

Effects of Bacterial Endophytes on Plant-Insect Interactions

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Abstract:

Insects must interact with bacterial endophytes when sharing a common host. To test the hypothesis that larvae of *Scaptomyza nigrita* detects and prefers *Cardamine cordifolia* infected with different strains of *Pseudomonas* bacteria. Thus, unitizing the JA/SA induced antagonism. When given the choice between an infected leaf and a non-infected leaf, larvae demonstrated a significant preference towards leaves infected with *Pseudomonas* bacteria. Plants in which leaves were treated with *Pseudomonas* strains showed a significant reduction in chlorophyll content. Suggesting that larval preference correlates with the effect the bacterium has on its host plant.

Introduction:

Insects account for a large fraction of earth's species, and the vast majority of these are herbivores that specialize on only one or a few related plant species. In addition to dealing with insect herbivores, plants encounter microorganisms that can strongly affect their fitness. Bacteria can live on as well as inside of plant leaves, and cause severe damage or, conversely, bolster plant immunity against other natural enemies (Cui *et al.* 2002, Dong and Mindrinos 1991). Although some strains of plant pathogenic bacteria have been extensively studied in the laboratory, the effects of naturally occurring bacteria on plant-herbivore interactions are still largely unknown. To the extent that plants are affected by bacterial pathogens, it is reasonable to expect that specialized herbivorous insects may be influenced by this interaction. Furthermore, specialist insects may themselves influence the plant-bacterial interaction through modifying plant defense signaling.

Many studies have shown that plant defenses can be suppressed or activated through plant responses to microbes (Bennett and Bever, 2009). For example, the Gram-negative phytopathogenic bacterium *Pseudomonas syringae* influences plant defenses against other strains of bacteria and herbivores. In one case, *P. syringae* manipulates signaling "cross-talk" between jasmonic acid (JA) and salicylic acid (SA) in *Arabidopsis thaliana*, which modifies the interaction with the generalist insect herbivore *Trichoplusia ni*. Typically, plants acquire systemic immunity against bacteria via the SA pathway and against herbivores via the JA pathway. However, these two pathways are mutually antagonistic across most land plants (Thaler *et al.* 2012). The reciprocally antagonistic nature of SA-JA cross talk had the effect of making plants more susceptible to herbivory upon bacterial infection (Cui *et al.* 2005). This is in contrast to certain strains of *P. syringae* that make host plants more resistant to insect herbivory, through activation of JA-mimicking anti-herbivore molecules (Tian and Yang, 2007). This demonstrates that co-occurring bacteria have the potential to strongly influence the outcome of plant-insect interactions in diverse ways. However, we do not know if these interactions are important in native systems.

Previous research at Rocky Mountain Biological Laboratory (RMBL) in Gothic, CO has shown that many diverse strains of *Pseudomonas* bacteria (including pathogenic *P. syringae*) occupy the leaves of heartleaf bittercress, *Cardamine cordifolia* (Brassicaceae). This native crucifer is also parasitized by the herbivorous

leaf miner *Scaptomyza nigrita* (Drosophilidae). Adult females use their ovipositor to create punctures in the leaves of the plant to feed from, and they deposit eggs within a subset of these punctures (Louda, 1984). Once hatched, larvae create feeding mines within bittercress leaves until they pupate. Both adult females and larvae very likely consume co-occurring bacteria, including pathogens, while making their meals out of bittercress tissues. Last summer at RMBL, I showed that infecting bittercress leaves with an avirulent strain of *P. syringae* reduced the feeding rate of adult female *S. nigrita* larvae. The extent to which *S. nigrita* larvae encounter bacteria in nature is unknown, and directly depends on where adult females choose to lay their eggs and whether the larvae themselves move within the same stem to infected or uninfected leaves compared to their original oviposition. It is known that larvae are able to move to different leaves on the same stem, from this behavior larvae may carry their own independent preference compared to adults.

Previous studies on bacteria responses have been conducted in lab settings, where insects have exposed to infection through artificial wounding. One study in particular determined that *Trichoplusia ni* larvae are able to detect as well as respond to microbes they encounter in their diet. As a result they are able to prime their immune system and decrease risk of serious infection, with the trade-off of negative affects to fitness traits (Freitak et al, 2007). Thus, the effects of bacteria like *P. syringae* can be studied within this native three-way interaction between *C. cordifolia*, *S. nigrita* larvae, to determine whether *S. larvae* can detect *Pseudomonas* strains and use infection to their advantage via SA-JA cross-talk or other mechanisms. Furthermore, the effect that a particular strain has on its host can be used as an indicator for avoidance or preference of larvae. In one study it was shown that *Pseudomonas syringae* caused disease development in certain biosynthetic mutants of *Arabidopsis thaliana*, a close relative to *C. cordifolia*. As a result chlorophyll content was drastically decreased to close half of its original level before inoculation of *Pseudomonas syringae* (Bent et al, 1992).

I sought to test the hypothesis that leaves infected with *Pseudomonas* spp. strains are more susceptible to leaf-miner larvae. I conducted manipulation experiments using larval choice and performance assays to determine if larval behavior varied in response to different strains of *Pseudomonas syringae*, *florescence*, and *viridiflava* that were isolated from the field at RMBL. Also, I tested for direct fitness effects of these three bacterial species on the photosynthetic physiology of bittercress based on chlorophyll content to determine how infection influences leaf-level function.

Methods:

Plant Collection

C. cordifolia used throughout this project were all collected or found along Copper Creek, Gunnison County, CO. Three transects were created along patches of *C. cordifolia* in a shaded area within the Copper Creek site. These parallel transects were between 6 and 13 m in length and spaced more than 1 m apart. Transect length differed based on transect location and abundance of *C. cordifolia*. Along each transect, 16 plants were labeled, each being 30cm apart from each other. These 16

plants were used as focal plants and two other plants with 5cm of these plants were also labeled and measured. Measurements taken on individual plants include: height (cm), number of leaves, number of flowers, number of fruits, number of *S. nigrita* adult female feeding punctures (stipples), number of *S. nigrita* mines, and any other noticeable damage. For each transect 48 plants were labeled and measured, totaling 144 plants. The first transect plants were excluded, thus only plants from transect two and three were used for detached leaf assay.

Larvae Collection and Bacteria

A total of 48 larvae were collected for larval choice assays and 54 for larval transfers. Larvae were collected from mined leaves from Copper Creek site and the second crossing along Copper Creek trail. Three strains of *Pseudomonas* bacteria were used for inoculation; *syringae*, *florescence*, and *viridiflava*. Each of these strains was originally collected from bittercress plants in the same field site in 2011 and I cultured these strains in the lab. Bacterial strains were identified based on corresponding number 8, 12, and 18 (*florescence*, *syringae* and *viridiflava*, respectively) (Humphrey, in preparation), which were identified using the bacterial 16S rRNA gene and the *rpoD* gene.

Transect Inoculations

Along each transect, sixteen plots were made each containing three plants, totaling 48 plants per transect. Two chlorophyll measurements were taken, one before inoculation and after bacteria inoculation. Using the statistical program R each plot was randomly selected for one of the four treatments; Mock (control), 8, 12, and 18. Four leaves on each individual plant were inoculated with bacteria at OD 600 of 0.02 or mock (10 mM MgSO₄ and sterile water), using a blunt syringe. Each leaf was inoculated with a volume of approximately 0.3 mL. Chlorophyll content was measured before and after inoculation on the same four leaves, using a SPAD meter (SPAD-502, Konica Minolta, Japan). Chlorophyll content was measured for each plant to determine how the three bacterial strains influenced leaf photosynthetic ability. Between the two transects chlorophyll content was measured for a total of 384 leaves. Two out of the four leaves inoculated from each plant were detached and brought back to the lab for larval choice assays after 72 hours of being inoculated.

Larval Choice Assay

To test the hypothesis that larva can detect bacteria and whether larvae prefer to feed within infected leaves to non-infected leaves, a choice assay was implemented. In this choice test, two leaves are placed in a container with a larva (Dorn & Cronin, 2001). Forty-eight containers with Phytigel (Sigma-Aldrich Corporation, St. Louis, Missouri) were used as a substrate for detached leaves, each containing two leaves from the transect inoculations that had been infected for 72 hours, one infected (bacteria strain) and one control (mock). Leaves were paired based on similar leaf size, and each strain was replicated sixteen times using larvae that I collected from Copper Creek the same day. Containers were placed under 32W fluorescent lights in research lab for 24 hours with larvae. After a 24-hour period,

mined leaf out the pair was recorded along with which leaf area mined, calculated using an imaging program *Image J*.

Statistics

I used a one-tailed exact binomial test to determine the probability of success for larval choice between control and strain infected leaves. Tests were one-tailed because strain infected leaves were expected to be preferred by larvae. A repeated measures ANOVA was used to calculate chlorophyll content before and post inoculations between the four treatments, to determine if there were significant differences over time.

Results:

Chlorophyll Content

Chlorophyll content varied significantly between the between the four treatment after inoculations. The amount of chlorophyll content present in the four leaves on individual plants before and post inoculation decreased, but not in the mock treatment. Bacterial strains 12 and 18 had the greatest reduction of chlorophyll, in comparison to strain 8 and mock treated (Figure 1). Chlorophyll content between the three strains was significantly different to that of the mock treated leaves (F-ratio=5.1226, p-value=0.0025).

Larval Choice Assay

When given the choice between an infected and non-infected leaf (Figure 2), larvae preferred leaves infected with *Pseudomonas* strains compared to mock (p-value= 0.0057). When comparing between paired strain replicates of strains 8, 12, and 18, two of the strains had a great portion of larvae chose the infected leaf (Figure 3). Although larvae preferred strain 8 infected leaves, it was not significantly different from the number of times they chose mock in the pair choice. In the case of strains 12 and 18 there was a significant preference towards leaves inoculated with strains 12 and 18 compared to mock (p-value=0.028, p-value=0.02246).

Leaf Area Mined

Leaf area mined was used as an indicator for larval performance when they chose an infected or uninfected leaf. It was determined that leaf area mined did not depend on the total leaf area. I did not find a statistically significant differences between leaf area mined among strains and mock, although there was a trend of strain 8 being less mined than mock (Figure 4).

Discussion:

This study tested the hypothesis that larvae are able to detect and prefer leaves infected with *Pseudomonas* bacteria. The result from the two main experiments supported this hypothesis as well as indicated that the effect certain bacterial strains have on the plant itself is an indicator for larval preference. Overall

these results are consistent with JA/SA antagonism, meaning the larvae are choosing leaves with lower herbivore defense in the presences of pathogens.

When given the choice between an infected leaf to that of a non-infected leaf, it was determined that larvae have their own preference similar to that of *S. nigrita* adults. In my previous study summer when adult females were given the choice between a plant inoculated with mock or strain 8, there was a significant preference feeding preference towards stain 8. Although larvae chose strain leaves, strain 8 in this case was not significant, out of the three strains it was less chosen out of 12 and 18. This might due to the effects strain 8 has on the plant itself, as was seen in chlorophyll content it was the least reduction out of the three. Perhaps larvae base their preference on virulence of *Pseudomonas*. From the results leaves inoculated with strain 12 and 18 had the greatest decrease, as well as the greatest number of leaves mined. These strains might create a stronger response of SA in the plant, thus down regulating anti-herbivore deference mechanisms that the larvae find more suitable. Also, leaves with more mines have a higher amount of bacteria present, which might reflect on the dispersal of bacteria.

We are in need of a better understanding of how ecological interactions between consumers of a common host play out in nature. In this case bittercress, which is closely related to model plant species, might provide insight into how other systems work. We would also like to know how prominent JA/SA antagonism influences *S. nigrita* preference and *Pseudomonas* presence. Future research with biosynthetic mutants of *Arabidopsis thaliana*, where plants either have one of the pathways blocked (like *coi1* or *sid2*) or genes underlying the antagonism blocked (like *npr1*) can be used to further determine what mechanism is responsible, from the plant's perspective, in mediating the preference of *S. nigrita* for leaves infected with *Pseudomonas*. Also, looking at the direct and indirect effects *Pseudomonas* has on both adult flies and larvae. By measuring *S. nigrita* fitness, might provide whether they can be secondary host for bacteria.

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Figures:

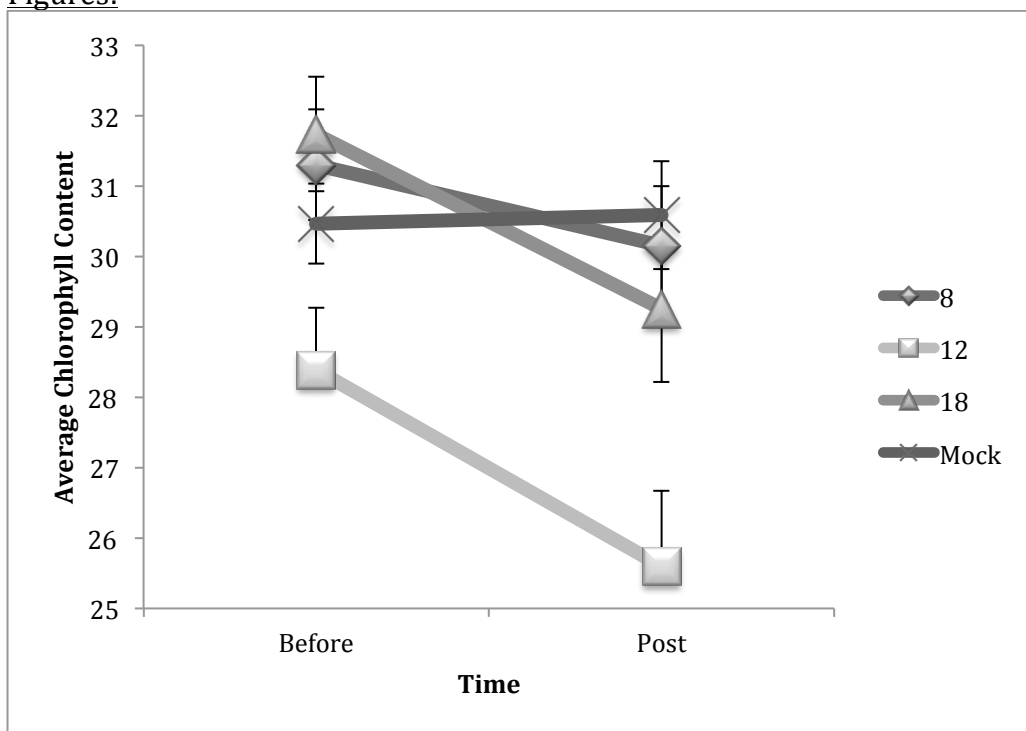


Figure 1: Average chlorophyll content measurements of four leaves inoculated with mock, 8, 12, and 18. Content measured over two periods before inoculation and after 72 hours post inoculations before leaves were retrieved for choice test. Significant reduction of chlorophyll among leaves treated with bacterial strains (F-ratio=5.1226, p-value=0.0025).

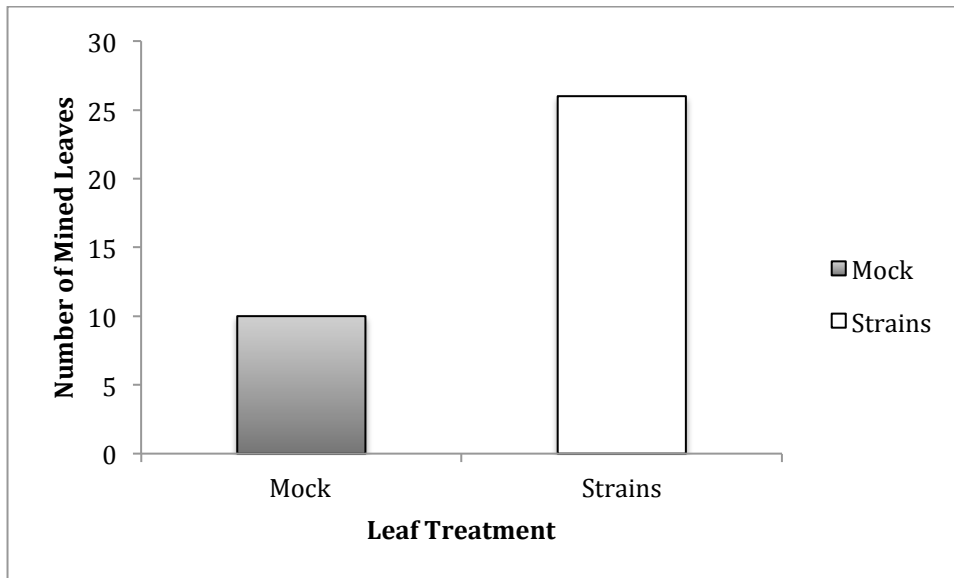


Figure 2: Number of mined leaves between mock and strain treated leaves, in larval choice test. Overall, larvae significantly preferred strain inoculated leaves (p-value=0.0057).

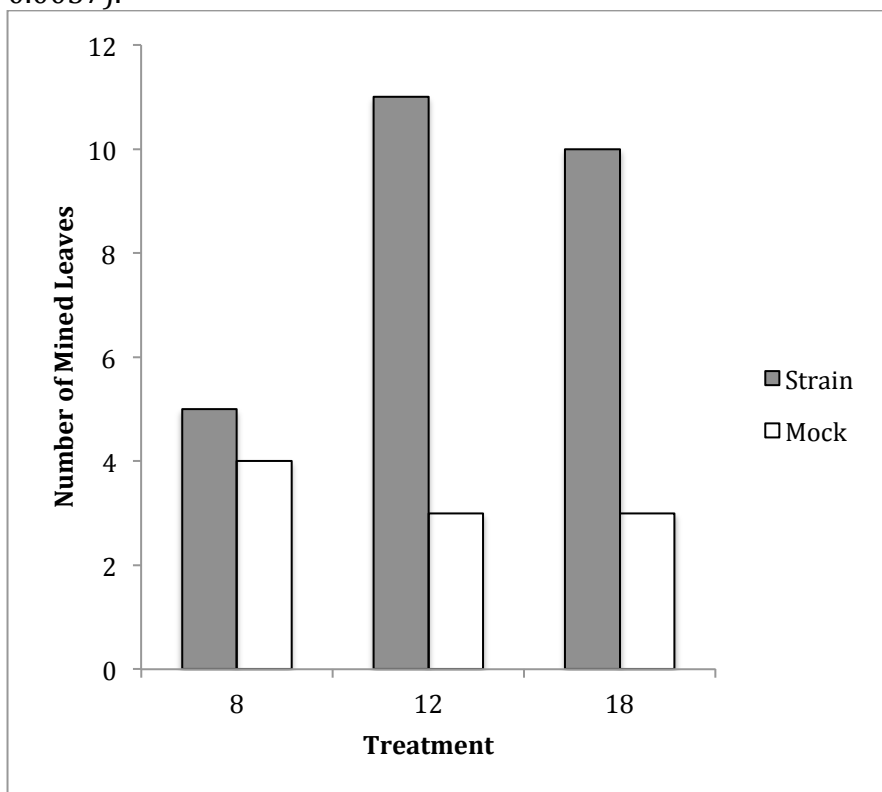


Figure 3: Number of mined leaves in paired treatment assays of strain 8, 12, and 18. Leaves inoculated with both strain 12 and 18 compared to their paired mock were significantly mined more (p-value=0.028, p-value=0.02246).

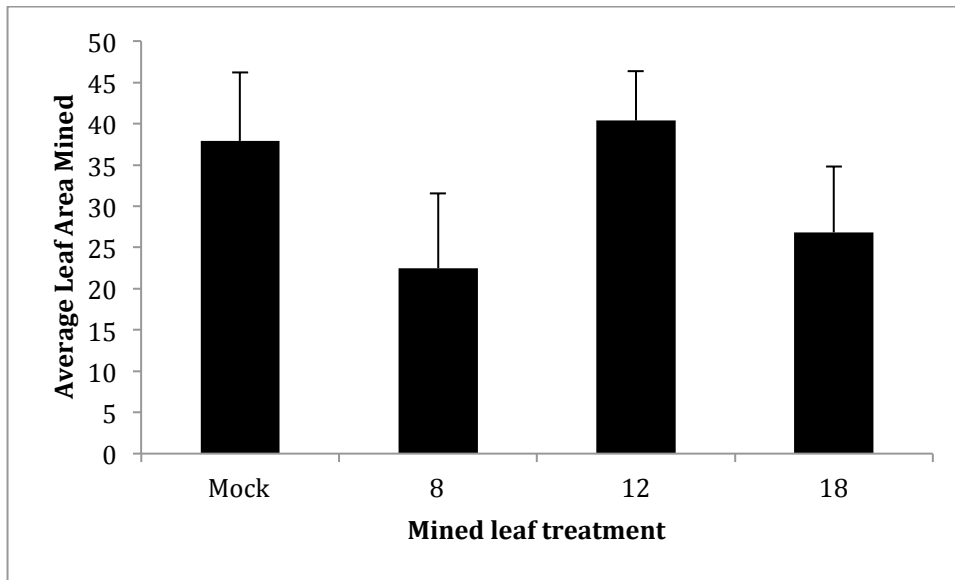


Figure 4: Average leaf area mined based on treatment after 24-hour period of feeding by larvae.