

Does Infection by Endophytic Bacteria Influence Plant Susceptibility to Herbivory in Nature?

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Independent Research

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Abstract

Host plant genotypes often interact with the genotypes of pathogenic bacteria in order to affect the susceptibility phenotype with regards to herbivory. Often specialist herbivores illicit host plant production of jasmonic acid while biotrophic pathogens induce a response of Salicylic acid, both of which slow growth of their target. Interestingly, production of either acid blocks the pathway of the other allowing specialist fly *Scaptomyza nigrita* and *Pseudomonas* bacteria to coinfect. A common garden was set up at Emerald Lake and three genotypes of *Cardamine cordifolia* were collected for planting. Three strains of bacteria as well as jasmonic acid, salicylic acid, and mock treatments were used to inoculate leaves prior to larval implantation to observe rate of larval growth in the host plant as an effect of treatment. The susceptibility to herbivores varied significantly among genotypes showing a plant response that did not differ significantly between JA and SA treatments, however, response to *Pseudomonas syringae*

differed significantly among genotypes. This was most likely due to avr gene crosstalk or coronatine production induced by bacteria.

Introduction

Often the scope of studies investigating the nature of species interactions is limited to that between two organisms, e.g. an herbivore and its host plant. However, multiple species interactions are more common than not, and every species involved has the potential to affect every other party directly or indirectly. For example, Karban *et al.* (1987) showed that volatiles transmitted through the air by one plant species can induce resistance against herbivores in another plant species. Rather than a two-way interaction, microbes often play a large part in mediating plant–herbivore interactions by directly affecting the plant and indirectly affecting herbivores (Biere *et al.* 2013). Thus, microbes offer a whole new factor to be incorporated into species interactions. Bittercress (*Cardamine cordifolia*, Brassicaceae), a native mustard that grows in large asexual patches, has been studied in relation to the drosophilid fly *Scaptomyza nigrita* (Collinge and Louda 1989). This fly and other herbivores that attack bittercress influence this plant's fitness and distribution across light quality gradient in the Elk Mountains of Colorado. However, bittercress is also infected by bacteria and it has recently been shown that *S. nigrita* and bacteria in the genus *Pseudomonas* often co-infect bittercress plants (Humphrey *et al.* in review). Interestingly, three strains of *Pseudomonas* seem to be common in bittercress leaves: *Pseudomonas syringae*, *Pseudomonas fluorescens*, and *Pseudomonas viridiflava*. One study showed that *P. fluorescens* was in higher concentrations on undamaged leaves, which adult flies preferred, however, *P. syringae* was in higher concentrations in damaged leaves, which larvae preferred (Humphrey *et al.* in review.) We know from laboratory studies of the related model plant *Arabidopsis thaliana* that prior infection by *P. syringae* bacteria influences subsequent herbivore performance elsewhere in the plant (in non-infected leaves) (Cui *et al.* 2002). However, we do not know if this scenario is important in nature or whether flies influence susceptibility to bacterial infection. However, the occurrence of both is positively correlated in natural populations of bittercress.

Many plant species produce salicylic acid (SA) in response to attack by biotrophic pathogens (those that proliferate in living tissue and cause damage to the host) such as *Pseudomonas* spp. bacteria that can result in systemic resistance to pathogens. In contrast, necrotrophic pathogens (those that proliferate in dead tissue) and specialist herbivores such as *S. nigrita* typically induce a jasmonic acid (JA) response that results in systemic resistance to herbivores. Interestingly, when JA is induced in controlled lab experiments, the SA pathway is suppressed (Thaler *et al.* 2012). Likewise, the JA pathway is often suppressed if the SA pathway has first been induced. Evidence for this reciprocal antagonism has been provided across a range of seed plants including the model species *A. thaliana*, *Solanum lycopersicum*, and *Nicotiana* spp., (Thaler *et al.* 2012). In lab experiments, bittercress has been shown produce JA-dependent

glucosinolates in response to *S. nigrita*, suggesting that this herbivore elicits a JA response in the plant (Humphrey *et al.* in review). In a previous field experiment, *S. nigrita* larval damage was higher when bittercress plants were pre-treated with SA and lower when plants were treated with JA relative to mock controls (Humphrey *et al.* unpublished data). This allowed us to see how, based on the strain of bacteria, the plant is able to mediate an indirect crosstalk between the microbe and specialist herbivore. In addition, previous lab work has shown that *Pseudomonas* infection alters the host choice behavior of *S. nigrita* adults and larvae in the predicted direction given a defense trade-off between SA and JA (Humphrey *et al.* in review). In lab experiments, *A. thaliana* plants containing a quadruple gene knockout for glucosinolate production (*cyp79b2cyp79b3 myb28myb29*) were planted adjacent to wildtype *A. thaliana* plants (Col-0) continuing to produce glucosinolates. Flies were released into these cages preferred feeding on the wildtype plants. This is probably because the adult flies require the presence of glucosinolates in order to find the proper host plant in the first place. So why then do adult *S. nigrita* flies choose nontoxic plants (containing high concentrations of *P. fluorescens*) to feed on? Probably because these flies are using chemoreceptors to detect toxic signals in order to identify the plant, allowing the fly to then choose among less toxic, and subsequently less damaged plants in a patch. Therefore, it is reasonable to see, via this mechanism, why both parties might often co-infect a plant because when a pathogen first attacks bittercress, the SA pathway will be induced and may prevent further infection. Subsequently, when a fly oviposits on the bittercress leaves, the JA pathway will be suppressed (because the SA pathway was first induced by the bacteria) allowing for increased larval growth. This is a hypothesis that I will in the field.

Another factor in this system is that bittercress genotypes vary in susceptibility to herbivores and pathogens (Humphrey and Whiteman, personal communication). It is also known that across diverse bittercress populations, there are many phytopathogenic bacterial species that are present in leaves damaged by herbivores of bittercress leaves (Humphrey *et al.* in review.) One study showed that rather than the additive effects of host and pathogen genotypes, it is the interaction between the genotypes of both the host and the pathogen that contribute most to an infection phenotype (Lambrechts 2010). Thus my goal here is to study how variation in plant genotype and bacterial genotype influence plant susceptibility to herbivory after systemic infection with bacteria.

Study Design

I tested the hypothesis that plant genotype, bacterial genotype, and plant genotype x bacterial genotype interactions influence subsequent plant susceptibility to herbivory from *S. nigrita*. I used three genotypes of plant along with three bacterial strains available in the lab from previous studies (and isolated from bittercress at Emerald Lake in 2012) including *P. syringae*, *P. fluorescens*, and *P. viridiflava*. Plants were then infested with larvae in systemic (non-

infected) leaves. We are testing the hypothesis that infection by leaf-dwelling bacteria affects the secondary susceptibility of bittercress by eliciting SA-induction in the plant. Through SA-dependent repression of the JA pathway, this causes the plant to mediate an indirect interaction between *Pseudomonas* spp. and *S. nigrita* that influences feeding by *S. nigrita*. More generally, I tested how plants are able to moderate chemical crosstalk between co-infections in a tripartite interaction. This experiment allowed us to measure SA-JA reciprocal antagonism in a natural setting through biochemical analyses which were then coupled with measurements of plant growth, larval growth, bacterial growth, rhizome weight, larval dry mass, larval wet mass, plant weight, leaf area mined, and susceptibility of the plant. This experiment also allowed us to test for the effects of bacterial strain and plant genotype on fly performance, and whether bacterial strains differ in impact, how variability in the plants can mediate these interactions and allow parties to indirectly affect each other, and whether the species of bacteria have an effect on herbivore-plant interactions? Genetic variability in the members of this tripartite interaction offers the opportunity to dissect how different strains of microbe being present are affecting the relationship between the genotype of the plant and (potentially localized) specialist herbivores. This experimental design incorporated the following variables: Genotype of Plant x Genotype of Bacteria x Species-Genotype Interactions.

Methods

Research was conducted in the year 2013 near the Rocky Mountain Biological Laboratory, Gothic, Colorado (Emerald Lake). ~1-10g rhizomes of three genotypes of bittercress (480 rhizomes total or 120 from each of three genotypes) were collected around Emerald Lake and were planted in a common garden near Emerald Lake with each plant being about six inches from the adjacent plant in in one large, common garden plot in eleven rows. Plant placement was randomized with respect to genotype. Each row had five groups labeled B-F with eight plants per group. The exceptions were rows 3-7, which were longer and were labeled group A. The dimensions of the garden were 6x12m with one meter between rows and one meter between groups. Plants eventually (outside the scope of the current project) will be genotyped in a lab to ensure distinct genotypes have been collected rather than phenotypically plastic individuals of a locally monotypic population however. Plants were collected from patches that are presumed to be made up of asexual clones because they are connected via rhizome mats. 120 rhizomes of each genotype were collected and planted in homogenized soil conditions in the field to account for differences in nitrogen, moisture, temperature, and sun exposure. Rhizomes took roughly four weeks to grow prior to research and were watered once daily. Plant stature and growth rate was measured, along with leaf number, leaf area, petiole length, chlorophyll content, and leaf thickness.

Bacterial strains were available from bittercress collected at Emerald Lake to researchers in the lab from previous and ongoing experiments including *P. fluorescens*, *P. syringae*, and *P. viridiflava*. These were used because abundances of these species were positively correlated with levels of *S. nigrita* herbivory levels in nature (Humphrey *et al.* in review.) Infiltrations of lab-grown bacterial suspensions were randomly assigned across plant individuals with respect to strain (or mock) to prevent bias. A randomized design with a single block was used in the common garden. 1-2 leaves per plant were infiltrated with the bacterial strains noted earlier, jasmonic acid, salicylic acid, or mock (sterile buffered water). The largest leaf in each cluster was not inoculated for larval implantation. After infiltration of 2.5 ml of bacterial suspension ($OD_{600}=0.002$) with the blunt-end syringe, bacteria (or mock) were allowed to grow for three days to allow for bacteria growth and potential plant response before implanting larvae on an adjacent leaf. A mock was used as a control to account for the possibility of injection allowing for colonization of bacteria or causing leaf wounding. The infiltrated leaves were clipped off of the plant three days post-infiltration. Next, a total of 200 larvae of *S. nigrita* were collected from Emerald Lake. About 20% more larvae than are required were collected in order to account for loss from previous experiments since larvae attached to leaf about 80% of the time. A single larva was placed on the largest leaf remaining and allowed to feed for two days.

The response variables measured included leaf area mined, bacterial growth, plant growth, rhizome mass, larval wet mass before/after, and larval dry mass before/after. Larvae were allowed to feed for three days before they were removed from leaves and were weighed based on wet and dry weight after the experiment. Weight of larvae before and after was recorded with a scale. I also measured leaf area mined, using Image J, after scanning leaves into the computer via digital camera. The caliper also allowed us to control for similar larval size during collection. This allowed the researchers to quantify the amount of infection by pathogenic bacteria and how this indirectly affected the response of the plant and its susceptibility to *S. nigrita* larvae. PhD candidate Parris Humphrey will analyze chemicals present in the leaves at subsequent stages in this experiment to determine the concentrations of endogenous defense hormones and tested for signal crosstalk occurring within the plant. Linear mixed models were used for statistical analyses. Plant genotype was analyzed as a random effect while fly genotype and bacterial genotype were analyzed as fixed effects. This is because the plant genotypes collected are a small sample of a larger population of genotypes and examining the variance between and among genotypes is the relevant question.

The preferences of adult flies may not be in congruence with larval preferences. The Whiteman group wanted to put together a separate experiment that would be able to evaluate larval choice when given the options of a treated versus untreated leaf. Thus, in opposition to implanting a larva on a leaf in which they were not given a choice as I did, performance is all we could accurately evaluate. However, when the larvae are put in an arena, this allowed them to choose which leaf they prefer and provides us with more insight into complexity of the tripartite interaction. For this experiment, about one hundred plants were collected from one rhizomatous

mat in North Pole Basin and were brought back to RMBL to be potted separately and left outside in a butterfly castle. Each plant was subjected to one of four treatments; *P. syringae*, *P. fluorescens*, *P. viridiflava*, and mock (saline solution.) Plants were infiltrated on the underside of two leaves on each plant via stomata. After 72 hours, systemic leaves, meaning leaves other than the leaves injected on the plant, were collected. Petiole was inserted into phytagel to decrease water loss. Sixty-five choice arenas were then made in petri dishes with a 2mm thick layer of phytagel (2%). One treated systemic leaf, and one control systemic leaf was placed on either left or right side of the phytagel and a larva was subsequently placed in the middle. Each systemic leaf that was collected was placed onto the left or right side of the phytagel randomly. Subsequently, a systemic leaf from a control plant was implanted on either the left or right side of the phytagel containing the treated systemic leaf. Larvae were allowed to choose a leaf and feed for 48 hours. Plates were then scored for preference and leaf area mined calculated using Image J.

Results

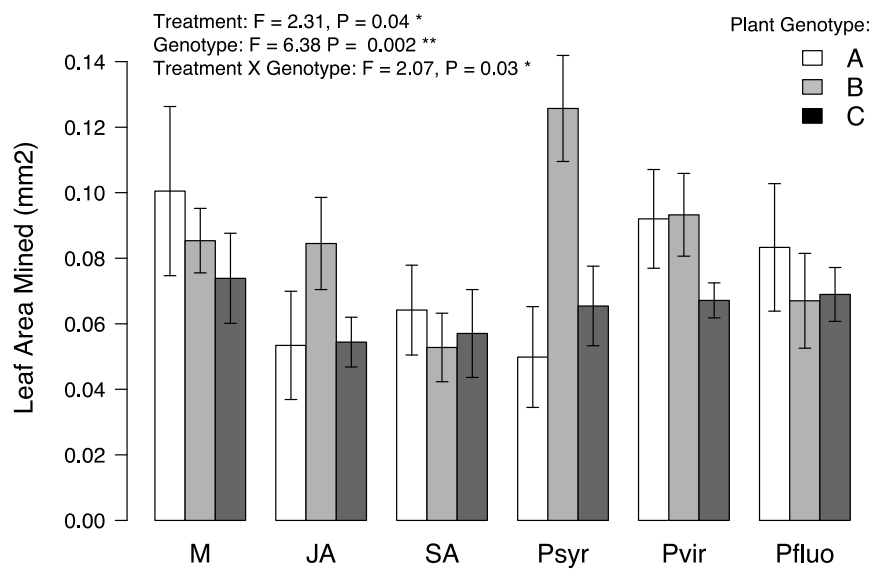


Figure 1. The impact of infection on herbivory depended on the strain of bacteria and the plant genotype*

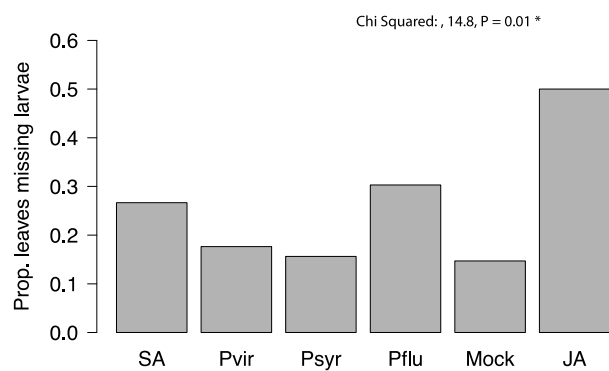


Figure 2. Number of larvae that left leaves after creating a mine by treatment type.

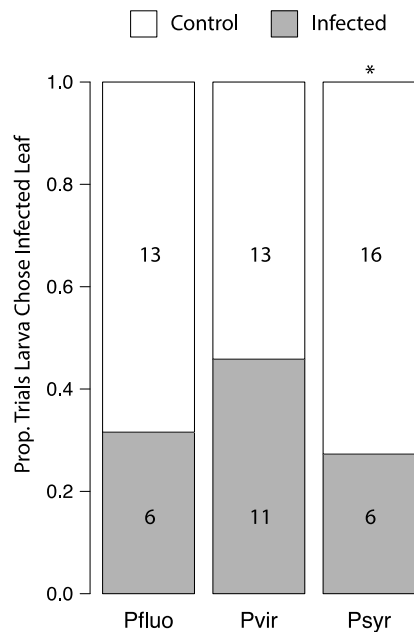


Figure 3. Number of larvae choosing between treatments offered in choice experiment. White represents control leaves and gray represents bacterial strain inoculated.

I found for the common garden experiment that among treatments of mock, jasmonic acid, or salicylic acid, there was a slight, but not significant difference among the three treatments in terms of herbivory. However, across genotypes, response to jasmonic acid differed most in genotype B with the opposite susceptibility phenotype than expected. When comparing among genotypes treated with bacterial strains in the garden experiment, I found that in treatments of *P. syringae*, the most prevalent strain in herbivore damaged leaves, there was a significant difference among genotypes (Humphrey *et al.* In Review.). Specifically, larvae ate more leaf area when feeding on plants of genotype B that were previously infected with *P. syringae*. Thus, the impact of infection on herbivory depended on the strain of bacteria and the plant genotype. There was not a significant difference in leaf area mined between treatments of jasmonic acid, salicylic acid, and mock. However, we recorded whether larvae had left the leaves, leaving behind only a mine, during time of collection. Plants treated with jasmonic acid showed a significantly higher incidence of larvae leaving the leaves among genotypes than in plants treated with mock or salicylic acid. The lack of difference in larval weight gain and leaf area mined with respect to SA and JA treatments was most likely due to the fact that larvae were all large at the time of collection and may not have had the ability to grow much more and thus show differing growth responses to treatments. Thus, larvae may have been past the point of being so responsive. However, I found that these larvae subjected to jasmonic acid ate fast and got out whereas larvae in salicylic acid treated leaves remained. Whether larvae in jasmonic acid treated leaves sought other plants or died after leaving, is unknown.

With regards to the choice experiment, larvae chose leaves at an equal rate between control leaves and leaves treated with *P. viridiflava*, marginal significance was apparent when

larvae chose more control leaves than those infected with *P. fluorescens*, and larvae chose control leaves significantly more than leaves inoculated with *P. syringae*.

Discussion

It is often observed that microbes influence plant-herbivore interactions in nature, but the focus has been on plant-fungal interactions. Although ubiquitous, leaf-dwelling bacteria have been largely ignored as agents affecting plant-herbivore interactions in nature. Here, I tested the hypothesis that prior infection with bacterial pathogens affected subsequent risk to herbivory in a native plant, and that this affect depended on plant genotype. I predicted that through inoculation of certain strains of bacteria such as *Pseudomonas syringae*, the most abundant strain in herbivore-damaged leaves in this system, that salicylic acid would be induced systemically in the plant, allowing for suppression of the jasmonic acid pathway (Thaler 2012.) Jasmonic acid is a plant hormone that results in the induction of antiherbivore toxins systemically, which has been shown to slow or halt larval development. In this light, larvae should have had a lower mass in those leaves treated with jasmonic acid as opposed to *P. syringae* or salicylic acid. What I found was a high degree of variation in the response to treatment with respect to genotype. When analyzing data in R, a plot was made showing the leaf area mined as the independent variable and treatment as the dependent variable. Bar graphs were subsequently split up among treatments to show response to each treatment by each genotype. The data showed that among JA, SA, and mock treatments, there was a slight, but not significant response difference in terms of herbivory. Interestingly though, data was collected whether larvae had left the leaf or stayed after mining; I found that in leaves treated with jasmonic acid as opposed to salicylic acid or mock, larvae left the leaves significantly more after mining than did the others. Larvae were collected late in the season meaning that they were exceptionally large; consequently, they probably did not have a long way to go in terms of growth before they needed to pupate, explaining the lack of significance in growth response. This said, we may have seen a more significant response had we collected larvae earlier in the season when they were smaller, and possibly more susceptible to plant defenses. Larvae that were implanted most likely responded to the jasmonic acid treatment by eating fast and getting out to search for new, less toxic leaves to pupate on.

Among genotypes, genotype B showed a different response to jasmonic acid than genotypes A or C with respect to subsequent mining . With regards to bacterial strain inoculated, plants from genotype B that were previously infiltrated with *P. syringae* showed the strongest response to herbivory. Specifically, genotype B showed a significantly higher response in leaf area mining to *P. syringae* than genotypes A or C, with genotype A having the lowest leaf mining response. These data supported that these large, isolated patches of bittercress, which reproduce asexually to produce rhizomatous mats of the same individual, vary significantly in their genetic composition and thus, their susceptibility or resistance to herbivory. This variation

probably is consistent with variation in both strains of bacteria. What this means is that microevolution is most likely happening within each of these ‘islands’ of genetically identical host plants allowing for interactions that are context dependent in terms of the susceptibility phenotype. For example, this study showed the opposite phenotype than expected using the JA-SA antagonism model. Two possible explanations are coronatine and avirulence genes in bacteria. Coronatine is a phytotoxin produced by certain *P. syringae* strains and acts as a jasmonic acid mimic, allowing for increased herbivore resistance and subsequently an opposite phenotype than predicted in SA-JA crosstalk by disrupting salicylic acid mediated responses in the host plant (Worley 2013.) Certain mats, individuals, or leaves in bittercress have a dark purple venation, which tend to be devoid of stippling, mines, or any other form of herbivore damage most likely because of the systemic resistance. This would be consistent if there is production of coronatine by *P. syringae*. What mediates this response and in what context it occurs is entirely unknown in this organism and is subject to further analysis in coming years. Another study done by Cui et al. (2005) showed that certain strains of *P. syringae* contain the *avrRp2* gene, which causes the plant to recognize and control the infection of this strain and induces resistance to *Trichoplusia sp.* herbivores on host plant *A. thaliana* (Cui et al. 2005.) Consequently, when the bacteria do not have this gene, plant susceptibility to herbivores is a result. Interestingly, when the host plant contains an *rps2* receptor, the avirulence gene in *P. syringae* will not induce resistance in the host plant. There is both variation in avirulence genes as well as host plant receptors allowing for a diversity of susceptibility phenotypes mediated by signaling crosstalk.

The larval arena experiment allowed larvae to have a choice in what leaves they were going to mine. In choice arenas that contained *P. syringae* inoculated systemic leaves and control systemic leaves, larvae chose the control leaves significantly more than the infected systemic leaves. This is probably an effect of either coronatine production or gene crosstalk. It is known that products downstream of the jasmonic acid pathway are toxic to larvae, thus, it would make sense that a jasmonic acid mimic such as coronatine would illicit an aversion from the larvae. Aversion to *P. fluorescens* was marginally significant whereas choice has divided equally between *P. viridiflava* systemic leaves and control systemic leaves. Because herbivore damaged leaves have the highest concentration of *P. syringae* strains, we expected a significant response from these arenas. However, the opposite choice than expected was executed probably as a result of the circumstances mentioned above.

Thus, susceptibility phenotypes in terms of herbivory are context dependent with regards to genotype of plant, bacteria, and probably herbivore. It can also be predicted that the presence of opposite phenotypes in our study are a result of gene by gene crosstalk in bacteria and plant mediation of herbivory or antiherbivore chemical mimics induced by particular strains of *Pseudomonas*. As a result of this study, we may need to begin looking at these clonal stands of host plants as particular environments experiencing their own tripartite microclimates that vary

genetically and thus phenotypically in response to variation in pressure of herbivory, and genetic composition of bacteria present.

Timeline

Date	Activity
June 26-June 27	Rhizome collection
June 28-June 29	Garden Plot Setup
June 29-June 30	Planting
July 1-July 31	Collect Data and Larvae Collection
July 21-July 25	Bacterial Infiltration
July 26-July 31	Larval Implantation
July 31-August 6	Analyze and Write Paper

Short-term and Long-term Conflicts

We removed around 480 rhizomes total or eighty rhizomes per plant patch. Because these patches are made up of hundreds of the same individual and the amount of stems needed to provide eighty ramets per patch is small compared to the entire patch, the effect on population size is negligible. We are decreasing seed input for each patch; however we are not decreasing the genetic diversity for future years because the patches are clonal. With regards to the fly larvae, we may be decreasing population size as well as genetic diversity by removing 200 larvae from Emerald Lake; however, given the fecundity of the flies, this is negligible as well.

Potential Conflicts

Growing a common garden up at Emerald Lake might have disturbed the local area to some degree from plant removal and modification of the soil for planting. At this time, I do not know whether anyone else I know of will be working up there as well besides other members of the Whiteman group.

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GIS Map



Literature Cited

- Biere, A., & Bennett, A. E. (June 01, 2013). Three-way interactions between plants, microbes and insects. *Functional Ecology*, 27, 3, 567-573.
- Collinge, S. K., & Louda, Svata M. (1989). *Scaptomyza nigrita* Wheeler (Diptera: Drosophilidae), a Leaf Miner of the Native Crucifer, *Cardamine cordifolia* A. Gray (Bittercress). DigitalCommons@University of Nebraska - Lincoln.
- Cui, J. (February 01, 2005). Pseudomonas syringae manipulates systemic plant defenses against pathogens and herbivores. *Proceedings of the National Academy of Sciences*, 102, 5, 1791-1796.
- Collinge, S K, and S M. Louda. "Herbivory by Leaf Miners in Response to Experimental Shading of a Native Crucifer." *Oecologia*. 75.4 (1988): 559-566. Print.
- Humphrey et al. Bacterial phyllosphere diversity and herbivory are linked in the wild. *Molecular Ecology*. 2012. (Unpublished: For Review Only)
- KARBAN, R., ADAMCHAK, R., & SCHNATHORST, W. C. (February 06, 1987). Induced Resistance and Interspecific Competition Between Spider Mites and a Vascular Wilt Fungus. *Science*, 235, 4789, 678-680.
- Lambrechts, L. "Dissecting the Genetic Architecture of Host-Pathogen Specificity." *Plos Pathogens*. 6.8 (2010). Print.
- Thaler, J. S., Humphrey, P. T., Whiteman, N. K., & Special Issue: Specificity of plant-enemy interactions. (May 01, 2012). Evolution of jasmonate and salicylate signal crosstalk. *Trends in Plant Science*, 17, 5, 260-270.
- Worley, J. N., Russell, A. B., Wexler, A. G., Bronstein, P. A., Kvitko, B. H., Krasnoff, S. B., Munkvold, K. R., ... Collmer, A. (January 01, 2013). Pseudomonas syringae pv. tomato DC3000 CmaL (PSPTO4723), a DUF1330 family member, is needed to produce L-allo-isoleucine, a precursor for the phytotoxin coronatine. *Journal of Bacteriology*, 195, 2, 287-96.