Characterisation of Novel Seed Endophytes in *Medicago sativa*

by

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1. Abstract

The microbiome of plants is essential in their development and health. Seed endophytes are microorganisms that live within the seeds of plants, the majority of which are believed to be non-pathogenic, although more research is needed to better understand the function of seed endophytes. They are present from the beginning growth stages and play many important roles. A variety of bacterial species have been found as seed endophytes. They have also been found to have common functions for colonization, benefiting plant growth, and antifungal properties. In this project, seed endophytes were isolated from surface sterilized *Medicago sativa* seeds on selective media for potassium solubilization, actinomycetes, and nitrogen free media. Sterilized seeds were germinated and planted in Yosida agar. The sterilized plants were then inoculated with the seed endophyte isolates and uptake of the isolates into the plant roots was measured. The methods were improved, germinating the seeds in sterile water often led to self toxicity and the seeds germinated at a much higher rate using the Murashige and Skoog Medium. Several

sources of contamination within the protocol were found and eliminated. The isolates also underwent DNA extraction and colony PCR for 16S rRNA gene sequencing. Using this method a *Pseudomonas* species was identified. Seed endophytes from *Medicago sativa* were characterized.

2. Introduction

2.1 Members of Seed Endophyte Communities

Seed endophytes are microorganisms that can colonize the seeds of plants without causing damage to the plant (Truyens et al., 2014). These microorganisms can be beneficial to the seeds in many different ways. A great variety of organisms colonize seeds. One hundred thirty-one bacterial genera have been found as seed endophytes, according to a meta-analysis by Truyens and colleagues in 2014 (Truyens et al., 2014). This included bacteria identified using many different methods such as 16S rRNA sequencing, which most commonly identified the phyla *Frimicutes*, *Actinobacteria*, and the genera *Pseudomonas*, *Enterobacter*, *Methylobacteria*, *Pantoea*, and *Sphingomonas* (Ferreira et al. 2008, Mastretta et al., 2009, Johnston-Monje and Raizada 2011, Liu et al., 2012). 16S rRNA sequencing is the most common genetic tool of phylogeny and taxonomy, it looks at the 16S rRNA gene is found in all bacteria and archaea, is large enough for bioinformatics and the function hasn't changed (Janda and Abbott, 2007). PCR DGGE was used to find *Stenotrophomonas, Ochrobactrum, Pseudomonas, and Enterobacter* species (Hardoim et al., 2012). *Bacillus, Pseudomonas, Burkholderia, and Curtobacterium* were isolated from seeds using fatty acid methyl ester profiles (Vega et al. 2005, Graner et al., 2003). Overall this meta-analysis found that the most common phyla present in bacterial seed endophytes are *Proteobacteria, Actinobacteria, and Frimcutes* across 25 plant species (Truyens

et al., 2014). A core microbiota of seed endophytes is conserved in maize and its ancestors from Mexico to Canada (Johnston-Monje and Raizada, 2011).

A study of fungal seed endophytes identified *Hormonema, Beauveria, Cladosporium, Geopyxis, Geomyces, and Sarcinomyces* from *Pinus monticola* seeds (Ganley and Newcombe, 2006). Pini and colleagues looked at the bacterial communities in *Medicago sativa* and found that *Alphaproteobacteria* dominated the plant tissues, but no bands of 16S rRNA were recovered from surface-sterilized seeds (Pini et al., 2012). MALDI-TOF has been used to find the phyla *Proteobacteria, Actinobacteria, Frimicutes,* and *Bacteroidetes* within surface sterilized *Medicago sativa* seeds (Lopez et al., 2017).

2.2 Vertical Transmission of Endophytes

Johnston-Monje and Raizada found that the conserved core microbiota is vertically transmitted between generations (Johnston-Monje and Raizada, 2011). Endophytes can enter seeds in several ways. Vascular connections to the parent plant can act as a path for endophytes to get into seed endosperm. Shoot meristems which can develop into ovules can be colonized by endophytes causing the seeds to be colonized (Truyens et al., 2014). This allows plants to pass beneficial bacteria onto their offspring which might help maintain the stability of endophyte communities. The endophytes passed down have been shown to be shaped by environmental stressors experienced by the parent plant (Truyens et al., 2014). GFP gene tagging has been used to show the transfer of *Pantoea agglomerans* from inoculated seeds to seedlings in *Eucalyptus* (Ferreria et al., 2008). Endophytes from seeds were also found in the same switchblade plants a year later (Gagne-Bourgue et al., 2012). Mitter and colleagues found that by adding *Paraburkholderia phytofirmans* to the plants' flowers it would then be included in the microbiome of that plant's progeny seeds (Mitter et al., 2017).

2.3 Location of Endophytes within the Seed

Endophytes have been found in the seed coat, crease tissue, and endosperm (Robinson et al., 2016). FISH has also been used to detect bacteria along the cell walls inside of seeds (Compant et al., 2011). They have also been found in the intercellular spaces of the root cortex and vascular system (Puente et al., 2009). Several different endophyte isolates were found using GFP throughout the vascular system (Johnston-Monje and Raizada, 2011).

2.4 Seed Endophyte Functions

Metagenomic and bioinformatics studies have looked at bacterial endophyte genomes for common genes and functions. Current evidence does not suggest that endophytes are not specific to a plant host, so it makes sense that there is a general strategy for host colonization. Prominent features found across many different species were flagella, plant-polymer degrading enzymes, protein secretion systems, iron storage, quorum sensing, detoxification of reactive oxygen species, nitrogen fixation and denitrification, transporters, transcriptional regulators, redox potential maintenance (Sessisch et al., 2012, Ali et al., 2014). Johnston-Monje and Raizada tested isolated seed endophytes for similar traits and found that the ones most commonly observed were phosphate solubilization and the production of acetoin/butanediol (Johnston-Monje and Raizada, 2011). As discussed below, many of these can benefit plant growth, such as iron storage, nitrogen fixation, and phosphate solubilization. Others may be useful for colonizing the plants.

Interestingly, all known protein secretion systems other than type III were found in endophytes. The type III section system is often used by pathogenic bacteria to control host response (Sessisch et al., 2012, Ali et al., 2014). Several different plant-ployer dragging enzymes have been found to help endophytes enter and move through the plant. Similarly, flagella have

been consistently found in seed endophytes; motility may likely be helpful for colonization. The presence of enzymes used for the detoxification of reactive oxygen species (ROS) have been found. Plants produce ROS when stressed, or as a response to colonization by microorganisms, so it seems these enzymes might help endophytes to survive (Sessisch et al., 2012, Ali et al., 2014). Transporter proteins for the MFS and ABC transport systems have been found in endophytes, which may allow them to take up nutrients from the plants (Ali et al., 2014). Many different translational regulators were also found, mostly global regulators that affect cellular metabolism, which may help respond to nutrients and the environment in the host (Ali et al., 2014).

2.5 Plant Growth Promotion Among Seed Endophytes

Seed endophytes can provide many different benefits to plants. Several studies have shown that they can stimulate plant growth. Many different plant growth-promoting traits have been found in seed endophyte isolates, such as indole-3-acetic acid (IAA) production, phosphate solubilization, siderophores production, nitrogen fixation, and 1-aminocyclopropane -1-carboxylate (ACC) deaminase in *Bacillus* species isolated from tomato seeds (Xu et al., 2014). IAA is naturally produced by plants to help with cell growth but can also be made by bacterial symbionts. It promotes plant growth by altering cell orientation, cell elongation and helping with organ development (Labeeuw et al., 2016). Plant-associated bacteria release organic acids, which solubilize phosphates into ortho-phosphate. Phosphate in the soil is insoluble making it unavailable to plants but when microbes convert it to soluble phosphate then it can be used by the plants (Otenio et al., 2015). Siderophores are used to uptake iron from the environment, which the plants can then use (Lurthy et al., 2020). Plants produce ethylene under stress conditions. Bacteria with the enzyme ACC deaminase can lower ethylene levels by

hydrolyzing ACC, the precursor of ethylene, into ammonia and α-ketobutyrate, which can make the plant more resistant to stress (Gupta and Pandey, 2019). Some bacteria can produce the enzyme dinitrogenase, which reduces N2 to fixed inorganic nitrogen, putting the nitrogen in a form that the plants can use (Li et al, 2017). One study found that when cactus seeds were inoculated with isolated source endophytic bacteria, they grew for a year with no fertilization getting all of their nutrients from endophytes, the same seeds sterilized with antibiotics they were unable to grow, but if sterilized seeds were inoculated then growth resumed (Puente et al., 2009). *Bacillus pseudomycoides* strain BM1 was isolated from the rhizosphere of alfalfa and has the ability to produce IAA, siderophores, lipase, cellulase, and pectinase. It was also found that inoculating the seeds with BM1 increased root and shoot length by up to 21.43% (Knezevic et al., 2021).

Seed endophytes have also been shown to have antifungal properties. Studies have found well-known toxins from seed endophyte isolates such as surfactins (C 13, 14, 15), iturins, mycobacillin (Gagne-Bourgue et al., 2012). Iturnin and surfactins are lipopeptides. They primarily work by binding to the cell membrane, forming ion channel-like complexes, and releasing ions from cells, causing cell death (Tran et al., 2022). Surfactins have been shown to have antifungal properties against several plant pathogens in particular (Vitullo et al., 2012). Mycobacillin binds to ATP transports on the plasma membrane, resulting in over-release and cell starvation (Tran et al., 2022). Rice seed endophyte isolates were found to inhibit the growth of three plant pathogens, *Curvularia, Fusarium oxysporum,* and *Pythium ultimum* (Ruiz et al, 2011). The isolated endophyte *Bacillus pseudomycoides* strain BM1 inhibited *F. graminearum, F. proliferatum,* and *F. oxysporum* in vitro. When seeds were inoculated, the number of seedlings infected *with F. oxysporum* were reduced by 25.41% (Knezevic et al., 2021).

2.6 Alfalfa as a Model Organism

Alfalfa (*Medicago sativa*) is a legume grown in temperate climates around the world and is often used in crop rotation. It is useful in crop rotation due to its symbiotic nitrogen fixing bacteria which can help add organic nitrogen to the soil (Pini et al., 2012). Alfalfa has also been investigated as a possible crop for biomass feedstock to be turned into bioenergy (Sanderson and Adler, 2008). Alfalfa has also been used in land restoration due to the deep taproot and nutrient cycling abilities. It is often used as a model species for studies of bacterial symbionts of plants, especially *Sinorhizobium meliloti* (Pini et al., 2012).

2. 7 Summary

The microbiome of plants is essential in their development and health. Seed endophytes are microorganisms that live within the seeds of plants, the majority of which are believed to be non-pathogenic although more research is needed to better understand the function of seed endophytes. They are present from the beginning growth stages and play many important roles. They have been found to be vertically transmitted between generations. A variety of bacterial species have been found as seed endophytes. They have also been found to have common functions for colonization, benefiting plant growth, and antifungal properties.

3. Objectives

The objectives of this experiment were to isolate and then characterize seed endophytes from *Medicago sativa*. There are many remaining questions about the functions of seed endophytes. This experiment used both molecular and culture based methods to try and address some of those questions. Molecular methods were used to genetically identify the isolates. Culture based methods were used to try and quantify the uptake of each isolate into sterile plants.

4. Materials and Methods

4.1 Biological Materials

Alfalfa Seeds (*Medicago sativa*), Isolates 1-6

4.2 Other Materials

Media Ingredients

Magnesium sulfate ($MgSO_4$), Calcium Carbonate ($CaCO_3$), Potassium Feldspar ($AlKO_8Si_3$), Dextrose ($C_6H_{12}O_6$), Ferric Chloride ($FeCl_3$), Agarose ($C_24H_38O_{19}$), Sucrose ($C_{12}H_{22}O_{11}$), Magnesium Sulfate ($MgSO_4$), Sodium Chloride (NaCl), Ferrous Sulfate ($FeSO_4$), Sodium Molybdate (Na_2MoO_4), Difco Bacto Actinomycete Isolation Agar dehydrated, Ammonium nitrite (NH_4NO_2), Potassium sulfate (K_2SO_4), Calcium chloride ($CaCl_2$), Manganese(II) chloride ($MnCl_2$), $(NH_4)Mo_7O_2$, Boric acid (H_3BO_3), Copper sulfate (C_4SO_4), Potassium phosphate (KH_2PO_4), Ethylenediaminetetraacetic acid (EDTA), LB broth mix, TSA mix, Murashige and Skoog Medium.

Lab Equipment

Distilled water, Bleach, 1.5ml Eppendorf Tubes, Sterile Petri dishes, 70% ethanol, sterile 1X Phosphate Buffered Saline, p20/p200/p1000 pipettes and sterile tips, PCR tubes, agarose, 1X TAE, gel box, 15ml tubes, sterile mortar, mason jars, Autoclave, heat block, 28^0C incubator, Thermocycler, Spectrophotometer, grow lights, nanodrop, and qubit.

Molecular reagents

Invitrogen 27F, invitrogen 1492R, Master Mix, DNA Ladder, DNA loading dye, Promega Wizard SV Gel and PCR cleanup kit, Qiagen DNeasy Ultra Clean Microbial kit, ITS 4, ITS 9, and Qubit reagents.

Table 1: Primers used in these experiments

Primer Name	Sequence
27F	5'-AGAGTTTGATCCTGGCTCAG-3’
1492R	5'-AGAGTTTGATCCTGGCTCAG-3’
ITS4	5’-TCC TCC GCT TAT TGA TAT GC-3’
ITS9	5’-GAA CGC AGC RAA IIG YGA-3’

4.3 Techniques

4.3.1 Culture-based Methods

4.3.1.1 Media Preparation- Alexsandrow’s Media/ Jensen’s Media/ Actinomycetes Media

1000ml of Alexsandrow’s Media (K solubilization) was made using 0.5g of Magnesium Sulfate, 0.1g of Calcium Carbonate, 2g of Potassium Feldspar, 5g of Dextrose, 0.005g Ferric Chloride, 20g of Agar. The 2g of Calcium Phosphate was left out because there was none in the lab. 1000 ml of Jesens Media (Nitrogen Free) was made using 20g of Sucrose, 1g of Potassium Phosphate, 0.5g of Magnesium Sulfate, 0.5g of Sodium Chloride, 0.1g of Ferrous Sulfate, 0.005g of Sodium Molybdate, 2g of Calcium Carbonate, and 15g of Agar (Jesen, 1941). Potassium Phosphate was substituted for dipotassium Sulfate. 1000ml of Actinomycetes Media was made using 22g of Difco Bacto Actinomycete Isolation Agar dehydrated, which contains 2g of Sodium Caseinate, 0.1g of Asparagine, 4g of Sodium Propionate, 0.5g of Dipotassium Phosphate, 0.1g of Magnesium Sulfate, 0.001g of Ferrous Sulfate, and 15g Agar. All three media were made by dissolving the ingredients listed above in 1000ml of distilled water on a hotplate, once dissolved they were placed within the autoclave (Buxton 8200) and the autoclave was set to the liquid cycle. Once the autoclave cycle finished they were all allowed to cool. Once cooled enough to touch the media was poured into sterile labeled petri dishes in the sterile media hood. Once the

plates were cooled and solidified they were placed into lalbed sleeves and stored in the refrigerator. All other media for this experiment was prepared the same way.

4.3.1.2 Seed Surface Sterilization and isolation of organisms

Fresh 10% bleach solution was made. About 30 Alfalfa seeds were added to a sterile 1.5 ml eppendorf tube. 70% ethanol was added to the tube to cover the seeds and left at room temperature for five minutes. The ethanol was poured off and 10% bleach solution was added to cover the seeds and left at room temperature for five minutes. The bleach solution was poured off and the seeds were rinsed five times with sterile water. 1X PBS was added to the tube to cover the seeds and they were left overnight at room temperature. The next day a sterile mortar was used to homogenize the seeds. A p200 was used to pipet 100 µl of homogenized material onto each plate. Three plates of each media (Alexandrow's, Jensen's, and Acintomyeyete) were used. Each of these plates plus one control per media were placed in a 28^0C incubator.

Six days later there was growth on all of the plates inoculated with homogenized seeds and no growth on the control plates. One organism was isolated from the Actinomycete media (Isolate 1). Two organisms were isolated from the Jensen's media plates (Isolate 2 and 3). Two organisms were isolated from the Alexdanrow's Media plates (Isolates 4 and 5). The organisms were isolated by touching a sterile loop to the colony and then performing a quadrant streak on the corresponding media. Each isolate was quadrant streaked twice. The new plates were incubated at 28^0C. The original plates were also incubated in order to look for slow growing organisms.

4.3.1.3 Media preparation- Yoshida Agar

Stock solutions were prepared for the Yoshida Agar in 50ml of distilled water. 2.84g of NH_4NO_2 for a 50X solution, 2.18g of K_2SO_4 for a 50X solution, 3.675g of $CaCl_2$ for a 50X

solution, 6.16g of $MgSO_4$ for a 50X solution, 0.204g of $MnCl_2$ for a 250X solution, 0.01g of $(NH_4)Mo_7O_2$ for a 250X solution, 0.143g of H_3BO_3 for a 250X solution, 0.003g C_4SO_4 for a 250X solution, 1.07g KH_2PO_4 for a 250X solution, and 1.55g of EDTA for a 250X solution. Regular EDTA was substituted for Ferric EDTA and KH_2PO_4 was substituted for H_2PO_4, $ZnSO_4$ was left out.

4.3.1.4 Seed Sterilization and Germination

A 3% bleach solution was made. Alfalfa Seeds were added to a sterile 1.5ml eppendorf tube. Sterile water was added to the level of the seeds. The tube was then heated on a 40^0C heat block (VWR scientific products Select heatblock) for thirty minutes. The water was removed and 70% ethanol was added to cover the seeds and left at room temperature for one minute. The ethanol was removed and the freshly prepared 3% bleach was added to cover the seeds and left at room temperature for five minutes. The bleach was removed and the seeds were rinsed with sterile water. The seeds were left in a small approximately 8 ml petri dish with sterile water and covered with foil for two days then left to sit under the grow lights for one day.

The original sterilized seeds were not germinating, possibly due to self toxicity. The seed sterilization was performed again as above but this time they were put in a normal approximately 20 ml sized petri dish at the end with sterile water.

4.3.1.5 Colonization Experiment 1

LB broth was made using the mix. 5ml of LB broth was added to sterilized 15ml tubes and then inoculated with each of the isolates. They were incubated in the 28^0C incubator for two days. The optical density of each isolate was measured using the spectrophotometer. They were then standardized to 0.01 OD. The Yoshida agar was made by using 1ml of 50X stock solutions and 200µl of 250X stock solutions and the media preparation as described above. Three plates

per isolate were prepared. Each plate was prepared by using sterilized tweezers to place one germinated seed on each plate. 100µl of each standardized isolate was pipetted onto the corresponding plant focusing on the root area. The plants were then placed under the grow lights for one week.

Tryptic Soy Agar was made using a mix and the media preparation as described above as a general media for the serial dilutions. Using sterilized tweezers the plants were placed into individual 1.5 ml eppendorf tubes and weighed, the weight was recorded. 500µl of 1X PBS was added to each tube; the tube was then vortexed for thirty seconds and poured off; this was repeated twice. 500µl of 1X PBS was added to each tube and the plant was homogenized with a sterile mortar. Once homogenized a serial dilution with 1X PBS was performed on each plant up to 10^{-6}. 100µl of each dilution was plated onto a TSA plate which was then spread with a sterile loop. The plates were then placed in the incubator at 28^0C. Two days later all of the plates were equally contaminated. A TSA plate and TSA plate with the 1X PBS were placed in the 28^0C incubator.

4.3.1.6 Seed Sterilization and Germination

Germination Agar was made using 2.22g Murashige and Skoog Medium and 5g of agar in 500ml distilled water and using media preparation as described above. Seed sterilization was performed as described above. About ten sterilized seeds were placed on each of the four plates using sterile tweezers. The plates were wrapped in foil and left on the bench for two days. Then the plates were placed under the grow lights for one day.

4.3.1.7 Colonization Experiment 2

Made LB broth and added 5ml to 15ml falcon tubes which were inoculated with the isolates and put in the 28^0C incubator for two days. The Yoshida agar was made again as

described above but put into mason jars that had been sterilized in the autoclave. The isolates in LB broth had the optical density measured and were standardized as described above. The germinated seeds were placed in the mason jars and the rest of the inoculation was performed as described previously. Had two control plants which were placed in the mason jars but not inoculated. The Mason jars were placed under the grow lights for one week and the serial dilutions were performed as described above.

4.3.1.8 Seed Sterilization

The seed sterilization used before germinating seeds was performed but at the end the seeds were homogenized and plated on LB agar. The plates were put in the 28^0C incubator for two days. The same procedure was done with new bleach at 3% and 10%.

The seed sterilization was performed as described above using 3% of the new bleach. Three plates of Murashige and Skoog Medium with 10 sterilized seeds each were placed under the grow lights for three days.

4.3.1.9 Germination Experiment

The seed sterilization was performed as described above using 3% of the new bleach. The isolates were grown in LB broth and standardized to 0.01 optical density using the spectrophotometer as described above. 15ml of each 0.01 OD isolate (other than isolate 4 which did not grow enough in the LB broth) were added to a sterile petri dish and twenty sterilized seeds were added to each dish along with a control petri dish with 15ml sterile water. The petri dishes were then left under the grow lights for one week.

4.3.1.10 Colonization Experiment 3

One 1L of Yoshida was made as described above. Six of the germinated sterilized seeds were placed into their own mason jars with Yoshida agar. Three were left as controls and three

were inoculated with 50 ul of 0.1 OD of isolate 2. The mason jars were left under the grow lights for one week. The plants were weighed and the homogenization and serial dilution were performed again as described above. The plants were homogenized in 200ul 1X PBS and 50ul of each dilution were plated on the LB plates and placed in the 28^0C incubator for two days.

In order to calculate the colony forming units (CFU) per gram the amount of colonies on each serial dilution plate was counted, anything over 300 colonies was considered too numerous to count (TNTC). The amount of CFU in 200ul was calculated by multiplying the amount on plates (amount in 50ul) by four. The CFU per gram was found by dividing the CFU in 200ul by the weight of the plant in grams.

4.3.2 Molecular Methods

4.3.2.1 Colony PCR and PCR Cleanup

After five days when the isolates had grown a colony PCR was performed. 1µl of invitrogen 27F, 1 µl of invitrogen 1492R, 12.5µl of Master Mix (Taq 2X Master Mix NEB #MO270S), 9.5µl dH_2O, were added to six PCR tubes. Each colony was touched using a sterile loop and then the loop was submerged in the corresponding PCR tube. The PCR tubes were put into the thermocycler (Applied biosystems GeneAmp PCR system 9700) and ran with the program: 94^0C- 3 minutes, (94^0C- 1 minute, 48^0C - 30 seconds, 72^0C - 1 minute) x35, 72^0C - 7 minutes, 4^0C. An agarose gel was made using 0.3g of agarose, 60ml of 1x TAE, combined in a 150ml flask and microwaved in thirty second increments until dissolved. The flask was left at room temperature until cooled and then poured into the gel box thing. The ladder was prepared by combining 8µl of dH_2O, 2µl DNA Ladder, 2µl loading dye in a PCR tube. After the cycle had run 5µl of DNA loading dye was added to each tube, and 5µl of each sample were loaded into the gel along with the ladder. The gel box was filled with 1x TAE and at 50 volts for thirty

minutes. Once done the gel was imaged using the UV gel box (Fisher scientific Transilluminator FBTIV-88).

PCR Cleanup was performed using the Promega Wizard SV Gel and PCR cleanup kit. 10µl of PCR product from isolate 2 was used. The kit protocol was followed. The product was sent to Genewiz for sequencing and the results were visualized using Ugene and identified using NCBI BLAST.

4.3.2.2 DNA Extraction

Used Qiagen DNeasy Ultra Clean Microbial kit according to protocol to extract DNA from each of the isolates. Working stocks of ITS 4 and ITS were made. 10µl of Master Mix, 0.5µl of ITS 4, 0.5µl of ITS 9, and 2µl of DNA extraction product were added to each PCR tube. The PCR tubes were added to the thermocycler and run using the program: 95^0C - 3 minutes, (95^0C - 30 seconds, 55^0C - 30 seconds, 72^0C - 30 seconds) x25, 72^0C - 5 minutes, 4^0C. Made and loaded gel as described above. The DNA extraction products were nanodroped (Thermo scientific nanodrop 2000c). The ITS PCR was performed again with a positive and negative control.

4.3.2.3 ITS PCR

A PCR was performed using the DNA extraction product. 0.5 µl of ITS 4, 0.5 µl of ITS 9, and 10µl of Master Mix, were added to 7 PCR tubes. 2µl of DNA extraction product from each isolate was added to the corresponding tube. The PCR tubes were put into the thermocycler (Applied biosystems GeneAmp PCR system 9700) and ran with the program: 95^0C- 3 minutes, (95^0C- 30 seconds, 55^0C - 30 seconds, 72^0C - 30 seconds) x25, 72^0C - 5 minutes, 4^0C. An agarose gel was made using 0.3g of agarose, 60ml of 1x TAE, combined in a 150ml flask and microwaved in thirty second increments until dissolved. The flask was left at room temperature

until cooled and then poured into the gel box. The ladder was prepared by combining 8µl of dH_2O, 2µl DNA Ladder, 2µl loading dye in a PCR tube. After the cycle had run 5µl of DNA loading dye was added to each tube, and 5µl of each sample were loaded into the gel along with the ladder. The gel box was filled with 1x TAE and at 100 volts for twenty minutes. Once done the gel was imaged using the UV gel box (Fisher scientific Transilluminator FBTIV-88).

The exact same protocol was repeated using only a positive and negative control.

4.3.2.4 16S PCR and PCR Cleanup

A PCR was performed using the DNA extraction product. 1.5 µl of invitrogen 27F, 1.5 µl of invitrogen 1492R, 12.5µl of Master Mix, 8.5µl dH_2O, were added to 8 PCR tubes. 1µl of DNA extraction product from each isolate was added to the corresponding tube. The PCR tubes were put into the thermocycler (Applied biosystems GeneAmp PCR system 9700) and ran with the program: 94^0C- 5 minutes, (94^0C- 30 seconds, 58^0C - 30 seconds, 72^0C - 1 minute) x25, 72^0C - 10 minutes, 4^0C. An agarose gel was made using 0.3g of agarose, 60ml of 1x TAE, combined in a 150ml flask and microwaved in thirty second increments until dissolved. The flask was left at room temperature until cooled and then poured into the gel box. The ladder was prepared by combining 8µl of dH_2O, 2µl DNA Ladder, 2µl loading dye in a PCR tube. After the cycle had run 2µl of DNA loading dye was added to each tube, and 5µl of each sample were loaded into the gel along with the ladder. The gel box was filled with 1x TAE and at 75 volts for twenty minutes. Once done the gel was imaged using the UV gel box (Fisher scientific Transilluminator FBTIV-88).

PCR Cleanup was performed using the Promega Wizard SV Gel and PCR cleanup kit. 20µl of PCR product from isolates, 1, 4, 5, and 6 was used. The kit protocol was followed. The PCR cleanup product was nanodropped and then a Qubit was also performed.

The 16S PCR was performed again exactly as described above. A PCR Cleanup was performed using the QIAGEN PCR cleanup kit, eluting in 30µl elution buffer and letting the elution buffer sit for one minute before centrifugation. The products were sent to Genewiz for sequencing and the results were visualized using Ugene and identified using NCBI BLAST.

5. Results

5.1 Isolation of organisms

Six different colonies were isolated from the surface sterilized alfalfa seeds. Isolate 1 grew on the Actinomycetes media, it was a small yellow round shiny colony. Two colonies were isolated from the Jensens Media. Isolate 2 was very small round white colonies, isolate 3 was a larger white fuzzy colony. Two colonies were isolated from the K Solubilization agar. Isolate 4 was a small round white colony, isolate 5 was a large fuzzy white colony with a slightly green center. There was no growth on any of the control plates. 5 days after the initial isolation another slow growing colony was isolated. Isolate 6 grew on the K solubilization agar and had small, tall, hard colonies that were gray with a white coating. These plates can all be seen in Figure 1.

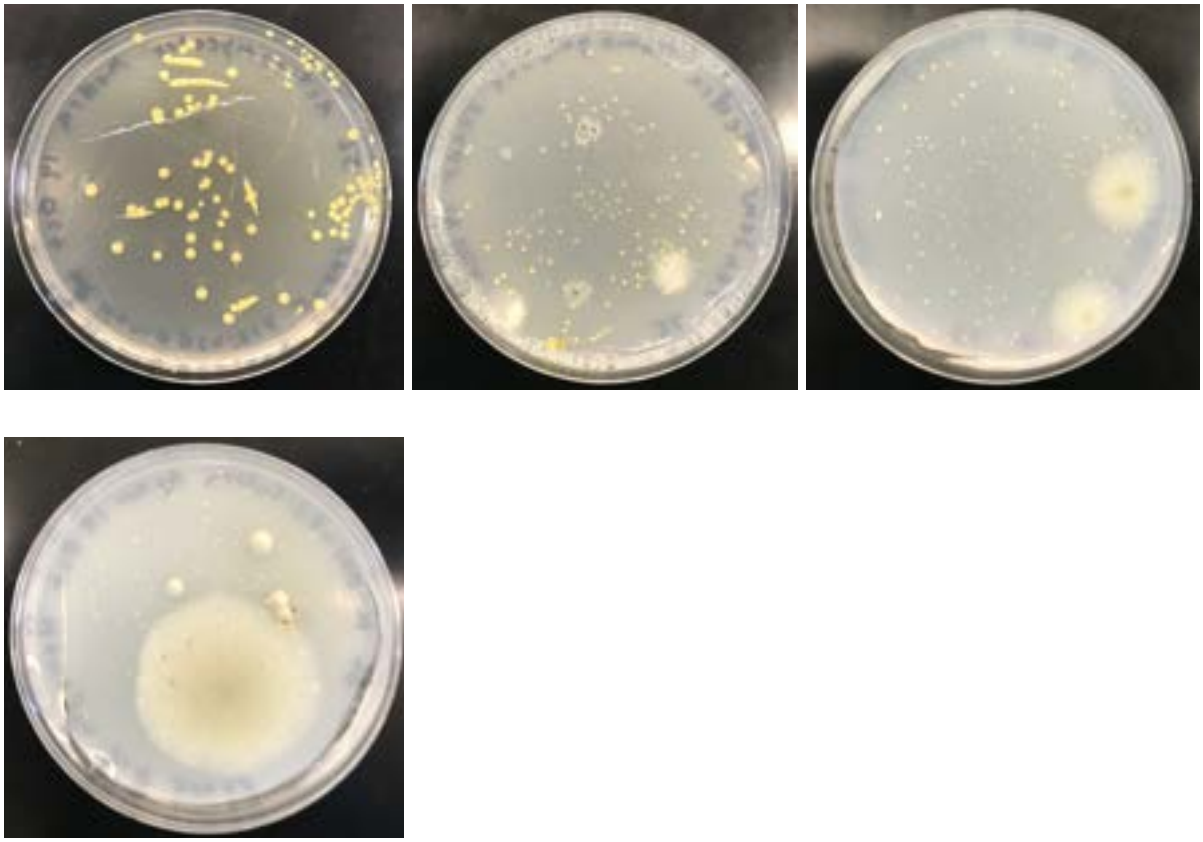


Figure 1: Left to right, Isolate 1 on Actinomycetes agar, Isolates 2 and 3 on Jensen's Media, Isolates 4 and 5 on K Solubilization Agar, Isolate 6 on K solubilization agar.

5.2 Seed Sterilization

After the alfalfa seeds were sterilized they were placed in an 8ml petri dish with sterile water and 4 of the 88 seeds germinated. More alfalfa seeds were sterilized and then placed in a 20ml petri dish and 28 of the 66 seeds germinated (Figure 2).

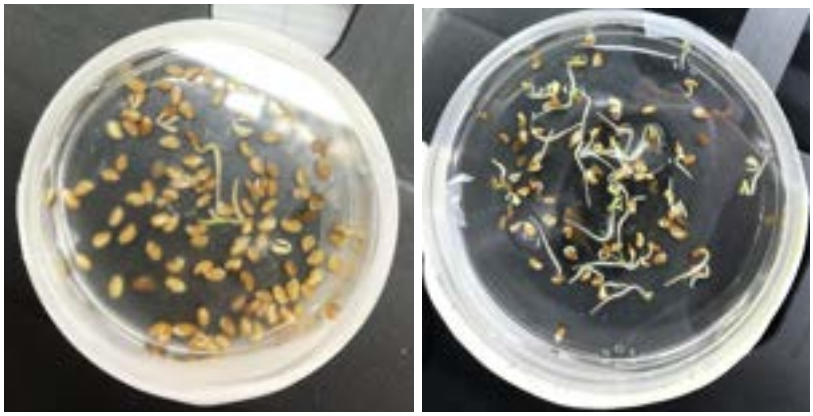


Figure 2: Left, Sterilized alfalfa seeds in an 8ml petri dish with sterilized water after 2 days in tin foil and 4 days under the grow lights. Right, sterilized alfalfa seeds in a 20ml petri dish with sterilized water after 2 days in foil and 1 day under the grow lights.

5.3 Colonization Experiment 1

After one week under the grow lights the inoculated alfalfa sprouts' roots and leaves had grown and the inoculated bacteria could be seen in a halo in the Yoshida agar surrounding the plant roots (Figure 3).

Figure 3: Alfalfa plants grown on yoshida agar and inoculated with isolates 4 and 5 after one week under grow lights.

In the first colonization experiment there was an even amount of contamination growth across all of the plates after two days in the 28^0C incubator. All plates of an isolate 6 replicate are shown as a representation but all of the plates looked very similar across all isolates and replicates (Figure 4). To find the contamination a plain TSA plate was incubated and no growth occurred, but there was growth on the plate with the 1X PBS used.

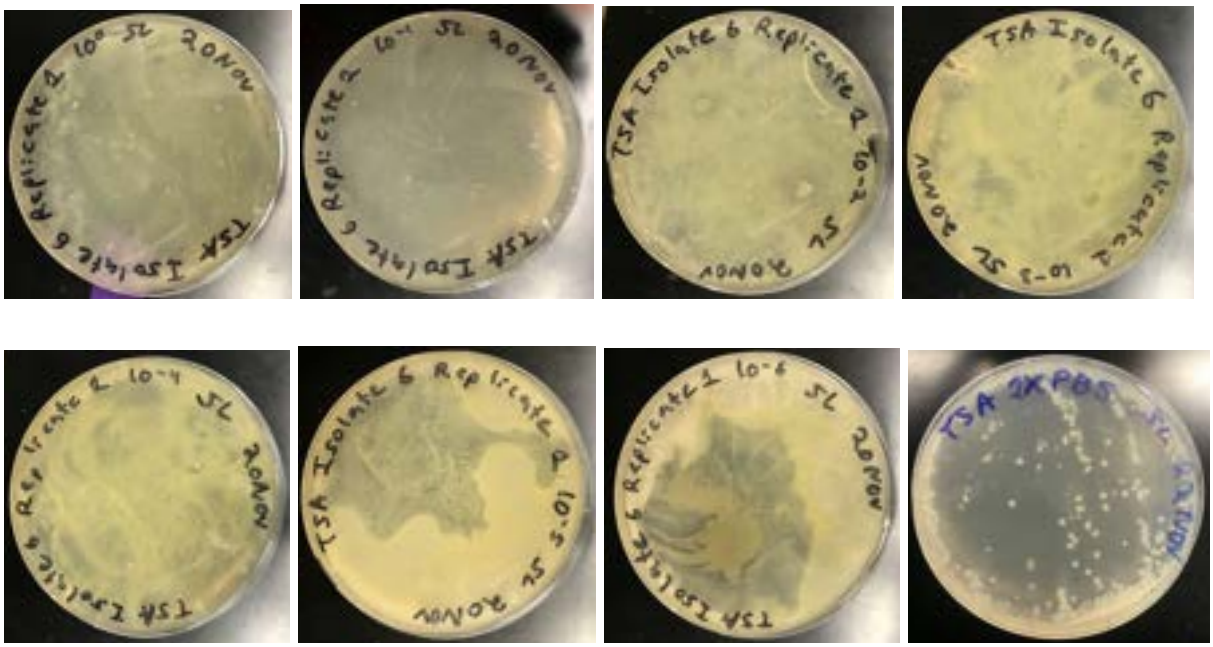


Figure 4: Serial Dilutions of homogenized alfalfa sprouts inoculated with isolate 6 on TSA after two days in the 28ºC incubator and 1X PBS on TSA after one day in the 28ºC incubator.

5.4 Seed Germination

Seeds were germinated using Murashige and Skoog Medium and 88% of the seeds germinated (Figure 5).

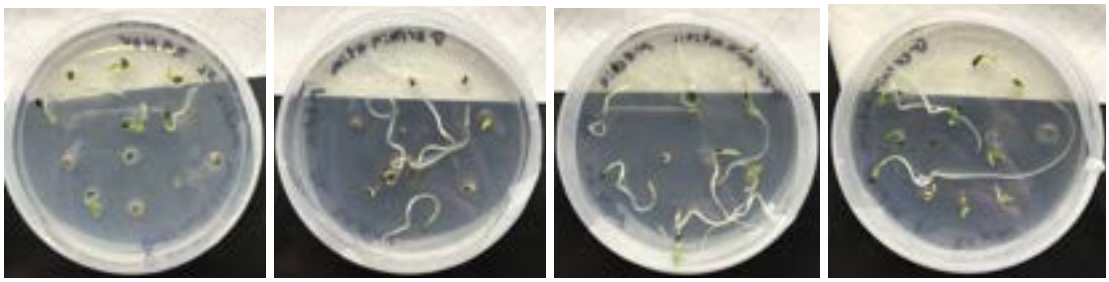


Figure 5: Sterilized alfalfa seeds germinated on Murashige and Skoog Medium after 2 days in tin foil and 3 days under the grow lights.

Table 2: Effectiveness of methods for germinating Alfalfa seeds

Method of Germination	Number of seeds germinated	Total number of seeds	Percent of seeds that germinated
Sterile water in 8ml petri dish	4	88	0.45%
Sterile water in 20ml petri dish	28	66	42%
Murashige and Skoog Medium	39	44	88%

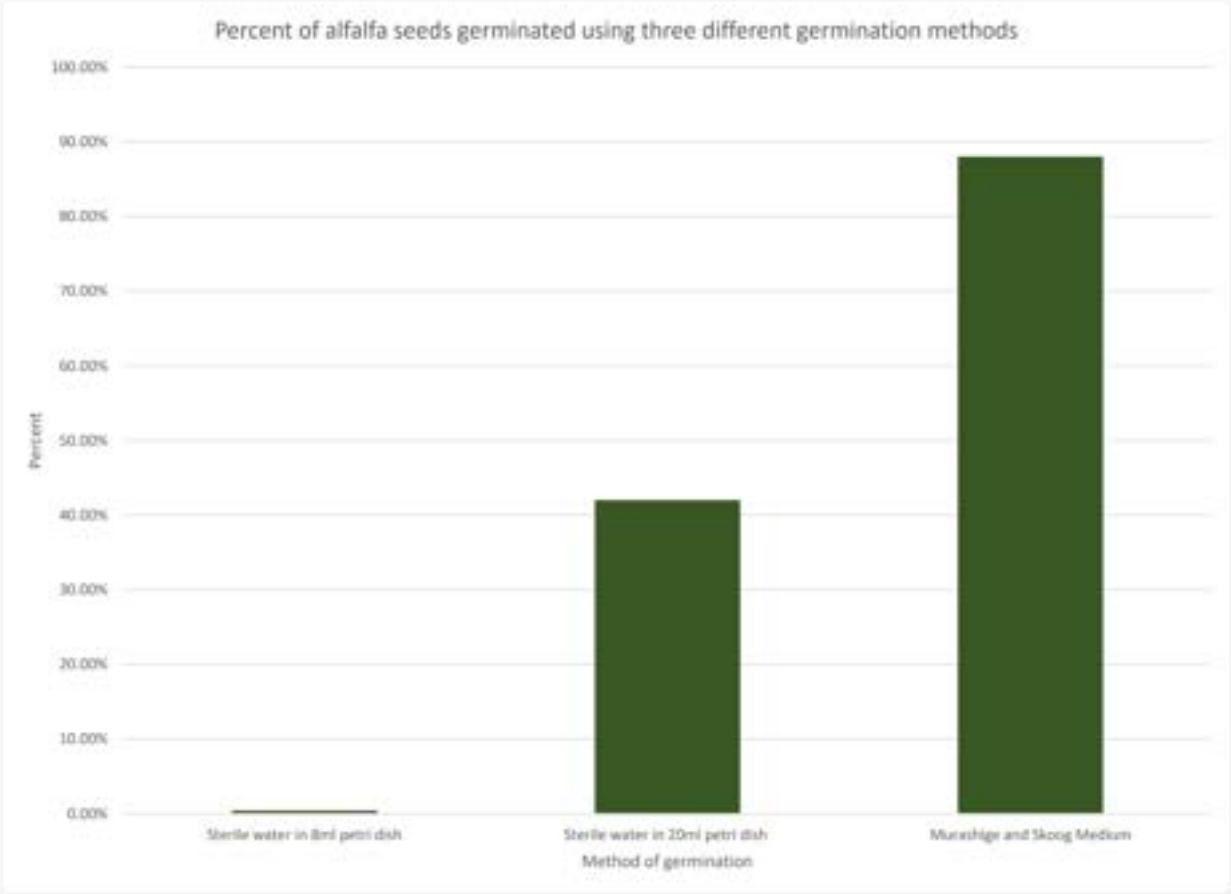


Figure 6: Percent of alfalfa seeds germinated using different methods

5.5 Colonization Experiment 2

In the second colonization experiment there was growth on all of the serial dilution plates including the control. There were multiple colonies with different colony morphology across all plates. The contamination was consistent with the dilutions, there was much more growth on the 10^0 plates than on the 10^6 plates.

5.6 Seed Sterilization

The alfalfa seed sterilization was performed exactly as before but the seeds were immediately homogenized and plated onto LB agar, there was growth on the plates. It was found that the bleach in the lab was one year expired. The serialization was performed again with new bleach at both 3% and 10% and there was no growth for either (Figure 7).

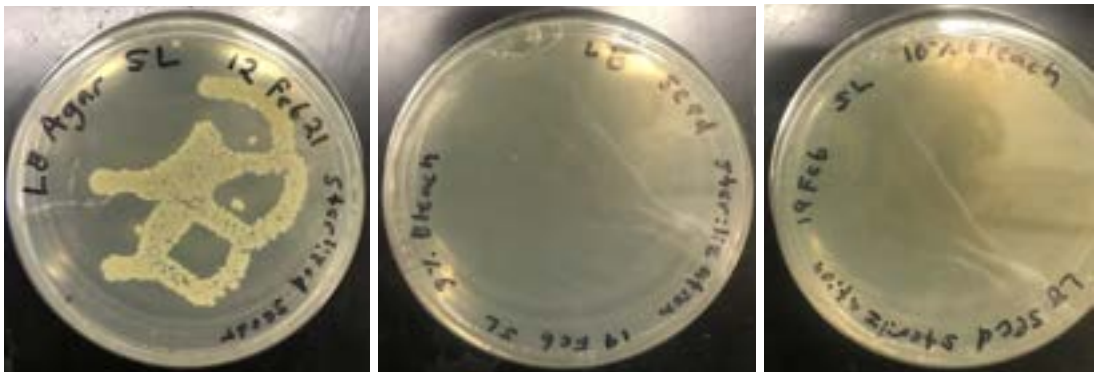


Figure 7: Left to right, homogenized seeds after seed sterilization using original bleach from the lab on LB agar after 2 days in the 28ºC incubator, homogenized seeds after seed sterilization using new bleach at 3% then 10% on LB agar after 2 days in the 28ºC incubator.

5.7 Germination Experiment

Sterilized seeds were germinated in 0.01 OD of each of the isolates under grow lights for one week. There was only germination in the control with sterile water. The sterilized seeds in the inoculants did not germinate (Figure 8).

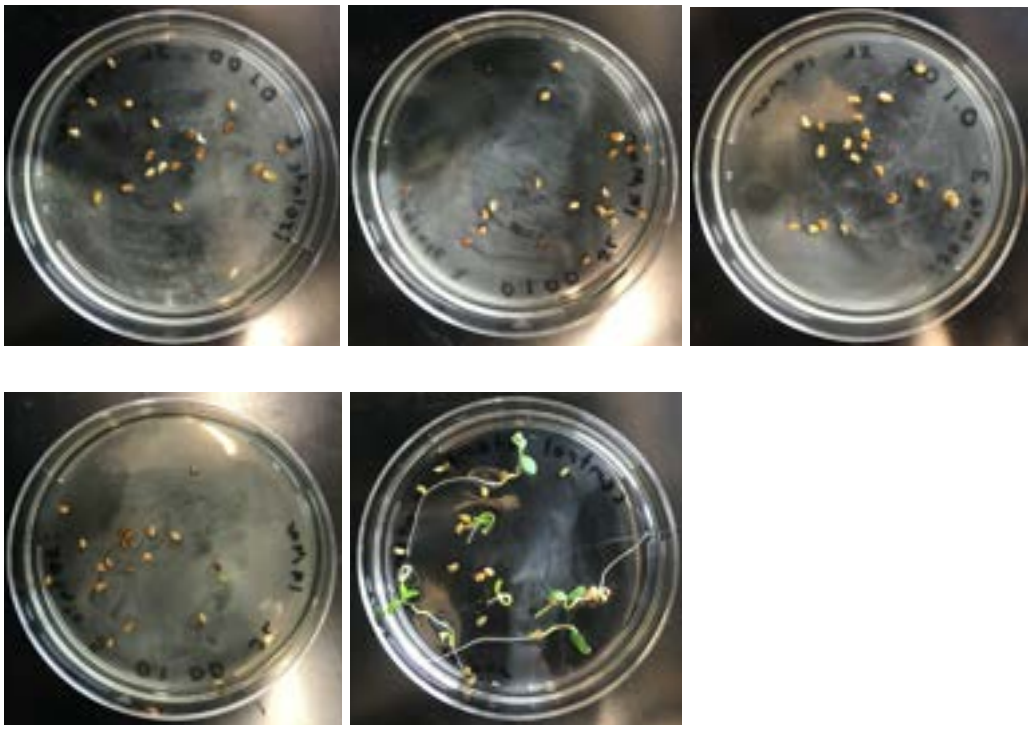
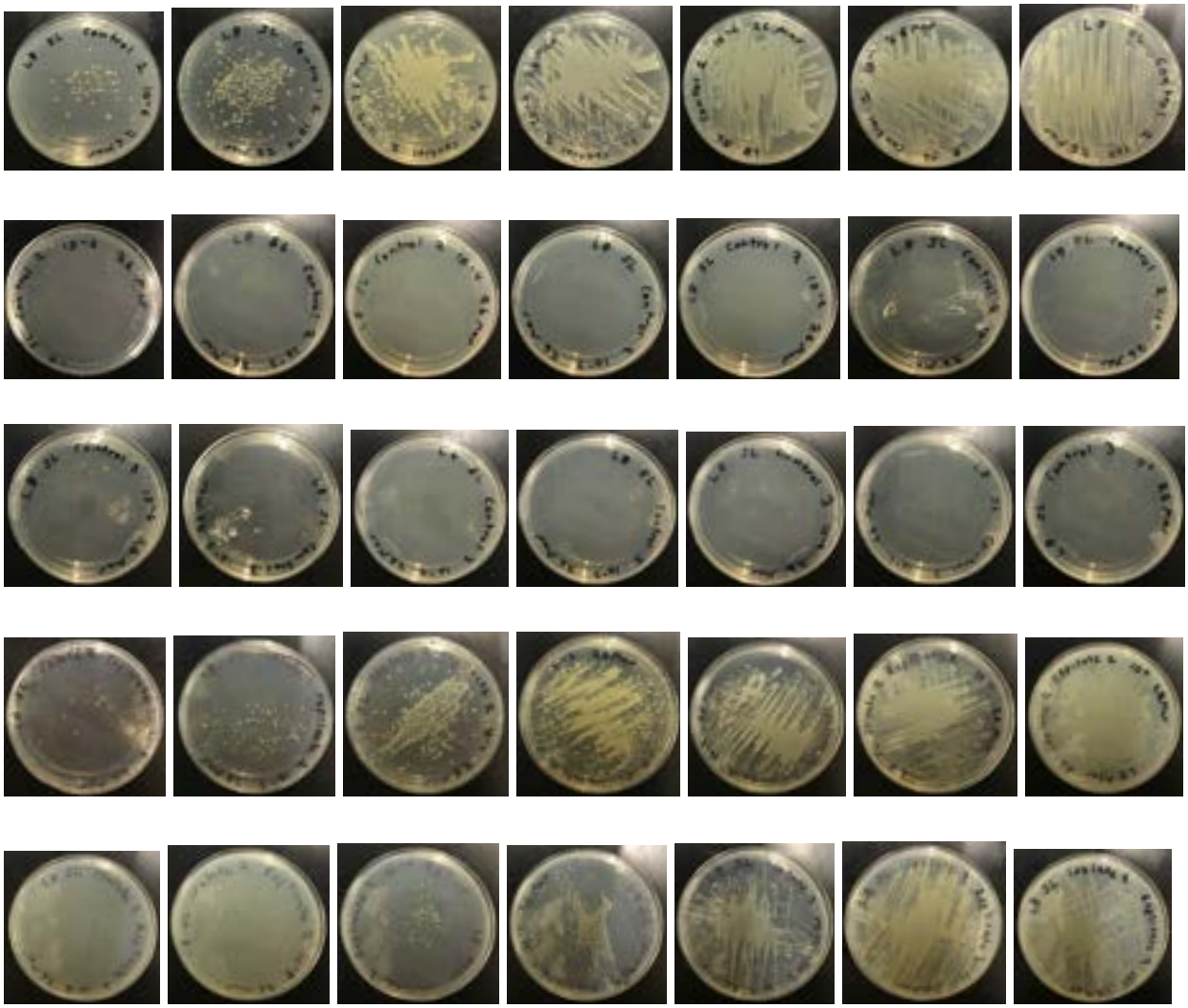


Figure 8: Left to right, 15 ml of isolates 1, 2, 3, 5, at 0.01 OD and control of sterile water. Each plates with 20 sterile alfalfa seeds after 1 week under grow lights

5.8 Colonization Experiment 3

In the third colonization experiment one of the controls got contaminated, the other two had no growth. For isolate 2, replicate 1 had 12 colonies on the 10^{-6} plate and 125 colonies on the 10^{-5} plate, replicate 2 had 18 colonies on the 10^{-5} plate and 178 colonies on the 10^{-4} plate, lastly replicate 3 had 2 colonies on the 10^{-6} plate and 46 colonies on the 10^{-5} plate. This can be seen in Figure 9.



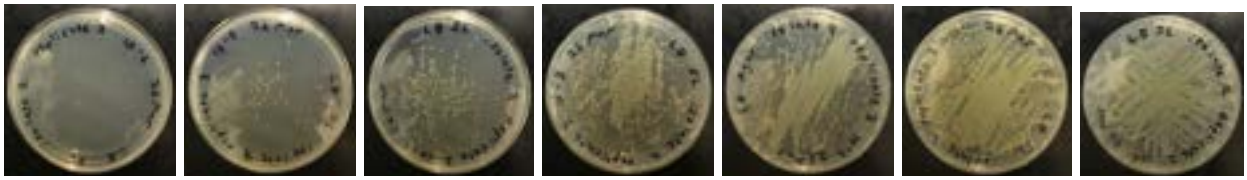


Figure 9: Results of third colonization experiment, serial dilutions on LB agar after 24 hours in 28^0C incubator. From top to bottom, Control replicates 1-3 and Isolate 2 Replicates 1-3. From left to right serial dilutions 10^{-6} - 10^0.

Table 3: Isolate 2 Colony Counts and CFU Calculations

					Colony Counts							CFU Calculations			
Isolated Microbe	Replicate Number	Eppendorf Tube Weight (g)	Eppendorf Tube with Plant	Plant Weight (g)	10^0	10^-1	10^-2	10^-3	10^-4	10^-5	10^-6	CFU on Plate	CFU per 50ul	CFU per 200 uL	CFU per gram
2	1	1.09	1.13	0.04	TNTC	TNTC	TNTC	TNTC	TNTC		125	12	12X10^-6	12,000,000	48000000	1600000000
	2	1.09	1.13	0.04	TNTC	TNTC	TNTC	TNTC		178	18	0	18X10^-5	1,800,000	7200000	180000000
	3	1.09	1.13	0.04	TNTC	TNTC	TNTC	TNTC	TNTC		66	2	2X10^-6	2,000,000	8000000	200000000
Control	1	1.09	1.11	0.02											
	2	1.09	1.13	0.04	0	0	0	0	0	0	0	0			
	3	1.09	1.11	0.02	0	0	0	0	0	0	0	0			

There was found to be $16x10^8$ CFU/g for replicate 1, $18x10^7$ CFU/g for replicate 2, and $2x10^8$ CFU/g for replicate 3 for isolate 2 (Table 3).

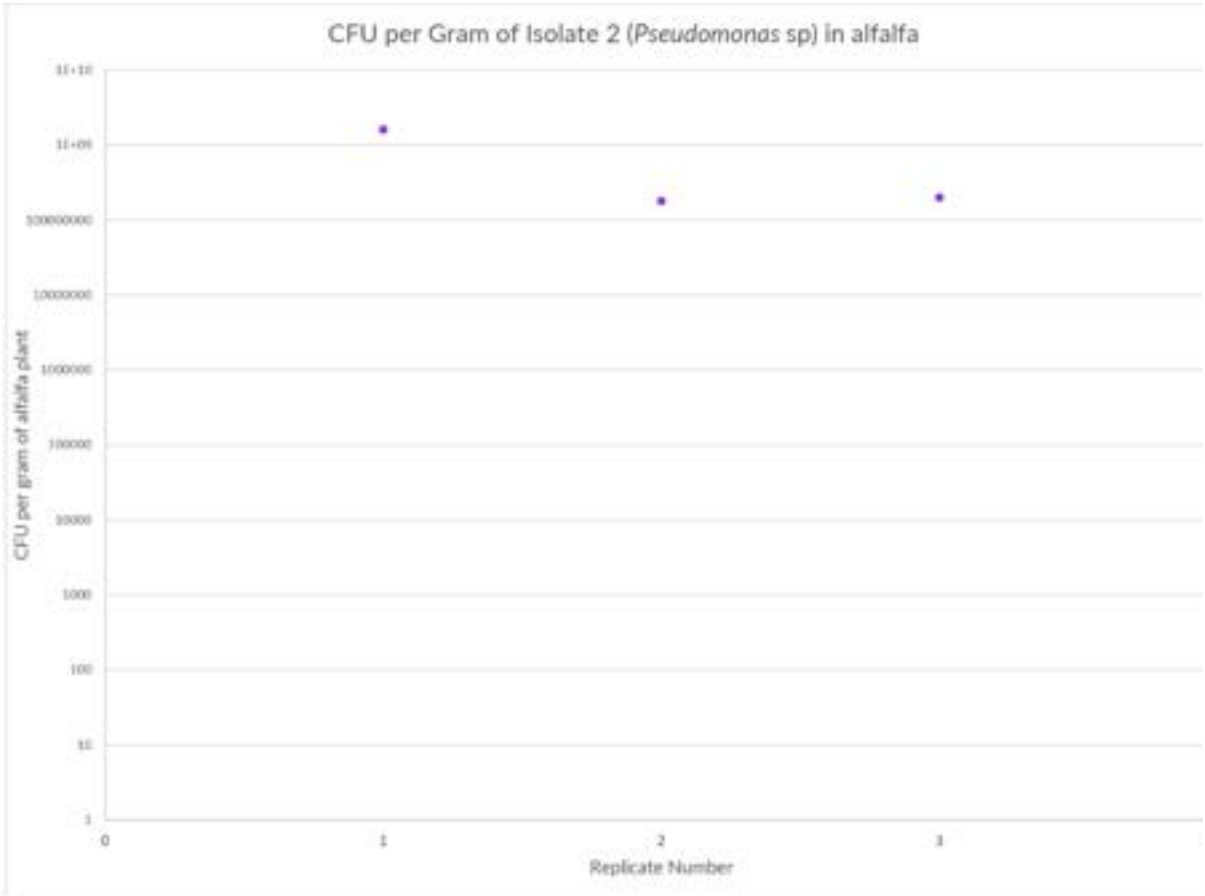


Figure 10: CFU per gram of isolate 2 in alfalfa

5.9 Colony PCR

A colony PCR was performed and a band was there for the positive control and for isolate 2 (Figure 11).

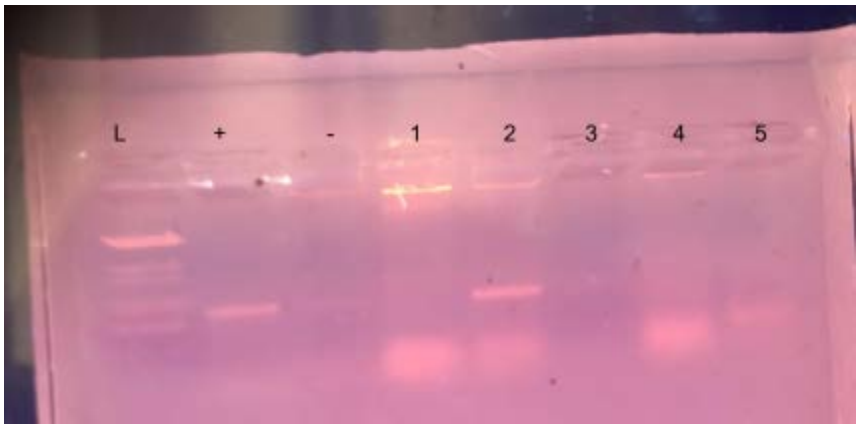
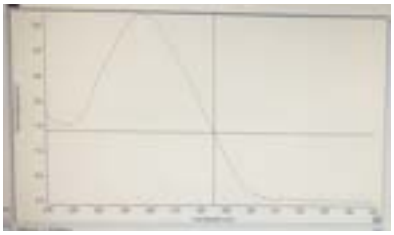


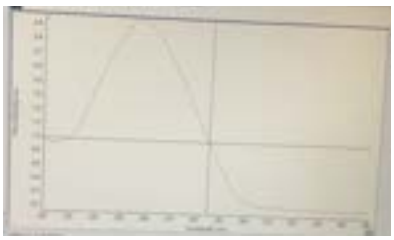
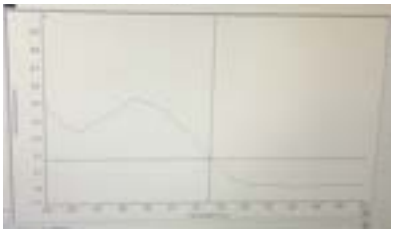
Figure 11: Gel from Colony PCR

5.10 DNA Extraction

A DNA extraction was performed and the DNA extraction product was nanodroped. The DNA concentration varied by isolates with some having very high concentrations of DNA and others with very little (Table 4).

Table 4: DNA concentration in extracted DNA product from each isolate

Isolate	Nanodrop DNA Concentration (ng/µl)	Graph
1	185	

2	132.7	
3	2.4	
4	242.9	
5	25	
6	56.3	

5.11 ITS PCR

An ITS PCR was performed and there were bands for isolates 1 and 4. The bands appeared to be between 600-400bp (Figure 12).

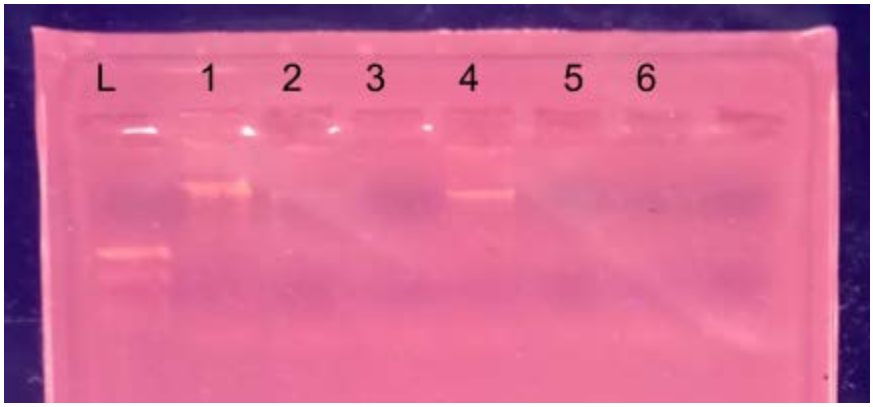


Figure 12: Gel from ITS PCR

The ITS PCR was performed again using just a positive and negative control; no bands for either control appeared (Figure 13).

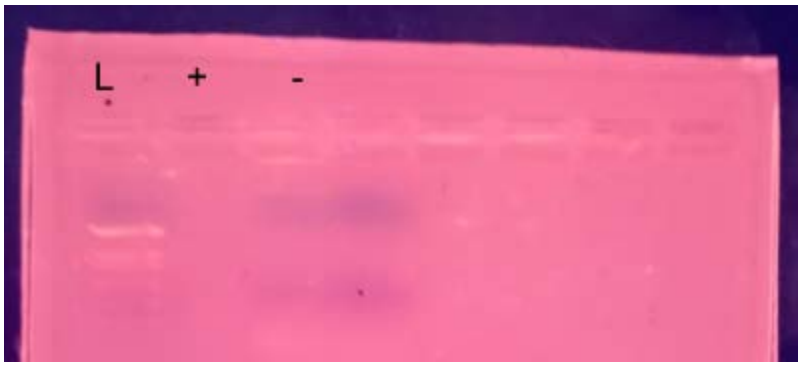


Figure 13: Gel from ITS PCR with only controls

5.12 16S PCR and PCR Cleanup

A 16S PCR was performed using the DNA extraction product, there was a between 2640bp and 1400bp band for the positive control as well as all isolates other than isolate 3 (Figure 14).

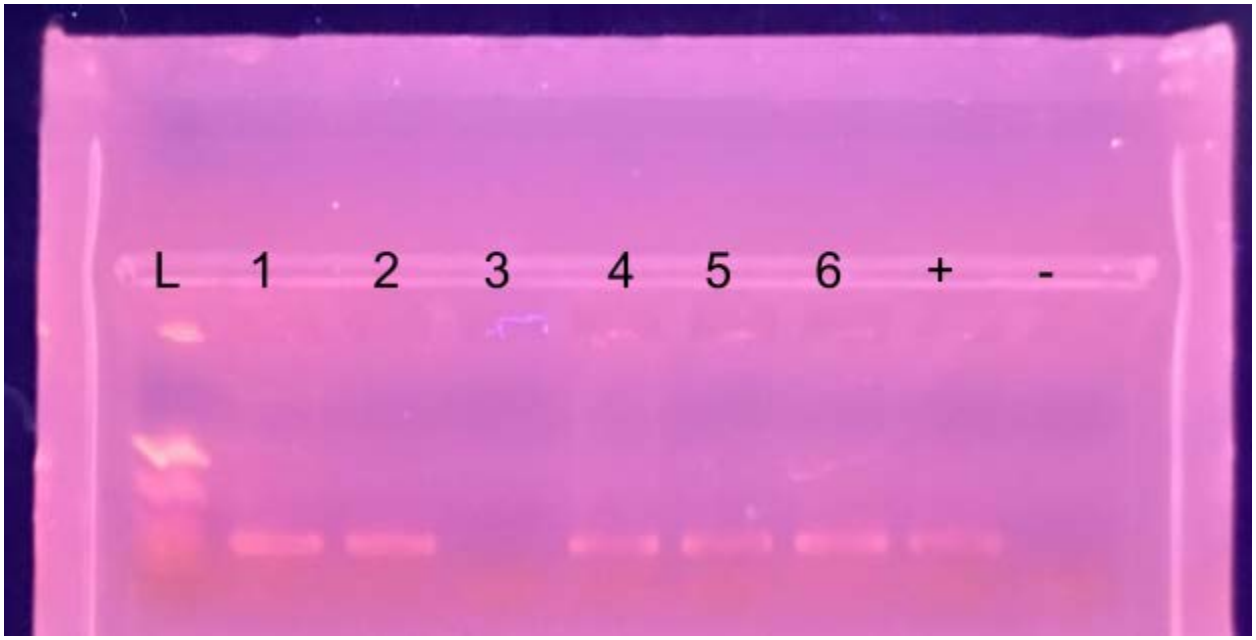


Figure 14: Gel from 16S PCR using DNA extraction product

The results from the 16S PCR Cleanup were nandroped and all isolates had very low concentrations of DNA. A Qubit was performed and very similar concentrations of DNA were detected for all samples (Table 5).

Table 5: DNA concentration from 16S PCR Cleanup

Isolate	Nanodrop DNA Concentration (ng/µl)	Nanodrop Graph	Qubit DNA Concentration (ng/µl)
1	3.9		3.09
4	3.3		2.96

5	2.0		1.93
6	7.2		3.30

The 16S PCR was performed again with a modified PCR Cleanup and the nanodrop DNA concentrations were higher, between 31 and 16ng/µl.

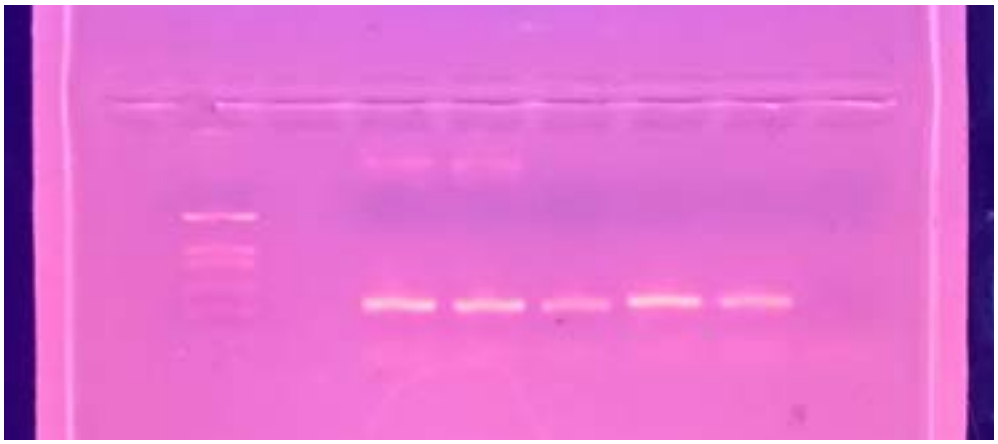
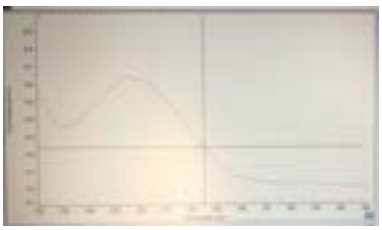
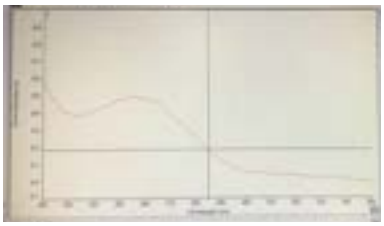
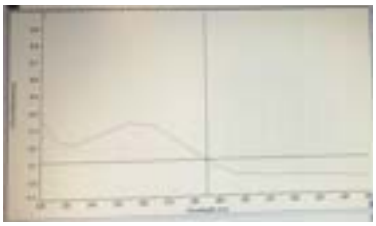
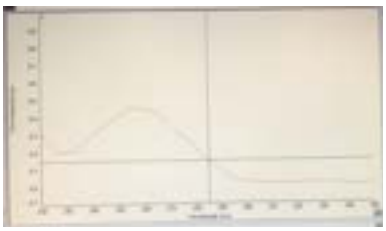


Figure 15: Gel from second 16S PCR using DNA extraction product

Table 6: DNA concentration from modified 16S PCR Cleanup

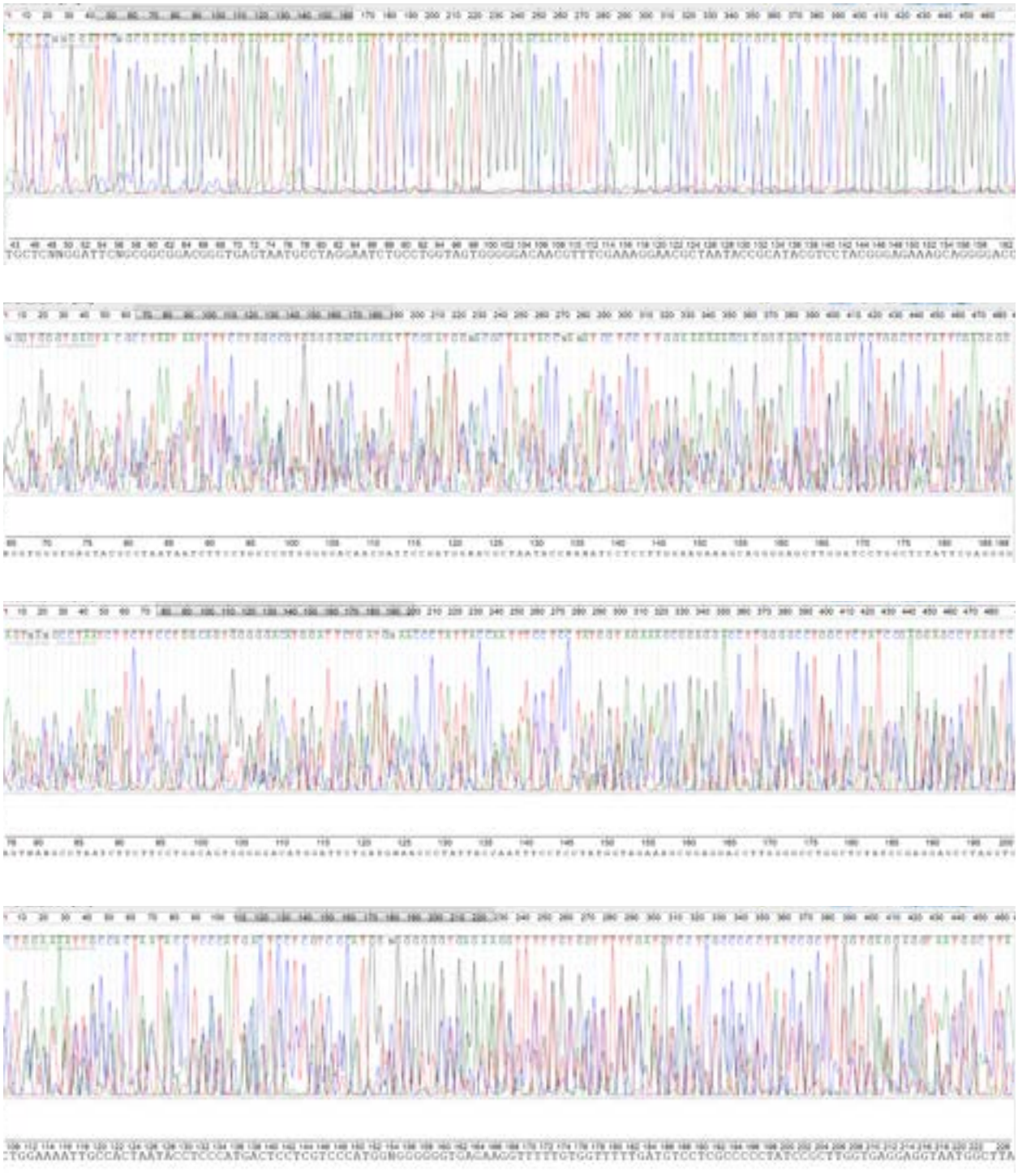
Isolate	Nanodrop DNA Concentration (ng/µl)	Graph

1	31.0	
4	23.9	
5	16.1	
6	22.0	

The PCR product from isolate 2 from the colony PCR was sent to Genewiz for sequencing and using NCBI BLAST was found to be a 99.35% match for several Pseudomonas partial sequences.

The PCR products for Isolates 1,4,5, and 6 from the 16S PCR were sent to Genewiz for sequencing. The samples were visualized using Ugene and appeared to be a little bit contaminated. The samples were identified using NCBI BLAST anyway. Isolate 1 had no

BLAST matches. Isolate 4 was a 93% match for *Pseudomonas monteilii*. Isolate 5 was a 77% match for *Pseudarthrobacter sp.*L1SW. Isolate 6 was an 83% match for *Pantoea agglomerans*.



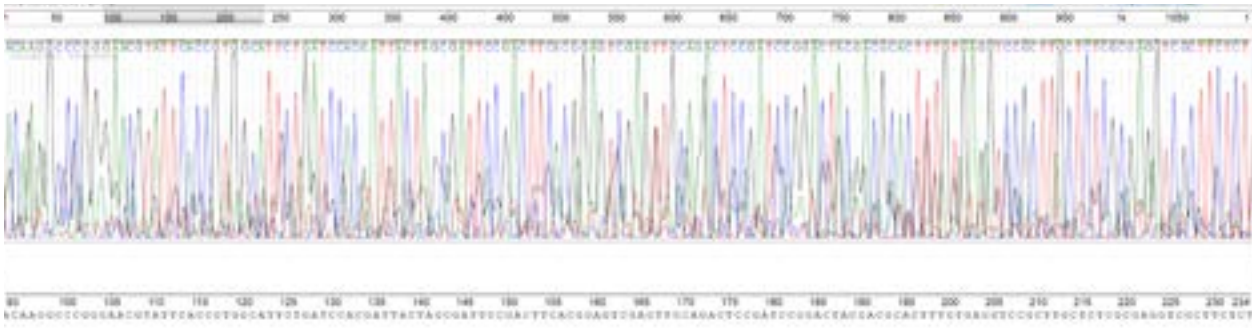


Figure 16: 16S sequences for (from top to bottom) isolate 2, 1, 4, 5, 6, visualized in UGene

6. Discussion

6.1 Summary

Seed endophytes from alfalfa were isolated and characterized. The germination of sterilized seeds was optimized and the best germination was found using Murashige and Skoog Medium. Two different sources of contamination were identified and eliminated in the colonization experiment, 1X PBS and expired bleach. It was found that isolate 2 (*Pseudomonas sp*) colonized alfalfa at an average of $6x10^8$ CFU/g after 7 days. Using colony PCR and 16S PCR the isolates were identified as a probable Acintomcyeytes species, 99.35% match for *Pseudomonas* species, a 93% match for *Pseudomonas monteilii*, a 77% match for *Pseudarthrobacter sp*, and an 83% match for *Pantoea agglomerans*. An optimal PCR cleanup protocol was also developed. The protocols for these experiments were performed for the first time at this institution and optimized. This created a functioning framework of experiments for this lab.

6.2 Culture Based Experiments

The germination of the alfalfa seeds was optimized. In the first attempt to germinate the surface sterilized seeds only 4 of the 88 seeds germinated. This may have been due to alfalfa

autotoxicity. Alfalfa are known to have autotoxicity where water soluble compounds are secreted which inhibit germination. These allelochemicals are likely produced in the shikimic acid or acetate pathway. They work by inducing oxidative stress and causing reactive oxygen species to build up which can result in membrane lipid peroxidation and even cell death (Zhang et al, 2021). When the alfalfa were germinated in a larger petri dish with less seeds 41% more of the seeds germinated. The highest percentage of seeds germinated when using Murashige and Skoog Medium, this is a medium for plant growth (Murashige and Skoog, 1962).

There were several sources of contamination that were found and eliminated. During the first colonization experiment the serial dilution plates were all equally overgrown with contamination. Since the contamination was the same throughout all of the serial dilution plates the contamination mostly likely occurred after the dilution had taken place. The most likely sources were either the TSA plates themselves or the 1X PBS which was used to dilute the samples. A TSA plate and a TSA plate with 50ul of 1X PBS were left in the incubator. There was no growth on the plain TSA plate but there was growth on the 1X PBS plate (Figure 4). This indicates that the 1X PBS was the source of this contamination. The lab is a shared space among both research students and students using the space for lab assignments for classes. 1X PBS is very commonly used in many different experiments. There were many different opportunities for the 1X PBS to become contaminated.

In the second colonization experiment there was still contamination but the contamination was serially diluted. There were several different types of colonies on each plate including the control but the distribution through the dilutions was what was expected. The contamination being diluted means that the contamination had to occur before the serial dilution step. It also means that the original source of contamination had been eliminated. In order to find

this new source of contamination the earlier steps within the protocol were evaluated. The first step is sterilizing the seeds which are then germinated. The seeds were sterilized according to the original procedure and immediately homogenized rather than germinated. The homogenization was plated on LB agar and there was growth so the original seed sterilization procedure was not working (Figure 7). The bleach used during this procedure was the bleach used to clean the labs. After examining all the reagents used to the procedure it was found that the bleach in the lab was over a year expired. The seed sterilization was performed again with fresh bleach at both 3% and 10% bleach concentrations and there was no growth on the plates (Figure 7). The issue with the procedure was the expired bleach and when fresh bleach was used the seeds were sterile so that source of contamination was eliminated. Since there was no growth on plates at both concentrations the issue was the bleach itself and not the concentration of the bleach so going forwards 3% of the fresh bleach continued to be used for this procedure.

The colonization experiment was repeated but less seeds germinated. This could be due to the fresh bleach in the sterilization protocol. Due to the amount of germinated seeds the colonization experiment was performed with only controls and isolate 2 since isolate 2 was able to be identified at that point. There was no growth on any of the plates for two of the controls but one of the controls had growth on the plates (Figure 9). The growth followed the serial dilutions and the colony morphology was the same as isolate 2. The serial dilutions and plating for the isolate 2 replicates was done before the controls. The control that was contaminated was the first control replicate so it is possible that the contamination occurred during the procedure. There was found to be $16x10^8$ CFU/g for isolate 2 replicate, 1. $18x10^7$ CFU/g for isolate replicate 2, and $2x10^8$ CFU/g for isolate 2 replicate 3 (Table 3). These values are similar to what has been

found for other *Pseudomonas* species colonizing alfalfa. *P. fluorescens* F113 has been found to colonize alfalfa roots at a level of $2.9x10^7$ CFU/g at day 7 (Villacieros et al., 2003).

None of the seeds germinated in the presence of 0.01 OD of the isolates (Figure 8). This could mean that the isolates are plant pathogens. Since all of them did not grow there also could have been an issue with the protocol or calculations. There was also an unexpected amount of turbidity in the water for 0.1 OD. This could indicate that the calculations were off or the spectrophotometer values were off. The spectrophotometer is quite old and infrequently used so it is very possible that the calibration is off. The control group of sterile water had an expected amount of germination which indicates that there was not an issue with the protocol. This experiment could be run again using a different spectrophotometer and having another person double check all calculations.

6.3 Molecular Experiments

A colony PCR is performed by touching a sterile loop to an isolated colony on an agar plate and using that as the DNA template for the PCR. When this was done there was only a band for one of the isolates, isolate 2 (Figure 11). Colony PCR is a lab technique usually used for rapid screening of yeasts or bacteria. Since it relies on using an isolated colony as the DNA template rather than DNA that has been extracted, often they do not work. This is because the membrane of the cells is unable to be broken open during the PCR. Since gram positive bacteria have a thick peptidoglycan cell wall they are harder to break open using colony PCR. The one organism that was amplified during the colony PCR was later found to be a *Pseudomonas sp.* which are gram negative. Other than isolate 4, the other isolates that underwent the colony PCR were later identified as gram positive organisms which might explain why they were not amplified by the colony PCR.

The first time that the ITS PCR was performed there were bands for some of the isolates but they appeared to be the wrong length (Figure 12). The protocol was done again with a positive and negative control and no bands appeared for either (Figure 13). It was confirmed that the primers used were correct and the DNA extraction products were nanodropped and other than isolate 3 all had fairly high concentrations. Later bands were found for all isolates other than isolate 3 during the 16S PCR, meaning that the isolates were all either bacteria or archaea. This would explain why there was no amplification during the ITS PCR as the ITS region is found in fungi (Martin and Rygiewicz, 2005). If none of the isolates were fungi then there is no ITS region to amplify.

A 16S PCR was performed using the DNA extraction products and there were bands for all isolates other than isolate 3 which is to be expected since the initial DNA concentration for that sample was very low (Figure 14). A PCR cleanup was performed using the Promega Wizard SV Gel and PCR cleanup kit and the DNA was eluted in 50ul sterile water. The DNA concentrations were very low using both the nanodrop and Qubit DNA concentrations between 1-3 ng/ul were found (Table 5). The PCR was performed again with the same results for the gel. The PCR cleanup was performed using the QIAGEN PCR cleanup kit and the DNA was eluted in 30ul of elution buffer and let to sit for one minute before the final centrifugation. After this change to the protocol DNA concentrations between 16-31 ng/ul were found (Table 6). For this kit DNA elution is most efficient under slightly basic conditions (pH between 7 and 8.5) with a low salt concentration (Kit handbook). DNA is more stable at this slightly basic pH because hydroxide ions can interrupt the hydrogen bonds in the DNA. It being more stable allows it to dissolve faster in the buffer which is important as the elution step is very quick. The pH of the

sterile water used for the initial elution was not tested and if the pH was too acidic that could have interfered with the elution and caused the low DNA concentrations initially found.

Isolate 1 was unable to be identified using molecular techniques. It was amplified using 16S primers meaning that it is either a bacteria or archaea. It was also isolated on actinomycetes selective media. Therefore it is very likely an actinomycetes species. Actinomycetes are a common endophyte with 123 actinomycete strains having been found in over 113 species of plants (Matsumoto and Takahashi, 2017). They have been found to have some plant growth promoting properties along with antibacterial and antifungal activity (Shan et al., 2018). Of 46 actinomycetes isolates from tea plants 93.5% produced indole acetic acid (IAA) and 21.7% produced 1-aminocyclopropane- 1-carboxylic acid (ACC) deaminase.

Isolate 2 was identified using DNA sequencing as a 99.35% match for *Pseudomonas* species. Isolate 4 was also identified as a 93% match for *Pseudomonas monteilii*. These isolates both have very similar colony morphology so it makes sense that they are both Pseudomonas species. *Pseudomonas spp.* have been found as a seed endophytes in several different plants using many different technologies such as 16S rRNA Sequencing, PCR DGGE, and fatty acid methyl ester profiles (Ferreira et al. 2008, Mastretta et al., 2009, Johnston-Monje and Raizada 2011, Liu et al., 2012, Hardoim et al., 2012, Vega et al. 2005, Graner et al., 2003). Pseudomonas have been found using both MALDI-TOF and 16S rRNA gene sequencing in alfalfa seeds (Lopez et al, 2017). *P. monteilii* has been isolated from surface sterilized *Salvadora persica* (the toothbrush tree) leaves, shoots, and roots (Korejo et al., 2019). It has been shown to have a zone of inhibition and the lysis of fungal hyphae against *M. phaseolina, F. solani, F. oxysporum and R. solani*, which are all root infecting fungi. It was also found to have a positive effect on sunflower growth. Similar results were found in Okra where *P. monteilii* was shown to reduce infection of

M. phaseolina, *F. solani*, and *P. decumbens* in okra roots alongside *Penicillin* (Urooj et al., 2020). In a mixture with *Penicillin* it was also found to improve okra height, weight and polyphenol production. *Pseudomonas fluorescens* F113 has been found to have several plant growth promoting features and siderophore production, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity, and inorganic phosphate solubilization (Lally et al., 2017). *P. fluorescens* F113 has been found to increase crop height, and stem, leaf and pod biomass in field experiments using *Brassica napus*.

Isolate 5 was identified as a 77% match for *Pseudarthrobacter sp.* L1SW complete genome. This species is unpublished and was uploaded directly to NCBI by Li, J at the School of Life Sciences and Biotechnology, Shanghai Jiaotong University (NCBI website). Another *Pseudarthrobacter species, Pseudarthrobacter phenanthrenivorans* strain MHSD1, has been isolated from surface sterilized leaves of *Pellaea calomelanos*, which is a species of fern (Tshishonga and Serepa-Dlamini, 2020). A different *Pseudarthrobacter* species has been identified in the rhizosphere of *D. antarctica* from King George Island in the Antarctic peninsula (Marian et al, 2022). *Pseudarthrobacter* have also been found in sterlized roots of tobacco plants (Shimasaki et al., 2021). While not much is known about the role of *Pseudarthrobacter* species as endophytes they have been found in a broad range of plants and environments. Isolate 3 was never able to be identified since there was a very low DNA concentration after DNA extraction. Isolate 3 had a very similar colony morphology to isolate 5 so it is possible that isolate 3 was also a *Pseudarthrobacter sp*.

Isolate 6 was identified as an 83% match for *Pantoea agglomerans*. *Pantoea agglomerans* YS19 isolated from rice plants has been shown to be a plant growth promoter (Shen and Song, 2006). It has been found to fix nitrogen and produce indole-3-acetic acid,

abscisic acid, gibberellic acid and cytokinin which are all phytohormones. It was also found to increase plant biomass as well as enhance the transportation of the photosynthetic assimilation product. The sugarcane endophyte *Pantoea agglomerans* strain 33.1 has been found to colonize all areas of the sugarcane plant including the rhizosphere, inside the roots and aerial plant tissues (Quecine et al., 2012). It has been found to produce indole acetic acid (IAA) and solubilize phosphate and it has been suggested to stimulate chitinase and cellulase production in plant roots.

The 16S identifications come with an important caveat. The 16S samples had relatively low similarity to the sequences they matched to in BLAST and when visualized in UGene they appeared to be contaminated. There were multiple overlapping peaks for all of these samples (Figure 16). Overlapping peaks most commonly mean that there were two template DNA sequences present in the reaction. The most obvious cause of this is contamination. The 16S reaction was run twice and both times the negative control did not have any amplification suggesting that contamination did not come from any of the reagents. One possible source of contamination would be intracellular bacteria, if two organisms with 16S regions are present then both would be amplified. Overlapping peaks can also mean that there are two priming sites present in the DNA template or too low of a primer annealing temperature was used (Nucleics DNA sequencing support, website). These isolates all came back as organisms that are known to be plant endophytes which could indicate that the identifications are correct. But all analysis on the identity of these organisms should be considered with the knowledge that these identifications could be incorrect.

6.4 Future Research

There are many interesting directions for future research. Now that a working protocol has been developed the colonization experiment could be repeated for the rest of the isolates. This is the first time these experiments have been run at this institution and now there is a framework for all of these experiments to be run again in the future. The colonization experiment could also be repeated for different lengths of time. Due to time constraints the colonization experiment was only conducted over 1 week but there can be differences in endophyte colonization over different phases of plant growth. It would be interesting to look at colonization over a month or two and look at the changes in colonization week by week. The colonization in different parts of the plant could also be measured. The roots, leaves, and stem could be homogenized separately and colonization levels in these different areas of the plant could be measured. FISH could be used to visually examine the location of these endophytes within both the seeds and the plant's roots as they colonize. Various biochemical assays could be used to look for plant growth promoting activities in the isolates such as indole-3-acetic acid (IAA) production, phosphate solubilization, siderophores production, nitrogen fixation, and 1-aminocyclopropane -1-carboxylate (ACC) deaminase production.

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