**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) biostimulants. These microorganisms operate through various modes of action, and the inoculation profile and load can be tailored to suit the target crop and inoculation method and 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 beads capable of delivering PGPB and arbuscular mycorrhizal fungi (AMF) that promote plant growth, stress tolerance, and produce shelf life. My research is aimed towards developing inoculated chitosan and alginate beads and testing their efficacy as biological fertilizers.

**Key words**: biostimulant, PGPB, AMF, chitosan, 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 current 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) and 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. 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. Soil inoculation is more effective at increasing yield than foliar inoculation (Li *et al.,* 2022). 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. 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, non-toxic, and simple to use (Bashan *et al.,* 2014).

A new inoculation method that has shown promise is encapsulation, the process of immobilizing biostimulants in droplet-derived beads which can then be dried for storage and transport. Encapsulation requires an encapsulation base, a protonation acid, a cross-linker (i.e., a dicarboxylic acid or other molecule with multiple negatively charged functional groups), and additives that can be introduced to help with maintaining microbial viability. 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 and can entrap microbial biostimulants in beads (Chanratana *et al.,* 2019). Another encapsulation base, alginate, is also a biostimulant that can entrap microbes. 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, as shown in **Figure 1** provided by Hao *et al.* (2017), 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 bead 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 bead morphology, water adsorption, and release characteristics. The degree of protonation affects the conformation the polymer takes, as shown in **Figure 2**, which will also likely affect bead characteristics (Dey *et al.,* 2016). This acid protonation also 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 3.** 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, as shown in **Figure X*,*** is made up of randomly distributed β-D-mannuronate and α-L-guluronate

As for cross-linking agents, the inorganic sodium tripolyphosphate (TPP) has traditionally been successful in forming inoculated beads with chitosan, and is gentle and non-toxic, making it ideal for inoculant production (Chanratana *et al.,* 2019). The capture efficiency of some, but not all, bioactive compounds depend on the cross-linker's pH, and understanding under what conditions the cross-linker enables high efficiency biostimulant capture is of high importance to inoculant production (Ghanem & Skonberg, 2001). There could be environmental concerns about developing sustainable agriculture solutions that use TPP, an inorganic substance attributed to be partially responsible for water body eutrophication (Greenwood *et al.,* 1997). Due to their 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 beads cross-linked with TPP. Should this be an issue, another option for cross-linking is the use of organic multi-carboxylic acids, such as glutaric, citric, or malonic acid.

Additives are a key component to increasing microbial viability when encapsulated and desiccated. Sugars, such as sucrose, glucose, and trehalose, can form biological glasses when desiccated which help prevent degradation of macromolecules in bacteria by replacing the hydrogen bonds on polar residues normally occupied by water, as well as physically immobilizing the macromolecules present in the cell, preventing protein aggregation, protein misfolding, and molecular crowding (Potts, 1994; Crowe *et al.,* 1998). Because of these properties, sugars are commonly used to increase desiccation tolerance (Strasser *et al*., 2009; Huang *et al.,* 2017). Antioxidants such as glutathione, ascorbic acid, and vitamin E help prevent oxidation and the ensuing reactive oxygen species (ROS) cell damage (Franca *et al.,* 2007) and would be worth experimenting with to understand how they interact with microbes in the encapsulation matrix following desiccation.

Desiccation is necessary for the successful commercialization of an encapsulation-based carrier material. 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, and 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 beads 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 beads, 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 priority effects and niche overlap (Verbruggen *et al.,* 2012).

Another route to sustainability is the use of low-water intensity farming, such as hydroponic farming. This approach 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.

Multiple studies have shown that combinations of PGPBs are more effective than lone PGPBs (Madhaiyan *et al.,* 2009; ; He *et al.,* 2019). The exact biostimulant load depends on the target crop and soil conditions. To this end, multiple microbes will be considered for use in a final biostimulant consortium, with emphasis on those that have already been proven to provide benefits to tomato plants:

*Azospirillum brasilense -* 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*, 2009).

*Azotobacter chroococcum -* 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 -* 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 -* 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 -* 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 root and shoot growth (Glick *et al,* 1997; Hall *et al,* 1996).

In summary, microbial viability and efficacy following chitosan 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, soil conditions, 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 release rate of desiccated chitosan beads formed with different protonation acids, and there have also been no studies done on the effectiveness of chitosan encapsulated-desiccated beads in maintaining microbial viability after extended storage. Addressing these will provide further insights into the potential for chitosan as a commercially viable biostimulant carrier.

1. **HYPOTHESES/ OBJECTIVES**

**Hypothesis 1 -** Combined soil and foliar *M. oryzae* application will increase salt stressed tomato fruit yield and fruit quality more than either soil or foliar inoculations.

Objective 1.1 - Determine the effect of *M. oryzae* inoculation methodon tomato plant growth and crop quality and yield.

**Hypothesis 2 –** Microbial bacterial granules (MBGs) created under ideal conditions will more reliably provide plant benefits than MBGs made under non-ideal conditions.

Objective 2.1 - Optimize MBG creation method to enhance efficacy and viability.

Objective 2.2 - Successfully maintain MBG viability for 1 year after desiccation.

**Hypothesis 3 -** Inoculation of tomato with MBGs will increase plant growth and fluorometric parameters (e.g., chlorophyll fluorescence, efficiency of photosystem II) more than other biostimulant application methods.

Objective 3.1 - Determine the effect of biostimulant inoculation method (liquid, MBG)

Objective 3.2 - Determine the effect of biostimulant inoculation timing (germination, transplantation)

**Hypothesis 4 –** Combined soil and foliar inoculation of microbial consortium will increase salt stressed tomato fruit yield and fruit quality compared to either soil or foliar inoculation.

Objective 4.1 - Determine the effect of microbial consortium inoculation method on tomato plant growth and crop quality and yield.

1. **METHODOLOGY**

**III.A. MBGs**

**III.A.1. MBG optimization**

Microbial Biostimulant Granules (BGs) will be created by protonating 5% chitosan (85% deacetylated) with the necessary amount of an acid to achieve the desired degree of protonation. A solution of 5% TPP will be created, and both solutions will be sterilized. Microbes collected in the stationary phase will be added to 5% protonated chitosan aliquots in concentrations that will facilitate the desired concentration after dilution. This microbial consortium and chitosan mixture can then be added to the TPP via dropwise addition using a peristaltic pump. After curing for 24 hours, the beads will be transferred to a vacuum desiccation chamber and allowed to dry until no water remains (approximately 24 hours).

Tests will be performed on chitosan protonation acid, chitosan protonation degree, dropwise addition nozzle size, cure time, desiccation method, and desiccation time to identify the optimal conditions for CBG creation. We will also be assessing the entrapment efficiency of bioactive compounds and microbes in CBGs, as well as the subsequent release rate under various conditions. Steps will be taken to increase the microbial viability of the CBGs as needed, starting with the addition of osmoprotectants (sucrose, vitamin C, salicylic acid). To facilitate successful microbial colonization upon rehydration, nutrients will be added to CBGs, paying special regard to Fe and Mo alongside other nutrients required by N-fixers but not provided by root exudates.

For each new iteration of BG and at set points in the creation process (freshly cured, 24H desiccation increments until completely dry, rehydration), the author will collect images and use them to approximate surface area and will also collect weight, volume, encapsulation efficiency, and release rate. Weight will be collected on a lab scale. Volume will be assessed by placing 10 CBGs in a 1mL graduated cylinder filled halfway with DI water. Encapsulation efficiency will be calculated by measuring the microbial concentration of (1) the microbial-chitosan solution prior to dropwise addition and (2) the cross-linking solution following dropwise addition. Release rate will be assessed by measuring the microbial concentration of the rehydration solution at set intervals after introduction of the desiccated CBGs.

**III.A.2. MBG longevity trial**

MBGs with seven different inoculations (five treatments comprising single bacteria inoculation of each species mentioned above, one treatment containing every bacteria, and one control treatment without bacteria) will be stored to assess their viability over time. Each sample will contain 50g of MBGs and storage conditions include temperature (-20, 4, 24°C) and atmosphere exposure (sealed and open). At set intervals (1 week, 1 month, 3 months, 6 months, 1 year) the microbial viability will be quantified via plate counting and the effect on seed germination will be examined by placing the MBG on a petri dish containing limited nutrient solution and a tomato seed.

**III.B. Greenhouse trials**

**III.B.1. Tomato inoculant stress 2023**

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) The sample size (n) was 32 tomato plants across four rows with eight plants per row. Row A was the control, with no treatment applied. Row B was subjected to soil inoculation with MBGs at transplantation by placing a few MBGs (~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. Nutrients and salt stress were continuously applied via nutrient solution. Tomato 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. Firmness was measured with an OA Supplies Fruit Pressure Tester.

The statistical power of this experiment is low, at 0.39 for an effect size of 0.4 with 4 groups, 8 replicates per group, and 0.05 significance level. However, this was the greatest number of tomato plants possible in the space available at the time.

**III.B.2. Tomato inoculant timing 2024**

The space available for the 2024 tomato inoculant trial is greater than that available for the 2023 trial, allowing for more replicates per group. The plants were grown in the Hydroponic Lab greenhouse at the KSU Field Station. The treatments used reflect objective 3.2. The treatments include inoculation at germination, inoculation at transplantation, inoculation at both germination and transplantation, and a control no inoculation group. Each group had 12 replicates, for a total sample size of 48 tomato plants. Plants were germinated on X and transplanted on X date. Plants were allowed to grow until X, 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.

**III.B.3. Tomato inoculant method trial**

This experiment will involve cultivating 144 tomato plants (cultivar BHN 589) for 40 days in the greenhouse outside of the KSU Science building. A graphical abstract of this experiment can be seen in **Figure 4.** There will be 10 treatments (control, liquid at germination, liquid at transplantation, liquid at both stages, MBG at germination, MBG at transplantation, MBG at both stages, liquid at germination with MBG at transplantation, and MBG at germination with liquid at transplantation) with 12 replicates per group. The inoculation is slated to include the entire microbial consortium listed above at 1x106 CFU/g each. Power analysis on this reveals a power of 0.90 for an effect size of 0.4, a significance of 0.05, 12 groups, and 12 replicates per group.

**III.C. Statistical analysis**

All statistical analysis were performed in the latest version of R, currently 4.3.3.

First, probability density function (PDF) and cumulative distribution function (CDF) 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 and Fv’/Fm’] which were just logit transformed and treated as normal). Variables were checked for homoscedasticity with Levene tests. Boxplots were constructed of each variable by treatment

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.

* Note: For the sake of brevity, only figures that show a **significant difference** are included. Non-significant graphs can be found at [github.com/zachpeagler/Tomatoinoculants/tree/main/figures](https://github.com/zachpeagler/TomatoInoculants/tree/main/figures) as both PNGs and SVGs.

1. **RESULTS**

**IV.A. MBG Optimization**

Authors have had success in creating inoculated MBGs and in qualifying that microbial viability can be maintained after desiccation via air-drying. Quantitative measurements for microbial viability have not yet been conducted. Preliminary results from nozzle tests show that smaller nozzle sizes produce beads with the least reagent waste. It also revealed microplastics in the MBGs. Plastic nozzles are a no-go, and future MBG creation should be done without plastic (especially 3D printed) parts contacting the granules. Preliminary results have shown that bead morphology can vary drastically even within a single batch made under identical conditions, as shown in **Figure 5**. Preliminary results for CBG desiccation show that desiccation reduces MBG weight by 90%, as shown in **Figure 6.**

**IV.B. Tomato Inoculant Trial 2023**

Stomatal conductance is graphed in **Figure 7**, and all treatments were found to be significantly different from the control using an lmer test when modeled with stomatal conductance as a response, treatment as a predictor, and relative humidity as a random effect.

Fruit weight across treatments is graphed in **Figure 8**, and foliar and joint treatments were found to be significantly different from the control using an lmer test when modeled with fruit weight as a response and treatment as a predictor. Total yield across treatments by blossom-end rot presence is graphed in **Figure 9*,*** and the joint inoculation was found to have a significantly higher portion of fruit with blossom-end rot than the control treatment using an lmer test when modeled with total yield as a response and treatment as a predictor.

Fruit sugar concentration across treatments is graphed in **Figure 10**, and foliar and joint inoculations were found to have significantly higher average sugar concentrations using an lmer test when modeled with sugar as a response and treatment as a predictor. However, when the sugar content is calculated as an absolute weight value rather than a relative percentage as graphed in **Figure 11**, the sugar content in the foliar and joint inoculation treatments is significantly less than the control, found using an lmer test and modeled with the sugar content as a response and treatment as a predictor.

IV.C. Tomato Inoculant Trial 2024

IV.D. Tomato Inoculant Method Trial

IV.E. MBG Decomposition

**V. INTEGRATIVE ASPECTS**

The proposed research integrates microbiology, mycology, plant biology, chemistry, statistics, and engineering. Microbiology is integrated through the culturing and application of five bacterial species, as well as through microbial community analysis, compatibility assays, and viability trials. Mycology is integrated through the propagation of arbuscular mycorrhizal fungi, the propagation of a non-mycorrhizal fungi, and the application of all three fungi in chitosan encapsulation. 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 CBG polymer matrix and further CBG optimization. Statistics is integrated via power analysis and experimental data analysis. Engineering is involved in the creation of the device that creates the CBGs, mainly through CAD modeling and 3D printing.

**VI. INTELLECTUAL MERIT**

There are many gaps in the literature surrounding chitosan encapsulation that need to be filled before chitosan can be considered for industrial commercialization. There have been no studies done on the release rate of desiccated chitosan beads formed with different protonation acids, and there have also been no studies done on the effectiveness of chitosan encapsulated and desiccated beads in maintaining microbial viability after extended storage. Addressing these will provide further insights into the potential for chitosan as a commercially viable biostimulant carrier.

**VII. BROADER IMPACTS**

Feeding an expanding human population more sustainably is of the utmost importance. By reducing our reliance on synthetic fertilizers, we can prevent further ecological devastation caused by fertilizer runoffs (I.e., harmful algae blooms) and the accompanying environmental and economic downturns. A cheap, hardy, non-toxic, and simple microbial fertilizer could revolutionize agriculture and generate billions in social benefit.

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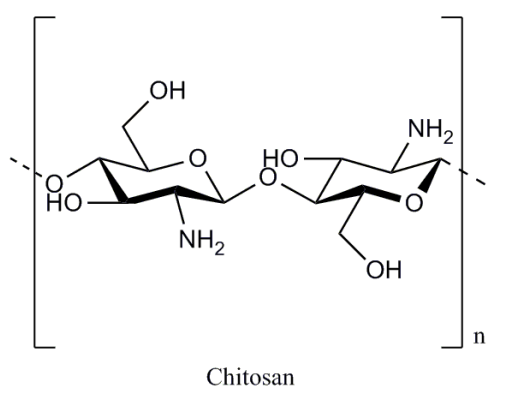
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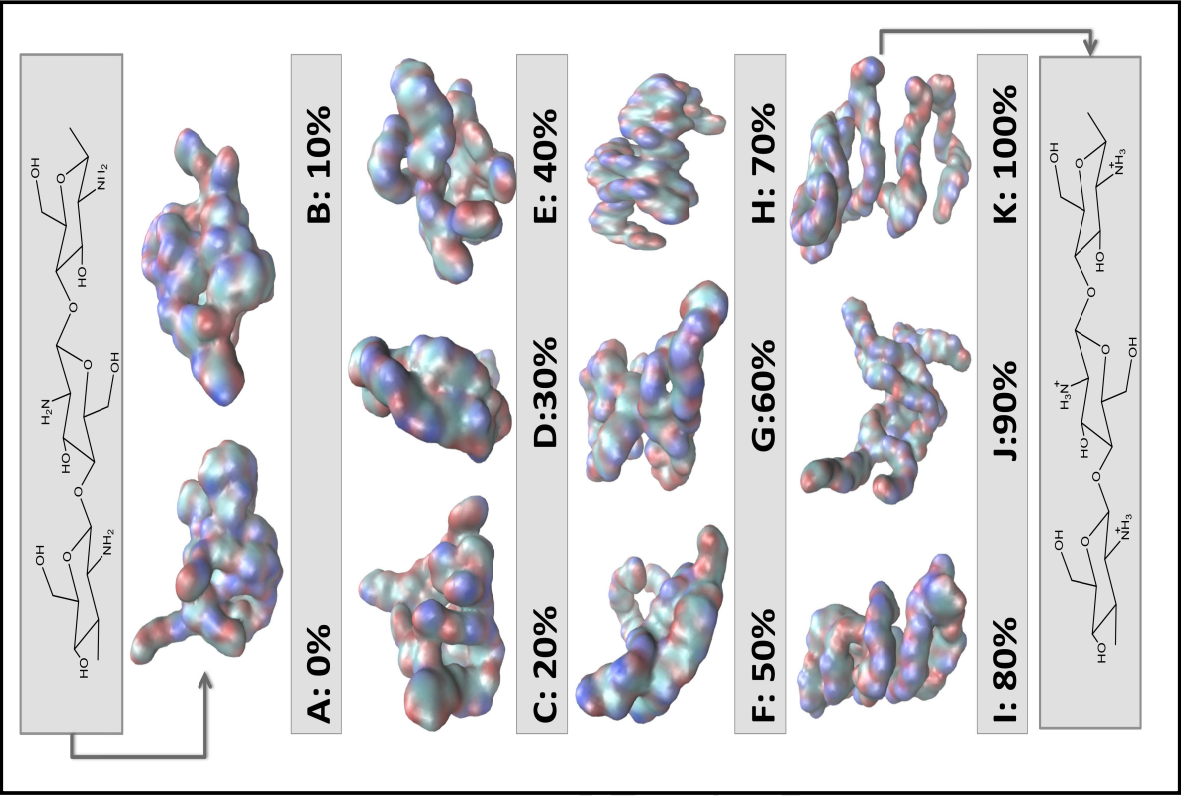
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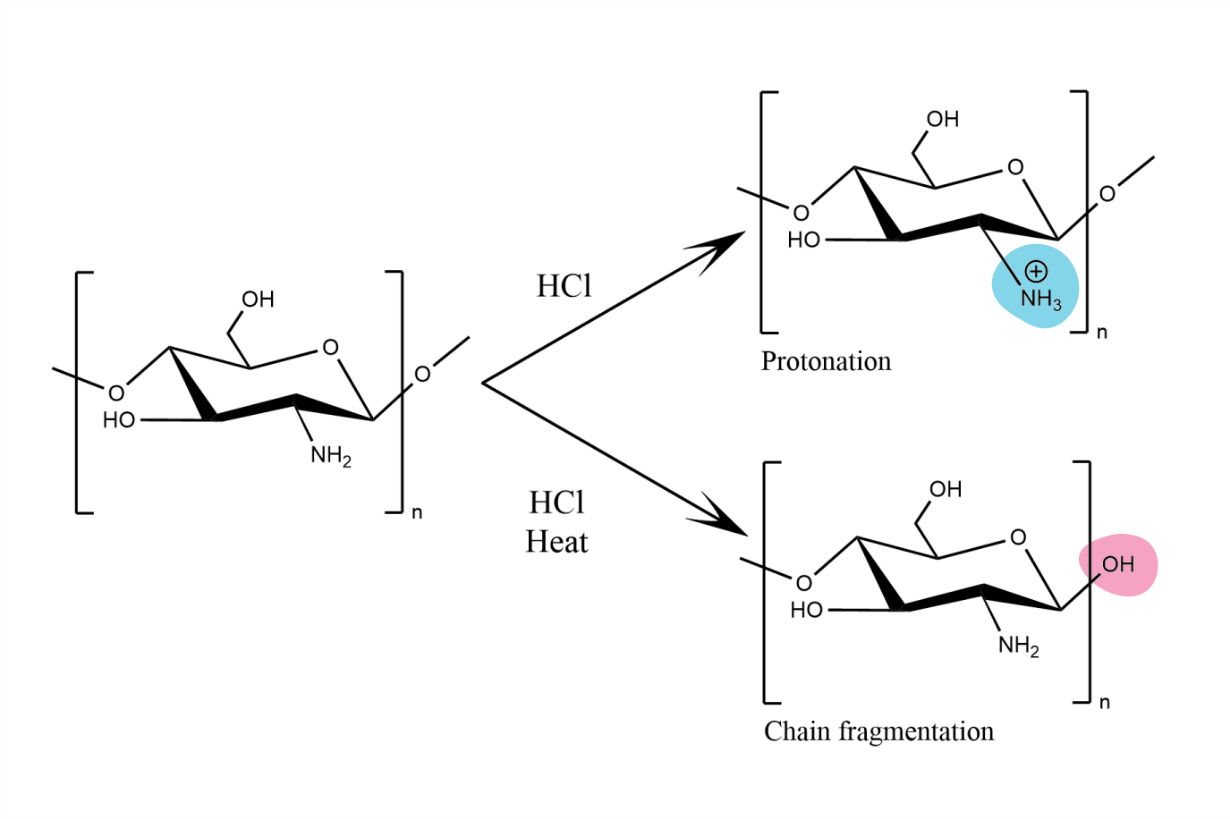
**IX. Figures**

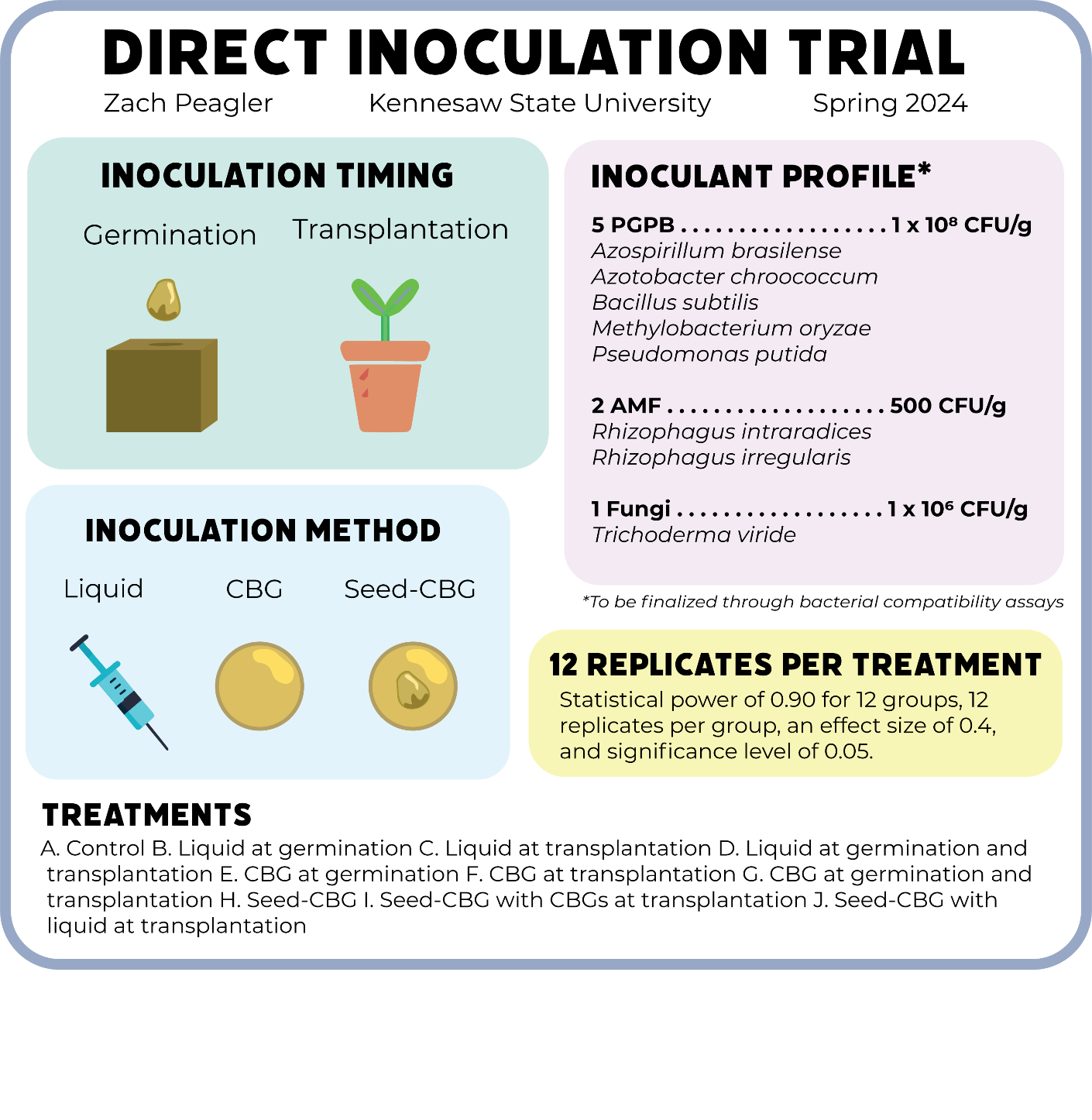


*Figure 1. Chemical structure of chitosan (Hao et al., 2017)*



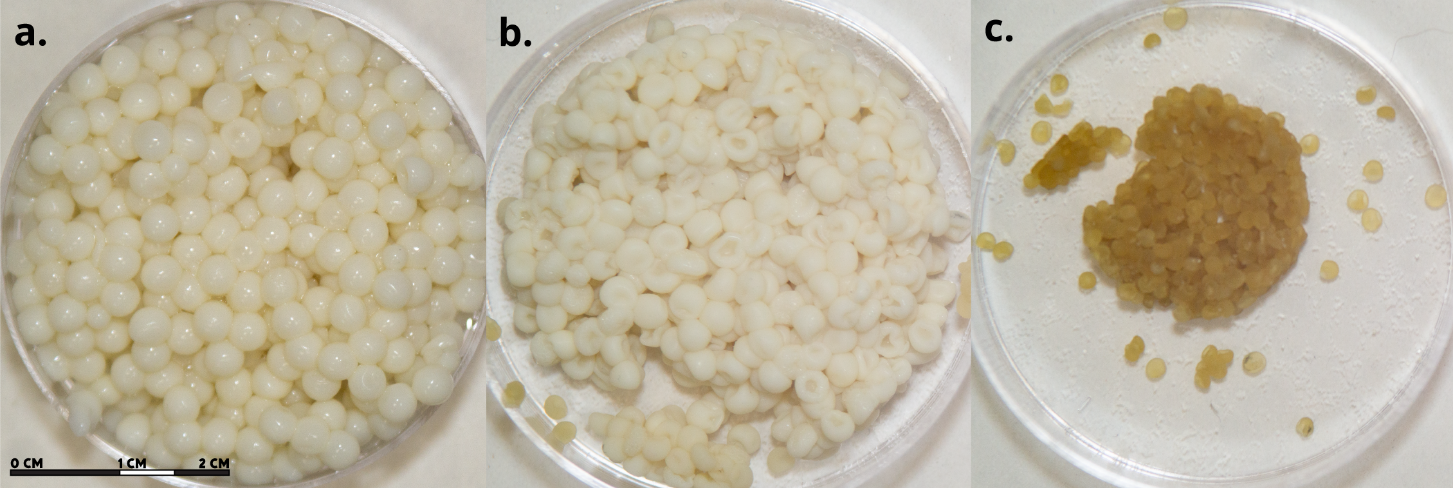
*Figure 2. Conformation of chitosan at different protonation levels, 0-100%. (Dey et al., 2016)*

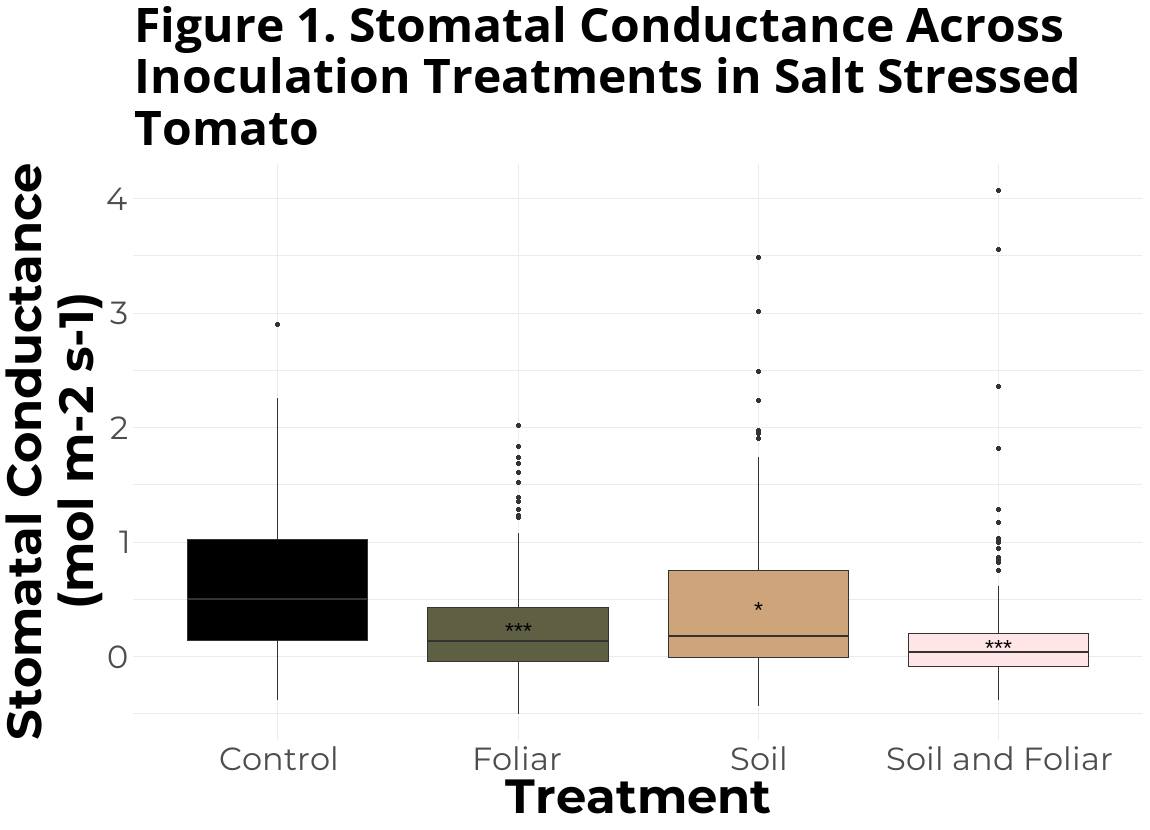
  
*Figure 3. Reaction of chitosan with HCl is temperature dependent*



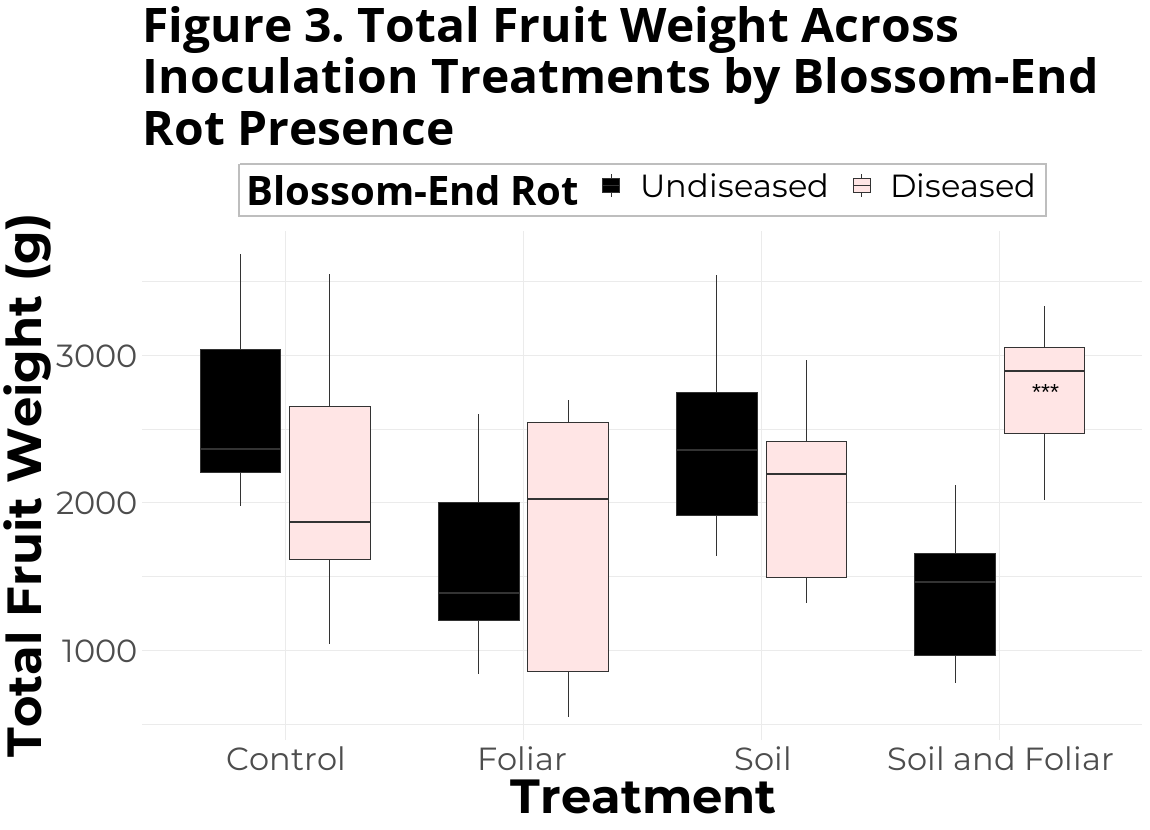
*Figure 4. Graphical abstract of Spring 2024 direct inoculation trial*

*Figure 5. Examples of variety in bead morphology created under the same conditions*

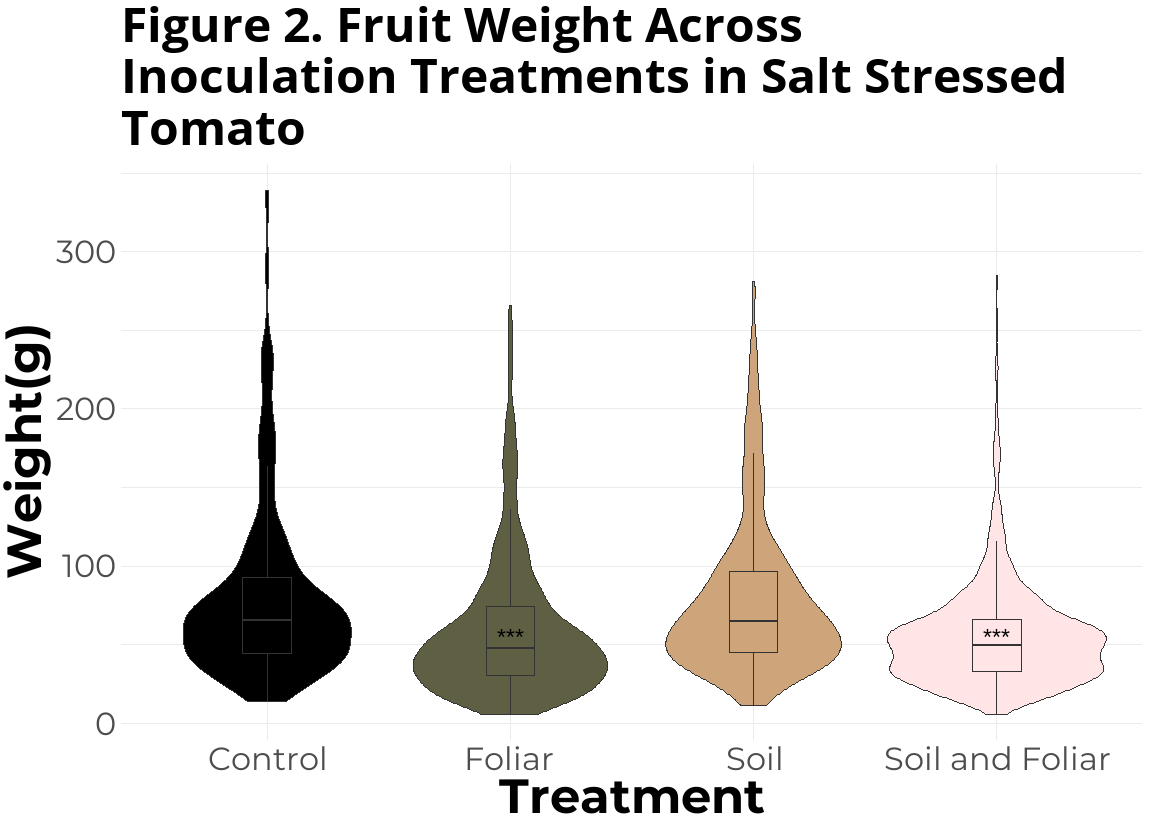
*Figure 6. Chitosan beads a. non-desiccated, b. desiccated for three days, c. desiccated for ten days, created through dropwise addition and air dried*



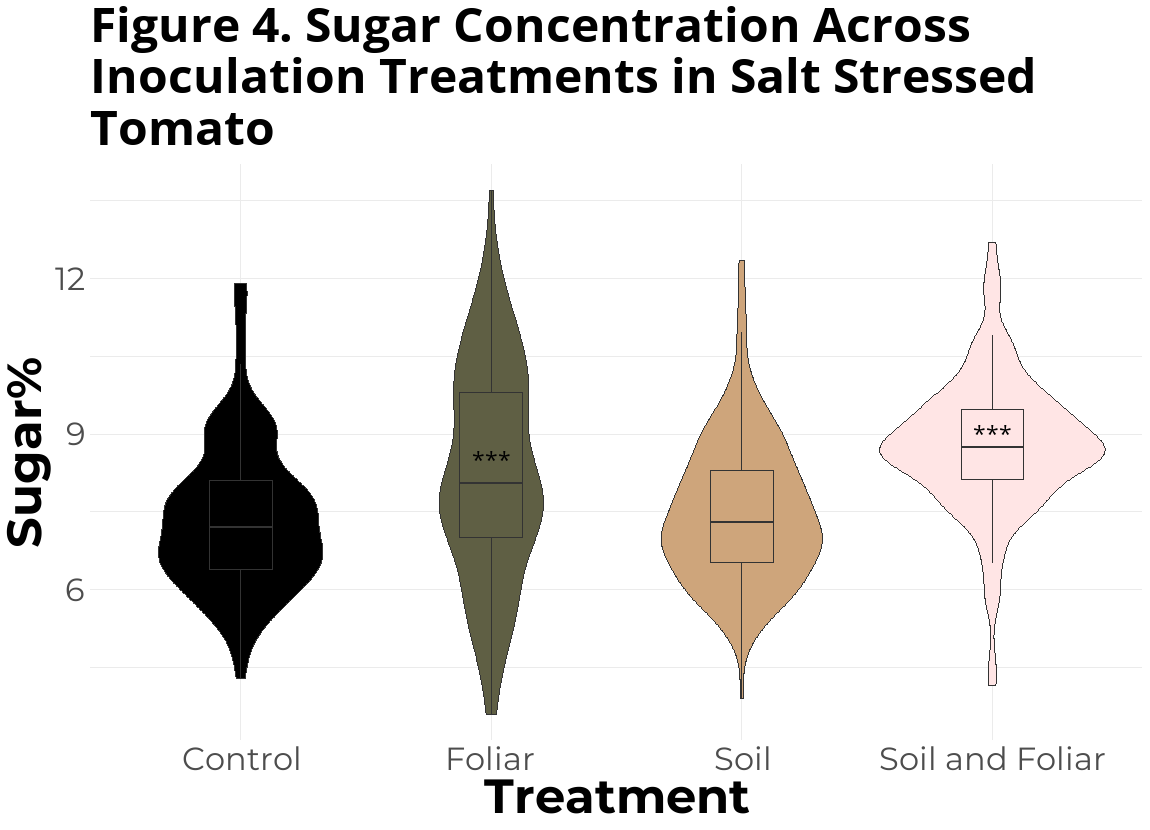
*Figure 7. Stomatal conductance (mol m-2 s-1) across inoculation treatments of salt stressed tomato*



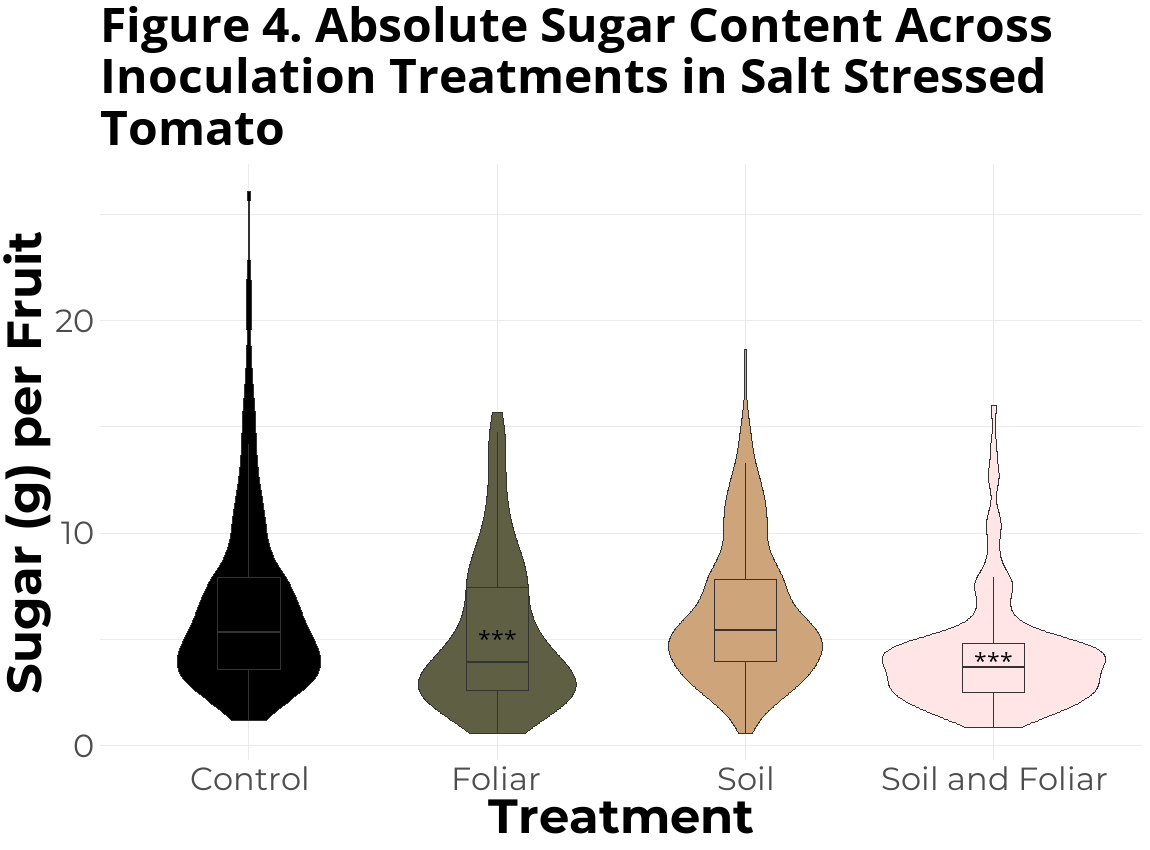
*Figure 8. Total fruit weight (g) by blossom-end rot presence across inoculation treatments of salt stressed tomato*



*Figure 9. Fruit weight (g) across inoculation treatments of salt stressed tomato*



*Figure 10. Sugar concentration (%) across inoculation treatments of salt stressed tomato*



*Figure 11. Sugar per fruit (g) across inoculation treatments of salt stressed tomato*