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SELECTION OF OPTIMAL RHIZOBIA STRAIN FOR *CROTALARIA LONGIROSTRATA*

A Major Qualifying Project Report

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Abstract

Chipilin (*Crotalaria longirostrata*) is a leguminous food crop native to Mexico and El Salvador. There is incentive to produce Chipilin for ethnic markets in the northeast; however, attempts to grow this plant in New England have not been profitable because Chilipin's optimal Rhizobium symbiont is unknown. Five Rhizobia strains were evaluated for their ability to form root nodules. Plant yield and minimum nitrogen fertilizer requirements were also measured across strains and three strains were identified to permit healthy chipilin growth in minimal Nitrogen fertilizer (5ppm). Nitrogenase activity was confirmed and quantified by a Ureide assay.

Table of Contents

Acknowledgements

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Background

Chipilin: Properties and Marketability

Chipilin (*Crotalaria longirostrata*) is a staple food crop for Central America because it can be farmed inexpensively and it is rich in protein, carbohydrates, fiber, calcium, iron, and vitamins A, B1, B2, and C. The leaf matter is considered the edible part of the crop and is conventionally prepared by boiling in soup, which allows the nutrients to be metabolized. Chipilin is native to very moist soil such as coastlines and marshland and grows best around seventy-five degrees Fahrenheit. Like most plants which prefer soil *C. longirostrata* would excel in a perlite hydroponic system which offers many scientific advantages over soil as a growth medium.

The University of Massachusetts (UMASS) Amherst Agriculture Experiment Station has been developing marketing promotions in order to inform the general public of Chipilin's nutritional benefits. Furthermore, Massachusetts has large Salvadoran and Guatemalan immigrant populations which embody a demand for inexpensive Chipilin in the US. With the demand for Chipilin rising, the plant holds tremendous promise for commercial production in Massachusetts; and lowering production costs would make this nutritious food more affordable and profitable.

A major expense for New England Chipilin farming is the cost of nitrogen fertilizer, $500/ton according to Haby (2008). However this cost can be bypassed to improve the marketability of the crop. In Central America, for instance, Chipilin can be mass-produced without nitrogen fertilizer because a bacterial symbiont specific to Chipilin is native to the soil in that region. These symbiotic bacteria span several families of the alpha-proteobacteria phylum but are commonly referred to by a single genus name: Rhizobium. They are capable of reducing plant nitrogen requirements because they can provide legumes with ammonia via the N_2 fixation pathway. By elucidating the optimal rhizobial strain, necessary nitrogen fertilizer requirements could be reduced and less fertilizer would need to be purchased..

In most farming applications for optimal yields chipilin is grown at 200 PPM (Vitosh, 2008). In order to elucidate the minimal aount of PPM which chipilin's could be sucessfully grown at a range of four different nitrogen concentations were used; 5,50,100, and 200. It was decided to water chipilins in a hydroponic manner using perlite (volcannic rock) which can absorb the water carrying minerals and vitamins. A 2-flat system is a modification of the Perlite Bag Drip irrigation system. It is a hydroponic design utilizing two types of flats, one perforated and one unperforated. The perforated flat rests within the unperforated flat and holds six perlite pots. Two liters of fertilizer solution is then added to the system so the pots are submerged about an inch deep in a reservoir of fertilizer solution. The perforated tray containing the plants can be easily lifted out of the reservoir to dump the old water and add new water containing all the required nutrients.

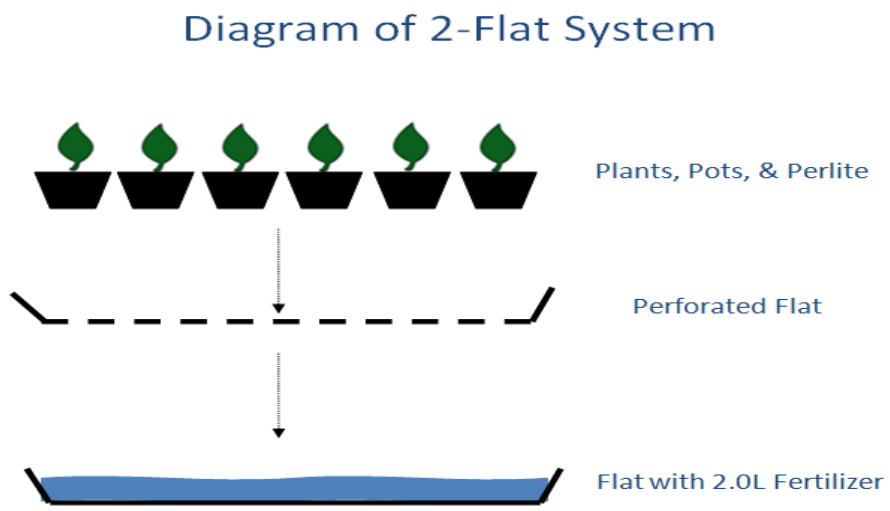

Figure one: A diagram of a modified perlite bag drip irrigation system called the 2-flat system.

Fertilizer solution was changed every three to four days for the remainder of the plant's growth. Each plant was inoculated on 2/9/10 and observations were taken until the plants were uprooted on 04/03/10 for analyses of crop yield, crop health, and bacterial infection. After inoculating the plants the Chipilin were immediately placed on a fertilizer cycle. After the first fertilization cycle the PPM was tested every day and after four days the PPM drastically dropped lower than 50% it's originally value. This would be indicative of the plant using the essential vitamins and minerals because these are the only

ones which affect the PPM. This is a good indicator of the water needing t be changed because the amount of nitrogen the plant received needed to be kept constant throughout the experiment and any amount of time the plant did not have adequate nutrients could be detrimental to the plant's health stunting plant growth or leading to chlorosis.

Inoculation

It was determined that that a range of $1x10^3$-$1x10^7$ CFU is enough to initiate an infection thread in unsterilized soil**.** Most farms typically use a concentration of about $1x10^3$-10^6 CFU's although for analytical purposes a concentration of 10^7-10^9 is used. However in sterile soil it has been reported that $3x10^4$ cells suffice to establish the inoculum strain in the majority of nodules and surpassing this concentration has no correlation with an increase in nodulation. Typically, concentrations greater than $3x10^4$ are reserved for outcompeting native rhizobia in unsterilized soil (Martensson and Gunnar, 1987). Formulating the correct inoculation dose depends on the growth characteristics of the rhizobium strain. More specifically, bacterial growth trends are used to calculate the number of living cells in a culture at a specific time. The growth curve developed in this experiment was attained by performing colony counts at 24 hour intervals of bacterial growth. Growth curves developed by this method require six to ten fold serial dilutions so it is possible to count each individual colony. Each colony corresponds to a single cell that was deposited there upon plating and is an accurate indication of the number of live cells in that dilution. The number of colonies counted at a certain dilution can be multiplied by its dilution factor to achieve the number of cells present in the initial, undiluted suspension. Rhizobia are sensitive at a wavelength of 600 nm and their absorbance readings can be a quantitative determination of cell concentration. However absorbance is not an accurate measure of infective units because optical density cannot distinguish between live, dead, and foreign bacteria

Harvesting the Plants

Chipilin is usually harvested after it reaches maturity at four to five months of growth. Three major properties of the plant should be measured to deduce symbiotic properties: leaf wet weight, plant height, nodule number, nodule weight, and xylem sap ureide levels. Leaf wet weight represents the crop yield of a particular weight, as the leaf matter is the only portion of the plant that is sold and consumed. The proportion of leaf wet weight to plant height was necessary to construct a health rating for each plant. Leaf weight to plant height provides an indication of the leaf density or lushness of the crop. The health rating was based on a scale of 1-4. A score of 1 corresponds to a dead plant, while a score of 4 corresponds to a lush, plant void of chlorosis. Chlorosis appears as yellowing or color loss of the leaf and it is an indicator of nitrogen deprivation. It was factored into the plant health score by deducting 0.5 points from the score attained from leaf weight/ plant height per level of chlorosis intensity. Two levels of chlorosis were scored; chlorosis 1 (-0.5 health points) corresponds to less than half of the leaf suffering from discoloration, and chlorosis 2 (-1.0 health points) corresponds to more than half the leaf mass suffering from discoloration. A visual depiction of the health scores is presented in Figure 2 below. Xylem sap was extracted in order to run a Ureide assay to determine the concentration of nitrogen transporting molecules. Nitrogen transporting molecules serve as indicators of Nitrogenase activity (see Ureide assay section of the introduction for more details). Nodule number and weight were calculated in order to determine a relationship between nodulation extent and plant performance. It is also necessary to harvest nodules in order to isolate bacterial enzymes indicative of nitrogen fixation by immunoblot detection.

Health Scores			
			
1	2	3	4

Figure 2: Photographic example of plants corresponding to a health score.

The data in this experiment was obtained by comparing nodulation extent, ureide activity, and crop quality. These properties are necessary to make correlations between nitrogenase activity and nodule number/weight. This correlation will show if increased nodule weight is an indicator of efficient nitrogen fixation. Comparing crop quality to ureide activity makes it possible to determine if there is a relationship between crop quality and nitrogenase activity. This data determines whether nitrogen activity is necessary for healthy plant growth. Also comparing this relationship at high and low fertilizer concentrations can elucidate the severity of chipilin's dependence on rhizobia at varying levels of nitrogen stress.
Comparing strains

Rhizobial Symbiosis

The symbiotic relationship involving rhizobia bacteria and host plants only occurs after low nitrogen levels in leguminous plants. If nitrogen levels are sufficient the legume will not partake in the infection thread process. For N_2 fixation to occur, the rhizobia must occupy the root hairs of the roots by forming an infection thread.

The infection thread starts by Legumes secreting plant metabolites called flavanoids, flavanones, and other exudates that are highly variable in structure. This high variability allows for the legume to

target specific rhizobia via flavanoids. After a flavanoid (ex. Naringenin and Quercetin) is bound to the Nod D protein, activating it, it causes the nod genes to begin transcription. After transcription finishes Nod proteins will have been created allowing for the secretion and construction of Nod factors. Nod factors will start a reaction in the epidermis (outermost layer of the roots, leaves, and stem) and are usually found in low concentrations.

All species of rhizobia hold specific adhesion protein molecules (rhicadhesin) found on the surface allowing the rhizobia to form a calcium complex with a root hairs which are attached to the epidermis. Root hairs are found connected to the epidermis and after the bacteria attaches to the root hair this allows for cells in the pericylce (bound externally by the endodermis and internally by the phloem) to stimulate the xylem to divide. Subsequently the bacterium penetrates one root cortical cell after another via a tip-growing structure, the infection thread. The infection thread will continue to grow and the nodule will elongate causing major reorganization of the microtubular cytoskeleton network in outer tissues (Taiz and Zeiger, 2006) The purpose of the infection thread in plants is for the nodule to reach the primordium cells. Rhizobia traveling down the infection thread (cellulosic tube) extend inward toward the root hair/primordium. The adjacent cells will be infected by the infection thread forming bacteroids and Symbiosomes, a vascular supply will be connected to a bacteria colony forming a nodule. A Symbiosome is a membrane bound compartment with metabolic parts and symbiont(s) found in the cytoplasm. After the bacteria are released into the cytoplasm they are then transformed into misshaped cells (bacteroids). The bacteroids will be surrounded by a plant membrane (peribacteroid membrane) to form structures called Symbiosomes. Symbiosomes are where nitrogen fixation occurs and also enables the production of leghaemoglobin.

Although rhizobia can be beneficial to the legume, it is not necessarily true that an infection thread is indicative of nitrogen fixation. Typically if the bacteroid is a reddish color this is indicative of legheamoglobin (a strong indicator of nitrogen fixation), and the host legume or rhizobium are able to abort symbiosis if the relationship becomes parasitic in either direction (Taiz, 2006)

Ureide

Ureide assay measures the activity of nitrogen transporting compounds quantitatively via a spectrophotometer set at different wavelengths for each compound. Ureide-N is responsible for transporting atmospheric nitrogen derivatives, while nitrate-N and alpha amino acid-N are responsible for transporting nitrogen obtained from the soil. Nitrate-N and Alpha amino acid-N transport one unit of nitrogen per molecule, while Ureide-N transports four. A Ureide assay was performed to measure the activity of the transport molecules via spectrophotometry to quantify what percentage of transporter bound nitrogen was obtained from the atmosphere. The calculation to obtain the percentage of atmospheric nitrogen bound to transporter molecules is shown below (Takahashi et al, 1993).

$$\%Nitrogen_{atmospheric} = 4*Ureide\text{-}N_{Activity}/(4*Ureide\text{-}N_{Activity} + Nitrate\text{-}N_{Activity} + alpha\text{-}Amino_{Activity})$$

Calculation 1: The formula to calculate the %activity of atmospheric nitrogen transporters

Project Purpose

The purpose of the project was to successfully inoculate enough Chipilin and grow plants infected with different strains of rhizobia at a range of nitrogen concentrations. Growth of plants will be harvested and measured for crop quality. The goal is to identify a rhizobial strain or strains which produced healthy and substantial crop yield(s) in minimal nitrogen conditions The performance of each strain was ranked and an optimum strain chosen. Xylem sap containing molecules necessary for nitrogen transportation was also extracted at harvest for Ureide analysis. Ureides assays were conducted to determine the percentage of the Nitrogen supply to the plant derived from the atmosphere as an indication of the plant's level of nitrogen fixation.

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Materials and Methods

Growing, Watering, and Fertilizing the Plants

Seeds were planted on three different dates (11/9/09, 12/7/09, and 1/4/10) to achieve three different age groups, each 28 days apart. They were inserted directly into dirt bulbs using tweezers and latex gloves to ensure no bacteria were transferred to the surface of the seeds. All plants were germinated using standard size flats (50 plants per flat) with a plastic wrap seal over the flats containing the seeds in the dirt pods dampened with rdH20. The first age group was kept in front of a large window at room temperature for the first month of growth and was then moved into a greenhouse for the remainder of the experiment. The second and third age groups were kept in the greenhouse with the first age group for the entirety of their growth. Age group 1 yielded 64 plants, age group 2 yielded 51 plants, and age group 3 yielded 64 plants for a total yield of 179 plants. On 1/8/10 (60 days after planting age group 1; 32 days after planting age group 2; 4 days after planting age group 3) the plants from each age group were transferred to larger plastic pots containing perlite. This date was chosen to prevent root rot of the 1st age group because their roots were found protruding from the bulb and exposed to light. On 2/6/10 144 of 179 plants were selected for experimentation based on health and age group.

There were four different Nitrogen levels to investigate; each nitrogen level was tested on six specific symbiosis types (rhizobial strain); 6 different plants (2 from each age group) were tested for each strain/nitrogen level combination (See Figure 1). This experimental design requires 24 2-flat systems, each holding 6 plants. Flats were watered every three days using 2L tap, by using a PPM meter the PPM of the water was measured for a few samples and was found to be approximately 85. The PPM meter reads parts per million and gives an idea of the quantity of minerals and metals in the water but not which ones. The flats were rearranged in the greenhouse once every 6-7 days to provide consistent light exposure among the plants.

Harvesting the Plants

Plants were harvested by removing them from the pot and dumping out the perlite in the trash and cutting the root system from the shaft of the plant. Some of the plants had pictures taken for the health key and also some of the nodules had pictures taken for reference. Subsequently the plant's height was measured, from the top of the plant to the beginning of the root system. Then the entire plant wet weight was determined and recorded. Then the leaves were removed by ripping off the leaf including the part attaching the root to the leaf and then the wet weight of the leaves was weighed. Lastly roots were separated from the dirt pod to access nodules for excision. All visible nodules were removed with tweezers, quantified, weighed, and dried in centrifuge tubes for later analysis.

Formulating Nutrients:

Three different types of nutrient solution were produced to meet the nutrient requirements of *C. longirostrata*. Nitrogen concentration was the variable factor for the experiment, and four different concentrations of nitrogen fertilizer solution were produced from Peters 10-0-0 "Magnitrate Special" concentrate. Nitrogen fertilizer was diluted in tap water according to the product manual to attain Nitrogen levels of 200, 100, 50, and 5ppm. Concentrations of phosphate, potash, and micronutrient levels were kept constant for each plant. The phosphate/potash fertilizer solution was prepared from General Hydroponics' 0-10-10 "Liquid KoolBloom Bulking and Ripening Formula" concentrate. The phosphate/potash fertilizer was diluted according to the product manual to attain a concentration of 40ppm for phosphate and potash each. The stock micronutrient solution used was 10x concentrated Murashige Skoog (MS) basal salt micronutrient solution. The concentrated formula was diluted to 1x concentrated and 1ml of 1x MS was added per L of total solution as described by Bartsev etal, 2004. See Table 1 for the fertilizer recipes for each solution.

N Concentration (ppm)	Nitrogen (g/L)	Phosphate/potash (ml/L)	Micronutrients (ml/L)
200	2.05	0.60	0.1
100	1.03	0.60	0.1
50	0.52	0.60	0.1
5	0.05	0.60	0.1

Table 1: This table depicts the amount of 10-0-0, 0-10-10, and micronutrient concentrates diluted in 1L of each fertilizer solution.

Before adding in the fertilizer, koolbloom, or micronutrients the calculations needed to be checked. This was performed by first determining the amount of salts and metals found in the water. A PPM meter was used to determine the total amount of salts and metals in the water. This value was recorded then the nitrogen fertilizer was added and stirred vigorously. Lastly the PPM was checked again and then the original PPM value with just water was subtracted from the PPM value of water and nitrogen fertilizer. This value should equal the desired calculated PPM. This same procedure was used for confirming the correct concentration of the phosphate/potash solutions.

Inoculation

Inoculation was performed before a reliable growth curve of all strains could be produced. Five-day old MAG suspensions of each strain were used to inoculate each plant. Each suspension was diluted to about 3.0ABS before inoculation. This absorbance was chosen because an optical density of 3.0 is roughly equivalent to $1x10^7$ units resembling rhizobia. Therefore, regardless of a large population of dead or foreign cells, it is probable that enough live cells ($3x10^4$) to initiate successful symbiosis would be present in the inoculum. The actual number of cells in each strain inoculum was later determined by in vitro growth analysis. After each suspension was diluted to 3.0ABS the inoculum was pipetted directly into the sterile dirt pod of each plant (Martensson and Gunnar, 1987).

In vitro Growth Analysis of Rhizobium

All strains were obtained from National Rhizobium Germplasm Resource in Maryland. Strain USDA3456 is a lyophilized (freeze dried) strain typically used for cowpeas. Strain USDA 3384 is also a lyophilized strain which is used as for several different *Crotolaria* and was isolated in Brazil. USDA 2370 is used to inoculate *R. leguminosarum*. Another stain USDA110 (ALICE) and comes recommended to inoculate with *B. Japonicum*. Lastly PN301 was obtained and is used usually to inoculate Crotolaria. Strains 3384, 2376, PN301, ALICE, and 3456 were individually cultured for seven days with gentle agitation at room temperature in 50ml conical tubes containing Modified Arabinose Gluconate (MAG) broth, prepared as done by Berkum and Beyene. Thirty milliliters of MAG sample was prepared by inoculating 29ml MAG broth with 1mL aliquots of each strain. A solution of 30ml sterile MAG broth was also prepared to serve as a negative control.

Cell plating and absorbance readings were conducted in 24 hour increments for 168 hours. To plate the cells, 1ml aliquots were taken from each strain suspension and serial dilutions were conducted, ranging from 10^{-1} to 10^{-7}. Using these previous made dilutions 100uL of each dilution was plated on corresponding labeled MAG agar and allowed to incubate for two days at room temperature. The control (Tube 6) was plated on a single plate without being diluted in order to maximize contamination detection. Each plate, representing a dilution range of 10^{-1}-10^{-7} for each strain, was counted after incubation and a CFU value was recorded for plates with distinguishable colonies. OD600 measurements were conducted on 1.0ml samples of each undiluted strain suspension immediately after plating. Sterile MAG medium used to tare the spectrophotometer. If too many cells were on the plates it was recorded as a lawn, which can be described as so many cells that single colonies could not be distinguished .

Homogenizing Solution

Assay was followed according to the procedure followed by Been and Bisseling, 2010. The assay calls for 1 gram of bacteroids to be ground up although there was not a gram from all the strains.

Approximately 0.5 ml from each sample was used and proportional volumes of reagents were used in order to lyse the bacteroids for enzyme analysis

Ureide Assay

The Ureide assay was followed according to the procedure followed by Takahashi et al, 1993. Instead of using single glass tubes for each bacterium a lesser dilution was used so the samples would fit in a 96 well plate. The original paper used 50 ul of sample and over 1 ml of reagents were added which would overflow the 9 well plate wells. The assay was modified to only use 5 ul of sample and proportional volumes of reagents were added which equaled over 200ul which is still a sufficient volume to produce a reading for the Spectrophotometer for the 96 well plate.

Results

The purpose of this experiment was to inoculate *C. longirostrata* with five different strains of rhizobia, and assess their individual performance as nitrogen fertilizer subsititutes. The strains were rated based on their average health, crop yield, atmospheric nitrogen usage, and infection efficiency.

Growth Curve and Inoculation

In order for successful inoculation in sterile soil, $3x10^4$ bacterial cells should be present. Thus, growth analysis was conducted for each strain of rhizobia to determine how many cells were inoculated. The optical density for each strain was recorded for the undiluted culture over seven days. The number of cells counted was also recorded for seven days. Plates were counted unless they were considered lawns and excluded from the data. Table 2 shows the results of the optical density readings. Table 3 shows the results of the cell count for each strain and Figure 3 depicts two types of growth curves: A) fluctuations of live cell numbers over time and B) fluctuation of optical density over time.

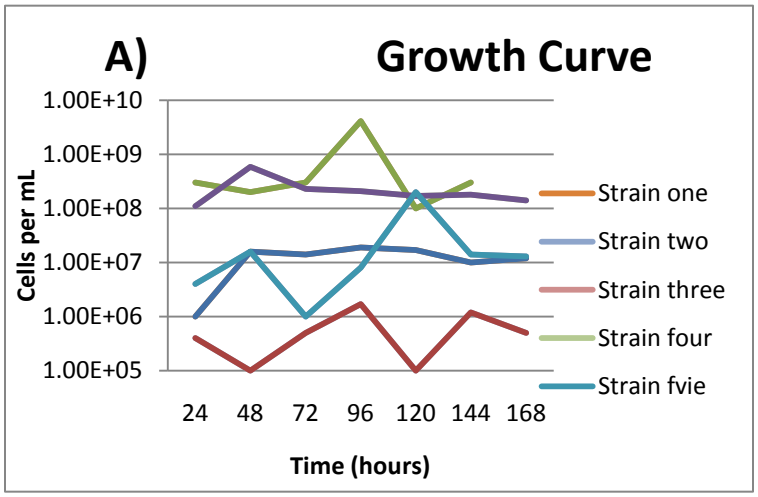
	Strain						
Time (hours)	3384	2376	PN201	Alice	3456	control	7
0	0.003	0.008	0.004	0.000	0.000	0.000	Blank
24	0.263	0.261	0.281	0.304	0.293	0.000	Blank
48	0.374	0.336	0.382	0.463	0.384	0.000	Blank
72	0.378	0.393	0.408	0.562	0.381	0.000	Blank
96	0.424	0.481	0.410	0.730	0.406	0.000	Blank
120	0.380	0.456	0.850	0.691	0.794	0.000	Blank
144	0.373	0.407	0.727	0.575	0.573	0.000	Blank
168	0.285	0.391	0.683	0.532	0.463	0.005	Blank
Strain had 0.1 subtracted from all the OD readings because a solution was made in order to compensate for the dirt. Solution was made straight from stock solution and diluted the same as the one which grew.							

Table 2: This table displays the optical density data of each strain's undiluted suspension over seven days.

Strain	Hours	Dilution (10^-x)							
		-1	-2	-3	-4	-5	-6	-7	-8
3384	24	Lawn	Lawn	Lawn	62	5	-	-	-
	48	Lawn	Lawn	Lawn	81	6	2	-	-
	72	Lawn	Lawn	Lawn	122	4	8	-	-
	96	Lawn	Lawn	Lawn	234	19	5	1	-
	120	Lawn	Lawn	188+lawn	200	17	3	-	-

	144	Lawn	Lawn	175+lawn	142	10	1	-	-
	168	Lawn	Lawn	134+lawn	117	12	3	1	-
2376	24	Lawn	Lawn	Lawn	4	-	-	-	-
	48	Lawn	Lawn	Lawn	1	-	-	--	-
	72	Lawn	Lawn	Lawn	5	-	-	-	-
	96	Lawn	Lawn	Lawn	17	1	-	-	-
	120	Lawn	Lawn	Lawn	16	-	-	-	-
	144	Lawn	Lawn	Lawn	12	-	-	-	-
	168	Lawn	Lawn	Lawn	5	-	-	-	-
PN201	24	Lawn	Lawn	109	10	2	-	-	-
	48	Lawn	Lawn	141	30	132	30	3	-
	72	Lawn	Lawn	173	110	13	15	2	-
	96	Lawn	Lawn	Lawn	152	48	14	3	-
	120	Lawn	Lawn	Lawn	Lawn	Lawn	197	41	4
	144	Lawn	Lawn	Lawn	Lawn	113	52	1	1
	168	Lawn	Lawn	Lawn	lawn	105	63	3	-
ALICE	24	Lawn	Lawn	Lawn	Lawn	105	11	-	-
	48	Lawn	Lawn	Lawn	332	173	59	2	-
	72	Lawn	Lawn	Lawn	361	182	23	3	-
	96	Lawn	Lawn	Lawn	lawn	212	21	2	-
	120	Lawn	Lawn	Lawn	Lawn	234	17	31	3
	144	Lawn	Lawn	Lawn	Lawn	232	18	1	-
	168	Lawn	Lawn	Lawn	lawn	228	14	28	-
3456	24	Lawn	Lawn	Lawn	Lawn	13	4	-	-
	48	Lawn	Lawn	Lawn	120	77	16	-	-
	72	Lawn	Lawn	164	341	8	1	-	-
	96	Lawn	Lawn	324	280	21	8	2	-
	120	Lawn	Lawn	Lawn	Lawn	Lawn	201	23	4
	144	Lawn	Lawn	Lawn	+1000	153	14	3	1
	168	Lawn	Lawn	Lawn	+1000	188	13	3	-
Control	24	-							
	48	-							
	72	-							
	96	-							
	120	-							
	144	-							
	168	-							

Table 3: This table displays seven days of cell count data from the five different strains at different dilution factors.



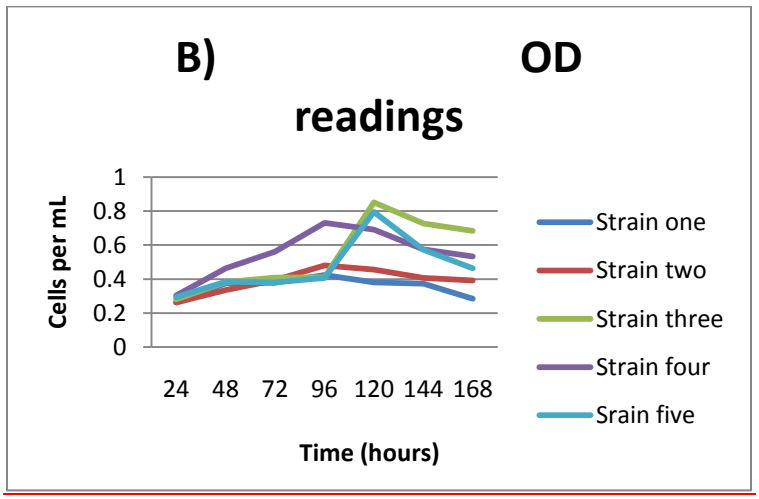


Figure 3: Graphical representation of A) cell number and B) optical density fluctuations over time for average cell counts of each strain of rhizobium.

In order for each strain to have an equal potential for nodulation, each plant must be inoculated with at least $3x10^4$ cells. With the data from the growth analysis it will be possible to quantify the number of bacteria used to inoculate each plant to determine whether each plant received a sufficient number of cells from each strain. Each strain was inoculated with 1ml of MAG suspension (0.30ABS) on the fifth day of bacterial growth. By using Equation 1, the number of live cells in the inoculum can be calculated for each strain.

$$\mathbf{Cells/ml_{inoculum}=(OD_{inoculum}/OD_{before\ dilution})\ *\ (cell/ml_{before\ dilution})}$$

Equation 1: This equation converts the number of cells in the stock suspension of each rhizobia to the number of cells that will be present in a 0.30ABS inoculation dose.

Cells/$ml_{inoculum}$ is the number of live cells in the inoculating dose of suspension. $OD_{inoculum}/OD_{before\ dilution}$ is the extent of dilution that the particular 120hr strain suspension underwent to reach an OD of 0.30ABS. Cell/$ml_{before\ dilution}$ is the average CFUs counted for a particular strain at the 120hr of culture before being diluted into the inoculum concentration of 0.30ABS. The number of cells used for inoculation of each strain type is shown in Table 4.

Strain	**$OD_{inoculum}$**	**$OD_{undiluted}$**	**Cell/$ml_{before\ dilution}$**	**Cells/$ml_{inoculum}$**
3384	0.302	0.38	2.23×10^6	1.77×10^6
2376	0.301	0.456	1.6×10^5	1.06×10^5
PN201	0.306	0.85	3.35×10^8	1.21×10^8
ALICE	0.298	0.691	1.17×10^8	5.05×10^8
3456	0.301	0.794	2.77×10^8	1.05×10^8

Table 4: This table shows the number of bacteria inoculated for each strain calculated by Equation 1. The data used to perform the calculation is also provided.

Harvesting Plants:

During harvesting, the nodule number, nodule weight, plant height, weight, leaf weight, and health observations were each recorded. These different characteristics were used to define infection efficiency, crop yield, and crop health; properties relevant to rhizobia efficiency. These properties were necessary to determine the effects of nitrogen concentration on infection efficiency and crop quality (combination of crop health and yield). They were also useful in determining the effects of infection efficiency on crop quality at different nitrogen concentrations. Furthermore, the properties of crop yield and health were important in comparing the effectiveness of different rhizobia strains at 5ppm. The data provided by the harvest is shown below in Tables 5, 6, 7, and 8 which correspond to plants harvested from the 5ppm, 50ppm, 100ppm, and 200ppm nitrogen concentrations respectively. Averages for each strain's health and yield were also calculated.

N_2	5ppm										
Strain	Age	Plant height (cm)	Plant wet weight (g)	Leaf wet weight (g)	Leaf weight/height	Health Score	Health Notes	Mean Health Score	Mean Crop Yield	Nodule#	Nodule wet weight
1	Young	17	0.63	0.38	0.022353	2	chlorosis 2			0	0
		15	0.49	0.28	0.018667	2				8	0.51
	Medium	18	0.84	0.46	0.025556	2	chlorosis 2			0	0
		42	2.01	0.6	0.014286	1.5	chlorosis 1			0	0
	Old	46	3.96	1.79	0.038913	3				1	0.64
		59	5.07	2.45	0.041525	3.5	chlorosis 1	2.333333	0.993333	47	0.85+0.74
2	Young	NA	NA	NA	NA	1	dead			0	0
		10.9	0.25	0.17	0.015596	1.5	chlorosis 1			0	0
	Medium	20.2	0.78	0.45	0.022277	3				2	0.52
		34.1	1.68	0.68	0.019941	2				7	0.56
	Old	52.6	5.63	2.75	0.052281	4				20	0.66
		41	3.03	0.9	0.021951	3		2.416667	0.99	5	0.55
3	Young	20.5	0.52	0.26	0.012683	1.5	chlorosis 1			0	0
		16.5	0.49	0.38	0.02303	3				7	0.53
	Medium	24.5	0.6	0.48	0.019592	2.5	chlorosis1			5	0.55
		NA	NA	NA	NA	NA	dried out			1	0.52
	Old	37	1.07	0.55	0.014865	2				0	0
		29.5	1.16	0.73	0.024746	2.5	chlorosis 1	2.3	0.48	14	0.6
4	Young	12.8	0.21	0.07	0.005469	2				2	0.51
		15.9	0.93	0.33	0.020755	3				7	0.52
	Medium	18.3	0.64	0.19	0.010383	1.5	chlorosis 1			0	0
		20.5	1.07	0.35	0.017073	1.5	chlorosis 1			0	0
	Old	38.5	1.14	0.2	0.005195	1	dead			0	0
		56.3	3.14	0.75	0.013321	2		1.833333	0.315	0	0
5	Young	15.5	0.66	0.42	0.027097	4				16	0.55
		24.5	0.91	0.59	0.024082	3				4	0.56
	Medium	10.7	0.2	0.15	0.014019	2				7	0.51
		23.2	0.78	0.56	0.024138	3				5	0.53
	Old	58.3	3.72	2.27	0.038937	3				12	0.71
		39.7	3.35	1.88	0.047355	4		3.166667	0.978333	14	0.63
6	Young	18	0.27	0.08	0.004444	1	dead			-	-
		16.5	0.47	0.21	0.012727	2				-	-
	Medium	19	0.46	0.16	0.008421	1	chlorosis 2			-	-
		20.5	0.49	0.24	0.011707	2				-	-
	Old	35	1.13	0.32	0.009143	1.5	chlorosis 1			-	-
		40	1.52	0.21	0.00525	1	dead	1.416667	0.203333	-	-

Table 5: Recorded harvesting data for the plants grown in 5ppm nitrogen fertilizer.

N_2	50ppm										
Strain	Age	Plant height (cm)	Plant wet weight (g)	Leaf wet weight (g)	weight/height (g/cm)	Health	Health Notes	Mean Health Score	Mean crop yield	Nodule#	Nodule wet weight
1	Young	NA	NA	NA	NA	NA	dried out			0	0
		NA	NA	NA	NA	NA	dried out			0	0
	Medium	NA	NA	NA	NA	NA	dried out			3	0.5
		NA	NA	NA	NA	NA	dried out			0	0
	Old	NA	NA	NA	NA	NA	dried out			0	0
		NA	NA	NA	NA	NA	dried out	2.5	0.65	9	0.55
2	Young	14.8	1.19	0.9	0.060811	4				0	0
		15.5	0.5	0.21	0.013548	1.5	chlorosis 1			0	0
	Medium	22.1	1.78	1.18	0.053394	3	chlorosis 2			0	0
		NA	NA	NA	NA	NA	dried out			2	0.52
	Old	37	2.06	1.04	0.028108	2.5	chlorosis 1			5	0.76
		36	2.22	1.27	0.035278	3		2.8	0.92	2	0.53
3	Young	20.5	1.14	0.29	0.014146	2				0	0
		24.5	1.41	0.89	0.036327	3				5	0.56
	Medium	24.5	0.9	0.42	0.017143	2.5				0	0
		21.5	1.8	0.3	0.013953	1.5	chlorosis 1			5	0.53
	Old	35	1.44	1.02	0.029143	3				0	0
		29.5	2.11	0.73	0.024746	3.5		2.583333	0.608333	0	0
4	Young	8.4	0.17	0.09	0.010714	2				0	0
		18.7	0.7	0.46	0.024599	2.5	chlorosis 1			8	0.56
	Medium	32.5	2.21	1.27	0.039077	3				0	0
		31	1.92	0.75	0.024194	1.5				2	0.56
	Old	38.2	1.94	0.61	0.015969	1.5				0	0
		NA	NA	NA	NA	NA	dried out	2.1	0.636	0	0
5	Young	9	0.2	0.08	0.008889	2				19	0.56
		12	1.53	1.08	0.09	4				0	0
	Medium	NA	NA	NA	NA	NA	dried out			21	0.54
		27.5	1.07	0.54	0.019636	2				4	0.5
	Old	34	1.78	0.9	0.026471	3				2	0.51
		28.6	1.95	1.03	0.036014	3		2.8	0.726	-	-
6	Young	12	0.12	0.01	0.000833	1				-	-
		17	0.77	0.51	0.03	2.5				-	-
	Medium	21	1.44	0.87	0.041429	3				-	-
		23	0.54	0.71	0.03087	3				-	-
	Old	31	1.43	0.6	0.019355	2.5	chlorosis 1			-	-
		47	2.77	1.23	0.02617	3		2.5	0.655	0	0

Table 6: Recorded harvesting data for the plants grown in 50ppm nitrogen fertilizer.

N_2	100ppm										
Strain	Age	Plant height (cm)	Plant wet weight (g)	Leaf wet weight (g)	weight/height (g/cm)	Health	Health Notes	Mean Health Score	Mean crop yield	Nodule#	Nodule wet weight
1	Young	11	0.3	0.19	0.017273	3				0	0
		10	0.58	0.39	0.039	3				0	0
	Medium	NA	NA	NA	NA	NA	Dried out			0	0
		18	0.879	0.51	0.028333	3				0	0
	Old	30	1.12	0.88	0.029333	3				0	0
		28	1.26	0.82	0.029286	2.5	chlorosis 1	2.9	0.558	0	0
2	Young	11.6	0.3	0.19	0.016379	2.5	chlorosis 1			0	0
		13.4	0.31	0.16	0.01194	2				0	0
	Medium	NA	NA	NA	NA	NA	Dried out			0	0
		20.1	1.21	0.68	0.033831	3				0	0
	Old	30.1	1.99	1.69	0.056146	3				0	0
		38.5	1.17	0.24	0.006234	1.5	chlorosis 1	2.4	0.592	0	0
3	Young	14.8	0.36	0.19	0.012838	2				0	0
		15.4	0.72	0.47	0.030519	3				0	0
	Medium	19.1	0.7	0.42	0.02199	3				0	0
		34.3	1.73	1.28	0.037318	3				0	0
	Old	36.5	1.1	0.27	0.007397	1.5	chlorosis 1			0	0
		38.6	1.4	0.51	0.013212	2		2.416667	0.523333	0	0
4	Young	11.1	0.41	0.18	0.016216	3				0	0
		10.9	0.31	0.14	0.012844	2				0	0
	Medium	NA	NA	NA	NA	NA	Dried out			0	0
		35	1.4	0.7	0.02	3				0	0
	Old	36.4	1.01	0.25	0.006868	1.5	chlorosis 1			0	0
		50.5	5.43	2.64	0.052277	4		2.7	0.782	0	0
5	Young	20.4	0.78	0.58	0.028431	3				0	0
		15	0.6	0.21	0.014	2				0	0
	Medium	28.9	1.48	1.12	0.038754	3				0	0
		20.4	0.76	0.3	0.014706	2				0	0
	Old	33	1.12	0.72	0.021818	3				0	0
		34.1	1.1	0.45	0.013196	2		2.5	0.563333	0	0
6	Young	NA	NA	NA	NA	NA	Dried out			0	0
		14.5	1.1	0.8	0.055172	3.5				0	0
	Medium	19.6	0.81	0.35	0.017857	3				0	0
		23.9	2.01	0.69	0.02887	3				0	0
	Old	30.6	1.03	0.44	0.014379	2				0	0
		58	5.98	3.76	0.064828	4		3.1	1.208	0	0

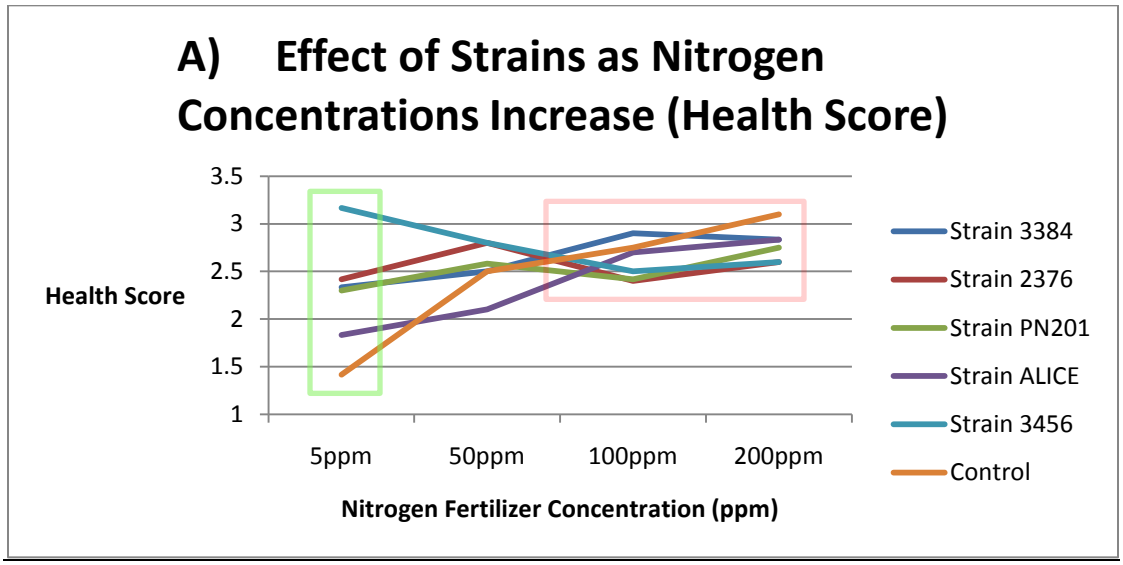
Table 7: Recorded harvesting data for the plants grown in 100ppm nitrogen fertilizer.

N_2	200ppm										
Strain	Age	Plant height (cm)	Plant wet weight (g)	Leaf wet weight (g)	weight/height (g/cm)	Health	Health Notes	Mean Health Score	Mean crop yield	Nodule#	Nodule wet weight
1	Young	18	0.68	0.3	0.016667	3				0	0
		11	0.36	0.2	0.018182	3				0	0
	Medium	35.9	1.86	0.98	0.027298	3				0	0
		20.8	0.71	0.3	0.014423	2				0	0
	Old	36	1.4	0.6	0.016667	3				0	0
		57	4.77	2.05	0.035965	3		2.833333	0.738333	0	0
2	Young	20.8	0.61	0.2	0.009615	2				0	0
		22.5	0.71	0.31	0.013778	2				0	0
	Medium	NA	NA	NA	NA	NA	dried out			0	0
		17.9	0.51	0.38	0.021229	3				0	0
	Old	39.8	2.4	1.28	0.032161	3				0	0
		30.4	1.9	1.03	0.033882	3		2.6	0.64	0	0
3	Young	18.2	1.3	0.82	0.045055	4				0	0
		11	0.4	0.13	0.011818	2				0	0
	Medium	20.3	0.76	0.3	0.014778	3				0	0
		28.7	1.56	0.8	0.027875	3				0	0
	Old	NA	NA	NA	NA	1	dead			0	0
		58.9	5.64	2.7	0.04584	3.5	chlorosis 1	2.75	0.95	0	0
4	Young	20.6	0.97	0.6	0.029126	3				0	0
		11.2	0.45	0.21	0.01875	2.5	chlorosis 1			0	0
	Medium	15.4	0.68	0.32	0.020779	2.5	chlorosis 1			0	0
		24.6	1.17	0.59	0.023984	3				0	0
	Old	34.8	1.89	1.31	0.037644	3				0	0
		40.2	2.9	1.58	0.039303	3		2.833333	0.768333	0	0
5	Young	NA	NA	NA	NA	1	dead			0	0
		9.7	0.21	0.08	0.008247	2				0	0
	Medium	28.4	1.43	1.09	0.03838	3				0	0
		13.6	0.42	0.29	0.021324	3				0	0
	Old	41.7	3.34	2.01	0.048201	4				0	0
		NA	NA	NA	NA	NA	driedout	2.6	0.8675	0	0
6	Young	10.9	0.28	0.1	0.009174	2				0	0
		20.8	0.77	0.48	0.023077	3				0	0
	Medium	18.6	0.71	0.35	0.018817	2.5	chorosis 1			0	0
		26.7	1.2	0.81	0.030337	3				0	0
	Old	26.9	0.98	0.63	0.02342	3				0	0
		42	1.99	1.36	0.032381	3		2.75	0.621667	0	0

Table 8: Recorded harvesting data for the plants grown in 200ppm nitrogen fertilizer.

Chipilin dependence on rhizobium can also be visualized with the harvesting data as shown in Figure 4. Figure 4 contains two line graphs depicting average chipilin yield and health over a large range

of Nitrogen concentrations for each strain. Strain 3384, 2376, and 3456 produce larger crop yields and health scores in low nitrogen levels rather than high nitrogen levels. Strain PN201 and Alice perform better in higher nitrogen concentrations, but in low levels they still outperformed the uninoculated plants. The negative control plant group produces the poorest crop yields and health at 5ppm but they perform more like the infected plants at higher concentrations. Furthermore, there is a great diversity of crop yield and health score averages among the different strains in low nitrogen levels (indicated by the green box). In contrast the discrepancy between individual strain performance diminishes as nitrogen levels increase(indicated by the green box). This data is depicted in a line graph below.



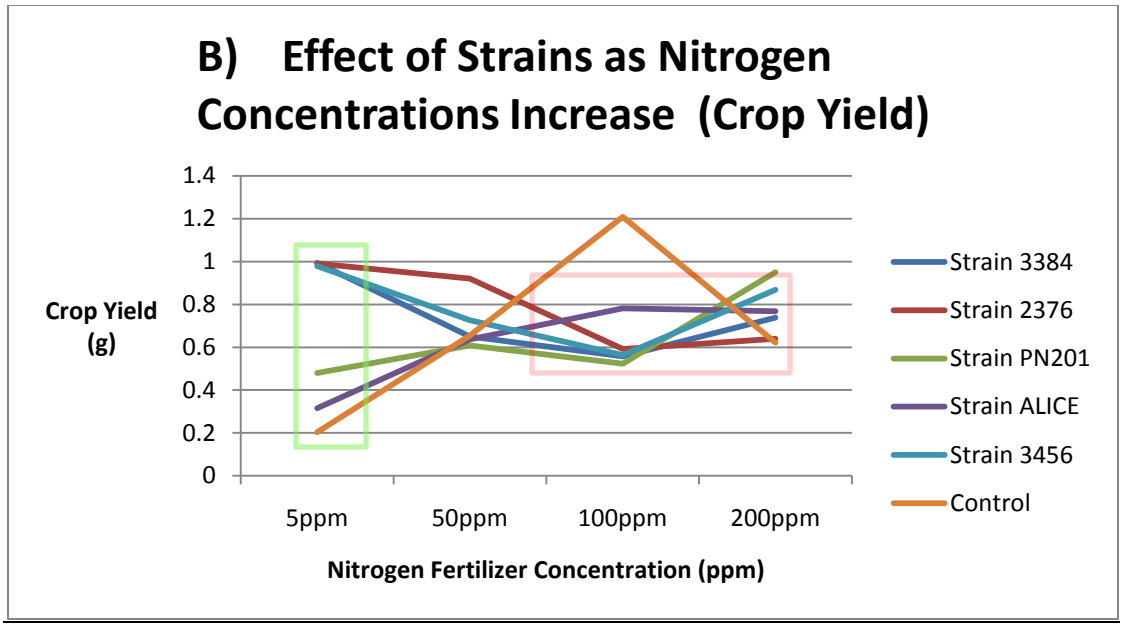


Figure 4: Line graph displaying the effect that rhizobia have on the plant's A)health score and B)crop yield as nitrogen levels increase. There was no value for strain 1 at 50ppm in either graph, but a point was included to make the graph easier to read.

Interpreting the harvest of the low nitrogen plants elucidated the most useful data in terms of the minimal nitrogen attainable with rhizobia. Of the six plants grown in a single strain group, the nodulated plants on average attained better health scores than the unnodulated plants in the group. The same is true for crop yields (excluding strain ALICE). Furthermore, plants nodulated with strain 3384, 2376, and 3456 outperformed the average crop yield and health of plants grown in sufficient Nitrogen levels, unlike the unnodulated plants which appeared extremely unhealthy and yielded much less than the plants grown in sufficient nitrogen. This large discrepancy between nodulated and unnodulated plant performance introduces another important characteristic of rhizobia, infection efficiency. This is the percentage of plants that developed nodules after being exposed to inoculum. Strain 3456 had the highest infection efficiency, 100%, Comparing nodulated to unnodulated crop yields and health scores at 5ppm may suggest that the plants are using rhizobial nitrogen fixation as a substitute for nitrogen fertilizer at varying levels of efficiency per strain (refer to Table 8).

	Nodulated plants only		Unnodulated plants only	
Strain	**Crop Yield (g)**	**Health Score**	**Crop Yield (g)**	**Health Score**
3384	1.51	2.83	0.48	1.83
2376	1.2	3	0.17	1.25
BRADY	0.53	2.67	0.405	1.75
ALICE	0.2	2.5	0.373	1.5
3456	0.978	3.17	NA	
Control	NA		0.203	1.42
*200ppm	0.764	2.74	0.764	2.74

Table 9: A Comparison of crop yield and health for unnodulated and nodulated plants grown in 5ppm Nitrogen fertilizer. *Average yield and health of all plants grown in 200ppm.

As observing the nodulated and unnodulated plants only from a certain strain group do not incorporate the infection efficiency factor, it is important to record the average of the whole group's performance to visualize the effects of infection efficiency. In Figure 5 for example, the nodulated plants only of strains 3384 and 2376 drastically appear to outperform the nodulated plants of strain 3456 in crop yield. However, the overall average yields are quite similar because strains 3384 and 2376 had lower infection efficiencies (50.0% and 66.0% respectively); Thus three plants from strain 3384 and two strains from 2376 did not become nodulated and produced poor yields which diminished the groups' average.

Due to the profound effects of infection efficiency, the primary mode of comparing crop yield and crop health values among strain type must include this factor. Therefore, the overall strain group average for health and crop yield (nodulated and unnodulated plants) was used to rank each strain as an optimal chipilin symbiont.

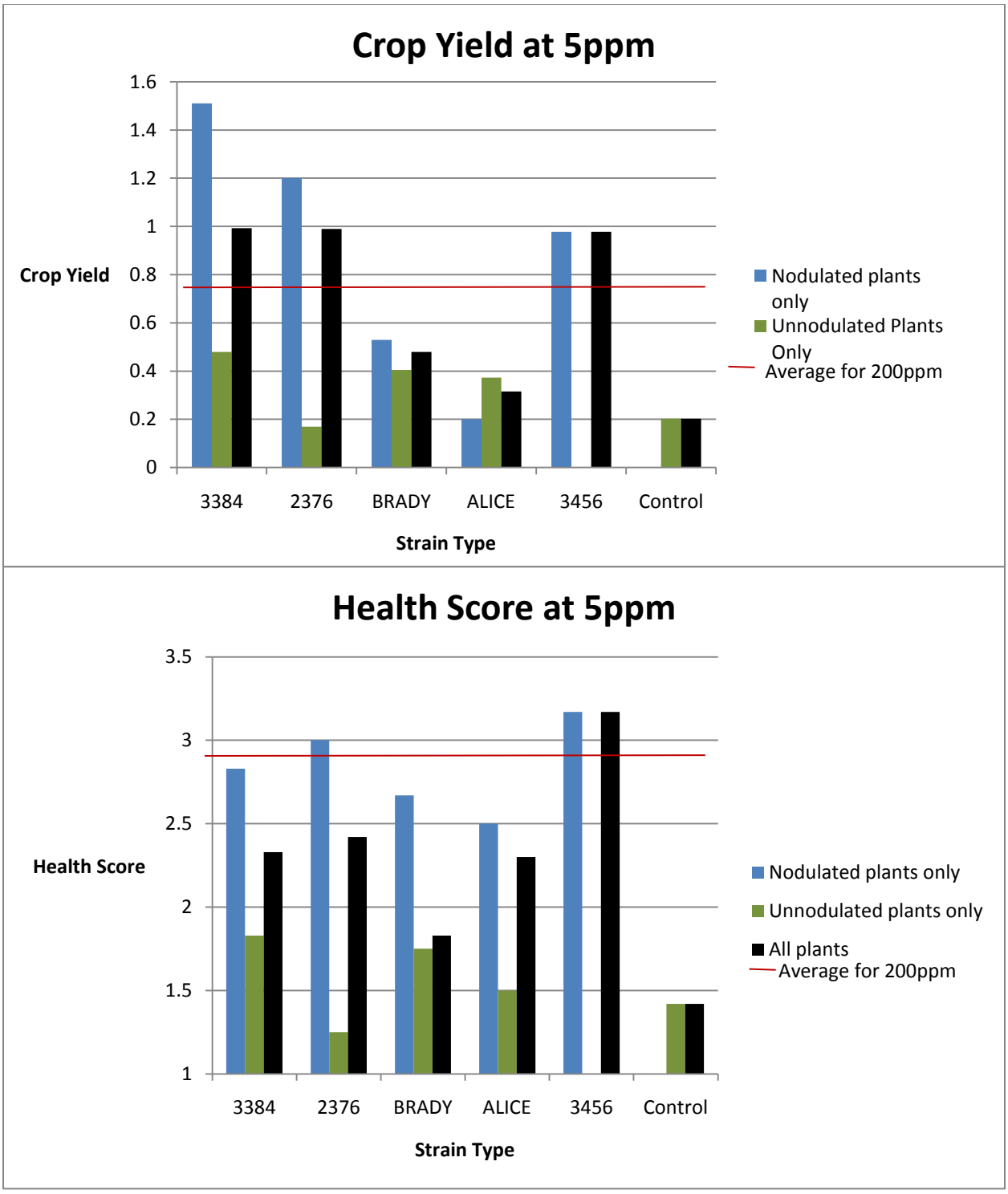


Figure 5: 5A is a line graph displaying the crop yield at 5 PPM for the nodulated plants, the unnodulated plants, and the plants at 200 PPM. This gives a graphical display of how the plants were affected by nodulation and how this helps them compared to higher nitrogen concentrations relative to crop yields. 5B shows the health scores at 5PPM for the nodulated plants, the unnodulated plants, and the plants at 200 PPM. This gives a graphical display of how the plants were affected by nodulation and how this helps them compared to higher nitrogen concentrations relative to health scores.

Ureide Assay

The Ureide assay identified the percentage of the total transport molecule-bound nitrogen in the plant was derived from the atmosphere. Nodulated plants from each strain at 5 and 50ppm were selected for Ureide testing and the results of the tests are shown in Table 10. A negative control was also used, and it contained the sap of several unnodulated plants. Strain 3456 displayed the highest percentage ureide-bound nitrogen for a single plant, and the highest average Ureide-bound nitrogen levels of all the strains at 5ppm. Strain ALICE was exluded from the 5ppm data because it was not possible to extract a large enough sap sample from the nodulated plants to run the assay.

Ureide Analysis												PPM
Strain	3456	3456	3456	PN201	PN201	2376	2376	2376	2376	3384	Control	5
	3456	3456	ALICE	ALICE	PN201	PN201	3456	2376	2376	3384	Control	50
Test #1												
Ureide	0.26	0.67	-0.003	0.088	0.023	0.083	0.054	0.068	0.123	0.092	0.021	5
	0.009	0.026	0.006	0.04	0.071	0.011	0.026	0.025	0.025	NA	0.05	50
Test #2												
Amino	0.055	-0.011	-0.016	0.005	-0.001	0.015	-0.001	0.012	0.023	0.019	-0.009	5
	0.008	0.003	-0.013	0.006	0.023	0.007	0.006	0.017	0.001	NA	0.029	50
Test #3												
Nitrate	0.484	0.441	-0.16	0.305	0.305	0.094	0.339	0.667	0.375	0.397	0.83	5
	0.007	0.412	-0.114	-0.083	0.183	-0.041	-0.088	0.146	0.015	NA	0.268	50
% Ureide Activity												
	33%	61%	17%	22%	7%	43%	14%	9%	24%	18%	5%	5
	0.375	0.058957	-0.04959	-1.08108	0.256318	-0.47826	-0.46429	0.132979	0.609756	NA	0.144092	50

Table 10: Results for quantifying transport molecule activity in a selection of plants from each strain group is displayed in this table. The average of ureide-N to total transport molecule-bound nitrogen was also calculated.

The data recorded in Table 10 is expressed in a bar graph in Figure 6. This graph also includes the crop yield and crop health for the individual plants tested in the Ureide assay. There was a relationship between crop yield and ureide activity which likely suggests that the ureide activity can substitute for nitrogen fertilizer to produce large crop yields. The relationship between ureide activity and crop health was less significant, however, plants expressing high ureide percentages were generally healthier than plants with very low ureide activities.

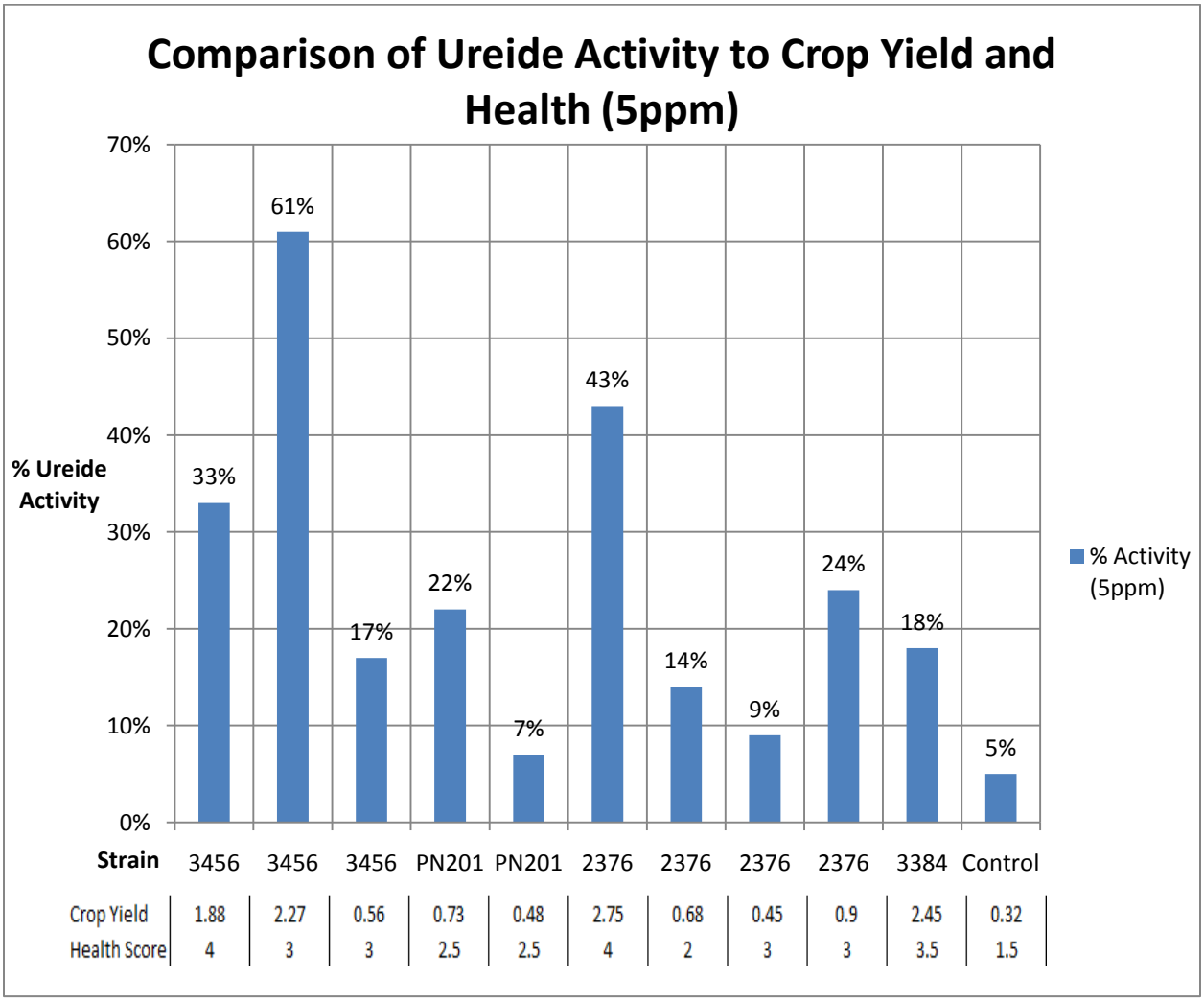


Figure 6: Column chart depicting atmospheric derived nitrogen percentage in chipilin plants grown in 5ppm nitrogen fertilizer. Health scores and crop yields are also shown for each plant tested for ureide percentage.

According to the summary in Table 11 strain 3456 was the optimal rhizobial strain because of the high infection efficiency, largest % Ureide activity, and the health score. Although two plants had higher crop yields the differences were negligible. Strains 3384 and 2376 had crop yields which were less than 0.1 larger than strain 3456. The average of all the plants was taken for the 200PPM and strain 3456 had a larger crop yield and a high health score than the 200PPM averages. Strain 3382 and 2376 were are of interest in comparison to the the 200PPM plants because they had a higher crop yield than 200PPM plants and comparable health scores.

Average including nodulated and unnodulated plants				
Strain	**Infection Efficiency**	**% Ureide Activity**	**Crop Yield (g)**	**Health Score**
3384	50.00%	18%	0.993	2.33
2376	66.60%	22.5%	0.99	2.42
BRADY	66.60%	14.5%	0.48	1.83
ALICE	33.30%	NA	0.315	2.3
3456	100.00%	37%	0.978	3.17
Control	0%	5%	0.203	1.42
*200ppm	0%	0	0.764	2.74

Table 11: This shows a summary of infection efficency, ureide activity, crop yeild , and health score of all the plants.

Discussion

Main Conclusions:

The major objective of this research is to maximally reduce the necessary amount of nitrogen fertilizer in Chipilin production. The data presented in this study support the hypothesis that specific rhizobial strains can reduce the fertilizer requirements of *C. longirostrata* by forty fold without reducing crop yield or plant health, See 5A and B. At low nitrogen fertilizer concentrations (5ppm) nodulated plants drastically outperformed unnodulated plants on the basis of crop yield and plant health. The 5 PPM concentration had a nodulated average ratio of crop yield to plant health of 0.906 grams to 2.92 centimeters. This is a better ratio than the 5PPM unnodulated average ratio of crop yield to plant health of 0.906 grams to 2.92 centimeters.

Furthermore, despite low nitrogen levels, nodulated plants produced crop yields and scored health ratings comparable to plants supplied with increasing concentrations of nitrogen fertilizer (50, 100, and 200ppm). Individual strain performance was analyzed at 5ppm and 200ppm to investigate whether a particular strain could be used as a nitrogen fertilizer substitute without diminishing crop quality. All five strains varied in their effect on crop yield and health, and strain 3456 was identified as the superior symbiont for Chipilin agriculture. This selection was based on four quantifiable properties: infection efficiency, crop yield, plant health, and N_2 fixation activity.

Host compatibility is an important factor in evaluating symbiosis. For example, a particular strain of rhizobium greatly reduces nitrogen requirements without diminishing crop quality; however it forms an infection with only 25% of the plants. There was a significant correlation between nitrogen concentration and crop quality for unnodulated plants, but not for nodulated plants infected with strains 3348, 2376, and3456. The plants that it failed to infect were more likely to die from nitrogen deprivation, produce unhealthy crop yields, or become victim of chlorosis. The effect of host compatibility can be visualized by comparing average yield and health of the visibly nodulated plants to the average yield and health of

all the plants of a certain strain (comparing the black and blue bars of figure 5). When interpreting the results for crop yield and strain health, host compatibility was included as a factor by not excluding unnodulated plants from the data. Host compatibility, for each strain was quantified by calculating infection efficiencies. All five strains tested positive for host compatibility at low nitrogen levels (5ppm). At 50ppm and 100ppm the strains showed an average reduction in infection efficiency of 20% and 43% respectively. At 200ppm no nodulation was identified for any strain. In severely limited nitrogen levels, the bradyrhizobium strain displayed the lowest rate of infection, 33.3% (2:6 nodulation: no nodulation). Strain 3384 had an infection efficiency of 50.0% (3:6 nodulation: no nodulation). Strains 2376 and Alice each had infection efficiencies of 66.6%, (4:6 nodulation: no nodulation). Strain 3456 was the most efficient strain, forming nodules in 100% of plants. The negative control showed no indication of infection.

Crop yield was quantified for each plant at harvesting time, and an average yield for each strain was calculated. Plants grown in 200ppm fertilizer had an average crop yield of 0.7643 grams. The greatest crop yields were attained from plants grown at 5ppm which were inoculated with strains 3384, 2376, and 3456. These three strains out-produced the average crop yield of plants grown in sufficient nitrogen by 0.23, 0.23, and 0.22g respectively. Strain Brady and Alice produced an average of 0.27 and 0.43g less crop than those grown at 200ppm.The negative control plants received no rhizobia inoculum and produced the least amount of crop, 0.561g less than the plants grown at 200ppm. By excluding unnodulated plants from the data, and thereby negating the factor of host compatibility, strains 3384 and 2376 greatly surpassed strain 3456 with crop yields of 1.510g, 1.200g and 0.978g respectively.

A successful infection thread and nodulation are not necessarily indicative of nitrogen fixation. Furthermore it has been reported that rates of nitrogen fixation do not necessarily correspond to the extent of nodulation in the roots. Therefore the activity of the nitrogen fixation process was assayed via the ureide assay. This assay colorimetrically determines the concentration of active atmospheric nitrogen transporter molecules allantoin and allantoic acid in the xylem sap of the plant. The activity was highest at

5ppm and strain 2376 and 3456 were found to have the highest levels of ureide activity, 0.960 and 0.820 respectively. Strains 3384 and Brady had activities of 0.098 and 0.094 respectively. Alice and the negative control had the lowest values of nitrogen fixation 0.084 and 0.041. Strains 2376 and 3456 had the highest Ureide activity and were also top of the top three in crop yields which supports that nitrogen fixation was the cause of the higher yield.

Health ratings were quantified on a scale from 1-4 and measures the density of leaf growth and the level of chlorosis for each plant. Like the crop yield, the plants with the best average health ratings were grown in 5ppm nitrogen fertilizer solution. Strain 3456 had the best average health rating of 3.17 (See figure 11), while strains 2376, 3384, and Brady had similar health ratings of 2.42, 2.33, and 2.30 respectively. The Alice and negative control plants scored the lowest health ratings, 1.83 and 1.42 respectively.The plants grown in sufficient nitrogen scored an average health rating of 2.74, higher than all of the 5ppm strains except for strain 3456. However, when unnodulated plants were excluded from the data, strains 2376 and 3384 attained health scores approaching that of strain 3456. Nodulated plants of strains 2376 and 3384 outperformed the 200ppm plants with a health score of 3.00 and 2.83 respectively. Nonetheless, strain 3456 still maintained a better average health score (3.17) than the nodulated plants of strains 2376 and 3384 even when the host compatibility factor was disregarded. A summary of the major conclusions is shown in Table 11.

Significance:

Analysis of each individual strain's performance at 5ppm made it possible to identify the best symbionts for chipilin production. At 5ppm, strain 3456 was the only strain to outperform 200ppm plants in both crop yield and health. Furthermore, strain 3456 had an infection efficiency of 100% making it the most compatible strain studied with *Crotalaria longirostrata*. It also had a high ureide activity suggesting that nitrogen fixation could be responsible for the success of these plants. Compared to the performance of other strains grown in 5ppm, strain 3456 had the highest average health score among all plants and

when unnodulated plants were excluded. Although strain 3456 surpassed the 200ppm plants with a relatively large crop yield, two strains outperformed it when the host compatibility factor is disregarded. Strains 3384 and 2376 attained the highest crop yields when unnodulated plants are excluded. However when the host compatibility factor is taken into account and the poor crop yields of the unnodulated 3384 and 2376 plants are included in the data, their average crop yield is not statistically greater than the crop yield of 3456. Furthermore, strains 3384 and 2376 did not surpass the 200ppm plants in crop health when host compatibility factor was considered. Strain Brady and Alice were not considered as an efficient *C. longirostrat* symbiont because they did not produce sufficient crop yields or health scores for consideration as a nitrogen fertilizer replacement. Refer to Figure 5 for crop yield and crop health comparisons among different strains when host compatibility factor is included in the data. Strain 3456 is the best symbiont studied for *C. longirostrata* because it allowed for healthy chipilin growth and high crop yields at miniscule nitrogen fertilizer availability. Furthermore it produced as high a crop yield as any of the other rhizobia strains while maintaining the highest health score.

Future Experiments:

After determining the most compatible rhizobial strain the next step would be determining what concentration of rhizobia would be the most economical. During the experiment the same concentration of bacteria was used for each strain. Using too few rhizobia for inoculation may result in decreased infection threads severely diminishing the ability of the plant to uptake nitrogen. This could be detrimental to large scale production considering the risk of drastically decreasing the amount of fertilizer. Therefore experimentation with different concentrations of inoculating doses would identify an optimal inoculation concentration.

Secondly in each of the experiments the same bacteria was used in an isolated system by itself. It could be possible that competitive, inefficient nitrogen fixers could outcompete a less competitive,

more efficient rhizobia. If this situation arises, it would be necessary to increase the inoculating dose of the desirable rhizobial strain.

When determining the adequate nitrogen concentration to add to the plants a large PPM range was investigated. A closer look at the lower PPM concentration (5-50) may be helpful in determining the most cost effective method for chipilin farming. Although the plants grow well at 5 PPM it could be plausible that the plants could give higher yields at a higher nitrogen concentration. The profit of the yield may outweigh the extra cost of the increased amount of nitrogen.

There were some limiting factors of this investigation which should be avoided in future experiments. For example, the bacterial growth curve was produced after inoculation so the quantity of rhizobia used to inoculate each strain type was not certain. Although it was estimated that the minimal number of bacteria to start an infection thread was used in the inoculums, and that excess bacteria only serve to outcompete foreign bacteria, much more concrete comparisons between strain efficiency could be obtained by inoculating each plant with the same number of bacteria. Furthermore, this experiment utilized plants which were not grown to full maturity. Due to space and time constraints it was not possible to grow the plants for five months while still having enough time to run analyses. By growing the plants to maturity, magnitudes of crop yield would be more relevant to large scale production purposes. Additionally, this investigation only utilized 6 plants per strain/nitrogen condition. A larger sample size would have produced much more concrete data. Lastly, when determining the adequate nitrogen concentration to add to the plants a large PPM range was investigated. A closer look at the lower PPM concentration (5-50) may be helpful in determining the most cost effective method for Chipilin farming. Although the plants grow well at 5 PPM it could be plausible that the plants could give higher yields at a higher nitrogen concentration. The profit of the yield may outweigh the extra cost of the increased amount of nitrogen.

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