Drew University

College of Liberal Arts

The Effects of *Rhizobia* Bacteria on Soil Nutrient Levels and Plant Productivity in *Pisum sativum* (Dwarf Snap Peas) and *Trifolium repens* (White Clover)

A Thesis of Environmental Science

By

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ABSTRACT

As the world is shifting towards green agriculture, the need for research in sustainable farming practices is increasing. One of the many important aspects to sustainable agriculture is the use of biofertilizers as an alternative to synthetic fertilizers. In order to understand the effectiveness of biofertilizers, I wanted to investigate the difference that the biofertilizer *rhizobium* has on two different legume species. The purpose of this study was to assess the effect of *Bradyrhizobium vigna* and *Rhizobium leguminosarum* strains viciae, phaseoli (a mixture of *rhizobia* species and strains) on plant productivity when applied to *Pisum sativum* (dwarf sugar snap peas) and *Trifolium repens* (white clover). This project was performed as a potted greenhouse experiment using soil from the organic urban farm Grow It Green Morristown and sourcing water from rain barrels to simulate a replicable environment utilizing sustainable practices. I analyzed the data for differences in plant height, root length, biomass, nodule formation, and total nitrogen content, as well as the nitrate-nitrogen content in the soil after the experiment. Many limitations and challenges arose throughout the experiment that may have influenced the data, which showed no significant differences in any of my parameters between treated and not treated groups. These results highlight the incredibly complex relationships that *rhizobia* bacteria hold with their symbiotic partners as well as with the environment. This research aims to expand our understanding of biofertilizer dynamics so that effective and proper implementation of them can be achievable in the greater agricultural landscape.

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INTRODUCTION

Green Revolution

Taking place between the late 1940's and 1980's, the Green Revolution began in Mexico as an effort to combat global malnutrition by increasing crop yield through science and technology (Rosset, 2000). With the backdrop of WWII, much of the world was suffering from widespread hunger and food shortages. In order to boost food production, governments invested in agricultural research that focused on producing high yielding varieties (HYVs) (Cabral et al., 2021). HYVs are plant varieties which respond highly to large inputs of chemical fertilizers and to heavy irrigation (Cabral et al., 2021). In the 1940s this research began with "indigenous and colonial programs for crop improvement," genetically modifying raw material, non-food crops for high yields that could be sold to industry for profit (Gollin et al., 2018). By the start of WWII, mass famine overtook developing countries in the Indo-Asia region, forcing the switch to technological advancements for an agricultural boost (Eliazer Nelson et al., 2019). At first, methods were to select HYVs from the fields of farmers, but later with the improvements of food crops (mostly cereals such as rice, wheat and sorghum) large-scale cross-fertilization programs were enacted (Gollin et al., 2018). Cross fertilization is an event where the male and female gametes (sex cells) of two different individuals are combined to create a new generation of plants (Britannica, 2024). This process creates genetic variation in the new generation, using the genes from the parent generation. Cross fertilization, already occurring in natural plant reproduction, was harnessed during this time to artificially select for favorable traits, consequently modifying the evolution of a species. Using this adaptive breeding technique, the Green Revolution was now able to select for larger, higher producing, and faster growing plant varieties that thrive in many different agro-ecological niches. The success of HYVs was so astounding that by the 1990s there was an estimation made claiming

that Green Revolution seeds were being used by approximately 40% of all farmers in the Global South (Rosset, 2000). Green Revolution practices were mostly taken up by regions existing on similar latitudinal planes with Asia adopting the highest use of HYVs and Latin America following closely behind (Rosset, 2000). One of the key characteristics of HYVs, however, that contributed to their success was their high responsiveness to concentrated synthetic fertilizers.

Because HYVs are highly responsive to synthetic fertilizers, the use of such applications also popularized during and after the Green Revolution (Eliazer Nelson et al., 2019). By the mid-1800s the scientist Justus von Liebig had published his "Theory of Mineral Nutrients," which recognized that the elements of nitrogen (N), potassium (K), and phosphorus (P) were all nutrients essential to the growth and success of most plants (University of Nebraska - Lincoln, 2015). This theory became the groundwork for agricultural chemistry and led to the understanding that unless replenished, these NPK nutrients could be depleted in a soil over time. Because of this, chemists Fritz Haber and Carol Bosch developed a way to convert atmospheric nitrogen into a form available to plants through what is now known as the Haber-Bosch Method (University of Nebraska - Lincoln, 2015). This process was necessary in the progression towards modern fertilizers because it takes atmospheric nitrogen (N2), which has a strong triple bond that plant struggle to break, and converts it into ammonia (NH_3), which no longer has such bond and can be easily utilized by plants (Department of Crop and Soil Sciences, 2005). From this, a series of innovations in the early 1900s by many scientists lead to the production of easy-applied synthetic fertilizers (University of Nebraska Lincoln, 2015). These synthetic fertilizers provide the soil with the three essential nutrients (NPK) and quickly became commercialized in the mid-1900s as a part of the global fight against famine (University of Nebraska - Lincoln, 2015). Because of their

success in the Green Revolution, the use of synthetic fertilizers has not since decreased (Figure 1)

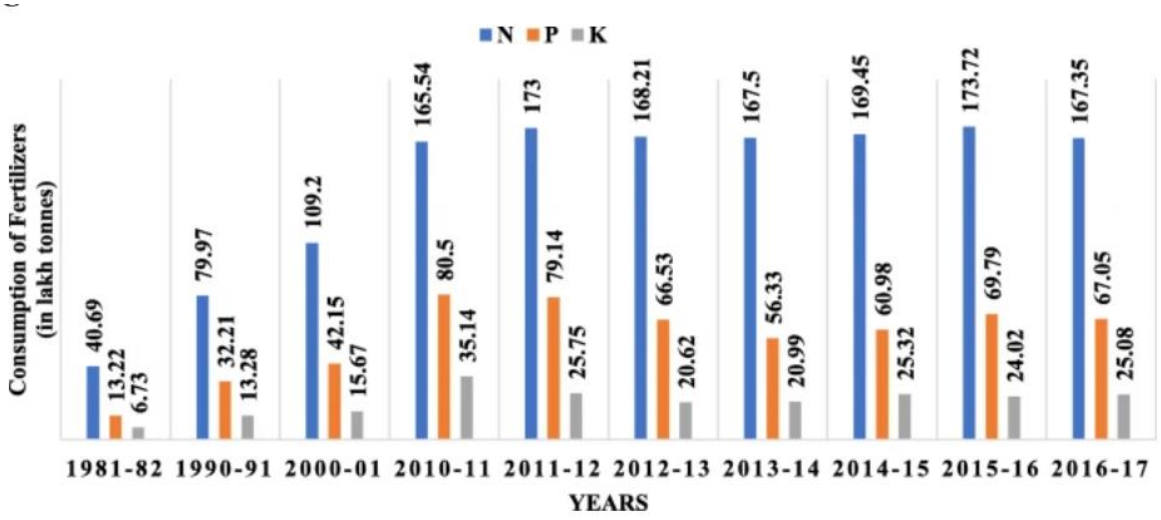

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Figure 1. Representation of the Annual Growth of NPK Fertilizer Consumption from 1981-2017 in India. Figure sourced from "The Impact of the Green Revolution on Indigenous Crops of India," by Eliazer Nelson et al. (2019).

Along with HYVs and synthetic fertilizers, chemical pesticides became popularized as well during the Green Revolution (Eliazer Nelson et al., 2015). Chemical pesticides are used to remove pests projected to be in competition with or a threat to crops, including organisms such as weeds, insects, fungi, bacteria, and rodents (National Research Council (US) Committee, 1993). By killing these pests, chemical fertilizers have protected crops and lead to dramatic yield increases for most major fruit and vegetable crops (National Research Council (US) Committee, 1993). An estimated loss of about 54% vegetable production, 78% of fruit production, and 32% of cereal production, would be suffered weeds, insects and infectious diseases without the use of pesticides (Tudi et al., 2021). During the Green Revolution, pesticide use in the U.S more than tripled, favoring herbicide use over fungicide and insecticide use (Fernandez-Cornejo et al., 2014). The success of pesticides

has meant that the use of such chemicals has not decreased since the Green Revolution (Figure 2).

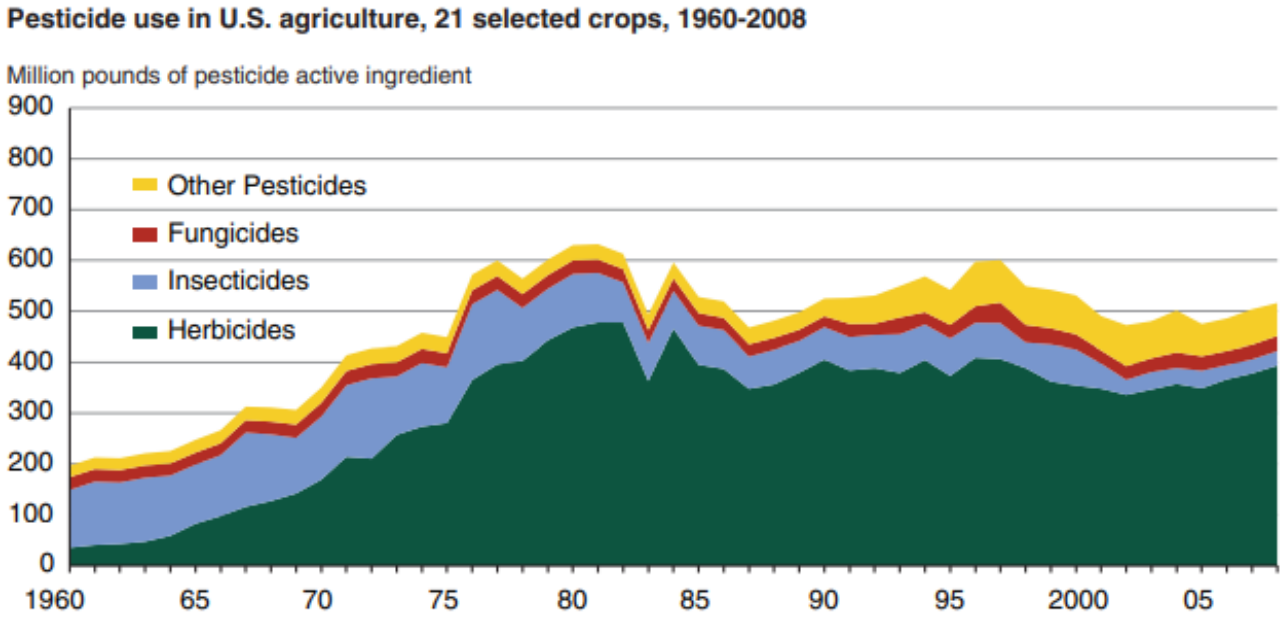

Figure 2. U.S. pesticide use by type between 1960 and 2008. Figure sourced from "Pesticide Use in U.S. Agriculture: 21 Selected Crops, 1960-2008," by Fernandez-Cornejo et al. (2014).

This success coupled with those of HYVs and synthetic fertilizers lead to increases of the highest yielding of the HYVs and consequently caused cultural food shifts in some parts of the world (Eliazer Nelson et al., 2015). In India in particular, crops such as rice, wheat, and coarse cereals have overtaken other indigenous crops in production (Figure 3). The differences between crops in both agricultural yield and shifting public tastes subsequently altered cultural diets in the area accordingly. Traditionally Indian cuisine consisted heavily of millets, but in the decades following the Green Revolution (1960-2017) due to production changes made during the movement, the World Health Organization (WHO) reported a decrease in the production of millets and an increase in the production of rice in India (John and Babu, 2021). Because of this rice became the new staple food in India (John and Babu, 2021).

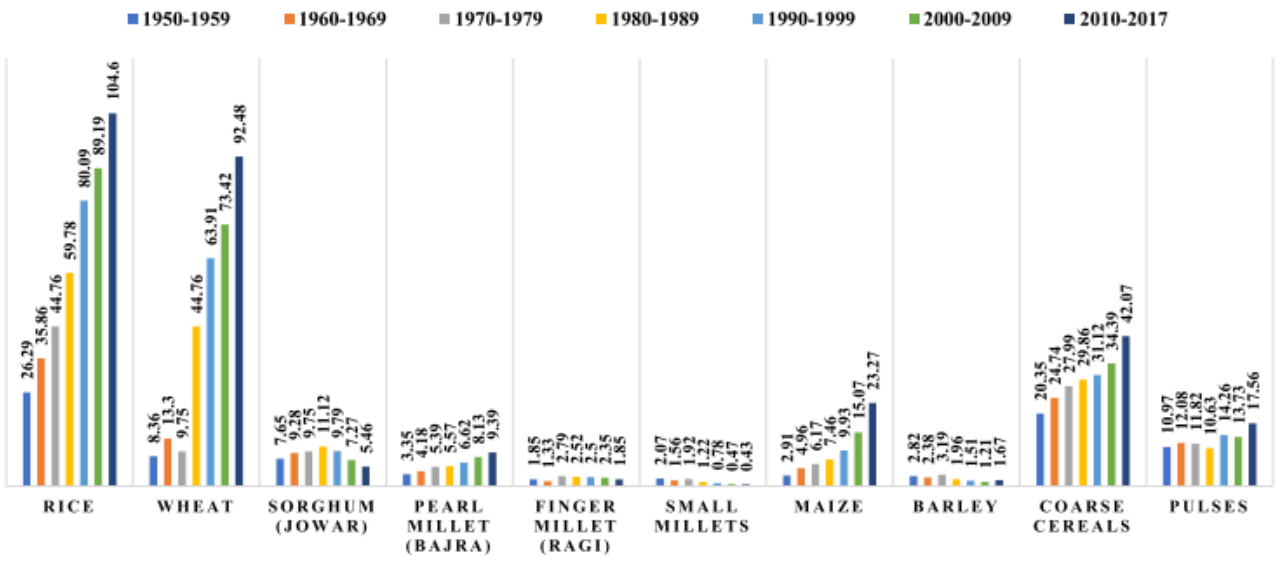

Figure 3. Food production of selected crops in India from 1950 to 2017 in millions of tons. Figure sourced from "The Impact of the Green Revolution on Indigenous Crops of India," by Eliazer Nelson et al. (2019).

Although they found their success during the Green Revolution, the applications of synthetic fertilizers have since proven to pose many environmental and human health risks (John and Babu, 2021). When it comes to synthetic fertilizers, high blanket applications of them mean that crops are not able to fully utilize the application and excess nutrients have the ability to leach/runoff into other systems (Tudi et al., 2021). The nutrient accumulation that occurs as a result of this often causes eutrophication in vulnerable water bodies. Eutrophication is the process where a water body becomes enriched in nutrients and causes harmful algal blooms, which deprive the entire system of oxygen and subsequently kill other organisms living within it: fish, aquatic plants, and other aquatic organisms (US Department of Commerce, 2019). When nutrients runoff from agricultural spaces they destroy ecosystems and even community spaces in it's path, leaching nutrients into species breeding grounds, recreational spaces, and water sources (Lazewski 2024). One of the main concerns about nutrient accumulation in water sources is the risk of health issues such as "blue baby syndrome," which is when high nitrate/nitrite levels in drinking water reduces

the oxygen carrying capacity of a person's blood causing young babies to fall sick and sometimes die (Lazewski, 2024). In addition to this, synthetic fertilizers also deplete soil health due to their lack of organic materials, where plants typically find their essential nutrients. Organic matter in a soil is incredibly important because it supports many soil properties, such as soil structure, pH, bulk density, and water retention/filtration (Lazewski, 2024). For plants, surviving off of synthetic fertilizers would be much like a person trying to survive solely off of vitamins, the essential macronutrients might be there, but other micronutrients and fibers are missing.

It was not only synthetic fertilizers that posed environmental and human health risks, chemical pesticides too have been found as problematic (John and Babu, 2021). Pesticides are often sprayed over crop fields, causing some to remain airborne and travel to areas not originally intended to be affected, but they can also leach/runoff into surrounding areas much like synthetic fertilizers do (Tudi et al., 2021). Because of this, pesticides have the potential to affect every living being on the surface of the Earth if used heavily and for long enough. Pesticides finding their way into every aspect of the environment is problematic because they are known to cause many health issues in humans (cancers, fetal anomalies, and more) as well as in other species (thinning eggshells, altered sex ratios, and decreasing prey to predator ratios) (Tudi et al., 2021).Synthetic pesticides often are indiscriminate and remove all organisms from the soil and surrounding spaces (aerial and aquatic) regardless of whether they pose threat or harm to the crops (Bahlai et al., 2010). Because of this indiscriminate nature of synthetic pesticides, the communities of many beneficial organisms, such as invertebrates and soil microbes, are compromised and depleted. These organisms are important to ecosystem health not only because they are essential to the foundations of many food webs, but also because they perform functions such as pollination, decomposition, and nutrient release, which are crucial ecosystem services (Morley et al., 2014). Therefore, an

alteration in their communities can consequently affect ecosystem factors and functions; harm to beneficial organisms could decrease fertilized plants, decomposition rates, nutrient release rates, and could even disrupt the structures of taxa further up the food chain (Morley et al., 2014).

Sustainable Agriculture

After the Green Revolution transformed agricultural practices globally, room was made at the scientific table for the more environmentally conscious approach that is sustainable farming. When discussing sustainable farming and agriculture it is important to understand what exactly that means. This can be a difficult concept to lay out because the term "sustainable agriculture" has been defined throughout the scientific community in so many ways that some explanations have become borderline contradictory. Today, sustainable agriculture is commonly referred to as farming in a way that protects the environment by minimizing the use of nonrenewable resources and expands natural resources by minimizing harmful wastes (National Agricultural Library, 2024). Less simplified definitions, however, describe sustainable agriculture more complexly. One of which defines it as alternative farming practices which minimize and/or eliminate harms to the environment by integrating biological, physical, chemical, and ecological principles (Tahat et al., 2020). Following these principles brought about the uses of cover cropping, companion planting, compost/ natural fertilizers, soil sciences, and more. To explain the phase compoundly and for the purposes of this material, the goal of sustainable food production is to build renewable food resources without depleting or destroying our environment. It is notable for using environmentally neutral or beneficial practices that do not exploit nutrients, or damage native species and ecosystems. Sustainable practices foster a healthy soil biome and cater to the needs of specific crops.

Cover Crops

Cover cropping is a method of sustainable farming that provides various benefits to the soil and the crops by intentionally growing (generally legume or non-legume grass) plants between main crop spaces (Gliessman, 2015). Cover crops are able to control erosion, improve water use efficiency and aeration, add to soil nutrients and prevent sun damage (USDA 2019). Much of these benefits are due to the root systems of these plants. The depth and range of roots can impact soil erosion by locking soil constituents together and preventing erosion both from wind and water runoff (Adetunji et al., 2020). The roots of the plants also are able to increase water filtration and soil aeration by burrowing into the soil and creating root channels (Gliessman, 2015). These root channels created by cover crops affect the soil's hydraulic properties by allowing water to more easily infiltrate the soil and reach the main crop(s) (Adetunji et al., 2020). To assist in soil aeration the root channels provide air space for soil biota to thrive, both macro- and microorganisms (Adetunji et al., 2020). Microbial populations can use these places as residences whereas larger fauna may take advantage of easier travel provided by the increased soil air content. Cover crops are able to add to soil nutrients and protect the soil from excessive sun exposure when they are uprooted and left on top of the soil (Adetunji et al., 2020). When these plants are left on top of the soil, they act much like mulch, creating a barrier between the sun and the soil, preventing the soil from drying out, and also releasing nutrients to the soil as they begin to decompose (Adetunji et al., 2020). Cover crops are therefore able to increase agricultural productivity because they are able to conserve important soil functions and interactions (Lehmann et al., 2020).

Biofertilizers

Biofertilizers have become a frontrunner in sustainable agriculture because of their ability to harness natural processes to increase productivity. There are many types of biofertilizers; there

is the application of organic matter, such as compost, manure, or cover crops, but there are also microbial biofertilizers, such as bacteria and fungi (Mahanty et al., 2016). Much like how humans have a biome of microorganisms in our digestive tract that help us process food, plants have a biome of microbes by their roots that help them digest nutrients from the soil; this area for plants is known as the rhizosphere (Barbu and Boiu-Sicuia, 2021). These microbes are known as biofertilizers. Biofertilizers are defined in the Romanian Journal for Plant Protection as "products derived from vegetal wastes and animal manures, minerals or microbial inoculants" (Barbu and Boiu-Sicuia, 2021). Microbial biofertilizers are present in many animal manures as well as in composts and are often administered to the soil via such, but they can also be bought in a more purified form by many retailers (Kour et al., 2020). Rather than directly adding nutrients to the soil the way synthetic fertilizers do, biofertilizers replenish the microorganism populations that naturally cycle nutrients on their own (Kour et al., 2020). Additional nutrients added to the soil are from the constituents that these microbes reside in: manure, compost, etc. (Gliessman, 2015). This process does away with the need for excessive synthetic fertilizer use, which as discussed before, is notable for its damage to the soil microbiome and leaching of nutrients. These are not threats when utilizing microbial biofertilizers. The most famous form of biofertilizers are the plant growth-promoting bacteria (PGPB). These are naturally occurring bacteria in the soil that form symbiotic relationships with plants (Mahanty et al., 2016). Symbiosis can be defined as any close and interactive relationship across species. This includes commensalism, parasitism, mutualism, and competition. When discussing biofertilizers however, the symbiotic relationship that takes place most commonly is mutualism, in which both the microbe and the plant benefit from the relationship (Mahanty et al., 2016).

Rhizobia and Legumes

Rhizobia is one of three groups of PGPBs (*rhizobia*, *frankia*, and *cyanobacteria*) that live in a symbiotic relationship with legume plants, increasing its plant productivity (Lindstrom and Mousavi, 2019). It does this by establishing itself in the root systems of the legume plants, forming nodules and fixing otherwise unavailable nitrogen in a process called biological nitrogen fixation (BNF) (Zahran, 1999). This process converts atmospheric nitrogen (N_2) into an ammonia (NH_3), or a nitrate (NO_3^-) form that can be taken up and used by the plants (Zahran, 1999). This is similar to the Haber-Bosh method which breaks the strong triple bond in N_2 and converts it into NH_3 during the production of synthetic fertilizers. However, BNF also makes the product of NO_3^-, which is more stable than NH_3 and is able to accumulate in the soil for later use (Lindstrom and Mousavi, 2019). *Rhizobia* is the generic name used for the now 18 genera group of BNF performing bacteria that have a mutualistic relationship with legumes (Lindstrom and Mousavi, 2019). Legumes are the entire *Leguminosae* family and are important food and forage dicotyledonous plants (peas, beans, or clover) that have dry fruits which develop from a single flower carpel and usually split into two halves with seeds attached to the seam of one half (Merriam-Webster.com, 2024). In the symbiotic relationship that occurs between *rhizobia* and legumes *rhizobia* formulate colonies on the roots (nodules) of their host and are supplied with carbon sources (Lindstrom and Mousavi, 2019). Nodulation occurs when *rhizobia* colonize the roots of legumes, resulting in the formation of bulb structures on the root hairs (Image 1). In return, the legumes receive NH_3 and NO_3^- provided by *rhizobia* (Image 2)(Lindstrom and Mousavi, 2019). The nitrogen fixation that occurs in *rhizobia* for the legumes is mainly attributed to the *nod, nif,* and *fix* genes. Nod genes encode for nod factors, signaling molecules important for the initial stage of nodulation within the root system (Lindstrom and Mousavi 2019).

Image 1. Picture of *rhizobia* nodules on legume roots. Image sourced from https://pixels.com/featured/pink-nodules-of-rhizobium-leguminosarum-drjeremy-burgess.html

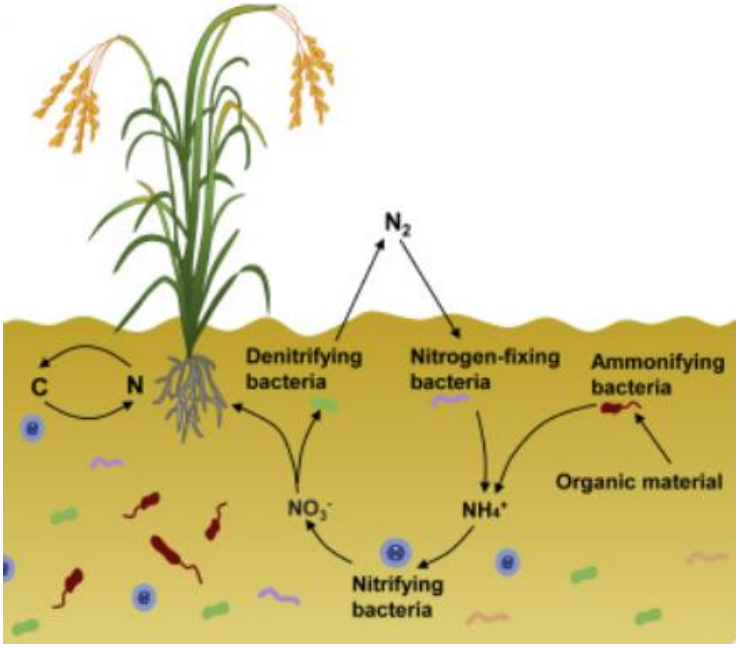

Image 2. Visual representation of the *rhizobia*-legume symbiotic relationship. Image sourced from "Biological nitrogen fixation in cereal crops: Progress, strategies, and perspectives" by Gou et al. (2023).

Objectives of the Study

The aim of my study was to analyze the physical and chemical changes that *rhizobia* bacteria make on inoculated legume plants and how that affects the plants' overall productivity.

Plant productivity is the measure of the success a plant has at growing and producing yields under certain conditions. In the case of my experiment, that measure is a compilation of various parameters testing the effect that *rhizobium* bacteria have on the physical and chemical properties of *Pisum sativum* (dwarf sugar snap peas) and *Trifolium repens* (white clover): biweekly plant heights, plant above and below ground biomass, nodule formation, nitrate-nitrogen content, and above and below ground lengths/heights. My goal is to better understand the impact that inoculating legume seeds has on plant growth in a potted greenhouse setting. I expect to see a higher overall productivity and nitrogen content in the plants receiving *rhizobia* inoculation than in plants not receiving *rhizobia* inoculation (control plants).

When choosing the legume plants that were to be studied, there were a few characteristics I was looking for. I wanted to find plants that had shorter growth periods, so that the experiment could be conducted in a timely manner. I also needed to ensure that the crops I chose would fare well in the climatic conditions of Manahawkin, NJ, where the experiment was run. Legume plants also are extremely diverse, so I wanted to choose two different varieties to better understand an overall effect. The dwarf sugar snap peas (also known as sweet peas) were the ideal candidate for measuring changes within a traditional agricultural crop when adding the *rhizobium* inoculant. This is because the dwarf sugar snap peas reach their adult stage in 8-10 weeks after germination (60-70 days) which can be collected and measured for yield. Dwarf sugar snap peas also have a short gestation period; it takes only 2-3 weeks for the seed to produce visible seedling. The white clover was chosen to measure the effect of *rhizobium* inoculation on a cover crop. White clover also has a short gestation period of four weeks. Both of these crops are suited for the temperate conditions of southern New Jersey.

METHODS AND MATERIALS

To analyze the effects of *rhizobia* bacteria on legume plants, I ran a 10-week potted greenhouse experiment testing soil and plant parameters before and after the 10-week growth period. I collected my soil from Grow It Green Morristown (GIGM) and tested it for starting parameters: bulk density, moisture, texture, pH, and organic matter. The soil was divided equally into 20 2-gallon plastic pots (9" diameter x 8.5" depth). These pots along with the seeds for both plant varieties, the *rhizobia* bacteria, and the compost were all purchased from Amazon.com. On Amazon.com, the pots were bought from the seller Seed Kingdom, the pea seeds were bought from the TomorrowSeeds Store, the clover seeds were bought from the Outsidepride Store, the *rhizobia* bacteria were bought from GARDENTRENDS, and the compost was bought from MSNOR. Ten of the pots were allocated for growing peas and the other 10 were allocated for growing clover. Each pea pot had four pea seeds planted in it to optimize successful germination. After germination in the peas, the largest from each pot was kept and any others that may have germinated were pulled up and removed from the experiment. In half of the pea pots all seeds within were inoculated with *rhizobia*, whereas in the other half of the pots none were inoculated. Each clover pot had a teaspoon's worth of clover seeds planted within it. Because clover is a cover crop, I wanted to simulate how it might naturally grow in abundance. In order to maintain evenness across the experiment however, after germination any clover pots with more than 10 plants within them were thinned down to contain only 10 each. Pots containing less than 10 plants were recorded and tracked. As was with the peas, half of the pots growing clover contained seeds that were inoculated with *rhizobia* whereas the other half contained non-inoculated clover seeds. The *rhizobia* that we inoculated with was a mixture of *Bradyrhizobium vigna* and the *Rhizobium leguminosarum* strains viciae and phaseoli. A ring of compost (~2.4g) was administered to the edges of each pot as

fertilizer to start off the experiment. This compost was not sterilized, but used directly as the manufacturer's (Visojon Biologics) directions suggests; I made this choice to maintain the practicality of the compost use to what a farmer/gardener would do. The pots were placed into a randomized block design with four rows inside a greenhouse. No other plants were grown in this greenhouse for the duration of the experiment. The pots were watered once every 1-3 days depending on the visual dryness of the soil with rainwater collected from the greenhouse's rain barrel system (Image 3). I used rain barrels to collect water for the experiment because I wanted to simulate the practical use of this biofertilizer while in combination with green agriculture practices, which would likely use some sort of water conservation.

Image 3. Image of rain barrel system used for collecting the water for the experiment.

The plants were left to grow for 10 weeks. The heights of the plants were recorded biweekly throughout this time. In the field, the terminated plants were uprooted: each was measured for the above ground height, below ground length, and the number of nodules in the root system. In the

lab, the plants were measured for their above and below ground biomass, and along with the soil they were measured for their nitrogen content.

Soil Collection

The soil that I used for this potted experiment was collected from Grow It Green Morristown (GIGM). GIGM is an organic and sustainable urban farm located in Morristown, NJ. GIGM is dedicated to engaging with the community by providing fresh produce and resourceful knowledge about sustainable and urban farming. At the farm, Grow It Green's organic and sustainable practices can be seen through the wildflowers used to attract pollinators, the chicken waste and harvest scraps composted for fertilizer, and the drip lines used to conserve water. Because of these practices, soil collected from Grow it Green Morristown was ensured to be free from excessive exposure to synthetic fertilizers, often found in industrial farming, and also from excessive pesticides and insecticides, often used to control urban landscaping. The soil was specifically collected from a plot that most recently supported raspberry shrubs, sunchoke plants, and a fig tree. No legumes in recent years have been grown in the section of land I collected my soil from. It is important to note that because GIGM uses natural compost in their fertilizer mixture, remnants from legume plants were likely within the compost. This means that naturally occurring *rhizobia* strains from the farm may have already been within the soil I collected and could have led to symbiotic relationships within my experiment, separate from the added inoculant. I collected my soil across two days: my first collection day was April 10th, 2024 and my second collection day was April 13th, 2024 (Image 4).

a. b. c.

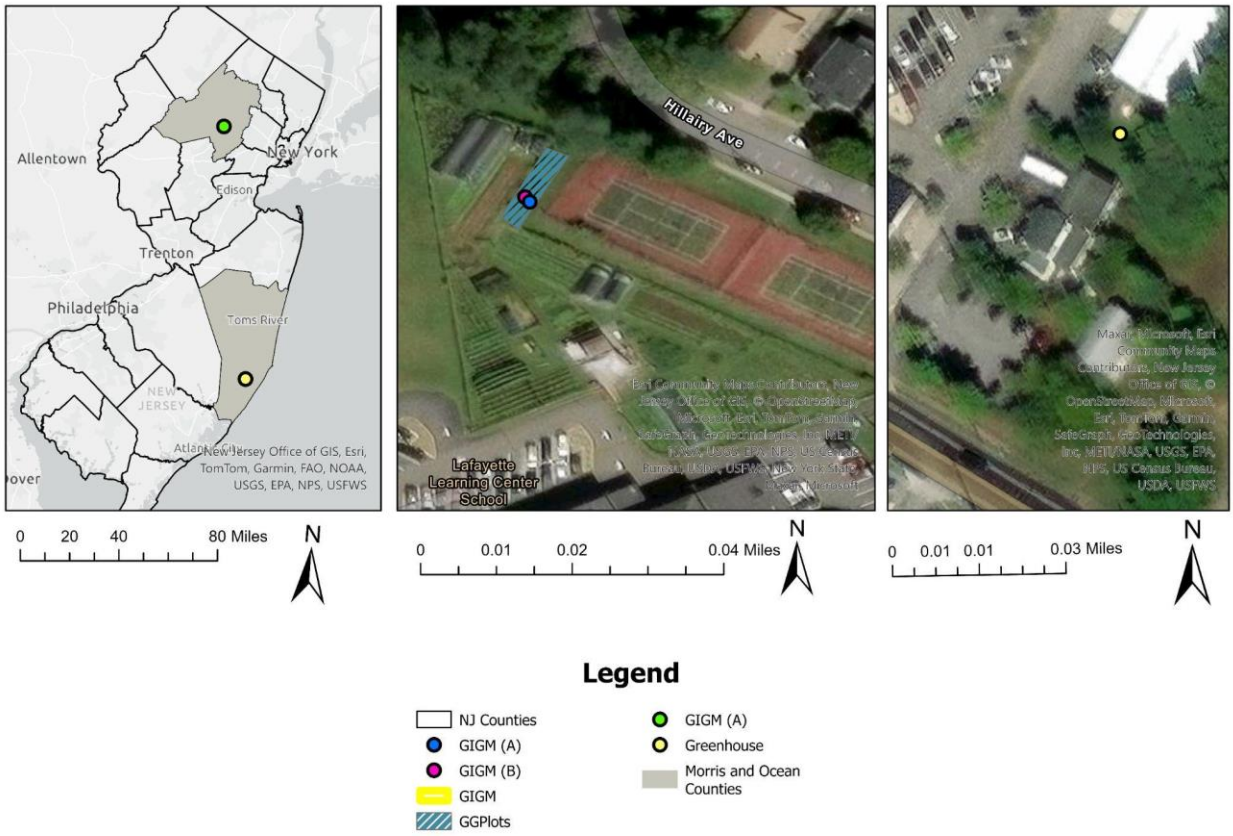

Image 4. Soil collection and experiment locations. These sites resided in two different counties, Morris and Ocean, in New Jersey (a). The soil was collected from two locations in a Grow It Green, Morristown plot (b). The greenhouse where the experiment was run was located in Manahawkin, NJ (c). Maps generated using ArcGIS Pro.

We collected the soil into plastic, unscented garbage bags to retain the moisture and contents of the soil during transport and testing. I gathered 22 gallons worth of soil for the 20 gallons needed in the experiment and an additional two for testing and error, using the two-gallon planter pots to measure. Considering both the heterogeneity of soil and the factor of having two separate collection points over two days it was important to ensure my starting soil for the experiment was uniform. In order to guarantee a consistent starting soil for all of my treatments, I

hand mixed my collected soil in a large container before distributing among pots (Images 5 and 6).

Image 5. Image of hand mixing collected soil in a large container.

Image 6. Image of evenly distributed soil into labeled pots.

Inoculation

In order to properly expose the treated seeds to *rhizobia,* I inoculated the seeds before planting them. Inoculation is the act of introducing a pathogen (in this case the *rhizobia* microbe) into another living organism (my selected legume species) (Meriam-Webster.com, 2024). For my experiment this process meant coating the seeds in the *rhizobia* inoculant at the appropriate ratio (Image 7). The manufacturer's instructions on the *rhizobia* inoculant package called for 10g of inoculant for every 133.4g of seed and just enough water to dampen the mixture and allow the *rhizobia* to cling to the seeds.

Image 7. Image of seed treatments. Treatments arranged from left to right include: inoculated peas, non-inoculated peas, non-inoculated clover, and inoculated clover.

Experimental Design

The experiment ran with four treatments, arranged in a randomized block design within a backyard greenhouse in Manahawkin, NJ. The four treatment groups I had included one control group and one group inoculated with *rhizobia*, for both species. Each treatment was repeated five times to increase sample size and reduce false-positive/false-negative risks. I coded each treatment

as T0-T3 (Table 1), and each repetition as R1-R5, with R1 being the first repetition and R5 being the fifth.

Table 1. Denotation for each treatment

	Treatment
T0	Control peas
T1	Control clover
T2	Peas inoculated with *rhizobia*
T3	Clover inoculated with *rhizobia*

We used a randomized block design to reduce bias and increase variability. Assorting the plants into organized rows according to treatment type would have left them vulnerable to variations in shade, sun exposure, drafts, pest exposure, etc. The randomized block (Tabel 2) was generated in an Excel spreadsheet using the number randomization feature.

Table 2. Completely Randomized Block Design generated in Excel

Block 1	Block 2	Block 3	Block 4	Block 5
T1R1	T0R2	T1R3	T0R4	T3R5
T2R1	T3R2	T3R3	T2R4	T0R5
T3R1	T1R2	T0R3	T1R4	T2R5
T0R1	T2R2	T2R3	T3R4	T1R5

I decided on running the experiment in a greenhouse to remove some weather factors and limit contact with pests during the experiment. Weather factors such as precipitation and high winds can be controlled by the greenhouse. Because of this I was able to ensure consistent and

even application of water to each pot throughout the experiment. However, weather conditions such as high humidity and high heat are not easily controlled via a greenhouse and may have affected the experiment. The location of the greenhouse was in Manahawkin, NJ (Image 4) because the timing of the experiment took place during a post semester period, during summer months. This location minimized commute times and allowed for more diligent monitoring from myself and from enthusiastic family members. The greenhouse was positioned so that the door was facing south (Image 4).

Field and Lab Studies Conducted

The parameters of my study included physical and chemical properties of both the soil, before and after the experiment, and the plants after termination (July 11th, 2024). Before the experiment I tested the soil for its starting bulk density, organic matter, moisture, pH, and texture. I did this to determine the state of the soil and ensure its capability to support my plants. After the experiment, the soil was tested for its available nitrogen (nitrate nitrogen) content. This would allow us to compare how efficiently plants with *rhizobia* took up available nitrogen compared to the control groups. Throughout the duration of the experiment the plant heights (cm) were recorded. At the termination of the experiment both the above ground height (cm) and the below ground length (cm) were recorded for each plant, as well as the number of nodules found in the root systems. Other parameters tested the plants for oven dried mass (g), and total nitrogen content.

(1) Soil Parameters

(1.1) Bulk Density

Bulk density (D_b) is a ratio measure of the soil's mass to its volume (g/cm^3) and can give us insight about porosity and compaction. These are factors that can affect plant root penetration, aeration, and water filtration. To measure this, I collected soil from the collection site at GIGM

into three metal cores (for repetition) all with a volume of 104.01cm^3 (Radius = 2.5cm, height = 5.3cm). The soil was transferred into separate metal cups so that they could be oven dried (Image 8). Oven drying the soil before measuring the mass eliminates the mass due to moisture in the soil, which is variable with weather/watering. The oven dried soil (ODS) was measured for its mass and divided by the volume of the metal cores used for collection (104.01cm^3).

$$D_b\ \% = \frac{oven\ dried\ soil\ (g)}{volume\ (cm^3)} \times 100$$

Image 8. Picture of the oven dried soil in metal cups.

(1.2) Soil Moisture

The soil moisture (SM) content is a measure of water held within the soil. This is usually portrayed as a percentage with the healthy SM range being between 21 and 80% water. I tested this by placing a set amount (5g) of mixed soil into five cups (for repetition) to be oven dried at a low temperature (105^{o}C) for 24 hours (Image 9). The purpose of the low temperature was to evaporate out the water without burning out any soil organic matter (SOM). The cups were each measured for mass before and after oven drying (subtracting the mass of the cup each time). The equation below was used to calculate the SM.

$$SM\ \% = \frac{oven\ dried\ soil\ (g) - wet\ soil\ (g)}{oven\ dried\ soil\ (g)} \times 100$$

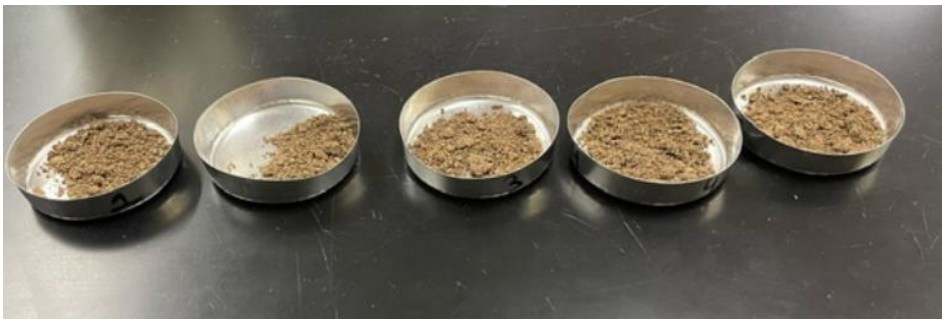

Image 9. Picture of oven dried soil for soil moisture experiment.

(1.3) Texture

The texture of a soil tells us the composition of the soil in terms of sand, silt and clay. Depending on the fractions for each particle size the overall texture can be classified as loam, silt, clay, sand, or an intermediate between these (Image 10).

Image 10. Soil Texture Chart (Groendendyk, 2015).

To test the texture of my soil, I followed the hydrometer method using an ATSM15H soil hydrometer from Fisher. This method consisted of uniformly suspending 40g of my soil into a 1000mL graduated cylinder containing a 9:1 ratio mixture of tap water and sodium

hexametaphosphate (dispersing solution). The hydrometer was carefully placed into the cylinder so that it was free-floating in the mixture and not touching the edges of the cylinder (Image 11). The scale on the hydrometer was read at the meniscus both 40 seconds after it was placed into the mixture and two hours after it was placed into it. The reading at 40 seconds indicated the sand content and the reading at two hours indicated the clay content. These values were recorded and used to calculate the amount of sand, silt, and clay my soil contained to determine the texture.

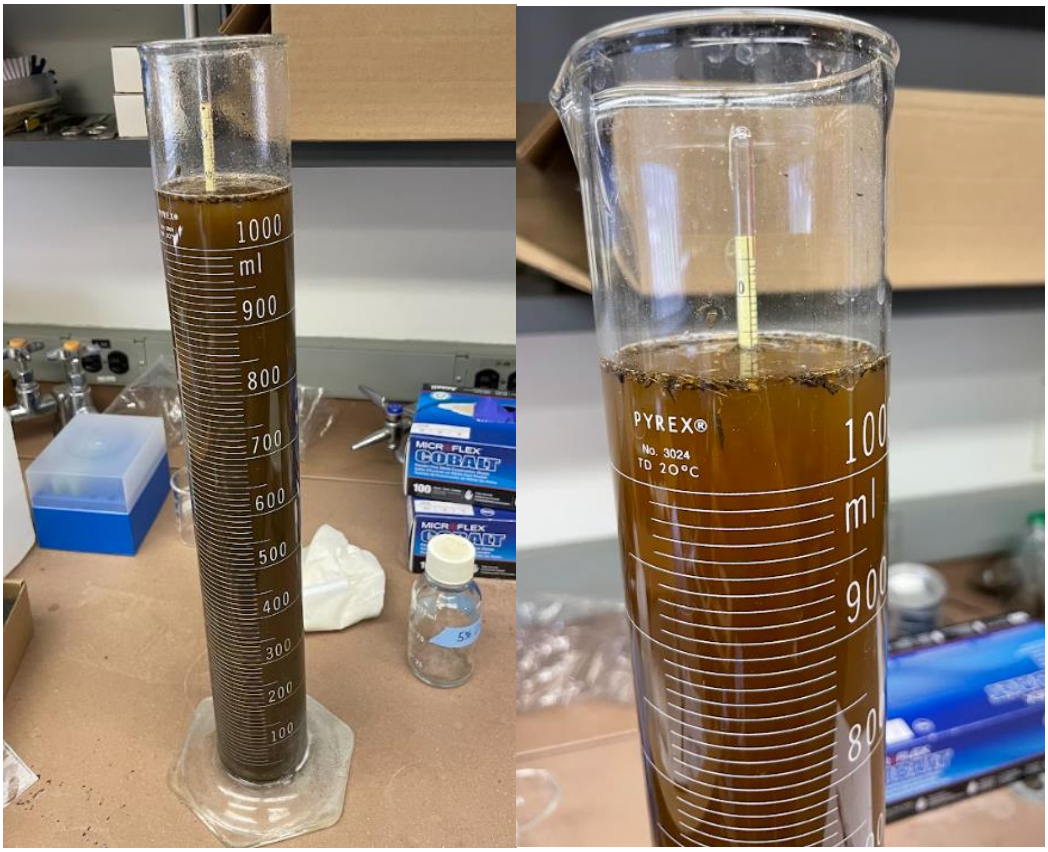

Image 11. Pictures of soil texture hydrometer method. The left image shows the state of the experiment after 40 seconds and the right image shows it after two hours.

(1.4) pH

The pH of a soil is a measure of its acidity or alkalinity. Soil pH determines how nutrients and minerals dissolve into the soil. These properties of the soil also determine how capable it is to meet the chemical and nutritional needs of an organism. The healthy range for pH in a productive

soil is between 5.5 and 7.5 SU. Legume plants specifically thrive in a slightly alkaline environment where the pH is between 6.2 and 6.8 SU. To calculate the pH for my soil I used a Vernier laboratory pH meter (Image 12).

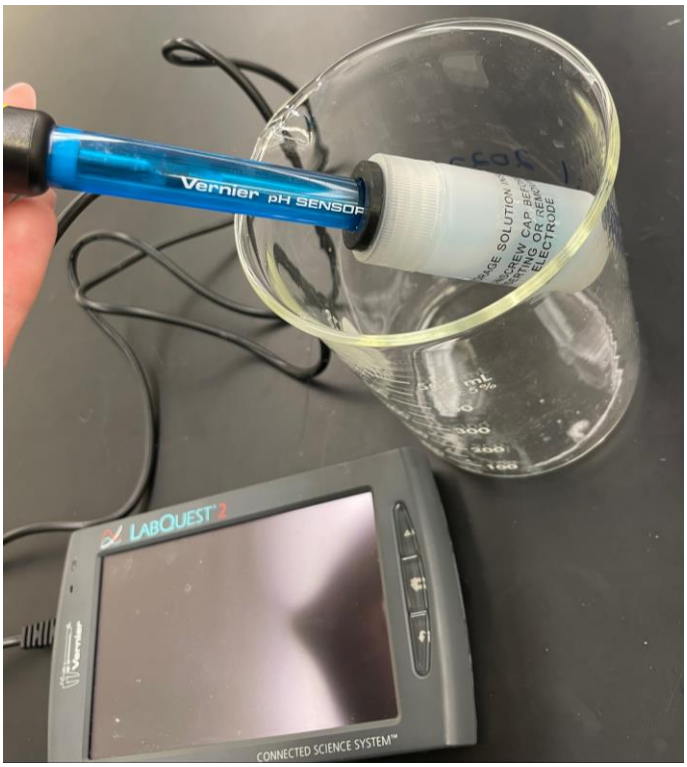

Image 12. Picture of the Vernier pH sensor used to determine soil pH.

(1.5) Soil Organic Matter

The SOM content is a measure of the fraction of mass attributed to organisms and organismal materials within a soil. This is usually portrayed as a percentage with a healthy range being between three and six percent. To calculate the SOM for my soil I placed an even amount (0.5g) of mixed soil (soil mixed from both collection points) into five cups (5 repetitions) to be oven dried at a high temperature (550^{o} C) for four hours (Image 13). The purpose of the high temperature was to burn off any and all organic matter within the soil to isolate the inorganic materials. These cups were each measured for their mass before and after oven drying (subtracting

the mass of the empty cup in both cases). The equation below was used to calculate the SOM from these masses.

$$SOM\ \% = \frac{wet\ soil(g) - oven\ dried\ soil\ (g)}{wet\ soil\ (g)} \times 100$$

Image 13. Picture of oven set up for soil organic matter experiment.

(1.6) Soil Nitrogen

In the soil, I measured the available nitrogen in the form of nitrate-N (NO_3^-). Available nitrogen is the form in which plants are able to readily take up and use for their development and growth. As I stated before, available forms of nitrogen in the soil include both nitrate nitrogen (NO_3^-) and ammonia (NH_3), however, because ammonia is a fairly unstable form of nitrogen, is easily converted into nitrate-nitrogen, and does not accumulate very well as NH_3 in the soil, I only measured the NO_3^- concentration in the soil. Some of the NH_3 is converted into NO_3^- during the NO_3^- isolation, and the remainder of it is dissolved from this process. To prepare the soil for extraction, a sample from each pot was laid out on a card to air dry (Image 14). By drying the soil in this way, I was able to conserve the components of the soil and only remove excess water from it. To isolate the NO_3^- from the rest of the sample I extracted all of the available nitrogen from the soil using a KCl extraction reagent, decolored it using Fisher G Caron Black, added an antimony

sulfate solution to isolate NO_3^- and dissolve impurities, and added a chromotropic acid solution (CTA) to develop a color (Jones Jr, 2001). The resulting sample was then run through a spectrometer (Image 15) to measure wavelengths (nm) and calculate for concentration in parts per million (ppm). I did this once for the soil in each treatment (collected at the end of the experiment), and ran the samples against two blanks (deionized water and KCl blanks) and three known standard solutions (5ppm, 10ppm, and 15ppm). The results of the spectrometer readings were inserted into the equation below to find the concentration (Jones Jr, 2001). ppm = mg/kg. Soil nutrient concentrations are measured in ppm because these levels are so exceptionally small that percentages and decimals would be incredibly miniscule (Spill Prevention and Response Division, 2009).

$$conc.N\ in\ soil\ (ppm) = conc.N\ in\ extract \times \frac{extract\ vol.}{wt.of\ soil}$$

Image 14. Picture of soil samples left out to air dry.

Image 15. Picture of spectrometer used for wavelength readings.

(2) Plant Parameters

(2.1) Plant Heights

The heights of the plants were measured both throughout the duration of the experiment and at the termination of the project. Because neither of my species grow horizontally, the vertical height (measured in cm) was measured from the base of the plant to the tallest point of the stems to document growth rate for each treatment. All of the heights were measured using a measuring tape at the beginning of the experiment, rounding each measurement to the nearest tenth of a centimeter. However, by week 5, the stems of the sugar snap peas began to curl, many of which were not taking to the supports placed in the pots next to them, making it difficult to measure these with a straight measuring tape. To record the heights for these plants I fit a string to the length of the stem, bending along with the stem's curves and marking where the tallest point of the stem ended. Measuring the length of the string allowed us to record the heights for the pea plants that curled from week five on (Image 16). Because of challenges in taking the photographs of this method, the string in Image 16 is not taut, but it was pulled tight when the data were recorded.

Image 16. String method for measuring the heights of curling pea plant stems

For the clover, because there were 10 plants per pot, the heights were measured for each plant within the pot and then averaged for an average height for the entire pot.

(2.2) Root Lengths

The root lengths indicate the success of below ground growth for each plant. I measured the root lengths at the termination of the experiment by uprooting each plant from their pots and measuring the length (cm) from the base of the stem to the longest point in the root system (Image 17). Because of the complex, fine, and delicate root systems of the clover it would have been extremely difficult to measure each individual length to average a mean. Instead, for the clover I just took the value of the longest root as the root length for each pot.

Image 17. Photos of uprooted plants. The left picture shows clover not treated with *rhizobia* (T1R2) and the right picture shows a pea plant treated with *rhizobia* (T2R3).

(2.3) Above Ground and Below Ground Dry Plant Biomass

The above and below ground biomass is a measure of the amount of plant mass within the shoots (above ground) and roots (below ground) of each plant. I measured this by separating the roots from the shoots (Image 18), oven drying them at 70^{o}C for 72 hours, then measuring the masses for each sample. Oven drying the samples before measuring the mass ensured that the moisture contents of the plants were not included in the biomass value.

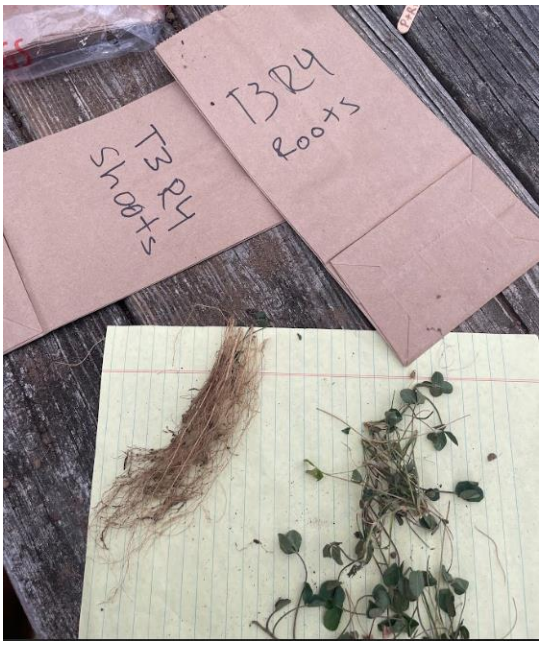

Image 18. Picture of separated roots and roots for plant T3R4.

(2.4) Plant Nitrogen

In plants the nitrogen content is measured as the total nitrogen present within the biomass. This is because the only nitrogen present within the plants would be the available nitrogen they picked up from the soil. Because the NO_3^- taken up by plants is used for protein building and in various other cellular processes, instead of testing for the values of each type of nitrogen within the plant I can just test for the total nitrogen. This process is very similar to the nitrogen detection process performed for the soil except for the fact that with the plant material I needed to do a digestion. I ground up the dried plant material from the above (shoot) and below (root) ground biomass test using a plant grinder and mortar and pestle accordingly (Image 19). The ground up material was digested in nitric acid and then in perchloric acid, to isolate the nitrogen within the samples. I diluted the samples with distilled water and added the KCl reagent. The samples were run through a spectrometer (Image 15) along with standard solutions and two blanks: KCl and deionized water.

$$conc.N\ in\ plants\ (\%) = \frac{conc.N\ in\ extract\ \times \frac{extract\ vol.}{wt.of\ plant}}{10{,}000}$$

Image 19. Picture of ground plant material. Shoots (left) were ground with an herb grinder and roots (right) were ground with a mortar and pestle.

(2.5) Nodule Count

The nodule counts on the root systems indicate success of *rhizobia* colonization on the legumes. Roots that had nodules indicated that successful symbiosis occurred between the *rhizobia* and the legume plants. I expected to find nodules on the plants inoculated with *rhizobia* before planting. If there are nodules on the control plant, not inoculated with *rhizobia*, that would indicate that there was preexisting *rhizobia* naturally occurring in my starting soil. I expected to see more nodules on the inoculated plants than on the control plants because they were directly exposed to the bacteria before being planted, whereas the control plants had a lower exposure (if any). I found the nodule count for each plant by carefully uprooting the plants from their pots, shaking off any excess dirt and counting the number I was able to see (Image 20). Formations were determined to be nodules if they exhibited a distinct oval or round knob shape.

Image 20. Nodules found on the root system in pot T3R2. Nodules are circled in red.

(3) Challenges

We conducted a 10-week study analyzing the effects of *rhizobium* inoculation of white clover and dwarf snap peas on soil pH, plant dry biomass, nitrogen content, and nodule formation, all in an effort to evaluate plant productivity. Plant productivity is the measure of the success a plant has at growing and producing yields under certain conditions. In the case of my experiment, that measure is a compilation of various parameters testing the effect that *rhizobium* bacteria have on the productivity of white clover and dwarf sugar snap peas. The study ran for ten weeks because the selected plants had growth cycles that landed their maturity between eight and ten weeks.

(3.1) Pests

In early stages of gestation, crops are the most susceptible to weeds. Unfortunately, my experiment was impacted by the unwanted growth of weeds. Due to the thinning process in the first few weeks of growth, some crops may have lost their place in the experiment in favor of weeds that began their growth looking extremely similar to them. The process of thinning is

important to ensure that the pots each contain an equal number of plants. In order to better maintain consistency throughout the experiment, it was crucial to standardize the number of plants within each pot. For the peas this resulted in one pea plant per pot. However, because clover is a cover crop that thrives in clusters rather than individually, 10 clover plants were conserved within each pot. The weeds which took residence in my pots were *Persicaria longiseta* (oriental lady's thumb) and *Robinia pseudoacacia* (black locust) (Image 21). These plants are common to the ecology of New Jersey, although the oriental lady's thumb is not native. It is unknown whether these plants were pre-existing constituents in the collected soil or if they were introduced to the pots during the experiment via wind travel or through the rainwater used to hydrate the plants. These weeds affected pots T1R5, T3R1, T1R4, T3R3, and T3R5, outcompeting the experimental plants until they were uprooted and removed from the experiment. The weeds were pulled up through hand weeding once it became undeniably evident that they were not the experimental plants.

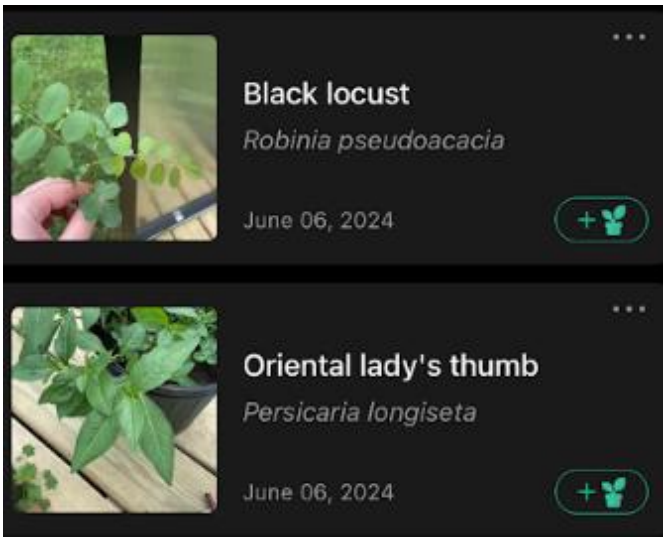

Image 21. Weeds found within the potted experiment. The phone application *PictureThis* was used to help identify weed species.

As is the nature of an outdoor greenhouse, native New Jersey wildlife found its way into the experiment in the form of eastern cottontail rabbits, *Sylvilagus floridanus*. During week four,

pot T3R2 (clover inoculated with *rhizobium*) was found to have been eaten down to its stems (Image 22).

Image 22. Picture of rabbit damage done to pot T3R2 in week 4.

Luckily by week five the clover had recovered, regrowing the leaves and resuming its growth. Even though this pot received considerable damage from pests, by the end of the experiment this pot's average height was the median height for the T3 treatment, leaving little reason to remove T3R2's data from the experiment. Because only one pot was affected and because it was in a position closest to the greenhouse door, no measures were taken to prevent further harm to this pot or any others. Because the door of the greenhouse was kept open after week four to provide additional ventilation, I suspect that rabbits were responsible for the damage made to the clover pot T3R2, which was easily accessible from the yard.

(3.2) Season and Heat Stress

The ideal growth seasons for dwarf sugar snap peas and white clover are in early spring and early fall. Because gestation began well into spring (late April), towards the beginning of summer, the elevated temperatures may have affected the plant growth speed, plant maturity, and the development of *rhizobia*. Heat stress and limited air flow was apparent within the greenhouse and the plants experienced a gradient of intensity dependent on their position within the block design. Pots positioned farther away from the door of the greenhouse may have experienced a higher intensity of these stressors as the growth within them seemed to struggle more than in pots closer to the door (Image 23). Environmental conditions surrounding the greenhouse caused partial shade cover over the southernmost half of the greenhouse (side with the door) during the morning hours and no shade cover for the northernmost half of the greenhouse (farthest from the door). I suspect that this differentiation in shade cover may have stunted the survival of the peas in pots T0R2 and T0R4 (which died in the fourth and sixth weeks of the experiment), as well as the growth of plants in pots T2R2, T2R3, T3R5, and T1R3 (all of which belonging to the farthest two rows from the door). The heights at the end of the experiment for both potentially stunted *rhizobia* treated pea (T2) pots were approximately 30% lower than the average height for the entire treatment group. For the potentially stunted clover pots, the ending height of T3R5 was 23% lower than the mean for the T3 group and the ending height of T1R3 was 56% lower than the mean for the T1 group.

Image 23. Picture of pot T2R1 on termination day. Heat damage can be seen by the discoloration of the plant.

Statistical Analysis

One-way analysis of variance (ANOVA) was carried out to test for differences in parameters between treatments for each species. Tukey–Kramer post hoc tests were performed to compare mean separation at $p < 0.05$ among treatments. Any differences between the mean values at $p < 0.05$ were considered statistically significant. Because the sample size was less than 30, two-tailed t-tests were also performed to test for differences between means for each parameter within each species. Cohen's D coefficients were calculated to measure effect sizes and test for practical significance. Practical significance for Cohen's d was considered very small when $d < 0.2$, small when $0.2 < d < 0.5$, medium when $0.5 < d < 0.8$, and large when $0.8 < d$. All statistical analysis was performed using functions in Microsoft Excel and SPSS.

RESULTS AND DISCUSSION

No results from the ANOVAs or t-tests for all tests between treated and untreated plants of the same species were statistically significant, but some practical significance was observed when measuring effect sizes (Table 3). This means that treatments of *rhizobia* showed no statistically

different effect on the plants' measured parameters as compared to the controls, however, some observed effect sizes were large enough to be considered significant in a real-world context. Trends and differences may have been affected by environmental factors as mentioned in the limitations above: heat, sun exposure, ventilation, pests, watering, etc. It should be noted that pots T0R4 and T0R2 were excluded from the mean height, mean root lengths, and dry biomass data in all weeks as they had died in the fourth and sixth weeks respectively. These low and absent points would skew the data for treatment T0.

Table 3. Independent Samples Effect Size

Species			Point Estimate
Sugar Snap Peas	above ground height (cm)	Cohen's d	.668
	below ground height (cm)	Cohen's d	-.065
	Root Nitrogen (%)	Cohen's d	1.617
	Shoot Nitrogen (%)	Cohen's d	.938
	Soil Nitrogen (ppm)	Cohen's d	1.194
	above ground biomass (g)	Cohen's d	.305
	below ground biomass (g)	Cohen's d	-.950
White Clover	above ground height (cm)	Cohen's d	-.015
	below ground height (cm)	Cohen's d	.203
	Root Nitrogen (%)	Cohen's d	-.918
	Shoot Nitrogen (%)	Cohen's d	1.409
	Soil Nitrogen (ppm)	Cohen's d	.202
	nodule count	Cohen's d	.905
	above ground biomass (g)	Cohen's d	.441
	below ground biomass (g)	Cohen's d	.874

Soil Parameters

The bulk density of my soil (0.81g/cm^3) was below the optimal range of 1.55-1.60 g/cm^3 for plant productivity (Diao et al., 2021). A low bulk density means that the soil is more porous,

more permeable (for both water and organisms), has more aeration, and has a higher water storage capacity than a soil with a high bulk density (Gliessman, 2015). Although tillage of soil creates porous spaces and decreases bulk density, excessive tillage exposes SOM and increases its decomposition (Gliessman, 2015). Soil that undergoes excessive tilling in and/or compaction from the use of heavy farming equipment would exhibit a higher bulk density because the "crumb structure" of the soil is compromised by both (the more the soil is disrupted, the more the porous spaces and structural components of the soil is deconstructed). A soil that maintains its SOM and is not subject to such practices typically exhibits a lower bulk density because its crumb structure is upheld (Gliessman, 2025). Our bulk density being below the optimal range ($0.81 g/cm^3$) means that there is high porosity, aeration, and permeability to our soil and suggests that there may be a high organic matter content maintained in the structure (Table 4).

The moisture content for my soil was within the optimal range of 10-45% for plant productivity (Datta et al., 2018). A healthy soil moisture (SM) content is important because SM influences plant growth, soil temperature, and chemical transport (Datta et al., 2018). A SM content below 10% would put vegetation at risk because of insufficient water and a SM content above 45% would put vegetation at risk of "drowning" or water logging the soil because of excess water (Gleissman, 2015). The moisture content of my soil, 20%, means that my soil has an ideal water content for supporting plants without the risk of water logging or drying out (Table 4). It is also mentionable that colloid sizes and soil texture can affect the water holding capacity of a soil (Gleissman, 2015). Sandy soils and soils with large colloids will not be able to retain as much water as clay soils and soils with small colloids because there are large pore spaces between particles in which the water can slip through/leach out (Gleissman, 2015). For clay soils and soils with small colloids, those pores are much smaller and the smaller spaces/particle sizes provide

water with more surfaces to adhere to, thus retaining much more water within the soil (Gleissman, 2015).

The texture for my soil is suitable for plant productivity as it is of a loam class (Diao et al., 2021). There are four types of loam soils (sandy loam, clay loam, silty loam, and a true loam) each of which are favorable for different types of crops and environments because of their slightly varying structure. The loam class is the most moderate of the soil textures and allows for the most moderate water filtration, soil porosity, soil mass, and water retention of all classes (Gliessman, 2015). More sandy soils have higher filtration rates and lower mass because the soil particles (colloids) are larger, do not pack as closely together and allow for water to filter through easier (Gliessman, 2015). Sandy loam soils would be favorable in more wet environments and for crops such as potatoes that need quick filtration to avoid root rot. More clay soils have higher water retention and heavier mass, however, because the colloid sizes are smaller, pack more tightly together, and hold onto water easier (Gliessman, 2015). Clay loam soils would therefore be more appropriate for drier environments and crops such as rice which thrives in heavy and wet soil. The texture of my soil (sandy loam) suggests that this soil is within the moderate loam class optimal for plant growth, but might need more watering than a true loam would (Table 4).

The organic matter content of my soil (20%) is above the optimal range of 3-6% for plant productivity (Fenton et al., 2008). When used for agricultural purposes, the original SOM content of the soil will steadily decline as it is broken down and used by crops for nutrients (Gliessman, 2015). Unless replenished externally, the SOM content will continue to fall until there is nothing left to break down, deeming the soil “infertile.” Because my soil was previously used for agricultural purposes and it has a SOM content above the optimal range (20%), this suggests that the soil was replenished with organic matter more than sufficiently (Table 4). Also noting that the

soil came from an urban organic farm that uses cover cropping and organic fertilizers, this data shows that these practices likely directly influenced this result.

The pH for my soil is below the optimal range of 6.0-7.5 for plant productivity (Msimbira et al., 2020). A soil is considered strongly acidic when its pH is 3 or less, at which soil microbes in particular begin to suffer and legume plants often lose their symbiotic partners (Gliessman, 2015). Soil acidification can occur naturally from nutrient leaching (losing basic ions) and also from acidic products produced by soil microbes. A soil is considered very alkaline when the pH is 8.5 or greater, at which point nutrient uptake by plants becomes hindered (Gliessman, 2015). A soil can become more basic from an accumulation of salts from runoff or from excessive/poor quality irrigation water. The pH of my soil (5.85) is not considerably lower than the optimal range and therefore suggests that there may be soil microbes and/or nutrient leaching in the soil prior to the experiment (Table 4).

Table 4. Selected Soil Physical and Chemical Properties

D_b (g/cm^3)	0.81±0.05
SM (%)	25.6±0.60
Texture	Sandy Loam
SOM (%)	20±0.00
pH	5.87±0.02

D_b = Bulk Density; SM = Soil Moisture; SOM = Soil Organic Matter; Values are expressed as mean ± standard error

Plant Parameters

There was no statistically significant difference found in the biweekly heights between treated and not treated groups, but some trends can be seen. Peas not treated with *rhizobia* consistently had taller heights than inoculated peas throughout the experiment with their final mean

heights being 74.1 cm and 59.5 cm, respectively (Figure 4). For both treated and not treated clover pots, the mean heights throughout the experiment were roughly the same with neither group persisting above the other. The end mean height for the not treated clover was 9.4 cm and the end mean height for the treated clover was 9.4 cm (Figure 5). The mean heights of the clover (both T1 and T3) for the second week was recorded as 0 cm because the plants were too small to accurately measure with a tape measure. The data for their heights at week two were therefore not included in the visualization (Figure 5).

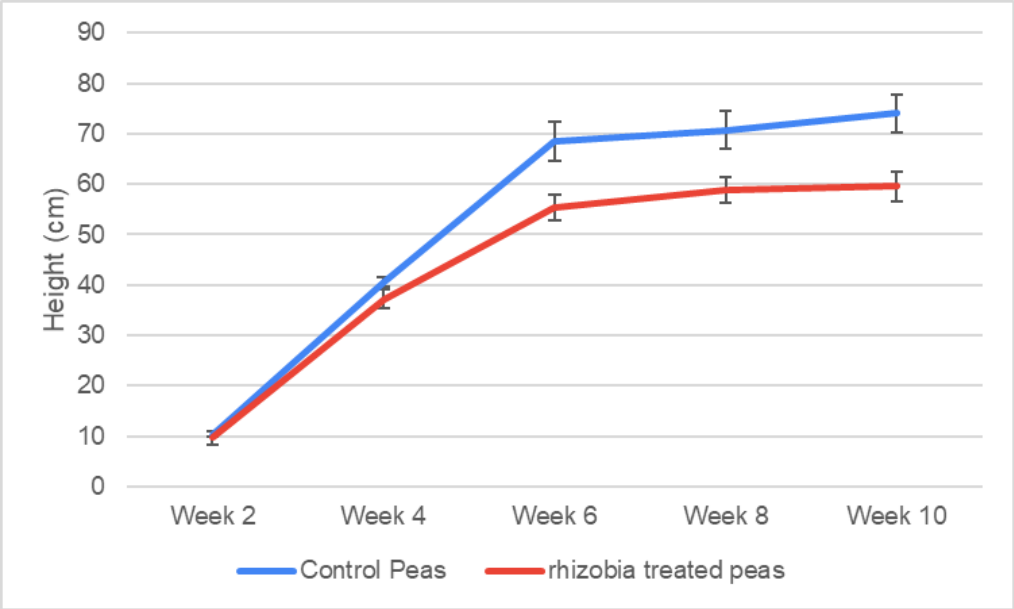

Figure 4. Line graph representing the mean biweekly heights (cm) for peas of each treatment. Error bars represent standard error.

Figure 5. Line graph representing the mean biweekly heights (cm) for clover of each treatment. Error bars represent standard error.

No significance was found for the data in Table 5; however, some practical significance (Table 3) and trends can be interpreted. There was not a statistically significant difference in the clover for below ground length of those with inoculation (mean = 21.1 cm, standard deviation = 6.1 cm) and those without (mean = 23.0 cm, standard deviation = 11.4 cm), $t(8) = 0.32$, $p = 0.76$. This result was corroborated with a low Cohen's d of 0.20 (Table 3), indicating only a small difference. There was not a statistically significant difference in the peas for below ground length of those inoculated (mean = 16.8 cm, standard deviation = 2.3 cm) and those without (mean = 16.7 cm, standard deviation = 2.5 cm), $t(6) = 0.09$, $p = 0.93$. This result is supported with a very low Cohen's d of 0.07 (Table 3), indicating a very slim difference between them. The mean root lengths of the clover may have been limited by the size of the pots. Many of the clover roots had reached the bottom of the pot and began to grow back up the sides of the container by the end of the termination day. These roots were flattened out when measured, but their growth may have been

different in a larger pot. The nodule counts for both treated and not treated peas was zero, so there is no difference to analyze. For the peas this means that inoculation was not successful and there were no established *rhizobia* colonies within the root systems of the pea plants. For the clover, there was no statistical significance in the nodule formation between clover treated with *rhizobia* (mean = 2.6, standard deviation = 2.1) and the control clover (mean = 10.4, standard deviation = 12.0), $t(8) = 1.43$, $p = 0.19$. This result was countered with a very high Cohen's d of 0.91 (Table 3), indicating that nodule formation in not inoculated clover is practically significantly higher than in inoculated clover. This result suggests that natural *rhizobia* may have been present in the collected soil to interact with the plants, separate from my treatment. The collection site of the soil had not recently supported legumes, but because of the organic practices of the farm *rhizobia* may have been a natural component to the soil. There is the chance that preexisting *rhizobia* species may have been different than those I inoculated my plants with and that they may have been preferable to the species of legumes I used in my experiment. If this is true, the inoculation performed on the clover may have blocked the plants from accessing naturally occurring *rhizobia* that may form a stronger and more efficient relationship with the plants.

Table 5. Selected Plant Parameters for Each Treatment

Parameter	T0	T1	T2	T3
Root length (cm)	16.67±1.12	22.98±2.39	16.82±1.07	21.12±1.75
Nodule Count	0	10.4±2.45	0	2.60±1.02

Values expressed as mean ± standard error. T0 = control peas; T1 = control clover; T2 = inoculated peas; T3 = inoculated clover.

The biomass for the shoots and roots of each treatment was not statistically significant, but some variation (Figure 6) and practical significance (Table 3) can be observed. There was no statistical significance in above ground dry biomass for inoculated peas (mean = 1.14, standard deviation = 0.40) and not inoculated peas (mean = 1.24, standard deviation = 0.09), $t(6) = 0.42$, $p = 0.69$. This result was corroborated with a small Cohen's d of 0.31 (Table 3), indicating a small difference. For the below ground dry biomass there was also no statistical significance for the control peas (mean = 0.09g, standard deviation = 0.08g) as compared to the inoculated peas (mean = 0.21g, standard deviation = 0.14g), $t(6) = 1.30$, $p = 0.24$. This result was partnered with a high Cohen's d of 0.94 (Table 3), indicating that the higher below ground biomass for the inoculated peas was practically significant. There was no statistical significance in the above ground biomass between the control clover (mean = 0.99g, standard deviation = 0.64g) and the inoculated clover treatments (mean = 0.75g, standard deviation = 0.43g), $t(8) = 0.70$, $p = 0.51$. This result was coupled with a medium Cohen's d of 0.44, indicating that the above ground biomass for the control clover can be considered moderately higher in a practical setting. According to the Cohen's d coefficient, the below ground biomass of the control clover (mean = 2.85g, standard deviation =2.73g) is 0.87 standard deviations higher (Table 3) than the below ground biomass for the inoculated clover (mean = 1.11, standard deviation = 0.69g). This result, however, was not statistically significant, $t(8) = 1.38$, $p = 0.20$).

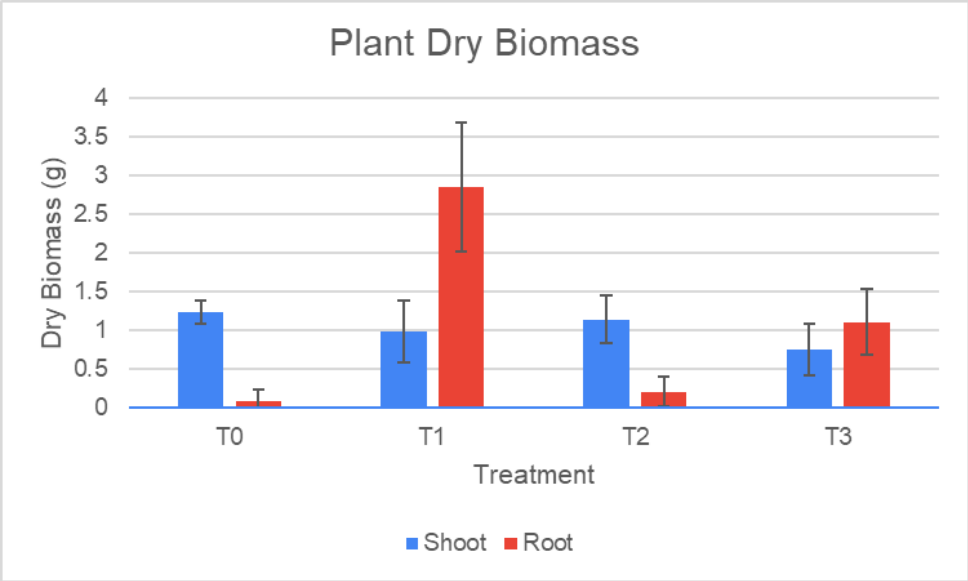

Figure 6. Bar chart of the mean dry biomass in the shoots and roots for each treatment at termination. Error bars represent standard error. T0 = control peas; T1 = control clover; T2 = inoculated peas; T3 = inoculated clover.

Nitrogen Content in Soil and in Plants

The nitrogen contents for the soil, shoots, and roots of each treatment at the termination of the experiment was determined and is reported in Figures 7 and 8. The nitrogen content within the soil only includes the nitrate-nitrogen content and is expressed as parts per million (ppm) because this is the form available for use by the plants and I only tested a 5g subsection of each pot. Because all forms of nitrogen within the plants had come from available forms in the soil, the nitrogen content for the plants is expressed as a percentage.

The nitrate-nitrogen content within the soil for each treatment showed a trend between treated and not treated groups of the same species (Figure 7). According to the Cohen's d coefficient, the mean nitrogen content in the soil for the pea pots not treated with *rhizobia* (mean = 27.06 ppm, standard deviation = 12.46 ppm) was 1.19 standard deviations higher than the nitrogen content in the soil for the pea pots that were treated with *rhizobia* (mean = 17.09 ppm,

standard deviation = 5.18 ppm). Although highly practically significant, this result was not statistically significant, $t(6) = 1.64$, $p = 0.15$. For the clover there was also no statistical significance between the nitrogen content in the soil for the control pots (mean = 24.21 ppm, standard deviation = 6.70) and the nitrogen content in the soil for the treated pots (mean = 22.49 ppm, standard deviation = 9.95 ppm), $t(8) = 0.32$, $p = 0.76$. This result is supported with a low Cohen's d of 0.20, indicating a small difference between the groups. These results suggest that in the pots containing treated plants the plants were able to take up more nitrate-nitrogen from the soil than in the pots not treated with *rhizobia*. The soil nitrogen content for both of the clover treatments is considered within the medium range of soil nitrate-nitrogen content of 10-20 ppm (Marx et al., 1999). The nitrate-nitrogen content for the soil of both of the pea treatments is considered high (20-30 ppm). The suggested healthy nitrate-nitrogen content for a productive soil is the medium range of 10-20 ppm. This means that my soil had more than enough available nitrogen in all treatments for the plants to take up and use for growth and development.

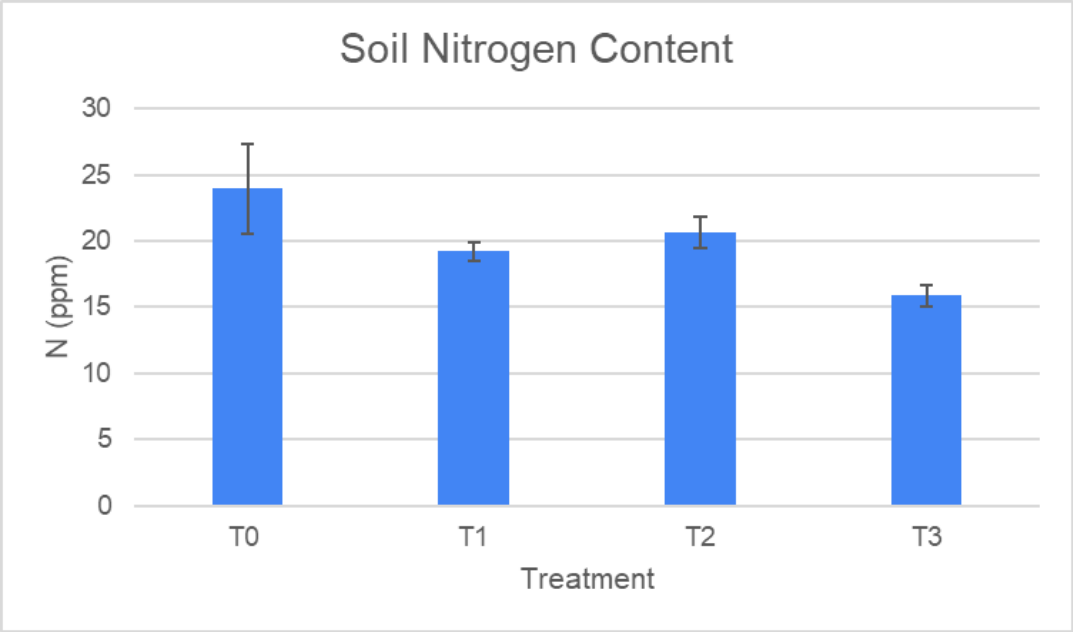

Figure 7. Bar chart of the nitrogen content (ppm) in the soil for each treatment after the duration of the experiment. Error bars represent standard error. T0 = control peas; T1 = control clover; T2 = inoculated peas; T3 = inoculated clover.

The mean nitrogen content for the plant shoots and roots for all treatments varied (Figure 8). Between treated and not treated groups there is no statistical significance, but some practical significance can be observed. For the nitrogen content in the roots of the peas there was a very high Cohen's d of 1.62, indicating that the nitrogen content in the roots of the not treated peas (mean = 6.56%, standard deviation = 1.70%) was 1.62 standard deviations higher than the nitrogen content in the roots of the inoculated peas (mean = 3.14%, standard deviation = 2.30%). Although highly practically significant, this result was not considered statistically significant, $t(6) = 2.21$, $p = 0.07$. There was also no statistically significant difference in the shoot nitrogen content between the control peas (mean =4.94%, standard deviation = 0.14%) and the treated peas (mean = 3.07%, standard deviation = 2.43%), $t(6) = 1.29$, $p = 0.25$). This result was countered with a high Cohen's d of 0.94, indicating that the shoot nitrogen content in the shoots was 0.94 standard deviations

higher in the control peas than in the treated peas). For the root nitrogen content in the clover, the Cohen's d indicates that the nitrogen content in the treated clover (mean = 9.13%, standard deviation = 9.12%) was 0.92 standard deviations higher than the nitrogen content in the roots of the control clover (mean = 2.96%, standard deviation = 2.68%). This result, however, was not statistically significant, $t(8) = 1.45$, $p = 0.19$. According to the Cohen's d for the nitrogen content in the shoots of the clover, the control clover (mean = 4.99%, standard deviation = 0.07%) had a content that was 1.41 standard deviations higher than the nitrogen content in the treated clover (mean = 2.89%, standard deviation = 2.11%). This result, however, was just barely not able to be considered statistically significant, $t(8) = 2.23$, $p = 0.056$.

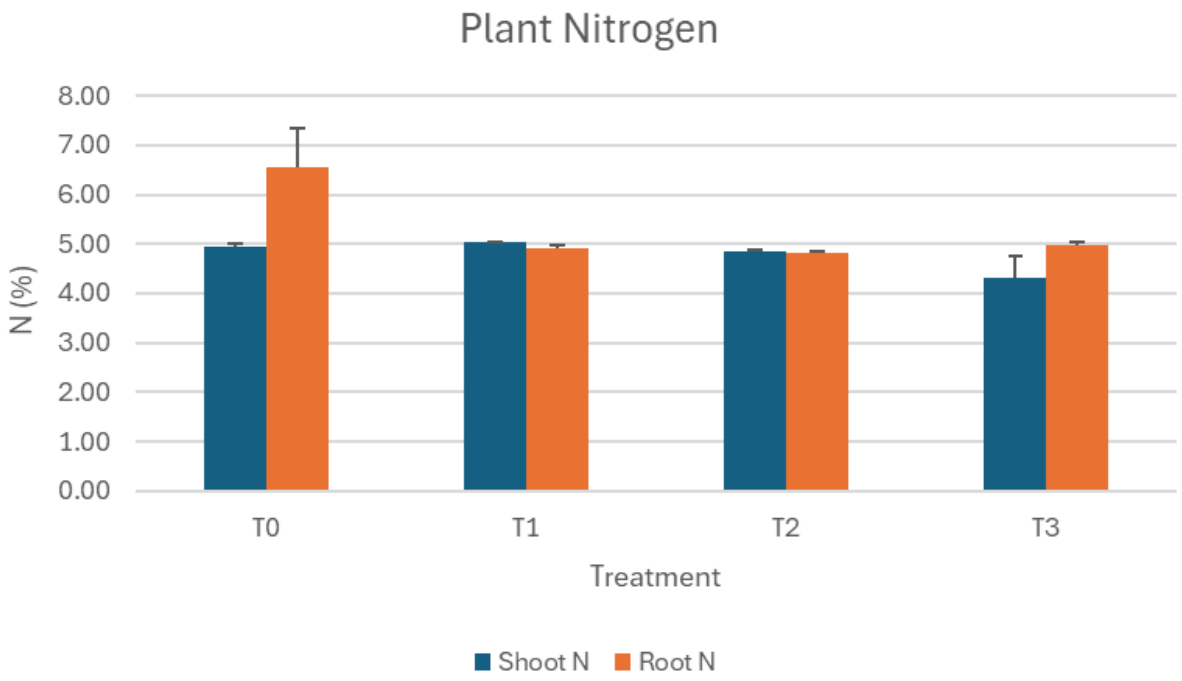

Figure 8. Bar chart of the nitrogen content (%) in the roots and shoots for each treatment. Values expressed as means and error bars represent the standard error. T0 = control peas; T1 = control clover; T2 = inoculated peas; T3 = inoculated clover; N = concentration nitrogen.

These trends suggest that the *rhizobia* treatment either was not needed to meet the nutrient needs of the plants, or it did not have a strong enough effect to overcome other growth-limiting factors: heat stress and pests. Because all of the pots received compost as a starter fertilizer at the beginning of the experiment, this application may have affected the results. When there is already a source of sufficient nitrogen within the soil, either applied or inherent to the soil, *rhizobium* will not fix atmospheric nitrogen due to the energy-expensive nature of the process.

CONCLUSION

Our understanding of the relationship formed between *rhizobia* bacteria and legume plants is complex and variable. Parameters accounting for productivity and success can be dependent on a number of chemical and physical conditions. The results from this experiment are important in building on our understanding of these symbiotic dynamics. Although I did not see significance between *rhizobia* treated and non-treated groups, these findings can be used to build on growing knowledge surrounding biofertilizer use.

Our results showed inconsistencies in all tested parameters except soil nitrogen content and biweekly heights for the peas. Although not statistically significant, I can assess the negative correlated trend between *rhizobia* application and soil nitrogen content and also the negative correlated trend in the biweekly heights for peas treated with *rhizobia*. The higher final nitrate-nitrogen content for the soil in the control pots, along with the nodule formation in the control clover, suggests that there may have been other *rhizobia* strains preexisting in the soil which interacted with the legumes. Because there was no nodule formation on any of the pea plants, this can only be said for the clover. The positive correlated trend between *rhizobia* application and mean biweekly pea heights indicates that the treated peas had a higher success in growth, in terms of height, consistently throughout the experiment when compared to the control peas. However,

these results may be due to marginal differences in sun exposure. Because the not treated peas exposed to the most sunlight (T0R2 and T0R4) were removed from the data (died early in the experiment), but the treated peas exposed to the most sunlight (T2R2 and T2R3) were not removed from the experiment, despite suffering heat and sun stress, the data may have been skewed in favor of the not treated peas. The practical significance observed from my data suggests that differences in much of my results is highly applicable in a real-world context. The variability of the setting and limitation of the sample size did show to have a significant effect on the results. If recreated without those limitations, the practical significance within my results projects that the same trends will be found. *Rhizobia* inoculation does not affect different legume species the same, creating a higher below ground biomass for the treated peas for instance than the control, and a higher below ground biomass in the control clover than in the treated clover.

From this experiment I have seen that the *Bradyrhizobium vigna* and *Rhizobium leguminosarum* strains viciae, phaseoli may not have been best suited for the environment, season, and/or legume species that I partnered them with, but further studies should be made to investigate why. Additional research is needed to explore the complexities and details of *rhizobia*'s relationships by diving into the influence that the variables within my study may have had on the experimental outcome. To analyze species specific partnerships, it would be beneficial to understand differences in the symbiotic relationship between differing *rhizobia* strains and differing legume species; if mutualistic benefits in symbiotic partnerships vary depending on species or strains, then farmers/gardeners should be aware of optimal pairings for the greatest agricultural success. Environmental compatibility is also extremely important for the realistic implementation of *rhizobia* as a biofertilizer; quantifying environments for viable *rhizobia* populations could expand successful green farming. Assessing the impact of soil conditions is

another aspect that could alter the nitrogen fixation of the *rhizobia* itself; studying how *rhizobia* functions under different soil and nutrient conditions could help us analyze the necessity of *rhizobia* inoculation for plant productivity. Long term studies are important in understanding the longevity of *rhizobia* as a sustainable alternative to synthetic fertilizers. These are only the conditions that occurred during my experiment, but others should be investigated to expand our understanding of the complex relationships' *rhizobia* strains have with the environment.

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