Isolation and Identification of

Plant Growth Promoting

Rhizobacteria on *Allium caeruleum*

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Introduction

Plant growth promoting rhizobacteria (PGPRs) are bacteria that live on the root and root hairs of plants. PGPRs either directly promote plant growth or provide protection against harmful environmental factors (Lugtenberg, et. al., 2009.) Some PGPRs are known as biofertilizers, and can increase the concentration of mineral nutrients and efficiency of nutrient uptake for plants (Vessey, 2002.) For limiting nutrients such as Nitrogen and Phosphorus, these bacteria can provide greater access to usable nutrients either by fixing nitrogen or solubilizing phosphorus (Orhan, et. al., 2006.) Other PGPRs help the plant by controlling pathogenic organisms such a fungi (Lugtenberg, et. al. 2009.) PGPRs also gain nutrients from the plant to survive, creating a symbiotic relationship.

The *Allium caeruleum* plant was chosen to examine the PGPRs present on the root system. This plant was chosen because it is abundant on the Loyola Marymount campus, has an extensive root system and is relevant to the nearby habitat. *A. caeruleum* is a species of onion, commonly known as the blue globe onion; it is native to California and produces clusters of bright blue flowers. The blue globe onion is a perennial monocot.

The techniques chosen to examine the PGPRs of the *A. caeruleum* included plating the isolated bacteria on certain medias that would indicate for specific PGPRs. The plates would test for plant growth promoting properties including phosphate solubilization, siderophore production, IAA production, cellulase production, and ACC deaminase activity.

The sixteen initial isolates were narrowed down to three for continued study. A genetic sample from the three chosen isolates was sequenced to determine their identity. The isolates were also inoculated individually onto a monocot (corn) to determine which promoted growth best.

This research was conducted in order to determine the most effective PGPRs for a monocot, like the *A. caeruleum*, as well as to determine what kind of growth promoting bacteria are present and common on the nearby plants and in the area.

A study done by the University of Agriculture in Pakistan referenced techniques that will be used in this experiment, including testing for ACC-deaminase activity and the effects of the PGPR on maize. The study found that significantly more growth was observed on the maize that was treated with bacteria producing ACC-deaminase (B. Shaharoona, et. al).

Materials and Methods

Selecting Plant for Rhizosphere Analysis

The plant, *A. caeruleum,* was collected from the east side of the Seaver building on the LMU campus. As a large and abundant monocot around campus, it was chosen for its ease of identification.

Isolating Bacteria from Rhizosphere

Five cm of root was removed from the plant, and placed into a test tube with 10 ml saline solution, then serially diluted 3 times by taking 1 ml and adding it to 9 ml of saline. One ml of the solution was added to an R2A agar plate twice (6 plates total) and spread plated. The plates were covered, set aside for 10 minutes for water to evaporate, and incubated at 30°C for 5 days.

Determining Colonies

Plates with between 20 and 200 CFU were counted, the CFUs were averaged together to give the population count.

Sixteen bacterial colonies were selected from the 10^{-2} dilutions, and the individual colonies were described in the lab notebook.

Biochemical Testing

A sample of each of the 16 chosen colonies was plated onto an agar plate with different biochemical indicators: 1 plate of Phosphate agar, 1 plate of Cellulase (CMC) agar, 1 plate of R2A + 5 mM L-tryptophan, 1 plate of ACC agar, and 2 plates of R2A (one to be used in CAS assay and one to be used as a control). The individual dishes were labeled according to the biochemical test, and incubated at 30°C for 5 days.

Soil Quantification

The weight of the wet soil was determined. The soil was left out to dry for 1 week. The dry soil was then weighed and water content was determined. The dry soil was added to 10 ml of water, and left to settle for 10 minutes. The pH of the water was then measured using a pH strip.

Testing for Plant-Growth Promotion

Sixty corn seeds (corn was selected because it is a monocot like *A. caeruleum)* were soaked in 5% sodium hypochlorite for 15 minutes, and rinsed well. The three bacterial isolates were each suspended in a tube of saline solution, and one tube of saline was kept as a control. Four sets of seeds were planted and each one watered with one of the four saline solutions. They were placed on a growth rack to germinate. After growing for two weeks, the plants were removed from the soil, their root and shoot lengths were measured. The plants were left out to dry for a week; the dry weight was then taken.

Retesting PGPR properties

A sample of each of the three chosen bacterial isolates were plated with different biochemical indicators—CMC agar, R2A for CAS agar, PO4 agar, and R2A + tryptophan--with a section of agar left empty as a control. They were then incubated at 30°C for 5 days. The CMC plate was flooded with Gram's iodine and inspected for halos. The PO4 plate was inspected for clearing halos. The CAS plate had CAS reagent added to it and was inspected for orange or

purple halos. A nitrocellulose membrane from the R2A+tryptophan plate was placed on a dish with Salkowski reagent, then inspected for red halos after 30 minutes.

Determining morphology

The colonies were examined under a microscope and the colony's shape, margin, surface texture, and color was described. Then, a sample from each colony was gram-stained and examined under a microscope to determine the shape of individual bacterium, and whether they were gram-positive or negative.

PCR amplification of 16s ribosomal DNA gene

Bacterial isolates were suspended in sterile water and lysed by a cycle of freezing and thawing. Universal bacterial primers 27F and 1492R amplified the 16s rDNA gene. The PCR product was processed by gel electrophoresis to confirm the presence of the amplified gene. The successful PRC product was submitted for sequencing.

Identification of bacterial strains

The sequenced strands of DNA were reversed, and the first 500 characters of the reverse compliment was run through BLAST (Basic Local Alignment Search Tool) to determine the identity. A phylogenetic tree was created to show the relationships between the three isolates, the three identifed bacterium and an outgroup (*Methanocaldococcus jannaschii)*.

Statistical testing of root growth

The root growth was examined for the corn plants treated with the three isolates (2, 3, 16) as well as the control group. This data was put through an ANOVA test to look for significance in root growth.

Results

The flowering plant, *Allium caeruleum,* was chosen from an area of dry soil (28.44% water) with a pH of 6. From the dilution of the root in saline solution, there were 5.2×10^{-4} Colony Forming Units per ml.

The experiment's data indicated that there were various types of plant growth promoting rhizobacteria (PGPRs) present on the root segment of the *A. caeruleum* plant, as displayed in Table 1 below. The test for phosphate solubilization was indeterminate for the 2/6/13 tests. Isolate 2 and 15 indicated cellulase production, while isolates 3, 11, and 16 indicated IAA production (auxin). Siderophores were not present in any of the isolates, while ACC deaminase activity was detected in isolates 2, 4, 5, 6, 8, 12, 14 and 15.

Isolates 2, 3, 5, 11, 14 and 16 were chosen to further examine because of their indicators (15 was not chosen because it appeared to be a fungus). From there, isolates 2, 3 and

Table 1. The bacterial isolates ("colony") and the PGPRs that were indicated for each isolate are displayed below. Isolate number 15 showed the strongest indicators for both cellulase production and ACC deaminase activity. Isolate number 2 also showed a strong indicator for ACC deaminase activity as well as some indication for cellulase production. The most common indicator was for ACC deaminase activity.

Colony	Siderophore production	IAA production	Cellulase production	Phosphorous solubilization	ACC deaminase activity
1	-	-	-		-
2	-	-	+		++
3	-	+	-		-
4	-	-	-		+
5	-	-	-		+
6	-	-	-		+
7	-	-	-		-
8	-	-	-	N.D.	+
9	-	-	-		-
10	-	-	-		-
11	-	+	-		-
12	-	-	-		+
13	-	-	-		-
14	-	-	-		+
15	-	-	++		++
16	-	++	-		-

16 were chosen to conduct further tests on. Isolate 2 showed a round shape, smooth margin, concentric surface and brown and cream color when viewed under a stereomicroscope. Isolate 3 had a round shape, smooth margin, smooth surface and a creamy white color. Isolate 16 had a round shape, smooth margin, smooth surface and a creamy yellow color (this data is shown in

Table 2 below). When gram-stained, all isolates 3 and 16 were gram-positive, while isolate 2 was gram-negative. Isolate 2 had filamentous tendrils and a pleomorphic shape. Isolate 3 had bacillus present in a streptobacillus arrangement. Isolate 16 had cocci in a streptococcus and staphylococcus arrangement.

Table 2. The colony morphologies of the three isolates (2, 3 and 16) are shown below. The shape, margin, surface and color were examined under a stereomicroscope.

	Shape	Margin	Surface	Color
Isolate 2	Round	Smooth	Concentric	Brown middle, cream border
Isolate 3	Round	Smooth	Smooth	Creamy white
Isolate 16	Round	Smooth	Smooth	Creamy with some yellow

The PGPRs for the three remaining isolates were examined for a second time (2/20/13). The results were somewhat inconsistent with the first examination. Table 3 shows that isolate 2 and 3 showed indicators for phosphorous solubilization which could not be determined after the original biochemical tests.

Table 3. The PGPRs related to the isolates are displayed below. IAA production was viewed found in isolates 3 and 16 even though they did not originally show an indicator for it. Phosphorous solubilization was also indicated for all isolates despite not having been indicated for in the previous tests.

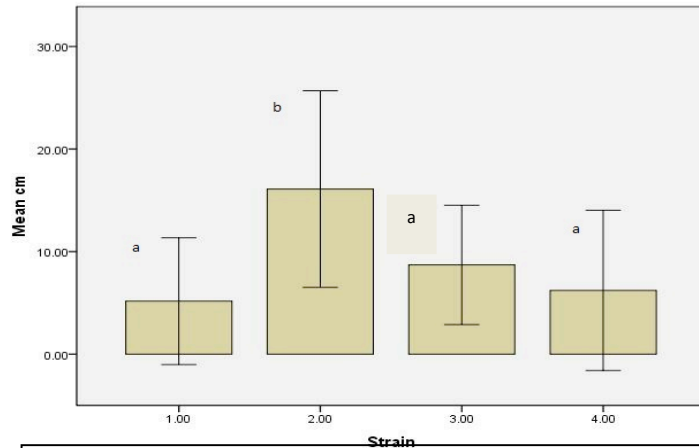| Colony	Phosphorous solubilization	Cellulase production	IAA production (auxin)	Siderophore production
Isolate 2	+	++	+	N.D.
Isolate 3	++	-	+	N.D.
Isolate 16	-	-	+	N.D.

Isolate 2 showed cellulase production and auxin production. Isolate 3 showed strong phosphorus solubilization. Isolate 16 did not show any PGPR characteristics. Both isolates 3 and 16 did not show auxin production in their second round of biochemical tests, although they tested positive for it on the first test. Siderophore production was indeterminate for all isolates.

The growth of the corn seeds was also examined. The plants inoculated with isolate 2 showed the most growth, with an average of 16.1 cm of root growth. Plants inoculated with isolate 3 had the second most growth, with an average of 8.7 cm of root growth, followed by the

plants inoculated with isolate 16, with an average of 6.2 cm of root growth. The control showed the least amount of growth, with an average of 5.2 cm of root growth.
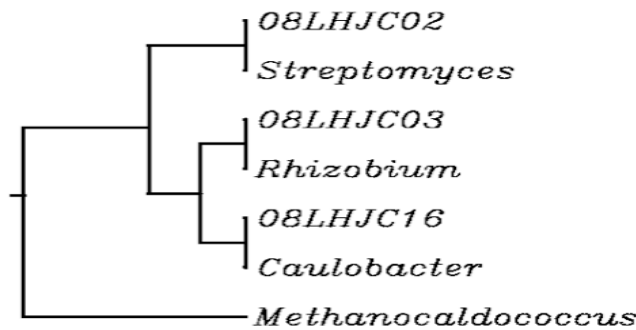
When the ANOVA test was performed a significance value was determined to be less than 0.05; therefore indicating significant differences between the test subjects, as displayed in Figure 1. Isolate 2 showed a significant difference between the other two isolates as well as the control; however isolates 3 and 16 did not show a significant difference between each other or the control.

Figure1. The results of the ANOVA statistical test for the root lengths of the corn plants, inoculated with the isolates and saline solution. Strain 1.00 indicates the control group, Strain 2.00 isolate #2, Strain 3.00 isolate #3 and Strain 4.00 isolate #16. A mean difference below 0.05 is shown for the comparisons between isolate 2, the control, isolate 3 and isolate 16, indicating a significant difference.

When the bacterial isolates were sequenced using the 16S rDNA gene, the resulting sequences identified isolate 2 as *Streptomyces phaeochromogenes*. Isolate 3 was identified as *Rhizobium etli* and isolate 16 was identified as *Caulobacter henricii*. The phylogenetic tree (Figure 2) that resulted from the three isolates, the three identified bacterium and the outgroup (*Methanocaldococcus jannaschii)*, reflected the results of the sequence results from the BLAST software.

Figure 2. Phylogenetic tree created from the identified bacteria DNA sequence, the isolates DNA sequences and an outgroup. The isolates are correctly matched with the identified bacteria, which corroborates the BLAST software data. 08LHJC02 indicates isolate 2, 08LHJC03 indicates isolate 3 and 08LHJC16 indicates isolate 16.

Discussion

The objective of this research was to isolate Plant Growth Promoting Rhizobacteria from a root sample, and evaluate its effect on *Allium caeruleum*. From analyzing the soil properties, it was determined that *Allium caeruleum* grows in relatively dry, somewhat acidic soil. According to the first round of biochemical tests, many of the microbes collected from the rhizosphere of *Allium caeruleum* were able to grow with ACC as their sole source of nitrogen.

During the first round of biochemical tests for PGPR properties, results for phosphorus solubilization were inconclusive. After retesting, both isolates 2 and 3 showed positive reactions to the phosphorus solubilization test. According to these results, isolates 2 and 3 affect *Allium caeruleum* by making phosphorus more available to the plant. As was shown in the initial biochemical testing, isolate 2 tested positive for cellulase production, meaning that this isolate can break down cellulose in the cell walls of a plant and use the carbon.

The corn plant inoculated with isolate 2 showed significantly more growth than the control. This makes sense because isolate 2 showed the most PGPR characteristics. Isolate 3 showed the second most PGPR characteristics and had the second greatest amount of growth. Isolate 16 showed more growth than the control plant, but not as much as the plants inoculated with the other two bacterial isolates. Isolate 2 was the most successful PGPR tested and had distinctive characteristics that the other two isolates did not. Isolate 2 was the only gram-negative isolate, and rather than a bacillus or coccus shape, the shape of individual bacteriums was pleomorphic with long tendrils. The tendrils increase the surface area of the cell, so perhaps this unique shape gives isolate 2 an advantage.

In future research, the three PGPR isolates will be inoculated onto the seeds of beans, corn, and chives. The plants, which include a dicot, a monocot, and a closely related monocot

respectively, will be allowed to grow for two weeks. Then root and shoot lengths will be

measured and dry weight will be taken to determine the effect of each of the isolates on the

different types of plants. In addition, the isolates will be plated on agar and tested for anti-fungal

properties--another PGPR characteristic.

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