

EFFECTS OF HORMONE CROSSTALK ON ENDOPHYTIC BACTERIAL COMMUNITIES

By

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**TABLE OF CONTENTS**

<b>ABSTRACT.....</b>	<b>4</b>
<b>INTRODUCTION .....</b>	<b>5</b>
<b>METHODS.....</b>	<b>9</b>
<b>RESULTS.....</b>	<b>17</b>
<b>DISCUSSION.....</b>	<b>21</b>
<b>ACKNOWLEDGEMENTS .....</b>	<b>25</b>
<b>REFERENCES .....</b>	<b>26</b>

## ABSTRACT

The plant hormones salicylic and jasmonic acid (SA and JA, respectively) both play a crucial role in the induction of plant defense system pathways and long-term pathogen resistance. Plants, which do not have an active cellular immune system like animals, instead rely on the release of specific molecules to mediate defense. In general, the SA pathway is activated by biotrophic pathogens and primarily induces antimicrobial responses, while JA is activated by necrotrophic pathogens and herbivory, and induces separate chemical responses. SA and JA are reciprocally antagonistic: activation of one pathway inhibits activation of the other. Here we explore how SA-JA inhibitory crosstalk is used by pathogens or herbivores to combat plant defense. We study the effects of hormone crosstalk on bacterial growth in two plant models: *Cardamine cordifolia* and *Arabidopsis thaliana*, which we treated to induce defense pathways. These plants were inoculated with endophytic bacteria isolated from field *C. cordifolia* plants, and the effects of hormone treatment on bacterial growth rates were measured. We show that JA-induced defenses, which are commonly associated with necrotrophic pathogens, affect varied biotrophic *Pseudomonas* strains both positively and negatively. Notably, we show that JA-induced defenses affect wild *P. fluorescens* strains more negatively than SA-defenses.

## INTRODUCTION

The plant defense system, unlike the system of animals, is not regulated by moving cells that directly combat threats. Plants have instead evolved a different defense strategy – using molecular defenses that are mediated by specific hormones without the need for mobile immune cells<sup>[1][2]</sup>. In both *Arabidopsis thaliana* and *Cardamine cordifolia* (bittercress), mustard species of the family Brassicaceae, along with many other plant species, the immune system is split into two main pathways, each mediated by different phytohormones<sup>[1]</sup>. The first, commonly considered the antimicrobial defense pathway, is mediated by the hormone salicylic acid (SA), and is thought to primarily defend against biotrophic attackers<sup>[1][2][3][4]</sup>. The SA-mediated pathway is also primarily responsible for the induction of Systemic Acquired Resistance (SAR) in plants<sup>[1]</sup>, helping combat repeated biotrophic pathogen threats more rapidly. Current literature focuses on SA being primarily activated and combating biotrophic pathogens, or pathogens that feed on live tissue<sup>[1][3]</sup>. The second defense branch, which is more closely associated with defenses against chewing insects and necrotrophic pathogens<sup>[1][3][4]</sup>, is mediated by the hormones jasmonic acid (JA) and ethylene (ET)<sup>[1][4]</sup>. JA and ET are both required for rhizobacteria-mediated induced system resistance (ISR)<sup>[1][2]</sup>, which is primarily focused in the roots of plants<sup>[1]</sup>. The joint interaction of these two branches gives these plants a complicated, but robust immune system that can combat an extremely wide variety of threats.

Notably, these two defense systems display a reciprocal antagonism<sup>[1][2][3]</sup>. In plants, the activation of one pathway inhibits the second in an inhibitory crosstalk that arises from molecular interactions between the two, along with a need to minimize the cost of immune system activation<sup>[3]</sup>. This means that when a plant pathogen induces the SA-mediated pathway,

the plant induces SA defenses at the risk of being unable to subsequently induce JA-mediated defenses. In nature, this comes at a significant risk – what if pathogenic endophytic bacteria are introduced at the same time as, or shortly after herbivory occurs? This reciprocal antagonism means that two threats introduced simultaneously could be detrimental to plants facing multiple attackers, and the question of who has the upper hand in these encounters – plant, pathogen, or herbivore – is poorly understood<sup>[3]</sup>.

In recent years, evidence has been presented that simultaneous attack can occur in nature, and some species can use the plant's immune crosstalk to their advantage. For example, the Colorado potato beetle has been shown to secrete symbiotic bacteria into plant wounds to elicit an SA response, which allows the beetle to feed without the plant being able to activate JA-mediated defenses against the beetle<sup>[5]</sup>. This also allows the beetle to continue feeding on nearby plants, as the JA response is not spread to neighboring plants<sup>[1][5]</sup>. Some herbivores bypass these defenses by themselves suppressing JA-mediated defenses<sup>[5]</sup>. On the other hand, some bacteria have evolved methods to suppress the SA pathway, and can thus continue to survive as plant pathogens without needing to combat the SA pathway<sup>[6]</sup>. As an example, some *P. syringae* strains are able to produce coronatine, a JA mimicking phytotoxin that seems to induce JA defenses, suppressing SA-pathway induction and increasing virulence<sup>[4]</sup>. This creates a vexing evolutionary battle for plant life – the inhibitory crosstalk of its defense pathways, coupled with the defense-bypassing mechanisms of plant herbivores and pathogens, may create an increased selective pressure for plant defense pathways to evolve to cope<sup>[1][3]</sup>. However, this evolution is not well studied and is the very reason why it is so

important to better understand the crosstalk-mediated interactions presented in plant-attacker models, especially when multiple attackers are present.

To counteract the effects of the inhibitory SA-JA crosstalk, evidence shows that the JA pathway is able to produce antimicrobial compounds to combat certain pathogens<sup>[1][2][3]</sup>. Likely candidates for this are the isoflavonoids phytoalexins, which are accumulated through JA-pathway activation<sup>[1][4][8]</sup>. Recent literature has shown that phytoalexins have an antimicrobial response. In *A. thaliana* and other crucifers, the phytoalexin camalexin has been shown to have an effect on bacterial and fungal pathogens<sup>[1]</sup>. However, phytoalexin responses tend to have pathogen-specific toxicity, and may thus vary in effectiveness on the wide array of endophytic bacteria found in nature<sup>[1][7][9]</sup>. But given the association that certain herbivores and endophytic bacteria display<sup>[10]</sup> and the large variety of pathogens, both biotrophic and nectotrophic, that plants must combat, it is possible that JA-mediated defenses have evolved more widespread antipathogenic mechanisms. Specifically, the JA and ET-mediated pathways of mustards produce a wide variety of both indolic and aliphatic glucosinolates, or mustard oil glycosides<sup>[1][11]</sup>. Glucosinolates have been widely implicated in antiherbivory in mustards<sup>[12]</sup> but may also be required for a mustard plant's innate immune response to pathogens<sup>[13]</sup>. Here we study the effect of JA and SA induction on a wide array of *Pseudomonas syringae* and *P. fluorescence* originally isolated from field experiments by Humphrey et. al.<sup>[10]</sup> in *C. cordifolia*, their original host. These field experiments established a baseline for our studies, showing the *P. syringae* was more closely associated with herbivory than *P. fluorescens*<sup>[10]</sup>. It was found that *P. syringae* was found in relatively higher abundance in herbivory-damaged plants, while *P. fluorescens* was negatively associated with herbivory. We thus hypothesize that these wild *P.*

*syringae* will be more adapted to thrive in JA-induced plants than wild *P. fluorescens*, which would not experience JA-induced conditions as often. We study the effect that JA and SA induction has on 12 different strains of *Pseudomonas* caught in the field (six each of *P. syringae* and *P. fluorescens*) in bittercress in the greenhouse, and focus on two of these (one each of *P. syringae* and *P. fluorescens*) in *A. thaliana* models in the laboratory. We used diverse *C. cordifolia* clones to mimic and study the effects that were previously observed in *C. cordifolia* field studies<sup>[10]</sup>. *A. thaliana* was used in the laboratory for three primary reasons: (1) it is an extremely well-studied model organism in the same family as *C. cordifolia*, with no notable differences in immune physiology found in the literature, (2) there is access to a wide selection of SA and JA mutant *A. thaliana* options that are to relatively simple to access, many with detailed genetic information and background research available and (3) *A. thaliana* can be grown in large quantities in a short timeframe, enabling well-replicated manipulative experiments. Using multiple *A. thaliana* mutants, we focused on better understanding of the antipathogenic mechanisms underlying JA-mediated defenses, and particularly the possibility of glucosinolates having antimicrobial effects distinct from innate immunity regulation and the effects of phytoalexins, especially in biotrophic *Pseudomonas* strains. Along with a wild type control, we used two SA-deficient mutants (one that is SA insensitive, and one that accumulates very small quantities of the hormone) to isolate the effects of the JA pathways in a way differing from previous literature<sup>[14]</sup> which focused on JA-deficient mutants, and hypothesize that these can be conferred resistance against certain biotrophic plant pathogens even without an SA system. A mutant *A. thaliana* that does not produce glucosinolates was used to similarly look for an antimicrobial effect keyed to glucosinolate production.

## METHODS

### ***Greenhouse Experiments***

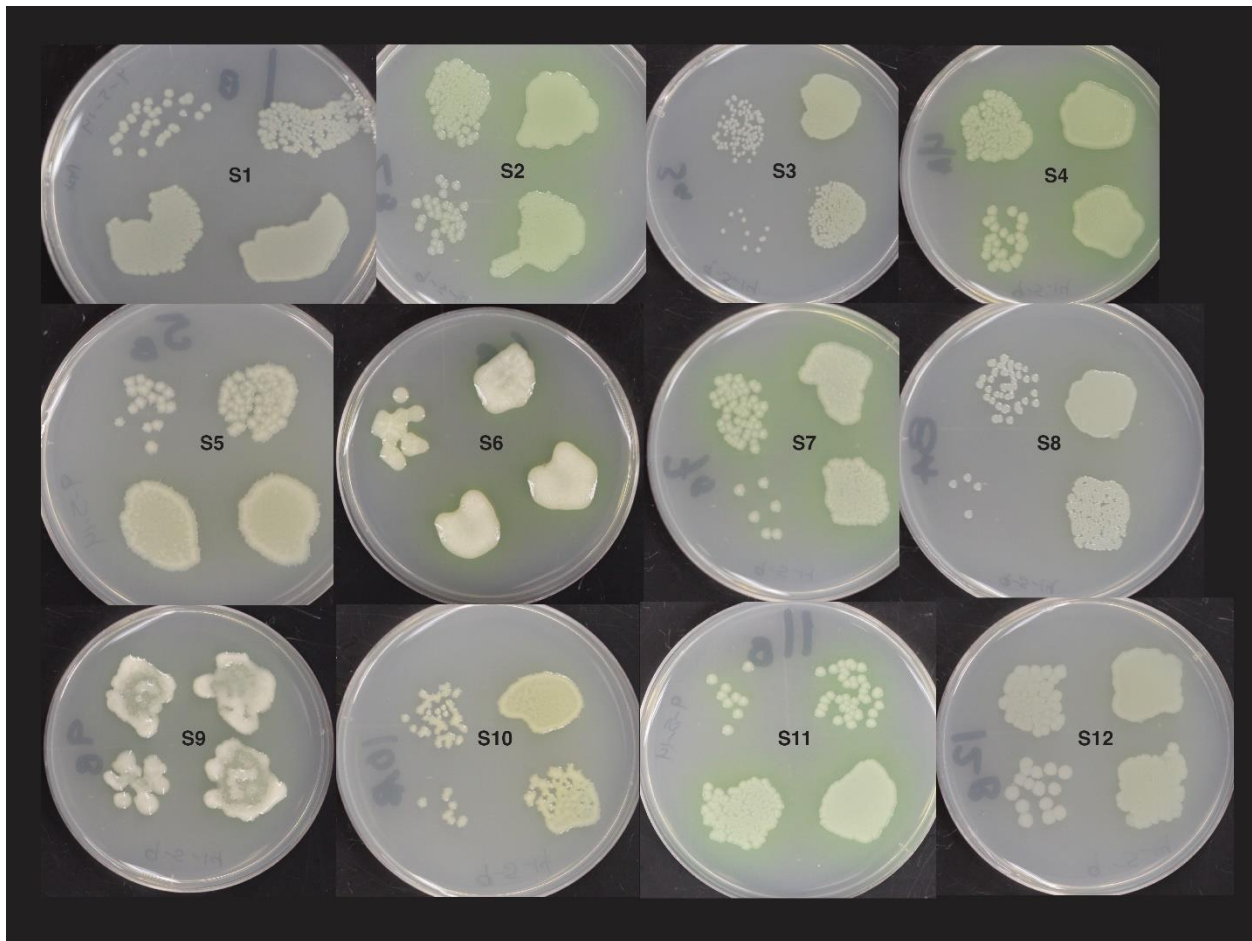
#### *Plant growth, treatment, and infection*

Diverse bittercress clones acquired from the field at the Rocky Mountain Biological Laboratory (RMBL) were planted and grown for approx. 6 weeks in the Biochemistry Greenhouse A at the University of Arizona, Tucson. Plants were kept consistently watered and were rotated around the greenhouse to ensure consistent light exposure and decreased variance due to local variations in humidity. Plants were grown one per pot – each pot was given a number code, and kept in a specific flat that contained a total of four pots. There were 16 flats total, for a total of 64 plants. Twelve leaves on each plant were randomly labeled 1-12 using a paint pen. Each plant was then randomized and sprayed until wet (visible droplets on leaves) with one of three treatments: 1mM JA (in H<sub>2</sub>O + 0.42% methanol), 1mM SA (in H<sub>2</sub>O + 0.42% methanol), or a mock treatment (H<sub>2</sub>O + 0.42% methanol). Plants were sprayed so that no cross-contamination occurred between treatments. During a two-day treatment period, 12 bacterial cultures were grown in Kings B medium. Bacterial cultures were taken from each of the 12 bacterial strains (6 *P. syringae*, 6 *P. fluorescens*) isolated from RMBL field experiments. These cultures were prepared for inoculation by diluting them into a solution of 10mM MgSO<sub>4</sub> to achieve OD<sub>600</sub> measurements of .002. Each strain was assigned an alpha-neumeric code (S01-S12) coinciding to the numbers marked on the leaves of plants. These solutions were made into four dilutions each, and were plated on agar gel plates to produce a morphological key for later colony counting (Figure 1). Plants were watered 2–3 hours prior to infection to facilitate opening of stomata to facilitate pressure infiltration. Bacterial suspensions were inoculated into

plants using blunt-end 1 mL syringes. Approx. 20 – 100  $\mu$ L bacteria suspension was infiltrated into leaves on each side of the medial vein. Bacteria were allowed to grow over three days before plants were harvested.

#### *Leaf collection and processing*

After the three-day bacterial growth period, leaves were collected using ethanol and flame sterilized instruments. Leaves were cut from the plant and had two equal sized discs removed using an increment borer. In cases where leaves were too small for two discs to be removed, a half leaf or whole leaf was collected. Leaves were photographed along with their assigned randomized treatment number and leaf discs, packed into an aluminum foil square



*Figure 1 Anonymized bacterial colony isolates. Each strain displays a unique morphotype allowing for visual confirmation of correct infection procedure during colony counting process.*

with the assigned treatment number written on top, and stored in liquid nitrogen until they could be transferred to a  $-80^{\circ}\text{C}$  freezer.

#### *Leaf processing for dilution cultures*

Samples collected from the greenhouse were weighed, then sterilized by using 95% ethanol (5 sec), into 70% ethanol (30 sec), and finally into three baths of sterilized water (1 min each). Samples were then dried on clean Kimwipes. Water baths were changed between each set of samples. Leaf discs were inserted into previously prepared 2mL centrifuge tubes containing 350  $\mu\text{L}$  10 mM  $\text{MgSO}_4$  and a sterilized 5 mm stainless-steel bead. Samples were placed into a bead beater in groups of 12 at 50 oscillations per second for 2 minutes. In cases where excessively large samples were processed, this period was increased or 50% more  $\text{MgSO}_4$  solution was used to accommodate a larger leaf. Samples were centrifuged for 8 seconds at up to 10,000 RPM, and then re-suspended. Once re-suspended, 120  $\mu\text{L}$  of solution from each tube was pipetted into the first row of a 96-well microplate, with 5 accompanying rows below the sample row filled with 180  $\mu\text{L}$  10 mM  $\text{MgSO}_4$ . From the first row, 20  $\mu\text{L}$  was pipetted into the next row using a multichannel pipette, ensuring that each solution was mixed by pipetting the solution 20 times before repeating the process into the next row. Dilutions were then transferred onto a marked agar gel plate, pipetting 40  $\mu\text{L}$  of solution starting with the most dilute solution. Plates were allowed to dry for 30 min—1 hr before being inverted. Plates were left to grow at room temperature for three days before counting.

#### *Colony counting and analysis*

Following three days of colony growth, plates were photographed and counted, using a dissecting microscope when needed. Figure 1 was used to verify morphotype visually. Two

dilutions out of six were counted from each plate. Photographs were taken as a reference in case of morphotype mismatch, since some morphotypes were very similar visually. Colony swabs were taken every three samples, with PCR and DNA analysis run on these samples to verify morphotype identity via DNA sequencing. Data was analyzed by converting colony counts to a rate of doublings using assumed original bacterial load per gram of leaf (standardized for all samples). Doubling rate was analyzed within each bacterial strain using an ANOVA. Doubling rate was tested using a linear mixed model to test whether the number of doublings of each *Pseudomonas* strain was impacted by the phytohormone treatment. Plant genotype was included as a random effect, while hormone treatment was included as a fixed effect. An individual model was run for each bacterial strain.

### **A. thaliana infection experiments**

#### *A. thaliana mutant choices*

For *A. thaliana* hormone testing, we chose three mutant and one wild type genotypes based on their immune systems. The chosen *wt* strain was *col0*, or Columbia, and is by far the most studied *Arabidopsis* genotype in the literature. *Col0* plants were expected to have a similar immune system to the *C. cordifolia* diverse clones tested previously. Two mutants were chosen with debilitated or diminished SA pathways: (1) *npr1-2*, a mutant with a non-functional *NPR1* gene, is able to produce and accumulate SA but is almost completely insensitive to the hormone, which debilitates both SA-JA crosstalk and SAR<sup>[15]</sup> and (2) *ics1*, a mutant with a nonfunctional *ICS1* gene, which is the key gene for the production of SA<sup>[16]</sup>. *ICS1* is one of two isochorismate synthase genes critical for SA production — *ics1* mutants are still able to produce and accumulate some SA, due to a functional *ICS2* gene. However, *ICS2* produces much less SA

(*ics1* mutants accumulate only ~5-10% of the SA of *col0* plants) compared to *ICS1*<sup>[16]</sup>. While available, an *ics1/ics2* double mutant was not used due to a significantly smaller phenotype<sup>[16]</sup>. These SA-deficient mutants can be compared to *col0* during both JA and null treatments as a measure of the antimicrobial effect of the JA pathway through two separate mechanisms. Lastly, the mutant *quad* was chosen due to it having four genes knocked out crucial to glucosinolate production, though it still produces camalexins. *Quad* plants cannot produce indolic or aliphatic glucosinolates, providing a model to compare to *col0* during JA induction treatments to measure the effect of non-phytoalexin glucosinolates<sup>[17]</sup>.

#### *Choice of Bacteria*

From the greenhouse experiment, we isolated two strains that were significantly affected by JA treatment – a *P. syringae* strain (02A) that displayed an increased growth rate in JA treatment, and a *P. fluorescens* strain (33E) that displayed a decreased growth rate. The *Pseudomonas syringae* strain Psm4326 cor<sup>-</sup> was added after pilot experiments to function as a sort of control — this lab strain is not found normally in nature and is less virulent than other *P. syringae* strains<sup>[18]</sup>. It also does not produce coronatine, a JA mimic discussed previously that 02A may produce<sup>[18]</sup>.

#### *Plant rearing and preparation for inoculation*

*A. thaliana* experimental protocols were refined based on extensive pilot experimentation on this same species. *A. thaliana* plants were planted in flats containing 40 plants, in packs of four, with each plant given its own peat pellet to grow individually. Each group of four plants was of a single genotype, and was given a number ranging from 1 to 14 for *col0*, 12 for *npr1*, 16 for *quad*, 10 for *ics1* plants for future randomization purposes. Plants were

grown for 4 weeks before treatment, being watered every 2-3 days. Fertilizer was used for the last two weeks of plant growth, as well as during hormone treatment and bacterial growth periods. Prior to treatment, three randomized leaves (generally the largest on each plant) were marked with a I, II, or III (for 2A, 33E, and Psm4326 respectively) to symbolize which bacteria they were to be inoculated with.

#### *Plant treatment and inoculation*

Plants were treated with either a mock solution (0.4mL 95% ethanol in 20mL sterile water) or 2mM JA solution (in 0.95% ethanol). Treatment was completed by spraying groups of four plants with one of the two treatments until droplets formed on all leaves, and allowing droplets to dry before re-randomizing plant locations within flats. A block setup was used, dividing plants into two blocks with two treatments each. This setup was abandoned due to damage caused by increased concentration of JA hormone solution, which was originally thought to be insect damage. The cause of damage to the plants was not understood until after mock treated plants from the first block were re-randomized into the second block and treated with either JA or mock solutions. JA-induced damage was again noted in the second block, but treatment continued.

Treatment period lasted for two days. During the treatment period, liquid cultures of 02A, 33E, and Psm4326 were grown overnight. At the end of the treatment period, these cultures were washed, homogenized, and diluted to an OD<sub>600</sub> of 0.001 in 10 mM MgSO<sub>4</sub>. These bacterial solutions were inoculated into *A. thaliana* leaves using the same protocol that was used in the greenhouse experiments, albeit requiring less force given the fragility of *A. thaliana* leaves. A second block design was used – samples were randomized between being allowed to

grow for three days or four, with the same number of *Arabidopsis* plants of each genotype within each block.

#### *Plant collection*

Leaf samples were collected on day three and day four of bacterial growth, depending on block. Plants within each block were chosen randomly, and leaves were harvested and weighed using sterilized instruments. Leaves were cut above the petiole, with larger leaves (~1.5–2") also being cut near the tip of the leaf to decrease size and possible variance in bacterial density. Weighed leaves were placed into marked 2mL centrifuge tubes and placed immediately placed in -80°C freezer for storage until processing. A total of 204 samples were collected per block, for a total of 408 samples.

#### *Leaf processing, dilution plating, and CFU counts*

##### *counts*

Leaves were processed in a similar manner to the greenhouse experiment, with two notable changes: first, 350  $\mu$ L 10 mM MgSO<sub>4</sub> and a sterilized 5 mm stainless steel bead were added just before processing, after the leaves

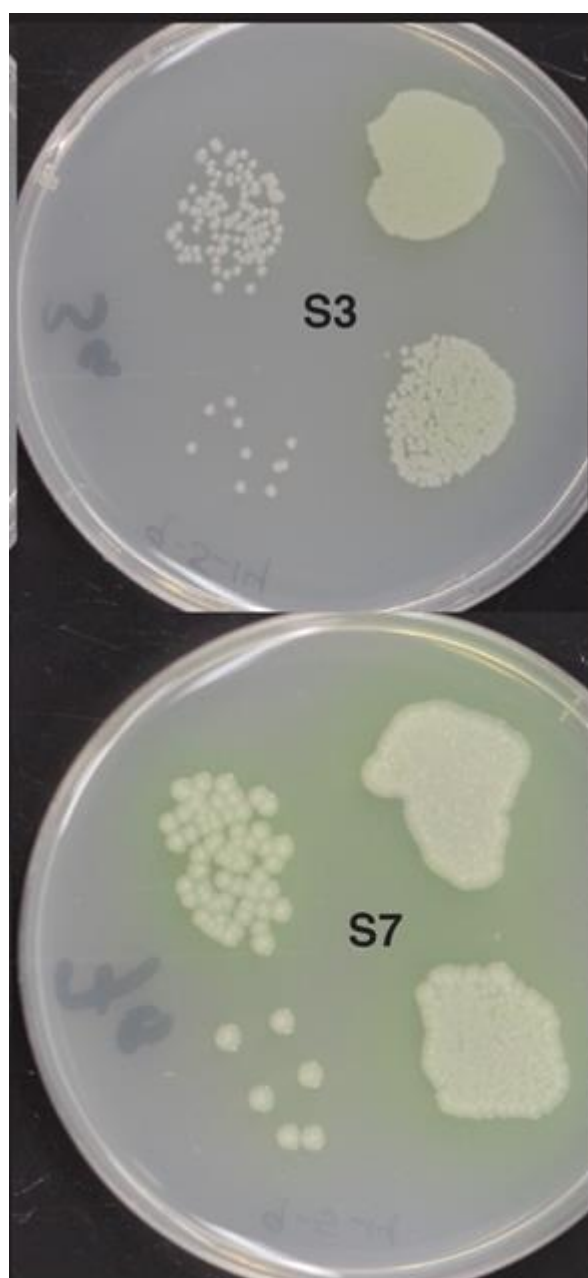


Figure 1. Morphologically diverse *Pseudomonas* colonies. S3 is the code for 2A, a *P. syringae* strain, and S7 is the code for 33E, a *P. fluorescens* strain.

were given a chance to thaw; second, due to time constraints, leaves were not sterilized in ethanol prior to processing – since the three bacteria used were morphologically distinct (Fig. 2, Psm4326 not included), they could be differentiated and counted even in cases of invasion, where a non-experimental endophytic bacteria grew along with the tested bacterium on the dilution plate. No negative controls were affected, so these invasions were most likely due to co-infiltration of the plant. Dilution plating was conducted using an identical processing and dilution protocol to the greenhouse experiment. After three days, plates were counted (same protocol as greenhouse experiments) and analyzed by calculating bacterial density in terms of Log Average CFU/gram between dilutions (a CFU is a colony forming unit, e.g. one “dot” in fig. 2). The first 204 samples were processed, and of these 183 samples were countable (no invasion confounding ability to count CFUs).

## RESULTS

### Greenhouse Experiment

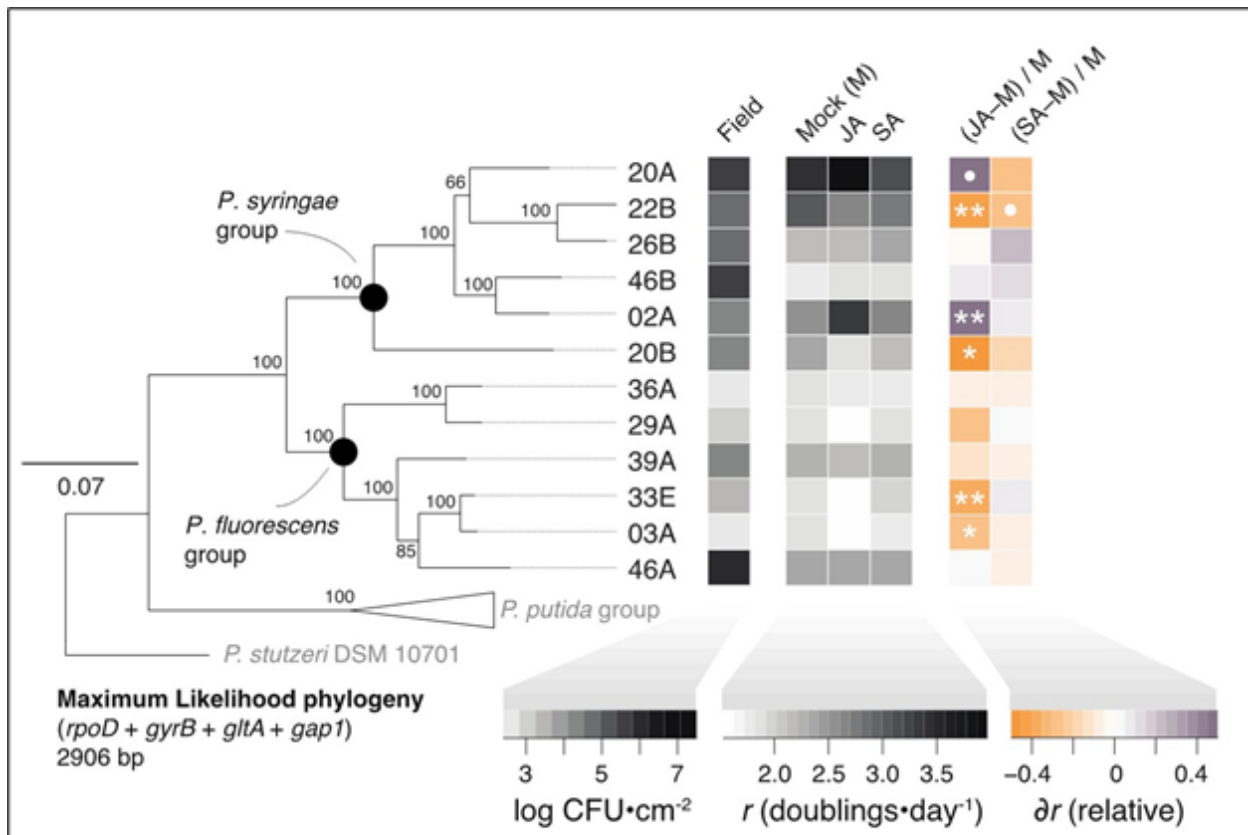


Figure 1. Compiled Greenhouse data. "Field" column shown log CFU bacterial growth based on leaf area in bacteria isolated during field experiments. Middle columns represent absolute rate of doublings, in doublings per day, in all three treatments. Right most columns compare rate of doublings between SA/JA treatments and mock treatment. \* represents  $p < 0.05$  \*\* represents  $p < 0.01$ . • represents  $0.05 < p < 0.1$  (Humphrey et. al. unpublished)

Colony counts taken during the greenhouse experiment were compiled and analyzed by calculating the log average bacterial density, which was converted to a rate of doublings. These values were compared between treatments by comparing differences between JA and SA treatment and mock treatment.

*P. syringae* strains were affected both positively and negatively through induction of JA treatment. However, no *P. fluorescens* strains were affected positively by JA treatment – all significant results show a negative effect compared to mock treatment. In only one studied

strain did SA induction have an (marginally significant) effect compared to mock treatment, implying that artificial SA induction does not significantly affect the internal environment of *C. cordifolia*. Given this data, the bacteria 02A and 33E were chosen for *A. thaliana* testing – 02A had the most significant increased growth due to JA treatment, and 33E had significantly decreased growth in JA treatment. 02A grew at much higher densities within plants compared to 33E, consistent with literature claims that *P. syringae* is normally more virulent than *P. fluorescens*, which seems to be commensal<sup>[19]</sup>.

**Table 1. Preliminary data – *A. thaliana* hormone treatment data, comparison btwn. treatment**

<b>Bacteria</b>	<b>Plant Genotype</b>	<b>Treatment</b>	<b>N</b>	<b>Log Average CFU/g</b>	<b>Tailed T-test</b>	<b>p</b>	<b>Significant? (p&lt;0.05)</b>
2A	col0	Mock	18	7.272	One-tailed	0.0217	Yes
		JA	3	8.068			
	npr1	Mock	6	7.004	Two-tailed	0.173	No
		JA	9	7.7			
	quad	Mock	11	7.503	Two-tailed	0.716	No
		JA	9	7.666			
	ics1	Mock	3	7.958	Two-tailed	0.918	No
		JA	8	8.055			
33E	col0	Mock	18	5.866	One-tailed	0.06	Marginally
		JA	3	6.87			
	npr1	Mock	5	6.236	One-tailed	0.344	No
		JA	7	6.0728			
	quad	Mock	10	6.158	Two-tailed	0.319	No
		JA	11	6.461			
	ics1	Mock	3	5.792	One-tailed	0.314	No
		JA	5	5.896			
Psm4326	col0	Mock	17	6.168	One-tailed	0.265	No
		JA	2	5.812			
	npr1	Mock	5	5.905	Two-tailed	0.844	No
		JA	7	5.972			
	quad	Mock	7	6.144	Two-tailed	0.82	No
		JA	7	6.269			
	ics1	Mock	3	5.895	Two-tailed	0.636	No
		JA	6	6.602			

### A. *thaliana* experimental results – preliminary data

A number of predictions was made for this *A. thaliana* hormone experiment based on greenhouse data, knowledge of *Pseudomonas* strains, and RMBL field data. They are as follows:

1. 02A was expected to grow at higher density than 33E and Psm4326 in all cases.
2. 02A growth was not expected to change with treatment in SA-deficient plants
3. 02A growth was expected to increase with JA treatment in *col0* plants
4. Bacterial density was expected to be higher in *npr1* and *ics1* in mock treatment than in *col0*
5. 33E was expected to decrease in density in SA-deficient plants (and *col0*) with JA treatment
6. Psm4326 was expected have increased growth density in JA treated *col0* than in mock treated
7. *Quad* plants were expected to show smaller effects sizes than *col0* under JA treatment in 33E

All preliminary data has been inputted into Tables 1 and 2. Table 1 shows a comparison between treatments within a plant genotype, while Table 2 compares the average bacterial density between genotypes within a specific treatment. All comparisons are made using a Welch's T-test, given an unequal variance an uneven sample size. T-tests are tailed based on the above hypotheses, and one-tailed tests that show significance in a different direction are

**Table 2. Preliminary data – comparison of bacterial density between plant genotypes.**

<b>Bacteria</b>	<b>Treatment</b>	<b>Comparison</b>	<b>p</b>	<b>T-test</b>	<b>Significant? (p&lt;0.05)</b>
2A	Mock	col0 vs npr1	0.158	One-tailed	No
		col0 vs quad	0.466	Two-tailed	No
		col0 vs ics1	0.248	One-tailed	No
	JA	col0 vs npr1	0.468	Two-tailed	No
		col0 vs quad	0.36	Two-tailed	No
		col0 vs ics1	0.965	Two-tailed	No
33E	Mock	col0 vs npr1	0.106	One-tailed	No
		col0 vs quad	0.0806	Two-tailed	Marginally
		col0 vs ics1	0.35	One-tailed	No
	JA	col0 vs npr1	0.161	Two-tailed	No
		col0 vs quad	0.421	Two-tailed	No
		col0 vs ics1	0.133	Two-tailed	No
Psm4326	Mock	col0 vs npr1	0.117	One-tailed	No
		col0 vs quad	0.939	Two-tailed	No
		col0 vs ics1	0.383	One-tailed	No
	JA	col0 vs npr1	0.767	Two-tailed	No
		col0 vs quad	0.48	Two-tailed	No
		col0 vs ics1	0.396	Two-tailed	No

not considered significant. Though our data is preliminary, a few of these expectations were met: (1) in all cases, 02A density was higher than 33E and Psm4326 – within treatments and within plant genotypes. (2 & 3) SA-deficient plants did not have a change in 02A density with treatment, while growth increased in *col0* with JA treatment. (5) JA treatment in *col0* did show a marginally significant difference in 33E growth, but this is discounted as a one-tailed T-test is used for a decrease in growth, while an increase in growth is observed.

The fact that this preliminary data shows some significance is very surprising – of the 21 *col0* plants processed, only 3 happened to be JA treated. This means that the difference in density was large enough in 02A-infected *col0* plants to be noticed with only 3 JA-treated *col0* samples. However, small sample size is an issue that seems to be affecting every single group. With 204 samples left to analyze, our current group sizes will double within each genotype, allowing us to notice differences that may not be currently apparent and removing ones that are due to random variance.

## DISCUSSION

The reciprocal antagonism that exists between the SA and JA pathways, combined with the complicated network of pathways that both of these phytohormones signal and interact with, creates a system that is difficult to very study. However, SA/JA inhibitory crosstalk also paints a fascinating story for researchers to unfold – this unique system of tradeoffs is a classic story of cost vs. benefit that on the surface seems to put the plant at a disadvantage<sup>[1][2][3][7]</sup>. However, this immune system pervades the entire plant kingdom, and that it hasn't created a damning fitness disadvantage is proof that more is at play than meets the eye<sup>[1]</sup>.

Our results show a number of interesting previews into the endophytic bacteria-plant interaction. The greenhouse data (fig. 3) in *C. cordifolia* has two implications important to our current study. First, SA-induction did not have a significantly different effect compared to mock in any of the twelve *Pseudomonas* strains studied. This implies that these bacteria already induce SA upon entry into the plant, which is expected – after all, SA is activated by biotrophic bacteria like both *Pseudomonas* species studied<sup>[1][3]</sup>. In only one of the twelve was the difference marginally significant, with a mild decrease in growth rate compared to mock treatment; a possible mechanism behind this decreased growth rate may be because some endophytic bacteria strains could be suppressing the activation of the SA pathway<sup>[6]</sup>, and are thus under decreased selective pressure to be able to resist antimicrobial compounds produced by SA pathway activated. The second result we see is a highly variable effect on growth in JA-treated plants when compared to mock treatment. This variation seems to coincide with strain association with herbivory – previous field research<sup>[10]</sup> noted that *P. fluorescens* was negatively associated with herbivory, which would normally induce the JA pathway<sup>[1][7]</sup>. All *P. fluorescens*

strains studied were either unaffected or negatively affected by JA induction in plants, consistent with a group that may be negatively associated with herbivory<sup>[10]</sup>. However, JA treatment had a much more variable result on *P. syringae*, which past field research notes had a positive association with herbivory. Here, two strains were unaffected, two strains had decreased growth rates, and two strains had increased growth rates. The mechanism behind this is not yet understood, but it implies that *P. syringae* strains vary greatly in how they deal with plant hormone-induced defenses, and it becomes questionable to characterize the strains of *P. syringae* studied as responding as a single species at all.

However, the most interesting result is the one that actually moves away from the primary literature: in most of the literature, JA-inducible defenses are said to be induced by necrotrophic pathogens and chewing insects<sup>[1][2][3][4][5][7]</sup>, and on a microbial level the JA pathway primarily targets necrotrophic pathogens<sup>[1][3][7]</sup>. While we cannot say that biotrophic pathogens induce a JA response, since we induced the response ourselves, we *can* state that the JA-mediated defense pathway has a significant effect on half of the biotrophic bacteria we studied. What is interesting is that a pathway that is consistently implicated in combating a specific type of pathogen (e.g. necrotrophic fungi, and that much of the literature states produces attacker-specific molecules)<sup>[1][7][9]</sup> seems to not only be able to combat a wide array of pathogens, but also pathogens that do not even activate that pathway<sup>[1][7]</sup>. While it may be that certain strains (e.g. 02A, which shows increased growth in both wild type models studied in JA treatment) are shown to be able to use that pathway to their advantage, in half of strains we studied activation of JA defenses was more detrimental to these biotrophic pathogens than activation of SA. This is completely separate from the implication that JA can confer long-term

resistance to pathogens, even biotrophs, through ISR<sup>[1][2][14]</sup>. Interestingly, the idea that JA activation confers resistance to biotrophic pests has been discussed since at least 2002<sup>[14]</sup>; however, the majority of the literature we have found does not discuss this in detail. Furthermore, while *P. syringae* is very well studied, very little (if any) literature discusses the effects of hormone treatments on *P. fluorescens*, even though we have shown that strains within this bacterial group are widespread within plant leaves and have effects on herbivore behavior in nature<sup>[10]</sup>.

Lastly, our preliminary data shows that *A. thaliana* is a good model organism to study the interaction of different pathogens with phytohormone-mediated defenses, at the very least to model the interaction compared to other mustards. *A. thaliana* is by far the most studied, has a huge number of available immune-deficient mutants, and is easy to raise, care for, and grow in large sample sizes. Our preliminary data shows that even in relatively small sample sizes *A. thaliana* begins to mimic the results we observed in a much larger sample of *C. cordifolia*, and we will be able to further research this claim once all our samples have been processed and analyzed. However, even with our preliminary data, we have shown that hormone treatments, in *col0* plants, can cause a strong immune response that leads to significant differences in growth, at least in O2A.

Interestingly, we also noted an experimental issue with the bacterium Psm4326 Cor-. Compared to O2A and 33E, Psm4326 was invaded and outcompeted on dilution plates significantly more often than either of the other two tested strains (O2A once, 33E six times, and Psm4326 14 times,  $p < 0.05$  using a  $\chi^2$  test). Psm4326 is effectively a “lab animal” – the strain has been kept in labs under relaxed selective pressures for years, which may have

affected its potential as a plant pathogen, especially in the Cor- sub-strain. However, in all groups, when countable, Psm4326 grew to similar densities to 33E, which may be due to *P. fluorescens*' commensal nature<sup>[19]</sup>.

There are a number of future studies that can and should be conducted to further test the validity of the results that we've observed. For one, a parallel experiment to the current *Arabidopsis* experiment we are running where each plant is only infected with one pathogen will allow us to test if different bacteria strains infecting the same plant on different leaves become confounding variable in studies on defense system effects. This is particularly problematic, for example, if one bacteria can induce an unexpected effect (e.g. a *P. syringae* that produces coronatine, eliciting a JA response in mock treatment) in the plant that effects the fitness of another, the experimental measurement of their individual growth would vary compared to an experiment where the two are inoculated in separate plants. However, it should be noted that there is evidence in the literature that this is not the case, and that a mimic-elicited response (like a JA response with coronatine) may not spread throughout the entire plant<sup>[4]</sup>. When considering this sort of experiment, one should also understand that in nature, co-invasion and competition within a host plant (and even within the very same leaf) is the norm<sup>[10]</sup>. Just as it is possible that having multiple strains in one plant may skew results, the opposite is also true – a multiple-inoculation protocol more closely mimics how the plant and bacteria must live in nature, and may thus provide results with greater relevance.

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