The Efficacy of Bacillus Subtilis in Promoting Growth of ABA-Deficient(CS156) and Wild-Type Arabidopsis Thaliana in Zinc-Contaminated Soil

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Introduction

With the advent and expansion of industrialization, pollutant levels in natural ecosystems are steadily increasing. One example of such pollutants is heavy metals, which are metals capable of causing harm to human health and the environment. Such metals can negatively impact the soil quality and therefore impact plant quality and growth (Mohamed et al., 2025). Additionally, these heavy metals can accumulate in plant tissues and enter the food chain, posing health risks to humans through the consumption of contaminated crops and livestock that have fed on these plants. Beyond direct dietary exposure, heavy metals can leach into groundwater, contaminate surface water, and accumulate in aquatic organisms, exposing humans through drinking water, fish consumption, and other environmental pathways (Ajala et al., 2025). Chronic exposure to heavy metals has been associated with neurological disorders, kidney and liver damage, developmental impairments, and an increased risk of certain cancers (Cheng et al., 2025). Therefore, an efficacious solution to ease the toxic consequences of heavy metals is crucial.

Abscisic Acid(ABA) is a phytohormone that plays an important role in plant development by inducing stomatal closure and prompting dormancy in plant seeds and buds. ABA has been shown to play an important role to decrease heavy metals toxicity (Zhao et al., 2023). During ABA biosynthesis, the enzyme xanthoxin dehydrogenase, encoded by the ABA2 gene in Arabidopsis thaliana, converts xanthoxin into abscisic aldehyde, a key intermediate in ABA production (Jia et al., 2022). Under heavy metal stress, increased ABA biosynthesis allows ABA to bind to PYR/PYL/RCAR receptors, which inhibit PP2C phosphatases and allow SnRK2 kinases—especially SnRK2.2, SnRK2.3, and SnRK2.6—to become activated. SnRK2.6 (OST1) controls guard cell ion channels that regulate stomatal closure, reducing water loss and limiting the uptake and translocation of toxic metal ions (Hu et al., 2020). This is illustrated in Figure 1.

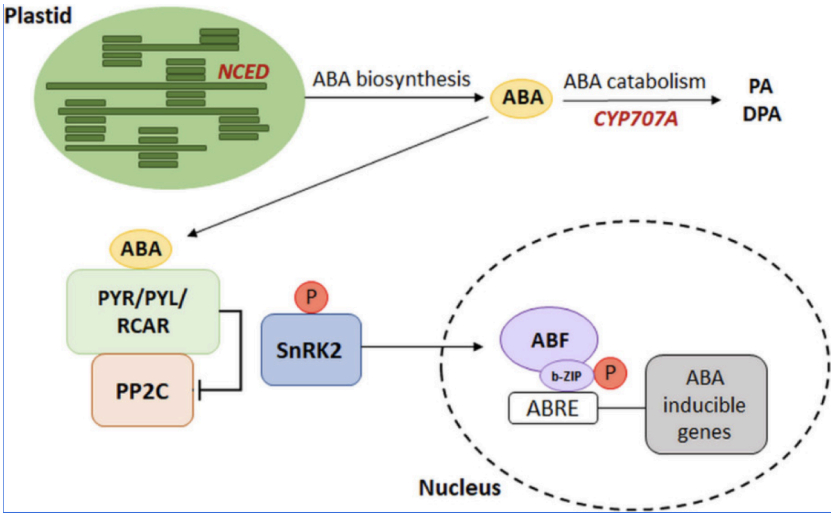

Figure 1: ABA Metabolism and Signal Transduction (Fidler et al., 2022)

Arabidopsis Thaliana has widely been utilized in plant research, as it has ideal and model attributes suitable for testing, such as ease of cultivation, rapid development, small size, etc. (Woodward and Bartel, “Biology in Bloom: A Primer on the Arabidopsis Thaliana Model System”). Numerous Arabidopsis mutants exist that lack functional genes within the ABA biosynthesis pathway, providing valuable systems for studying hormone-regulated stress responses. One such mutant is ABA2-1, a mutant that causes Arabidopsis to be incapable of the conversion of 2 intermediaries of ABA (Fujii & Zhu, 2009). In particular, it is missing the AT1G52340 gene(ABA2 gene) that regulates the conversion.

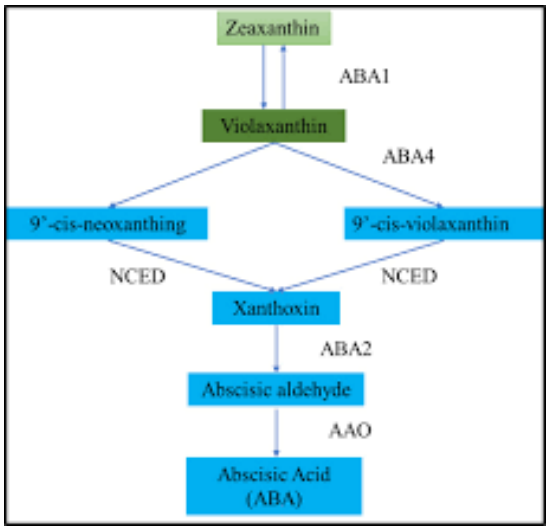

Figure 2: Pathways in which ABA can be synthesized in *Arabidopsis Thaliana* (Gurme et al., 2023)

In addition to endogenous plant mechanisms, plant growth promoting rhizobacteria have emerged as promising biological tools for environmental remediation. Bacillus Subtilis is a gram-positive bacterium found in multiple environments. It is ubiquitous in soil environments, and is a known plant growth-promoting rhizobacteria(PGPR). A PGPR aids in plant growth by enhancing nutrient availability, regulating plant hormones, and protecting against pathogens. They achieve this through direct mechanisms like nitrogen fixation and mineral solubilization (Ehinmitan et al., 2024). Recent studies show that it has the special property of being able to purify soil with high metal concentrations (Mahapatra et al., 2022). An interesting method of increasing endogenous ABA levels is through inoculation with plant-growth-promoting rhizobacteria such as *Bacillus Subtilis*, which can stimulate ABA production in the plant under stress conditions. Ultimately, this reduces the heavy metal content in plants (Pan et al. 2023).

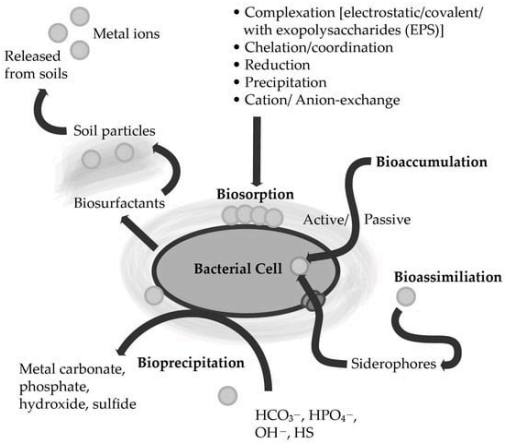

Figure 3: Mechanisms of bacterial bioremediation (Wróbel et al., 2023)

Based on this context, this experiment aims to observe whether inoculation with Bacillus Subtilis can increase endogenous ABA levels and promote plant growth in zinc-contaminated environments. Specifically, this study explores if bacterial inoculation can restore ABA signaling and promote normal growth in the Arabidopsis ABA2-1 mutant through stimulation of a secondary ABA production pathway and by increased uptake of Zinc by *B. Subtilis*.

Hypothesis

I hypothesize that the Wild type strain treated with *B. Subtilis* will grow the best among the other three treatment groups as it has a functional AT1G52340 gene in addition to high levels of Abscisic acid expression.

Materials/Methods

The materials list for this experiment included the following standard items: nitrile laboratory gloves, lab goggles, beakers, lab tape, Erleynmeyer flasks, scoopula, analytical and standard balances, and distilled water. Additionally, all-purpose soil potting mix, both types of arabidopsis seeds(wild and ABA2-1 mutant), soil pots, P100, P1000, and styrofoam plant potters were utilized to support the sowing and growth of the plants. Finally, B. Subtilis stock plates, LB broth, 70% ethanol, 30% bleach, inoculating loops, and a lighter were used in the microbial aspect of the experiment. All laboratory procedures followed benchmark STS rules, specifically adhering to the OSHA Laboratory Standard regulations.

On day 1 of the experiment, the arabidopsis seeds were prepared for germination. Particularly, the seeds were placed in a dark, refrigerated environment for 3 nights to stimulate germination. Additionally, I inoculated 2 Liter bottles of LB broth with B. Subtilis.

On day 2, 48 pots were filled with damp soil and Zinc Sulfate, following a concentration of 1250 mg kg^{-1}. In one pot, there was roughly 1.85 grams of soil and 0.0025 grams of $ZnSO_4$, measured with standard and analytical balances, respectively. ~55 microliters of inoculated broth were added to 24 pots via a P-100 micropipette. Afterwards, 2-3 seeds of WT seeds or MT seeds were pipetted onto 12 pots with bacterial broth present and 12 pots without inoculum. In all, this resulted in 12 plants of each of the 4 groups, totaling 48 plants.

In the following days, I measured the plant's stem height and average leaf diameter using a millimeter ruler. I also observed leaf color and lateral growth as qualitative factors showcasing the health and wellness of the seedlings.

At roughly the middle of the experiment(18 days), I analyzed the growth rate of the seedlings by taking the height of the plant at that day and dividing it by the number of days

elapsed. Correspondingly, I calculated the growth rate of the seedlings at the end of the experiment to discern if there was a disparity between initial and final development and to determine if the bacteria would cause this in stressful environments. All of the data collected was analyzed via t-tests.

In all, each group(wild type with bacteria, WTB; wild type no bacteria, WTNB; mutant with bacteria, MTB; mutant with no bacteria, MTNB) had 12 plants, divided along 3 sets of quadpots.

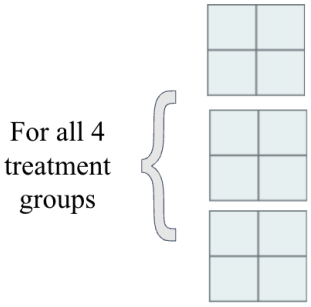

Figure 4: Experimental set-up with quadpots

Variables

Independent Variable	Wild strain and Mutant strain, Bacteria
Dependent Variable	Stem height, Average Leaf Diameter, leaf color, rate of growth
Constants	Type of broth, amount of soil, type of bacteria, amount of zinc sulfate
Replicates	12 soil pots per treatment
Statistical Analysis	T-tests & Standard Deviations

Results

Table 5: Average Leaf Diameter Measurements at 18 days Across Treatment Groups(Raw Data)

	WTB	WTNB	MTB	MTNB
Plant 1	0.2, 0.1	0.1, 0.2	0.2, 0.3	0.05, 0.05
Plant 2	0.3, 0.2	0.4, 0.4	0.4, 0.1	0.1, 0.05
Plant 3	0.4, 0.4	0.3, 0.2	0.08, 0.06	0.1, 0.05
Plant 4	0.4, 0.3	0.5, 0.4	0.05, 0.05	0.05, 0.05
Plant 5	DNG	0.2, 0.1	0.05, 0.05	0.3, 0.2
Plant 6	0.5, 0.4	0.5, 0.4	DNG	0.2, 0.3
Plant 7	0.2, 0.2	0.4, 0.5	DNG	0.3, 0.4
Plant 8	0.4, 0.3	DNG	0.3, 0.3	0.2, 0.1
Plant 9	0.4, 0.2	0, 0.1	0.05, 0.05	0.03, 0.4
Plant 10	0.5, 0.6	0.5, 0.3	0.3, 0.2	0.05, 0.05
Plant 11	0.05, 0.05	0.5, 0.4	0.3, 0.4	DNG
Plant 12	0.6, 0.4	0.4, 0.4	0.2, 0.2	0.4, 0.1

Table 5 displays the leaf diameter of all 48 plants at 18 days of measurement. The diameter was measured from the base of the leaf to the apex via a millimeter ruler. The purpose of measuring leaf diameter along with stem height was to observe if B. Subtilis aided in both types of plant growth(primary and secondary). Primary growth makes plants taller and longer, while secondary growth makes them thicker and wider. DNG denotes seedlings that did not germinate, and were not included in the final processing of the data.

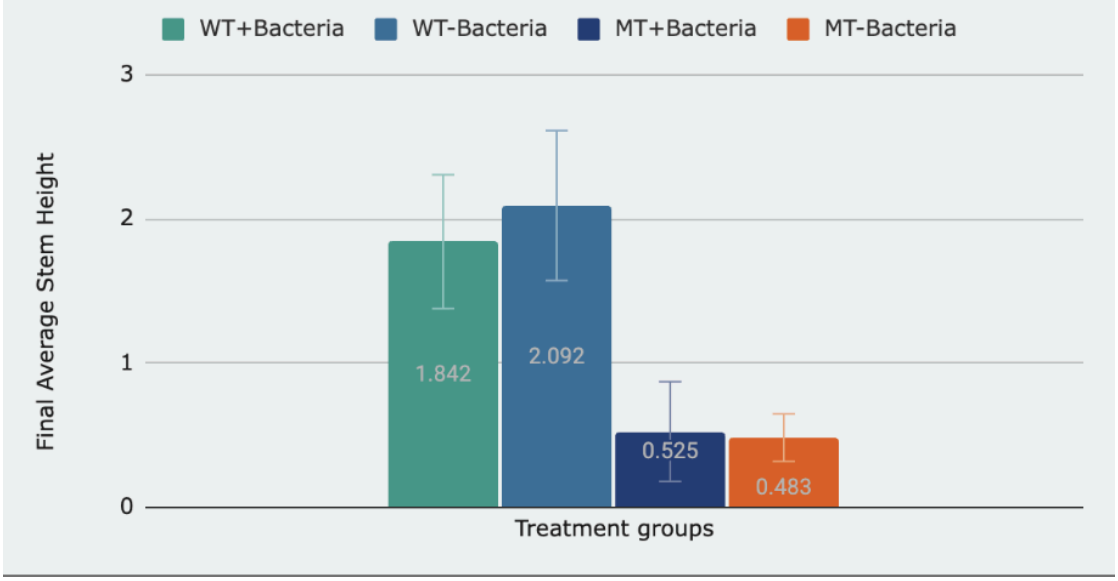

Figure 6: Average Stem Height in Different Soil Arabidopsis types at 19 days(cm)

Figure 6 describes the average stem height of the different treatment groups at 18 days. The stem height was measured from the base of the stem to the apex using a millimeter ruler. The values displayed within each column is the average stem height of all of the plants. The seedlings that did not germinate were not included in the average. The 95% confidence intervals validate the results of the t-tests: there was no significant statistical difference between WTB and WTNB, and between MTB and MTNB; however, there was a difference between WTNB and MTB, and MTB and WTB. This is because there is no overlap between the confidence bars.

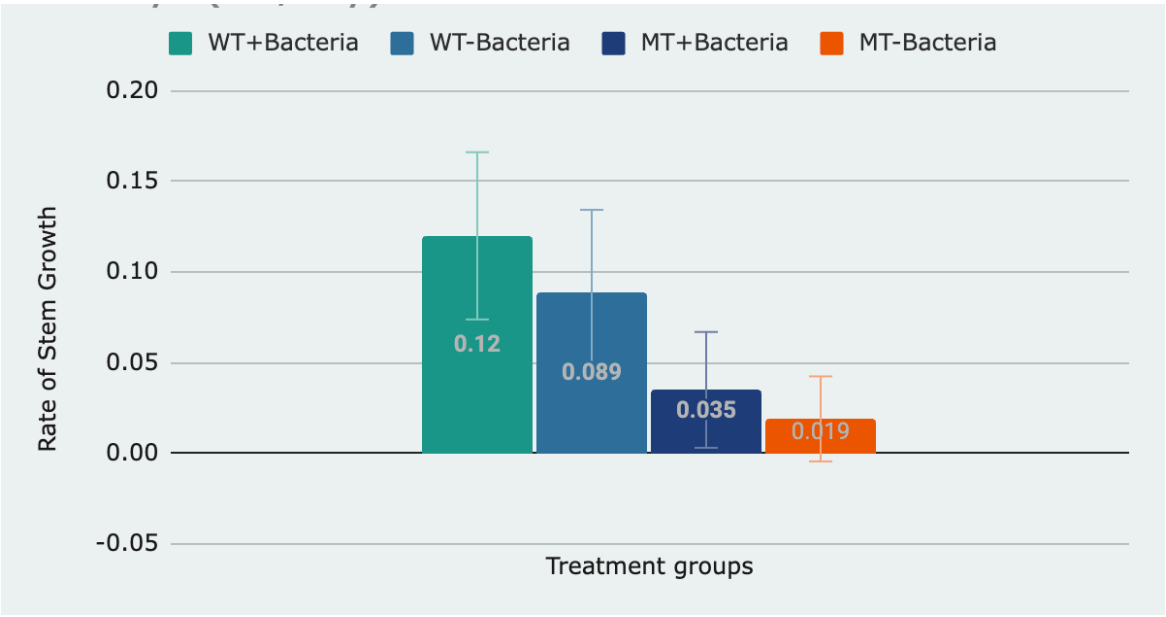

Figure 7: Average Rate of Growth of All Experiment Groups at 18 days(cm/day)

Figure 7 showcases the average rate of growth of all treatment groups at 18 days. The rate of

growth(ROG) was calculated by dividing the stem height at 18 days of the plants by the number of days elapsed(18). The values within the columns are the average ROG of the plants, averaged between all the plants of that group. The significant difference results for the rate of growth generally followed the results from stem height at 18 days; importantly, the 95% intervals for the WTNB and MTB groups support the t-test that there was no significant difference between the MTB and WTNB groups for the rate of growth at 18 days. This is because the interval bars intersect/cross.

Table 8: T-Test Values of All Treatment Groups Across Every Measurement at 18 days

Treatment Group	WTB and WTNB	WTNB and MTB	WTB and MTB	MTB and MTNB
T-Test Values for Stem Height	0.8267019365	0.000827467521	0.0002130736589	0.1880897732
T-Test Values for Leaf Diameter	0.9442746557	0.0221469566	0.02792217736	0.6153234532
T-Test Values for Rate of Growth	0.5536354177	0.08177278889	0.02190894396	0.5627678814

Table 8 displays the processed data of all of the quantitative measurements(stem height, leaf diameter, rate of growth) of the experiment at 18 days. All of the following conclusions are based on if the calculated t-test value was greater or less than the alpha value, 0.05, indicating the presence of a statistical difference. There was no significant difference in both stem height and leaf diameter between the WTB and WTNB groups and the MTB and MTNB groups, as the t-test values are greater than 0.05. However, there was a significant difference between the WTNB and MTB and WTB and MTB groups for leaf diameter and stem height, since the computed t-test values are less than 0.05. Importantly, there was no difference in rate of growth between the WTNB and MTB groups, as the t-test number was greater than the alpha value. Following the trend for stem height and leaf diameter, WTB and WTNB had no statistical distinction, along with MTB and MTNB. Finally, there was a significant distinction between the WTB and MTB.

Table 9: T-Test Values of Stem Height at 39 days of all Treatment Groups				
Treatment Group	**WT +bacteria(WTB) and WT -bacteria(WTNB)**	**WT-bacteria(WTNB) and MT+bacteria(MTB)**	**WT+bacteria(WTB) and MT+bacteria(MTB)**	**MT+bacteria(MTB) and MT-bacteria(MTNB)**
T-Test Values for Stem Height	0.3689397661	0.000000278210	0.000000062798	0.000677174677

Table 9 describes the processed data of the final stem height of all treatment groups. There was no significant difference in the WTB and WTNB group. Nevertheless, there was a difference statistically between WTNB and MTB, WTB and MTB, and MTB and MTNB, since the calculated t test value is less than the alpha value, 0.05. Notably, MTB and MTNB had a significant statistical difference, an important contrast compared to other measurements.

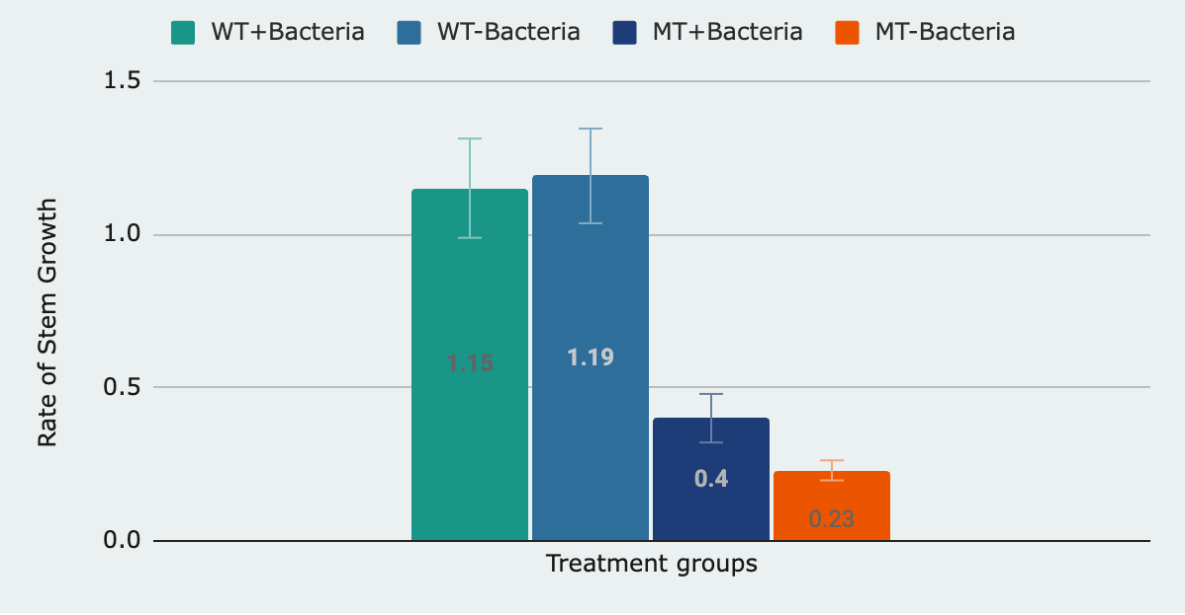

Figure 10: FInal Rate of Growth of All Experimental Groups along 39 days(cm/days)

Figure 10 displays the final growth rate at 39 days of each of the 4 treatment groups. The value inside the column is the average rate of growth of each treatment group. Due to the lack of overlap for the 95% confidence intervals between the WTNB and MTB groups, there is a

significant difference in terms of the **final** growth rate of the 2 groups. This is an important difference in comparison to the initial growth rate(18 day growth rate). Additionally, there was a significant difference between the MTB and MTNB groups.

Figure 11: WTNB Quadrant at 39 days of Growth

Figure 11 showcases a WTNB quadrant of plants on the 39th(final) day of data collection. Comparatively, the WTNB group had lighter colored leaves compared to all groups and greater primary and secondary growth versus the mutant groups.

Figure 12: MTB plant at 39 days of growth, emphasized dark color

Figure 12 displays a typical MTB plant. This photo was taken on the final day of measurement, and showcases its darker color compared to the WTNB and MTNB groups, which did not receive bacteria. However, this group and the MTNB group displayed limited/stunted growth, due to the removal of the gene AT1G52340.

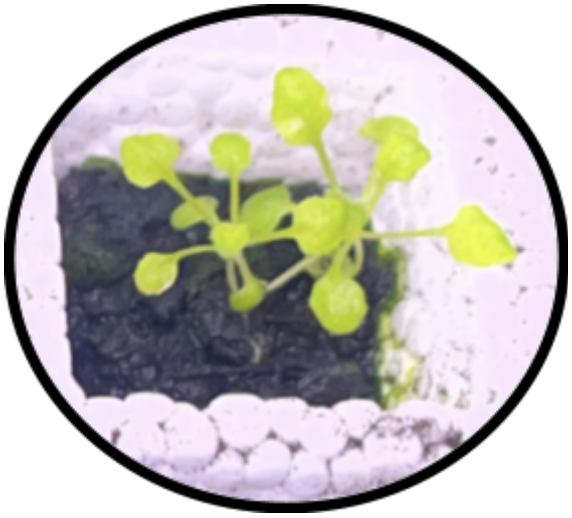

Figure 13: MTNB seedling at 18 days of growth, highlighting light color

Figure 13 shows a standard MTNB seedling, taken at 18 days of growth. This photo emphasizes the light color of the MTNB plants, a general pattern across the groups without bacteria present. Additionally, the MTNB group had characteristic “stunted” growth compared to the WT groups due to the removal of the AT1G52340 gene, which caused it to have decreased **absolute** vertical and horizontal growth.

Figure 14: Arabidopsis Plant in Soil Pots under Microscope

Figure 14 displays the presence of trichomes of the WTB group at 18 days. This image was captured via a microscope, which was used to observe the trichomes effectively. Importantly, trichomes are triggered in response to a foreign pathogen, indicating that the plant had an effective immune response.

Figure 15: Vertical growth of WTB at 39 days

Figure 15 showcases a typical WTB plant at 39 days. It emphasizes the vertical growth of the WTB groups; compared to the mutant groups, the wild arabidopsis had greater lateral and vertical growth, as they were not genetically modified.

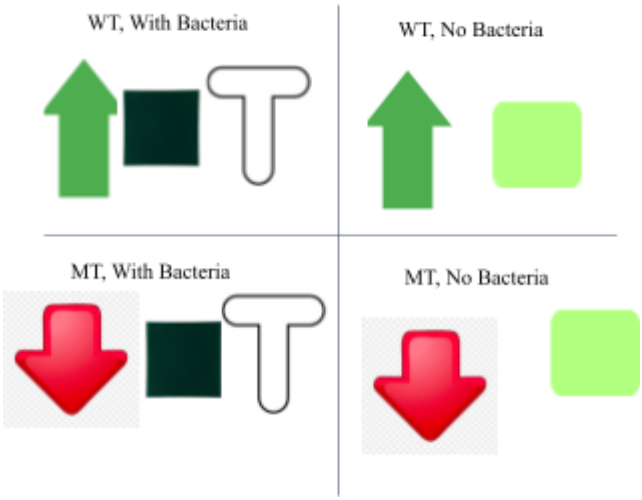

Figure 16: Visual Presentation of Observations

Figure 16 describes the summarized qualitative observations of all the treatment groups at 39 days. The green upward arrow symbolizes increased growth, while the red downward arrow represents stunted growth. Stunted growth was characteristic across both groups of mutants. Additionally, the dark square represents darker leaves, while the light square illustrates lighter colored leaves. The WTB and MTB groups had darker colored leaves, while the groups without

bacteria had lighter leaves. Finally, the capital, “bubble-letter” T represents if the plant had trichomes present at day 39; these hairs were only present for the groups with bacteria supplementation.

Discussion/Conclusion

2 sets of data were measured at 18 and 39 days to determine if *B. Subtilis* would have a different effect on initial versus overall growth. Initially, the WTNB group had the greatest stem height of the groups, at an average of 2.09 centimeters. However, the WTB group had the highest growth rate, at 0.12 centimeters per day. This supports that the bacteria aided the plants growth by accelerating growth as the days progressed. Additionally, The WTNB and MTB groups had no statistical difference at 18 days for the growth rate, indicated by the p-value being more than 0.05. This supports the idea that the bacteria may have compensated for the lack of ABA production in the MTB by making the plant produce more ABA using an alternate pathway of ABA synthesis, instead of relying on the primary method of aba production via xanthoxin dehydrogenase(ABA2 enzyme). This was not supported for lateral/secondary growth, however, and was only supported by primary/vertical growth.

The mutants generally had decreased growth compared to the wild type plants. This is because the mutant plants did not possess a functional AT1G52340 gene, which governs the ability of the plant to produce ABA(the primary pathway). Excising this gene caused the plant to have to rely on secondary means of production to create ABA, causing the plant to spend increased energy and nutrients on production of ABA.

WTB did not have the greatest growth rate as the experiment progressed(towards the latter half of the experiment), as the WTNB group eventually had the greatest growth rate, at 1.19 cm/day. This supports the idea that the bacteria had varying effects on the seedling’s growth: initially, it facilitated the WTB plant’s development via production of more ABA to counter the toxic environment. However, as the experiment commenced, the bacteria may have begun competing with the plants for nutrients or imposed an increased metabolic burden by requiring the plant to sustain the plant–microbe interaction. These long-term costs may have reduced the growth-promoting effects observed during the early stages of development.

Overall, inoculation with bacteria at 18 days increased the rate of growth of both types of Arabidopsis plant compared to identical groups without bacteria. Even at 39 days, bacterial inoculation helped the mutant plant have a greater rate of growth compared to the MTNB group. This is likely since *B. Subtilis* acted as a PGPR, enhancing plant growth through various mechanisms.

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Additionally, the plants inoculated with bacteria had trichome presence and had darker colored leaves compared to their non-innoculated counterparts. This potentially indicates that these plants had an effective immunogenic response, as trichomes “spike” up in response to foreign pathogens. Additionally, the darker leaves suggest that the inoculum caused an increase in chlorophyll production and nitrogen uptake of the plant. These effects are vital for the health of the plant, as as increased chlorophyll content enhances photosynthetic efficiency, allowing the plant to produce more carbohydrates and energy for growth, while improved nitrogen uptake supports the synthesis of proteins, nucleic acids, enzymes, and chlorophyll itself.

Overall, the hypothesis was somewhat supported, since the WTB had the highest growth rate of my plants at 19 days; yet over time, the addition of bacteria injured the plant, decreasing the growth rate until eventually, the WTNB overtook it.

Errors and Extensions

Initially, some seeds did not germinate, which prompted the supplementation of additional seeds as a replacement. This may affect the data, as both the initial and final growth rates depend on the number of days since germination. As the seeds were planted on different days, the number of days since germination differs, thereby affecting the validity of the growth rates. Supplementary seedlings were not planted past day 2 of germination.

Additionally, this experiment assumed that *B. Subtilis* always had a positive effect on the plant. In reality, increased inoculation could have enduring negative effects on plant growth, as excessive bacterial colonization may create competition for nutrients, alter microbial community dynamics, or trigger plant stress responses. Consequently, the beneficial effects observed in this study may not persist under higher inoculation concentrations or over longer periods of time

A modification to the methodology of experiment to improve data validity would be to add more seeds to all quadrants and have more replicates. This strengthens the credibility of the data while eliminating the need for the addition of seeds.

A possible extension of this experiment is to test for other types of abscisic acid deficient mutants. This verifies that bacterial remediation via B. Subtilis inoculation is applicable to other pathways involved in ABA synthesis, as the plant contains many secondary and tertiary routes of production.

Additionally, the use of a less detrimental gene that is still involved in resistance to heavy metals/abiotic factors is another potential extension. The removal of the AT1G52340 gene in the ABA2-1 had deleterious effects on the mutant, including stunted growth and increased evaporation. Ideally, the removal of a gene that subsequentially doesn't induce deleterious effects would allow for better and consistent testing conditions across all types of Arabidopsis plants.

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