**Master’s Thesis**

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**TITLE**

Optimization of biostimulant encapsulation and its efficacy on tomato

**ABSTRACT**

Finding sustainable ways to feed an ever-expanding population while reducing our reliance on synthetic fertilizers is of the utmost importance to humanity. To accomplish this, new sustainable agriculture techniques are required. One technique that has garnered attention is the use of plant growth promoting bacteria (PGPB) as organic biostimulants. These microorganisms operate through various modes of action, and the inoculation profile and load can be tailored to suit the target crop. Biostimulants have been shown to provide tangible benefits to the plant ranging from increased stress tolerance to reduced nutrient requirements. However, while commercial biofertilizers do currently exist, their widespread implementation has been hindered by inconsistent field results and a lack of economic feasibility stemming from sub-optimal carrier materials. One promising avenue is the use of an encapsulating carrier material, such as chitosan or alginate. These biopolymers can form inoculated granules capable of delivering PGPB and arbuscular mycorrhizal fungi (AMF) that promote plant growth, stress tolerance, and produce shelf life. The goal of this research is to elucidate the effects of encapsulated biostimulant inoculation on tomato plants and to improve the methodology for encapsulated biostimulant granule creation.

**Key words**: biostimulant, PGPB, AMF, chitosan, alginate, encapsulation, desiccation, tomato

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1. **BACKGROUND**

The human population has recently surpassed 8 billion and is expected to exceed 9.8 billion by 2050 (UN, 2017), and this expanding population requires an expanding food supply, with food production projected to increase by 70% from 2005 to 2050 (Noel *et al.*, 2015). The predominant approach to maximizing crop yield is the use of synthetic fertilizers, chemically manufactured sources of nitrogen, phosphorous, and potassium, which have had environmental impacts including groundwater contamination, water body eutrophication, ecosystem disruption, greenhouse gas emission, and stratospheric ozone depletion (Conley *et al.,* 2009; Rosa & Gabrielli, 2022). These synthetic fertilizers are also expensive, accounting for 20% to 36% of a farm’s budget, with this cost nearly tripling in the past two years (Schnitkey *et al.,* 2021; Brownlie *et al.,* 2023). A recent report has stated that microbial fertilizers have the potential to generate billions in social benefits be reducing the emissions from synthetic fertilizers (UC Innovation Commission, 2023). Sustainable alternative solutions to synthetic fertilizers are necessary to alleviate the environmental and economic toll of synthetic fertilizers while also feeding an expanding human population (Fitter, 2012).

One possible solution is the use of biostimulants, which include microorganisms such as plant growth promoting bacteria (PGPB), arbuscular mycorrhizal fungi (AMF), and biologically derived compounds (humic acid, biochar, kelp extract, etc.) that promote plant growth, improve crop quality and yield, and increase plant stress tolerance. Applications of microbial biostimulants have been found to reduce fertilizer requirements by 25% and increase crop yield by 17.9% on average (Adesemoye & Kloepper, 2009 ; Li *et al.,* 2022). While biostimulants show promise as a potential alternative for or amendment to synthetic fertilizers, microbial biostimulants are currently held back from widespread implementation due to logistical constraints arising from sub-optimal carrier materials and a lack of awareness from farmers. Carrier materials are necessary for inoculant production, as without a carrier most PGPB populations decline rapidly after inoculation (Bashan *et al.,* 2014).

Current carrier materials can be classified as either wet or dry, with dry inoculants (peat, clay, lignite, etc.) having longer shelf lives, lower contamination risk, and cheaper storage and transportation costs than liquid inoculants (Bashan *et al.,* 2014). Dry carriers can be applied directly to the soil or dissolved in water for a foliar inoculation, while wet carriers are applied almost exclusively as a foliar inoculation. In a broad review (Li *et al.,* 2022), soil inoculation has generally been shown to be more effective at increasing yield than foliar inoculation. Dry inoculants have shown inconsistent results in the field, shown unreliable performance in different environments, are often expensive, and are easily contaminated, preventing their implementation.(Bashan *et al.,* 2014) To achieve the next step towards widespread biostimulant use, inoculation methods must prove that they can reliably maintain microbial viability and efficacy in the field after long-term storage and exposure to environmental stressors, all while being cheap, safe, and easy to produce and use (Bashan *et al.,* 2014).

A new inoculation method that has shown promise is encapsulation, the process of immobilizing biostimulants in droplet-derived granules which can then be dried for storage and transport. Encapsulation requires an encapsulation base and a cross-linker. One encapsulation base that has shown great promise is chitosan, itself a biostimulant that has been shown to increase plant growth and stress tolerance in tomato plants and can entrap microbial biostimulants in granules (Chanratana *et al.,* 2019). Another encapsulation base, alginate, is also a biostimulant that can entrap microbes (Joe *et al.,* 2012). Encapsulation has been shown to increase microbial viability in the presence of desiccation stress, as well as allow for a controlled release of the inoculant (Schoebitz *et al.,* 2013). Studies have shown that both bacteria and fungi can be encapsulated and maintain their viability, but many of these studies have been done with alginate rather than chitosan, despite chitosan having been shown to be more effective at maintaining microbial viability than alginate (Declerck, 1996; Bashan & Gonzalez, 1999; Chanratana *et al.,* 2018). Studies also suggest that encapsulation is necessary for PGPBs and AMFs to achieve maximum effectiveness and viability while also being practical for implementation by farmers (Bashan *et al.,* 2014; Berninger *et al.,* 2018).

Chitosan, a biopolymer derived from crustacean shells and fungi, is made up of repeating N-acetylated glucosamine units, generally 85% deacetylated. When combined with an acid, the deacetylated amine groups protonate, becoming NH3+. This allows the amine groups to act as binding sites for negatively charged functional groups, like those found on cross-linking agents. The acid used to protonate the chitosan, as well as the degree of protonation, have large impacts on the final granule formation and strength, and are likely to alter the microbial release rate (Fanaee & Filiaggi, 2023). Acetic acid is commonly used as a protonation agent throughout the literature (Chanratana *et al.,* 2018; Fanaee & Filiaggi, 2023). No tests have been done with organic acids besides acetic acid to protonate chitosan, and protonation with other organic acids (formic, propionic, butyric, etc.) may prove useful in altering granule morphology, water adsorption, and release characteristics. The degree of protonation affects the conformation the polymer takes, as shown in **Figure 1**, which will also likely affect granule characteristics (Dey *et al.,* 2016). Sterilization of encapsulation components is important to remove any possible prior microbial contamination, and this acid protonation makes sterilization difficult, as the protonated chitosan cannot be autoclaved without undergoing chain fragmentation, as at elevated temperatures the chitosan is likely to undergo acid hydrolysis, as shown in **Figure 2.** This means that the production of chitosan-encapsulated inoculants will need to either find an order of operations that does not autoclave protonated chitosan or find an alternate method for the sterilization of the protonated chitosan solution. Finding an order of operations to not autoclave chitosan may prove challenging, as chitosan needs to be in an acidic solution to be soluble in water.

Alginate, a biopolymer derived from algae,is made up of randomly distributed β-D-mannuronate and α-L-guluronate units. Unlike chitosan, alginate does not require a protonation step prior to encapsulation and does not suffer from chain fragmentation when autoclaved in solution, making it significantly easier to process, while eliminating the cost of the acid. It is also an order of magnitude less expensive than chitosan. The literature surrounding the use of alginate and chitosan is unclear as to which is better as an encapsulation agent for microbial biostimulant immobilization.

As for cross-linking agents, the inorganic sodium tripolyphosphate (TPP) has traditionally been successful in forming inoculated granules with chitosan, and is gentle and non-toxic, making it an excellent choice for inoculant production (Chanratana *et al.,* 2019). For alginate cross-linking, calcium chloride has been shown to reliably produce granules. The capture efficiency of some, but not all, bioactive compounds depend on the cross-linker's pH, and on the understanding under what conditions the cross-linker enables high efficiency biostimulant capture is of high importance to inoculant production (Ghanem & Skonberg, 2001).

Desiccation is necessary for the successful commercialization of an encapsulated microbial biostimulant. Desiccation can increase the encapsulated microbial density, stop bacterial metabolism, and lower transportation and storage costs, but desiccation is also known to be one of the main causes of unviability in inoculants (Meng *et al.,* 2008; Berninger *et al.,* 2018). Microbes are not inherently tolerant to desiccation. Desiccation induces oxidative stress from ROS buildup and is known to damage membranes, proteins, and DNA of cells, with protein damage thought to be the leading cause of desiccation-induced cell death (Franca *et al.,* 2007; Tapia & Koshland, 2014). Previously, desiccation has been shown to induce protein aggregation, which subsequently induces molecular crowding, lipid peroxidation, ROS production, and leakage in the cell (Wang *et al.,* 2012; Bednarska *et al.,* 2013; Tapia & Koshland, 2014). In addition, the removal of water can cause protein misfolding and changes to the protein’s structure, which can lead to cell damage.

Despite multiple endogenous mechanisms for dealing with desiccation stress, finding methods to increase desiccation tolerance in microbes is key to successful inoculant production (Greffe & Michiels, 2020). Air drying is a simple technique that involves exposing the inoculated granules to air at ambient pressure or under a vacuum (Santivarangkna *et al.,* 2007) and is highly effective at a low cost. There are many different approaches to air drying, ranging from simple airtight containers filled with a desiccant, such as silica gel, all the way to complex vacuum heated chambers with shaking plates (Berninger *et al*., 2018). The inoculant density heavily influences desiccation time, with more densely packed inoculants requiring longer drying times. Air drying also enables the microbes in the inoculant to produce endogenous responses to drought stress, increasing their viability.

In addition to questions associated with developments of inoculated granules, the timing at which the inoculants are introduced to the plant has been shown to greatly influence the inoculation efficiency, with some strains showing increased performance with seed inoculation, and others showing increased performance with soil inoculation (Ciccillo *et al.,* 2002; Afzal *et al.,* 2011). Biotic factors also influence inoculation efficiency and can be inhibited by factors like microbiome mass effects, priority effects, and niche overlap (Verbruggen *et al.,* 2012). The mass effect is a phenomenon where microbes in sufficiently high concentrations can overcome unfavorable conditions to survive and influence their environment. The priority effect is a phenomenon where the microbes that are introduced to an ecosystem *first* can outcompete microbes that under other circumstances could outcompete them and fill a particular niche. These effects are critical to consider when considering the use of microbes to increase plant health and crop yield.

In summary, microbial viability and efficacy following encapsulation-desiccation and subsequent storage hinge upon many factors such as encapsulation base, cross-linker, and dropwise addition conditions, as well as desiccation method, osmoprotectant addition, storage conditions, and more. There are many gaps in the literature surrounding chitosan encapsulation that need to be filled before chitosan can reasonably be considered for industrial commercialization. There have been no studies done on the effectiveness of chitosan encapsulated-desiccated granules and their effect on plants, especially in low-water intensity farming. Low-water intensity farming reduces the potential for nutrient runoff and produces greater yields, however this often comes at the cost of crop quality (Stamatakis *et al,* 2003). Salt stress produces greater crop quality, often at the cost of yield by inducing blossom-end rot, which results in unmarketable fruit (Rosadi *et al.,* 2014). By combining these techniques with the application of biostimulants, it should be possible to offset the quality-quantity tradeoff, achieving a large yield of high-quality fruit. Previous research has shown that individual PGPB, such as *Methylobacterium oryzae*, leads to increased fruit quality and yield in both foliar and soil applications (Chanratana *et al.,* 2019). Multiple studies have shown that combinations of PGPBs are more effective than lone PGPBs (Madhaiyan *et al.,* 2010; He *et al.,* 2019), with the exact biostimulant load depending on the target crop and soil conditions, warranting further research. The overarching objective of my research was to test chitosan as a carrier material for PGPBs, ranging from a single species (*M. oryzae*) to a mixture of multiple species, and test the impact of timing and method of application on plant growth and health. Specifically, I addressed the following hypotheses and objectives:

1. **HYPOTHESES / OBJECTIVES**

**Objective 1.** Determine the effect of *M. oryzae* inoculation method on tomato plant growth and crop quality and yield.

**Hypothesis 1.1** – Because of the mass effect, combined soil and foliar *M. oryzae* application will increase salt stressed tomato plant fluorescence parameters, fruit yield, and fruit quality more than either soil or foliar inoculations.

**Objective 2 –** Determine the effect of biostimulant granule (BG) inoculation timing on tomato plant growth and crop quality and yield.

**Hypothesis 2.1 –** Because of the priority effect and the mass effect, BGs with the full microbial consortium applied at both germination and transplantation will increase fluorescence parameters, fruit yield, and fruit quality more than either germination or transplantation inoculations.

**Objective 3 –** Determine the effect of biostimulant inoculation method (liquid, BG, uninoculated granule) on plant growth and fluorescence parameters.

**Hypothesis 3.1 –** Because these bacteria have been shown to increase tomato health and encapsulation will aid in bacterial delivery, inoculation of tomato plants with BGs will increase plant growth and fluorescence parameters more than liquid inoculation or uninoculated granules.

**Objective 4 –** Determine the effect of polymer and decomposition media on the decomposition rate of BGs.

**Hypothesis 4.1 –** Because of differences in the physical properties of alginate and chitosan granules as a result of the cross-linking reaction, BGs made of alginate will decompose more quickly than BGs made of chitosan.

1. **METHODOLOGY**

**III.A. Biostimulant Granules**

**III.A.1. Biostimulant Granule Creation**

Biostimulant granules (BGs) with chitosan were created by first autoclaving 250 mL of DI water, to which was added 5% chitosan (85% deacetylated), which was then protonated with 0.3 M HCl. A solution of 5% TPP was created and autoclaved. Microbes collected in the stationary phase were added to 50 mL protonated chitosan aliquots at a concentration of 5x106 cfu/mL. Bacterial concentration was achieved by culturing bacteria in their respective media for 72 hours, taking measurements with a spectrophotometer at OD600, comparing that to a known calibration curve, and diluting accordingly. Inoculated aliquots were combined to form a single inoculated chitosan solution with each bacteria at 1x106 cfu/mL. The microbial chitosan mixture was then added to the TPP via dropwise addition on a lab bench sterilized with 70% ethanol 15 cm away from a Bunsen burner using a peristaltic pump and a 2mm nozzle, with the TPP submerged in an ice bath. After curing for 30 minutes, the granules were transferred to a vacuum desiccation chamber and allowed to dry for 24 hours at 30°C and 0.5 atm. The varying morphology in dried chitosan BGs created under the same conditions can be seen in **Figure 3.** A comparison of dried and undried chitosan BGs can be seen in **Figure 4.**

To create BGs with alginate, a similar process was followed but using 2% alginate as the encapsulation base and 2% calcium chloride as a cross-linker. Both solutions were prepared, then autoclaved, and from there made in the same manner as the chitosan BGs sans ice bath.

The following microbes were used in this research, with emphasis on those that have already been proven to provide benefits to tomato plants. This combination of microorganisms (at 1x10^6 cfu/mL each) will be referred to as the “microbial consortium”.

*Azospirillum brasilense Sp7 -* PGPB that benefits the plant via nitrogen fixation, siderophore production, and by increasing lateral root growth (Sahoo *et al*, 2014; Li *et al*, 2005), and has been shown to increase plant stress tolerance (Casanovas *et al*, 2002). It has been shown to increase crop yield and plant nitrogen, phosphorous, and potassium content (Askary *et al*, 2009). It has also been reported to work well with *Methylobacterium oryzae* (Madhaiyan *et al*, 2010).

*Azotobacter chroococcum 43 -* PGPB that operates via nitrogen fixation, phosphate solubilization, and vitamin, indole acetic acid (IAA), gibberellin (GA), hydrogen cyanide (HCN), siderophore, and cytokinin (CK) production (Abd El-Fattah *et al*, 2013; Revillas *et al*, 2000; Wani *et al*, 2007). Shown to increase germination rates and aboveground biomass and crop quality and yield in maize (Zahir et al, 2005).

*Bacillus subtilis* (type strain) *-* PGPB that has been shown to improve fruit quality and yield in tomato (Mena-Violante & Olalde-Portugal, 2007; Kokalis-Burelle *et al*, 2002) and shown to increase metabolite production (Sharaf-Eldin *et al*, 2008). Shown to solubilize phosphate, fix nitrogen, produce IAA, CK, GA, HCN, and antibiotics, as well as exhibiting phytase activity (Ahmad *et al,* 2008; Arkhipova *et al,* 2005; Yao *et al,* 2006). It has been used as a biocontrol agent against aphids and pathogenic bacteria (Kokalis-Burelle *et al,* 2002).

*Methylobacterium oryzae CBMB20 -* PGPB that has been shown to improve fruit quality and yield in tomato in both foliar and chitosan encapsulated inoculations (Chanratana *et al.,* 2019). Operates through phytohormone (auxin and cytokinin) production, stress reduction via ACC deaminase production, increased nutrient availability through nitrogen fixation, and as a biopesticide (Chauhan *et al*., 2015).

*Pseudomonas putida 90 -* PGPB that increases plant growth by solubilizing phosphate and producing IAA and siderophores (Hariprasad & Niranjana, 2009). Shown to inhibit ethylene production (Mayak *et al,* 1999). Shown to significantly increase tomato fruit macro- and micronutrient content (He *et al,* 2019). Shown to increase potassium, magnesium, and calcium uptake and decrease sodium uptake (Yao *et al,* 2010). Also shown to increase plant root and shoot growth (Glick *et al,* 1997; Hall *et al,* 1996).

**III.A.2. Bacterial Granule Decomposition Trial**

Soil was collected from the KSU Field Station and brought back to the lab. BGs were created with one of two polymers (chitosan or alginate) and one of two inoculation treatments (full microbial consortium and uninoculated) and were stored in petri dishes containing one of two mediums (soil or filter paper). Ten grams of soil or a piece of filter paper was added to the petri dish, to which was then added ten grams of water and ten BGs. Initial weight was recorded. Ten identical samples were prepared for each treatment group. A subset of samples was measured for weight once a week over ten weeks, with measured samples being discarded. Upon measurement, BGs were removed from the petri dish, patted dry with a paper towel, and weighed. Statistical power for this trial was 0.99 for an effect size of 0.4 with 80 groups and 10 replicates per group at a 0.05 significance level.

**III.B. Greenhouse Trials**

**III.B.1. Tomato Inoculant Location Trial**

This trial was performed to assess the effect offoliar, soil, and joint inoculation of *M. oryzae* on salt stressed tomato plant health and crop quality and yield. This trial took place in the hydroponic greenhouse at the KSU field station (34.0622° N, 84.6034° W). Tomato seeds were germinated in Oasis cubes and fertilized for three weeks before being transplanted into Dutch buckets containing Mother Earth™ Coco + Perlite Mix, copper-lined root barriers, and Mother Earth™ Hydroton Original clay pellets. The sample size (n) was 32 tomato plants across four rows (A to D) with eight plants per row. Row A was the control, with no treatment applied. Row B was subjected to soil inoculation with BGs at transplantation by placing a few BGs (~5 g) beneath the roots when transplanting. Row C was subjected to foliar inoculation with a spray outside the greenhouse prior to transplantation. Row D was subjected to both soil and foliar treatments as described. The unit of replication for bacterial treatments are the tomato plants. The unit of replication for sugar and weight measurements are individual tomatoes. However, because the individual tomatoes are pseudoreplicates (we aren’t applying the treatment to the individual tomatoes, but rather the plant that those fruit comes from) they were summarized by plant prior to analysis.

Plants were inoculated at the time of transplantation. A nutrient mix composed of 1.32 g/L Jack’s 0-12-26, 0.075 g/L magnesium sulfate, and 0.912 g/L calcium nitrate was mixed in 1000-liter batches and fed into the Dutch buckets. To salt stress to the plants, sodium chloride was added in 50g batches until the nutrient solution reached an electrical conductivity of 3.5 mS/cm. Plants were allowed to grow for 7 months before teardown, with tomato fruits harvested upon ripening, as assessed by color via visual analysis. Initial fruit data was collected at the time of fruit harvesting. Fruit was weighed, assessed for blossom-end rot, and catalogued with row and pot information. Weight measurements were performed with an OHAUS Scout SCA210, and blossom-end rot was assessed by visual observation of the fruit. Authors used a Li-COR Li-600 and PhotosynQ MultispeQ V2 to gather biweekly plant fluorometric parameters. Sugar concentration was measured with a Fisherbrand Brix refractometer. Firmness was measured with an OA Supplies Fruit Pressure Tester (penetrometer). Upon trial completion, 3 leaf samples were taken from each plant and tested for stomatal density using the methods outlined in (GTAC 2016).

The statistical power of this experiment was 0.39 for an effect size of 0.4 with 4 groups and 8 replicates per group at a 0.05 significance level. This is not very powerful, but this was the greatest number of tomato plants possible in the space available to the author at the time.

**III.B.2. Tomato Inoculant Timing Trial**

The space available for the tomato inoculant timing trial was greater than that available for the tomato inoculant location trial, allowing for more replicates per group, thus increasing statistical power to 0.59 for an effect size of 0.4 with 4 groups and 12 replicates per group at a 0.05 significance level. The plants were grown in the hydroponic greenhouse at the KSU Field Station (34.0622° N, 84.6034° W). Tomato seeds were germinated in Oasis cubes and fertilized for three weeks before being transplanted into Dutch buckets containing Mother Earth™ Coco + Perlite Mix, copper-lined root barriers, and Mother Earth™ Hydroton Original clay pellets. A nutrient mix composed of 1.32 g/L Jack’s 0-12-26, 0.075 g/L magnesium sulfate, and 0.912 g/L calcium nitrate was mixed in 1000-liter batches and fed into the Dutch buckets. The objectives of the trial reflect Objective 4.1.

The treatments include inoculation at germination, inoculation at transplantation, inoculation at both germination and transplantation, and a control no inoculation group. All inoculation groups used the entire microbial consortium listed above encapsulated in chitosan. For inoculation at germination, a single BG was placed atop the seed in the Oasis cube. For inoculation at transplantation, approximately 5 grams of BGs were placed in contact with the plant roots. The joint inoculant group effectively received a double dose, as they received inoculation at both germination and transplantation. Each group had 12 replicates, for a total sample size of 48 tomato plants. Plants were germinated on May 1st, 2024 and transplanted on May 23rd, 2024. Plants were allowed to grow until October 19th, 2024, at which point they were taken down with samples collected for stomatal density measurements. Biweekly measurements were taken with a Li-COR Li-600 and two PhotosynQ MultispeQ V2.0s. Fruit were harvested when ripe, as assessed by color and firmness, and were taken back to the lab for analysis. Weight measurements were performed with an OHAUS Scout SCA210, and blossom-end rot was assessed by visual observation of the fruit. Sugar concentration was measured with a Fisherbrand Brix refractometer. Firmness was measured with an OA Supplies Fruit Pressure Tester (penetrometer).

**III.B.3. Tomato Inoculant Method Trial**

This experiment involved cultivating 144 tomato plants (cultivar BHN 589) for 40 days in a greenhouse. There were 4 treatments (control, liquid inoculation, uninoculated chitosan granule, inoculated chitosan granule) with 36 replicates per group. The inoculation included the entire microbial consortium listed above at 1x106 CFU/g each. Seeds were surface sterilized before planting, and inoculation was performed at the time of germination. For liquid inoculants, approximately 10 mL of the liquid microbial consortium was pipetted directly atop the seed. For granular treatments, approximately 5 grams of granules were nestled in contact with the seed. Inoculants were not re-applied throughout the trial. Plants were grown in 6-inch diameter plastic pots with Miracle-Gro potting soil. No fertilizer was applied. Plants were top-watered every other day and allowed to grow for 40 days, with regular height measurements taken with a ruler and fluorescence measurements taken with a Li-COR Li-600. At the end of the growing period, plants were destructively sampled for aboveground and belowground length and mass, as quantified with a ruler for length and an OHAUS Scout SCA210 for mass. The statistical power for this trial was 0.98 for an effect size of 0.4 with 4 groups and 36 replicates per group at a 0.05 significance level.

**III.C. Statistical Analysis**

All statistical analysis were performed in R version 4.4 (R Core Team, 2025) using the packages MASS (Venables & Ripley, 2002) for statistical transformations, vegan (Oksanen *et al.,* 2025) for dimensionality reduction, and multcomp (Hothorn *et al.,* 2008) for pairwise comparisons. Graphs were generated using ggplot2 (Wickham, 2016) for plotting and scico (Pederson & Crameri, 2023) for accessible color palettes. Throughout the course of the statistical analysis, the author created several R functions that culminated in the production of an R package, ztils (Peagler, 2025), which was also used in this analysis. To account for pseudoreplication because we applied our treatments to the *plants* rather than the individual *fruit*, fruit variables were averaged or summarized between each plant prior to analysis.

First, probability density function and cumulative distribution function graphs were created for each variable, and one-sample Kolmorgorov-Smirnov (KS) tests were performed against all exponential family functions. This was not done for variables which are known to be ratios [e.g. PhiPS2] which were just logit transformed and treated as normal. Variables were checked for homoscedasticity with Levene and Bartlett tests.

Linear models or generalized linear models were made for each variable, depending on the results of the PDF, CDF, and KS tests, with post hoc tests being performed as needed. For models that include a continuous predictor variable, prediction plots were made from these models, comparing the observed data with the predictions. For models with only treatment as a predictor, boxplots were made that included annotations for significance.

* Note: For the sake of brevity, no exploratory figures are shown, and only figures that show a **significant difference** are included. Exploratory and non-significant graphs can be found at <https://github.com/zachpeagler/Thesis/tree/main/figures> in both PNG and SVG format.

1. **RESULTS**

**IV.A. Bacterial Granule Decomposition**

Summarized data from this trial is shown in **Table** 1, and all polymer treatments were shown to have a significant decrease (p < 0.001) in mass over time. Change in mass over time is significantly different between polymers, with chitosan having a significantly greater change (p < 0.001) in mass over time compared to alginate, as shown in **Figure 5.** This was calculated by a linear model with logit transformed proportion change in mass as a function of elapsed weeks and polymer. This model has an R2 of 0.1417, meaning that it accounts for 14.17% of the variation in the change in mass.

**IV.B.1 Tomato Inoculant Location**

Stomatal conductance is graphed in **Figure 6**, and all treatments were found to be significantly lower (p < 0.01) than the control using a linear mixed model, modeled with stomatal conductance as a response, treatment as a predictor, and relative humidity as a fixed effect. This model has an R2 of 0.4889, meaning that it accounts for 48.89% of the variance in stomatal conductance. Plant stomatal density significantly decreased (p < 0.01) in the soil treatment as compared to the control, as shown in **Figure 7** and tested with a linear model of the log-adjusted mean stomatal density as a function of treatment. This model has an R2 of 0.1934, meaning that it accounts for 19.34% of the variance in stomatal density. For Photosystem II Efficiency (PhiPS2) the best fitting model as determined by the Akaike information criterion (AIC) was a linear mixed effect model, shown in **Figure 8**. Date was transformed into an integer, days from germination and used alongside treatment as predictor variables in the model, with device as a random effect, and logit-transformed PhiPS2 as the response variable. This model found that the soil inoculation was significantly higher than the control (p < 0.01), the foliar inoculation was significantly lower than the control (p < 0.0001), and the joint inoculation was significantly higher than the control (p < 0.0001), with a post hoc pairwise comparison revealing that all inoculation treatments were significantly different from each other.

Summarized fruit data can be seen in **Table 2**, with asterisks denoting significant differences from the control. Total fruit count across treatments is graphed in **Figure 9**, and joint treatments were found to be significantly lower (p < 0.001) than the control when modeled as a generalized linear model with fruit count as the response, treatment as a predictor, and Poisson as a link function. The pseudo-R2 for this model is 0.1701, indicating that the model accounts for 17.01% of the variation in total fruit count. Marketable fruit count is graphed in **Figure 10**, and foliar and joint treatments were found to be significantly lower (p < 0.01) than the control when modeled as a generalized linear model with marketable fruit count as the response, treatment as a predictor, and Poisson as a link function. The pseudo-R2 for this model is .2042, indicating that the model accounts for 20.42% of the variation in marketable fruit count.

Fruit mass across treatments is graphed in **Figure 11**, and joint treatments were found to be significantly lower (p < 0.001) from the control when modeled as a linear model with log-adjusted fruit mass as a response and treatment as a predictor. This model has an R2 of 0.3142, meaning that it accounts for 31.42% of the variance in fruit mass. Fruit sugar concentration across treatments is graphed in **Figure 12**, and joint inoculations were found to have significantly higher (p < 0.01) sugar content than the control by a linear model with sugar as a response and treatment as the predictor. This model has an R2 of 0.2419, meaning that it accounts for 24.19% of the variance in fruit sugar content.

**IV.B.2. Tomato Inoculant Timing**

All treatments had a significantly lower (p < 0.001) stomatal conductance compared to the control as determined by a linear model of the log adjusted stomatal conductance as a function of treatment and relative humidity, as seen in **Figure 13.** This model has an R2 of 0.5242, meaning that it accounts for 52.42% of the variance in fruit sugar content. For efficiency of photosystem II (PhiPS2), control and germination were significantly lower than the transplantation and combined germination/transplantation groups, as shown in **Figure 14**,as calculated by a linear random effect model with PhiPS2 as a response, treatment as a predictor, days from germination as a fixed effect, and device as a random effect. This model has an R2m of 0.0249 and an R2c of 0.5078, meaning that without the random effect it accounts for 2.49% of the variance in stomatal conductance and with the random effect it accounts for 50.78% of the variance in stomatal conductance. No significant differences were found in fruit parameters.

**IV.B.3. Tomato Inoculant Method**

Tomato plant height was found to be significantly lower in the inoculated BG group as compared to the control, as shown in **Figure 15** and determined by a linear model of log-adjusted height as a function of date and treatment. This model has an R2 of 0.8314, meaning that it accounts for 83.14% of the variance in height. However, a post hoc pairwise comparison revealed that the inoculated BG group height was not significantly different from the uninoculated granule or liquid inoculant treatments. Fluorescence environmental variables were scaled and their dimensionality reduced using principal component analysis. The first two principal components were then used as predictor variables alongside treatment in a pair of linear models, one with log transformed stomatal conductance as a response and the other with logit transformed photosystem II efficiency as a response. These models revealed no significant difference between treatments. Aboveground and belowground mass and length were used to calculate root:shoot mass and root:shoot length ratios. These ratios were then logit transformed and used as the response variables in linear models with treatment as the predictor. These models revealed no significant difference between the treatment groups.

1. **DISCUSSION**

Hypothesis 1.1 stated that salt stressed tomato plant fluorescence parameters, fruit yield, and fruit quality would increase due to combined soil and foliar *M. oryzae* application. The results indicate that the soil and joint inoculation significantly increased PhiPS2 and the foliar inoculation significantly decreased PhiPS2, with all treatments being significantly different from one another, while the stomatal conductivity significantly decreased in all treatments. In addition, the stomatal density significantly decreased in soil inoculation as compared to the control, but the soil inoculation was not significantly different from the foliar or joint inoculations. This is contrary to the expectation laid out in the hypothesis and paints an interesting picture where soil inoculations seem to be associated with a *structural* change in the stomates that may be related to the decreased stomatal conductance, while the foliar and joint inoculations seem to be associated with a purely *behavioral* change in the plants that is driving their decreased stomatal conductivity. This could be due to *M. oryzae* producing ACC deaminase which results in lowered ethylene emissions in tomato (Yim *et al.,* 2004). Ethylene has an important role in tomato stomatal conductance and can inhibit stress-induced stomatal closures (Chen *et al.,* 2013). Reductions in ethylene signaling from an uptick in ACC deaminase production could partially explain reductions in stomatal conductance in inoculated tomato plants. Another possible reason for the decreased stomatal conductivity may be due to the plant hormone auxin. Auxins are an essential regulatory hormone for plants, are critical to many different elements of plant health, and exist in plants in a range, levels outside of which can be detrimental to plant health. Salt stress is known to increase auxin content in tomato plants (Ho *et al.,* 1995) as is *M. oryzae* (Chauhan *et al*., 2015). Thus, it is possible that a combination of salt stress and *M. oryzae* inoculation could increase tomato plant auxin content outside of the healthy range, thereby reducing stomatal conductance.

When looking at the fruit, the joint inoculation exhibited a significantly smaller fruit mass than the control, as well as a significantly greater fruit sugar content. The joint inoculation also exhibits a significantly greater total fruit count than the control, with the foliar and joint inoculations exhibiting a significantly lower marketable fruit count as compared to the control. In summary, joint foliar and soil inoculations of *M. oryzae* produce a higher total yield of smaller, sweeter fruit, while all inoculations reduce stomatal conductivity, with only soil inoculations reducing stomatal density in salt stressed tomato plants, and both foliar and joint inoculations decrease the marketable fruit yield.

Hypothesis 2.1 stated that tomato plant fluorescence parameters, fruit yield, and fruit quality would increase more with the full microbial consortium at germination and transplantation as compared to inoculations at either germination or transplantation. The trial accompanying this hypothesis, the tomato inoculant timing trial, produced results that do not align with the hypothesis. All inoculations significantly decreased plant fluorescence parameters, which are indicative of inhibited photosynthesis and plant growth. This contrasts with findings by Madhaiyan *et al.* (2010) who reported increased tomato plant growth associated with synergistic inoculation of *M. oryzae CBMB20* and *A. brasilense CW903.* There could be a few reasons for this. This trial utilizes five bacteria, rather than two, which could interfere with each other rather than synergize, especially seeing as no research has been done using these specific bacteria in tandem. This trial also uses a different strain of *A. brasilense,* Sp7 rather than CW903.

Neither fruit yield nor fruit quality saw any change due to inoculation. This is in contrast with existing literature which has found that tomatoes co-inoculated with up to four bacteria increased fruit yield and decreased blossom-end rot occurrence (He *et al.* 2019). All the inoculants in this trial were encapsulated in chitosan using the methods outlined in III.A.1. which poses several potential explanations: Because the granules were created on a lab bench near a Bunsen burner, there is a high likelihood of contamination. This could be addressed by moving granule creation to a Biosafety cabinet or a completely sealed reaction chamber. Also, the desiccation process has a chance of reducing microbial viability. This could be addressed via the addition of osmoprotectants such as sucrose or trehalose. In summary, both germination and transplantation inoculations decrease plant fluorescence parameters without having an impact on fruit yield or quality.

Hypothesis 3.1 stated that tomato plants inoculated with BGs would see increased growth and fluorescence as compared to liquid inoculation or uninoculated granule application but was not supported by the data. This is in opposition to results found by other researchers, who have found that *A. brasilense, B. subtilis, M. oryzae,* and *P. putida* have synergistic effects and have been correlated with increased growth in tomato (He *et al.,* 2019; Madhaiyan *et al.,* 2010). There are several possible explanations for this. It’s possible that the combined effects of the bacteria in the microbial consortium produced too many phytohormones (gibberellins, auxins, and cytokinins), inadvertently increasing the stress of the tomato plant and cancelling out any potential benefit they could have provided. It’s possible that all inoculant treatments were outcompeted by native soil microbes, seeing as the soil was not sterilized. It’s also possible that top-watering physically separated the inoculants from the seeds, preventing them from influencing the plants. It’s also possible that, due to the plants being inoculated at seed, the bacteria were not able to successfully establish on/near the seed and were no longer present by the time the plant had begun to grow and produce root exudates, the main nutrition source for the bacteria. The BGs were created using the methods outlined in III.A.1, the potential explanations for which have already been outlined above. In summary, tomato plants grown with inoculated chitosan granules exhibited decreased plant growth compared to control, uninoculated chitosan granule, and liquid inoculant treatments, but exhibited no change in fluorescence parameters or resource allocation.

Hypothesis 4.1 stated that granules made of alginate would decompose more quickly than granules made of chitosan. The results of the bead breakdown trial showed that chitosan beads break down significantly faster than alginate beads, contrary to the hypothesis. One possible reason for this is the differing bond strengths between the two polymer matrices. The Ca-O bond present in the alginate-calcium matrix has a bond strength of 464 kJ/mol (Cottrell, 1958) and each Ca2+ can bind to two carboxylic acid sites on alginate, for a theoretical total of 928 kJ/mol across a single Ca2+ ion and the N-O bond present in the chitosan-tripolyphosphate matrix has a bond strength of 201 kJ/mol (Cottrell, 1958), with the tripolyphosphate having five O- reaction sites for a theoretical total of 1005 kJ/mol. However, the conformation of chitosan-tripolyphosphate cross-linking makes it highly unlikely for any reaction sites on tripolyphosphate other than the two on the ends to be in use, for a realistic bond energy of 402 kJ/mol across a single tripolyphosphate ion. Another confounding factor may be the methodology of this trial. The experiment saw non-desiccated BGs placed in sealed petri dishes with 10 mL of water and either soil or paper, and granule mass was weighed upon plate creation and after a set interval. The problem here is that mass does not equate to decomposition, especially in a situation where the granules are in a hydrated state. It’s possible that any observed differences in mass are due to changes in granule hydration than true decomposition. As such, it may not be appropriate to call this decomposition. In summary, chitosan granules *dehydrate* more quickly than alginate granules when in a hydrated environment.

Going forward, future research should consider the use of alginate as an encapsulation base over chitosan. A pain point for future research to alleviate is the trial length. Tomatoes are nice for their commercial value and ease of growth but take several months to provide fruit data. To hasten development, future research should consider using faster-growing plants, such as turf grass or leafy greens. Of critical importance is the advancement of encapsulation methods, which should look for ways to decrease contamination and increase microbial viability, such as the use of a biosafety cabinet for granule creation and the addition of osmoprotectants. While 3D printed parts are excellent for rapid prototyping, future research should consider relegating the use of 3D printed parts to roles where they will not directly come into contact with the BGs. Microplastic contamination is a real concern with direct 3D printed part exposure, especially for something that is going to be applied directly to the environment.

There could be environmental concerns about developing sustainable agriculture solutions that use sodium tripolyphosphate, an inorganic substance attributed as being partially responsible for water body eutrophication (Greenwood *et al.,* 1997). Due to its cross-linked nature with chitosan, it is unlikely that such solutions are susceptible to runoff in the manner traditional synthetic fertilizers are, but more testing is required to examine the runoff potential of encapsulated granules cross-linked with tripolyphosphate. Both sodium tripolyphosphate and calcium chloride are advised against uses in food, drugs, pesticides, and biocides (Calcium chloride SDS; Sodium tripolyphosphate SDS). While tripolyphosphate is not considered hazardous, calcium chloride is, with a category 2 eye irritation warning. Thus, future development should strive to identify better crosslinkers, as commercialization with these crosslinkers represents a real environmental risk.

Overall, the results found herein largely contrast with the results prevalent in the literature, finding that inoculations of *M. oryzae* to salt-stressed tomato decrease photosynthesis and marketable fruit yield while increasing fruit quality, and that co-inoculation of *M. oryzae* with *A. brasilense, A. chroococcum, B. subtilis,* and *P. putida* in chitosan granules has a confounding effect on tomato plant growth and no effect on tomato fruit yield or quality. Future research is recommended to incorporate arbuscular mycorrhizal fungi alongside synergistic plant-growth promoting bacteria, and to implement stringent protocols to reduce the risk of contamination. Future work is also highly recommended to use alginate over chitosan, and to identify a better cross-linker for alginate than calcium chloride, which poses an environmental safety risk. The population is growing, and agriculture must grow with it. Finding ways to grow more food with less environmental impact is critical to maintaining the health of the planet, and while the results herein indicate that this is not the route to that goal, this work lays the foundation for further biostimulant research that will facilitate sustainable agriculture for all.

**VI. INTEGRATIVE ASPECTS**

This research integrates microbiology, plant biology, chemistry, statistics, and engineering. Microbiology is integrated through the culturing and application of five bacterial species. Plant biology is integrated via the cultivation of tomato plants, the measurement of fluorometric parameters, and the harvest and analysis of tomato fruit. Chemistry is involved with the creation of the BG polymer matrix and further BG optimization. Statistics is integrated via power analysis and experimental data analysis. Engineering is involved in the creation of supporting lab materials and devices, such as microcentrifuge tube racks and cuvette holders through CAD modeling and 3D printing.

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**VIII. Figures**

**Table 1.** Change in mass over two polymers for non-inoculated granules stored for 10 weeks

|  |  |  |  |
| --- | --- | --- | --- |
| **Polymer** | **Mean Initial Mass Per Bead (g)** | **Mean End Mass Per Bead (g)** | **Mean Mass Change (%)** |
| Alginate | 0.041 g | 0.024 g | -41.1%\*\*\* |
| Chitosan | 0.031 g | 0.019 g | -38.5%\*\*\* |

**Table 2.** Summarized fruit data for inoculation location treatments in salt stressed tomato plants inoculated with *M. oryzae.*

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Treatment** | **Total Fruit Count** | **Marketable Fruit Count** | **Mean Fruit Mass (g)** | **Total Fruit Mass (kg)** | **Mean Sugar Content (%)** | **Fruit w/ BER (%)** |
| Control | 477 | 180 | 79.30 ± 54.41 g | 37.83 kg | 7.38 ± 1.41 % | 45.91% |
| Soil | 453 | 171 | 78.85 ± 49.43 g | 35.72 kg | 7.50 ± 1.40 % | 45.70% |
| Foliar | 445 | 134\*\* | 59.77 ± 45.36 g | 26.60 kg\*\*\* | 8.25 ± 2.06 % | 50.34% |
| Soil+Foliar | 598\*\*\* | 133\*\* | 55.30 ± 33.59 g\*\*\* | 33.07 kg | 8.77 ± 1.30 %\*\* | 60.87%\* |

**Table 3.** Summarized fruit data for inoculation timing treatments in tomato plants inoculated with microbial consortium granules*.*

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Treatment** | **Total Fruit Count** | **Marketable Fruit Count** | **Mean Fruit Mass (g)** | **Total Fruit Mass (kg)** | **Mean Sugar Content (%)** | **Fruit w/ BER (%)** |
| Control | 578 | 531 | 118.01 ± 69.05 g | 31.17 kg | 6.41 ± 1.58 % | 8.13% |
| Germination | 487 | 446 | 127.32 ± 80.78 g | 28.02 kg | 6.70 ± 1.55 % | 8.42% |
| Transplantation | 472 | 440 | 127.83 ± 75.42 g | 32.79 kg | 6.75 ± 1.34 % | 6.78% |
| Germ+Trans | 547 | 504 | 105.73 ± 74.69 g | 28.11 kg | 7.04 ± 1.66 % | 7.86% |

|  |
| --- |
| A group of different colored shapes  AI-generated content may be incorrect. |
| **Figure 1.** *Protonation dependent chitosan conformations (Dey* et al. *2016)* |

|  |
| --- |
|  |
| **Figure 2.** *Temperature dependent reaction of chitosan with HCl* |
| A group of round objects  AI-generated content may be incorrect. | |
| ***Figure 3.*** *Dried chitosan BGs at 400x magnification.* | |

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| A close up of food  AI-generated content may be incorrect. |
| ***Figure 4.*** *Chitosan BGs during different stages of the drying process. (a) fresh, (b) 24 hours, (c) 72 hours* |

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| ***Figure 5.*** *Observed data (points) vs predicted (lines with 95% confidence interval) for mass change over time in biostimulant granules composed of two polymers* |

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| ***Figure 6****. Observed data (points) vs predicted (lines with 95% confidence interval) for stomatal conductance in salt stressed tomato plants inoculated with M. oryzae.* | |
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| ***Figure 7****. Mean stomatal density by M. oryzae inoculation location in salt stressed tomato plants.* |

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| ***Figure 8****. Observed data (points) vs predicted (lines with 95% confidence interval) for photosystem II efficiency in salt stressed tomato plants inoculated with M. oryzae.* |

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| ***Figure 9****. Total fruit count across inoculation location in salt stressed tomato plants inoculated with* M. oryzae |

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| ***Figure 10.*** *Marketable (non-BER) fruit count across inoculation location in salt stressed tomato plants inoculated with* M. oryzae |

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| ***Figure 11****. Mean fruit mass by M. oryzae inoculation location in salt stressed tomato plants* |

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| ***Figure 12****. Mean sugar content by M. oryzae inoculation location in salt stressed tomato plants.* |

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| *Figure 13. Observed data (points) vs predicted (lines with 95% confidence interval) for stomatal conductance along relative humidity by inoculation timing of a microbial consortium in tomato plants* |

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| ***Figure 14****. Observed (points) vs predicted (lines with 95% confidence interval) for Photosystem II Efficiency measured with two devices across days from germination by microbial consortium inoculation timing in tomato plants* |

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| ***Figure 15****. Observed (points) vs predicted (lines with 95% confidence interval) for plant height across days from germination by microbial consortium inoculation timing in tomato plants* |