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Characterization of PGPRs in the Rhizosphere of the *Trachelospermum jasminoide*

Introduction

The rhizosphere, where interactions between the soil, microorganisms, and plants take place, houses various plant growth promoting rhizobacteria, also known as PGPRs, which have been found to beneficially impact plant growth. Some PGPRs found in the root zone enhance plant growth by improving plant nutrition through the production of organic substances, such as acids and protons, which aid in the mobilization of phosphate from inorganic and insoluble phosphate (Lucy et. al 2004). In addition PGPRs have the ability to generate plant hormones, such as auxin and ethylene, and even degrade xenobiotics or other chemical substances that may harm the plant. For example PGPRs can prompt plant defense responses and eliminate pathogens in order to protect the plant (Antoun et al. 1998).

In this study the numerous beneficial mechanisms of PGPRs were studied using inoculated strains of bacteria from the rhizosphere of the *Trachelospermum jasminoide*. The *Trachelospermum jasminoide*, also known as the star jasmine, is a flowering angiosperm with five petal flowers, making it a dicot. With the impressive benefits of PGPRs we hypothesized that the isolated bacterial strains would enhance the growth of the pea plants in comparison to the control group. Further research on the advantages of various PGPRs could provide farmers with natural substitutions for harmful chemicals and pesticides used to promote growth and eliminate pathogens.

Materials and Methods

Isolating the Bacteria

The *Trachelospermum jasminoide*, also known as the star jasmine, was identified on the west side of the chapel on Loyola Marymount University's campus. Small sections of the root, approximately one to two inches, were collected with a twenty gram soil sample from the rhizosphere of the plant. Five to ten centimeters of the root sample was then placed in sterile media and three serial 1:10 dilutions were performed. 100 ul of solution was then plated twice from each of the three dilutions, resulting in two

10^{-2} dilution plates, two 10^{-3} dilution plates, and two 10^{-4} dilution plates. After completion the six plates were placed into the incubator agar side up at 30 degrees Celsius for one week. Finally, the soil sample was weighed to ensure a sample of approximately 20 grams was present in order to measure the percent moisture content.

Identifying Bacterial Colonies

The number of colony forming units per milliliter on the 10^{-4} agar plate was calculated after counting the number of present bacterial colonies. Next sixteen distinct colonies were isolated and identified, using a morphology table, including shape, margin and surface characteristics. The sixteen identified bacterial colonies were then streaked on six various media including, a R2A, R2A + CAS, R2A + TRP, ACC, phosphate, and cellulase plate in order to detect plant growth promoting characteristics amongst the plated bacterial colonies. The bacteria were plated in separate quadrants in order to create a grid of the sixteen bacterial colonies on each plate. Finally the six plates were placed into the incubator agar side up for one week at 30 degrees Celsius.

Testing for Plant Growth Promoting Properties

Following incubation, the six various media were analyzed for plant growth promoting properties amongst the bacterial colonies. The plant growth promoting properties tested for during the analyzation process included cellulase activity, ACC deaminase activity, phosphate solubilization, siderophore production, and auxin production. Cellulase production was detected by flooding the plate with gram's iodine and identifying existing halos after five minutes (Bric et al. 1991). ACC deaminase activity was confirmed by identifying bacterial growth on the plate (Penrose et al. 2003). Phosphate solubilization was signified by clear halos surrounding the plated bacteria colonies (Rodriguez et al. 2000). Siderophore production was tested for by pouring an agarose and CAS mixture on the R2A + CAS plate and identifying purple and orange halos after a total of thirty minutes (Perez-Miranda et al. 2007). Finally, auxin production was determined by identifying red halos after putting a nitrocellulose filter on the prepared Salkowski saturated membrane for thirty minutes (Bric et al. 1991). The biochemical characteristics of the sixteen colonies were recorded on a table with "+" indicating the presence of the plant growth promoting property and a "-" indicating the absence of that property. Finally four bacterial

colonies with several present properties were chosen, plated on separate agar plates using the streak method and then placed into the incubator at thirty degrees Celsius for one week.

Preparing Plants for PGPR Testing

In order to test the effects of the two bacterial strains on plant growth, three black boxes, eighteen grow baskets and a foil packet of wicks were obtained to hydroponically plant pea seeds. Each basket was filled with pumice and a sterile wick was pulled through in order to relay nutrient solution from inside the box to the pea plant. Once the baskets were set up two sterilized pea seeds were planted in each basket and covered with pumice. Next 1 Liter of nutrient solution was added to each box and 0.1 Liter was poured over the pumice covered seeds. Finally the hydroponic pea plants were placed under heat lamps for one week in order to promote growth in a controlled environment.

Adding the Bacterial Strains to the Hydroponic Plants

After one week of inoculation under the heat lamps, the growth of the hydroponic pea plants was observed and extra seedlings were cut if two were found growing in one basket. Bacterial inoculum for the two bacterial strains was prepared by obtaining the absorbance of the bacteria and creating an appropriate saline solution, using the absorbance as a guide for the ratio of bacteria to saline. Next the three plant boxes were divided into three groups, one control, one containing the 13MRNV05 strain, and one containing the 13MRNV16 strain. For the control group 1 mL of pure saline solution was pipetted into each basket, while 1 mL of the corresponding bacterial inoculum was pipetted into the other two boxes. Once again the hydroponic pea plants were placed under heat lamps for growth for one week.

Performing Statistical Analysis of the Dry Root and Shoot Weights.

After one more week of inoculation under the heat lamps, the hydroponic pea plants were removed from their boxes and the shoots were separated from the roots. Next the shoots and roots were wrapped in paper towels and placed inside a magazine in order to dry them out. After the shoots and roots were dried the masses were calculated and entered into a two separate bar graph, one for the root masses and one for the shoot masses. Using anova the p values were calculated in order to compare the average masses of the shoots and roots of the three hydroponic pea plant groups.

Preparing Bacterial Strains for PCR Analysis and Gel Electrophoresis

Freeze and thaw cycles were conducted on the bacterial isolates in order to perform PCR amplification of the 16s rRNA gene of strains 13MRNV05 and 13MRNV16, using a “universal” 16s rRNA gene primer. Once amplification of the gene was completed using a thermal cycler, the amplified bacterial strains were submerged in a glycerol solution, in order to prevent cell damage, and placed in a freezer at -80 degrees Celsius. Next the amplified bacterial strains were mixed with loading dye and inserted into the designated electrophoresis gel in order to separate the substances based on the rate of movement. A UV light box was then used to visualize the DNA and a photograph of the gel was taken for permanent record.

Molecular Identification of Bacterial Isolates

Using the 16s rRNA gene sequences from the PCR amplification, a BLAST search was performed in order to find a genus and species match for the bacterial isolates. Next the sequences were entered into the MUSCLE program for proper alignment and then into GBlocks for trimming. Finally the GBlock information and output group sequence were entered into Mesquite in order to create a phylogenetic tree.

Testing Strain for Antibiotic Production

Two TY plates were obtained for isolate 13MRNV16 in order to test for antibiotic production against bacteria. First the isolate was streaked in a vertical line on each plate and placed in the incubator at 30 degrees Celsius overnight and then into the refrigerator for one week. After a week three bacterial strains, including *P. aeruginosa*, *E. coli*, and *S. aureus*, were streaked from both edges of the plate to the isolate in three separate horizontal lines. Once again the two plates were placed in the incubator overnight and then into the refrigerator until observation. Next two Nutrient Yeast Dextrose Agar (NYDA) plates were obtained in order to test strain 13MRNV16 for antibiotic production against fungi. The isolate was first streaked on half of each plate and placed in the incubator for one week. Next the other half of each plate was streaked with a different fungus. The first plate was streaked with *Fusarium oxysporum* while the second plate was streaked with *Rhizopus stolonifer*. The two plates were then placed into the incubator for one week until observation.

Second Round of PCR Analysis

Using the same procedure as before, PCR analysis of strain 13MRNV05 was performed once again in order to determine the genus and species of the isolate.

Preparing Plants for Hoagland's Phosphorous Test

In order to test the effect of strain 13MRNV05 on plant growth, two black boxes, twelve grow baskets and a foil packet of wicks were obtained to hydroponically plant pea seeds. Each basket was filled with pumice and a sterile wick was pulled through in order to relay Hoagland's solution from inside the box to the pea plant. Once the baskets were set up two sterilized pea seeds were planted in each basket and covered with pumice. Next 1 Liter of Hoagland's solution with insoluble phosphate was added to each box and 0.1 Liter was poured over the pumice covered seeds. Afterwards bacterial inoculum for the strain 13MRNV05 was prepared by obtaining the absorbance of the bacteria and creating an appropriate saline solution, using the absorbance as a guide for the ratio of bacteria to saline. Next the two plant boxes were divided into two groups, one control and one containing the 13MRNV05 strain. For the control group 1 mL of pure saline solution was pipetted into each basket, while 1 mL of the corresponding bacterial inoculum was pipetted into the other box. Finally the hydroponic pea plants were placed under heat lamps for two weeks in order to promote growth in a controlled environment.

Performing Statistical Analysis of the Root and Shoot Measurements

After two weeks of inoculation under the heat lamps, the hydroponic pea plants were removed from their boxes and the shoots were separated from the roots. The shoot and root lengths were then recorded in centimeters and entered into two separate graphs, one for the root lengths and one for the shoot lengths. Next the shoots and roots were wrapped in paper towels and placed inside a magazine in order to dry them out. After the shoots and roots were dried the masses were calculated and entered into a two separate bar graph, one for the root masses and one for the shoot masses. Using anova the p values were calculated in order to compare the average masses of the shoots and roots of the two hydroponic pea plant groups as well as the average lengths of the shoots and roots of the two groups.

Results

Isolation of Rhizobacteria

The *Trachelospermum jasminoide* was found to have ten various colonies which were identified from the six dilution streak plates. After review of ten various colonies from the soil, which was found to

have a pH of 4.5, two bacterial strains, 13MRNV05 and 13MRNV16, were isolated. After observation and gram staining the colony morphologies of the isolates were then recorded, as shown by Table 1 and Table 2. Both isolates were found to be gram positive.

Table 1. Displays gram staining results of two isolates.

Isolate	Gram Stain	Shape	Arrangement
13MRNV05	Gram Positive	Bacillus	Staphylococcus
13MRNV16	Gram Positive	Bacillus	Staphylococcus

Table 2. Displays morphology of two isolates.

Isolate	Shape	Margins	Surface	Color
13MRNV05	Irregular	Lobate	Smooth	Milky Beige
13MRNV16	Round	Smooth	Smooth	Chalky White

Screening for PGPR characteristics

Isolates 13MRNV05 and 13MRNV16 were chosen to due testing positive for several biochemical tests. Isolate 13MRNV05 was found to test positive for cellulase activity, phosphate solubilization and ACC deaminase. Next isolate 13MRNV16 tested positive for cellulase activity and ACC deaminase. Both isolates were found to have the most positive results out of the other fourteen bacterial strains that were originally tested for PGPR properties.

Table 3. Displays results of biochemical tests for both isolates.

Isolate	Auxin	Cellulase	Phosphate	ACC	CAS
13MRNV05	-	+	+	+	-
13MRNV16	-	++	-	+	-

The dried roots and shoots of the hydroponic pea plants were recorded for all three groups. Using the average masses of the roots and shoots for each group, the p value for the roots was found to be 0.4041 and the p value for the shoots was found to be 0.2157.

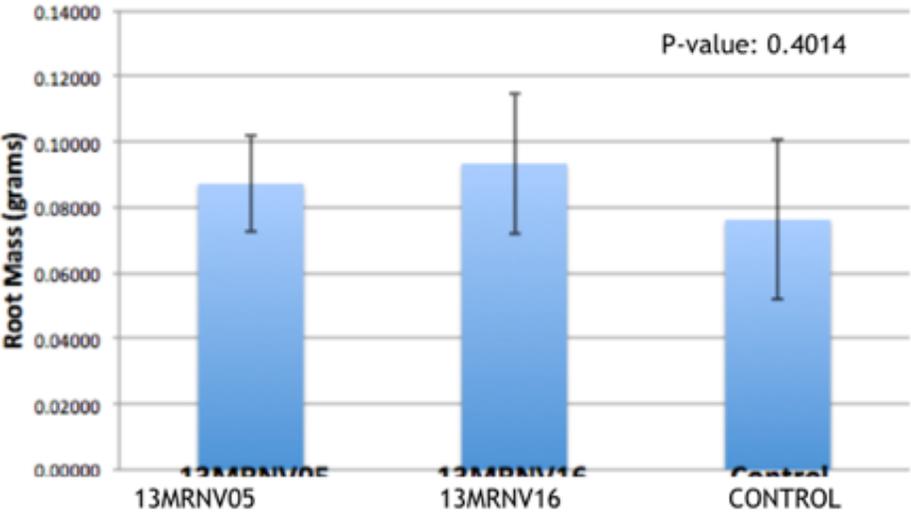


Figure 1. Displays average mass of roots for three hydroponic pea plant groups and calculated p value.

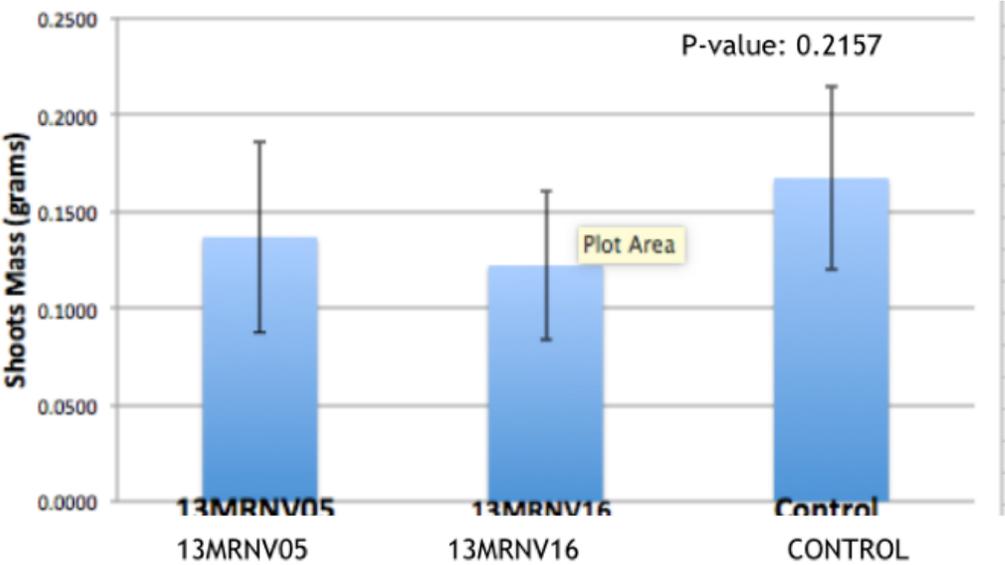


Figure 2. Displays average mass of shoots for three hydroponic pea plant groups and calculated p value.

Unfortunately PCR amplification was not completed for strain 13MRNV05 due to contamination, but through BLAST analysis strain 13MRNV16 was found to be *Streptomyces prunicolor*, since it had a 99% match.

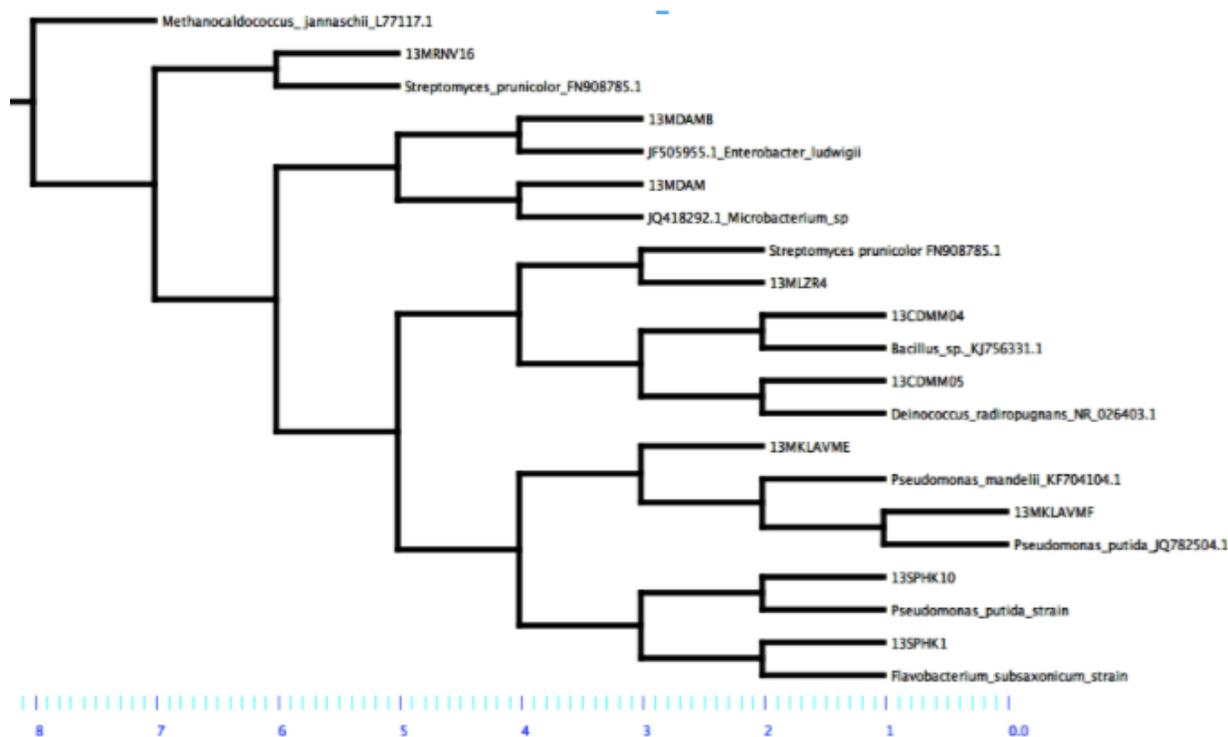


Figure 3. Displays that 13MRNV16 is most similar to *Streptomyces prunicolor* based on the 16S rRNA sequence.

Testing Strain for Antibiotic Production

Streptomyces prunicolor was found to have antibacterial properties against all three tested bacteria, since it inhibited the growth of the P-aeruginosa, E. Coli, and S. aureus bacteria. In addition *Streptomyces prunicolor*, was found to have anti fungal properties against *Fusarium oxysporum*, since it inhibited the growth of the fungus. Unfortunately, results for the assay containing *Rhizopus Stolonifer* were inconclusive since the fungus never grew on the plate.

Second Round of PCR Analysis

Once again PCR analysis of strain 13MRNV05 was not completed.

Performing Statistical Analysis of the Root and Shoot Measurements

The dry root and shoot weights of the hydroponic pea plants were recorded for both groups. Using the average masses of the roots and shoots for each group, the p value for the roots was found to be 0.413 and the p value for the shoots was found to be 0.352.

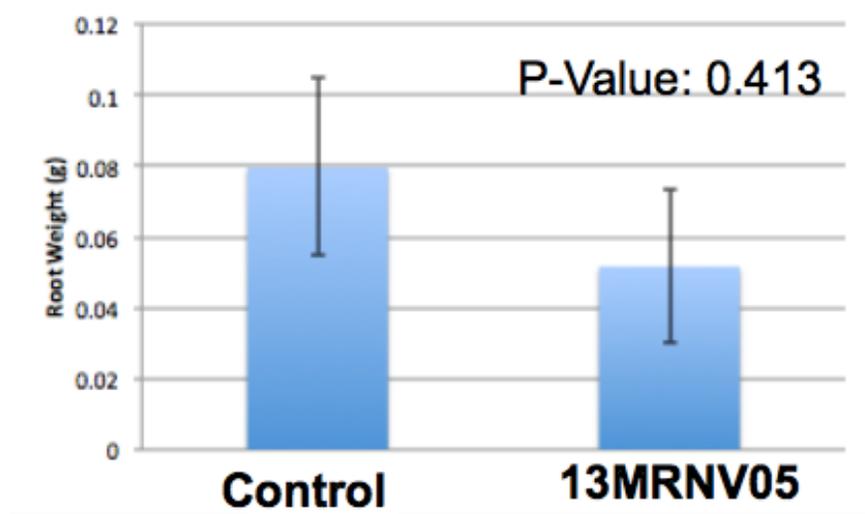


Figure 4. Displays average mass of roots for both hydroponic pea plant groups and calculated p value.

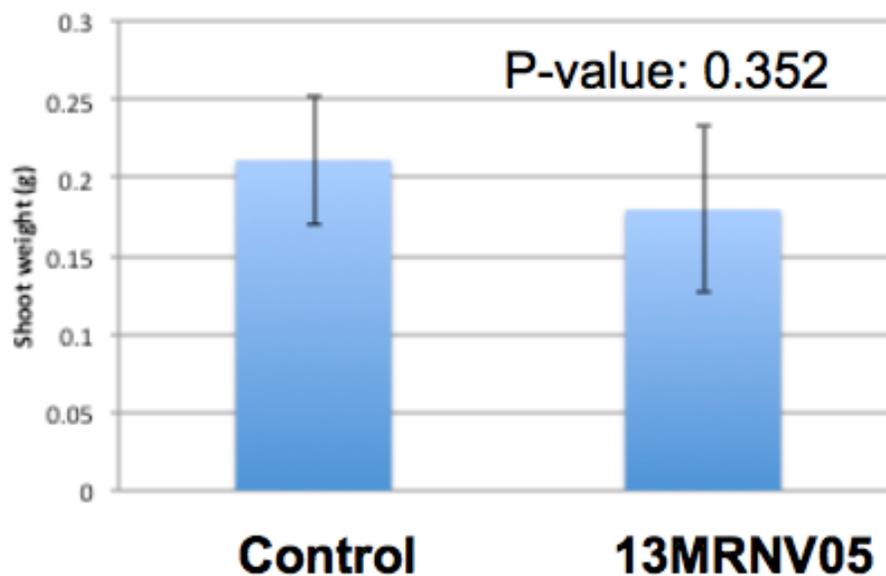


Figure 5. Displays average mass of roots for both hydroponic pea plant groups and calculated p value.

In addition the root and shoot lengths of both hydroponic pea plant groups were recorded. Using the average lengths of the roots and shoots for each group, the p value for the roots was found to be 0.394 and the p value for the shoots was found to be 0.309.

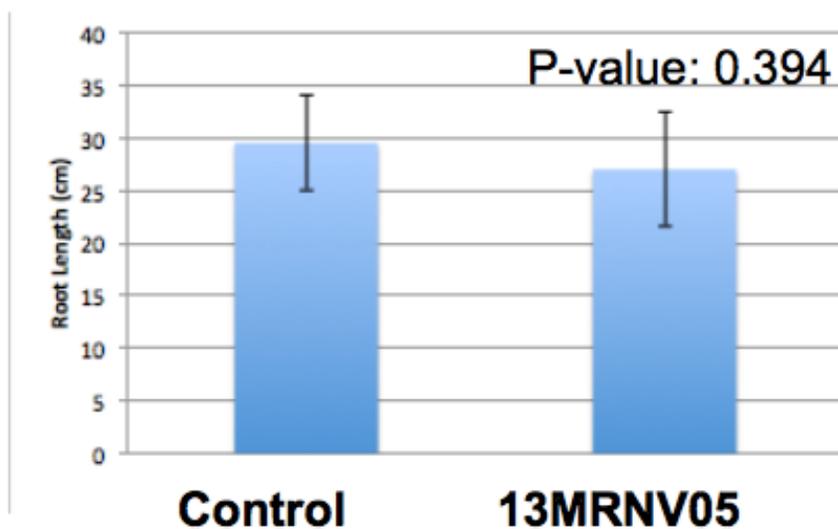


Figure 6. Displays average length of roots for both hydroponic pea plant groups and calculated p value.

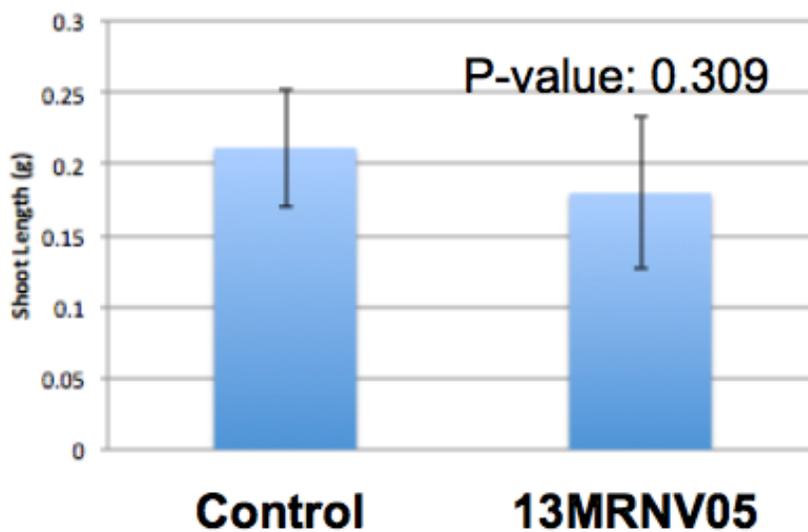


Figure 7. Displays average length of shoots for both hydroponic pea plant groups and calculated p value.

Discussion

After analyzing the results it was concluded that there were no statistical differences between the average masses of the shoots or roots of the three hydroponic pea plant groups, disproving the hypothesis that our two bacterial isolates promote plant growth. Although the first isolated strain, 13MRNV05, tested positive for cellulase activity, phosphate solubilization and ACC deaminase, it tested negative for auxin production and siderophore production. The plant hormone, auxin, regulates plant development and has been found to promote cell elongation, while siderophores help plants acquire usable iron, an element that is not found abundantly in the soil in its usable form (Aloni et. al 2003). The absence of these two properties in the 13MRNV05 strain may have contributed to the lack of conclusive results and clear statistical differences between the control and the 13MRNV05 group. Similar to the 13MRNV05 strain, the 13MRNV16 strain tested positive for cellulase production and ACC deminase, but did not test positive for phosphate solubilization, auxin production and siderophore production. The ability to solubilize phosphate allows bacteria to provide plants with readily available phosphorous, a necessary macronutrient to plants (Rodriguez et al. 2000). The absence of these three properties in the 13MRNV16 strain provides an explanation for the lack of statistical variation between the average root and shoot masses of the control and 13MRNV16 group. Therefore further research must ensue in order to explore the severity of the impact of specific plant growth promoting properties on plant growth.

In addition through DNA analysis strain 13MRNV16 was found to most likely be *Streptomyces prunicolor*, while data for 13MRNV05 was inconclusive since DNA analysis was not performed. No PGPR properties were found specifically for *Streptomyces prunicolor*, but some species belonging to the genus *Streptomyces* have been found to be anti fungal biocontrol agents that can inhabit plant pathogenetic fungi (Yi-ying and Gui-lan 1990). In addition *Streptomyces* have the ability to produce bio active secondary metabolites such as antivirals, immunosuppressants, and antibiotics (Procópio et al. 2012). Due to the antibiotic producing properties of *Streptomyces*, *Streptomyces Prunicolor* was tested for antibiotic production against bacteria and fungi. Since it was found to have antibacterial properties against all three tested bacteria, including *P-aeruginosa*, *E. Coli*, and *S. aureus*, and anti fungal properties against *Fusarium oxysporum*, it can be concluded that *Streptomyces Prunicolor*, is most likely a antibiotic

producing bacteria. This correlates with the previously stated studies that explain how species belonging to the genus *Streptomyces* have been found to be anti fungal biocontrol agents and produce antibiotics.

Furthermore due to the similar morphological characteristics of the two isolates, PCR analysis was performed once again to see if strain 13MRNV05 is a part of the same genus as 13MRNV16. Unfortunately, results were inconclusive since PCR analysis was not completed once again. Failure may be due to recombination, which leads to rearrangement of DNA, especially by crossing over in chromosomes, thus negatively affecting PCR analysis or contamination by multiple DNA samples from various bacteria (Zhang and Hewitt, 2003).

Finally, the Hoagland's Phosphate Test was performed on hydroponic pea plants in order to determine if strain 13MRNV05 would promote plant growth due to its ability to solubilize phosphate, since it tested positive for phosphate solubilization. Unfortunately, after analyzing the results it was concluded that there were no statistical differences between the average masses of the shoots and roots or between the average lengths of the shoots and roots of the hydroponic pea plant groups, disproving the hypothesis that our bacterial isolate would promote plant growth due to its ability to solubilize phosphate. This correlates with our previous discovery that strain 13MRNV05 does not promote plant growth.

The failure of the PCR analysis on strain 13MRNV05, which could have been due to recombination or contamination as discussed above, may have lead to the lack of statistical variation between the average masses and lengths of the control group and the group containing isolate 13MRNV05. Both contamination and recombination could essentially alter the effect of the bacteria by changing the DNA composition. Therefore, further research could be conducted to isolate a pure culture of strain 13MRNV05 in order to determine the genus and species, thus providing insight into the properties of the isolate.

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