UNIVERSITY OF HOHENHEIM

Hans-Ruthenberg Institute of Agricultural Sciences in the Tropics and Subtropics Management of Crop Water Stress in the Tropics

Effects of Microbial Inoculants on Growth and Salinity Tolerance of Hydroponically-Grown Tomatoes

Master Thesis

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SP SOURCON PADENA

HYPOWAVE

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Abstract

Hydroponic production offers huge benefits over conventional geoponic production, but relies on large volumes of clean water. Increasing plant tolerance of salinity could unlock new marginal water sources for hydroponics. One strategy for maintaining crop yield in spite of salinity is the use of plant growth promoting microorganisms (PGPM). The microbes form relationships with plants which improve plant performance in a variety of ways. The aim of this work is to further our understanding of the effects of two strains of bacteria and one fungus known to benefit crop plants, working towards the optimization of this relationship.

Our experiments used tomato (*Solanum lycopersicum L.* var. Sweeterno) seedlings in deep-pool hydroponic setups. The design may be helpful in future efforts to identify or characterize PGPM, minimizing the required time and inputs, while maximizing resolution.

Our experiments found that the *Bacillus megaterium* and *Pseudomonas brassicacearum* strains increased plant biomass, height, and nutrient solution uptake in tests without salt. They did not increase plant performance under salt stress. The *Trichoderma harzianum* strain was detrimental to plant performance in our experiments. This has implications for our understanding of the plant relationships and life strategies of these microbes.

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1. Introduction

1.1 Hydroponics

Earth's human population is projected to grow to 9.1 billion by 2050. The productivity of arable land is projected to decrease due to increased temperature, aridity, salinity, etc. while the total amount of land available will remain the same (FAO 2023). If we do not want to plow under broad swaths of the remaining uncultivated land, we will need to increase the amount of food that we grow per unit area, in the face of these environmental challenges. One tool that may allow for this intensification is hydroponics.

1.1.1 Background information on hydroponics

Hydroponics, a term coined by Dr. W. A. Setchell from "*hydro*, water, and *ponos*, labor" (Setchell, via Gericke 1937) is used to describe a suite of related soilless cultivation methods, where plants take up dissolved nutrient and water as an artificial nutrient solution. The obviation of soil from the growth setup as the source of nutrients and water, or at least the medium of their conductance, enables a very high degree of flexibility and control in and over the growth environment and inputs. Inputs, including mineral fertilizers and water, but also including light, CO2, and temperature, can be varied according to the needs of the plants. Thus, Hydroponic techniques enable the production of more crops, with fewer inputs, on less land, often in less time (total time seed to harvest) (Corrêa et al. 2008)

1.1.2 Advantages of hydroponics

Each of the plant's needs – if unmet – can potentially curtail growth and limit productivity. In conventional production systems, the availability of water and light, as well as that of each of the plant's required nutrients, can limit yield. Hydroponics enables growers to precisely dose out inputs directly to the plants. In hydroponic production, water is abundantly available, and the local depletion of nutrients in the volumes surrounding the roots, which is characteristic of soil-based rhizospheres, is washed away by the movement of fresh solution. This spatial uniformity throughout the hydroponic system allows individual plants to be served the optimum mix of nutrients. Furthermore, by growing inside greenhouses, growers can control the levels of light, heat, and CO_2 in the plants' growth environment, and exclude herbivorous pests and weeds, and limit intra-crop competition to optimal levels (Jo and Shin 2020, Son, Kim, and Ahn 2020). Barbosa et al. found that

hydroponics yielded 11±1.7 times the amount of lettuce that a geoponic system produced (measured in kg/m^2/year), with 13±2.7 times less water (Barbosa et al. 2015).

The benefits of hydroponics fall broadly into two categories: input conservation and control of the growth environment; these two enable a meta-benefit – spatial and temporal intensification. The delivery of nutrient via solution, without the mediation of soil, supports finely calibrated dosages of nutrition to be dispensed evenly throughout the growing system.

1.1.3 Limitations of hydroponics

A set of challenges accompany the novel benefits of hydroponic production. Many of them, e.g., the infrastructure cost of building a system, the genetic optimization of crops for the new growing conditions, and the energy costs of running an indoor farm, are outside the scope of this thesis.

It is possible to exert some control over the temperature of a greenhouse in the hot sun with a combination of coordinated shading and ventilating, or even via active cooling with heat pumps or air conditioning. But these can be energetically costly, and a single runaway temperature event can wreak serious damage to a crop of plants that have not been heat-tempered (Bäurle 2016). In the study by Barbosa et al. mentioned above, they found that the massive benefits to yield and water use efficiency were offset by huge increases in energy use – hydroponics used 87±11 times more energy than conventional production, with most of that energy used to control the temperature in the growth environment (Barbosa et al. 2015). The increase in energy costs in Europe related to the Russian invasion of Ukraine has driven several large European vertical farm firms out of business, and forced others to reduce their production to save energy (Cadogan 2023).

Water cannot be escaped as a requirement for plant cultivation. Although hydroponics are far more efficient in water usage than conventional systems, they lack the soil medium present in geoponic cultivation, which can buffer plants against harmful solutes that would otherwise be taken up by the plants. Depending on the source of the water for the hydroponic system, salts may be present (Antolinos et al. 2020).

Disease poses a stark challenge in hydroponic systems, as pathogens can wash throughout growing systems' shared volumes of solution. The standard management strategies of keelping things clean, maintaining cool solution temperature to limit pathogen growth, and sacrificing the entire crop in the event of an identified infection, as laid out in the Cornell University Handbook for Controlled

Environment Agriculture (Brechner & de Viliers 2013) are costly. Inoculation of the crops with beneficial microbiota that control pathogen growth or ameliorate plant responses to infection has shown promising results (Forsyth et al. 2008; Mishra, Ellouze, and Howard 2018). These pathogenic considerations are not the subject of the current investigation, but the experimental setup used and refined through the trials described in this thesis could offer some advantages to future investigations of biocontrol by beneficial microbiota – e.g., self-contained nutrient solution volumes, materials that can be effectively washed and sanitized.

1.2 Problem statement

Salt-stress is the hydroponic problem to which this thesis investigates seeks a solution. Working tangentially with the EXALT program at the University of Hohenheim, in the working group 490g of the Hans-Ruthenberg Institute for Tropical Agricultural Science, the author was able to share resources and techniques for investigating salt in hydroponics with research that was already underway.

1.2.1 The problem of salinity in hydroponic systems

Salt stresses plants. How much the plants are stressed depend on the plants involved and the environment. Salinity stress, with its accompanying negative plant growth outcomes, arises from two chemical phenomena. Salt in solution on one side of a membrane, raising the osmolarity of that solution, reduces the rate at which water will move through the membrane. The plant, needing to draw water up through its roots to all of its tissues, is stressed by the need to create a concentration gradient down which the water will move, or – insofar as it cannot overcome the osmolarity of the outside solution to create such a gradient – the plant suffers from water stress (Parihar et al. 2015). The second way in which salt causes stress for plants is through ionic effects. Sodium ions compete with potassium ions in plants' transport systems and interferes in potassium's role as signaling molecule and osmolyte (Kronzucker et al. 2013). Salinization of soils and water supplies therefore poses a threat to crop yields in geoponic systems, but what about hydroponics?

Salt can enter a hydroponic system from two different sources: the water used for the solution, if desalinized seawater or another salty or brackish water source is used (Tarroum et al. 2022; Moncada 2020), or from the chemical contents of the nutrient solution, as the plants take up the nutrients and leave the ions from which the nutrients have dissociated behind to build up (Antolinos et al. 2020; Endut et al. 2009; Carmassi et al. 2005). In the latter situation, salt buildup is highly dependent on

both the formulation of the nutrient solution components and the rate at which old solution washes out of the system (Carmassi et al. 2005). Salt tolerance is of concern for hydroponic production not only as a guard against negative growth outcomes, but also as an enabling capability that would allow growers to use more marginal water sources. Being able to maintain yields, using marginal water sources without having use energy to desalinate them, could lead to both economic and ecological benefits (Cifuentes-Torres et al. 2021; da Silva Cuba Carvalho, Bastos, and Souza 2018; Richa et al. 2020).

1.2.2 The potential of PGPM to address these challenges and improve hydroponic crop yields

Plant Growth Promoting Microorganisms (PGPM) – sometimes discussed in subsets PGPB (Bacteria), PGPF (Fungi), or PGPR (Rhizobacteria) – form mutually beneficial relationships with plants, often interfacing at, through, or inside plants' root tissues. They have a range of effects, and of modes of action (Dimkpa, Weinand, and Asch 2009; Malgioglio et al. 2022; Lopes, Dias-Filho, and Gurgel 2021). The application of PGPM – which has been practiced and studied in geoponic production for decades (Vessey 2003) – to hydroponics has several distinct potential advantages. Soil is highly heterogenous. This leads to an enormous diversity of microorganisms, each existing within the spatial, chemical, aerobic, temperature, and/or hydrological constrains of their niche. The homogeneity of the nutrient solution, and the degree to which it can be altered by growers, makes PGPM a particularly potent effector in hydroponic production (Mourouzidou et al. 2023; Dhawi 2023).

1.3 Research questions

1.3.1 What is the impact of PGPM on hydroponic crop growth, yield, and salinity tolerance?

There is a growing dual recognition that PGPM can be provide significant benefits to plant growth specific to the conditions of hydroponic production, and that these same specific conditions could give growers an unparalleled level of control over the environment in which the plants and microbes live together – and therefore power to specifically (Glick 1995; Mourouzidou et al. 2023). The free-flowing environment of a hydroponic setup could offer inoculants huge benefits in terms of motility and access to roots, boosting infection rates and effects. Infection by PGPM, which act on and with plants through a variety of different mechanism. Many PGPM can affect plant hormone levels through the production of ACC deaminase, IAA, and Cytokinin, as well as through other chemical exudates (Grover et al. 2011; Sun et al. 2020). In the face of salt stress, PGPm have been found to

decrease ethylene-related stress responses (through the production and activity of ACC deaminase), increase compatible solute production, and induce antioxidant enzyme production in their plant associates (Gamalero and Glick 2022) The mechanisms of these interactions, and how they can best be brought to bear to improve plant performance in hydroponic conditions is a field of growing academic study.

1.3.2 How do different types of PGPM affect hydroponic plant health and productivity?

The range of potential microbial allies which might be recruited is vast. Like tools in a vast toolbox, however, not all PGPM work in all settings, on all crops. Mourouzidou et al conducted a meta-analysis of PGPM that have been studied, *Trichoderma*, *Bacillus*, and *Pseudomonas* were among the most commonly studied clades of PGPM (Mourouzidou et al. 2023). However, effective use of PGPM may require setting-specific experimentation as plant-microbe and microbe-environment interactions increase the complexity of application (Stegelmeier et al. 2022).

1.3.3Which PGPM did we choose?

We chose two bacteria and a fungus: *Pseudomonas brassicacearum* 3Re2-7 (supplied by the Italian biostimulant company Sourcon Padena), *Bacillus megaterium* Ni-5-SO-11 (from Hans-Ruthenberg institute), and *Trichoderma harzianum* (DSM 32006) (also from Sourcon Padena).

All three strains have been evaluated for the key molecular markers of beneficial plant symbionts.

Pseudomonas brassicacearum has been found to promote plant growth by several different mechanisms, including ABA content and transpiration (Bresson et al. 2013). *Pseudomonas brassicacearum* 3Re2-7 has been genetically investigated in depth by Nelkner et al. and was found to encode for ACC deaminase and riboflavin synthase (Nelkner et al. 2019). Gislason & de Kievit later found that it was closely related with the opportunistic pathogen *P. corrugate* (Gislason and de Kievit 2020).

Bacillus megaterium has been found to benefit plants via phosphate solubilization by excreting lactic and malic acid, and by producing the plant hormone Indole-3AA (Taha et al. 1969). *B. megaterium* Ni-5-SO-11 was found to affect the Na/K ration in rice leaves by Weinland et al., a potential indication that it could have an effect on salt stress (Weinand, Asch, and Asch 2023).

Trichoderm harzianum has been found to produce siderophores, solubilize phosphate, and produce the plant hormone Indole-3AA (Mastouri, Björkman, and Harman 2010; Zhang et al. 2013). Ahmad et al. and Yedida et al. found that *Trichoderma harzianum* benefited crops – mustard and cucumbers, respectively – against salt stress(Ahmad et al. 2015; Yedidia et al. 2001). Yedida et al. conducted the experiments hydroponically.

1.3.4 What are the optimal conditions for using PGPM in hydroponics?

A goal of this research is to move towards identifying the optimal conditions for PGPM. Different organisms have different life strategies, different interactions with plants. This leads to results that may be highly inconsistent.

The experiments discussed here represent novel settings for these specific strains of bacteria. The *Bacillus* strain was tested for its anti-nematode functions in rice, and later for its function improving the iron tolerance of rice (Weinland et al. 2022). The *Pseudomonas* is part of a commercial biostimulant formulation by Sourcon Padena. The formulation is marketed as being beneficial to many horticultural crops, including tomatoes, in a number of environments, including fertigation, but it had not yet been studied in a deep-pool hydroponic system, according to Sourcon Padena Chief Scientist, Karin Mai (personal interview, October 2022).

1.4 Objectives

The objectives of this research are as follows:

To evaluate the effects of the chosen *Bacillus*, *Pseudomonas*, and *Trichoderma* strains on hydroponic crop growth, yield, and salinity tolerance. To compare the performance of different types of PGPM in hydroponics. And, to identify the optimal conditions for using PGPM in hydroponic systems.

1.5 Significance of the study

1.5.1 Advancement of knowledge on the use of PGPM in hydroponics.

PGPM are another variable element being added into growers' and researchers' calculations. The more information that we have about the properties of different PGPM, and their interactions –

PGPM : plant, PGPM : environment, PGPM : stressor, PGPM : plant stress response – the easier and more effectively they will be able to make those calculations.

1.5.2 Establish testing practices to explore the potential of other PGPM varieties.

This thesis lays out an experimental design that is flexible, simple, and yet powerful. In its final form – that used for experiment 4, it minimizes labor while maximizing statistical resolution. If taken as a model, it could be beneficial for future work regarding PGPM, where a fast, high-throughput screening process could help increases our 'library' of potential responses to abiotic stresses.

2. Materials and Methods

2.1 Materials

2.1.1 Tomatoes

The tomato that was chosen for this study was the Rijk Zwaan variety Sweeterno (RZ F1 72-733); the seeds were provided for free by Rijk Zwaan. These tomatoes are indeterminate table tomatoes and are bred to be resistant to blossom end rot (Rijk Zwaan catalog, 2022). The plants were F1 hybrids. While the choice of this variety – and the fact that it is bred for commercial production – meant that experimental results could more directly be tied with potential commercial outcomes, interindividual variation was higher than it might have been with a more controllably inbred line.

2.1.2 Inoculants

Pseudomonas brassicacearum 3Re2-7, obtained from Sourcon Padena. *P. Brassicacaerum 3Re2-7* was isolated from the rhizosphere of potato plants in Germany. It was identified as having biocontrol potential as a PGPB, and thereafter characterized by genetic sequencing as haing many gene of interest (Nelkner et al. 2019; Gislason & Kievit, 2020).

Bacillus megaterium Ni-5-SO-11, obtained from the Hans-Ruthenberg Institute, University of Hohenheim. The *Bacillus* strain was originally isolated from the rhizosphere of Taiwanese rice plants and identified as a biocontrol agent against nematodes in rice by Padgham & Sikora, 2006. It was later used by Weinland et al. to test the induction of iron toxicity resistance in rice plants (Weinland et al. 2022).

Trichoderma harzianum (DSM 32006) was obtained from Sourcon Padena. It is an active ingredient in several of their commercial biostimulants.

2.1.3 Hydroponic materials: nutrient solution, pots, and aeration

The inputs in each pot (in experiment 1, 2, and 3) were: 2,500ml of 50% INTEGAR nutrient solution, 35ml of 1.0E6 cfu/ml bacterial inoculation solution (35 ml sterile Ringer in control), and 50ml 3M NaCl solution (50ml deionized water in control).

The solution that was used for all experiments was the INTEGAR (Institute für Technologien im Gartenbau) tomato nutrient solution. The recipe is included in the first appendix. This solution is used by all researchers working with tomatoes in the 490G Marginal Waters working group; its selection for these experiments therefore makes comparison between experiments, and sharing resources, easier.

Experiments 1, 2, and 4 were carried out in 2.5-liter pots made of opaque, black plastic. Experiment 3 used 1-litre pots, made of semi-translucent white plastic, wrapped in aluminum foil to keep light out of the solution.

The air hose was a 'bubble wand' configuration – a loop on a stick. A short single length of tubing ran from the pump before being split into two. These two split-off lines were joined into a large loop, with offshoot tubes going to individual setups. The T-junction joints that went to each individual bubbling tip were larger in interior diameter across the top of the 'T' going along the loop, and thinner splitting off to the pots. Thus the air could more easily move along the loop than down the offshoots, and the pressure down each offshoot was more equal than it would have been with a linear, branching setup.

In experiments 3 & 4, the air pump was run on a timer that turned it on and off every 15 minutes.

2.1.4 Growing environment

Plants were grown under high pressure sodium vapor lamps. The lamps were set to a 12-hour day/night, turning on from 8:00 to 20:00. The lamps were hung a high as possible, to decrease the variability, and to reduce the intensity of the heat felt by the plants. Measurements of light intensity from the lamps were taken at night. Although there was a significant difference between the exterior

(outer positions along the edge) and interior (inner 4 x 4 square) in terms of lamp light intensity, measured at night, this did not meaningfully impact the plants (see section 2.5: statistical methods).

2.2 Methods

2.2.1 Inoculation

2.2.1.1 Culturing bacteria

Both varieties of bacteria (*Pseudomonas* and *Bacillus*) were obtained as live cultures on Tryptic Soy Agar (TSA) in petri dishes at the beginning of the series of experiments being described here. Beginning the process of growing the population that would be used for inoculation, colonies were picked from the plate and grown overnight in a small volumes (~5ml) of Tryptic Soy Broth (TSB). This, and all other overnight incubations, took place at 24˚C with the shaker tray at 180 rpm. In addition to being used in the first experiment, this first overnight culture was used to make a glycerol-suspended bacterial stock, frozen at -80˚C, to be used as the source of bacteria for the future experiments. A 50% glycerol solution (1:1 glycerol to deionized water) was mixed in a 1:1 ratio with the TSB-grown bacterial stock. Thereafter, scrapings from the frozen stock were used to inoculate the small volume TSB cultures for the first overnight growth step.

After the first overnight incubation, the small volume culture was transferred to a large Erlenmeyer flask with a large volume of TSB. The volume was determined via a rough overestimation of the volume that would be needed to inoculate the hydroponic setups. In determining how much TSB to use, the following relations were assumed: estimating 1.0E9 cfu/ml at OD600 of 1.0 (Coleman et al. 2010; Collin et al. 2022), 1ml of liquid culture media is needed per liter of nutrient solution that will need to be inoculated to achieve an inoculant concentration of 1.0E6 cfu/ml. Some extra TSB volume was used in this stage, as the final volume would be measured out after the OD reading was taken. Growing too few bacteria, in too small a volume of TSB, could be problematic if it required an adjusted, longer growth period, which could have led to some cells altering their growth strategies. Our goal was to use cells in a uniformly exponential growth stage.

After growing for about 20 hours at 24˚C, agitated at 180rpm, the large volume cultures were ready to be process to the inoculating solution. Samples of the large volume cultures were measured for their Optical Density (OD) at λ600nm. Sample ODs between 1.1 and 1.2 (estimated 1.0E9 cfu/ml) were considered acceptable; solutions that exceeded the target density were diluted iteratively with

fresh TSB until the target OD was reached. The appropriate volume (calculated as ml matching total number of liters of nutrient solution to be dosed) was divvied out among four 250ml Nalgene centrifuge bottles, and spun at 4,500 g for 12 mins to pellet it without damaging the cells. The supernatant was poured off, the pellet resuspended in ¼ Ringer solution. The cells were mixed homogeneously into the whole volume of Ringer solution (35ml x number of treatment pots). The freshly resuspended inoculants in Ringer solution were brought directly to the greenhouse and 35ml were dosed out to treatment plants. Sterile Ringer solution was dosed to control plants in the same volumes.

The large volume growth culture and the Ringer solution inoculant were serially diluted, and the E-5 and E-6 dilutions were plated in triplicate on 100% Tryptic Soy Agar (TSA), and grown for 24-36 hours at 24˚C. The colonies on these plates were counted to evaluate the concentration of active bacteria in the inoculant solution.

As a backup to the OD600 measurements, E-5, E-6, and E-7 dilutions of the final overnight culture and the inoculation Ringer solution suspensions of both *Bacillus* and *Pseudomonas* were plated in triplicate on 100% TSA, and incubated at 25˚C for two days. The Ringer solution was found to have approximately ¼ the density of viable cells compared to the media. These counts found that the solutions were to be less dense than the target. The real inoculation densities for experiment 4 were approximately 1.2E7 cfu/ml for *Bacillus* and 1.6E8/ml for *Pseudomonas*, rather than 1.0E9. It is possible that these plates' results represent an undercounting, since the same procedure for experiment 3 led to empty *Bacillus* plates.

2.2.1.2 Preparation of *Trichoderma* from spore powder

Trichoderma was obtained as a spore-containing powder with the concentration of 1.0E9 CFU/g. This was weighed out, then dissolved in water with a magnetic stirrer. The bottle containing the mixture was shaken up between each dose, to limit possible variation as the spores settled to the bottom.

In all cases, after inoculation plants and inoculants were left alone for ≥48 hours without further treatments (salinity or glucose) being applied to allow more complete infection. This time varied slightly among experiments but was consistent within experiments.

2**.2.2 Germination**

Plant growth prior to treatment occurred in three sequential settings. First, seeds were arranged flat between sheets of wet filter paper until the radicle emerged. Then they were folded into a filter paper envelope, arranged vertically with the bottom of the envelope in water, until the petioles emerged from the top of the envelope. At that point they were transplanted into hydroponic setups and grown until they were large enough to begin the experiment – at 1 or 2 true leaves.

Seeds were sprouted between two sheets of Macherey Nagel 710/714 (75g/m^2) filter paper inside germination boxes (25x25x5cm). The filter paper was wetted with deionized water until fingers touching the paper came away visibly wet, but there was no standing water on the surface of the paper. Up to 100 seeds were distributed evenly across the paper, arranged evenly to avoid crowding as the radicles sprouted, then the second wetted sheet was laid on top (Figure 1).

Before any radicles appeared, the lid of the germination box was arranged with no gap. After radicles appeared, the lids were flipped over to allow airflow through the 2mm gaps which are built into one side of each lid. To avoid pooling and potentially heterogenous water supply conditions, the germination boxes were kept as level as possible throughout the germination process.

The boxes were kept in a dark, warm environment (under thick blanket or opaque box), until radicles had emerged from the enough of the seeds to continue the experiment, but before stem elongation began among the bulk of the individuals. Uniformity of conditions was important to achieving the highest possible temporal and developmental uniformity among individuals at this and other steps. Higher uniformity of treatment and development meant that less culling and fewer seeds were needed to get a uniform study population.

The now-germinated seeds were transferred from the germination boxes to the inside of an ‘envelope’ of filter paper made by folding a large square sheet into 12^{ths}, resulting in 12 layers measuring 19.3 x 14.5 cm. Ten seeds were lined up horizontally across the inside of the folded, wetted envelope, 2cm from the top edge, with the emerged radicle oriented downwards (Figure 1). These envelopes were then arranged side-by-side in a box with 1-2 cm of deionized water in the bottom. This allowed water to wick up from the pool at the bottom of the box to replace the water evaporating from the paper. The envelopes, lined up in their reservoir box, were situated under a high pressure sodium vapor grow lamp on a 12h on/off cycle (Figure 2).

On the choice of paper use for germination:

Filter paper was chosen because it wicks water without becoming sodden. This allows air (most importantly the component oxygen) to enter the paper matrix. It also maintains its structural integrity, even when wet for an extended period. This allows the envelopes to keep standing up throughout the sprouting period, only being held in place laterally by a rack of parallel bars. In an improvised setup, a compound arrangement of paper towel and printer paper sheets yielded satisfactory results, with the paper towels providing the mixture of water and air and the printer paper providing the structure. Filter paper varies by weight and thickness. The envelope folding setup used in this work was designed around the properties of the filter paper that was available to hand. Thicker paper might achieve the same results with fewer or no folded layers.

After the cotyledons emerged and the seed was no longer providing nutrients to the plant, a narrow-neck wash bottle was used to water the seedlings from above with 50% INTEGAR nutrient solution. Care was taken to wash the nutrient solution down the inside of the envelopes, without disturbing the arrangement or orientation of the seeds, to limit the growth of algae on the envelopes. This was a balancing act between providing enough nutrients for long enough that no seedling would experience nutrient depravation, while trying to limit algal growth. Generally, the period of fertilization in the envelopes did not last longer than five days, as the plants were then moved to the nursery hydroponic systems.

When the roots had reached the bottom of the envelope, and the cotyledons had emerged from the top and spread, the seedlings were transplanted to hydroponic setups.

Ten seedlings were transplanted into each hydroponic pot, each wrapped gently in thin strips of 1cm open cell foam. The hydroponic setups were filled with 20% INTEGAR solution. Air was bubbled into each pot via hoses fed from an air pump through 200µl pipette tips. The small diameter of the hole in the pipette tip led to smaller bubbles with more surface area per volume of air.

After a few days growing ten-to-a-pot in hydroponic setups, when the plants had one or two true leaves, individuals were assigned to their pots for treatment (this was consistent within experiments but not necessarily between them). The extremes of the bell curve were pared off; the largest, most developed plants, and the smallest, least developed plants, as well as any that had any deformities, were culled. The experimental population was then selected from this more homogeneous group, and individuals were assigned to treatments blindly.

To hold them in place, seedlings were wrapped in strips of open cell foam, approximately 1 x 2 x 10cm, inserted into holes in the lid on the pot (Figure 2). The foam did not touch the solution and does not act as media, only as physical support for the plant.

The exception: in experiment 2, plants were germinated on filter paper as above, then the seeds with emerged radicals were planted in sand, watered, then fertilized with 50% INTEGAR nutrient solution after the shoots emerged from the sand. This took the place of the 'envelope' and the 'nursery hydroponic' stages. The plants were allowed to grow to 4 - 5 leaves before beginning treatment. The structural role that the roots played in supporting the growing seedlings in the sand led to different root structures that were never totally outgrown as the roots developed. For experiments 3 and 4, we returned to the germination practices of experiment 1, described above.

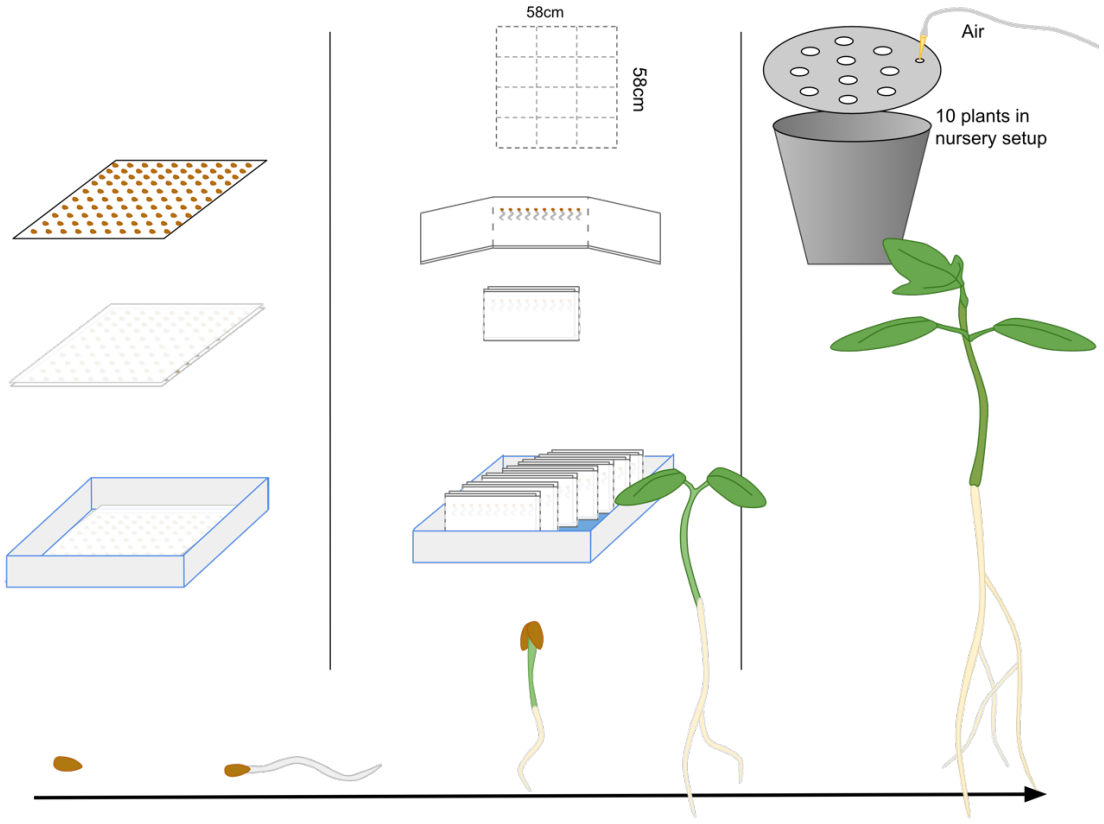

Figure 1. Diagram illustrating the arrangements and setup used at different stages of the germination process.

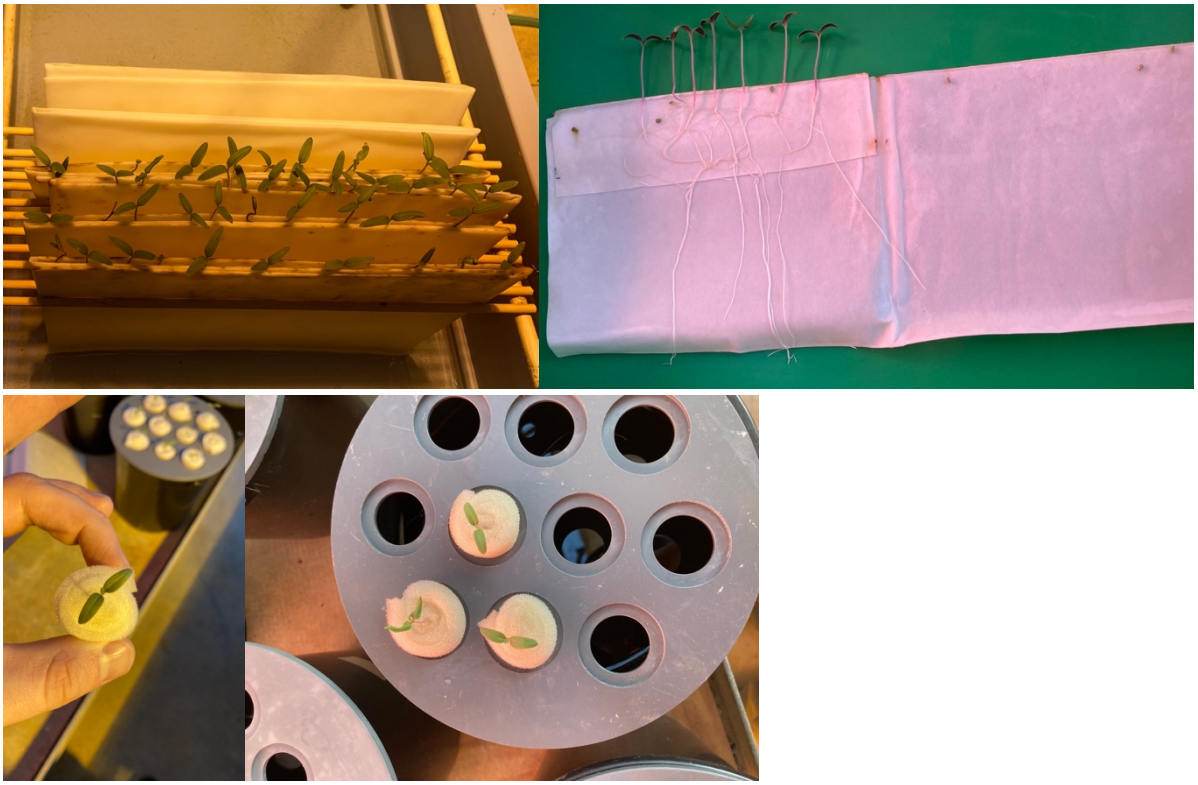

Figure 2. Images of seedlings in various stages of the germination process, highlighting (top left) the seedlings growing in the filter paper envelopes; (top right) the seedlings are transplanted when their roots reach the bottom of the envelope; (bottom left) the seedlings are rolled in 1cm thick open-celled foam before being transplanted into the nursery hydroponic settings; (bottom right) a nursery hydroponic pot with 3 of the 10 holes filled with newly-transplanted seedlings.

2.2.3 Treatment

After the sprouted seedlings have developed their first true leaf, and when the inoculants are prepared, the seedlings were transplanted from the 'nursery' hydroponic setup into the treatment setups (Figure 3). Seedlings were transplanted blind with respect to treatment. After seedlings were inoculated (see section 2.1.1) they were allowed to grow for ≥48 hours before any further treatments (salinity of glucose) were applied to allow infection to occur without interference.

Figure 3. The setup used in experiment 4, with one plant per pot, pots covered with aluminum foil to limit evaporation, and paper towels on the pots to limit reflection.

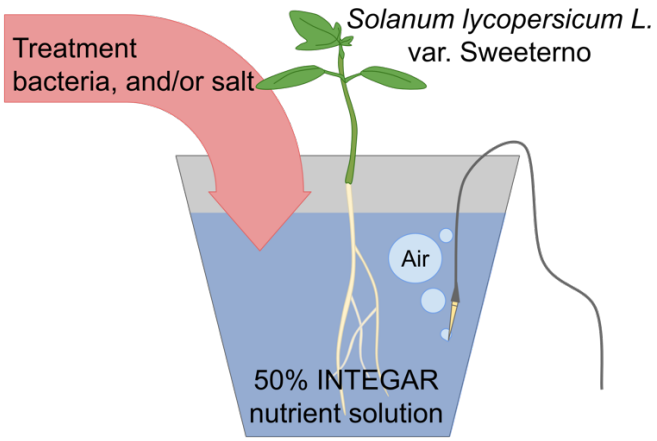

Figure 4. Diagram showing the deep-pool hydroponic setup used for treating the plants.

3-plant- vs. 1-plant-per-pot:

Experiments 1 and 2 used 2.5L pots with three plants in each pot. Each pot was an independent replication, with the data from the trios of individual plants combined for each datapoint. This was to limit variation. Taking the average of a measurement of three individuals as the measure for each replication helped to dampen the inter-individual variations of the F1 hybrid seeds. However, this setup offers a harsh tradeoff between statistical power and the length of experimental treatment that

could be undertaken. There was not time for effects to build up to levels that could overcome the noise, although that noise was ‘muffled’ by the reporting the average of the three plants. Competition within pots may have led to more noise; there were usually one large plant and two smaller ones in each pot. The three-plant-per-pot approach was abandoned in experiments 3 and 4 in favor of more replications and longer growing periods.

1-dose design:

Inoculation, treatment, and growth took place in the same volume of solution (Figure 4). It was adopted to avoid losing inoculants down the drain, by not changing the solution. At the end of the experiment, the residual mass of the solution in each pot was measured, and samples were taken of each nutrient solution, to calculate the uptake of dissolved nutrients and salt. This design enabled insights into the nutrient uptake from the solution, complementing the measurements from the tissues. However, it limits the length of time available for treatment. And, few nutrients remained in the nutrient solution at the end of the experimental period, so insights on differences in uptake between treatments may have been limited. Furthermore, there is a risk that nutrient depravation stress may become an unintentional treatment condition, if the experiment continues beyond the time when the nutrient solution is depleted.

2.2.4 Harvest and Measurements

Figure 5. Image from the harvest of experiment 4, showing the leaves being split into petioles (on table) and blades.

2.2. Measurements

Pre-harvest: SPAD

Soil-Plant Analysis Development (SPAD) measurements were taken using a Konica Minolta SPAD-502Plus. Measuring the difference between the transmittal rates of red (650nm) and infrared (940nm) light, SPAD gives an indirect measure of chlorophyll concentration in the subject leaves (chlorophyll absorbs red-wavelength light) and a proxy indicator of plant nitrogen uptake and nutrition status. Measurements were taken from the 3rd and 6th leaves (or highest leaf of sufficient size). Four measurements were taken from across the terminal leaflet, avoiding veins and any holes or necrosis; these four measurements were averaged to the value recorded for each leaf.

Harvest measurements: Fresh weight (total, shoot, and root), height, number of leaves, leaf area (exp 4 only)

At harvest, the plants were photographed with a phone camera (whole plant, stem form high angle, roots from low angle). The roots were rinsed by swishing them around in a 10L container of deionized water (water changed regularly throughout the harvest), then squeezed dry in microfiber towels. The fresh weight of the entire plant was massed, the root was excised at the visible start of the root tissue, and the remaining stem was massed. The root fresh weight was calculated as the difference between the two. The height of each plant was measured against a ruler from the bottom of the stem – where the root was cut off.

At harvest, the plants were split into five types of tissues: roots, stems, petioles, old leaves, and young leaves. Here and throughout this paper, 'petiole' is used to describe the petiole, petiolule, and rachis, i.e. all the parts of the leaf that are stem-like and are not the leaf blade (Figure 5). The specificity of this division varied between the experiments: in experiment 2 the plants were divided into 3 parts –roots, stems, and leaves (including petioles and all other parts that branch off the stem); in experiment 4 the plants were divided into 15 parts – roots, stem, petioles (including petiole, petiolules, and rachis) 1-6, and leaves (leaf blade) 1-7.

In experiment 4 a leaf area meter was used to measure the combined leaf area of the leaflet blades (separated from the petioles) of each leaf.

These tissues were separated into paper tissue-drying bags (white, non-waxed). They were dried in a drying oven for more than 48 hours at 55˚ or 60˚ c, until brittlely dehydrated. They were then massed with a Precisa XB 220A balance. In experiment 1 & 2 , the three individual plants' tissues were massed separately; the pot average values were used for statistical analysis as the data outputs for each replication.

Preparation of samples for tissue ion concentration analysis:

After being massed, the tissue samples were prepared for ion extraction as per Asch et al. (Asch et al. 2022). For experiment 1 and 2, the individual tissues, (roots, old leaves, etc.) were combined with the like tissues from the other plants in each pot into a 20ml polyethylene scintillation flask. In these flasks they were ground to powder by steel bearings in a ball mill. A tenth of a gram of this powder was mixed into 10ml of deionized H_2O in 15 ml centrifuge tubes, and this mixture was then autoclaved at 120˚C for 60 minutes. In experiments 3 and 4, with individual tissues from individual plants, some masses were to small to use the ball mill technique, due to material losses in the process of separating out the balls. Tissues weighing less than 0.25g were milled with ceramic beads in 1.5ml

screw-top tubes. After milling, the tissue, beads, and tube were all autoclaved together. The autoclaved mixture was centrifuged at 3500 times gravity for 10 minutes, to produce a clear supernatant. This supernatant was then diluted 1:10 to get the concentration into the range of the flame photometer. Samples were run sequentially, organized by tissue, through the flame photometer, taking readings for first Na^+ then K^+ ions. These aqueous samples were also analyzed for Cl- in the autoanalyzer (Asch et al. 2022).

A flame photometer, fueled by bottled propane gas, was used to measure ion concentrations. Na^+ samples were measured against a 6-point standard curve (0, 12.5, 25, 50, 75, and 100 mgNa/l). K^+ samples were measured against a 7-point standard curve (0, 12.5, 25, 50, 75, 100, and 150 mgK/l).

Nutrient solution sample measurements: (pH, Electroconductivity, osmolarity, [NO_x-N], [NH_4-N], [PO_4-P], [Na+], [K+], [Cl-]

The pH of the remaining volumes of nutrient solutions was measured with a WTW 3310 sensor, calibrated at pH 4 and 7 before the measurements were taken.

Pots were massed, dry, before the beginning of the experiment. After the harvest, the pots and the remaining solution were massed together, and the mass of the residual nutrient solution was calculated as the difference between the mass of the pot and the total mass of both.

Nutrient solution samples were then taken from the residual nutrient solution. These samples were taken in a swooping motion through the pot with the sampling vessels in forceps and are therefore taken to be representative of the whole volume of the solution. These samples were taken singly – one for each pot, except for experiment 4 when they were taken in duplicate.

[NO_x-N], [NH_4-N], and [PO_4-P] were measured in parallel via autoanalyzer as per (Asch et al.)

2.5 Statistical Methods

Data were analyzed with ANOVA, followed by post-hoc Tukey's honest standard deviation tests. The independent variable terms included in the model varied by experiment. Model generation and evaluation was done in R.

Experiment 1: PGPM, Salt (y/n), row, column, interior/exterior, and light level

Experiment 2 *Trichoderma*: *Trich.* dose (1-way ANOVA)

Experiment 2 salt: Salt dose (1-way ANOVA)

Experiment 3: PGPM, Salt (y/n), row, column

Experiment 4: PGPM, Salt (y/n), row, column, interior/exterior, and light level

The Akaike information criterion (AIC) was used to test the model fit. Models were generated for all combinations of independent variable, then compared via the AIC for relative goodness of fit. The model with the lowest AIC scores were judged to be the model of best fit. Although many spatial variables were included as possible independent variable in the models, the ANOVAs that best fit the data (as determined by comparisons of AIC test results) were two-way ANOVAs of PGPM and Salt (y/n). Post-hoc Tukey's honest standard deviation tests were then carried out for the ANOVA results (Bevins, 2023, Julkowska et al. 2019).

3.Results

3.1 Experiment 1

3.1.1 Experiment 1 design and aims

Experimental design: Experiment 1

Experiment 1 was designed to examine the effects of six different inoculant treatments on plant growth, in conjunction with 2 different salt treatments. The inoculant treatments were Control (C), *Bacillus* (B), *Pseudomonas* (P), *Trichoderma* (T), *Bacillus* and *Trichoderma* (BT), and *Pseudomonas* and *Trichoderma* (PT). The salt treatments were control (no initial) and Salt (S). The salt concentration used 0.04M NaCl, the inoculant target concentrations in the nutrient solution was 1.0E6 cfu/ml for *Bacillus* and *Pseudomonas* and 4.0E5 cfu/ml for *Trichoderma*. Thus 12 treatments were used: C, CS, B, BS, P, PS, T, TS, BT, BTS, PT, and PTS. Each of these treatments had 4 independent replicates, for 48 total pots. Two extra pots were added to complete the array of fifty, serving as informal negative controls (no Ringer solution), filling out the 5•10 grid, and supplying an extra individual when one was crushed in the transplanting process.

The combination treatments – *Bacillus*-*Trichoderma* and Pseudomonas-*Trichoderma* – had had such huge ranges of effect, with some plants comparable to respective control plants but others nearly dead, that it was decided that the labor involved in preparing the tissue-ion-extraction samples would not be worthwhile.

3.1.2 Experiment 1 results

The first experiment yielded few significant results. No clear pattern was uniformly discernable between salt-dosed and salt-free (Figure 6). The highest mean total dry weight was the *Trichoderma*-*Bacillus*-salt (TBS) treatment, but the results were not significant and the complexity of measuring paired inoculants led to this treatment being discontinued in future experiments.

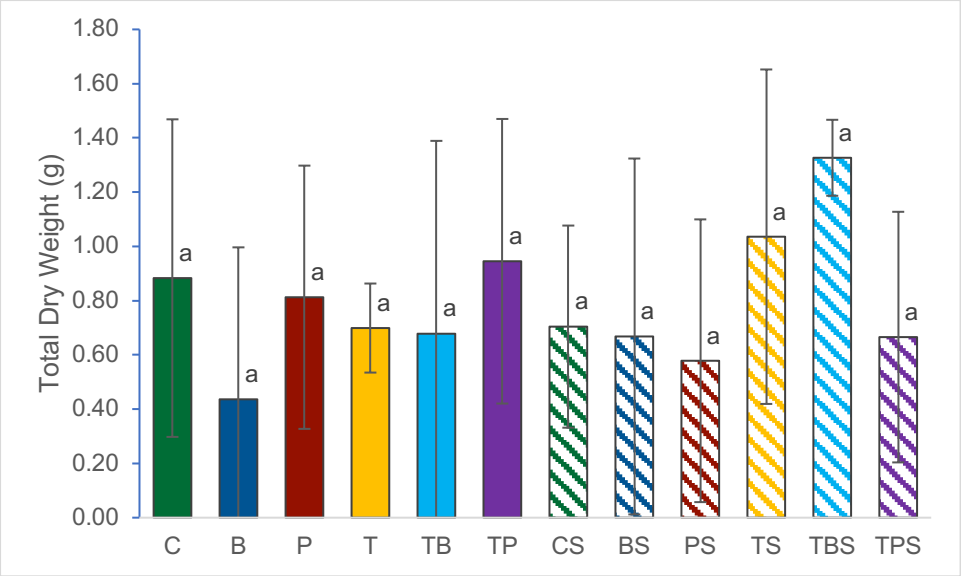

Figure 6. Bar graph showing the total dry weight in grams of each treatment. No significant differences between groups by Tukey's HSD test.

Trichoderma leaves were pale, with the variegated pattern of dark veins associated with magnesium deficiency. A SPAD measurement taken one week into the experiment had treatment Tc significantly lower than Pc, though not significantly different from Cc. This difference decreased as the plants grew, although several *Trichoderma*-treated individuals remained stunted throughout the course of the experiment.

Nutrient solution uptake did have some significant results between treatments (B – highest, TS – lowest), but no significant differences from control (C) (Figure 7)

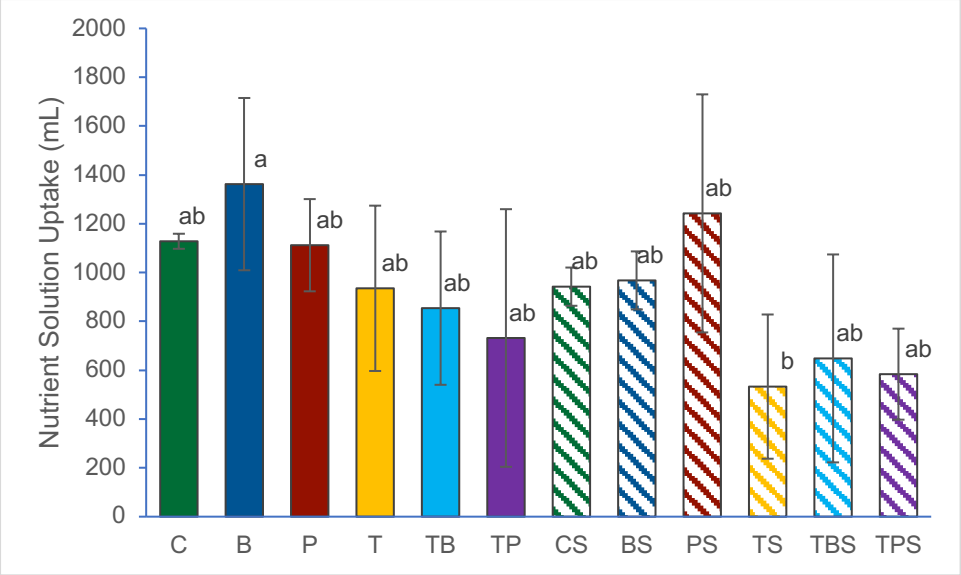

Figure 7. Bar graph showing experiment 1 nutrient solution uptake. Errors bars show 1 standard deviation. Letters indicate groupings by Tukey HSD test.

These two issues: the lack of clear signs of stress from the salt-dosed plants, and the various but detrimental effects of *Trichoderma* led to the formulation of the research questions of the second experiment. What concentration of NaCl provides the optimal level of stress for these young plants over the short treatment period? And what is the optimal dosage concentration of *Trichoderma* spores for the treatment?

3.2 Experiment 2

Experiment 2 was really two separate experiments conducted at once: an investigation into the effects of increasing salt concentration and an investigation into the effects of different *Trichoderma* spore inoculant concentrations. These two investigations shared a pool of control plants, i.e. plants that were dosed with neither salt nor *Trichoderma*. All three sets (control, salt treated, and *Trichoderma* inoculated) were grown and harvested together. But the treatments were not combined and the possible interaction between treatments could not therefore be examined.

3.2.1 Experiment 2 design and aims

Experiment 2 was designed to investigate the dosage effects of NaCl and *Trichoderma* on our experimental tomato varieties in the experimental setup. To this end a linear progression of NaCl concentrations was prepared: 0mM, 0.04mM, 0.08mM, 0.12mM, and 0.16mM NaCl. Pots were dosed with solid (granular chrystaline) NaCl, with the dosages for each pot weighed into containers

in the lab, then emptied into the hydroponic setups in the greenhouse and allowed to dissolve into the nutrient solution.

Trichoderma powder was prepared according to the procedures described in Methods section 2.1.2. Three dosages were prepared: 4.0E5 cfu/ml (H), 4.0E4 cfu/ml (M), 4.0E3 cfu/ml (L), and 0.0 cfu/ml (C).

The investigation of *Trichoderma* spore inoculant concentrations was motivated by the fact that the plants in experiment 1 that had the visibly poorest growth performance also had the most obvious *Trichoderma* infection, while pots with a less obvious *Trichoderma* presence performed better. Thus the *Trichoderma* treatments started with the experiment 1 dosage level of 4.0E5 cfu/mL (termed "High"), and decreased tenfold for the "Medium" (4.0E4 cfu/ml) and "Low" (4.0E3 cfu/ml) doses.

At harvest in experiment 2, the plants were split into three tissues: roots, stems, and leaves. 'Leaves' here therefore include the tissues that were split out into 'petioles' in other experiments (see "Tissue separation" table in Methods). For experiment 2, the nutrient solution dosage and volume were the same as experiment 1.

3.2.2 Experiment 2 results

3.2.2.1 Salt dosages

Experiment 2 yielded a succinct summary of the effects of increasing salt concentration.

Total biomass decreased with increasing salt concentration, although, as in experiment 1, no significant difference was observed between control plants and those grown with 0.04M NaCl solution. And there was no significant difference between plants grown with 0.12M and 0.16M solution, presumably because growth was effectively stunted at 0.12M, without room below it for the effects of 0.16M to be distinctly observed (Figure 8).

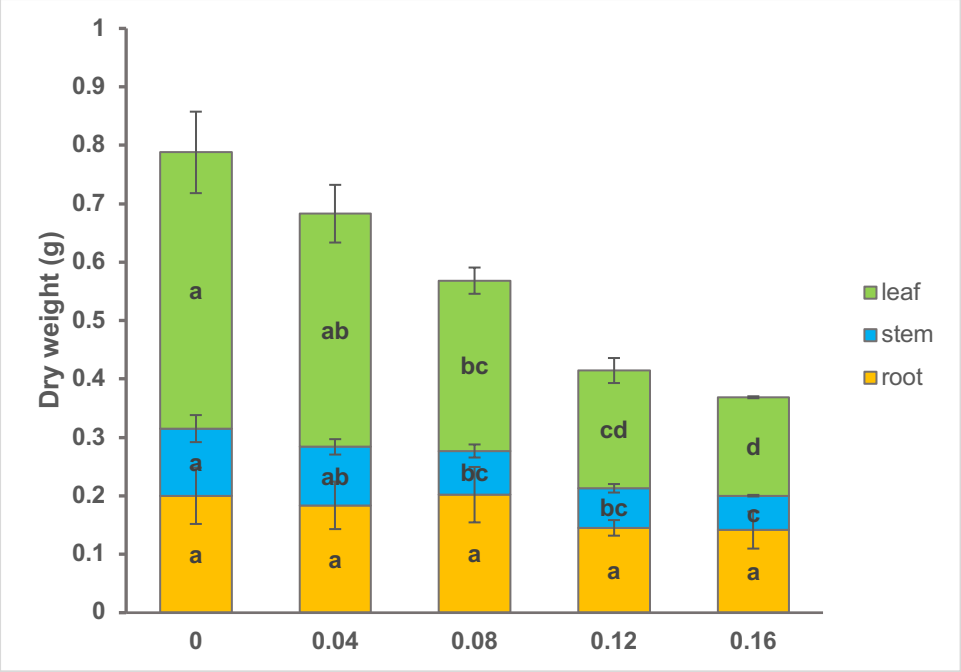

Figure 8. Bar graph showing the additive dry mass of the root, stem, and leaf tissues for the treatments with 0.0M through 0.16M NaCl, experiment 2. Error bars show 1 standard deviation; letters indicate Tukey HSD groupings.

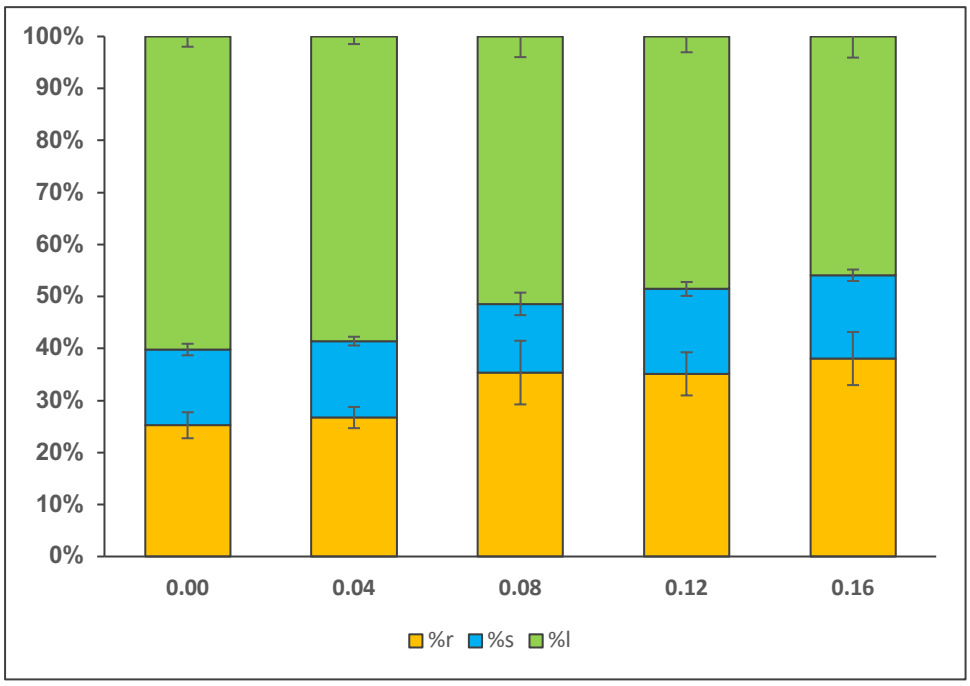

Figure 9. Bar graph showing the fraction of the total dry weight made up by each tissue: roots, stem, and leaves. Error bars indicate 1 standard deviation for the data below the error bar.

Root biomass was not significantly affected by increasing salt concentration. As a result, root biomass as a proportion of the total dry weight increased with increasing salt concentration, making a linear pattern of root:shoot ratio over increasing salinity (shown in Figure 9 as root dry weight as a percentage of the total). Root fresh weight was significantly affected by salt, but the roots lost less water in the drying process than the other tissues. Roots from the two highest salt treatments had significantly lower fresh weights than the roots of control, 0.04M, and 0.08M pots, but that difference decreased in the dry weight measurements such that it is no longer significant.

Of the three tissue categories measured, leaves suffered the most under salt stress. Under the saltiest conditions, their dry weight was less than a third that of control. The 0.16M plants also had, on average, two fewer leaves than their control or 0.04M counterparts (Figure 10).

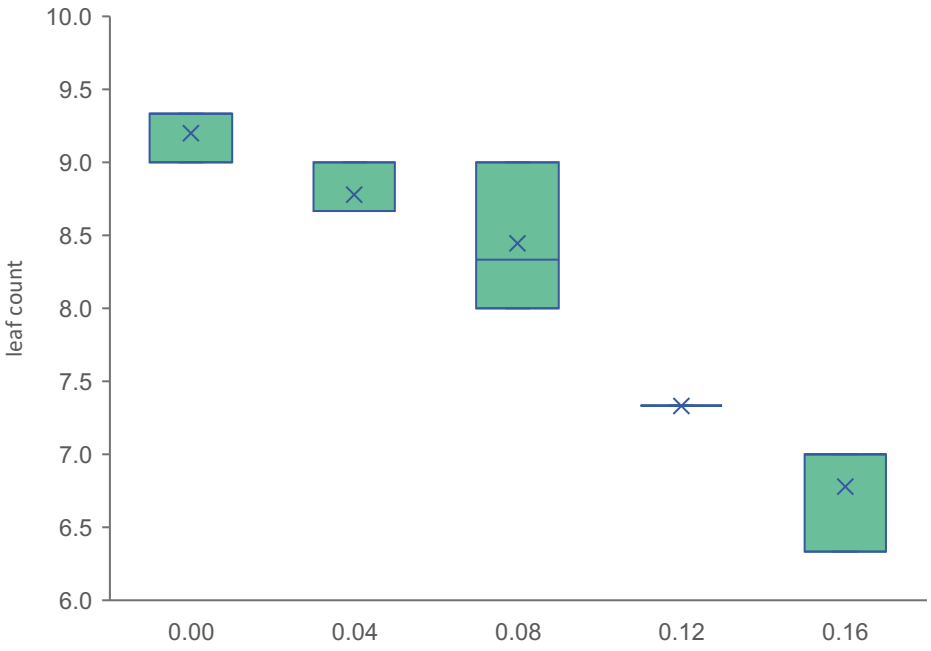

Figure 10. Box whisker plot showing leaf count across salinity treatments (0.0M to 0.16M NaCl) in experiment 2. Central line indicates median, 'x' indicates mean.

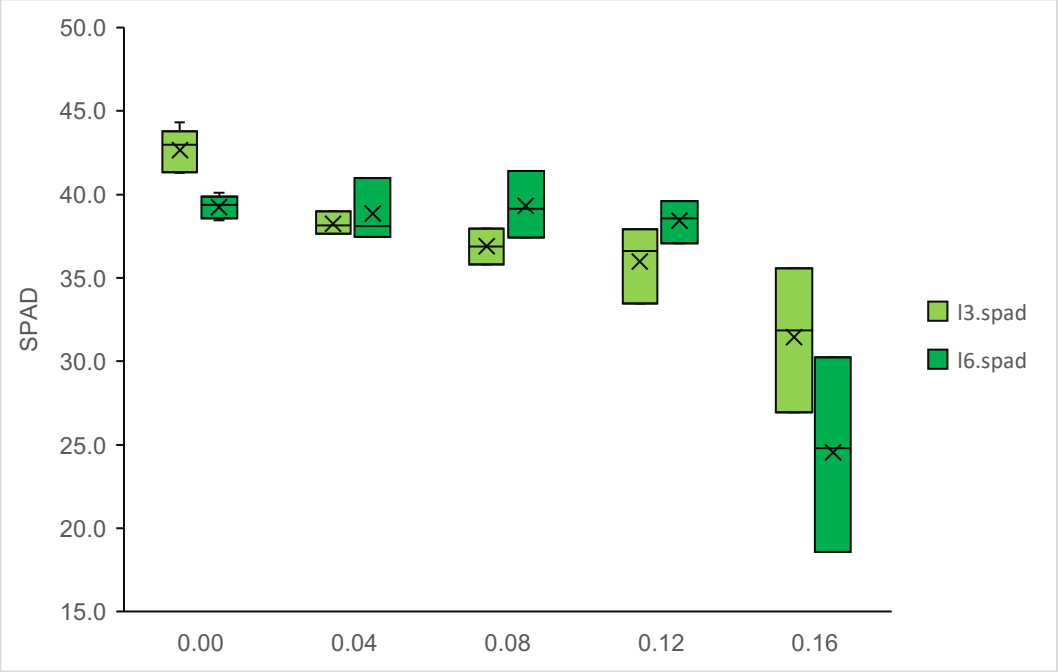

Figure 11. Box and whisker plot showing SPAD readings for leaf 3 and leaf 6, experiment 2. Central line shows median, 'x' shows mean.

Leaf chlorophyll content, as measured by SPAD, was affected negatively by salt, although this effect was greater in older leaves than younger ones (leaf 3 vs. leaf 6) (Figure 11). While the SPAD readings from the third leaves decreased in a more stepwise fashion, the SPAD readings of the younger, sixth leaves consistently averaged around 40 until the final level of salt treatment, where the average dropped to 24.8. This is one of the few measurements where the effects of 0.12M NaCl solution were distinctly and significantly different from the effects of 0.16M treatment.

Tissue ion concentration measurements show significant differences among treatments, but also among different tissues. Root K^+ concentrations decreased steadily with increasing NaCl.

R^2 value of 0.9718 on the group means over the increasing NaCl treatments. Stem K^+ concentrations decreased step by step with increasing NaCl concentration, although the differences between treatments got smaller as NaCl concentration increased, such that 0.08M, 0.12M, and 0.16M were not significantly different from one another. A linear trendline of the decreasing group means over increasing NaCl treatments had an R^2 of 0.8754.

Leaf potassium concentrations dropped quickly from control to 0.04M to 0.08M, but then levelled off, with the K^+ concentration of the leaves of plants grown in 0.16M NaCl solution increasing to the point that the mean is no longer significantly different from the control.

Root sodium levels increased sharply between control and 0.04M treatment, then plateaued, with all the salt treatments' means significantly different from control, but not different from one another. Stem and leaf Na^+ concentrations saw more gradual, and higher, increases with increasing NaCl concentrations. In interpreting these results, it should be recalled that the petioles were not separated from the leaf blade at harvest. Their tissues, which sequester Na+ to prevent it from doing damage within the leaf blade, are therefore included in the 'leaf' measurements in experiment 2. This may explain the steady increase (R^2 value of 0.9642 of mean Na^+ tissue concentration over increasing NaCl treatment) in 'leaf' tissue Na^+ ion concentration with increasing NaCl treatment, above the level that is detrimental to leaf functioning.

3.2.2.2 *Trichoderma* dosages

In the first experiment, *Trichoderma* inoculation had a negative effect on the plants. Their roots were stunted and bristly, branching out from a thick central taproot. This led to the hypothesis that the *Trichoderma* was eating the roots. *Trichoderma* ssp. are a very intensely studied clade of PGPM, and a lot of research has been done documenting their beneficial. The negative results that we had seen in experiment 1 were therefore hard to understand. Experiment 2 was set up to examine the question of whether this outcome was density-dependent on the part of the fungus. To this end, three dosages were prepared, beginning with the previous treatment dose (4.0E5 cfu/ml of nutrient solution) at the high end, and decreasing logarithmically (4.0E4 cfu/ml for medium, 4.0E3cfu/ml for low).

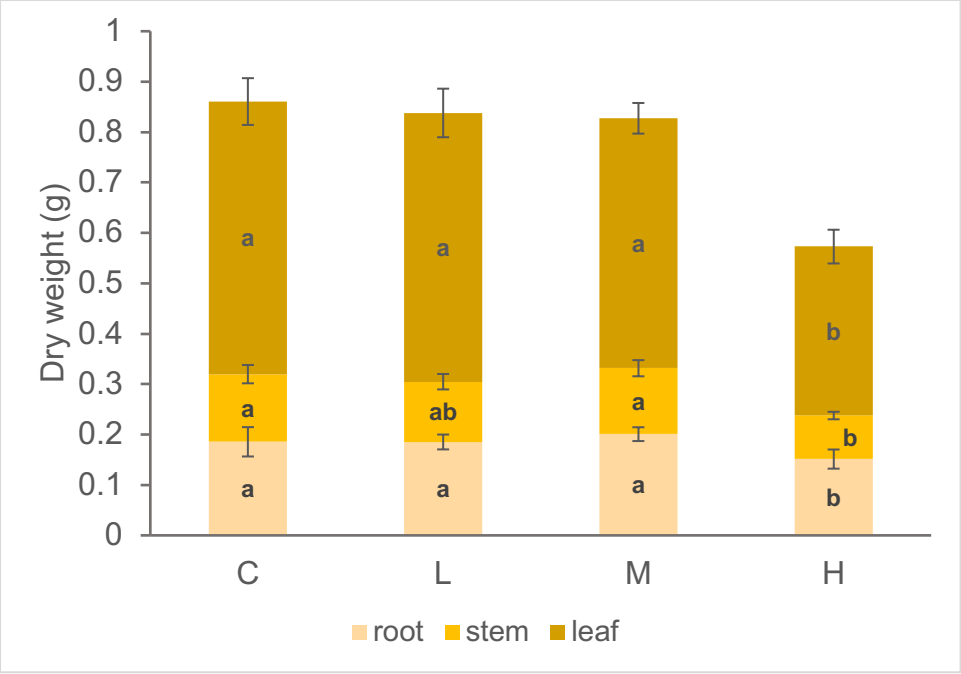

Figure 12. Bar graph showing the additive masses of root, stem, and leaf tissues across Trichoderma dose treatments. Error bars indicate 1 standard deviation; letters designate Tukey's HSD groupings.

Biomass decreased with increasing *Trichoderma* dosage concentration. Significant differences were measured between control and high dose treatments in leaf mass, stem mass, and root mass, as well as total dry weight (Figure 12). Low and Medium dose treatments did not differ significantly from control in any specific tissue or in dry weight totals.

Table 1. SPAD readings for leaf 3 and leaf 6, experiment 2, Trichoderma dosage treatments.

	Leaf 3			Leaf 6		
Treatment	SPAD	SD	Tukey HSD	SPAD	SD	Tukey HSD
Control	42.93	1.43	a	42.19	0.66	a
Low	42.32	0.63	a	41.52	3.35	a
Medium	43.64	0.68	a	41.76	1.72	a
High	43.11	3.43	a	32.33	12.30	b

SPAD measurements of leaf 3 did not vary significantly, but leaf 6 SPAD reading decreased at the highest dosage levels (4.0E5 cfu/ml) (Table 1). Leaf 6 SPAD for high-dose plants was approximately 37% lower than for those that received control, low, and medium doses. The high dose was found to be significantly detrimental to the young plants, as measured by biomass and SPAD. Lower doses were not seen to have any impact on their plants compared to control plants.

The main conclusions from experiment 2 were: that the detrimental effects of salinity increased apace with increasing doses of NaCl, and that *Trichoderma* had negative effects on plant growth at the treatment dosage of 4.0E5 cfu/ml. These findings are useful for the design of future experiments, and interesting as this is distinctly counter to the findings of the broad swath of literature on the topic, which have found *Trichoderma* to be beneficial.

3.3 Experiment 3

In the previous two experiments, conclusive observations of trends were inhibited by high rates of variability in plants' responses to inoculants. It was hypothesized that the inoculants' interactions might have been influenced by the availability of chemically available energy, i.e. by the lack of any carbon in the nutrient solution except the plant root exudates – and the plant roots themselves. Based on the phenotype of many of the *Trichoderma*-infected plants' roots, it was hypothesized that the

fungus may have been supporting itself by turning mildly pathogenic. It was decided to test these hypotheses by providing the inoculants with an external source of carbon.

3.3.1 Experiment 3 design and aims

Experiment 3 was carried out in a different hydroponic setup than the others. Instead of 2.5l pots, 1l pots were used, with one plant in each pot. I also had fewer degrees of freedom, with 3 replications of each treatment, except control, which had 4 plants –just in case.

To this end, the nutrient solution was adapted into a functional 'minimal media' as one of the treatments. Minimal media is a microbiological media that has a minimum of necessary nutrients, in their most basic, available forms needed to sustain wild microbial life; it does not contain amino acids, but rather chemical nutrient salts. The necessary mineral and chemical nutrients were already contained in INTEGAR formulation hydroponic nutrient solution, but a carbon source was lacking (the hypothesized root of the question that was being investigated). Glucose was selected as the added carbon source because it is used in the reference Davis Formulation minimal media, and because it is the carbon source used in the propagation media of all three treatment microbiota and was therefore certainly known to be biologically available to them.

Glucose was dosed out by mass directly into the treatment setups in a ratio of 2.500g glucose to 2.5l solution (0.006938M); this is the ratio used in the Davis formulation of minimal media.

3.3.2 Experiment 3 results

Glucose, added into the nutrient solution, was found to be massively deleterious to plant growth. All plants infected by *Pseudomonas* that were dosed with glucose died within 24 hours of the sugar being added. Control with glucose, *Bacillus*-infected with glucose, and *Trichoderma*-infected with glucose plants survived, but were significantly smaller than their glucose-free counterparts (Figure 13). Leaf, petiole, and stem tissues were significantly smaller in glucose-treated plants than control, regardless of inoculant treatment (Table 2). Root tissues did not show significant differences, except for *Pseudomonas*-glucose (Figure 13). Both glucose and inoculant treatments had significant effects on nutrient solution uptake (Figure 15). Control-, *Bacillus*-, and *Pseudomonas*-infected (C, B, P) plants took up the most, and glucose-treated, *Pseudomonas*- and *Trichoderma*-inoculated (Pg & Tg) plants took up the least. Both glucose and inoculant treatments also had significant effects on plant height (Figure 14).

The osmotic effects of the added glucose can be ruled out as a main cause of the negative growth results, since the osmotic concentration of the treatment glucose in solution was only 0.006938 osmol/L. The salt treatment in the same experiment added 0.12 osmol/L (these calculated osmolarity values are borne out by osmolality measurements of freezing point depression), and yet the plants dosed with salt performed better than those dosed with glucose. Control (no inoculant) plants treated with 0.00684M glucose were significantly smaller than control plants treated with 0.06M NaCl (2-sample t-test, p-value 0.0072).

The microbial behavior of the inoculants was altered by the addition of glucose. As mentioned above, the combination of *Pseudomonas* and glucose led to immediate plant death. The nutrient solution that remained was stained a bright orange-red color by the glucose-fed *Pseudomonas* (Figure 17). The behavior of *Bacillus* and *Trichoderma* was also altered when they were fed sugar: both formed thick, gloopy mats on the plants' roots and on the outside of the containers (Figure 16).

The ability of glucose to boost the salinity-tolerance-inducing capabilities of *Trichoderma* was negligible. All *Trichoderma*-salt-glucose treated plants barely grew beyond the size that they were when treatment began (Bsg and Psg treatments were not carried out).

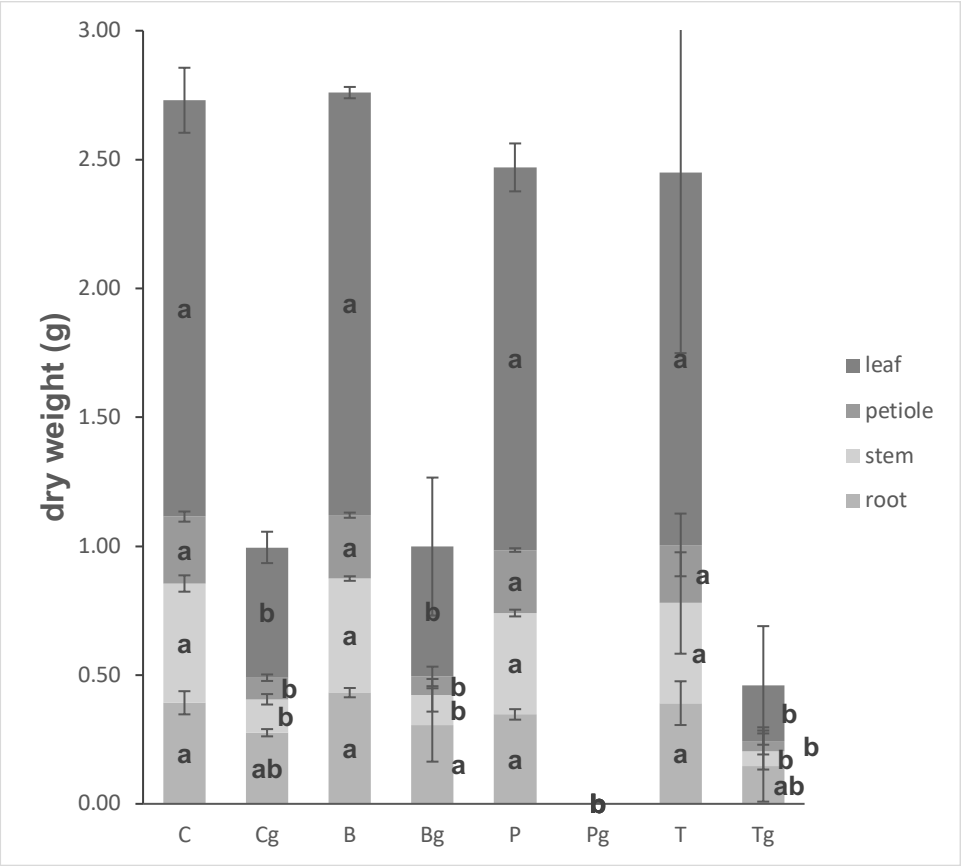

Figure 13. Bar graph showing the additive masses of root, stem, and leaf tissues across inoculant and glucose treatments, experiment 3. Error bars indicate 1 standard deviation; letters designate Tukey's HSD groupings.

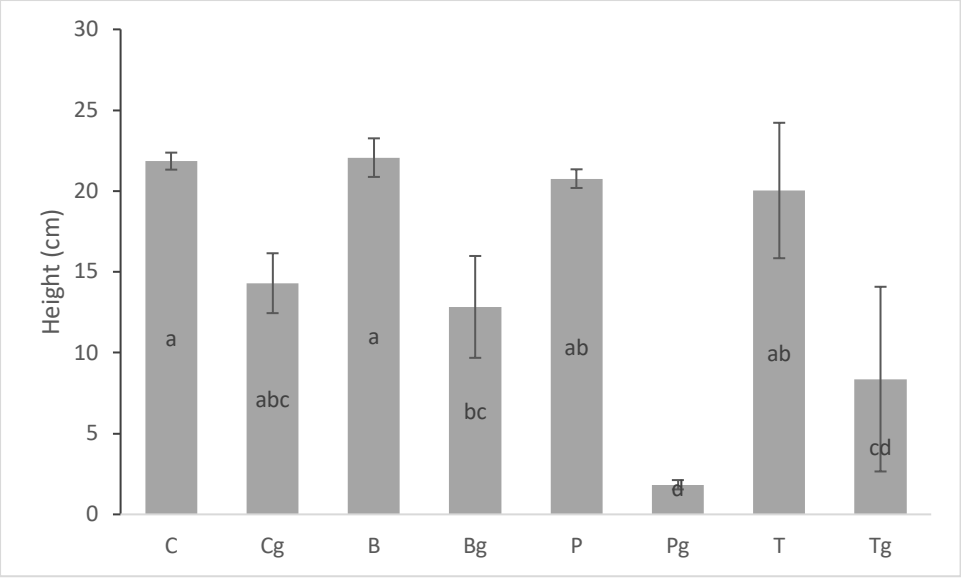

Figure 14. Bar graph showing plant height (cm) across inoculant and glucose treatments, experiment 3. Error bars indicate 1 standard deviation; letters designate Tukey's HSD groupings.

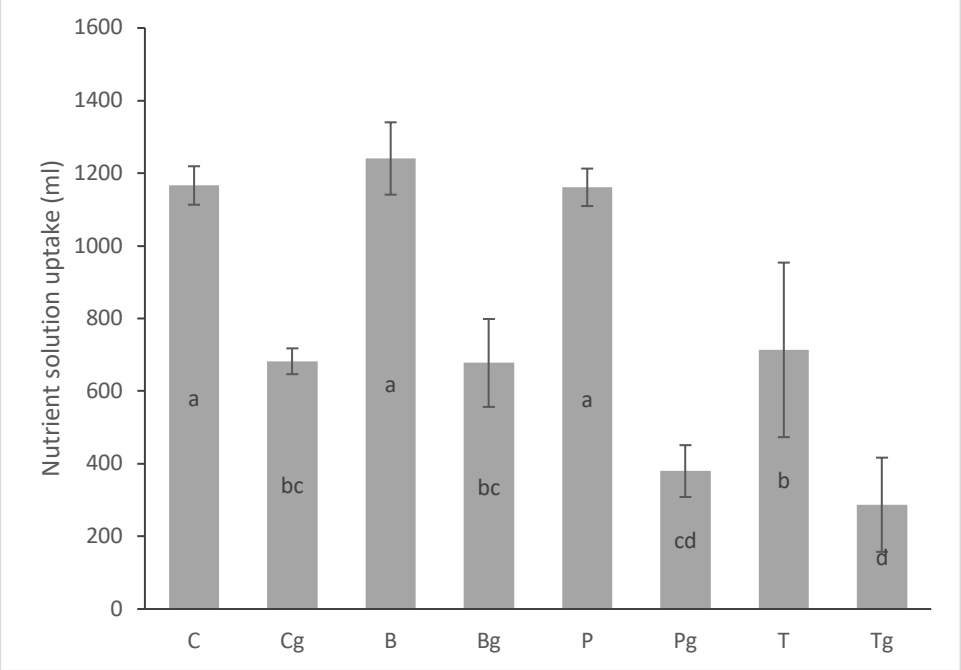

Figure 15. Bar graph showing nutrient solution uptake (mL) across inoculant and glucose treatments, experiment 3. Error bars indicate 1 standard deviation; letters designate Tukey's HSD groupings.

Table 2. Dry masses (in grams) of the tissues by treatment, experiment 3.

	Treatments								
Dry mass (g)	C	Cs	Cg	B	Bg	P	Pg	T	Tg
Leaf, young	0.61± 0.103 a	0.23± 0.044 ab	0.16± 0.018 c	0.64± 0.033 a	0.11± 0.073 c	0.64± 0.022 a	0.00± 0.0 c	0.54± 0.329 ab	0.05± 0.072 c
Leaf, old	1.00± 0.045 a	0.47± 0.047 bc	0.35± 0.048 cd	1.00± 0.055 a	0.39± 0.195 cd	0.85± 0.076 ab	0.01± 0.004 d	0.91± 0.375 a	0.16± 0.159 cd
Petiole, young	0.08± 0.017 a	0.05± 0.007 abcd	0.03± 0.005 cd	0.08± 0.008 ab	0.02± 0.011 d	0.09± 0.003 a	0.00± 0.000 d	0.07± 0.043 abc	0.01± 0.016 d
Petiole, old	0.17± 0.008 a	0.09± 0.006 bc	0.06± 0.008 c	0.16± 0.009 ab	0.06± 0.027 c	0.15± 0.008 ab	0.00± 0.000 c	0.15± 0.079 ab	0.03± 0.037 c
Stem	0.46± 0.032 a	0.43± 0.033 bc	0.13± 0.020 c	0.44± 0.009 a	0.12± 0.063 c	0.39± 0.013 ab	0.00± 0.001 c	0.39± 0.197 ab	0.06± 0.070 c
Root	0.39± 0.045 a	0.21± 0.018 a	0.28± 0.014 ab	0.43± 0.018 a	0.31± 0.142 a	0.35± 0.020 a	0.00± 0.000 b	0.39± 0.085 a	0.15± 0.138 ab
Total dry weight	2.73± 0.205 a	1.48± 0.138 ab	1.00± 0.091 bc	2.76± 0.008 a	1.00± 0.511 bc	2.47± 0.124 a	0.01± 0.005 c	2.45± 1.097 a	0.46± 0.490 bc

Figure 16. Images of Bacillus-glucose treated (left), control (center), and Trichoderma-glucose (right) treated plants at harvest, experiment 3. The air tubes included in the Bg and Tg photos shows the slimy buildup characteristic of the these treatments (also on the roots).

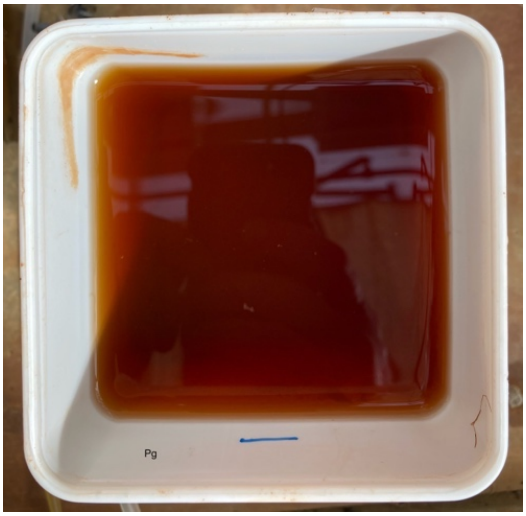

Figure 17. Image of the Pseudomonas-glucose-treated nutrient solution at the end of the experiment, stained a vivid orange color. All Pg-treated plants died within 24 hours of the addition of glucose.

3.4 Experiment 4

The research questions that experiment four was designed to answer were the same, central questions that had been investigated in experiment 1, and which motivated the whole project: how plant-growth-promoting microorganism affect the growth and salinity tolerance of hydroponically grown tomatoes.

3.4.1 Experiment 4 design

Experiment 4 was designed with the lessons learned in the previous experiments in mind. To minimize the amount to labor required and maximize the number of replicates, *Trichoderma* was excluded from the list of treatments. It had never produced positive results in terms of plant growth outcomes. Thus, treatments were limited to 6 (C, B, P, Cs, Bs, Ps), and the number of replications was increased to 6. These 36 pots were arranged in a Latin square, with one replication of each treatment in each row and each column. As had been decided in the design of experiment 3, with reference to the results of experiment 2, salt treated plants were dosed with 0.06M NaCl.

As in Experiment 3, one plant was grown in each pot, but the 2.5L pots from experiments 1 & 2 were used (rather than the 1L pot used in experiment 3). Both decisions were aimed at increasing the growth time available for treatment before the plants outgrew their systems.

The scope and scale of measurements was increased for this final experiment. The leaf and petiole tissues that had been grouped into various configurations in previous experiments were separated into individual tissues (see Table 2.X). Furthermore, the leaf area of each of these individual leaves was measured.

3.4.2 Experiment 4 results

In salt-free conditions, *Bacillus* and *Pseudomonas* increased plant biomass compared to control (B and C not significantly different by HSD, but significantly different by 2-way t-test). Salt dosed plants were significantly smaller than salt-free ones, but there were no significant differences among salt-dose plants (Figure 18). The same pattern was visible for nutrient solution uptake (Figure 19). Leaf area measured a significant impact of salt treatment, but not of any inoculant treatment (Figure 20). Height also showed significant salt-induced decreases, but no significant differences among

inoculation treatments (Figure 21). Leaf 3 SPAD measurements did not vary significantly. But leaf 6 SPAd measurements increased among salt-dosed plants. *Bacillus*-salt-treated (Bs) plants had the highest young leaf SPAD readings, and control had the lowest (Figure 22). Root fraction (root tissue mass as a fraction of the total dry weight) showed significant salinity effects. Across inoculation treatments, salt-treated plants had more root tissue as a fraction of the whole plant. Salt significantly affected specific leaf area (cm^2 of leaf area per total g dry weight; 2-way ANOVA p-value >F: 1.24E-5) and leaf density (g leaf dry weight per leaf area; 2-way ANOVA p-value >F: 1.54E-6; Figure 24. Box and whisker plot showing leaf density, calculated as lea dry weight (g) per leaf area (cm^2), experiment 4. Central line shows median, 'x' shows mean. HSD groups a: P, B; ab: c; b: Ps, Cs, Bs.). By breaking the plants into more individual tissues, a more granular understanding of tissue mass allocation is enabled. This allows a greater resolution of analysis (Table 3). For example, the only significant difference among salt treatment tissue masses is leaf 5, where Bs is significantly larger than Cs (Table 3).

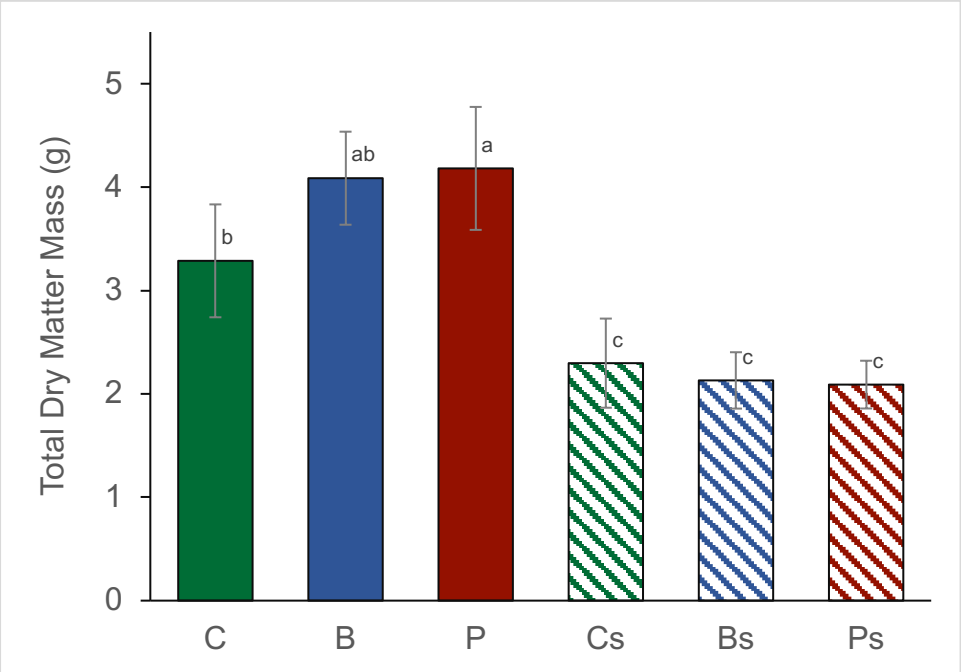

Figure 18. Bar graph showing total dry matter (g) across inoculant and salinity treatments, experiment 4. Error bars indicate 1 standard deviation; letters designate Tukey's HSD groupings.

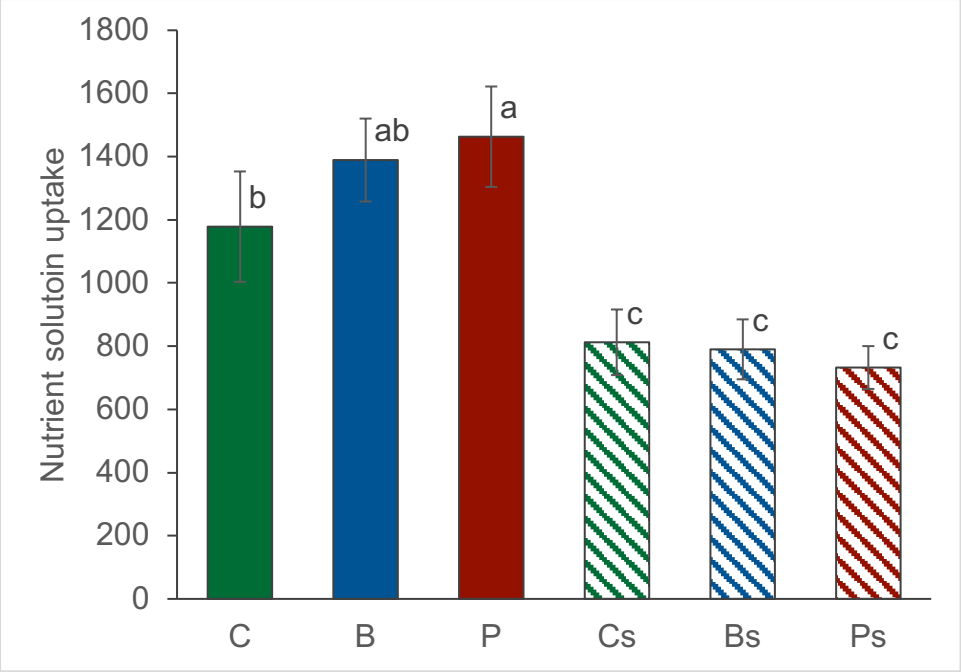

Figure 19. Bar graph showing the mean nutrient solution uptake (ml) across inoculant and salinity treatments, experiment 4. Error bars show one stand deviation; labels show the mean groupings by Tukey's HSD test.

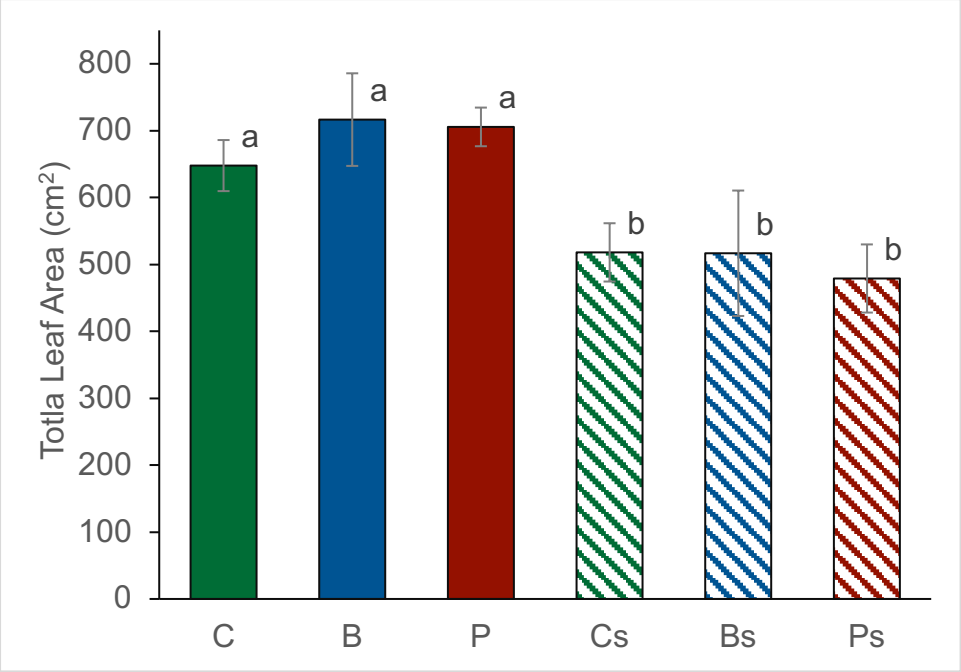

Figure 20. Bar graph showing the mean total leaf area (cm^2) across inoculant and salinity treatments, experiment 4. Error bars show one stand deviation; labels show the mean groupings by Tukey's HSD test.

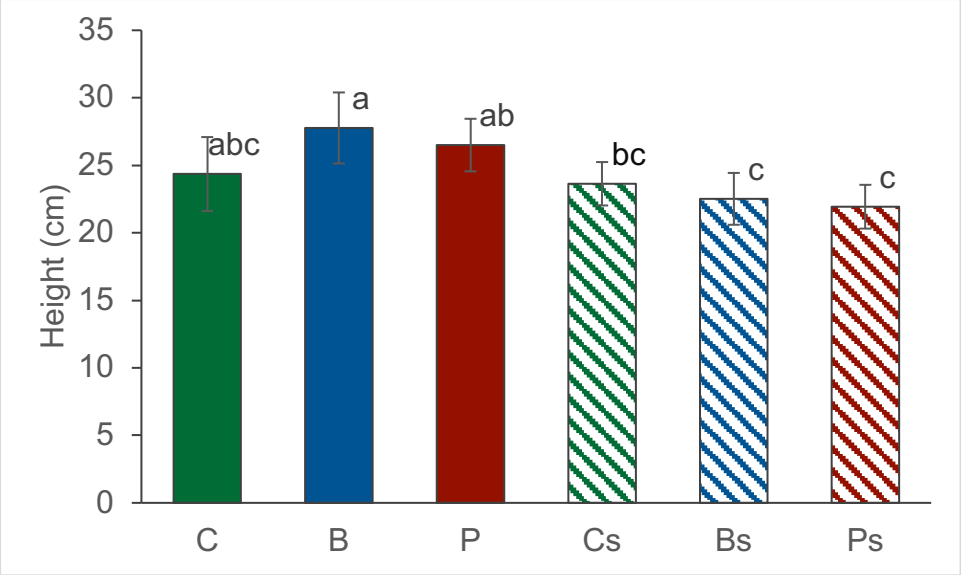

Figure 21. Bar graph showing the mean height (cm) across inoculant and salinity treatments, experiment 4. Error bars show one stand deviation; labels show the mean groupings by Tukey's HSD test.

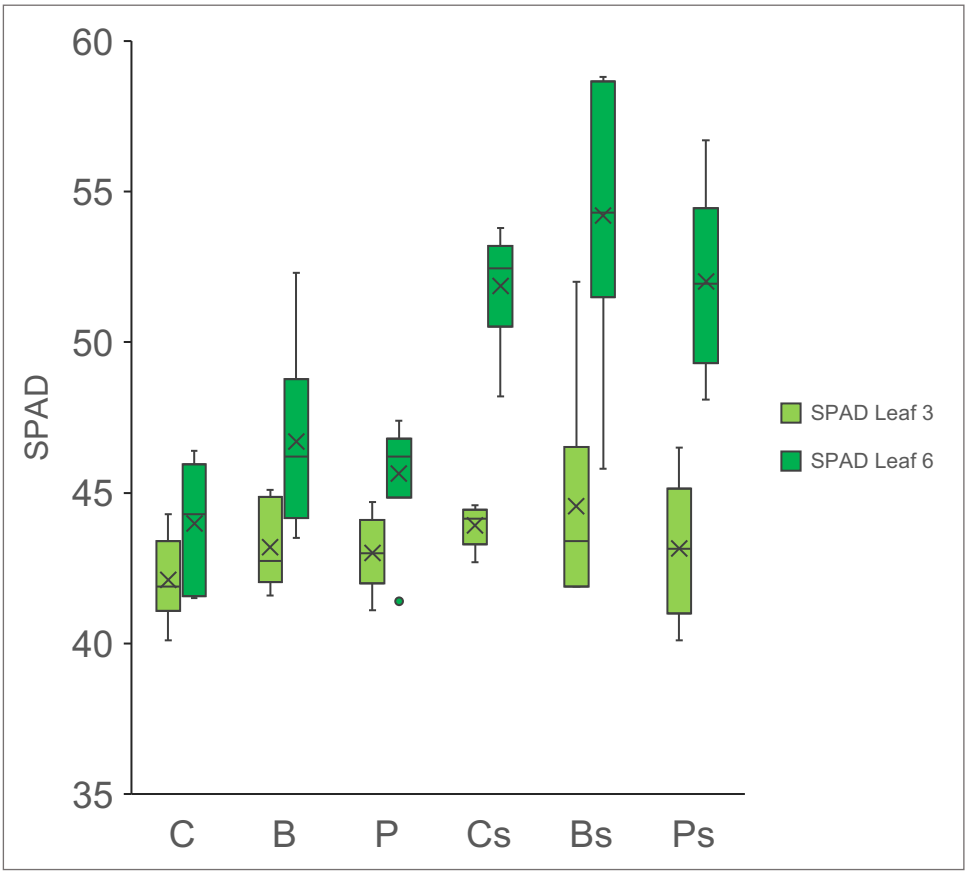

Figure 22. Box and whisker plot showing SPAD readings for leaf 3 and leaf 6, experiment 2. Central line shows median, 'x' shows mean. Leaf 3 HSD groups a: all. Leaf 6 HSD groups a: Bs; ab: Ps, Cs; bc: B; c: P, C.

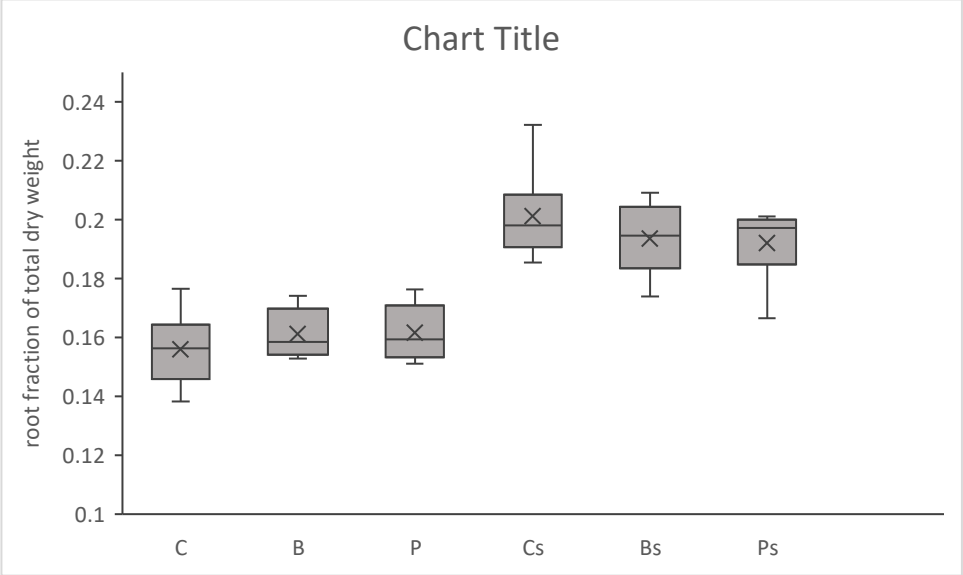

Figure 23. Box and whisker plot showing root fraction of total dry weight, experiment 4. Central line shows median, 'x' shows mean. HSD groups a: Cs, Bs, Ps; b: P, B, C.

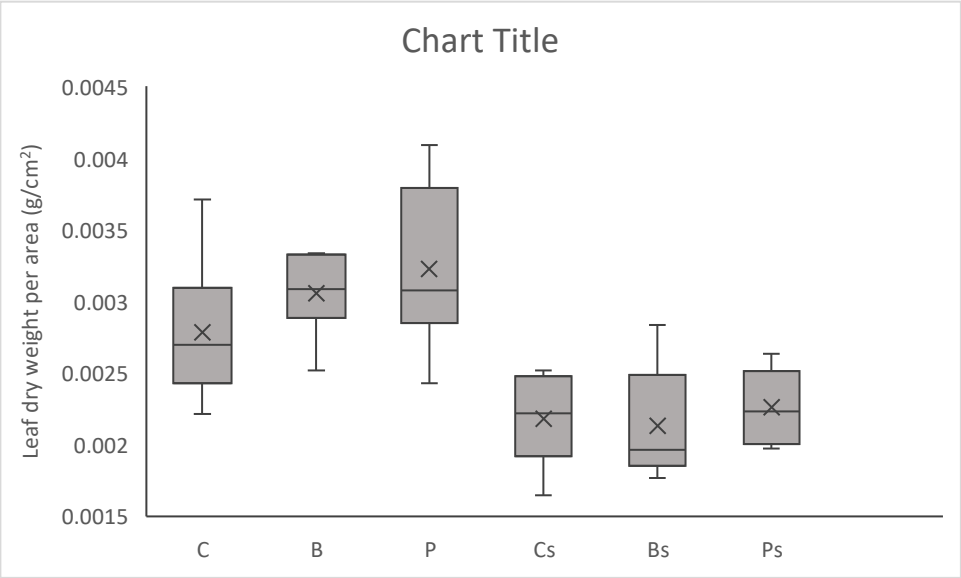

Figure 24. Box and whisker plot showing leaf density, calculated as lea dry weight (g) per leaf area (cm^2), experiment 4. Central line shows median, 'x' shows mean. HSD groups a: P, B; ab: c; b: Ps, Cs, Bs.

Table 3. Dry weight of tissues by treatment, experiment 4

Tissue	C			B			P			Cs			Bs			Ps		
	$\bar{X}$ (g)	SD(g)	HSD	$\bar{X}$ (g)	SD(g)	HSD	$\bar{X}$ (g)	SD(g)	HSD	$\bar{X}$ (g)	SD(g)	HSD	$\bar{X}$ (g)	SD(g)	HSD	$\bar{X}$ (g)	SD(g)	HSD
Root	0.514	0.110	b	0.659	0.087	a	0.672	0.084	a	0.458	0.075	b	0.412	0.065	b	0.401	0.056	b
Stem	0.564	0.110	b	0.745	0.091	a	0.728	0.087	a	0.430	0.097	bc	0.394	0.097	c	0.370	0.048	c
Petiole 1	0.022	0.004	abc	0.032	0.009	a	0.026	0.006	ab	0.019	0.007	bc	0.017	0.006	bc	0.014	0.004	c
Petiole 2	0.074	0.010	a	0.080	0.011	a	0.086	0.014	a	0.051	0.009	b	0.047	0.015	b	0.042	0.013	b
Petiole 3	0.110	0.012	b	0.138	0.016	a	0.138	0.013	a	0.074	0.015	c	0.066	0.007	c	0.065	0.005	c
Petiole 4	0.114	0.016	a	0.130	0.020	a	0.140	0.019	a	0.070	0.012	b	0.068	0.010	b	0.064	0.008	b
Petiole 5	0.061	0.007	ab	0.082	0.020	a	0.081	0.018	a	0.040	0.011	bc	0.034	0.006	c	0.037	0.010	c
Petiole 6	0.024	0.004	abc	0.032	0.008	ab	0.034	0.006	a	0.019	0.008	bc	0.018	0.006	c	0.020	0.009	c
Leaf 1	0.132	0.040	ab	0.168	0.019	a	0.171	0.019	a	0.120	0.040	ab	0.108	0.034	b	0.088	0.029	b
Leaf 2	0.309	0.049	ab	0.328	0.061	a	0.331	0.093	a	0.198	0.048	c	0.203	0.045	bc	0.181	0.058	c
Leaf 3	0.421	0.110	b	0.556	0.074	ab	0.562	0.122	a	0.252	0.065	c	0.225	0.027	c	0.236	0.031	c
Leaf 4	0.484	0.116	a	0.550	0.097	a	0.572	0.130	a	0.248	0.043	b	0.245	0.034	b	0.250	0.023	b
Leaf 5	0.259	0.036	b	0.336	0.080	ab	0.355	0.069	a	0.151	0.058	c	0.148	0.021	b	0.158	0.027	c
Leaf 6	0.133	0.033	ab	0.155	0.036	a	0.183	0.031	a	0.098	0.018	b	0.085	0.026	b	0.091	0.040	b
Leaf 7+	0.065	0.021	a	0.094	0.033	a	0.102	0.033	a	0.070	0.035	a	0.060	0.017	a	0.072	0.034	a
Total D.W.	3.287	0.598	b	4.086	0.493	ab	4.181	0.651	a	2.297	0.472	c	2.130	0.299	c	2.090	0.253	c

Note: Tukey's HSD test performed for each tissue. Groupings are comparable along rows, not down columns.

4. Discussion

4.1 Effects of salt.

This sketches a Portrait of salinity tolerance for *Solanum lycopersicum L.* var. Sweeterno, and is the Starting point for a discussion ways to maximize of salt tolerance.

Salt stresses plants. How plants react to this stress depends on the plant – its genes and stress responses – and the environment – how much salt there is, how much water there is, and how much of each is taken up via transpiration (Cuartero and Fernández-Muñoz 1998). The initial concentration of salt used to induce salt stress in the seedlings was chosen with references to the work of Puppala et al. (2023, not yet published) who were in the same working group, and working with the same variety of tomatoes, germination practices, and nutrient solution. They found significant effects at 0.03M, a lower concentration of NaCl than was used in experiment 1, but their trials lasted longer, with the effects compounding over time until it was measure in mature plants. In a hydroponic system, the list of things that can affect salt uptake include the ambient temperature and humidity, the plants' rate of photosynthesis and growth, and the flow rate of the solution around the roots. The results of the experiment 1were muddied by the fact that many of the growth parameters did not show significant differences between salt and control treatments. The plants were small – so was the stress treatment time. The aim of the second experiment was to find a level of salinity that would induce crudely measurable stress responses from the seedlings without choking them of nutrient solution through the increased osmolarity. The young plants have less leaf surface area over which to evaporate water to generate a transpiration gradient. Indeed, the young plants did wilt immediately after being dosed with salt, but by the next morning they had returned to full turgor – presumably having adjusted the osmolyte concentration of their tissues in response to the increased osmolarity of the nutrient solution.

4.1.2 Salt on biomass totals

The response to salt stress that was seen in the experiment was in line with the literature results: The plants invested in root tissue (Figure 8, Figure 18), and limited solution uptake (Figure 19) (Cuartero and Fernández-Muñoz 1998). Maggio et al. conducted research hydroponically exposing tomato plants to a range of salinities. Our EC values (mS/cm) at 0.06M match the inflection point in the curve described by Maggio et al. where the decrease in biomass per additional mS/cm began to level

off (Maggio et al. 2007). While our data broadly concur with the rate of decrease, in experiment 2 when a range of salinity levels was tested, we found the decrease to be more linear than that of Maggio et al.

Tomatoes are able to store Na+ in petioles and different varieties or landraces may invest in petiole tissue, or benefit from having proportionally larger petiole tissues, as a salt-stress mitigation technique to keep the salt away from the leaf blade in a less active and sensitive organ (Taleisnik 1989, Cuartero and Fernández-Muñoz 1998). In experiment 4, there was not a significant change in the proportion of the total mass made up by the petiole with the introduction of salt. The petioles were significantly smaller under salt stress, but they decreased in step with the rest of the plant. Petioles had much higher Na^+ concentrations, compared to the other plant tissues, and these salt treatment differences in tissue Na^+ concentration were the highest of the different tissues. González-Fernández found that although the concentration of Na+ as a function of tissue dry matter is higher in petioles than other tomato plant tissues, this distinction disappears when measured as a function of fresh weight. The water holds the same range of concentrations, there's just more water per unit dry matter in the petiole (Gonzalez Fernandez 1996). We cannot comment directly on this, as we did not measure the fresh weight of the individual tissues. However, our petioles also had higher K^+ ion concentrations than the other tissues, in both salt and non-salt treatments. This understanding could be in line with our findings. If petioles hold more water per unit dry biomass, resulting in a higher K^+ concentration per unit dry biomass, under salt stress the competition between Na^+ and K^+ ions leads to the displacement of K^+. All potentially without a massive or disproportionate increase in the concentration per volume of water in the tissue.

4.1.3 Effects of salinity on chlorophyll content (SPAD)

Salinity influences chlorophyll in two main ways. First, salt affects the plant's ability to take up water and nutrients from the soil, potentially limiting the nitrogen (and to a lesser extent magnesium) nutrition that the plant needs to build chlorophyll proteins (Cuartero and Fernández-Muñoz 1998). Second, salt stress limits transpiration, leading to a build-up of reactive oxygen species (ROS) as the Calvin cycle is CO_2-limited (Hossain and Dietz 2016). These ROS damage leaf tissue. As such, SPAD is a meaningful indicator of this aspect of plant health. Interestingly, many of our plants increased their SPAD under salt stress.

Moles et al. characterized the maintenance of leaf chlorophyll content as a marker of salt tolerance in the salt-hardy 'Ciettaicale' landrace. But this maintenance was in the context of decreasing SPAD readings with increasing salinity i.e. a less steep decline, not an increase (Moles et al. 2016). In experiment 2, SPAD decreased at the highest dosage of salt (Figure 11), but in other experiments salt had a positive effect on SPAD readings, at least in young leaves (Figure 22). Leaf Na^+ was significantly positively correlated with Leaf 6 SPAD across treatments in experiment 4. Our findings were not unique: Souri and Todhidloo also found higher SPAD readings in their salt stress tomatoes (0.10M NaCl), grown hydroponically on sand, which they attributed to the smaller leaf size (Souri and Tohidloo 2019). Others have found the opposite, however, with both geoponic and hydroponic salinity experiments (Ullah et al. 2020; Islam et al. 2011; Shimul et al. 2014; Carbajal-Vázquez et al. 2022).

4.1.4 Effects of salinity on root/shoot ratio: higher is better?

Experiment 2 saw a linear increases in the root tissue and decreases in leaf tissue as a percentage of total dry weight with increasing NaCl dosage (Figure 8, Figure 9). In the other experiments, salt had a negative effect on plant growth, but did not affect the allocation of dry matter by tissues as a percentage of the whole (Figure 13, Table 2, Table 3).

As mentioned in the material and methods, the plants used in the second experiment were older when they began their treatment (4-5 leaves). It is unclear whether the differences between the results of Experiment 2 and others (different patterns of SPAD responses and proportional increase in root tissue as percentage of total) is due to the doses involved and the relative statistical resolution of the experimental designs of the different experiments, due to these differences in the plant material used. If exp2 plants began treatment older and less nutrient stressed (having spent longer in an environment with abundant nutrients) than those in the other experiments, the patterns of allocation could understandably vary, which could explain the different SPAD responses.

The interaction between plants and PGPM evolved in soil-based systems, facing stressors that occur for plants growing in soil with strategies that tend to work in soil. For example, in the face of salt stress in a geoponic system, PGPM associated with the plants have been found to produce chemical signals that lead to increased plant investment in the roots (Gamalero and Glick 2022). This resulting greater root growth increases the plant's ability to draw the needed water, and potentially to find areas of the soil where there is less salt. This strategy does not work in hydroponics. The same

ubiquity and uniformity of solution conditions that alleviate the necessity for root hairs means that there is nowhere to grow to avoid the salt. Investment in root tissues by hydroponically grown crops has been shown to be adaptable. Kang & Van Iersel (2004) found that root/shoot ratio decreased with increasing nutrient concentration in hydroponic systems (Kang and Iersel 2004). It is not clear to what degree root growth in hydroponically grown plants can be regarded as a vestigial function, maladaptive in a system where the nutrients flow to the roots and all available recourse might otherwise be put towards yield.

Comparisons of plants' roots between hydroponically- and soil-grown samples can be difficult, since different amounts of materials may be lost, depending on the sampling method used and amount of care taken. However, there are clear morphological differences between tomato plants grown hydroponically and those grown in soil. In deep-pool hydroponic setups, the roots have no structural function – the plants are supported from above at the level of the lid of the container, and, as the plant grows, from wires or lines up which the plants are trained. The non-structural needs of the plant are met by long, fine, silky roots. Root hairs, appendages of the root epidermis which serve the double function of foraging for nutrients in the soil and increasing the root's surface area were found to be totally absent from healthy roots of hydroponically tomatoes in our experiments (control (center) image Figure 16).

The effect of salt on root : shoot ratio (expressed in terms of root biomass as a percentage of the total biomass) is well established (Cuartero and Fernández-Muñoz 1998). Increasing salinity elicits increased root tissue relative to total biomass. A couple of closely related experiments support this trend: El-Hassanin et al. (2020) conducted a similar experiment using tomatoes, hydroponically grown to six weeks under different salt treatment regimes (El-Hassanin et al. 2020). They found increasing root/shoot ratios with increasing salt. Miceli et al. used various biostimulants to mitigate the effects of salinity in hydroponically grown tomatoes. They found that root/shoot ration increases with salinity, and is not impacted significantly by microbial biostimulant (Miceli, Moncada, and Vetrano 2021).

In experiment 2, it was observed that the roots increased as a fraction of the total biomass with increasing salt. There was no significant difference between the mass of the roots under the different salt treatments, but their relative proportion of the total biomass increased as the rest of the plant decreased with increasing salt concentration, relative to the control. This was also seen in the other experiments where salt was a factor. The salted plants were smaller, with higher root : shoot ratios.

The presence or type of inoculant were not seen to affect this ratio, this is comparable with Miceli et al (2021). Thus, our results support the literature trends.

4.2 *Bacillus* and *Pseudomonas* increase biomass in non-salty conditions

When not treated with salt, young tomato plants benefit from inoculation with the target *Bacillus* and *Pseudomonas* strains. They have more biomass (Figure 18). They take up more nutrient solution (Figure 19), and more nutrients from that solution – specifically nitrate and potassium, though this trend was not significant and might have been better captured before the nutrients had been depleted from the solution. If the plants were grown to fruit-bearing stage, this would result in higher, better quality yields (Rodríguez-Ortega et al. 2019). *Bacillus* and *Trichoderma* will benefit tomatoes in hydroponic setups without salt. The mean total dry weight of *Bacillus* was 124.3% that of control, and *Pseudomonas* was 127.2% of control. The assumption is that plants will grow optimally, unless limited. It is difficult to tell what factor might have been the short stave in Leibig's barrel; considering that both control and bacterial inoculants were grown under the same conditions, it is difficult to ascertain what shortcoming could be made good by the inoculants to push their host plants beyond the control growth pattern. However, it seems clear that salt inhibits these bacteria. And the benefits are not evenly experienced across the plant tissues.

4.2.1 Bacteria do not benefit leaf growth

As shown in table x, significant differences in plant growth between control and bacteria-inoculated plants were observed for root, stem, and petiole tissues. There were no significant differences between inoculant treatments with respect to leaf growth. Leaf growth is of critical economic importance. Leaf mass and area are linked to yield by many studies, e.g. (Jo and Shin 2020; Rodríguez-Ortega et al. 2019). More leaf tissue and more leaf area enable more photosynthesis and net primary productivity.

Pseudomonas brassicacearum is known to affect abscisic acid (ABA) levels and to be able produce ACC deaminase, which collectively affect plant stress responses (Bresson et al. 2013; Nelkner et al. 2019; Singh, Ma, and Shadan 2022). *Bacillus megaterium* has been found to benefit plants by producing the plant hormone Indole-3AA which is the most common auxin-class plant hormone(Taha et al. 1969). IAA controls cell division, and therefore has a role in the coordination of plant growth. IAA-producing PGPM can influence plant growth coordination by producing the hormone according to their own set of responses to conditions (Glick 1995). The mechanisms

governing these interactions under stress may be more clearly elucidated, but in the role of performance enhancers, it is less clear what tradeoffs are being run differently (compared to control) to get these results. Miceli et al. found that microbial biostimulants did significantly increase leaf mass in both absolute terms and relative to total dry weight (Miceli, Moncada, and Vetrano 2021). But we, in a very similar experimental setup, though with different microbial and tomato varieties, did not.

It may be that an external factor is creating a tradeoff, or a threshold value of leaf area was reached, leading to increased allocation to other tissues. For example, if the light was too intense, and/or the plants had taken up all the nitrogen, would that explain these larger plants with the same-sized leaves? In-depth reporting of light conditions is not common in the literature for experiments not directly concerned with lighting research questions, so it is difficult to make direct comparisons.

4.3 Salt erases these beneficial effects

When the plants are treated with salt, they do not benefit from the inoculation of these target PGPBs (Figure 18). It is not clear whether this is because these PGPBs do not improve plants' salinity tolerance, or whether the salt treatment limited their ability to affect changes in the plants (Malgioglio et al. 2022). In the face of salt stress, PGPM have been found to decrease ethylene-related stress responses (through the production and activity of ACC deaminase), increase compatible solute production, and induce antioxidant enzyme production in their plant associates (Gamalero and Glick 2022). Our bacteria were known to produce ACC deaminase. An interesting next step could be measuring the effects of PGPM on plant concentrations of ROS-scavenging enzymes, as per Mondal et al (Mondal et al. 2023)The improvement of plants' salinity tolerance was a target for this research, to identify a method that would enable the use of more marginal water in hydroponic solution. These PGPM do not seem to be the answer to this problem.

4.3.1 Does the salt kill the PGPB?

One of two things seems to be happening when inoculated plants are salt stressed: either the bacteria die, or they remain present but do not help the plant. It is not clear which is the case. An independent persistence trial was considered but not carried out, in any case it is not clear whether the bacteria not being able to survive alone would necessarily mean that it could not survive within the sheltering confines of the plant and provide support from there.

There have been approaches to identify PGPB after implementing a screening process mimicking the stress environment that they hope the bacteria will be able to ameliorate e.g. (Castiglione et al. 2021). In a discussion with Prof. Dr. Castiglione, he expressed the opinion that it was not surprising that bacteria that were not known to be salt tolerant themselves might not endow their hosts with salt tolerance (Castiglione, personal discussion, September 2023).

Pseudomonas bacteria are known to colonize interior plant tissues (Germaine et al. 2009). *Bacillus* is well known establish endophytic relationships within plants' tissues. Both bacterial species are known to find their ways into plant tissues, where the salt would presumably be less dangerous (Hardoim et al. 2015; Hardoim, van Overbeek, and van Elsas 2008; Kandel, Joubert, and Doty 2017). If the bacteria were able to shelter within the plant tissues, they may have survived the salinization of the nutrient solution. In that case, it is not clear why they were not able to affect the plants growth in a noticeable way. A couple of individual tissue measurements showed significant differences between Bs and Cs plants, for example leaf 5 dry mass was significantly higher in the *Bacillus*-dose salt-treated plants (Table 3).

The experiment approach of having inoculation and treatment in the same volume of nutrient solution does not allow for the selection of endophytic relationships. Weinland et al., in previous , work with the *B. megaterium* strain, inoculated the subject rice plants in one volume of nutrient solution before transferring them to another for treatment (Weinand, Asch, and Asch 2023). This step functioned as a low-rigor selection for endophytic colonization. Without the means to screen plant tissue samples for bacterial genetic material, we cannot say whether, or where, the bacteria might have persisted through the treatment.

4.4 Glucose

In experiment 3, we examined the effects of adding glucose to the nutrient solution. The proximate motivation for this investigation was the hope of changing the behavior of *Trichoderma*, to get effects closer to those reported in the literature. This approach was informed by a discussion in the hobbyist hydroponics sphere. The addition of available carbohydrates to hydroponic nutrient solution is the subject of YouTube videos and web articles with large audiences (Plant Medicine 2020; Woodstream n.d.; Garden Talk with Mr. Grow It 2020). Growers who are aware, at least in a general sense, of the benefits of PGPM, will sometimes include molasses (suggested rates between 5 and 15 ml per 4 liters of nutrient solution) dissolved in the nutrient solution, as 'food' for the bacterial

community and for the plants. This has been taken up to some degree in the academic sphere (Darmawan et al. 2020)(Li et al. 2020). Dr. Julia Karadjov's 2013 white paper seeks to bridge the gap between hobbyist theories and the scientific evidence. In it she suggests that Carbohydrates, or a mix of different carbohydrate forms, may improve plant growth outcomes in hydroponics (though she warns against molasses, as it may gum up the system)(Karadjov 2013). Plants do have receptors for carbohydrates in their roots, though the mechanisms involved in the uptake and utilization are not clear (Xia and Saglio 1988).

This study cannot shine any light on the mechanisms involved, but the findings were quite clear in terms of observed plant growth outcomes. All the glucose-treated plants in experiment 3 grew very badly. The glucose regime in experiment 3 was within the range of the hobbyists' molasses dosage of 1 teaspoon (5ml) - 1 tablespoon (15ml) per gallon, and it also within the range of the commercial carbohydrate additive product Bud Candy®'s prescribed additive concentration (Garden Talk with Mr. Grow It 2020; "Bud Candy | Carbohydrate Enhancer" n.d.).

We reproduced minimal media conditions in the nutrients solution, wherein the bacteria had available everything that they needed for growth. This radically changed their behavior, with respect to the plants and to the setup environment in general. *Pseudomonas* killed the plants as soon as the glucose was dosed out (within 24 hours they were all dead and brown) and stained the solution a bright orange (Figure 17). *Pseudomonas* spp. have been known to opportunistically attack their host plants (Xia and Saglio 1988). The *Bacillus* and *Trichoderma* clearly changed their growth patterns as well (Figure 16). The *Pseudomonas* strain used in these experiments is known to be closely related to the opportunistically pathogenic strain *P. corrugate* (Gislason & Kievit, 2020). The addition of glucose clearly triggered the activation of pathogenic traits, although it is not clear how or why.

4.5 Trichoderma

One of the major questions that persisted throughout the series of experiments was the effects of *Trichoderma* on the plants. The strain of *Trichoderma harzianum* (DSM 32006) used is the active ingredient in the commercial product MobiSol prepared by Soucon Padena. The literature on the effects of *Trichoderma* on horticultural plant growth, e.g. López- Bucio et al. 2015, suggests that *Trichoderma* is nearly universally beneficial to plants. The benefits of *Trichoderma* infection, as salinity tolerance, biocontrol, growth enhancement, and many other positive outcomes is well documented in the literature (e.g. Mastouri, Björkman, and Harman 2010; Zhang et al. 2013; López-

Bucio, Pelagio-Flores, and Herrera-Estrella 2015; Ahmad et al. 2015; Cano 2011). But *Trichoderma* was observed to be detrimental in our experiments where it was a treatment.

The first hypothesis was that the *Trichoderma* were perhaps overcrowded in the nutrient solution. In experiment 2, a set of treatments were drawn up that decreased the inoculation concentration logarithmically: 'High' was 4E5 cfu/mL, 'Medium' was 4E4 cfu/ml, 'Low' was 4E3 cfu/ml. This progression (as opposed to a linear decrease) was chosen due to the exponential nature of many biological properties and processes; ten-fold dilutions of inoculant were thought more likely to lead to differentiable results than linear decreases – the differences seemed to be on the level of orders of magnitude. Experiment 2's investigation showed that this decision may have been off base. The growth outcomes under the low and medium regimes were indistinguishable from the control.

The dose chosen for the first experiment (4E5 cfu/mL) is in the same order of magnitude as the 10^5 *Trichoderma* cfu/mL preparation used by Yedida et al. in 2001 and Brotman et al. in 2013 in their investigation of the effect of *Trichoderma* on the salinity tolerance and responses of cucumber and Arabidopsis, respectively (Yedidia et al. 2001; Brotman et al. 2013). Both these papers reported positive effects.

The second hypothesis was that the *Trichoderma* may have been hungry, lacking any energy source in the nutrient solutions except for the plant tissue. Experiment 3 was an effort to change the behavior of the *Trichoderma* by providing it with a source of carbohydrates. This had no clear effect on the *Trichoderma* – Tg performed as worse than T, and as badly as Bg and Cg – and had a clear pathologizing effect on *Pseudomonas* (see above).

Conclusion

The aim of this study was to evaluate the effects of the chosen *Bacillus*, *Pseudomonas*, and *Trichoderma* strains on hydroponic crop growth, yield, and salinity tolerance. And, in doing so to compare the performance of different types of PGPM in hydroponics and to identify the optimal conditions for using PGPM in hydroponic systems. The study found that the subject *Bacillus* and *Pseudomonas* strains can effectively and significantly benefit hydroponic tomato plants, but that these microbes did not benefit the plants' salt tolerance.

The results of this study found inoculation by the subject *Trichoderma* strain to be uniformly detrimental to plant growth outcome. This is very different from the expected results, given the positive impacts of a wide variety of *Trichoderma* strains across a wide variety of crop plants and test conditions reported in the literature. Further research is required to clarify the characteristic of the testing environment that led to this very different response from the fungal inoculant.

One of the aims of this experiment was the design of a set of streamlined, low-input testing practices to explore the potential of other PGPM varieties. The final design – small-volume deep-pool hydroponic systems, with young plants treated individually – was found to be the most efficient and powerful setup available for research with limited resources.

This research did identify a novel application of plant-growth-promoting bacteria improving the growth of inoculated plants, compared to control plants grown with the same inputs. Insofar as this improvement is reflected in the yield, this approach could benefit the sustainability and efficiency of this style of production. The study fell short of its aim to find a means of improving plant salt tolerance. This might have enabled the expansion of hydroponic practice to include more marginal water sources, potentially widening the range of conditions in which it could be practiced, improving food security and reducing the environmental impact of production.

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Appendix

Nutrient solution recipe

Partial Solution	Macro nutrients		(mg)	Element
A	Di - Ammonium hydrogen phosphate	(NH4)2H2PO4	4.5	NH4-N
			4.9	P
B	Calcium nitrate Tetrahydrat	Ca (NO3)2 x 4 H_2O	83.2	Ca
			58.1	NO3-N
C	Potassium nitrate	K NO3	104.8	K
			37.5	NO3-N
D	Potassium sulfate	K_2 SO_4	26.9	K
			11.0	S
E	Potassium dihydrogenphosphat	KH2 PO4	25.9	K
			20.5	P
	Magnesium chloride hexahydrate	MgCl2 6H2O	0.0	Mg
			0.0	Cl
F	Magnesium sulfate heptahydrate	Mg SO_4 x 7H_2O	16.8	Mg
			22.1	S
	Calcium chloride dihydrate	CaCl2 2H2O	5.9	Ca
			5.2	Cl
	Potassium chlorid	KCL	5.2	K
			4.8	Cl
	Micro nutrients			
G	Ethylendiamin tetraacidic acid ferrous sodiumsalt trihydrate	$C_{10}H_{12}FeN_2NaO_8$	0.8	Fe
G	Manganese sulfate Monohydrate	Mn SO_4 H2O	0.6	Mn
G	Zinc sulfate 7 hydrate	Zn SO_4 x 7H2O	0.3	Zn
G	Copper sulfate pentahydrate	Cu SO_4 x5H2O	0.06	Cu
G			0.03	S
G	Ammonium heptamolypdate tetra hydrate	(NH4)6Mo7O24 · 4 H2O	0.05	Mo
			0.006	NH4-N
G	Boron acid	H_3 BO_4	0.5	Bo

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