, D. M., Raaijmakers, J. M., McSpadden Gardener, B. B., & Thomashow, L. S. (2002). Microbial populations responsible for specific soil suppressiveness to plant pathogens. In *Annual Review of Phytopathology* (Vol. 40). https://doi.org/10.1146/annurev.phyto.40.030402.110010

Westphal, L., Scheel, D., & Rosahl, S. (2008). The coi1-16 mutant harbors a second site mutation rendering PEN2 nonfunctional. *Plant Cell*, *20*(4). https://doi.org/10.1105/tpc.107.056895

Wiermer, M., Feys, B. J., & Parker, J. E. (2005). Plant immunity: The EDS1 regulatory node. In *Current Opinion in Plant Biology* (Vol. 8, Issue 4). https://doi.org/10.1016/j.pbi.2005.05.010

Yarwood, C. E. (1956). Obligate Parasitism. *Annual Review of Plant Physiology*, *7*(1). https://doi.org/10.1146/annurev.pp.07.060156.000555

Yuan, J., Zhao, J., Wen, T., Zhao, M., Li, R., Goossens, P., Huang, Q., Bai, Y., Vivanco, J. M., Kowalchuk, G. A., Berendsen, R. L., & Shen, Q. (2018). Root exudates drive the soil-borne legacy of aboveground pathogen infection. *Microbiome*, *6*(1). https://doi.org/10.1186/s40168-018-0537-x

Zamioudis, C., & Pieterse, C. M. J. (2012). Modulation of host immunity by beneficial microbes. In *Molecular Plant-Microbe Interactions* (Vol. 25, Issue 2). https://doi.org/10.1094/MPMI-06-11-0179

Zipfel, C. (2014). Plant pattern-recognition receptors. In *Trends in Immunology* (Vol. 35, Issue 7). https://doi.org/10.1016/j.it.2014.05.004

Supplemental Figures

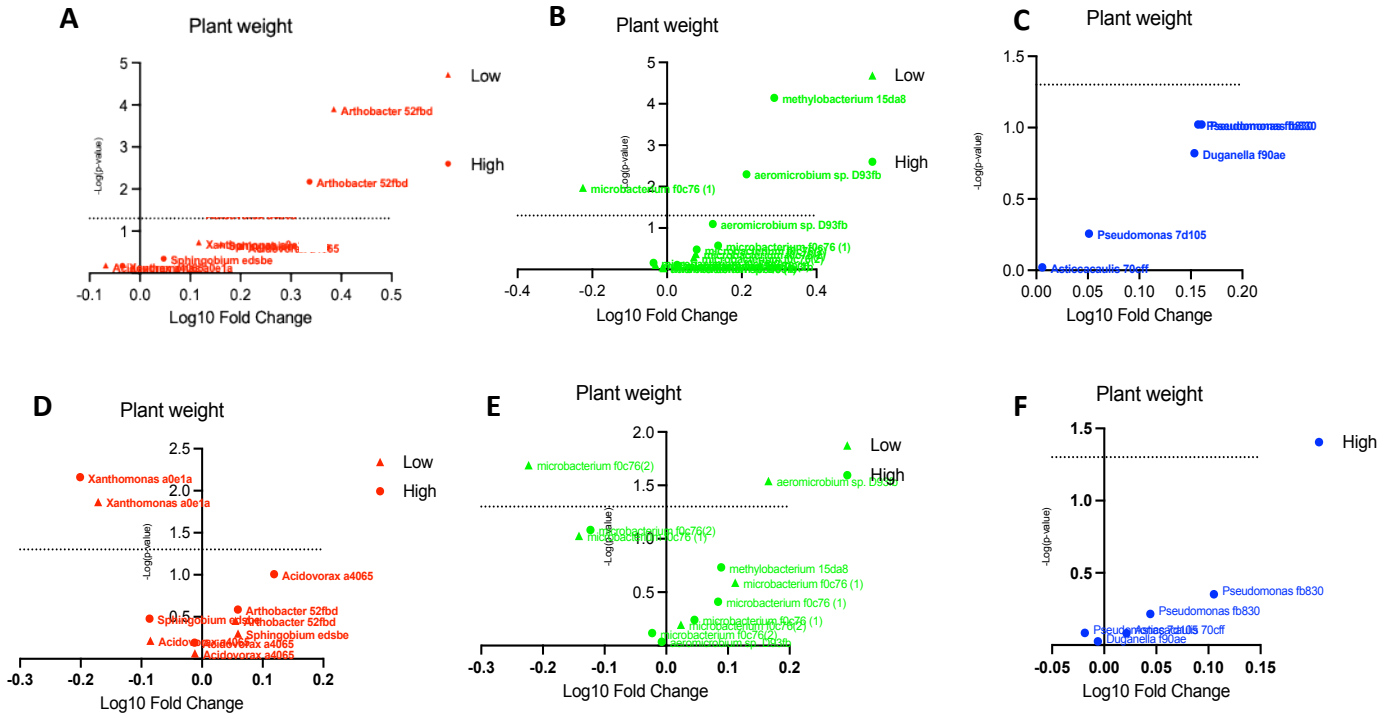
Supplemental table 1. Overview of plant immunity mutants

Mutant name	Description	Reference
Enhanced disease susceptibility 1 (Eds1)	Defective in all disease resistance pathways	(Falk et al., 1999)
Salicylic Acid Induction-Deficient 2(Sid2-1)	Defective in Salicylic acid biosynthesis	(Nawrath & Métraux, 1999)
Nonexpressor of PR genes1-1 (Npr1-1)	Defective in Salicylic acid signaling	(Cao et al., 1994)
Coronatine-insensitive 1 allele 16 (Coi1-16)	Defective in Jasmonic acid signaling	(Westphal et al., 2008)

Supplemental table 2. Overview of the twelve bacterial isolates that were tested in a gnotobiotic bioassay.

The first column indicates the Genus name and ASV. The second column indicates the isolate name. The third and fourth column specifies in which experiment the bacterial isolates were enriched. The fifth column shows the categorization of the bacterial isolates.

Genus* and *asv	**Isolate name**	**UU_MPIPZ**	***9- passage experiment***	**Category**
Acidovorax sp. a4065	*CN-YEM-16*	Enriched		Core
Sphinogobium sp. edsbe	*4c17*	Enriched		Core
Arthrobacter sp. 42fbd	*LC-R2A-7*	Enriched	Enriched	Core
Xanthomonas sp. a0e1a	*LC-LB-21/WCS2014-23r*	Enriched	Enriched	Core
Methylobacterium sp. 15da8	*CN-YEM-22*		Enriched	Associated
Aeromicrobium sp. d93fb	*PM-R2A-31*		Enriched	Associated
Microbacterium sp f0c76 (1)	*CN-YEM-23*		Enriched	Associated
Microbacterium sp.f0c76 (2)	*CN-YEM-9*		Enriched	Associated
Pseudomonas sp. fb830	*PN-YEM-6*		Depleted	Unaffected
Pseudomonas sp. f0c76	*CN2-GNA-2*		Unaffected	Unaffected
Duganella sp. f90ae	*PN-TSA-11*		Unaffected	Unaffected
Asticcacaulis sp. 70cff	*PN-R2A-26*		Unaffected	Unaffected



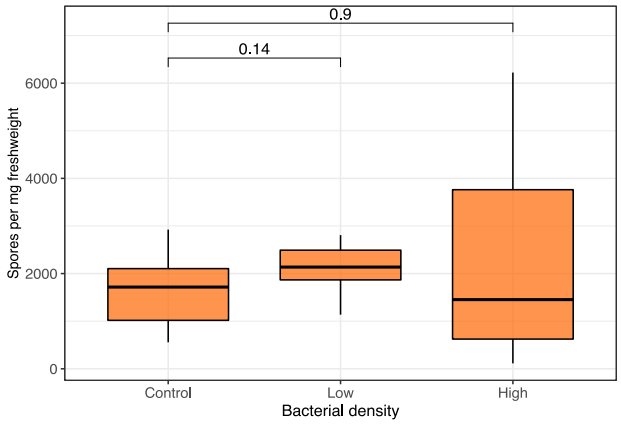
Supplemental figure 1 Two Hpa-Core bacterial isolates influences plant growth

A-C Scatter plot of Log10 fold change fresh weight (horizontal axis) of 17 DAG Arabidopsis plants that were inoculated at 10 DAG with: a single bacterial isolate or co-inoculated with gno*Hpa* and a single bacterial isolate. **D-E** Scatter plot of Log10 fold change fresh weight of 17 DAG Arabidopsis plants that were inoculated at 10 DAG with: only gno*Hpa* or co-inoculated with gno*Hpa* and a single bacterial isolate. Y-axis indicates the $-^{10}$log-transformed *P*-value of the comparison (Wilcoxon test). The dashed line in the graph represents *P*-value = 0,05, all data-point above this line are statistically significant. • Indicates a bacterial density of OD_{600} = 0,02 and ▲ indicates a bacterial density of OD_{600} = 0,0002. Red represents *Hpa*-Core bacteria, green represents *Hpa*-associated bacteria and blue represents unaffected or depleted bacteria. All labels contain bacterial *genus* and *asv.*

Supplemental figures 2 Gno*Hpa* Sporulation of all twelve isolates

Boxplots showing sporulation of arabdiopsis plants at 17 DAG inoculated with only gno*Hpa* (control) or co-inoculated with gno*Hpa* a single isolate at a (Low) or High (High) bacterial density. The titles indicate which bacterial isolate was inoculated. *P*-values are shown in the graphs using a Wilcoxan signed-ranked test. Bars indicate minimum and maximum numbers in a set.

Acidovorax a4065



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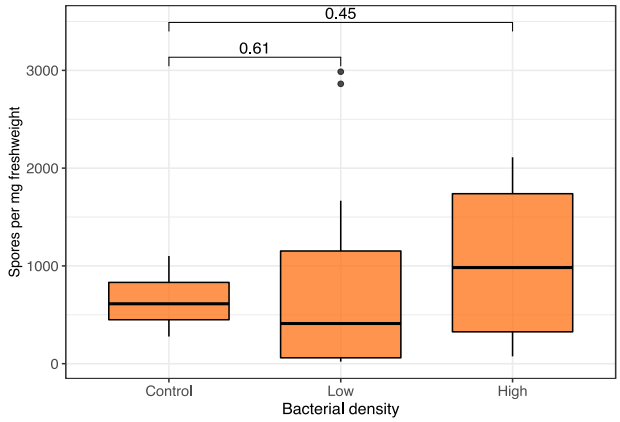
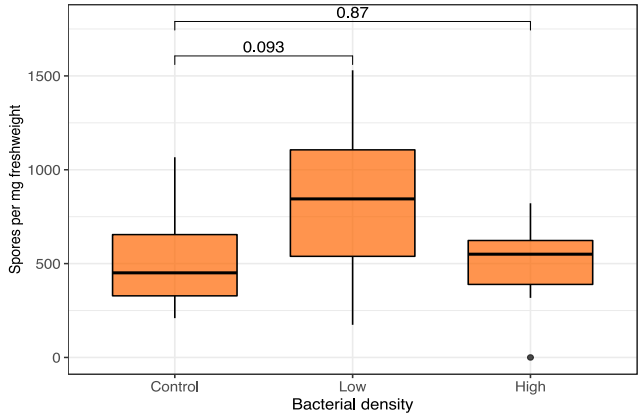
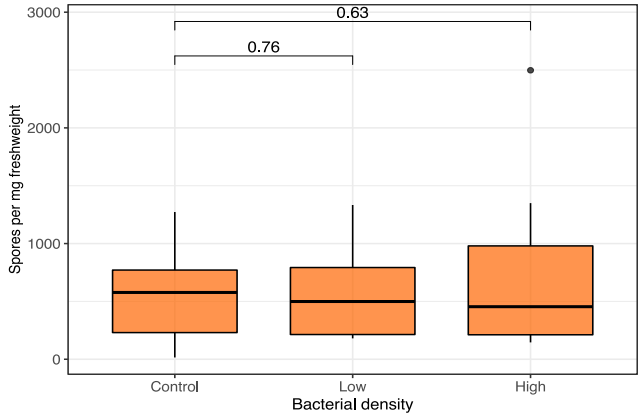


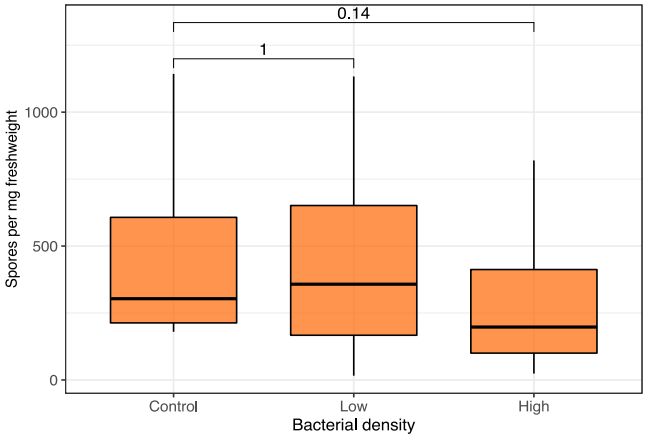
Sphingobium edsbe



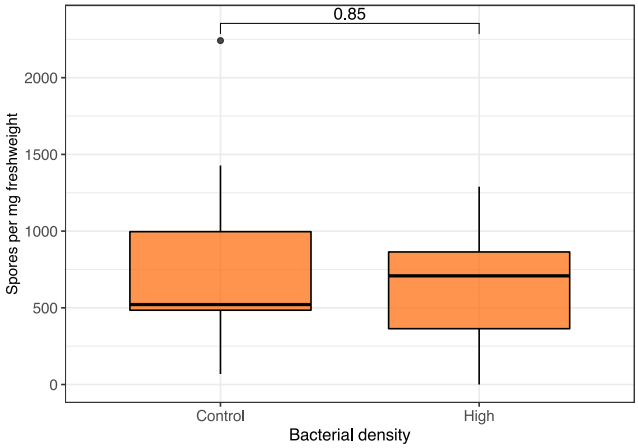
Arthrobacter 42fbd



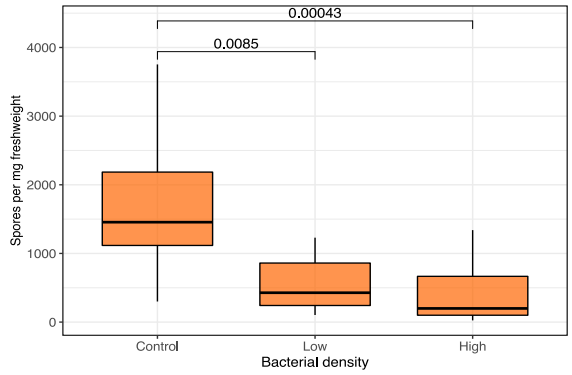
Xanthomonas a0e1a



Methylobacterium



Aeromicrobium d93fb



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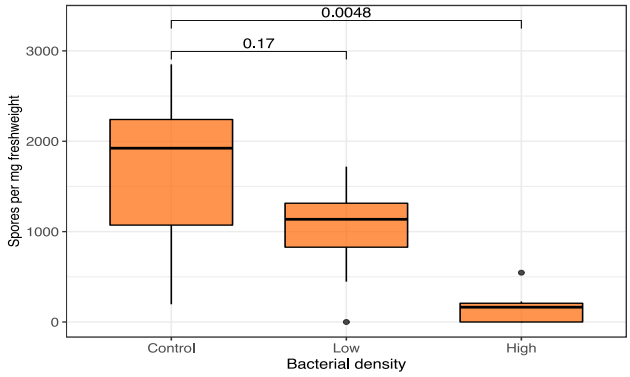
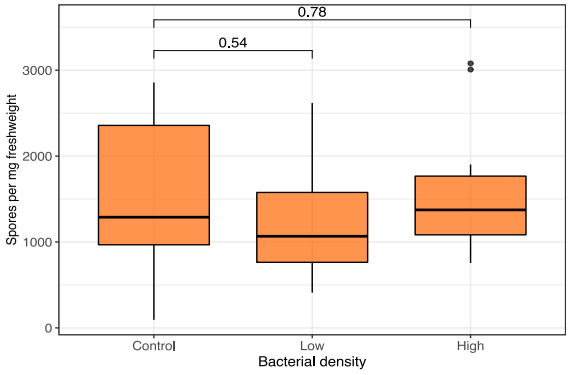


Microbacterium f0c76 (1)



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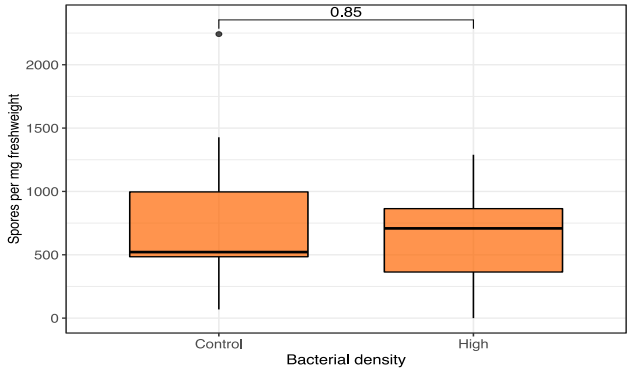

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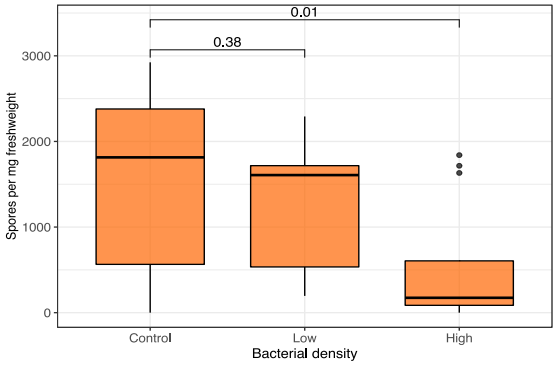

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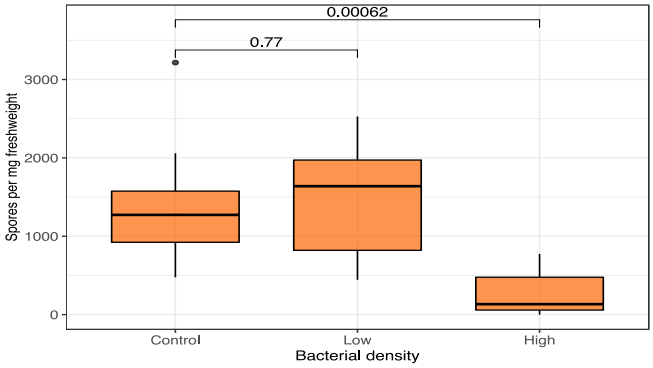
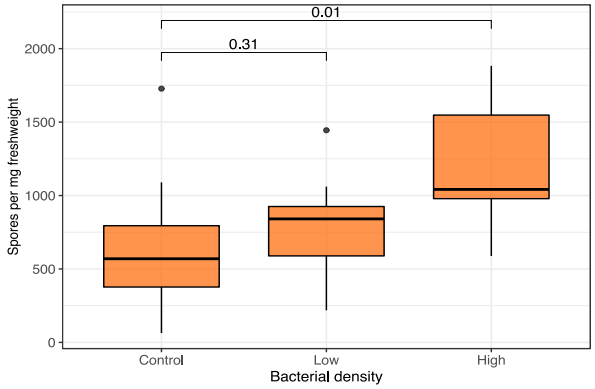


Pseudomonas fb830



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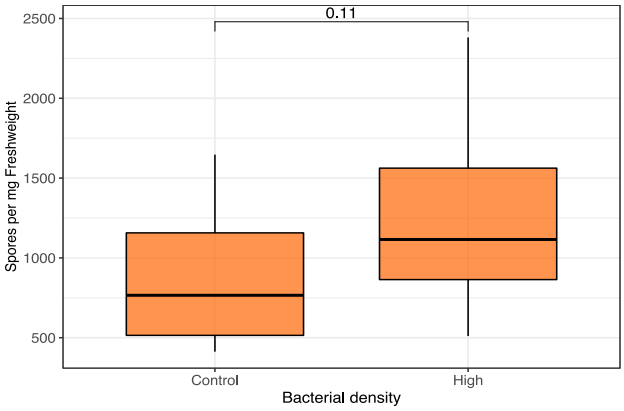

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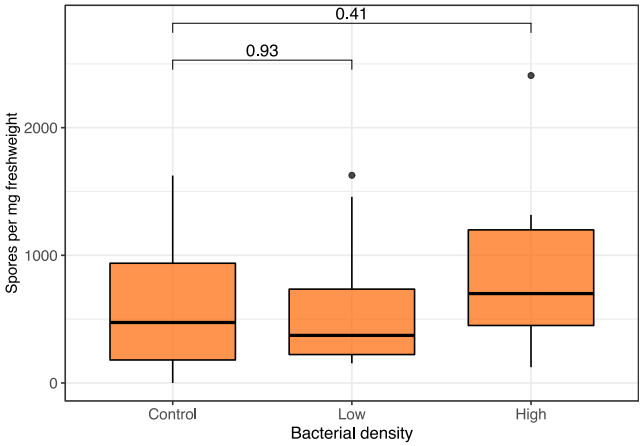
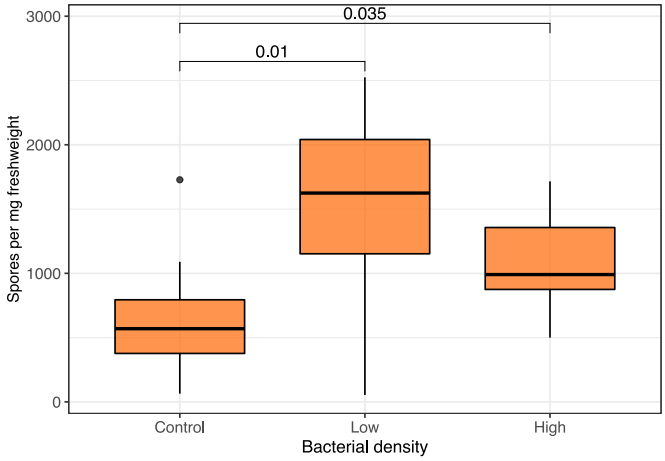


Duganella f90ae



Asticcacaulis 70cff