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Article

The effects of early probiotic supplementation on the germination of *Arabidopsis thaliana*

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SUMMARY

The agricultural industry uses fertilizer to produce enough food for the world's robust and growing population. However, the use of fertilizers is associated with an increase in soil degradation, which will lead to a decrease in crop production within the next decade. This soil degradation is due, at least in part, to the long-term use of oil-based synthetic fertilizers. These fertilizers deplete the soil of nutrients and bacteria essential to plant growth. It is critical to find solutions to support crop production to sustain the robust global population. As a result, this study was conducted to investigate how probiotic bacteria, like *Rhizobium leguminosarum*, *Bacillus subtilis* and *Pseudomonas fluorescens*, can impact the growth of *Arabidopsis thaliana* when applied to the seeds. The effect of multiple probiotic genera on early root growth was evaluated through the generation of various bacterial solutions composed of different quantities and combinations of probiotic bacteria, treating *Arabidopsis* seeds with these probiotic solutions and placing the seeds on agar plates to germinate with the evaluation of early root growth. We hypothesized that solutions with multiple bacterial species compared to those with only a single bacterial species would promote seed germination more effectively. The results suggest that there was no statistically significant difference in the total root lengths of each group, but there was a statistically significant difference in the number of root branches. Overall, treatment groups with probiotic species of bacteria promoted root branching more than treatment groups with no bacteria. Multiple species of these bacteria, especially the groups containing *R. leguminosarum*, stimulated more root branching than treatment groups with one species of bacteria. Further research on how different bacterial genera affect root characteristics may support the development of alternative and sustainable bacteria-based fertilizers, which can aid in reversing the effects of soil degradation.

INTRODUCTION

The pressure to produce enough food intensifies as the world's population continues to increase over time. The exponentially increasing global population is projected to surpass 9.8 billion people by 2050 (1). With more people comes the need for more food, and since agricultural commodities are the world's main source of nutrition, it is important that agricultural output keeps pace with the growing population. However, various environmental stressors, including the looming threat of climate change, have put the agricultural industry under immense strain. Another related environmental stressor is the impact of soil degradation on the agricultural industry (1). Farming an adequate amount of food becomes more difficult with the slew of deleterious effects resulting from soil degradation. These detrimental outcomes include the depletion of vital nutrients and the elimination of critical bacteria and fungi that synthesize essential organic material for plants from the soil (2). This soil degradation is due in part to the overuse of oil-based synthetic fertilizers that are typically used by commercial farmers to produce enough food for the robust global population. Utilizing these traditional synthetic fertilizers can cause severe soil degradation over time. This ultimately reduces the quality of the soil, decreases the amount of land viable for farming and impedes the ability of the soil to sustain plant life. About a third of the world's arable soil is degraded, and current degradation rates set the lifespan of conventional agriculture at a mere 60 years (3). This imminent future disaster calls for a change in the way we cultivate our food.

The use of biofertilizers is one of the most promising solutions for this impending crisis. Biofertilizers are live microorganisms that are added to the soil to enhance plant growth. This "living fertilizer" supplements the microbial community already present in the soil environment, referred to as the soil microbiome, instead of stripping the soil of its naturally occurring substances. A common example of microbes that enhance soil are growth-promoting rhizobacteria (PGPR) that assist plant growth when applied to the seeds or surfaces of a plant by colonizing the rhizosphere (4). These probiotic bacteria (bacteria with abilities that have positive health impacts) form a symbiotic relationship with the plant and survive while promoting plant growth. Using microorganisms as a form of fertilizer can boost crop production and enrich the nutritional quality of the products of commercial agriculture (5). In addition, biofertilizers aid in the restoration of the soil and other environmental damages associated with the use of these synthetic fertilizers. Unlike traditional fertilizers, biofertilizers are non-pollutant and inexpensive since they take in atmospheric nitrogen instead of depleting natural resources (6). This can be especially significant in poorer areas of the world where the need for a low-priced and effective way of maintaining crop production

is ever-present. Utilizing microorganisms to supplement plant and soil microbiomes in an effort to boost crop production is a sustainable and practical alternative to the synthetic oil-based fertilizers that jeopardize the Earth's ability to maintain the environment and our population. For example, a study that evaluated the effect of diverse microbial communities on plant growth found that more species of *Pseudomonas* helped plants grow more so than fewer species of *Pseudomonas* (7). Thus, further researching the potentials of PGPR can advance developments for sustainable forms of biofertilizer. These developments can help lessen the environmental damages that have occurred due to the overuse of traditional fertilizers. Although research has been conducted to evaluate the effectiveness of different probiotic bacteria, the full extent of the capabilities of these microorganisms remain unknown.

This led to us to ask: how can adding different amounts of probiotic bacteria from different genera to *Arabidopsis thaliana* seeds impact early root development when germinated on agar? We selected *Arabidopsis* for this experiment because, in addition to its fast growth cycle, there is an abundance of scientific knowledge pertaining to its specific genes and traits (8). This made *Arabidopsis* an ideal organism with which to conduct this experiment because procedures were carried out in a relatively timely manner, and the results obtained from this study can be modified and applied to other plants of greater agricultural significance. Additionally, studying *Arabidopsis* allowed us to examine the effects of microbes on studying seed germination, which is the first stage of a plant's growth cycle and is therefore vital for crop production and the establishment of a stable source of food for the world's population (9). Lastly, germinating the seeds on plain agar without any nutrients or outside factors successfully isolated the effects of the probiotic bacteria present. We utilized *Bacillus subtilis*, *Pseudomonas fluorescens*, and *Rhizobium leguminosarum* in this experiment, as they are three bacterial genera shown to have probiotic effects that positively impacted plant growth in previous studies (10, 11). We included two negative controls in this experiment. Our water-only group verified that the seeds could germinate on their own without the presence of bacteria for each trial. We included *Escherichia coli* as another negative control to distinguish the effects of a bacterium not known to be probiotic from the effects of bacteria known to have positive effects on plant growth like the species that made up the experimental groups. We organized different quantities and combinations of the three probiotic species selected into treatment groups to evaluate the effect of varying numbers of genera on plant growth. We hypothesized that a seed treated with a bacterial community composed of a larger number of bacterial species originating from multiple genera would have a greater impact on plant growth than communities made with a fewer number of species. The data collected from this experiment indicated that the number of probiotic bacterial species from different genera did not have a significant impact on total root length but more so on the number of root branches. Treatment groups with greater numbers of probiotic bacteria species promoted root branching more than treatment groups with one species. This signifies that the amount of probiotic species can play a substantial role in the development of root growth characteristics.

RESULTS

To determine the effect of multiple probiotic genera on early root growth, we conducted an experiment that involved the generation of different bacterial solutions to treat *Arabidopsis* seeds and monitor their growth. We grew overnight cultures of bacteria in nutrient-rich media, centrifuged the cultures to isolate the bacterial cells, and resuspended the cells in phosphate buffered solutions. We soaked sterilized seeds in combinations of bacterial solutions for 30 minutes and placed them on plain agar to germinate for two weeks. After, we measured total root length and the number of root branches with ImageJ software and analyzed these numbers using an ANOVA test.

We compiled abbreviations and descriptions for each treatment group **(Table 1).** "Bs" represents *B. subtilis,* "Pf" represents *P. fluorescens,* and "Rl" represents *R. leguminosarum*. Groups with multiple species of bacteria are denoted with these abbreviations connected with a plus sign, so "Bs + Pf" represents the group with *B. subtilis* and *P. fluorescens*, "Bs + Rl" represents the group with *B. subtilis* and *R. leguminosarum*, "Pf + Rl" represents the group with *P. fluorescens* and *R. leguminosarum*, and "Bs + Pf + Rl" represents the group with all three genera of bacteria. The negative control group with distilled water is portrayed as "water-only," and the negative control group with *E. coli* is named after that species.

The results obtained from this experiment can contribute to the knowledge needed to develop a viable alternative to traditional fertilizers and to making agriculture more sustainable for the sake of both environmental and global survival.

Water-only	*E. coli*	Bs	Pf	Rl	Bs + Pf	Bs+ Rl	Pf + Rl	Bs + Pf + Rl
Distilled Water	*E. coli*	*B. subtilis*	*P. fluorescens*	*R. leguminosarum*	*B. subtilis* *P. fluorescens*	*B. subtilis* *R. leguminosarum*	*P. fluorescens* *R. leguminosarum*	*B. subtilis* *P. fluorescens* *R. leguminosarum*

Table 1: The 7 experimental groups and the control groups that make up the different treatment groups are depicted in this table. Groups 1-7 are composed of different quantities and combinations of the three probiotic bacteria. The negative controls are the water-only and *E. coli* groups.

Total Length

We measured the total root lengths of the plants for each of the different treatment groups and calculated the average length per group. We found that the water-only and *E. coli* negative control groups had similar mean root lengths of 2.73 cm and 2.48 cm, respectively **(Figure 1)**. For the groups with treatments of only one species of bacteria (groups Bs, Pf, and Rl), the mean lengths were 2.54 cm, 3.52 cm, and 3.84

cm. Treatments consisting of combinations of two probiotic bacteria (groups Bs + Pf, Bs + Rl, and Pf + Rl) had mean lengths of 2.26 cm, 4.71, cm and 5.75 cm. Lastly, the treatment group featuring all three bacteria (group Bs + Pf + Rl) had a mean root length of 4.94 cm. The average root lengths of the experimental groups, apart from group Bs + Pf, were larger than those of either of the control groups. Additionally, groups Bs + Rl and Pf + Rl had larger mean lengths than groups Bs, Pf, and Rl. Group Bs + Pf + Rl had an average total length that was larger than every treatment group except for group Pf + Rl.

However, it is important to note that the data values for each treatment group were relatively inconsistent, as demonstrated by the ranges of root length **(Figure 1)**. After analyzing the total lengths with an ANOVA test, we determined that the differences in root length among the different treatment groups were not statistically significant ($P = 0.15$). Therefore, we could not draw definitive conclusions about the effectiveness of the quantity of probiotic bacteria from different genera on promoting plant growth based on total root length alone.

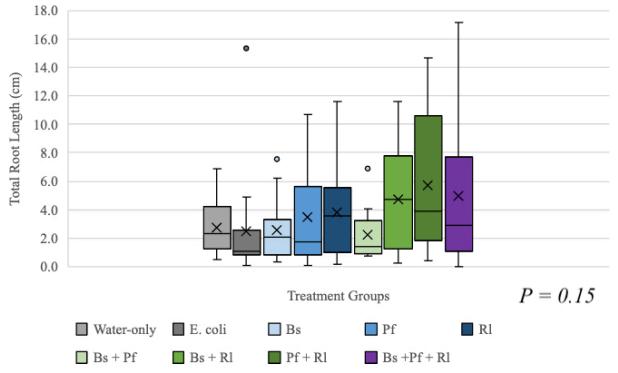

Figure 1: The total root lengths among treatment groups are depicted in this box and whisker plot. The "X" on each box denotes average values. The endpoint caps on each whisker indicate maximum and minimum values with the whiskers demonstrating the overall variability in each group. The lone dots represent outliers in the data set.

Number of Root Branches

We also counted the number of root branches per plant in each treatment group. The water-only and *E. coli* negative control groups had average branch counts of 0.714 and 0.526 branches, respectively **(Figure 2)**. Single species treatment groups (Bs, Pf, and Rl) had mean branch counts of 1.25, 2.50, and 3.13 branches. Treatment groups composed of combinations of two bacteria (Bs + Pf, Bs + Rl, and Pf + Rl) had average branch counts of 0.67, 2.87, and 3.11 branches. Finally, the treatment group consisting of all three probiotic bacteria (Bs + Pf + Rl) had an average branch count of 3.15 branches, which is the largest mean branch count out of all the treatment groups. The presence of probiotic bacteria led to higher numbers of root branches compared to the two control groups overall, but based on these values, there did not seem to be a distinct pattern between the average number of root branches and the number of bacterial species from different genera in the treatment groups. However, groups Rl, Bs + Rl, Pf + Rl, and Bs + Pf + Rl were the groups with the highest average branch counts and were the only groups to contain *R. leguminosarum*. Within these specific groups, the mean number of branches increased as the number of bacterial species in the treatment groups increased.

Just like the previous aspect of growth, the range of branch counts among the plants within each treatment group varied, as there was at least one plant with no branches in every group **(Figure 2)**. We analyzed the branch counts of each treatment group with an ANOVA test, which indicated that the differences in the average branch numbers among the different treatment groups were statistically significant ($P = 0.02$). Due to this, we concluded that both the presence and quantity of bacteria from different genera had an effect on the number of branches in this experiment.

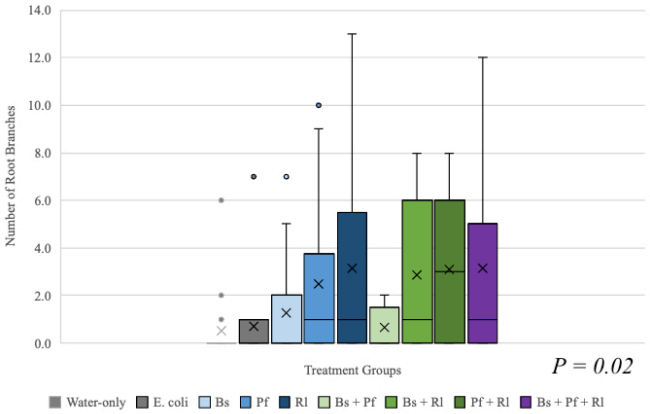

Figure 2: The number of root branches among treatment groups are depicted in this box and whisker plot. The "X" on each box denotes average values. The endpoint caps on each whisker indicate maximum and minimum values with the whiskers demonstrating the overall variability in each group. The lone dots represent outliers in the data set.

To further analyze the effectiveness of probiotic bacteria from multiple genera on root branching, we made multiple comparisons among the treatment groups. We performed a post-hoc Tukey HSD test to determine the statistical significance between specific treatment groups. This analysis concluded that between individual groups, there was no statistical significance among the results, as all the overriding *p*-values were greater than 0.05.

DISCUSSION

Based on our results, we rejected the hypothesis that a greater number of probiotic bacteria from multiple genera would lead to an increased promotion of root growth. The differences in total root lengths between the treatment groups were not statistically significant and could not be accurately utilized for further investigation, which led to the rejection of the original hypothesis. However, due to the overall

significant *p*-value for the number of root branches and the insignificant *p*-values comparing the number of root branches between groups, we chose to qualitatively analyze the results with pictures of the roots from the different groups. When examining specific plants in each of the treatment groups, it was clear that while the total lengths of the roots did not vary significantly, the roots of each plant in the different groups were distinct. An example of a plant from the water-only negative control group consisted of one continuous root that did not branch off in any direction **(Figure 3A).** In contrast, the main root of a plant from the group treated with all three genera of probiotic bacteria diverged into seven smaller roots that branched out into different directions **(Figure 3E)**. The roots of other treatment groups containing probiotic bacteria separated in a similar fashion **(Figures 3B, 3C, and 3D)**. When considering the different treatment groups and the specific species of probiotic bacteria that they were composed of, the diverging of the roots into the surrounding area of the agar plate appeared to be promoted by the presence of a greater number of probiotic species, as seen with the different groups containing *R. leguminosarum*. By qualitatively analyzing root characteristics in this way, we found that the quantity of probiotic bacteria from multiple genera may not significantly impact the growth of the roots with respect to total length but rather impact the way the roots grow and develop from germination.

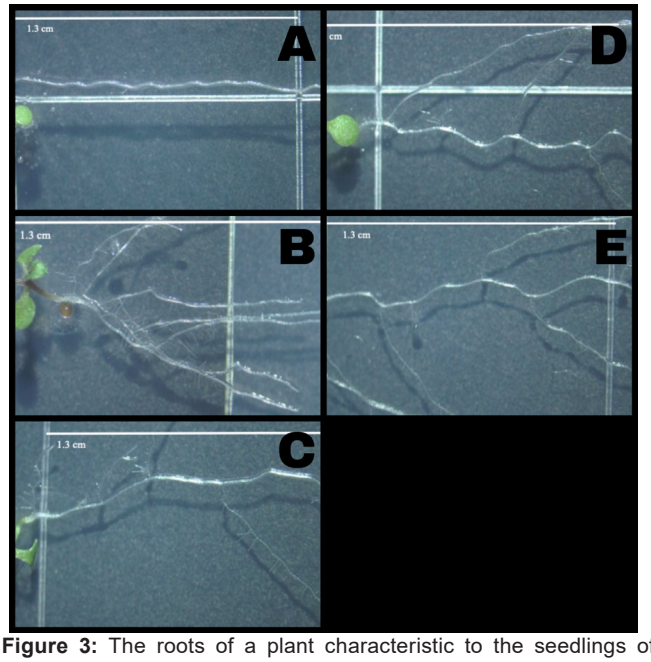

Figure 3: The roots of a plant characteristic to the seedlings of select treatment groups. A: distilled water; B: *R. leguminosarum*; C: *B. subtilis* and *R. leguminosarum*; D: *P. fluorescens* and *R. leguminosarum*; E: *B. subtilis*, *P. fluorescens*, and *R. leguminosarum*

Given that the original hypothesis was rejected overall, it is crucial to evaluate the various sources of error that were present in this experiment and the limitations that impacted the results. There was extensive variation in root length and the number of root branches between individual plants within the same treatment group, and there was also an unequal and inadequate sample size for each of the treatment groups. It was difficult to predict whether every seed would successfully germinate. During the two-week germination period, some of the seeds in each of the treatment groups failed to grow, and since we only analyzed the seeds that germinated for this study, the sample sizes for each group were unequal and relatively small by the conclusion of the data collection period. The small sample sizes of each group were not enough to counteract the variability present in the data sets, which led to a decrease in the validity of the results. Additionally, we could not capture the entirety of some of the plants with the camera-microscope setup due to the magnification settings of the microscope available. Therefore, when measuring the roots with ImageJ, we had to measure each picture separately. Since usage of the tracing tool stopped at the grid lines present in each picture, there is the possibility that there was some overlap from picture to picture, leading to slight errors in measurement. Lastly, although we carried out the process of cultivating the bacteria in the liquid media to create the probiotic solutions as efficiently as possible, we did not validate that the bacteria from every species survived in all the different treatment combinations. Thus, the validity of the results may have been impacted regarding the components of each treatment group.

Similar to how the probiotic bacteria in this experiment significantly impacted the number of root branches, further investigation of how probiotic bacteria can impact different root characteristics can be beneficial to increasing the knowledge in this field. The branching of roots into the space around the plant increases the amount of area that the roots extend into the soil. This allows the plants to both anchor themselves better into the soil for more support and gain nutrients from a greater area. These aspects portray the importance of further investigation of root characteristics like branching, root hairs, and the overall path of the roots and how that can be advantageous in increasing crop production and quality of growth. Additionally, since *R. leguminosarum* was associated with the four groups that had the greatest mean numbers of root branches, conducting similar studies with different probiotic bacteria from multiple genera that are related to *R. leguminosarum* could lead to significant developments. For example, *Rhizobium* is a genus that is categorized as an intracellular PGPR unlike *Pseudomonas* and *Bacillus,* which are both genera that are extracellular PGPR; *Bradyrhizobium*, *Sinorhizobium*, *Azorhizobium*, *Mesorhizobium*, and *Allorhizobium* are all bacterial genera that are considered to be intracellular PGPR and assist plant growth from inside the cells of the plants (12). Investigating the effects of *Rhizobium* bacteria in combination with bacteria from other intracellular PGPR genera that can work together from inside the cells of plants could be important and potentially increase the magnitude of the effect that *R. leguminosarum* had on the root branching in this study. Knowing more about how the specific development of roots can be manipulated by probiotic

bacteria from multiple genera can lead to advancements in the development of alternative and sustainable bacteria-based fertilizers, which can aid in reversing the effects of soil degradation. By supplementing the soil with probiotic bacteria, these bacteria-based fertilizers can help to alleviate the stress of the increasing global population on food production and enrich the agricultural industry. Continuing to find more uses for probiotic bacteria in this way can enhance the way we grow our food and extend the life span of agriculture for our planet.

MATERIALS AND METHODS

Cultivation of Bacteria

All bacteria cultures and the media and materials used to cultivate them were obtained from Carolina Biological Supply Company. We carried out proper laboratory precautions, such as wearing gloves, while preparing the bacteria and during any steps of this experiment in which these bacteria were utilized to ensure the safety of ourselves as the researchers. To prevent contamination that would affect the results of this experiment, we also created sterile conditions.

P. fluorescens, *B. subtilis* and *E. coli* were streaked on nutrient agar plates, and *R. leguminosarum* was streaked on *Rhizobium X* plates. We used a sterile plastic inoculation loop to pick up single colonies and spread them on their respective plates for cultivation. *B. subtilis* and *E. coli* were incubated at 30°C, and *P. fluorescens* and *R. leguminosarum* were incubated at 25°C. We streaked each species of bacteria on new plates weekly to keep a growing population of bacteria alive and prepared for when we were ready use them.

Proceeding this, we transferred each bacteria culture into a liquid medium. Nutrient broth was used for *P. fluorescens*, *B. subtilis*, and *E. coli,* and *Rhizobium X* broth was used for R. leguminosarum. The bacteria grew overnight on a rocking platform at a speed of 60 rpm. We then measured the optical density of each bacterial sample using a spectrophotometer at 600 nm with a 10 mL cuvette. After using a centrifuge to spin down and isolate the bacterial cells of each culture for 30 seconds at a speed of 6000 rpm and removing the remaining supernatant with a micropipette, we combined the cells with a phosphate-buffered saline (PBS) (13). The bacterial cell solutions were spun at the same speed for an additional 30 seconds and then suspended in PBS again.

These solutions served as the different treatments for the *Arabidopsis* seeds. We created these with either one, two, or three species of the probiotic bacteria in addition to the water-only and *E. coli* negative controls **(Table 1)**. For treatments with multiple species of bacteria, we took equal measurements of each solution to create a 500 µL mixture.

Seed Treatment

The *Arabidopsis* seeds were provided by Flinn Scientific, and we thoroughly sterilized them before experimentation. The seeds were rinsed with 500 µL of distilled water in a 1.5 mL tube by pipetting up and down, after which the distilled water was removed. This process was repeated with the same amount of a 5% sodium hypochlorite solution and again with 70% ethanol (14). Lastly, we rinsed the seeds with distilled water. We soaked the sterilized seeds in 500 µL of the different treatments for 30 minutes. After the seeds were treated, they were placed on clear square plates with sterile tweezers. Prior to seed placement, we filled the plates with a 0.8% agarose solution that set into a gel.

Germination and Growth

The square plates containing the seeds were placed on a rack at a 45° slant under a fluorescent light to germinate. We set the fluorescent light to be lit for a 16-hour photoperiod over a two-week span so that the seeds could fully germinate and develop into seedlings (15). The square plates used to germinate the seeds featured grid lines marking 1.3 cm, which we used as a scale to measure the root lengths of the different seedlings. We positioned a camera above a light microscope to take pictures of the seedlings. At the end of the two-week germination period, we took pictures of each seedling using this camera setup **(Figures 3A-E)**. The total number of seeds treated for each group was recorded in addition to how many of these seeds germinated by the end of the two-week period **(Table 2)**.

	Seeds Treated	Seeds That Germinated	Seeds That Did Not Germinate
Water-only	37	19	18
E. coli	19	14	5
Bs	24	16	8
Pf	24	16	8
Rl	30	24	6
Bs + Pf	24	9	15
Bs + Rl	27	15	12
Pf + Rl	19	9	10
Bs + Pf + Rl	29	13	16
Total	233	135	98

Table 2. The number of seeds treated and the numbers of seeds that did and did not germinate for each treatment group are depicted in this table. Seed numbers of the entire experiment for each of these categories are totaled at the bottom of this table.

Data Analysis

The pictures were analyzed using the imaging software ImageJ. We obtained digital measurements with this program by first using the 1.3 cm grid lines on the plates to set a scale of approximately 1950 pixels per cm and then using a freehand line tool to trace the section of the root that needed to be measured. We added the lengths of each root segment together to calculate the total root length for each seedling. The number of root branches, characterized by how many times the root diverged, was also recorded. We included the length of each root branch in the total length measurements. We then analyzed these data values to determine if the differences in total length or the number of root branches were statistically significant among the various treatments. Using a one-way Analysis of Variance (ANOVA) test, we compared the means of each group collectively. With a significance level of 0.05, we identified the *p*-values, which indicated whether the different treatment groups had an effect on plant growth. *P*-values less than 0.05 denoted statistical

significance between the differences in growth among the different groups.

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