Evaluation of Physiological Traits Expressed *in vitro* and Effects on Plant Growth by *Bacillus* Endophytes

by
Jewel Galloway

A thesis presented to the Honors College of Middle Tennessee State University in partial fulfillment of the requirements for graduation from the University Honors College

Fall 2020

Evaluation of Physiological Traits Expressed *in vitro* and Effects on Plant Growth by *Bacillus* Endophytes

by Jewel Galloway

APPROVED:

_____

Dr. Stephen Wright, Thesis Director
Professor, Biology Department

Dr. John DuBois, Second Reader
Professor, Biology Department

Dr. Ryan Otter, Thesis Committee Member
Professor, Biology Department

_____

Dr. Dennis Mullen, Thesis Committee Chair
Professor, Biology Department

Acknowledgements

I want to thank Dr. John DuBois for his assistance throughout the greenhouse study. I would also like to acknowledge the Undergraduate Research Center for funding this research through an Undergraduate Research Experience and Creative Activity (URECA) grant. Most of all, I want to thank Dr. Stephen Wright for being a wonderful mentor and professor who always supports my academic goals.

Abstract

Bacillus species are an important group of rhizobacteria that have been shown to enhance the growth of plants and protect against diseases caused by plant pathogens. This study investigated the mechanisms used by *B. atrophaeus* and *B. thuringiensis* to promote plant growth and explored their potential as biofertilizers and biocontrol agents for application in agriculture. Effects of each bacterial strain on the growth of *Brassica rapa* were evaluated under greenhouse conditions with sterile soil. Plant growth promotion traits including nitrogen fixation, phosphate solubilization, and siderophore production were assessed using *in vitro* microbiological techniques. Additionally, *in vitro* methods were used to evaluate antagonistic activities against two soil-borne pathogens, *Fusarium oxysporum* and *Agrobacterium tumefaciens*. To verify nitrogen fixation activity, the nitrogenase gene, *nifH*, was amplified by PCR. Plants inoculated with *B. atrophaeus* had significantly greater biomass and increased length compared to the control. In the *in vitro* assays, *B. atrophaeus* exhibited antimicrobial and nitrogen fixation activity while *B. thuringiensis* was positive for siderophore production. These bacteria could potentially be used as biofertilizers and biocontrol agents in conjunction with currently used agrochemicals to reduce the environmental costs associated with synthetic chemicals.

Table of Contents

Acknowledgements……...iii

Abstract…….....iv

List of Tables……..……...vi

List of Figures……..……vii

List of Appendices…….....viii

Introduction……..……..1

Materials and Methods……..5

Results……...11

Discussion…….21

References…….24

List of Tables

Table 1: Greenhouse Conditions…….6

Table 2: PCR Program: *nifH*……...9

Table 3: Summary of Plant Growth Promotion Activity by *Bacillus* Strains……...20

List of Figures

Figure 1: Average length of *Brassica rapa* control and treatment groups at the end of the experiment……..11

Figure 2: Average above ground dry biomass of *Brassica rapa* control and treatment groups at the end of the experiment……...12

Figure 3: *Brasscia rapa* inoculated with *Bacillus* species…..……..13

Figure 4: *Bacillus* species cultured on GNFM agar with BTB as an indicator…….14

Figure 5: *Bacillus* species cultured on O-CAS medium…….....15

Figure 6: Pikovskaya plate showing no phosphate solubilization for *Bacillus atrophaeus* or *Bacillus thuringiensis*……16

Figure 7: *In vitro* inhibition of *Fusarium oxysporum* by *Bacillus atrophaeus* on TSA……17

Figure 8: *In vitro* inhibition of *Agrobacterium tumefaciens* by *Bacillus atrophaeus*…...18

Figure 9: Agarose gel showing DNA fragment produced by PCR amplification of the *nifH* gene……19

List of Appendices

Appendix A: Length of *Brassica rapa* After Four Weeks…….……...29

Appendix B: Dry Mass of *Brassica rapa* After Four Weeks……30

Appendix C: O-CAS Media……..31

Appendix D: Buffers…….32

Introduction

Synthetic chemicals, such as fertilizers and pesticides, have become increasingly relied upon as sources of essential nutrients and phytopathogen control in modern agriculture. As application and production continue to rise, their detrimental impacts on the environment rise as well. Excess nitrogen fertilizer contaminates local water supplies or is converted by soil microorganisms into nitrous oxide, a potent greenhouse gas (1). Moreover, further chemical application has limited value as it has an inverse relationship with declining uptake efficiency by plants (2, 3). Bioavailability of some nutrients also does not increase with application of agrochemicals as evidenced by phosphorous fertilizers which precipitate and become insoluble after addition to soil (2, 3). Furthermore, populations of microorganisms needed to provide nutrients for plant growth decline due to the build-up of toxic residues (4, 5). Inoculation of plant growth-promoting rhizobacteria (PGPR) presents a sustainable approach to increase crop productivity, while reducing production costs and harm to the environment (6).

Plant growth-promoting rhizobacteria reside in the rhizosphere, the region of soil in which diverse microbial activity is affected by compounds secreted from plant roots (7). These bacteria confer beneficial effects on plant health directly by mobilizing nutrients or indirectly by production of phytohormones and suppression of plant pathogens (8). Among PGPR, bacteria in the genus *Bacillus* are highly culturable and represent the most important species currently used in bio-based agricultural products (9). *Bacilli* are tolerant to a variety of stressors, such as heat and desiccation, due to their spore-forming abilities (9). Endospores are dormant structures that are non-reproductive. Furthermore, endospores enable the colonization of most soil environments and their high

stability allows for long-term storage rivaling agrochemicals without undesirable side effects (2).

Nitrogen (N), phosphorous (P), and iron (Fe) are critical nutrients for plant productivity; however, their accessibility in soil is limited. Using the enzyme nitrogenase, certain strains of *Bacillus* can convert atmospheric dinitrogen into ammonia, a form of N that can be utilized by plants (9). Moreover, *Bacilli* promote the acquisition of largely insoluble P by secreting organic acids that lower the soil pH and aid in solubilizing inorganic phosphates (7, 10). One study reported that *Bacillus* inoculants lowered soil pH to values between 6.2 and 5.5 (11). To overcome Fe limited conditions, organic compounds called siderophores are produced by some *Bacilli*. Siderophores bind to Fe^{3+} making Fe available for plant uptake. Consequently, this inhibits pathogen growth as their Fe supply becomes insufficient (9). In addition, synthesis of hormones by *Bacillus* species, such as indole-3-acetic acid, increases the number of lateral and adventitious roots, thereby indirectly promoting nutrient acquisition and overall plant health (12).

Bacillus species have been well-studied for their production of structurally diverse antimicrobials (12). Non-ribosomal peptides (NRP), the most abundant being cyclic lipopeptides, are among the antimicrobials known for their anti-bacterial and anti-fungal properties (13). Furthermore, *Bacilli* inhibit fungal pathogens by secreting a variety of lytic enzymes, such as chitinases and proteases, that degrade their cell wall (13). Described as contributing to *induced systemic resistance*, these bacteria change the physiological state of the plant to heighten defenses such as cell wall reinforcement and oxidative burst against pathogens (12).

In this study, the *Bacillus* species that were investigated are *B. thuringiensis* and *B. atrophaeus*. These were selected based on preliminary studies concerning their ability to affect plant growth in rapid-cycling *Brassica rapa*, an important model organism with diverse applications in biology education and research (14). *Bacillus thuringiensis* is antagonistic against a wide range of insects due to the production of parasporal crystal proteins. As such, it has been extensively studied and commercialized as a bioinsecticide (15). Recently, several studies have demonstrated that strains of *B. thuringiensis* have the potential to control many bacterial and fungal pathogens and promote plant growth as a biofertilizer due to factors described above (9). Furthermore, recent genome sequencing of strain GQJK17 *B. atrophaeus* revealed secondary metabolic gene clusters that are likely responsible for enhancing plant growth and controlling pathogens (16, 17). Many of these clusters function in the production of lipopeptides including surfactin which induces biofilm formation and has been reported as characteristic of *B. atrophaeus* in previous studies (13, 16). Though the mechanism of biofilm formation in biocontrol is not fully understood, studies have suggested that biofilms aid in colonization of plant root surfaces, a critical step in promoting plant health (18).

In a greenhouse experiment, *B. thuringiensis* and *B. atrophaeus* were evaluated for effects on plant growth. To understand their effects, they were evaluated for the following traits associated with plant growth promotion: siderophore production, phosphorus solubilization, and nitrogen fixation. Furthermore, PCR was conducted to assess presence of the nitrogenase gene, *nifH*. In addition to evaluating impacts on plant growth, the *Bacilli* were evaluated for anti-fungal and anti-bacterial activity. The fungal and bacterial pathogens that were used in this study included *Fusarium oxysporum* and

Agrobacterium tumefaciens. These soil-borne pathogens were chosen due to their effects on a variety of crops including Brassicas that are important to the global agricultural economy (6, 19).

Materials and Methods

Bacterial Strains

The following organisms were obtained from the American Type Culture Collection: *Bacillus atrophaeus* (9372), *Bacillus thuringiensis* (10792), *Bacillus cereus* (13061), *Serratia marcescens* (94A-429), and *Bacillus pumilus* (700814). *Azotobacter vinelandii* and *Agrobacterium tumefaciens* were purchased from Ward's Science (West Henrietta, NY). *Escherichia coli* B and *Fusarium oxysporum* were purchased from Carolina Biological (Burlington, NC). An environmental strain of *Bacillus pumilus* isolated by Dr. Stephen Wright was also included in the study.

Media Preparation

Both glucose nitrogen-free mineral (GNFM) agar and Pikovskaya's agar were purchased from HiMedia Laboratories through VWR (Atlanta, GA). Tryptic Soy Agar (TSA) and Tryptic Soy Broth (TSB) were purchased from Becton, Dickinson (Sparks, MD). All media were prepared as instructed by the manufacturer.

Greenhouse Experiment

Seeds of rapid-cycling *Brassica rapa* (Wisconsin Fast Plant, standard strain; Carolina Biological) were surface sterilized by soaking in 1% household bleach (Clorox®) for five minutes followed by 95% ethanol for three minutes and rinsed with sterile distilled water five times. After drying in a laminar air-flow hood, three seeds were plated onto TSA to confirm sterilization success. The rest of the seeds were stored at 4°C for 24 hours. Each *Bacillus* strain was cultured in TSB, and seeds were soaked in the bacterial broths for one hour prior to planting in autoclaved Miracle-Gro® Moisture Control® potting mix. Control seeds soaked in sterile Phosphate Buffered Saline (PBS) were also planted. PBS

composition is listed in Appendix D. Fifteen four-inch pots containing four seeds each were used for each test group and placed randomly on a bench in the greenhouse. The number of pots was determined based on a preliminary study in which up to five of ten pots per group did not have surviving plants. For the duration of the study, plants were watered 3-4 times per week, and fertilizer and insect repellants were not used. Furthermore, temperature, relative humidity, and light varied at different times of the day (Table 1). After 27 days, plant height was measured by combining the length of the tallest stem and branching stems greater than 1 cm. Shoots were then individually dried in open plastic bags weighing 2.40 g each at 37°C for 3 days and weighed. One-way analysis of variance (ANOVA) was conducted to compare the effects of bacterial and control treatments.

Table 1. Greenhouse Conditions.

Time	Temperature	Relative Humidity	Lights
400	22°C	70%	On
1800	20°C	70%	On
2100	20°C	50%	Off

Nitrogen Fixation

The *Bacillus* species, *Azotobacter vinelandii* (positive control), and *Escherichia coli* (negative control) were streaked on GNFM agar to assess nitrogen fixation activity. After incubation at 28°C for 7 days, bromothymol blue (BTB) (Acros, through Fisher Scientific) was added to the plates as an indicator. Observation of a dark blue or bluish green color change in the green medium indicated nitrogen fixation activity.

Siderophore Production

Siderophore production was observed using the Overlaid Chrome Azurol S (O-CAS) assay (20). All media components are listed in Appendix C. This method was chosen over the traditional CAS assay because it is non-toxic to Gram-positive bacteria. Each *Bacillus* strain was cultured on TSA before being overlaid with 10 mL of the prepared CAS medium. *Bacillus cereus* was used as a positive control. Within 15 minutes, observation of an orange or purple halo around a colony indicated siderophore production.

Phosphate Solubilization

Phosphate solubilizing activity of the *Bacillus* species was determined using Pikovskaya's agar medium. Each test strain and *Serratia marcescens* as the positive control were spot inoculated onto plates and incubated for 7 days at 28°C. Phosphate solubilization was visualized by a clear halo around bacterial growth.

Anti-fungal

Fusarium oxysporum was cultured on TSA at 28°C for 3 days. Following this, a small amount of fungal growth was cut and placed in the center of a new TSA plate. Each *Bacillus* strain was inoculated the same distance from *F. oxysporum* prior to incubation at 28°C for 5 days. *Bacillus pumilus* (ATCC) was also inoculated as a negative control. Observation of a zone of inhibition around the bacterial colonies indicated anti-fungal activity.

Anti-bacterial

To evaluate anti-bacterial activity, a line of *Agrobacterium tumefaciens* was prepared in the center of a TSA plate. Next, *B. atrophaeus*, *B. thuringiensis*, and *B. pumilus*

(environmental isolate) as the positive control were inoculated perpendicular to *A. tumefaciens*. After incubation at 28°C for 2 days, the plate was evaluated for presence of a zone of inhibition around growth indicative of bacterial antagonism.

DNA Extraction

Genomic DNA was extracted from each test strain and *Azotobacter vinelandii* (positive control) using the Genomic DNA Mini Kit Bacteria Protocol (IBI Scientific, through Sigma-Aldrich, St. Louis, MO). Cultured bacterial cells were transferred to a 1.5 mL tube and centrifuged for 1 minute. The cells were resuspended in 200 µL of 2X Proteinase K buffer containing 20 µL of Lysozyme at a concentration of 50 mg/mL. Following a 10-minute incubation period at 70°C, 200 µL of GB Buffer was added to the sample. Next, 200 µL of absolute ethanol was combined with the sample, and the lysate was transferred into a GD Column in a 2 mL Collection Tube. The sample was centrifuged for 2 minutes; afterward, the flow-through was discarded and the GD Column placed in a new 2 mL collection tube. To wash the sample lysate, 400 mL of W1 Buffer was added to the GD Column and centrifuged for 30 seconds. The flow-through was again discarded and the GD Column was placed back into the collection tube prior to being centrifuged once more for 3 minutes. Next, the GD Column was placed in a 1.5 mL tube, and 100 µL of preheated Elution Buffer was added to the column and allowed to be absorbed by the matrix. Last, to elute the DNA, the sample was centrifuged for 30 seconds.

Amplification of *nifH* Gene

The extracted DNA from each bacterial species was amplified using the designed primers F5'-TGTAGATTTCCTGGGCCTTG-3' and R5'-GACTCCACCCGCCTGATCCT-3'. Primers were obtained from Eurofins, through Fisher Scientific. These primers targeted a

316 base pair fragment. For each sample, the following reagents were added to a 0.5 mL tube: 2 µL extracted genomic DNA, 34.5 µL sterile dH_2O, 37.5 µL Master Mix (with Taq DNA polymerase) obtained from Promega (Madison, WI), and 0.5 µL each of forward and reverse primers at a concentration of 200 ng/µL. The tubes were then centrifuged for 3-5 seconds and placed in the thermal cycler. The PCR program used is listed in Table 2. Sterile dH_2O was used in place of extracted DNA for the negative control.

Table 2. PCR Program: *nifH*.

PCR Step	Temperature	Duration	Cycles
1	93°C	2 min	1
2	93°C	30 sec	1
3	55°C	45 sec	1
4	72°C	1 min	1
5 (Go to Step 2)			30
6	72°C	5 min	1
7	4°C	Forever	
8		End	

Gel Electrophoresis

Agarose gel was prepared by combining 0.5 g NuSieve agarose (FMC BioProducts, Rockland, ME) and 40 mL 1 X Tris-acetate-EDTA (TAE) buffer in an Erlenmeyer flask and heating for 30-40 seconds in a microwave. Once cooled, the agarose solution was poured into a sealed tray with an 8-well comb in place and allowed to solidify. The comb was then removed, and the gel was placed into the gel box. Next, 250 mL 1 X TAE containing approximately 80 µL ethidium bromide was added to cover the gel. The Biomarker low DNA ladder (BioVentures, Murfreesboro, TN) containing 1.5 µL buffer and 8.5 µL ladder was loaded to one well, and 8.5 µL of each sample PCR product

containing 1.5 μL blue sample buffer were loaded to wells. The gel was run at 75 volts for 1 hour and 15 minutes. Afterward, the gel was removed and observed by UV transilluminator. TAE buffer composition is listed in Appendix D.

Results

Greenhouse Experiment

Bacillus atrophaeus significantly increased the growth of *Brassica rapa* compared to the control ($P < 0.05$; Figures 1, 2). No significant differences were detected for length or dry weight between the other treatment groups (Figures 1, 2). The effect of each group on the growth of *B. rapa* can be seen in Figure 3.

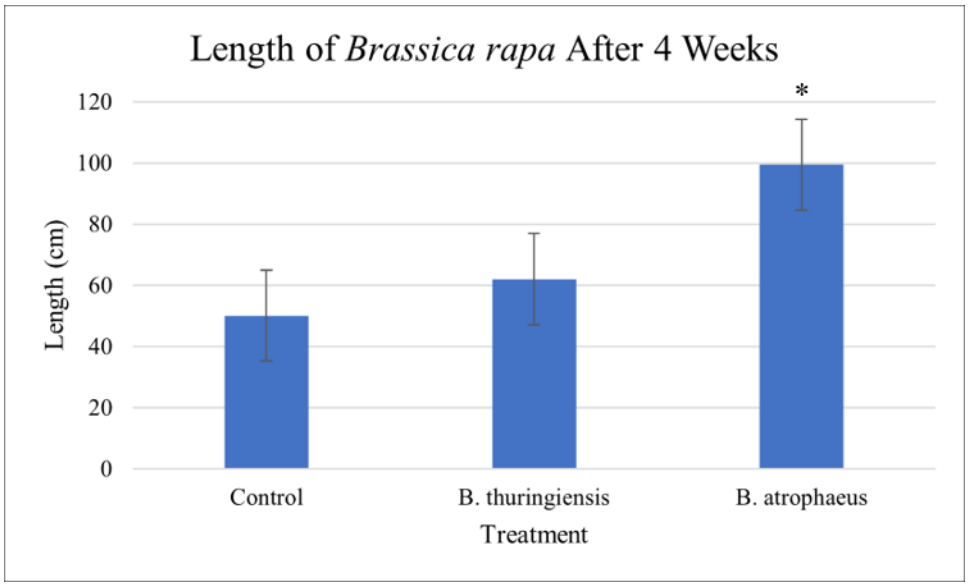

Figure 1. Average length of *Brassica rapa* control and treatment groups at the end of the experiment. Plants inoculated with *B. atrophaeus* showed a significant difference compared to uninoculated control plants ($P = 0.012$). Significant difference $P < 0.05$ is indicated by asterisk (*). Error bars represent standard error.

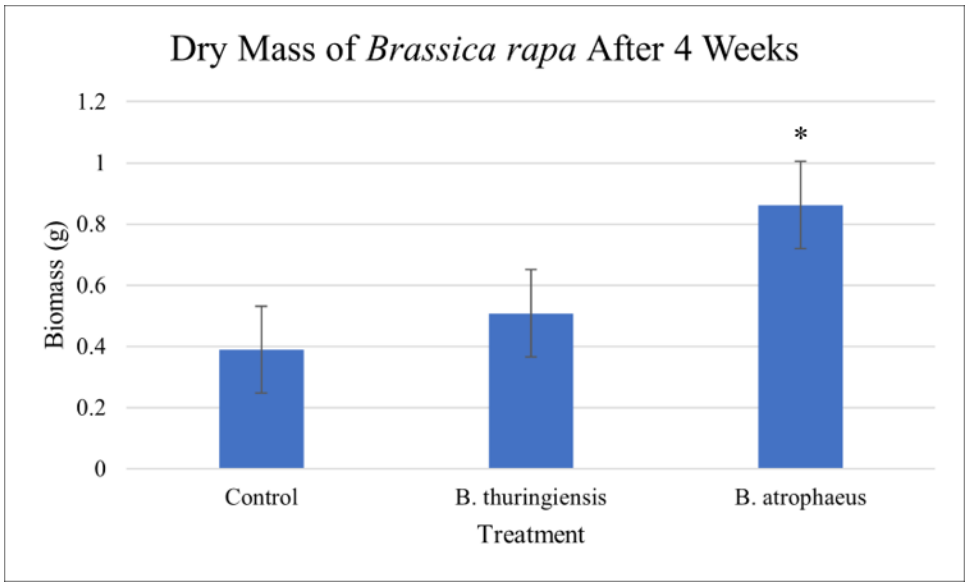

Figure 2. Average above ground dry biomass of *Brassica rapa* control and treatment groups at the end of the experiment. Plants inoculated with *B. atrophaeus* showed a significant difference compared to uninoculated control plants ($P = 0.028$). Significant difference $P < 0.05$ is indicated by asterisk (*). Error bars represent standard error.

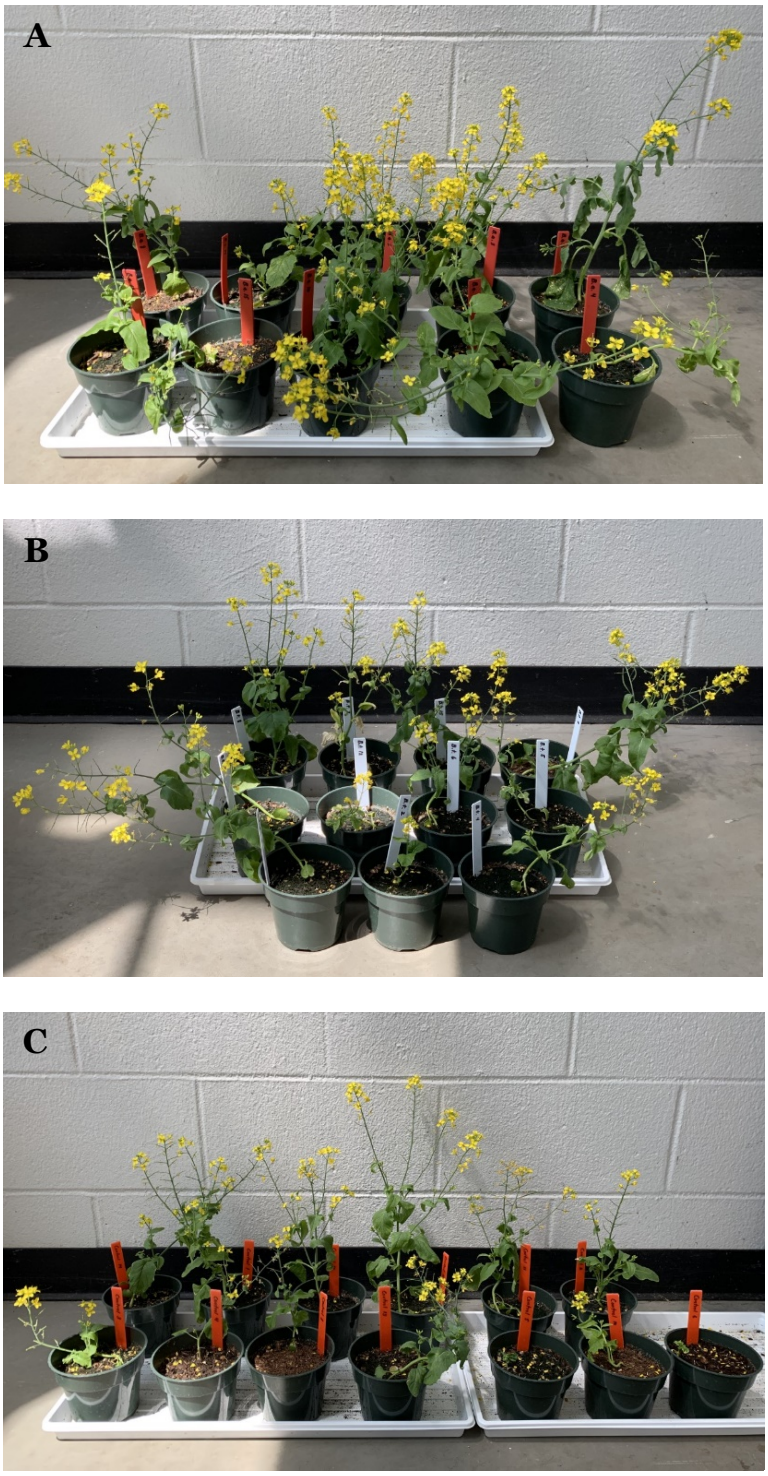

Figure 3. *Brassica rapa* inoculated with *Bacillus* species. Plants inoculated with *B. atrophaeus* (A) exhibited more extensive growth than plants inoculated with *B. thuringiensis* (B) or PBS as the control (C).

Nitrogen Fixation

Nitrogen fixation activity for each test strain as well as the positive and negative controls are shown in Figure 4. *Bacillus atrophaeus* was positive for nitrogen fixation activity, producing a dark blue color change in the medium. However, it is unclear whether *B. thuringiensis* exhibited nitrogen fixation activity.

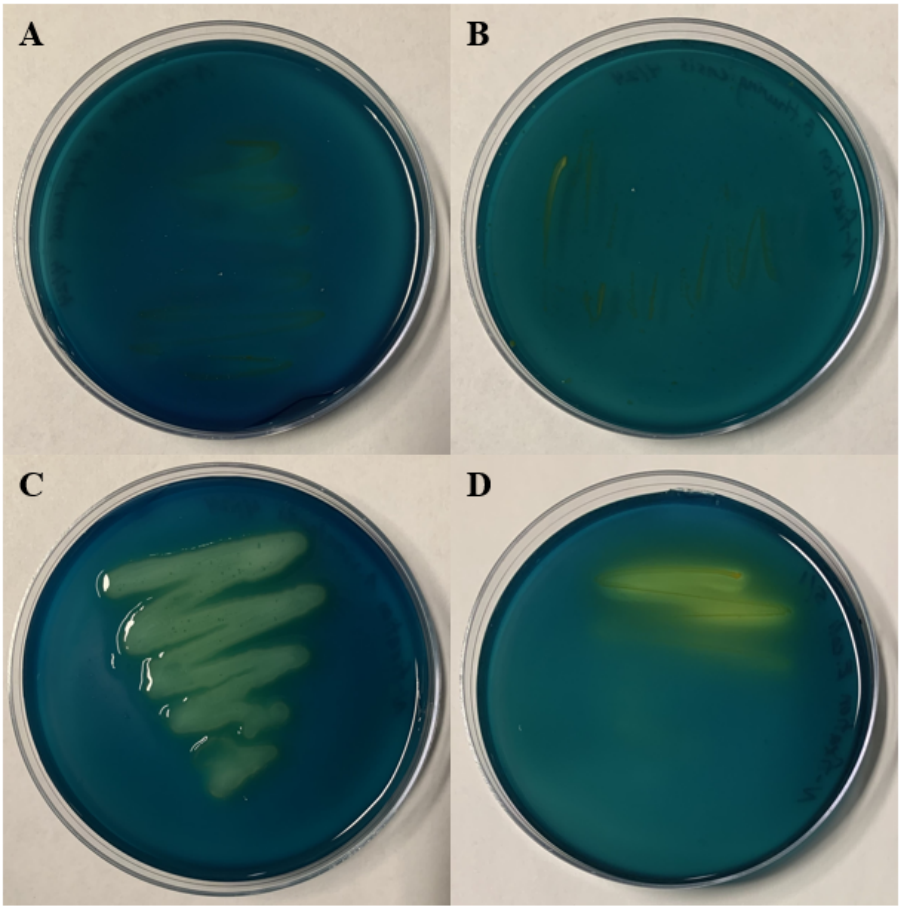

Figure 4. *Bacillus* species cultured on GNFM agar with BTB as an indicator. Plate A shows *B. atrophaeus* and plate B shows *B. thuringiensis*. *Azotobacter vinelandii* (plate C) is the positive control and *Escherichia coli* is the negative control (plate D).

Siderophore Production

Figure 5 shows the O-CAS assays for each test strain and the positive and negative controls. *Bacillus atrophaeus* was negative for siderophore production as no color changes around colonies were observed. *Bacillus thuringiensis* produced a blue color change in the medium and was recorded as positive for siderophore production.

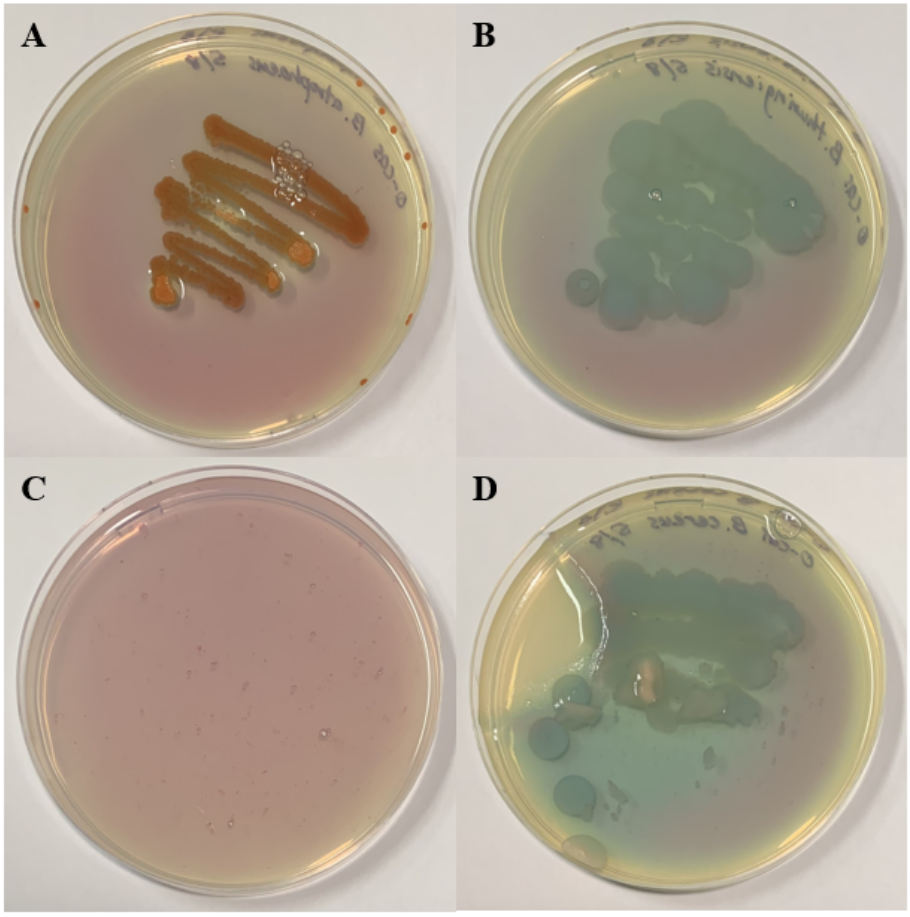

Figure 5. *Bacillus* species cultured on O-CAS medium. Plate A shows *B. atrophaeus* and plate B shows *B. thuringiensis. Bacillus cereus* (plate D) is the positive control. Plate C has no microorganisms.

Phosphate Solubilization

No solubilization was observed for *B. thuringiensis* or *B. atrophaeus* (Figure 6).

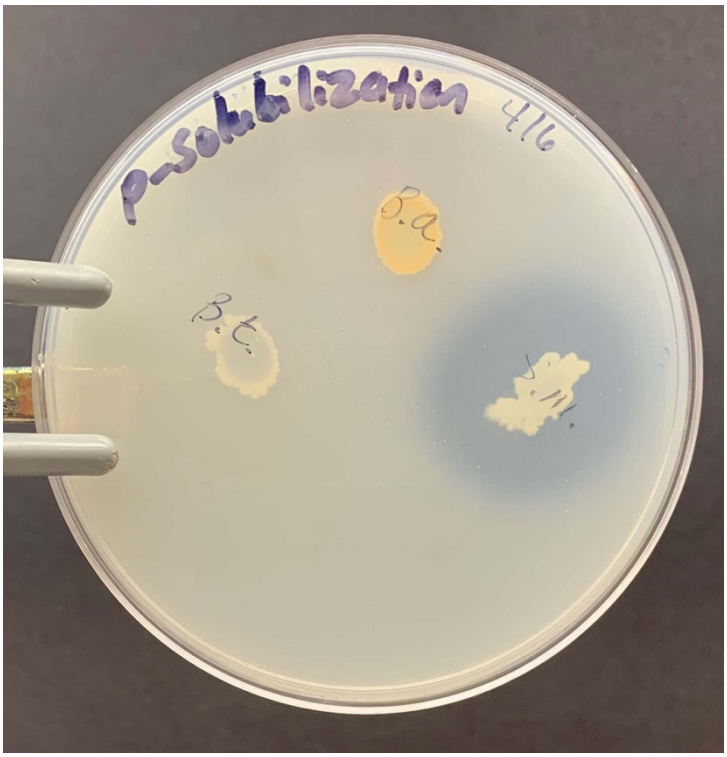

Figure 6. Pikovskaya plate showing no phosphate solubilization for *Bacillus atrophaeus* or *Bacillus thuringiensis*. *Serratia marcescens* was used as the positive control (evident at right).

Anti-fungal

Only *Bacillus atrophaeus* exhibited antagonism against *Fusarium oxysporum* (Figure 7).

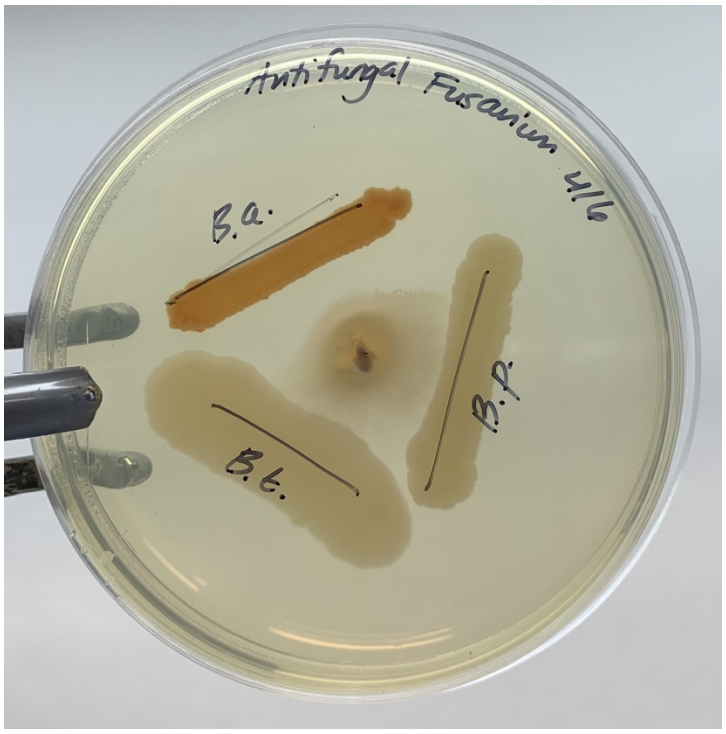

Figure 7. *In vitro* inhibition of *Fusarium oxysporum* by *Bacillus atrophaeus* on TSA. *Bacillus thuringiensis* did not inhibit the pathogen. *Bacillus pumilus* (ATCC) was used as a negative control.

Anti-bacterial

Only *Bacillus atrophaeus* exhibited antagonism against *Agrobacterium tumefaciens* (Figure 8).

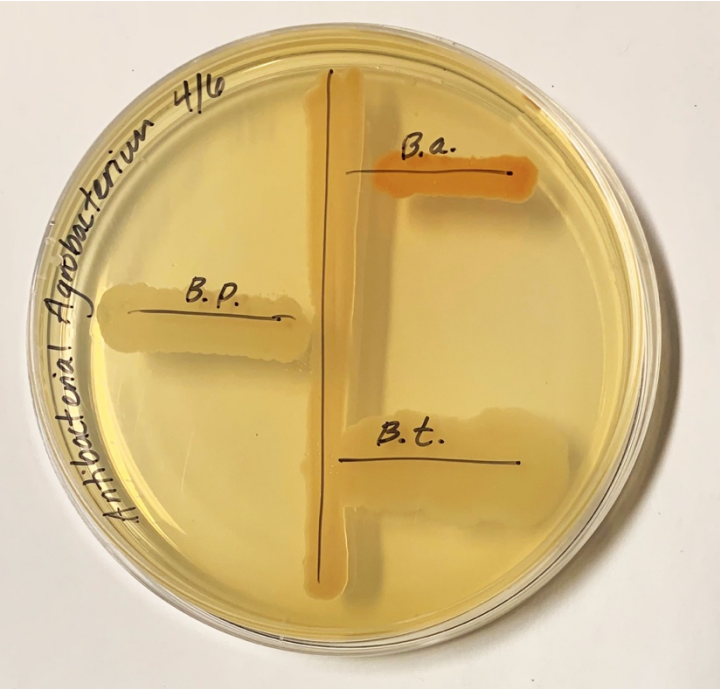

Figure 8. *In vitro* inhibition of *Agrobacterium tumefaciens* by *Bacillus atrophaeus*. *Bacillus thuringiensis* did not inhibit the pathogen. *Bacillus pumilus* (environmental isolate) was used as a positive control.

PCR Amplification of *nifH* Gene

Azotobacter vinelandii was positive for the designed primers, F5'-TGTAGATTTCCTGGGCCTTG-3' and R5'-GACTCCACCCGCCTGATCCT-3' (Figure 9). *Bacillus thuringiensis* and *B. atrophaeus* did not produce a DNA fragment.

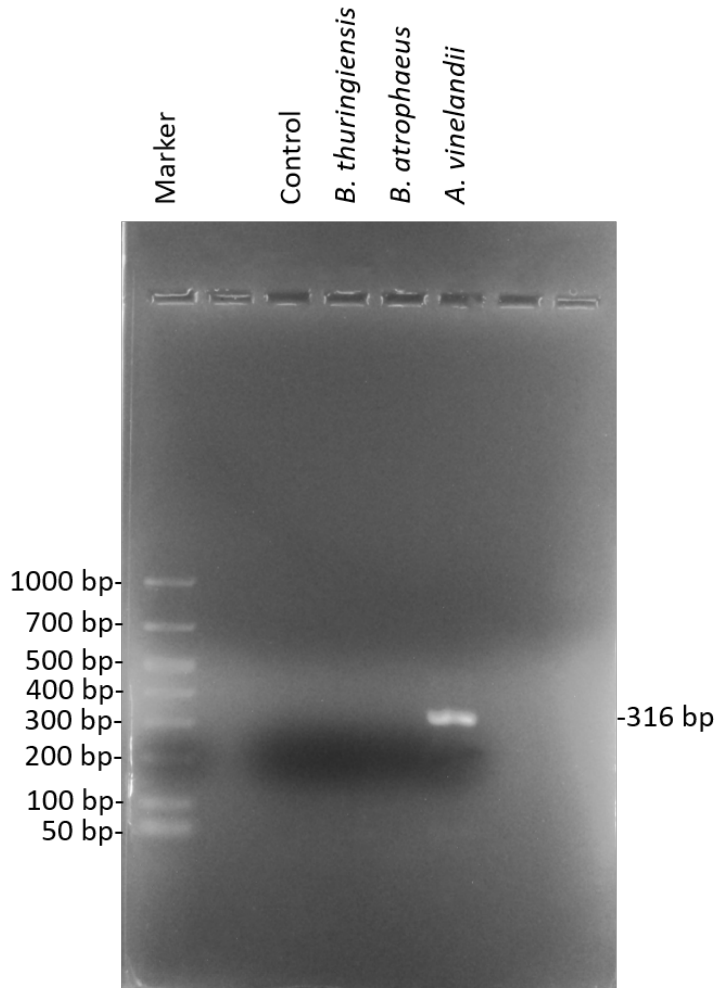

Figure 9. Agarose gel showing DNA fragment produced by PCR amplification of the *nifH* gene. Expected size of DNA fragment was 316 base pairs (bp). Sizes of the marker (BioMarker) are noted at left in bp.

Summary of Results

Table 3. Summary of Plant Growth Promotion Activity by *Bacillus* Strains.

Strain	Phos	Nit	Sid	Antif	Antib	nifH
B. atrophaeus[1]	–	+	–	+	+	–
B. thuringiensis	–	–	+	–	–	–

Phos = phosphate solubilization; Nit = nitrogen fixation; Sid = siderophore production; Antif = antifungal activity; Antib = antibacterial activity; nifH = *nifH* gene. + = trait exhibited; – = trait absent. [1]Significant difference from the control for length ($P = 0.012$) and above ground dry biomass ($P = 0.028$) of *B. rapa*.

Discussion

This objective of this study was to evaluate the mechanisms used by *B. atrophaeus* and *B. thuringiensis* to promote plant growth and investigate their biocontrol activities against *F. oxysporum* and *A. tumefaciens*. The two endophytes were evaluated using *in vitro* microbiological techniques as well as under greenhouse conditions. *Bacillus atrophaeus* was the only strain to significantly increase the growth of *B. rapa* in the greenhouse study.

Results from the antimicrobial assays demonstrated that *B. atrophaeus* has antagonistic activities over the growth of *Fusarium oxysporum* as well as *Agrobacterium tumefaciens*. This aligns with previous studies which identified *B. atrophaeus* as effectively inhibiting plant diseases caused by *Fusarium* species, such as root rot and Fusarium wilt (16, 17). However, *Bacillus thuringiensis* did not inhibit either of the pathogens.

Furthermore, the nitrogen fixation assay did not clearly show whether *B. thuringiensis* had nitrogen fixation activity. The color of the growth medium most closely resembled the negative control plate. As such, it would be useful to perform an Acetylene Reduction Assay (ARA) in future studies which is a quantitative method for determining nitrogenase activity. Moreover, *B. atrophaeus* produced a dark blue color in the GNFM agar, indicating nitrogen fixation activity, but did not produce a *nifH* PCR product. Previous studies have reported that many primer sets have a low coverage of *nifH* sequences which may be the case in this study (21). Additionally, as the nitrogenase enzyme subunits are encoded by *nifH*, *nifD*, and *nifK* genes, *nifD* and *nifK* should be targeted and amplified in future studies concerning the nitrogen fixation activity of *B.*

atrophaeus (21). Lastly, the discrepancy between the nitrogen fixation assay and *nifH* results may indicate that other genes in the *B. atrophaeus* genome are involved in nitrogen fixation activity.

For the O-CAS assay, the color of the medium was pink instead of blue, possibly due to pH sensitivity of the medium. Therefore, observation of any color change in the pink medium was noted as a positive result for siderophore production. Only *B. thuringiensis* was positive for siderophore production, producing a blue color change in the growth medium. This result is strengthened as *Bacillus cereus* also produced a blue pigment in the medium and is a well-known siderophore producer. However, in future studies, siderophore production activity should be re-evaluated for both *B. thuringiensis* and *B. atrophaeus*. Furthermore, chemical assays could be utilized to determine the type of siderophore produced, such as catecholates or hydroxamates.

Overall, this study suggests that *B. atrophaeus* has the potential to promote plant growth in *B. rapa*, possibly through the mechanism of nitrogen fixation. It has been reported that strains of *B. atrophaeus* can increase plant growth in other important crops as well, such as *Zea mays L.* and S*olanum lycopersicum* (16). Therefore, in combination with the exhibited antimicrobial activity, this strain of *B. atrophaeus* could potentially be a useful tool in agriculture as a biofertilizer or biocontrol agent. *B. thuringiensis* could also possibly be beneficial as a biofertilizer as it can produce siderophores. Further investigations under field conditions as well as inoculation in different plants would be useful to evaluate the full plant growth-promotion potential of *B. atrophaeus* and *B. thuringiensis*. Though *Bacillus* species may not be able to fully replace agrochemicals,

the combination of agrochemicals with these endophytes would reduce the degradative effects agrochemicals often have on the environment and human health.

References

1. Khan Z, Guelich G, Phan H, Redman R, Doty S. 2012. Bacterial and yeast endophytes from poplar and willow promote growth in crop plants and grasses. ISRN Agronomy [Internet]. [cited 17 January 2020]; 2012:1-11. Available from: https://doi.org/10.5402/2012/890280.
2. Hashmi I, Paul C, Al-Dourobi A, Sandoz F, Deschamps P, Junier T, Junier P, Bindschedler S. 2019. Comparison of the plant growth-promotion performance of a consortium of Bacilli inoculated as endospores or as vegetative cells. FEMS Microbol Ecol [Internet]. [cited 16 January 2020]; 95(11):1-14. Available from: https://doi.org/10.1093/femsec/fiz147.
3. Adesemoye AO, Torbert HA, Kloepper JW. 2009. Plant growth-promoting rhizobacteria allow reduced application rates of chemical fertilizers. Microb Ecol [Internet]. [cited 17 January 2020]; 58(4):921-929. Available from: https://www.ncbi.nlm.nih.gov/pubmed/19466478.
4. Aktar MW, Sengupta D, Chowdhury A. 2009. Impact of pesticides use in agriculture: their benefits and hazards. Interdiscip Toxicol [Internet]. [cited 17 January 2020]; 2(1):1-12. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2984095/.
5. Islam S, Akanda AM, Prova A, Islam MT, Hossain MM. 2016. Isolation and identification of plant growth promoting rhizobacteria from cucumber rhizosphere and their effect on plant growth promotion and disease suppression. Front Microbiol [Internet]. [cited 12 January 2020]; 6:1-12. Available from: https://www.ncbi.nlm.nih.gov/pubmed/26869996.

6. Kandel SL, Firrincieli A, Joubert PM, Okubara PA, Leston ND, McGeorge KM, Mugnozza GS, Harfouche A, Kim SH, Doty SL. 2017. An in vitro study of bio-control and plant growth promotion potential of Salicaceae endophytes. Front Microbiol [Internet]. [cited 17 January 2020]; 8:1-16. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5347143/.
7. Kumar A, Kumar A, Devi S, Patil S, Payal C, Negi S. 2012. Isolation, screening and characterization of bacteria from rhizospheric soils for different plant growth promotion (PGP) activities: an in vitro study. Recent Res Sci Technol [Internet]. [cited 15 January 2020]; 4(1):1-5. Available from: https://pdfs.semanticscholar.org/e1b1/a4df85235581b5b094b82ac4b88f2ca08a59.pdf.
8. Carvalhais LC, Dennis PG, Fan B, Fedoseyenko D, Kierul K, Becker A, von Wiren N, Borriss R. 2013. Linking plant nutritional status to plant-microbe interactions. PLoS One [Internet]. [cited 12 January 2020]; 8(7):1-13. Available from: https://www.ncbi.nlm.nih.gov/pubmed/23874669.
9. Cherif-Silini H, Silini A, Yahiaoui B, Ouzari I, Boudabous A. 2016. Phylogenetic and plant-growth-promoting characteristics of bacillus isolated from the wheat rhizosphere. Ann Microbiol [Internet]. [cited 12 January 2020]; 66(3):1-10. Available from: https://pubag.nal.usda.gov/catalog/5479262.
10. Saeid A, Prochownik E, Dobrowolska-Iwanek J. 2018. Phosphorus solubilization by Bacillus species. Molecules [Internet]. [cited 12 January 2020]; 23(11):1-18. Available from: https://www.ncbi.nlm.nih.gov/pubmed/30404208.

11. Jamal Q, Seong LY, Deok JH, Young KK. 2018. Effect of plant growth-promoting bacteria *Bacillus amylliquefaciens* Y1 on soil properties, pepper seedling growth, rhizosphere bacterial flora and soil enzymes. Plant Protect Sci [Internet]. [cited 7 April 2020]; 54:129-137. Available from: https://www.agriculturejournals.cz/web/pps.htm?type=article&id=154_2016-PPS.
12. Lopes R, Tsui S, Goncalves P, de Queiroz MV. 2018. A look into a multifunctional toolbox: endophytic *Bacillus* species provide broad and underexploited benefits for plants. World J Microbiol Biotechnol [Internet]. [cited 15 January 2020]; 34(7):1-10. Available from: https://doi.org/10.1007/s11274-018-2479-7.
13. Saxena AK, Kumar M, Chakdar H, Anuroopa N, Bagyaraj DJ. 2020. Bacillus species in soil as a natural resource for plant health and nutrition. J Appl Microbiol [Internet]. [cited 7 January 2020]; 1-12. Available from: https://doi.org/10.1111/JAM.14506.
14. Musgrave ME. 2000. Realizing the potential of rapid-cycling Brassica as a model system for use in plant biology research. J Plant Growth Regul [Internet]. [cited 8 February 2020]; 19(3):314-325. Available from: https://link-springer-com.ezproxy.mtsu.edu/article/10.1007%2Fs003440000036.
15. Raddadi N, Cherif A, Boudabous A, Daffonchio D. 2008. Screening of plant growth promoting traits of Bacillus thuringiensis. Ann Microbiol [Internet]. [cited 12 January 2020]; 58(1):47-52. Available from: https://link.springer.com/article/10.1007%2FBF03179444.

16. Ma J, Wang C, Wang H, Liu K, Zhang T, Yao L, Zhao Z, Du B, Ding Y. 2018. Analysis of the complete genome sequence of *Bacillus atrophaeus* GQJK17 reveals its biocontrol characteristics as a plant growth-promoting rhizobacterium. Biomed Res Int [Internet]. [cited 15 January 2020]; 2018:1-9. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6038694/.
17. Rajaofera MJN, Wang Y, Jatoi ZA, Jin P, Cui H, Lin C, Miao W. 2020. Bacillus atrophaeus HAB-5 secretion metabolites preventing occurrence of systemic diseases in tobacco plant. Eur J Plant Pathol [Internet]. [cited 12 January 2020]; 156(1):158-172. Available from: https://link.springer.com/article/10.1007/s10658-019-01873-1.
18. Chen Y, Yan F, Chai Y, Liu H, Kolter R, Losick R, Guo JH. 2013. Biocontrol of tomato wilt disease by Bacillus subtilis isolates from natural environments depends on conserved genes mediating biofilm formation. Environ Microbiol [Internet]. [cited 5 February 2020]; 15(3):848-864. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3904073/.
19. Subramoni S, Nathoo N, Klimov E, Yuan ZC. 2014. Agrobacterium tumefaciens responses to plant-derived signaling molecules. Front Plant Sci [Internet]. [cited 10 February 2020]; 5:1-12. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4086400/.

20. Pérez-Miranda S, Cabirol N, George-Téllez R, Zamudio-Rivera LS, Fernández FJ. 2007. O-CAS, a fast and universal method for siderophore detection. J Microbiol Methods [Internet]. [cited 8 February 2020]; 70:127-131. Available from: https://www-sciencedirect-com.ezproxy.mtsu.edu/science/article/pii/S0167701207001315.
21. Gaby JC, Buckley DH. 2012. A comprehensive evaluation of PCR primers to amplify the *nifH* gene of nitrogenase. PLoS One [Internet]. [cited 3 October 2020]; 7(7):1-12. Available from: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0042149#s1.

Appendix A:

Length of *Brassica rapa* After Four Weeks

Control (cm)	***Bacillus thuringiensis* (cm)**	***Bacillus atrophaeus* (cm)**
64	117.6	137
19.8	116.8	63.5
117	49.8	133
85.6	9	107.5
40.5	69	122
23.7	41	134
91.4	23.5	133
74.3	64	33
2.9	15.5	84
2.4	99.5	47
16.2	76	
70.7		
42.1		

Appendix B:

Dry Mass of *Brassica rapa* After Four Weeks

Control (g)	***Bacillus thuringiensis* (g)**	***Bacillus atrophaeus* (g)**
0	0.86	1.08
0	0.07	0.39
0.64	0.63	1.12
0.12	0.22	0.8
0.14	0.15	0.98
0.2	1.38	0.56
1.08	0.3	1.6
0.63	0.64	0.25
0.56	0.8	0.19
0.57	0.09	1.65
0.14	0.45	
0.61		
0.37		

Appendix C:

O-CAS Media

O-CAS: Chrome Azurol S (CAS)…….60.5 mg
hexadecyltrimethyl ammonium bromide (HDTMA)…..72.9 mg
Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES)…….30.24 g
Agarose……...9 g
1 mM $FeCl_3 \cdot 6H_2O$ in 10 mM HCl 10 mL

All media components were purchased from IBI Scientific, through Sigma-Aldrich.

Appendix D:

Buffers

PBS: NaCl……....8.0 g
KCl……..0.2 g
Na_2HPO_4……...1.15 g
KH_2PO_4…….…..0.2 g
dH_2O……..to 1 L

All PBS reagents were purchased from Fisher Scientific (Fair Lawn, NJ).

10 X TAE: Tris base……..9.6 g
0.2 M EDTA…….10 mL
glacial acetic acid….2.3 mL
dH_2O……to 100 mL

Tris base was purchased from BioRad (Hercules, CA). EDTA was purchased from IBI Scientific, through Sigma-Aldrich. Glacial acetic acid was purchased from Acros, through Fisher Scientific.