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The Effects of Priming *Eruca sativa* Seeds with Short-Chain AHL C6-HSL at Bard Farm

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The Effects of Priming *Eruca sativa* Seeds with Short-Chain AHL C6-HSL at Bard Farm

Senior Project Submitted to
The Division of Social Studies of Bard College

by Shannon Ryan

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Abstract

Many gram-negative bacteria use quorum sensing to assess population densities and cooperate with another. The quorum sensing autoinducers *N*-acyl homoserine lactones (AHLs) have been found to cause altered gene expression patterns in plants, resulting in increased root and shoot growth as well as induced pathogenic resistance in various species. Researchers have begun exploring the ways AHLs may be used in agricultural systems to reduce the use of environmentally harmful synthetic fertilizers and pesticides. In this study, the effect of priming arugula (*Eruca sativa*) seeds with the short-chain AHL C6-HSL was investigated at Bard Farm in Annandale-On-Hudson, New York. Leaf lengths of AHL-primed, water-primed and control plants were compared to investigate the effect of AHL seed priming on arugula growth. The study sought to also analyze the effects C6-HSL may have on the bacterial diversity and dominant functional groups present in root and root soil samples collected from the plants, though metagenomic analysis ultimately could not be performed due to COVID-19 related delays in DNA sequencing. Soil moisture and temperature was measured three times over the course of the experiment to contextualize results. Soil samples of the experiential plot were also collected before and after the in-field portion of the experiment for metagenomic analysis. The study ultimately found C6-HLS seed priming to have no significant effect on leaf length ($P=1.57$). Metagenomic analysis will be performed once the sequenced samples are available.

Introduction

In order to monitor population density and coordinate communal activities, bacteria need to communicate with one another. Quorum sensing is the main way bacteria communicate, which involves the exchange of compounds known as signal molecules. Quorum sensing was first described in 1979 in *Vibrio fischeri* and *Vibrio harveyi*, and this communication system was thought to be characteristic of these bacterial species specifically (Nealson & Hastings, 1979). Further research in cell-to-cell communication has revealed that most bacteria communicate through quorum sensing, and that these communication systems are not only much more common than we initially thought, but also much more complex. A wide array of different classes of chemicals are utilized by bacteria, over 250 genes can be affected by an AHL molecule and many species of bacteria are able to release and perceive more than one type of signal (Whiteley *et al.*, 1999). This ability to communicate with other bacteria in the environment is now known to be critical for bacterial survival (Miller & Bassler, 2001).

In quorum sensing systems signal molecules are released by individual bacteria and accumulate in the surrounding environment as the number of cells increases. Once a threshold concentration is exceeded, the signal molecule enters the cell and binds to a transcriptional activator protein responsible for the transcription of certain target genes. In this way, quorum sensing allows bacteria to regulate the expression of genes responsible for various actions such as swarming, production of antibiotics, infection of a host and creation of a biofilm (Brelles-Mariño *et al.*, 2001).

LuxIR-type quorum sensing is the primary mode of communication between gram negative bacteria, which utilizes N-acyl homoserine lactones (AHLs) as signal molecules. Interestingly, AHLs have been found to not only affect bacteria, but also plants. In many cases,

plants have adapted to utilize AHLs for their own benefit. The presence of AHLs in the root environment has been shown to increase root and shoot growth in various plants, and even induce pathogenic resistance (see Schikora *et al.*, 2016 for review). These reactions seem to be AHL-specific, with different AHLs causing different reactions in plants. Furthermore, in some cases plants have been found to exude AHL-deteriorating substances in order to disrupt the quorum sensing of pathogenic bacteria (Scott *et al.*, 2006). Some plants can even synthesize their own AHL-like substances to alter their root microbiome (Lakshmanan *et al.*, 2012).

These findings have brought attention to the importance of AHL-plant interactions when considering plant health. Researchers studying AHL-plant interactions have begun exploring the uses of AHLs in agriculture, as they have the potential to be a sustainable replacement for synthetic pesticides and plant growth promoters. However, research on AHL-plant interactions is still limited. Moving forward, more research needs to be performed in agricultural fields on crops in order to better our understanding of the role AHLs may play in sustainable agriculture.

LuxIR-Type Quorum Sensing

LuxIR-type quorum sensing in gram negative bacteria has become integral to our understanding of the interactions between plants and soil bacteria. In this system, the LuxI protein produces acyl-homoserine lactones (AHLs), which serve as autoinducers. As individual bacteria release AHLs, the concentration increases in the environment as a function of increasing cell population (Waters & Bassler, 2005). Once a threshold concentration is reached, the autoinducer will bind to a receptor protein in the bacteria, which activates the transcription of target genes necessary for communal actions. A bacterial species may have various receptor proteins controlling different behaviors (Teplitzki *et al.*, 2000). The binding of an AHL to the

receptor may also increase the synthesis of the AHL, or induce the synthesis of a different AHL (Li & Tian, 2012).

Rhizospheric Bacteria in Agriculture

The majority of agricultural systems in the United States currently rely on the use of synthetic fertilizers and pesticides to maximize plant growth and health, though these compounds are known to have detrimental effects on the environment (Chopra *et al.*, 2011, Wang *et al.*, 2018). Application of synthetic nitrogen fertilizer to agricultural fields is currently the most cost-effective way to maximize crop yield, and since fertilizer is so cheap, many farmers choose to over-apply to avoid the possibility of a shortage of nitrogen in their fields. Though this may help to ensure an adequate harvest, the nitrogen not taken up by crops will be lost to the environment through groundwater-leaching, run-off into surface waters or nitrous oxide emissions to the atmosphere. This release of reactive forms of nitrogen into the environment is known to cause various biological and ecological hazards, including eutrophication of waterways, which devastates aquatic ecosystems and can be harmful to humans, and the contribution of nitrous oxide to the atmosphere, which contributes to the increasing levels of greenhouse gases reinforcing climate change (Stark & Richards, 2008). Pesticide use is similar to nitrogen fertilizer in that it helps to ensure adequate crop yields though ultimately has lasting effects on the surrounding environment. After being applied to fields, pesticides can similarly enter waterways through agricultural runoff and leaching. Once in the environment, pesticides can cause loss of species diversity and soil health deterioration (Fleeger *et al.*, 2003).

Researchers have begun exploring the ways naturally occurring plant-microbe interactions already present in nature can be harnessed in order to reduce the use of synthetic

fertilizers and pesticides (Wu *et al.*, 2009). The soil environment surrounding the roots of the plant, known as the rhizosphere, is known to house various species of bacteria who have formed beneficial relationships with plants (Lareen *et al.*, 2016). Many of the bacteria have been found to cause increased plant growth. Though these bacteria don't provide the nitrogen necessary for plant survival, they can be utilized to lessen the amount of fertilizer necessary in an agricultural system. Research on the potential uses of plant-microbe interactions in agriculture is currently focused on a group of rhizospheric bacteria known as plant growth-promoting bacteria (PGPB). PGPB reside in the rhizosphere and are naturally capable of enhancing nitrogen use efficiency, improving plant performance in water-stressed conditions, and inducing resistance to disease in plants, making them perfect for agricultural use (Dimka *et al.*, 2009, Grover *et al.*, 2011). Studies have explored the use of PGPB to reduce the need for synthetic fertilizer and found the bacteria to be useful in agricultural systems. In a 2008 study, Adesemoye and colleagues found that inoculating tomato plants with PGPB strains *Bacillus amyloliquefaciens* IN937a and *Bacillus pumilus* T4 allowed for a 25% reduction in synthetic fertilizer use with no loss in yield. Similarly, another study found that inoculation of sunflower seeds with PGPB strain YAS34 resulted in increased nitrogen use efficiency in the plant. Sunflowers treated with the PGPB also showed a significant increase in shoot and root dry mass by up to 50% and 70% respectively, under both normal and water stressed conditions (Alami *et al.*, 2000). PGPB can also be used to increase a plant's resistance to pathogens. This was seen in a study in which *Pseudomonas* strains with biocontrol activity were used in agricultural systems to induce resistance against the pathogenic fungus *Venturia inaequalis* in *Malus domestica* (Gau *et al.*, 2002). Burr and colleagues (1996) also found over 100 bacterial isolates to inhibit *Venturia inaequalis*, which causes apple scab (Burr *et al.*, 1996).

AHLs in Agriculture

With a better understanding of the mechanisms of AHL-plant interactions, AHLs have the potential to be used in agriculture in ways comparable to PGPB. Various studies have found the presence of AHLs to increase root and shoot growth in plants. Von Rad and colleagues found C6-HSL to increase root and shoot growth in the model plant *Arabidopsis thaliana*, which was in agreement with other research on AHL-plant interactions (Schenck *et al.*, 2012, Schickora *et al.*, 2011).

AHLs have also been found to enhance natural defense mechanisms against pathogens in plants. In a 2006 study, Schuegger and colleagues found that C6-HSL and C4-HSL produced by *Serratia liquefaciens* MG1 and *Pseudomonas putida* IsoF increased resistance to the fungal leaf pathogen *Alternaria alternata* in tomato plants. Bacteria of the same species' which were incapable of synthesizing the beneficial AHLs were significantly less effective in inducing resistance in the plants, displaying the necessity of AHLs in inducing pathogenic resistance (Schuegger *et al.*, 2006). A similar study found that the benefits afforded to cucumber, tomato and bean plants through colonization with the beneficial bacteria *Serratia plymuthica* depend strongly on AHL signalling (Pang *et al.*, 2009). Schikora and colleagues found that oxo-C14-HSL increased barley's resistance to the pathogenic fungus *Golovinomyces orontii*. In order to ensure the induced resistance was an effect of the AHLs reaction with the plant as opposed to the AHLs ability to effect the pathogen's fitness, the researchers cultivated the pathogenic bacteria in the presence of the AHL, and found no change in bacterial fitness (Schikora *et al.*, 2011).

Studies have also found that various plant species are capable of synthesizing and exuding compounds that mimic AHLs in order to alter the activity of rhizospheric bacteria and compose a microbiome that benefits the plant. A study by Tepliski and colleagues (2000) found

that the exudates of pea, rice, soybean, tomato, crown vetch and *Medicago truncatula* contained substances that mimicked AHL signals and were able to cause altered gene expression in bacteria. These findings coincide with previous research which found that the furanones produced by the red algae *Delisea pulchra* are capable of inhibiting AHL-regulated behaviors by binding to the AHL receptor protein in *Serratia liquefaciens* and other bacterial species (Manefield *et al.*, 1999).

These observations have inspired recent research exploring the possibility of engineering plants capable of exuding AHLs in order to decrease losses to disease in agriculture. Scott and colleagues (2006) found that after introducing two AHL synthase genes in tomato plants, *lasI* from *Pseudomonas aeruginosa* and *yenI* from *Yersinia enterocolitica*, the plants successfully secreted short and long chain AHLs into the rhizosphere. These plant-produced AHLs may be used to disrupt bacterial quorum sensing necessary for infection, making crops less likely to fall victim to pathogenic disease (Scott *et al.*, 2006). Studies have also explored the possibility of manipulating plants to express AHL-lactonase, which is able to break down AHL molecules used by pathogenic bacteria, resulting in increased pathogenic resistance (Zhang *et al.*, 2003).

AHLs ability to affect growth and pathogen resistance in plants make them a possible biological alternative to man-made agricultural chemicals such as fertilizer and pesticides. Unfortunately, research that has been done on AHL-crop interactions has shown that these interactions are often species specific, and the majority of findings have been based on studies of the model plant *Arabidopsis thaliana*. Though studies on model plants help us to better understand the mechanisms through which AHLs affect plants, the effect AHLs have on crops will likely differ from the model plants. Also, the most common AHL application method used in these studies (application of AHLs to roots of plants) does not work for in-field usage of

AHLs. Other application methods need to be explored. Furthermore, most of the research done on AHLs has been performed in laboratories, and very few of the field studies have a crop species as their subject. Research in this field needs to be directed toward in-field crop trials, as AHLs have shown potential to be used as biological crop growth promoters, bactericides and fungicides.

Moshynets *et al.* (2019) is currently the only study to explore this potential through in-field trials using pure AHLs in a seed priming technique on a crop. The study assessed whether winter wheat seeds primed with a C6-HSL solution would vary significantly from the control in terms of seed germination, plant development and productivity. The study found that the AHL seed priming resulted in increased germination of seeds in *in vitro* trials, and increased biomass, crop structure and productivity (amount of “good grain”) in field trials. Interestingly, the study assessed the effects the AHL had on the plant’s rhizosphere, which is an analysis often left out of AHL-plant interaction studies. Bacterial populations in five functional groups in root soil samples were found to be significantly different when treated with C6-HSL (Moshynets *et al.*, 2019).

Moshynets and colleagues’ study on winter wheat serves as an important step toward agriculturally-focused AHL research. In-field trials are key to advancing this research, as it is currently unclear how field conditions effect AHL-plant interactions. Environmental factors such as precipitation, temperature and the natural microbiome of soil need to be considered in future research. It is therefore important that knowledge gained from AHL-crop studies are based on in-field trials. Furthermore, the study utilizes a technique that is already familiar to many farmers. Seed priming is commonly used in agriculture and AHL seed priming could therefore be an easy transition for farmers interested in utilizing the benefits of AHLs in their fields.

The current study seeks to build off of the findings of Moshynets and colleagues (2019) and investigate the effect priming *Eruca vesicaria* seeds with C6-HSL may have on the plant's growth and rhizosphere at the Bard Farm in Annandale-on-Hudson, New York. AHL seed priming has potential to be successful, and the current study seeks to extend this research to a different species of crop (Moshynets *et al.*, 2019). *Eruca vesicaria* was chosen as the subject of this study due to its short lifespan and its tolerance to cold weather conditions. C6-HSL was chosen as the AHL for seed priming due to its ability to increase growth in the model plant *Arabidopsis thaliana* in various studies, and its use in Moshynets et al (2019) (Moshynets *et al.*, 2019, Schenck *et al.*, 2012, Schickora *et al.*, 2011, von Rad *et al.*, 2008).

Current State of AHL-Plant Interaction Research

Mathesius and colleagues (2003) provided the first analysis of the effects AHL molecules have on plants. The study found that the application of the AHLs 3-oxo-C12-HL and C6-HSL to the roots of *Medicago truncatula* resulted in a change in accumulation of over 150 proteins. The authors were able to identify 100 of these proteins by peptide mass fingerprinting and found that 25% had functions pertaining to pathogenic defense. Furthermore, the study showed that one third of the differentially accumulated proteins correlated to a specific AHL, meaning that the plant was reacting differently to different AHLs, which was later supported by other studies (Götz *et al.*, 2007, Mathesius *et al.*, 2003, Ortíz-Castro *et al.*, 2008, Schenck *et al.*, 2012, Schuhegger *et al.*, 2006, von Rad *et al.*, 2008).

The findings of Mathesius et al. (2003) opened the door for a plethora of other AHL-plant interaction studies. The majority of these studies have been done on the model plant *Arabidopsis thaliana*. These studies have been successful in forming the foundational research necessary to

further our understanding of AHL-plant interactions. One of the major findings of this research pertains to the relationship between the length of the carbon chain of an AHL and the effect it has on *Arabidopsis thaliana*. Short-chain AHLs (C4-, C6- and C8-) tend to increase plant growth rate and primary root elongation (Bai *et al.*, 2012; Liu *et al.*, 2012; Schenk *et al.*, 2012, von Rad *et al.*, 2008). Long-chain AHLs (C12- and C14-) tend to induce pathogen resistance (Schenk *et al.*, 2014; Schenk & Schikora, 2015; Schikora *et al.*, 2011).

Upon noticing this pattern in other studies, Schenck *et al.*, (2012) tested the effects five AHLs ranging from 6 to 14 carbons had on *Arabidopsis thaliana* in a laboratory setting. The study confirmed a negative correlation between length of lipid chain and shoot biomass. The authors speculate that this may be due to the inability of the plant to transport AHLs with longer carbon chains throughout the plant (Schenk *et al.*, 2012). Von Rad *et al.* also found that C6-HSL but not C10-HSL was translocated from root to shoot in *Arabidopsis* (Von rad *et al.*, 2008).

While the findings of model plant studies are interesting, research has found that AHL-plant interactions tend to be species specific, so these studies tell us very little in regards to AHL interactions with crops. For instance, C6-HSL is one of the most commonly used AHLs in experiments and has been found to evoke different responses from different plants. Von Rad *et al.* 2008 found that application of C6-HSL to the roots of *Arabidopsis thaliana* resulted in changes in the expression of several genes associated with cell growth, though very few defense-related genes, and the AHL ultimately did not increase the plant's resistance to *Pseudomonas syringae*. This coincides with the findings of Scheck *et al.* 2012 and Schikora *et al.* 2011. Both studies found that an AHLs ability to promote plant growth decreased with increased lipid chain length, while the AHLs ability to induce pathogenic resistance increased with increased lipid chain length (Scheck *et al.*, 2012, Schikora *et al.*, 2011). However, the same

pattern does not seem to hold true for other plant species, as Schuhegger *et al.* 2006 found that both C4-HSL and C6-HSL played a significant role in the systemic resistance of tomato plants against the fungal leaf pathogen, *Alternaria alternata*. Interestingly, Heidel *et al.* 2010 found that application of C6-HSL to tobacco plants actually decreased the plants defense to *Manduca sexta* larvae.

AHL Seed Priming

Moshynets and colleagues (2019) took research a step further by exploring the potential of AHLs as seed primers. Priming seeds is a process through which seeds are soaked in water to partially hydrate the seed, initiating germination before planting in order to increase germination rate, germination percentage and advancement to maturity (Welbaum *et al.*, 2015). The study assessed how priming seeds with a C6-HSL solution affects seed germination, plant development, productivity and rhizosphere microbiology in two varieties of winter wheat. Importantly, the study involved both *in vitro* trials and field trials. The study found that AHL seed priming had significant positive impacts on seed germination, biomass at the tillering stage, crop structure, and grain yield and quality (Moshynets *et al.*, 2019). Bacterial populations in five functional groups in root soil samples were found to be significantly different under different treatments.

My Senior Project

For my Senior Project, I was interested in further exploring the potential of priming seeds with AHL solutions. In order to assess the effect C6-HSL would have on arugula plants, I used a variation of the priming method used in a study by Moshynets and colleagues (2019). The seeds

were planted at the Bard Farm in Annandale-on-Hudson, New York and allowed to grow for three weeks before leaf measurements were recorded and root soil and root samples were collected. The study sought to move AHL-plant interaction research in the direction of in-field crop trials and to gain a better understanding of the effects applied AHLs may have on plant rhizospheres.

Methods

Experimental Design and Study Site

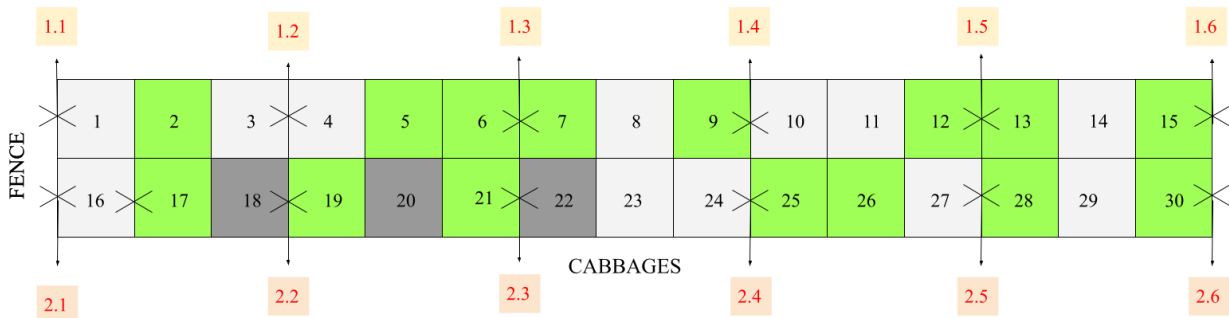


Figure 1: Experimental design diagram. Green grid units represent AHL-primed units while light grey grid units represent water-primed units and dark grey units represent control (un-primed) units.

“X”s mark plot soil sampling sites.

The study took place on a tilled 30 by 2 foot plot at Bard College Farm in Annandale-on-Hudson, New York. A grid of wooden stakes and twine was created over the plot to divide the plot into 30 grid units in which five or more arugula seeds were planted. The treatment of each grid unit was decided randomly using R. Due to human error, three grid units were planted with unprimed seeds, which serve as a control in the study. 12 sampling locations across the plot were sampled before the seeds were planted and after the arugula was harvested to allow comparison of root and rhizosphere bacterial communities to the soil microbiome of the plot. Soil moisture and temperature were recorded at the sampling locations three times during the experiment to contextualize results.

Seed Trials Before Experimentation

Arugula was chosen as the subject of the study due to its fast growth and ability to withstand colder temperatures. Different species of arugula seeds were tested the summer before

the experiment began in order to choose the fastest growing species. Speed to maturity was prioritized in order to ensure the plants would grow to maturity before the first frost. Two varieties of arugula seeds, *Eruca sativa* and *Diplotaxis tenuifolia*, were purchased online from Hudson Valley Seed Co. The seeds were planted in separate plastic 12” pots, then both pots were labelled with their species written on a ribbon. *Eruca sativa* sprouts emerged after 6 days while *Diplotaxis tenuifolia* sprouts took 15 days to sprout. *Eruca sativa* was therefore chosen as the subject of the study.

Seed Priming

A modified version of the seed priming protocol used in Moshynets *et al.* (2019) was used to prime the arugula seeds. First, a C6-HSL concentrate was created by adding 0.00103 g of C6-HSL powder purchased online from Santa Cruz Biotechnology. The C6-HSL priming solution was then created by adding 1 ml of C6-HSL concentrate to 99 ml of water. 85 seeds were placed in each of two sterile beakers labeled “C6-HSL” and “Control.” 85 seeds were primed though only 75 were planned to be planted to account for possible error. The C6-HSL priming solution was poured into the beaker labeled “C6-HSL” and 100 ml of water was poured into the beaker labeled “Control.” The seeds soaked for 3 hours and were then dried in the beakers for 72 hours at 25° C.

Unfortunately, the seeds stuck to the beaker after the drying period. The seeds were removed with a sterile spatula and placed in a sterile 50 ml collection tube to be brought to the field. The removal of the seeds from the beaker resulted in some of the seeds being compromised, making them unusable in the study. 78 control seeds and 83 treatment seeds were successfully removed and taken to the field.

Plot Preparation

The experimental plot was tilled by the Bard Farm manager Rebecca Yoshino the day before the grid was installed. Stakes for the creation of the grid were made by sawing thin planks of wood. Three parallel columns of stakes were then placed along the plot to serve as the foundation of the grid. The stakes were each placed 1 foot apart using a measuring tape, creating 30 1x1 foot grid squares. Finally, twine was tied around each of the stakes connecting them to create a 30x2 unit grid.

Seed Planting

Each grid unit was randomly assigned a treatment using R random number generator. The generator was limited to produce numbers between 1 and 30. The grid units correlating with the first 15 numbers randomly generated were determined to be control units while the rest were determined to be treatment units. On October 11, 5 or more seeds were planted in each grid unit. 4 seeds were planted 1 inch from each corner of the grid unit, and 1 seed was planted at the center of the unit, using a measuring tape for measurements. Grid unit 1 received more than 5 seeds due to human error. Because of this, there were not enough water-primed seeds to plant in grid units 22, 20 and 18, so these grid units were seeded with unprimed seeds taken directly from the seed packet. These unprimed seeds act as a control to assess if priming with water also has an effect on the microbiome and plant growth.



Photo 1: The plot with grid applied.

Monitoring Sprout Growth

Sprouts were first observed on October 18, 2020. Remy was applied to the plot on October 15th in order to protect the plants from frost on October 17th. The final number of harvested AHL-primed, water-primed and control plants was 62, 45 and 12, respectively. The number of plants grown in each grid unit is shown in Figure 7 in the appendix. No watering or fertilization was performed during the experiment. The farm manager Rebecca Yoshino had stopped watering her crops due to the soil being very wet and advised me to do the same.



Photo 2: The plot with remay applied.

Plot Soil Sampling

Soil samples from 12 locations across the plot were collected before the seeds were planted and after the arugula was harvested to allow comparison of root soil samples to overall plot soil samples. Each sample was taken using a sterilized spoon and collection tube. Sterilized gloves were worn during collection to avoid contamination. The samples represent the top 2 inches of soil. After sampling, the tubes were brought to the lab and stored at -80°C . These sampling sites are labeled with an “X” in Figure 1.

Soil Temperature and Moisture Measurements

Soil temperature and moisture were measured three times over the course of the experiment; October 20th, October 27th and October 20th. The measurements were taken at the

same points the plot soil samples were taken, which are labeled with an “X” in Figure 1. The *Aquaterr™ EC-350 Digital Soil Moisture, Temperature and Salinity Meter* was used. The instrument was first calibrated in the lab before being taken into the field. The end piece of the instrument was cleaned off with a towel between measurements, as is instructed in the user manual. The instrument was inserted about 2 inches into the soil at each sampling site and temperature and moisture measurements were taken after the reading stabilized.

Leaf Length Measurements and Rhizosphere and Root Sampling

Leaf measurements and root and root soil sampling was done one grid unit at a time. All of the leaf measurements for plants in a grid square were taken first. The midrib of the longest leaf of each plant was measured in centimeters using a ruler and then recorded in a field notebook. After all the leaf measurements in a grid unit were recorded, a plant was removed from the soil using a sterilized trowel, with effort not to disturb the root system of the plant. The plant was then picked up by the leaves while wearing sterilized gloves, and the root system was removed using sterile forceps and collected in a sterile tube. The next plant was then removed and the root system was collected, following the same steps. These samples were separated into root samples and root soil samples at a later date in the laboratory.

DNA Extractions:

The root and rhizosphere samples first had to be separated from one another, as the roots and root soil of each plant were collected together in a single collection tube in the field. The samples were separated by pouring the contents of the collection tube onto a sterile petri dish and using sterilized forceps to separate the roots from the root soil. The roots of all C6-HSL primed

plants were pooled together and placed in one sterile collection tube while the water primed and unprimed seeds were pooled together and placed in another tube. The pooled root samples were then cut into pieces about one centimeter in length using a sterile scalpel on a sterile petri dish. The rhizosphere soil samples were not pooled and were individually processed after removal of the root systems.

ZymoBIOMICS™ DNA Miniprep Kit was used for all of the DNA extractions. Lysis of the cells was first performed using 250 mg of soil or root sample and the ZymoBIOMICS™ Lysis Solution. Since a Disruptor Genie bead beater was used, the samples were shaken for 20 minutes as instructed in the kit's manual. Lysis of the root samples was performed at a lower speed as is instructed in the appendix of the kit manual. The lysed samples were then centrifuged and the sample was filtered various times using different filters provided in the kit. The DNA was then eluted and put through a final filter before being transferred to a centrifuge tube. A control was also prepared using the extraction kit in an effort to detect any possible contamination of kit materials. The DNA was stored at -80°C before being sent to Juniata labs in Huntingdon, PA on March 23rd, where the samples would be sequenced.

Results

Soil Temperature and Moisture

Soil temperature across the plot did not vary much spatially, though the temperature did vary over time. The average temperature at each site over time is shown in Figure 2a.

Temperature between sites did not differ by more than 2.5°F on October 20th, 1.1°F on October 27 and 1.9°F on October 31. Average temperatures over time at each site were within 0.9°F of one another. However, soil temperature decreased over time across the plot, which is reflected in Table 1. Over the three weeks between the first and last soil temperature measurements, the temperature of each site decreased by an average of 10.4°F.

On the other hand, soil moisture varied spatially and temporally. Average moisture levels at each site over time are presented in Figure 2b. The data is presented in ranges to illustrate where the major differences in moisture content are located on the plot. Moisture measurements for each site over time as well as averages are shown in Table 2.

Figure 2a

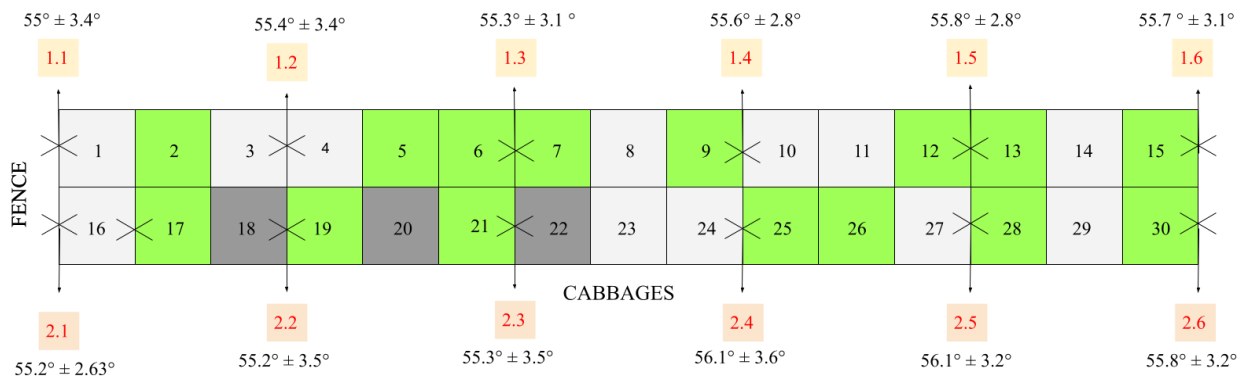


Figure 2b

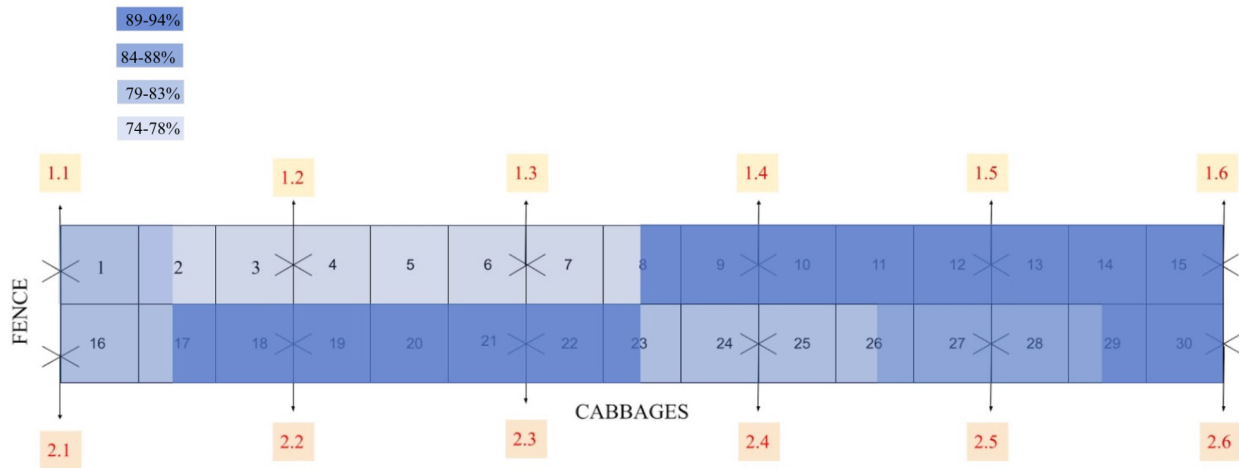


Figure 2a: Average temperature measurements at each sampling site with standard error. Measurements were taken three times over the course of the experiment. Figure 2b: Average moisture content separated into four ranges.

Table 1a

Site	Date			Average
	10/20	10/27	10/31	
1.1	60.2°	56.6°	48.8°	55.2°±3.4
1.2	61.1°	55.7°	49.3°	55.4°±3.4
1.3	60.5°	55.6°	49.8°	55.3°±3.1
1.4	60°	56.5°	50.3°	55.6°±2.8
1.5	60.8°	56.6°	50.1°	55.8°±3.1
1.6	60.2°	57.1°	49.7°	55.7°±3.1
2.1	59.3°	56.1°	50.3°	55.2°±2.6
2.2	60.1°	57.1°	48.4°	55.2°±3.5
2.3	60.2°	56.6°	49.1°	55.3°±3.3
2.4	61.8°	57°	49.5°	56.1°±3.6
2.5	61.2°	57°	50.1°	56.1°±3.2
2.6	60.6°	57.2°	49.7°	55.8°±3.2

Table 1a: Temperature measurements taken over the course of the experiment and averages.

Table 1b

Site	Date			Average
	10/20	10/27	10/31	
1.1	96.8%	62.9%	83.5%	81.1%
1.2	75.3%	64.3%	89.6%	76.4%
1.3	92.9%	57.7%	73.9%	74.8%
1.4	101.5%	79.2%	88.3%	89.7%
1.5	103%	85.2%	84.1%	90.8%
1.6	101.5%	88.9%	89.9%	93.4%
2.1	97%	63.5%	83.7%	81.4%
2.2	99.2%	85.1%	87.9%	90.7%
2.3	102.2%	82.2%	83.2%	89.2%
2.4	71.1%	80.9%	88%	80%
2.5	95.7%	71.9%	87.6%	85.1%
2.6	99%	89.6%	77.4%	88.7%

Table 1b: Moisture content measurements taken over the course of the experiment and averages.

Preparation of Microbial Data:

If the microbial DNA sequences were available, The DADA2 pipeline would first be used to prepare bacterial sequences for metagenomic analysis. The set of pipelines that would be used was put together by Gabriel Perron. First, the sequences would be filtered for quality, as errors in sequencing can easily happen. The reads would then be truncated to ensure each sample has at least 200 reads. Dereplication of the sequences would then combine all identical sequences into a single “unique sequence” with an abundance corresponding to its frequency in the data. Error inference models would then be created to visual the PCR error in forward and reverse reads. The core sequence-variant inference algorithm would then be applied to the dereplicated data in order to identify and separate unique sequence variants from errors. This will produce the final number of identified taxa and their abundance, and a sequence table will be created.

Chimeras, or sequences formed from multiple sequences put together, would then be removed. Taxonomy would then be assigned and a taxa table would be created to be used in order to create a phyloseq object.

In order to create the phyloseq object, the metadata file created throughout the study would next be uploaded to R to provide contextual data pertaining to each of the samples. A phyloseq object would then be created using the taxa table, OTU table and metadata file. Chlorophyll and mitochondrial DNA would then be removed from the data to ensure the analysis only includes bacterial DNA. The sequences would then be pruned to ensure the number of reads is similar across all samples. Finally, exploratory bar plots would be produced to show overall differences between the bacterial diversity of the samples. The phyloseq object would then be used in order to analyze and visualize the data later on.

Effect of Treatment on Leaf Length

There was not a significant difference between the leaf lengths of plants that were primed with C6-HSL, those that were primed with water and those that were unprimed (Figure 3, $P=1.57$). This was found through first creating a box and whisker plot to visually assess the differences between the treatments, and then through a t-test.

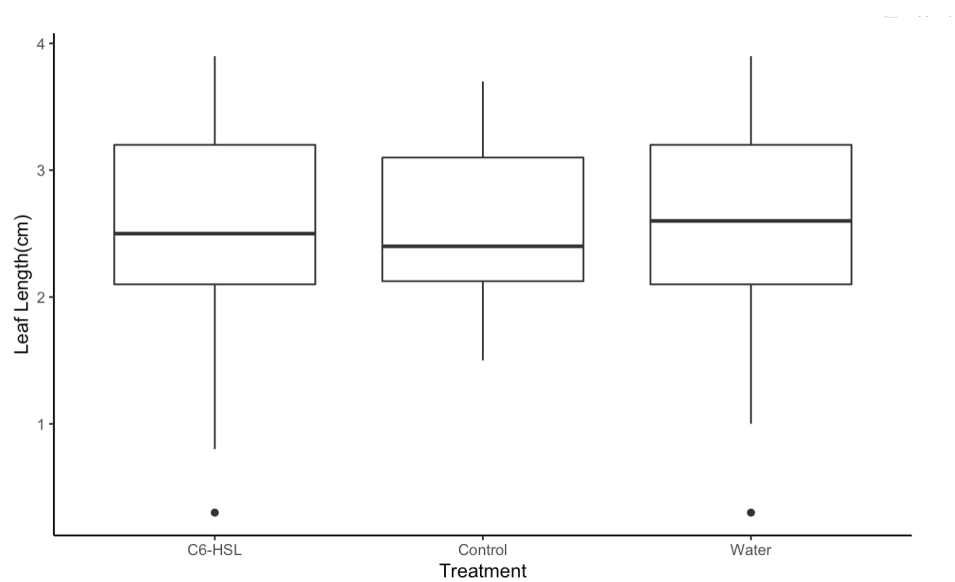


Figure 3: Box and whisker plot portraying the leaf lengths of plants in each treatment. Lines represent the median, and tails indicate first and third quartiles.

Metagenomic Analysis of the Plot

Soil moisture and temperature data would be used to contextualize the bacterial analysis of the plot soil samples taken before the sowing of the arugula seeds and after harvest. Temperature remained relatively constant across the plot, so differences between the bacterial diversity between sites would most likely not be attributed to soil temperature. However, differences in bacterial diversity over time may be affected by temperature. The effect the decrease in temperature had on the microbial diversity would be explored through comparing the bacterial diversity of the samples taken before the experiment to the samples taken after the experiment. This comparison would be done through the creation of a scatter plot and linear regression. A figure similar to Figure 4 would be produced. The variability in moisture content would be expected to affect bacterial communities in different samples across the plot as well as over time. A scatterplot and linear regression would also be used to analyze the relationship between moisture and bacterial diversity, which would also look similar to Figure 4.

Metagenomic analysis of the plot soil samples would be used to contextualize the root and rhizosphere bacterial samples. The diversity and dominant families in the plot overall would be compared to the root and rhizosphere samples to assess if the presence of the arugula plants and possibly the presence of the AHL had an effect on the bacterial communities. This could be done through a principal coordinates analysis similar to Figure 5. Furthermore, if it was found that temperature and moisture conditions affect the bacterial communities in the plot soil samples, it would be assumed that they would also affect the root and root soil bacterial communities in a similar way. This would be important in understanding what differences were caused by treatment versus environmental conditions.

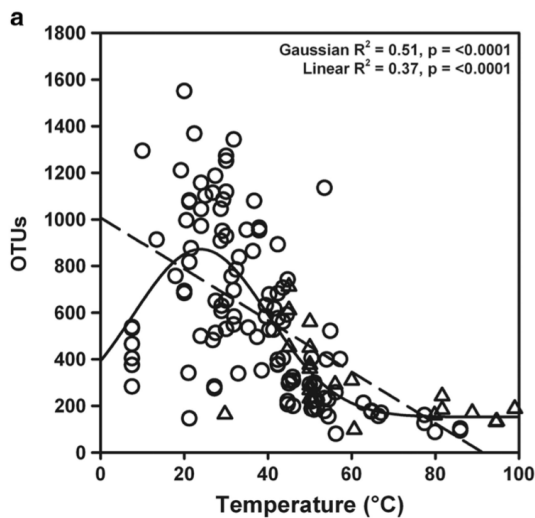


Figure 4: Scatter plot and linear regression from Sharp *et al.* 2014. Data and axis labels do not represent the current study, the figure is solely used to display a linear regression.

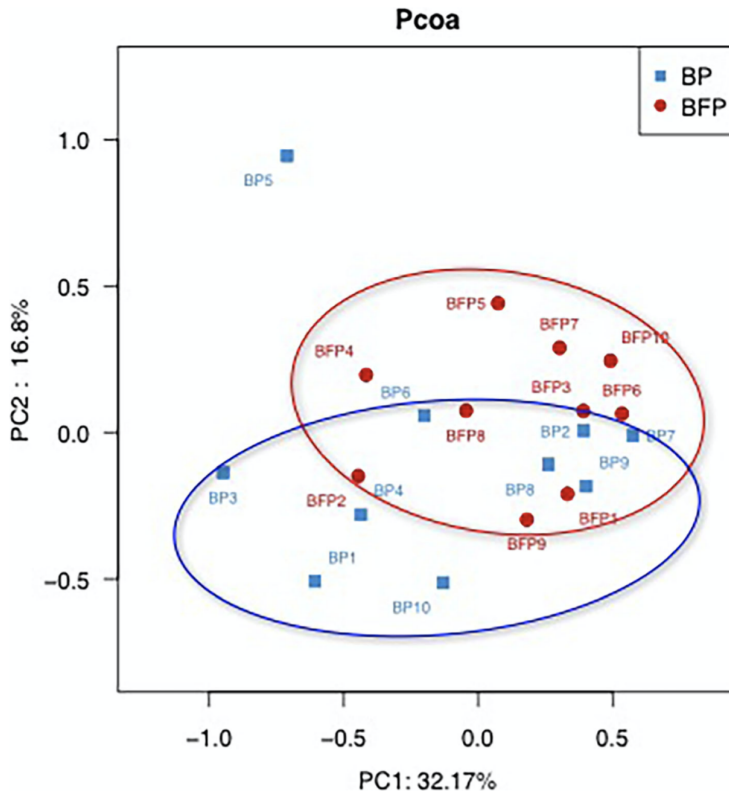


Figure 5: PCoA from Chen *et al.* 2019. Data and axis labels do not represent the current study, the figure is solely used to display a PCoA.

Metagenomic Analysis of Roots and Rhizosphere

The root samples and rhizosphere samples would be used to analyze the effects that treatment had on microbial diversity and dominant functional groups. The alpha diversity of the samples would be plotted against the treatment of the samples in a box plot similar to Figure 6. A principle coordinate analysis could also be used to analyze the difference between the bacterial diversity associated with different treatments. A t-test would be performed to determine if treatment had a significant effect on alpha diversity. If treatment was found to have a significant effect on alpha diversity, the dominant families present in different treatment samples would be compared. Through research, the functions of these families could be defined and compared.

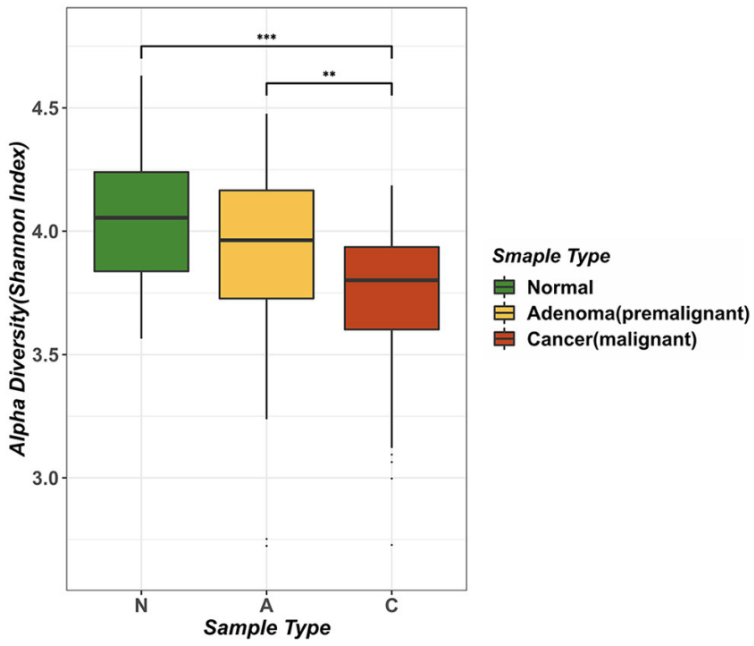


Figure 6: Alpha diversity box plot from Ai *et al.* 2019. Data and axis labels do not represent the current study, the figure is solely used to display an alpha diversity box plot.

Discussion

Previous research on AHL-plant interactions has focused on *in vitro* experiments performed on model plants. Most of these studies have applied AHLs directly to the root systems of the evaluated plants. This study sought to better understand the effect of C6-HSL used as a seed-priming solution on arugula leaf growth and the bacterial communities of the plant's root system and rhizosphere. Leaf lengths of AHL-primed, water-primed and unprimed seeds were compared, though the metagenomic analysis of the diversity and dominant functional communities of the plants has been delayed due to the COVID-19 pandemic.

The study did not find a significant difference between the leaf lengths of the plants grown ($P=1.57$). Though microbial analysis has not been completed, it is assumed that the microbial diversity and dominant functional groups in root and rhizosphere samples would be affected by variation in moisture content across the plot and possibly by temperature over time, as is supported by previous research (Borowik & Wyszowska, 2016, Kim *et. al* ,2008). Without the bacterial data, it is unclear if the AHL had an effect on the roots and rhizosphere of the plants since this was the first study of AHL effects on arugula and only the second study exploring AHL seed priming. Importantly, however, the completion of this study has shed light on the gaps that exist in current AHL-plant interaction research, particularly pertaining to the use of AHLs in agriculture.

Effect of C6-HSL on Shoot Growth

This study found that priming *Eruca sativa* seeds with C6-HSL ultimately did not have an effect on the plant's leaf length. This is in contrast with previous research which has found C6-HSL to increase shoot growth in *Arabidopsis thaliana* (Ortiz-Castro *et al.*, 2008, Schenk *et*

al., 2012, Schikora *et al.*, 2011, von Rad *et al.*, 2008). However, this is not the first study in which C6-HSL did not have an effect on shoot growth. Schuhegger and colleagues found that C6-HSL did not affect the growth of tomato plants, and instead induced pathogenic resistance (Schuhegger *et al.*, 2006). Other research on AHL-plant interactions has found that these interactions are species specific, so it is not surprising that arugula did not react to C6-HSL the same way *Arabidopsis thaliana* has.

Unmeasured Possible AHL-Plant Interactions

It is possible that the AHL had an effect on the plant that was not measured in this study. Many studies have found C6-HSL to have a significant impact on the root systems of various plant species (Liu *et al.*, 2012, Schikora *et al.* 2011, Schenck *et al.*, 2012, von Rad *et al.*, 2008). The roots of the arugula grown in this study were not measured, though this would be an interesting avenue for further study. A future version of this study could include a protocol such as the one described in Pan & Bolton (1991). The protocol involves the usage of a flatbed scanner to create high resolution images of root systems which allow researchers to measure root diameter and area. Other studies have simply used a ruler to measure root length (Liu *et al.*, 2012, Ortiz-castro *et al.*, 2008).

Furthermore, the ability of AHLs to induce pathogenic resistance is well documented and may be an undetected advantage of C6-HSL seed priming. Neither this study nor Moshynets and colleagues' study analyzed the effect C6-HSL may have on crops facing pathogenic stress, though previous research has shown that this is an avenue of research worth exploring. As previously stated, C6-HSL was found to induce pathogenic resistance in tomato plants by

Schuhegger and colleagues (2006). Future research could include an in-field study of the capacity of C6-HSL to induce pathogenic resistance in crop species.

AHLs have also shown potential to improve plant performance against environmental stresses. Moshynets and colleagues found that the winter wheat primed with C6-HSL performed better in water limited conditions compared to control plants. Though plant resistance to stress was not the focus of their study, it was observed that during the second season of the experiment C6-HSL primed plants maintained a high productivity in lower than average rainfall conditions, while the control plants experienced reduced productivity. Tolerance to environmental stresses could be yet another avenue of research pertaining to AHL seed priming.

Degradation of AHLs in the Environment

There is also a possibility that the AHL may have degraded over time, which may have contributed to the lack of effect C6-HSL had on plant growth. Since AHLs are produced and received at critical times, AHLs must degrade if they aren't taken up by other bacteria, or else they would remain in the environment and possibly initiate unintended action. A 2005 study found that the degradation rate of C6-HSL in the environment is highly dependent on pH and temperature. C6-HSL was found to be more stable at colder temperatures and more acidic pH conditions (Delelande *et al.*, 2005). The AHL remained stable for the longest time period under conditions of -20°C and a 7-8 pH level. Though pH levels were not recorded in this study, the temperature in the experimental plot ranged from 16.6°C to 8.9°C over the duration of the experiment, which is far from the documented optimum temperature. However, temperatures in the study by Moshynets and colleagues (2019) ranged from $\sim 20^{\circ}\text{C}$ to $\sim -5^{\circ}\text{C}$ and the AHL seemed to have had an effect on the plants grown in the study. Temperature may have had an

effect on the degradation rate of the AHL, though not enough in-field research on AHL-plant interactions has been done to understand the effect environmental factors have on AHLs.

To better understand the end location of the AHL in the experiment, future research could include evaluation of the internal physiological effects of C6-HSL. Various other studies have tracked AHL movement through plants to better understand the mechanisms through which AHLs affect plants. A better understanding of this would make clear if the AHL simply did not have an effect on plant growth or if the AHL was not taken up by the plant at all, due to error in seed priming protocol or degradation of the AHL. A protocol such as the one used in a study by Sieper and colleagues (2013) could potentially be used in this research. The study used radioactively labeled AHLs to track the location of the AHL during the experiment (Sieper *et al.*, 2012).

Effect of Moisture:

It is expected that the variability in moisture over the plot will have an effect on the bacterial diversity of the samples. Studies have found that the bacterial diversity and activity of organisms is significantly impacted by soil moisture (Borowik & Wyszowska 2016, Kim *et al.*, 2008). Both excessively wet and excessively dry soils have been found to lead to a decrease in biomass of bacteria (Borowik & Wyszowska, 2016). The soil in this study had high moisture levels, which would likely have an impact on the soil microbiome. The effect of moisture on the microbiome would need to be considered while assessing the effect C6-HSL had on the microbiome.

Future studies could attempt to better control the moisture content of the soil in C6-HSL experiments. However, it is important to note that the moisture content of agricultural soils

naturally varies, which will need to be considered if a C6-HSL seed priming solution were to be used by farmers. Furthermore, It is also currently unclear what effect moisture has on AHLs. The high moisture content in this study may have caused the C6-HSL to leach into the soil profile, possibly far enough away from the plants to deter an interaction between the AHL and the plant. Environmental factors such as moisture and temperature are important in the analysis of AHL-plant interactions, though they are often left out of previous research.

Effect of AHLs on the Plant Bacterial Communities:

There has not been much research on the effect AHL application has on the rhizosphere of the plant. Moshynets and colleagues investigated the effect C6-HSL had on the rhizosphere of winter wheat and found the functional groups to differ significantly from control plants. However, a metagenomic analysis of the effect AHL application has on the natural microbiome of an agricultural field has not been performed.

Early AHL-plant interaction research hypothesized that AHLs cause increased growth and pathogenic resistance in plants by altering the rhizosphere of the plant. It has since been found that AHLs directly affect plant cells in various ways which can cause changes in growth and resistance to pathogens (Schikora *et al.*, 2016). However, it is still possible that the application of AHLs alters a plant's rhizosphere. For instance, Moshynets and colleagues mention the possibility that stimulation of plant growth by C6-HSL may increase photosynthate exude, which may in turn increase beneficial bacteria populations in the rhizosphere (Moshynets *et al.*, 2019).

Conclusion

Though the study is currently incomplete and far from perfect, effort was made to design an experiment in which AHL-plant interactions are utilized in an agricultural system. The study investigated an alternative application method of AHLs which could be used by farmers.

Ultimately, the study found that C6-HSL in the form of a seed priming solution did not affect leaf length of arugula plants. However, AHL-plant interaction research is still a relatively new field, and exploratory studies such as this one can only move research forward. Future studies could build upon this experiment by further investigating the effects of AHL-seed priming on other species of crops. Further research should also look into the possible effects of AHL-seed priming other than plant growth.

Appendix

Figure 7

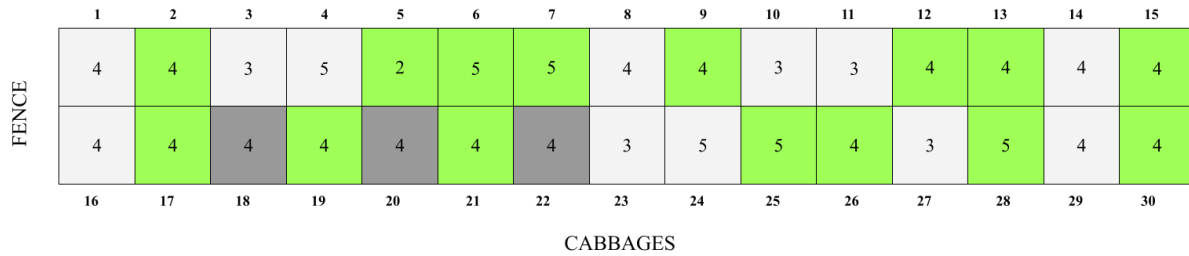


Figure 7: Number of sprouts grown in each grid unit is presented inside each grid square. The number of the grid square is written outside the box. The treatment of the plants grown in the grid unit is represented by the color of the square. Green represents AHL primed plants, light grey represents water primed plants and dark grey represents plants that were not primed.

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