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Identification of Rhizobia Species that can Establish Nitrogen-Fixing Nodules in Crotalaria Longirostrata

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IDENTIFICATION OF RHIZOBIA SPECIES THAT CAN ESTABLISH NITROGEN-FIXING NODULES IN *CROTALARIA LONGIROSTRATA*

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Abstract

Legumes are notorious for their ability to fix and use atmospheric nitrogen by forming a symbiosis with a rhizobia bacteria species. Dr. Frank Mangan and the UMASS Extension farm in Deerfield, MA wish to transplant and grow the El Salvadoran leguminous crop *Crotalaria longirostrata*, or "Chipilín," in New England to supply homeland crops for American immigrants. In order to better establish this crop in its new environment, six different rhizobia strains, some known to nodulate other Crotalaria species, were tested to find a strain that could effectively nodulate Chipilín and eliminate the need for an outside source of nitrogen. Over four months, 190 Chipilín plants were grown from seeds, maintained, and inoculated with cultured rhizobia strains. The effects of nitrogen application were also analyzed by applying different levels of nitrogen fertilizer to each inoculated plant. Upon harvest, nodule growth was discovered on every subset of inoculated plants and Western Blots were used to probe for leghemoglobin, a protein indicator of nitrogen fixation.

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Authorship Page

Throughout the creation of this document all team members put forth a collaborative effort to generate and revise the various sections.

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Chapter 1 Introduction

Members of the Fabaceae plant family, known as legumes, are able to form mutually beneficial symbiotic plant nodules with rhizobia that provide the bacteria with plant-derived carbohydrates in exchange for fixed nitrogen. Although over ninety percent of legumes can be nodulated, these relationships are also highly specific, as only specific strains of rhizobia can form effective nodules with each legume.

Dr. Frank Mangan of the UMASS Research Farms in Deerfield, MA has been working with the organization World Crops to transplant certain legumes and other crops popular within immigrant communities from countries like El Salvador and Brazil to New England. World Crops has proven that there is also an added economic incentive for New England farmers to grow immigrant crops because many people who immigrate to the United States are willing to pay more for homeland crops. Over the past summer, he attempted to transplant the crop *Crotalaria longirostrata*, more commonly known as Chipilín, to his farm in Deerfield, but was unable to sustain the crop without providing it with superfluous amounts of nitrogen fertilizer. Unfortunately, the amount of nitrogen fertilizer that farmers would have apply in order to grow Chipilín would make the crop unprofitable.

However, Chipilín has been found to form and derive nitrogen from effective symbioses with rhizobia in countries like El Salvador and Mexico where the crop naturally grows. Unfortunately, there are several laws that prevent or require several months of approval and paperwork to import bacteria strains from other countries making it very difficult to identify or obtain the El Salvadorian or Mexican strain of rhizobia.

This study focused on identifying a rhizobia strain that could effectively nodulate and provide a source of nitrogen to Chipilín and thus eliminate the need for nitrogen fertilizer. Over seven months, 120 Chipilín plants were germinated from seeds, inoculated with one of six rhizobia strains and maintained on a watering and fertilization schedule. After 80 days, the plants were harvested and the nodules were removed from each plant for further analysis. Effective nitrogen fixation was investigated through wet weight, dry weight, microscopy, and Western blot staining for the leghemoglobin, a protein indicator of symbiosis, analysis.

It was initially hypothesized that higher levels of nitrogen fertilization would inhibit the formation of nodules and that *Crotalaria longirostrata* would be best nodulated by a strain of *Bradyrhizobium* because nearly all members of this genus are known to form symbioses with this type of rhizobia. When coralloid nodules were found on every set of plants, including the negative control, it was later hypothesized that the source of contamination was the Chipilín seeds.

Although each group revealed the presence of globular-shaped and coralloid-shaped nodules, the pink-color of the coralloid nodules suggested that they were more effectively fixing nitrogen. Western blot results confirmed the presence of leghemoglobin in all nodules, however, it was impossible to conclude which set expressed the protein the most. Since all plant groups produced coralloid nodules, including the negative control, it is likely that an outside source of rhizobia equally contaminated all sets of plants. Future studies should further culture the coralloid nodules in order to investigate this strain of rhizobia.

Chapter 2 Literature Review

2.1 World Crops

In 1996, the University of Massachusetts founded, "World Crops," an organization that researches the market potential, production costs, and distribution system of immigrant crops, like taioba and Chipilín (Mangan et al.). According to Dr. Frank Mangan, United States immigrants comprise a large portion of the fresh fruits and vegetables consumer pool. Mangan believes that New England farmers could profit from and also benefit the exponentially increasing immigrant population by producing and marketing fruits and vegetables native to immigrants' home countries (World Crops).

Although World Crops initially only researched crops native to Puerto Rico and the Dominican Republic, since then they have investigated Asian, Brazilian and Latin American crops. Over the years, World Crops has also collaborated with "Flats Mentor Farm," or FMF, and introduced these immigrant crops to a group of Hmong farmers in Lancaster, MA as a profitable source of revenue (Mangan et al.).

Recently, Mangan and other collaborators experimented with Chipilín seeds exported from the Salvadoran Ministry and then hired Liliana Murillo, an agronomist from El Salvador, to help market Chipilín and pipián to American immigrants. World Crops is currently trying to develop a way to produce Chipilín, which is natively a perennial crop, as an annual crop in New England that dies off with the winter frost. (Mangan et al.). Unfortunately, initial trials were hampered by the garden pest known as the "potato leaf hopper," but the team was eventually able to negate its presence using Agribon and AG Insect Barrier row cover and PyGanic

pesticide. World Crops is now trying to identify the rhizobia strain that will nodulate the legume and provide the plant with a sufficient amount of fixed nitrogen (Hazzard).

2.2 Crotalaria longirostrata

2.21 The Crotalaria Genus

The genus Crotalaria contains over 600 dicot plant species, including Chipilín, that are mainly concentrated in Africa, but span tropical and mountainous regions as well. Crotalaria species are generally green, leafy bushes that are used as food or plant fertilizer ("Crotalaria longirostrata").

Most of these plants form symbioses with rhizobia species because they belong to the Fabaceae or, legume family. Until recently biologists thought that only species of the Bradyrhizobium Proteobacteria branch could nodulate Crotalaria plant species. However, Dreyfus et al. discovered that a few Crotalaria form symbioses with a highly specific methylobacterium species, which they named, "*Methylobacterium nodulans*," or *M. nodulans*. Interestingly, *M. nodulans* likely gained the ability to fix nitrogen through horizontal gene transfer with a Bradyrhizobium species because its NodA gene is similar to that of the Bradyrhizobium bacteria branch (Dreyfus et al.).

2.22 Crotalaria longirostrata, Chipilín

Although the perennial angiosperm crop Chipilín (USDA), or *Crotalaria longirostrata*, is relatively unknown in the United States, it flourishes in southern regions like El Salvador and Guatemala where people refer to it by other names like Chipilíno, Chepil, and Parrajachel. Dr. Sinclair first discovered the small shrub while traveling through Southern Mexico in 1891

Kingdom	*Plantae* – Plants
Subkingdom	*Tracheobionta* – Vascular plants
Superdivision	*Spermatophyta* – Seed plants
Division	*Magnoliophyta* – Flowering plants
Class	*Magnoliopsida* – Dicotyledons
Subclass	*Rosidae*
Order	*Fabales*
Family	*Fabaceae* – Pea family
Genus	*Crotalaria* L. – rattlebox
Species	*Crotalaria longirostrata* Hook. & Arn. – longbeak rattlebox

Figure 1: Taxonomy of the plant *Crotalaria longirostrata* (USDA)

This figure shows the different levels of taxonomy of Chipilin, beginning with the kingdom and ending with the species

(Morton). Since then, Chipilín has been characterized by its woody stem and upright branches that support small alternating green dicot leaves.

Despite its underwhelming popularity, Chipilín is highly nutritious because it is rich in calcium, iron, riboflavin, thiamine, ascorbic acid, and niacin (Morton). Apart from its vitamin content, its leaves are high in protein, and its stalks-with-leaves are high in fiber (Arias et al.). Unfortunately, Chipilín is not easily digested; Arias et al. believe that Chipilín might contain plant tannins which typically make plants indigestible.

Figure 2: Tortillas made with Chipilin (Dardón)

This figure shows Chipilin tortillas that are available for sale in some American markets

Crotalaria longirostrata encompasses a variety of uses in different countries. In Honduras it is used in soup as well as in Guatemala, where it is also used in stews and omelettes; people in El Salvador use Chipilín as an herb and in white cornmeal (Morton).

However, it can be highly toxic because it accumulates alkaloids and gamma-glutamyltyrosine which are poisonous to animals. In fact, people in some countries, like Guatemala, use it as a means to eliminate animals that are dangerous. Hawaiians also overlook its nutritional value and treat Chipilín as an invasive weed (Morton).

2.3 Geological History of El Salvador

El Salvador, the major producer of Chipilín, is located in Middle America between Guatemala and Honduras, two countries that also widely market Chipilín. Though its latitudinal coordinates, 13-14.5 degrees north, place El Salvador in the middle of the tropics, the country's higher elevation lends to an "intermediate tropical zone" (Sheets) its highlands span from 1500-1800 m. The country's highlands span elevations from 1500-1800 m, but its interior reaches elevations as high as 2000 m. As a result average rainfall and temperature vary across the country. El Salvador's Lowlands and capital city reach higher temperatures, in the 80s and 90s (F), and receive lesser precipitation, around 1700 mm, than mountainous regions, but receive higher precipitation than deep valleys (Britannica).

Figure 3: Map of El Salvador and Surrounding Countries (Britannica)

Figure 3 shows a map of El Salvador, and its surrounding countries, Guatemala to the Northwest, and Honduras to the Northeast

Geologists agree that much of this climatic diversity is likely also related to the eruption of "Volcan Ilopango" (Ilopango Volcano), which erupted over the Western portion of El Salvador in the late 3rd Century (Sheets). As a result, volcanic deposits, lava, and alluvium, enrich much of the nation's soil, but isn't considered useable for agriculture because this also makes it prone to

erosion. Most of El Salvador's agriculture occurs near the Southern coast because its constant high-temperatures and the country's seasonal rainfall, or "temporales," support it ("El Salvador" Britannica).

2.4 Rhizobia Infection and Nodulation

Nodulation is an important process that occurs as a relationship between many legumes and soil-dwelling rhizobium bacteria in a symbiotic root-microbe interaction. Experiments and research investigating the molecular processes behind rhizobial processes have revealed insight on several of the mechanisms responsible for the plant-bacteria interaction. Two model legumes have been the focus of research: *Medicago truncatula* and *Lotus japonicus* (Mathesius 2009). Although not everything is known about this symbiotic relationship, much is now understood of the interaction of rhizobia and legumes. One of the major and most-agreed upon findings is that the processes involved with the symbioses are extraordinarily complicated and that substantial further exploration of the field is warranted.

There are six groups of bacteria that are known to nodulate several leguminous plant species and they are the *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* (Fauvert & Michiels). Recently, another group of bacteria, the *Methylobacterium* has also been observed to be capable of rhizobial symbiosis (D'Haeze & Holsters). These bacteria colonize the root hair tip regions of the plant and eventually, through a complicated series of molecular interactions, form nodules on the root hairs in which they fix atmospheric nitrogen to a form of nitrogen that is usable by the plant in exchange for the ability to receive carbohydrates from the plant. Interestingly enough, the interactions are highly specific, and the species of rhizobia available in the area surrounding a plant must be a certain species in order for any nodules to form (Fauvert & Michiels).

Rhizobial symbioses have been observed to be initiated by the host organism, the plant, through the excretion of molecules known as "flavonoids." Experiments have shown that these molecules are generally produced by the plant only when soil nitrogen levels are low, perhaps to conserve energy, in an effort to attract nearby rhizobial bacteria and essentially form rhizobial colonies at plant root hairs (Cho & Harper). As soon as this colonization occurs at the root hair tip, the root hair tip is divided into three different zones, Zones I-III, each containing a different cellular polarization and function (D'Haeze & Holsters). This lack of nitrogen is thought to trigger the plant to follow the "central phenylpropanoid pathway," and the "acetate-malonate pathway," both of which combine to result in the production of the plant metabolites called "flavonoids." The molecular composition and number of flavonoids that are produced by a specific plant vary between species, and are generally chalcones, flavones, isoflavones, or coumestrans. Each flavonoid interacts differently between species of rhizobia, and can induce certain genes in one bacteria, but inhibit those similar genes present in another (Cooper).

Scientific literature agrees that the production of very small amounts of these molecules, even micromolar to nanomolar concentrations, triggers a series and variety of biological events if the correct rhizobia have congregated near the plant roots (Cooper). The major molecular event that ensues following their excretion which sets the plant on the path to successful symbiosis, is the activation of "nod genes" within the rhizobia population that has gathered at its root hairs. The group of genes within the plant referred to as "nod genes," include nod, nol and noe genes, and have found to be induced by a total of 30 different flavonoids among plant species, each flavonoid(s) specific to the plant and bacteria (D'Haeze & Holsters) (Cooper).

The most prominent series of response elicited by the induction of nod genes involves the production of what are known as "nod factors," or "lipochito-oligosaccharides." These molecules

were first discovered in 1990, as produced by the bacteria *Sinorhizobium meliloti*. The precise means by which this is done are not certain, though research thus far has suggested that the induction of these "nod genes" within the bacteria requires the presence of a coinducer. In most cases this coinducer has been found to be, "NodD," a protein that is constantly produced by bacteria. This complex is thought to bind to sites within the bacterial genome known as "nod boxes," which are similar in function to "TATA" boxes within the human genome. The NodD protein is thought to gather at the site of the nod box first, creating a slight bend in the DNA site; upon binding of the appropriate flavonoid, the bend becomes much more severe and the complex at this promoter region results in the co-induction and expression of the nod gene. The transcription the nod factor encoded by the nod gene at hand then follows (Cooper).

The number of nod factors that can be transcribed by a specific rhizobia species varies greatly between each bacterial species, within a span of 2 to 60 nod factors (D'Haeze & Holsters). Though two nod factors can have vastly different functions within the bacteria or host plant, the basic structure and molecular basis of each are strikingly similar. Each nod factor consists of a backbone made of oligosaccharide, a fatty acid, and a certain combination of substituent groups. A nod factor derives its specificity through the number of beta-1,4-linked N-acetyl-D-glucosamine residues in the backbone, whether the fatty acid is saturated or unsaturated and which substituent groups and how many of each are included in the nod factor (Cooper).

Although, the production of nod factors is one of the greatest responses elicited by the excretion of flavonoids by the host plant, several other interactions are triggered by their release. Another major pathway initiated by flavonoids, are those that lead to the production of certain bacterial proteins. The protein, "NodO," which is known to be specific to only a couple of rhizobial species, is transcribed by the nodO gene through co-induction by a flavonoid and

NodD, in similar manner to the co-induction of nod factors (Fauvert & Michiels) (Cooper). Through scientific testing, NodO has been identified to play a substantial and necessary role in the recognition of nod factors by the host plant. Findings demonstrated if the gene encoding NodO was mutated, nodulation is not observed in the specimen. It is for this reason Sutton et al. proposed this protein contributes to the calcium spiking that occurs later in symbiosis (Fauvert & Michiels). Though the presence of NodO has only be observed in less than a handful of rhizobial species, it is likely that other rhizobia produce proteins similar in function, demanding further research on its function and molecular importance.

At some point in between the production of flavonoids by the plant host and the bacterial response to produce nod factors, a molecular interaction must take place where the host plant and rhizobia in a sense, "recognize" each other as compatible candidates to form a mutual symbiosis. Though thorough research and field studies on the topic have been performed, with some probably still underway, the exact method has not been proven, and only possible mechanisms have been proposed. Evidence has shown that the most probable host-recognition mechanism involves a bacterial receptor consisting of polysaccharides on the surface of the rhizobial cell. Lectins produced by the host plant are thought to interact and bind with these receptors, which by some undefined means trigger for the rhizobia to enter the plant. Scientists have experimented with the deletion of bacteria receptors that bind with lectins and have found it inhibits nodulation (D'Haeze & Holsters). This hypothesis is also supported by the fact that flavonoids produced by the plant often are involved with the alteration of bacterial surface polysaccharides structurally and molecularly, and it would therefore not be unlikely that some step also occurring towards the beginning of the symbiosis involved the same molecules (Cooper).

Although the majority of evidence thus far supports a theory involving surface polysaccharides as receptors for plant lectins, the "sym 10" bacterial molecule has also been identified as a receptor of interest (D'Haeze & Holsters).

From this point on, the flavonoids continue to promote the transcription of nod genes and other genes induced and co-induced by flavonoids. The proteins and nod factors that result from this change in gene expression within the bacteria begin to trigger their own cascade of events within their own cells and those of the host plant. The initial molecular activity that follows the release of nod factors is the induction of nodulin genes within the host plant. Again, due to the complexity of the symbiotic reaction, the specific function of these genes and their products are not yet completely understood (Cooper). The main genes classified as nodulin genes are the ENOD and PSENOD12 genes that are present, or genes similar to these, within several plants. The activation and initial transcribed products of these genes have been documented to arise as late as two days after the initial inoculation by rhizobia and are thought to play a significant role in appropriate molecular processes involved with initial steps of rhizobial infection (D'Haeze & Holsters).

Nod factors, however, have several more roles apart from the induction of plant nodulin genes, and are thought to interact with plant cells to some degree at every step of the infection. It is at this point that the zones of the root hair tip begin to take on different roles within the root hair.

A recent study has identified the rhizobia that have colonized the root hair tip as the "infection organiser," identifying it as the source of cause for the changes in polarity that are observed in these zones. As infection organiser, the rhizobia colony is attributed to putting out a

series of molecular signals that trigger the cytoskeleton of the plant cells to begin changing its morphology. One of the most important cytoskeletal changes reported to occur is the formation of "cytoplasmic bridges" within the outer cortical cells of the root hair which comprise the area which will later become the "infection thread" (Catoira et al.). Zone I is classified as the portion of the root hair that contains all root hair cells that are still growing, or are in the G_1 phase of the cell cycle. Zone III contains all mature cortical cells that have fully differentiated and stopped proliferating. Cells that are in Zone II, or the "susceptible zone," are all cells that have stopped growing, and are stuck G_2 of the cell cycle. Most symbiotic activity is observed within this zone, and it is mainly at this site that the nod factors interact with plant cells. At this time, this zone is characterized by a distinct, specific polarity where most organelles and vacuoles are in these cells. The initial influx of nod factors results in the reinitiation of growth within Zone II and erratic changes in calcium levels within these cells. The exact pattern of these fluctuations varies between symbioses, but eventual stabilized oscillations of calcium are observed to occur in all species. These variances in calcium levels as a whole are referred to as "calcium spiking" (D'Haeze & Holsters). DMI3, seemingly downstream of the calcium fluctuation, may be responsible for sensing calcium levels by

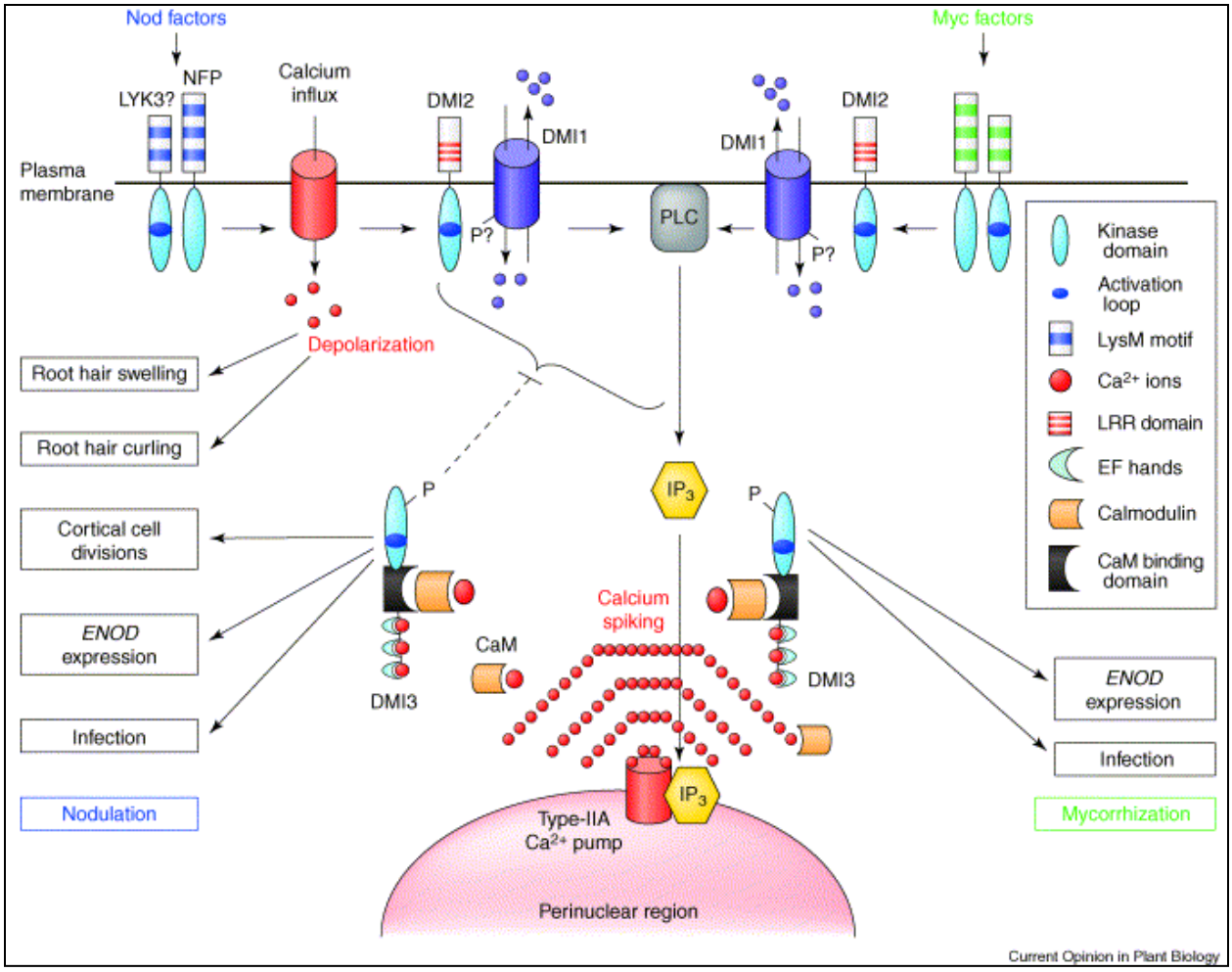


Figure 4: Proposed Model of the Nod-factor Signaling Pathway (Riely)

binding calcium and calmodulin (CaM) bound calcium, which begins autophosphoylation and kinase activation, and also acts as a negative regulation of the calcium response. Activated DMI3 allows for the phosphoylation of downstream targets which allows nodules to develop (Riely). Studies have shown that the presence of nod factors are thought to interact with the plant HCL gene (identified in alfalfa, but thought to be present in other plant species as well) or LYK3 to initiate this series of events (Catoira et al.) (Riely). Figure 2 shows the likely scenario of this molecular cascade. Figure 2, below, shows a proposed model of the Nod-factor signaling pathway within the plant roots.

When oscillation of calcium levels is observed, which can occur from 20 to 60 minutes after the initial change in calcium levels, the membrane of the cell is depolarized and then the area around the cell slowly increases in pH. As with other specifies involved with rhizobial symbiosis, the exact mechanisms surrounding these processes cannot yet be fully explained. Still, it is likely that these changes in ion levels and pH level are involved in some way with the correct gene expression required for rhizobial symbiosis with the host plant (D'Haeze & Holsters).

Subsequent to this, "root curling," or the formation of what are known as "Shepherd's crooks," occurs. This change occurs soon after the hair terminates growth, but then begins to 'grow' in a direction different than archetypal due to interaction with the rhizobia that causes the root hair to swell and grow (Geurts). This curling of the root hair essentially traps all neighboring rhizobia between the plant cells that enclose them, allowing the bacteria to degrade the cell walls and then break through the cell membrane in combination with increased turgor pressure to begin the formation of an "infection thread" (Catoira et al.). As this infection thread begins to form in the outer cortical cells, the rhizobia continue to follow the infection thread through each adjacent plant cell. This entry allows the bacteria to go around the plant's innate defenses. Simultaneous to this, the inner cortical cells, which are located in Zone II, begin to replicate again, exiting the G_2 phase of the cell cycle. As these cells begin to divide again, they start to form the nodule primordium. The infection thread continues to travel through the outer cortical cells until they meet with the inner cortical cells and deposit the rhizobia within the nodule primordium that have developed into nodules. When the bacteria reach the nodules, they begin to develop into their differentiated form known as, "bacteroids" (D'Haeze & Holsters).

When the bacteria differentiate into bacteroids, the rhizobial symbiosis is considered to

be complete. Even after the nodules have formed, nod factors and bacterial proteins are thought to be continually produced to ensure that the plant defense mechanisms against the rhizobia are not activated and the symbiosis is maintained (D'Haeze & Holsters). After the infection of rhizobia in the formed nodules the complete, the bacteria can then begin to convert free-formed nitrogen from the air into ammonia (fixed nitrogen) which the plant can then utilize in the form of amino acids. In exchange, the plant exports carbon into the rhizobia. Coordination of both partners is required for the exchange, and may be limited at times of sufficient ammonia supply (Mathesius 2009).

Still, many aspects and specificities surrounding the rhizobial symbiosis are not clear. Studies have been able to identify several other proteins and molecules that are certain to play some sort of a role in the symbiosis, yet their exact function and whether they are necessary for the symbiosis have not been determined. One of the major groups of proteins that is thought to be involved in the symbiosis, but perhaps not entirely necessary, is the hoponoids. The usual role of these proteins within bacterial cells is to provide structure and stability to the cell membrane. During the formation processes of a rhizobial symbiosis, increased expression of the genes encoding for the production of hopanoids is observed. However, the presence of the actual hopanoid proteins within the cell has not been seen, and thus their function, or existence cannot be identified or confirmed (Cooper).

2.4 Nitrogen Regulation

Nitrogen is one of the exchange components of the rhizobia-legume symbiosis. The rhizobia bacteria can fix organic nitrogen in the soil into ammonium nitrate (NH_4^+), which can be used by the legume in amino acid synthesis. The source of organic nitrogen can come from several places. The earth's atmosphere, for example, consists of 78% nitrogen. This nitrogen can

enter the soil through rainfall, which adds about 10 pounds of nitrogen to the soil per acre each year. Decomposition of plants also forms soil organic matter which is about 5 percent nitrogen. Generally about 1 to 3 percent of this organic nitrogen is converted per year by microorganisms, like rhizobia, to form usable ammonium (NH_4^+). If legume roots are well nodulated, the legume plant does not benefit from the addition of nitrogen in the form of fertilizer, which is most commonly in the forms of manure or commercial fertilizers (Barbarick).

2.41 Nitrogen Transformations

Nitrogen exists in several forms and undergoes chemical and biological reactions to alter structure. Organic nitrogen (N_2) can be changed into ammonium nitrogen (NH_4^+) through mineralization. Organic nitrogen makes up over 95 percent of soil nitrogen. It cannot be used in plants, but can gradually become ammonium due to soil microorganisms. Mineralization is the process used by rhizobia bacteria in the symbiosis with legumes (Barbarick).

Ammonium nitrogen (NH_4^+) can be transformed into nitrate nitrogen (NO_3^-) through nitrification or to ammonia gas (NH_3) through ammonia volatilization. Ammonium can change rapidly into nitrate, which is the form of nitrogen most easily used by plants. It is also the type of nitrogen most associated with nitrogen pollution. Ammonia gas is produced through ammonia volatilization when soils have a high pH. This gas, and nitrogen, is then lost back into the atmosphere (Barbarick).

Ammonium nitrogen (NH_4^+) or nitrate nitrogen (NO_3^-) can both change into organic nitrogen through immobilization. This process entail nitrogen forms becoming entrapped in the microbial tissue of decomposing plant residues. Nitrate nitrogen (NO_3^-) can also transform into gaseous nitrogen through denitrification. Gaseous nitrogen can be lost to the atmosphere when

nitrate nitrogen converts to the gaseous form due to insufficient air within the soil, which causes microorganisms to use the oxygen from nitrate in its place (Barbarick).

2.42 Nitrogenase

Rhizobia bacteria utilize nitrogen from the atmosphere and fix it into ammonia (NH_4^+), which may then be exported into the plant's TCA cycle to form amino acids:

$$N2 + 8H + 2H+ \rightarrow 2NH4+ + H2$$

(Slonczewski). The enzyme nitrogenase plays an essential role by catalyzing the initial step to nitrogen fixation (Ohki). Nitrogenase is synthesized by both the free-living form of rhizobia and the plant-bound bacteroids (Beringer et al). Production is therefore independent to symbiosis with legumes, but the production and resulting effect differ slightly between each.

The success of the symbiosis is directly dependant on how the integration of fixed nitrogen is controlled and expressed by nitrogenase. The regulation of this enzyme varies between free-living and plant-bound forms of rhizobia. In free-living bacteria glutamine synthetase, an enzyme involved in the assimilation of fixed nitrogen, plays a key role in controlling nitrogenase (Beringer et al.). High ammonia (NH_4^+) concentrations cause glutamine synthetase to be repressed, and even low concentrations of ammonium added to a culture cause rapid inhibition of nitrogen fixation that only resumes when the ammonium is exhausted and the nitrogenase can resume its activity (Munoz-Centeno). Only negligent amounts of ammonia cause the activated form of the enzyme to be synthesized. This active form of the enzyme is a positive inducer of nitrogenase synthesis. In plant-bound bacteroids, ammonia (NH4+) or nitrate

(NO_3^-) also act as repressors of nitrogenase, but the concentrations required for repression of the enzyme are much greater than that required for free-living form of the bacteria (Beringer et al.).

Since nitrogen fixation in the bacteroids is dependent on the energy provided by respiration, they are also dependent on the influx of oxygen into the cell. The movement of oxygen into the cell is carried out by the gradients of oxygen concentration in the gas spaces between cells or the oxygenation of the oxygen carrier leghemoglobin. The influx of oxygen into the cell is causes a dilemma. Oxygen is required to produce energy, but a certain threshold of oxygen causes irreversible damage to the nitrogenase enzyme. Even small amounts of oxygen can cause a temporary, reversible inhibition of the enzyme. Legumes have developed mechanisms to balance oxygen (maintaining low oxygen concentration, while providing oxygen fluxes to bacteroids to support respiration rates) using concentration gradients. The first is a physical barrier surrounding the interior of the nodule which prevents gas diffusion. The second mechanism developed by both legumes and bacteria is the pigment leghemoglobin, which facilitates oxygen diffusion within bacteroid cells (Denison). The production of leghemoglobin is truly an example of symbiosis, as both the host and bacteria are required for synthesis, and it's presence is required for significant nitrogenase activity to occur (Beringer et al.).

2.43 Leghemoglobin

Leghemoglobin, as described above, is a red pigment protein that acts as a high affinity oxygen carrier within bacteroid cells. Its function is quite essential for the fixation of nitrogen to occur. Without the influx of oxygen that this pigment transports throughout the bacteroid, the oxidative respiration process of the bacteroids would suffer. With the concentration of oxygen mismanaged, or the high free oxygen content would inactivate the nitrogenase enzyme. It has

been shown that nodules lacking this important protein invariably lack nitrogenase activity, and therefore lack nitrogen-fixing capability (Beringer et al.).

The synthesis of this protein reflects the remarkable symbiosis between legumes and rhizobia. The globin component is produced by the plant. The plant genes encode the primary structure of the protein (the globin part), but the protein remains unexpressed until interaction with the rhizobia species. The rhizobia species not only allows for expression of the protein to occur, but also allows for the synthesis of a prosthetic group of the structure. This prosthetic group expresses enzymes δ-aminolaevulinic acid synthase and ferrochelatase which allow for biosynthesis of the haem component of the protein structure. The two components, synthesized separately, can combine spontaneously to form the function leghemoglobin protein. This collaboration therefore means that leghemoglobin is only found in legume roots infected with rhizobial bacteroids, and not in either free-living bacteria or uninfected legume tissue. As a major product of the symbiosis, leghemoglobin can quantitatively account for up to forty percent of the total soluble nodule protein (Beringer et al.).

Chapter 3 Methodology

3.1 Experimental Design

The following chart illustrates the intended experimental design.

#	Strain	Nitrogen	Experiment	Inoculation Set
10	Brady	Ø	Strain-Nitrogen	Set 1
10	3384	Ø	Strain-Nitrogen	Set 1
10	3456	Ø	Strain-Nitrogen	Set 1
10	Ø	Ø	Strain-Nitrogen	Set 1
10	Brady	1x	Strain-Nitrogen	Set 1
10	3384	1x	Strain-Nitrogen	Set 1
10	3456	1x	Strain-Nitrogen	Set 1
10	Ø	1x	Strain-Nitrogen	Set 1
10	Brady	5x	Strain-Nitrogen	Set 1
10	3384	5x	Strain-Nitrogen	Set 1
10	3456	5x	Strain-Nitrogen	Set 1
10	Ø	5x	Strain-Nitrogen	Set 1
2	Brady	Ø	DF Competition	Set 1
2	3384	Ø	DF Competition	Set 1
2	3456	Ø	DF Competition	Set 1
2	Ø	Ø	DF Competition	Set 1
2	Brady	1x	DF Competition	Set 1
2	3384	1x	DF Competition	Set 1
2	3456	1x	DF Competition	Set 1
2	Ø	1x	DF Competition	Set 1
2	Brady	5x	DF Competition	Set 1
2	3384	5x	DF Competition	Set 1
2	3456	5x	DF Competition	Set 1
2	Ø	5x	DF Competition	Set 1
3	Alyce	Ø	Strain-Nitrogen	Set 2
3	2376	Ø	Strain-Nitrogen	Set 2
3	101	Ø	Strain-Nitrogen	Set 2
3	A	1x	Strain-Nitrogen	Set 2
3	2376	1x	Strain-Nitrogen	Set 2
3	101	1x	Strain-Nitrogen	Set 2
3	A	5x	Strain-Nitrogen	Set 2
3	2376	5x	Strain-Nitrogen	Set 2
3	101	5x	Strain-Nitrogen	Set 2
2	A	Ø	DF Competition	Set 2
2	2376	Ø	DF Competition	Set 2
2	101	Ø	DF Competition	Set 2
2	A	1x	DF Competition	Set 2
2	2376	1x	DF Competition	Set 2
2	101	1x	DF Competition	Set 2
2	A	5x	DF Competition	Set 2
2	2376	5x	DF Competition	Set 2
2	101	5x	DF Competition	Set 2

Each number of a specific group of plants was given a letter (a-j) for individual plant identification purposes. The total number of plants in experimental rotation is 189, with 36 extra plants (Ø Rhizobia, 1x fertilization). The Strain-Nitrogen experiment tests the variable of both applied rhizobial strain and N concentration content in fertilizer application. The Deerfeild Completion Study (DF) tests the strain and fertilizer content as well as competition within native soil.

The greenhouse where the plants were grown was set up as follows along an easterly facing wall:

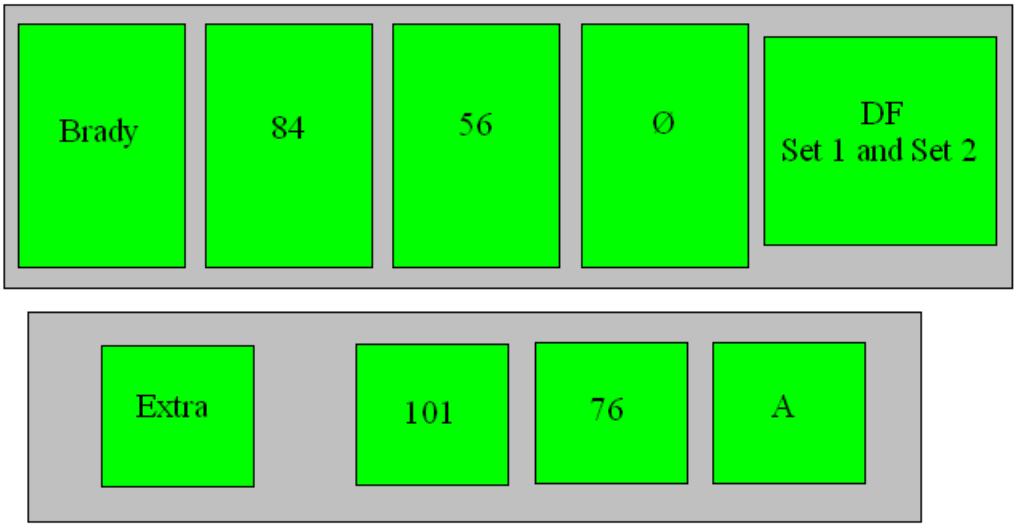


Figure 5: The placement of plants as groups within the greenhouse.

Within each indicated block of Rhizobial strain, plants of all fertilizer applications were kept.

3.2 Germination

3.21 Chipilín

Chipilín seeds were kindly provided by Dr. Frank Mangan (Department of Plant, Soil & Insect Sciences - University of Massachusetts Amherst).

3.22 Germination

Chipilín germination took place over a period of four weeks, with the majority of seeds planted in the first two weeks. Two sizes of Jiffy-7 Peat Pellets were used for germination: large and small. To prepare for seed insertion, the dried peat pellets were placed in trays with drainage capacity, and reconstituted by the addition of water until pellet reached final growth sizes (large peats: 1.5 inches diameter x 2.5 inches height, small peats: 0.75 inch diameter x 1 inch height). This process took between 10 and 20 minutes, and trays held 55 large peats and 112 small peats. The first set of peat pots (4.5 trays of large peats) were seeded in the greenhouse and subsequent seeding was done in a controlled temperature setting (Goddard Hall 206 laboratory, GH206) due to unexpected cold temperatures in the greenhouse.

Figure 5: Chipilín seeds

The seeding procedure was completed as follows. A hole of approximately 0.5 inch depth was created in reconstituted peat pots with tweezers. Approximately 3 to 5 seeds were then placed within each peat pot. Seeds were then covered with peat material. Peat pots were then placed in trays to await germination. During subsequent germinations/later time periods, saran wrap was placed over trays to retain moisture and heat throughout the period.

Seedlings were grown in a controlled temperature setting (in GH206). The first set of plants seeded in the greenhouse were moved to this location approximately 50 hours after planting. All others were seeded, germinated, and experienced initial growth in this single location. In this location, the temperature remained fairly stable, and artificial lighting was placed to give additional warmth and light to the germinating seeds and seedlings.

3.23 Plant Maintenance

While in germination period, the peat pots were watered until moist 4 to 5 times per week until germination was complete. Once germinated the seedlings were subsequently watered 3 times each week until moved into the greenhouse. The seedlings were moved to the greenhouse (right, which maintained an approximate 70^{o}C daytime temperature) after the germination stage, when most were at the 2 leaf stage and approximately 3 to 5 cm tall. All plants were less than 4 weeks old at this time. Once in the greenhouse, the seedlings were watered 2 to 3 times each week.

For the duration of the growing period, plants were observed three times per week, noting change in the color of the leaves, height of the plants, and any additional plants that grew alongside the main Chipilín plant. Some plants grew secondary Chipilín plants during this period, and others, especially those involved in the Competition study, grew other, unidentified plants/weeds. These secondary plants were extracted upon observation.

3.3 Transplantation

All plants were transplanted into sterile, perlite material 9½ weeks after first seeds were planted. To do this, one 4 inch pot was filled half way with perlite material. This medium was watered to compact the perlite material. The mesh material encasing the peat of the peat pot was carefully removed and the plant was placed into the pot, on top of the perlite material. perlite was then used to fill pot to approximately 2 cm under rim of pot, encasing the peat material in perlite material. The pots were then watered again (inconsiderable amounts) to compact the medium again. A total of 215 plants were transplanted.

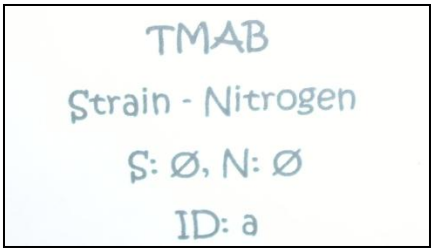
All pots were labeled with [1 1/3” x 4”] labels with appropriate plant designation. The label components consisted of:

Experimenter Label

Study

Strain, Nitrogen Application Details

Individual ID



Each label was secured with 2” width clear tape to encase and preserve label from water.

Approximately 4 weeks subsequent to the perlite transplant, the plants destined for the Competitive Deerfield Soil Study (DF Comparison Study), were transplanted into soil kindly bequeathed by the UMASS extension farm located on River Road in Deerfield, MA by Dr. Frank Mangan (Department of Plant, Soil & Insect Sciences - University of Massachusetts Amherst). The soil was taken directly from the farm where Chipilín crops had grown the previous summer months. The soil had 300 lb/acre of nitrogen containing fertilizer applied throughout the duration of this growing period. The appropriate plants were carefully extracted from the perlite medium. The excess perlite was mixed with Deerfield soil to obtain an approximate 1:1 ratio. The pot was filled with this soil

Figure 7: UMASS Extension Farm, Deerfield, MA

mixture and the plant was then carefully transplanted back into the pot with this soil mixture. The pots were watered at this time to compact the soil mixture.

3.4 Inoculation

3.41 Rhizobia

Rhizobia species were kindly provided by several sources for this investigation. Dr. Frank Mangan (Department of Plant, Soil & Insect Sciences - University of Massachusetts Amherst) kindly provided ALYCE Rhizobia, a strain commonly used on cowpeas. Becker Underwood (ISO Rep Marita McCreary, QC Manager Padma Somasageran) provided Bradyrhizobium sp. PNL0i. And lastly, Patrick Elia (USDA ARS, Soybean Genomics and Improvement Laboratory, National Rhizobium Germplasm Resource, Maryland) kindly provided USDA110 (a leguminosarum type strain), USDA2370 (The Rhizobium), USDA3456 (a misc cowpea strain), USDA3384 (a broad spectrum inoculant used on several Crotolaria and isolated in Porto Alegre Brazil). Strains USDA3456 and USDA3384 were lyophilized and required reconstitution as done using Mr. Elia's instructions.

Table 1: Common annotations used in the following methodology/discussion are as follows

Name	Annotation
Dr. Frank Mangan's Cowpea Rhizobia	Alyce/A
Bradyrhizobium sp. PNL0i	Brady/B
USDA110	110
USDA2376	76
USDA3456	56
USDA3384	84

3.42 Media

Modified Arabinose Gluconate (MAG) media, as suggested by Patrick Elia (USDA ARS, Soybean Genomics and Improvement Laboratory, National Rhizobium Germplasm Resource, Maryland), was used for growth and storage of rhizobial strains over the course of the project. The method for formation and included correspondence may be seen in Appendix C.

3.43 Growth Curve

To measure the concentration of cells within a specific volume over a specific period, a growth curve was created.

For each strain being tested, 25ml of MAG media was placed into a 50ml conical tube. Each conical tube was then inoculated with either a loop of the bacteria as grown on a MAG agar or with 250µl of cultivated stock media. Conical tubes were then placed on "shaker" at room temperature. At each 24 hour interval period, the Optical Density (OD) reading was taken at 600nm on a Jenway 6305 Spectrophotometer with Plastibrand 1.5ml semi-micro disposable cuvettes. A serial dilution was also created with 100µl of 3 dilutions being plated on MAG agar for each strain each day for 7 days (168 hours). Plates were left at room temperature for growth period. Plates were observed, and resulting colonies were counted on Day 5 after culture inoculation.

This procedure was followed several times. Two growth curves were created for strains Brady, 3384, 3476, and 2370 (See Appendix F). For the first growth curve, D1 (Day 1), D2, and D3 plates were counted 6, 5 and 4 days after plating respectively. D4, D5, D6, and D7 were placed at 4^{o}C after 7 days of growth and observed and counted 9 days subsequent to this. For the second growth curve created, the procedure was followed as stated.

Another growth curve was created for Alyce and 110 strains. See Appendix F for results. This growth curve was performed slightly differently than the previous two. OD readings were not taken throughout. The trial also only lasted for 5 full days (120 hours).

3.44 Plant Inoculation

To prepare inoculants to directly apply to plants, a slightly altered technique to rhizobia growth in MAG media was taken. To begin inoculation, 1 full loop of rhizobia from completed growth trials was taken and reconstituted in 1ml of MAG media in an appropriately labeled 15ml conical tube for each strain. This inoculated media was allowed to grow overnight at room temperature under constant motion on a shaker. 24 hours later, the full 1ml contents were used to inoculate appropriately labeled 300ml MAG media in 1000ml flasks. This flask was covered with a foam stopper and foil and then placed on the New Brunswick Scientific I24 Incubator Shaker Series at 190RPM and 25^{o}C for 120hours (as determined by previous growth trials).

At 120 hours, the fully inoculated media was applied to approximately 2 to 3 month aged plants. 5ml of the inoculant was applied to the base of each appropriate plant. To prepare plants for inoculation, plants were not watered before application, and subsequent watering was completed with minimal water application.

Plants were inoculated in two groups. The first set, aged 6 to 10 weeks, included plants inoculated with strains of Brady, 3456, and 3384, and the negative control (MAG media). Day 0 of the trial is the date of this first set of inoculations. The second, smaller set- aged 8.5 to 12.5 weeks, inoculated 18 days following (Day 18), included Alyce, 110, 2370.

3.5 Fertilization

All plants were fertilized twice during the 60 day growth period. The first fertilization was applied on Day 22 and the second was applied on Day 46.

All plants received 5ml of 0-10-10 (*nitrogen- phosphorus*- potassium) Koolbloom liquid fertilizer with at concentration of approximately 175ppm. Plants were grouped in 3 different concentrations of nitrogen application. The nitrogen 10-0-0 solution was created using Peter's Excel water soluable fertilizer. The three concentrations tested, and applied in 5ml increments, included 0ppm (Øx), 250ppm (recommended application: 1x), and 1250ppm (5x). The nutrient content of Øx fertilizer was created using 10x Murashige and Skoog basal salt micronutrient solution.

3.6 Harvest

The plants were harvested on Day 60 after inoculation. The first set was there for harvested on Day 60, with plants aged 14.5 to18.5 weeks old. The second set was harvested on Day 78, with plants aged 17 to 21 weeks.

To harvest plants, roots were carefully excavated from perlite or soil medium. Observations on plant growth and nodule formation were made at this point. Using tweezers nodules (with small pieces of root attached) were excised from roots. Plants and remaining root segments were then weighed to determine fresh weight. Weight of nodules was also taken at this time so total weight of plant could be determined. Nodules were stored using the Nodule Preservation Vial as described by Somasegararn in the Handbook for Rhizobia (1994). Remaining plant material was placed in labeled plastic bags for organization until dry weight procedure could be initiated no less than 5 hours after harvest.

3.7 Applied Analysis

3.71 Dry Weight Determination

Dry weight was determined using an oven no less than 5 hours after harvest. Plants were dried at 65^{o}C over a period of at least 48hr (Somasegaran and Bohlool).

3.72 Protein Extraction

Dried nodules were reconstituted in dH_20 overnight. The nodules were then sterilized as outlined by Somasegaran (1994) using ethanol, bleach and dH_20 rinses.

Extraction of protein from the nodules was completed by using the plant extraction procedure as outlined by Ott et al. (2005). The only exception to this procedure was to change the Bradford Protein Assay to Peirce 660 Protein Assay due to high concentration of 100x Triton in the protein extraction buffer, which interfered with the colormetrics of the Bradford Assay.

3.73 Western Blot Analysis

Western blot analysis was performed as proposed in Current Protocols in Molecular Biology (Ausubel et. al). The primary antibody anti-LHb and was kindly provided by Carroll Vance (USDA/ARS, ARS Research Leader and Location Coordinator; University of Minnesota, Agronomy and Plant Genetics; Minnesota) and used in a 1:1000 dilution. The secondary antibody was Peroxidase Goat Anti-rabbit IgG (H+L) manufactured by Zymed Laboratories and also used in a 1:1000 dilution. The ladder used to visualize protein movement was EZ-run Rec Protein Ladder by Fisher Scientific.

The gels made for running the electrophoresis were created to be 12% Acrylamide using a 40% Acrylamide solution instead of a 30% Acrylamide (adjusting the protocol appropriately). All gels were run at 120V for approximately an hour and a half on mini gels. For the first trial,

the protein sample was diluted 3:1 with SDS-Page buffer. The second trial used a combination of 1:3 and 3:1 dilutions of protein for high and low determined concentrations as noted from the Peirce 660 assay. In the third trial, all protein samples were diluted to be approximately 200 to 300 ul/ml as determined by the Peirce 660 assay and a sample of 30ul was applied to the gel. For all trials, reconstituted dry leaf sample was used as a negative control. All samples were dry loaded onto the gel before buffer was applied to the gel interior.

After the proteins were blotted in a semidry transfer at 65mA for 1 hour, the gels were stained overnight with GelCode Blue reagent to visualize protein and ladder formation. The membranes were immersed in TMB Membrane Peroxidase Reagent to visualize the secondary antibody. The membranes were then dried at room temperature to eliminate background staining.

3.74 Plasmid Profile Assay

The Plasmid Profile Assay was performed as outlined by Somasegaran (1994) with the following exceptions. The assay was completed on mini-gels, and therefore all measurements (time/voltage/volume application) was divided to allow this. Cybergreen was added to the lysed bacteria cultures, and a blue light was used in addition to the Ethidium bromide and UV light combination for visualization of the plasmid. The ladder used to visualize plasmid size was the Hyperladder I manufactured by Bioline.

The main differences between the trials were applied amounts of reagents (lysed bacteria/cybergreen/Eckhart A, Eckhart B, and Eckhart C respectively), voltages, and times run for each voltage. The first trial was run in a similar manner to the book, but on a mini gel, which caused issues in size of the wells, voltage times, etc. The second trial, with 2 gels, was run with ¼ the amount of reagents (10, 10, 25ul as compared to 40, 40, 100ul respectively) and

approximately ¼ the voltage (3mA for 1 hour, and 10mA for 50 minutes). The third trial was most successful. It was run with the same amount of reagents as trial 2, but with a voltage of 2mA for 25 minutes, and a voltage of 10mA for 1.5 hours. It is suggested that in a future experiment, the times/voltages be manipulated to see what works best at the mini-gel level for this particular experiment.

3.75 Culture Nodules

The standard rhizobial stains were cultured in MAG media from conserved sources for 2 days and then 100ul was plated without dilution onto MAG agar plates. The plated cultures grew 2 days before final observations.

To test the hypothesis that the media inoculated plants (the negative control) and perhaps the other inoculated plants were at least partially infected due to the seeds carrying some form of rhizobia, the seeds themselves were used to grow a culture. To do this, approximately 20 seeds were placed in 5mL media and allowed to culture for 4 days. 100ul of the infected media was placed on a MAG agar plate, and allowed to grow for 24 hours. The resulting culture was then streaked onto 2 separate MAG plates. These plates were grown for 24 hours and then observed for morphology.

To observe the morphology of the bacterial strains that infected the harvested nodules, a sample of nodules (only from Set 1) were rehydrated overnight in dH_2O, sterilized and then slightly crushed before being placed in 5mL MAG media in a 15mL conical tube. The strains were grown for 5 days, and then 100ul was plated without dilution on MAG agar. Morphology was observed 2 days after plating.

Chapter 4 Results and Analysis

4.1 Inoculation

4.101 Growth Curve as Determined for all Strains

The average CFU values for Brady, USDA 3384, USDA 2376, and USDA 3456 determined over two growth curve trials and the CFU values for USDA 101 and Alyce determined over one growth curve trial are reflected in this graph. Brady, USDA 3384, and USDA 3456 were chosen as inoculants of the main set of plants because they revealed similar growth patterns and CFU values at Day 5. Later in the project, smaller subsets of plants were inoculated with USDA 2376, USDA 101, and Alyce in order to assess the nodulation patterns of these strains as well.

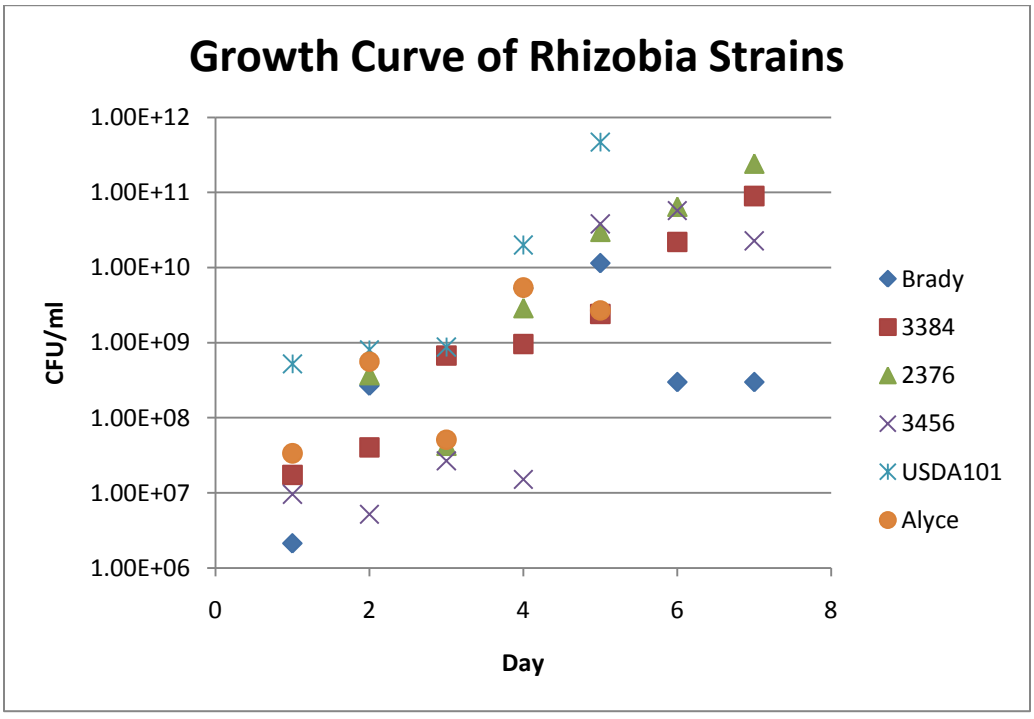


Figure 4.101: Determined growth curve for all strains

4.102 Inoculated Values

The average CFU values for the rhizobia cultures used to inoculate the first set of plants, Brady, 84, and 56, were much higher than those used in the later smaller subset groups even though all cultures were grown to Day 5. However, this discrepancy most likely is a reflection of the different growth conditions that each set was exposed to; Set 1 was grown in a flask at 25 degrees Celsius shaking at 190 RPM, whereas Set 2 was grown in conical tubes on an inverter at room temperature.

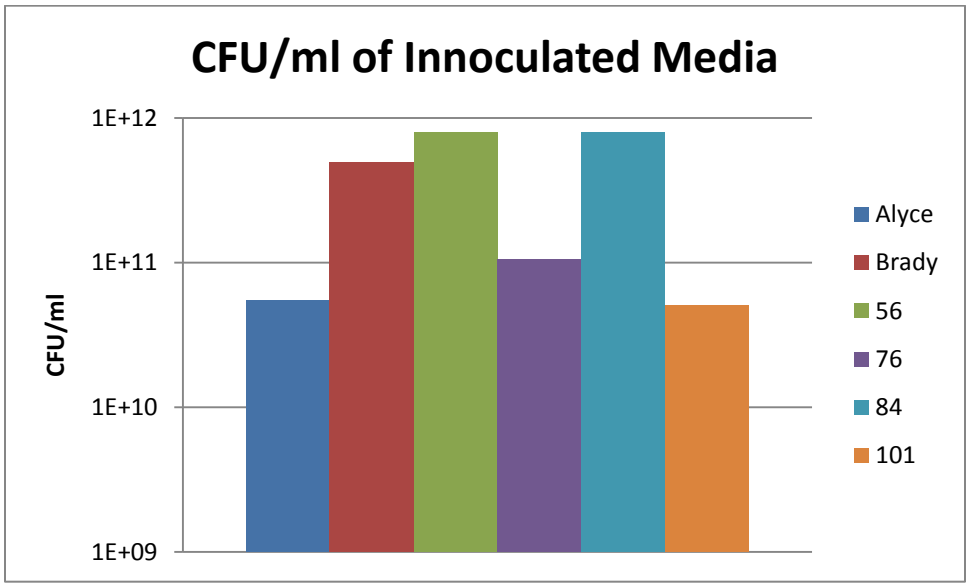


Figure 4.102: The concentration of inoculated media applied to each set of plants.

4.2 Harvest Results

4.201 Pearson Correlation Study of Average Values

Correlation studies were performed on the above combination of factors using raw data from Appendix J. Significant correlation is indicated as values approach a correlation coefficient of 1. Very little correlation was observed in any of the groups, save for Plant height vs. Plant wet weight which yielded a correlation coefficient of approximately 0.93.

Correlations		
Factor 1	**Factor 2**	**Correlation Coefficient**
Nodule dry weight	Plant dry weight	0.345646398
Plant height	Plant dry weight	0.071725802
Plant height	Nodule dry weight	0.520573982
Nodule wet weight	Plant wet weight	0.47612402
Plant height	Plant wet weight	0.926445155
Date from innoculation	Plant height	0.555351148

Table 4.201: Calculated Pearson Correlation Coefficients

4.202 Correlation between Constant Weights of Plant Shoot and Nodules

Nodule total dry weight and plant shoot total dry weight raw data (see Appendix J) were plotted against each other to evaluate correlation between the two factors. This graph shows very correlation between the two, supporting its correlation value of 0.35 in Table 4.201.

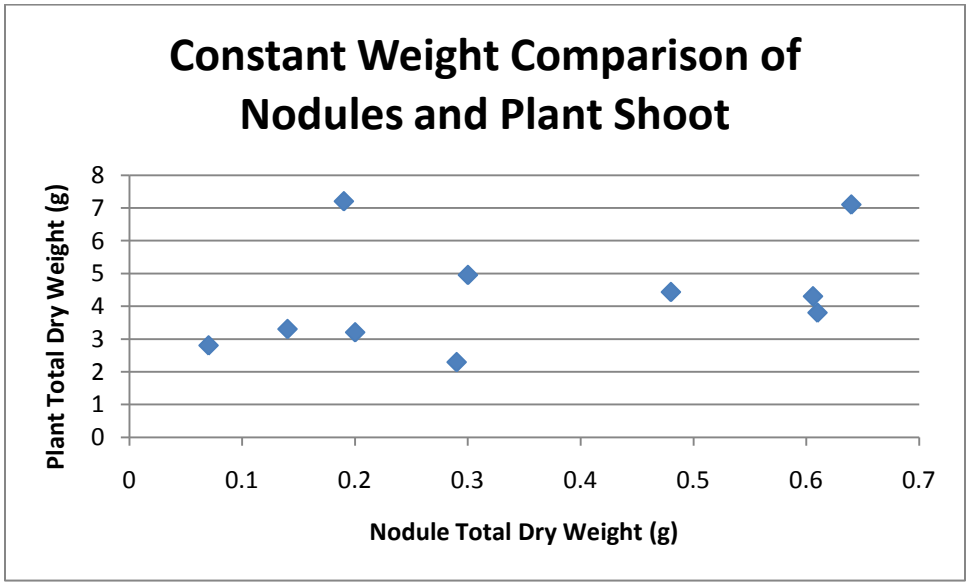


Figure 4.202: Constant weight comparison of nodules and plant shoot

4.203 Nodule Fresh Weights

The average nodule fresh weights for each group of inoculated plants are plotted based on the nitrogen treatment they received. USDA 3456 had a significantly higher average nodule weight than most other plant inoculant groups across all three nitrogen treatments. However, increasing the concentration of nitrogen application did not appear to affect the weight of nodules.

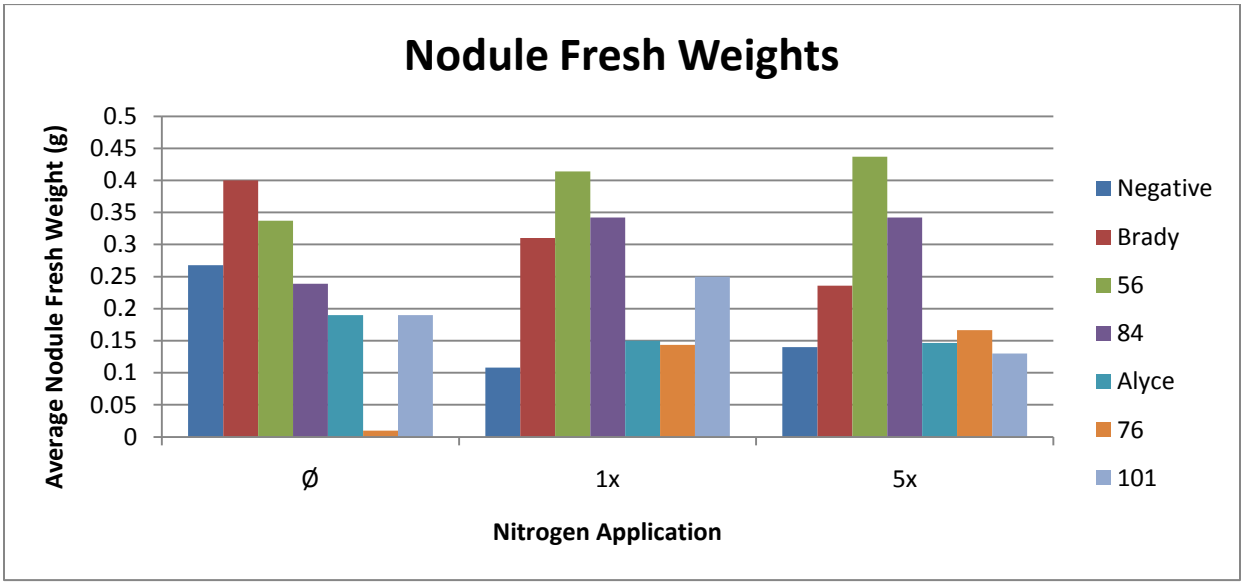


Figure 2.03: Nodule fresh weights at harvest

4.204 Fertilizer Effect on Nodulation

The average fresh weight of nodules of all plants, regardless of inoculant, are separated into three groups based on nitrogen application. Increasing the concentration of nitrogen application did not appear to affect the average weight of nodules across all groups cumulatively.

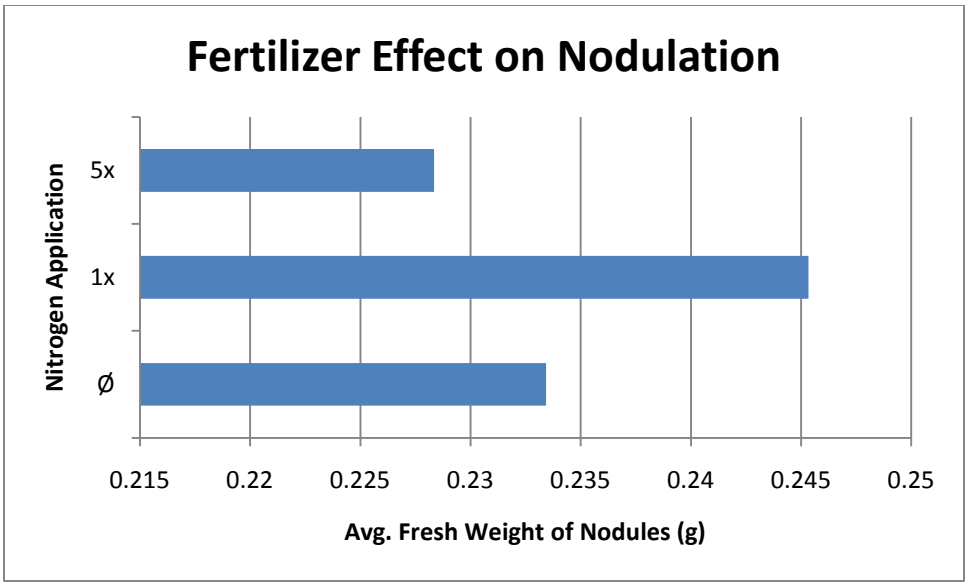


Figure 4.204: Fertilizer application effect on nodulation

4.205 The effect of Nitrogen Application on Plant Height

The average plant heights for each group of inoculated plants are plotted based on the nitrogen treatment they received. Increasing the concentration of nitrogen application did not appear to affect the height of plants.

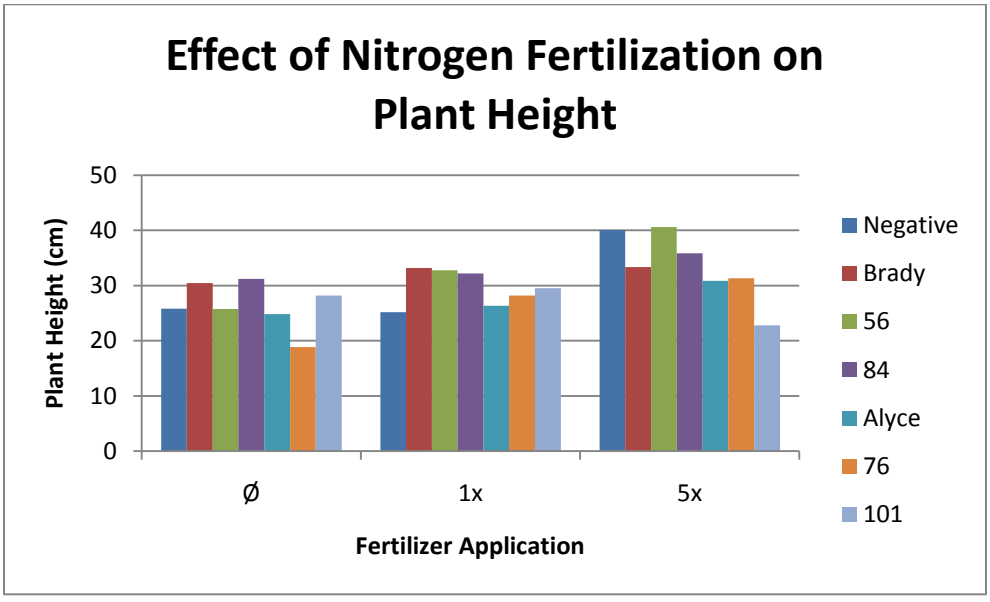


Figure 4.205: Effect of nitrogen application on plant height

4.206 The Overall Effect of Nitrogen Application on Plant Height

The average plant heights across all inoculant subsets are separated into three groups based on nitrogen application. As nitrogen application was increased, the average plant height seemed to increase as well. Thus, this suggests that the increase in nitrogen was not enough to inhibit nodulation, but was effectively taken up by the plant and encouraged plant growth.

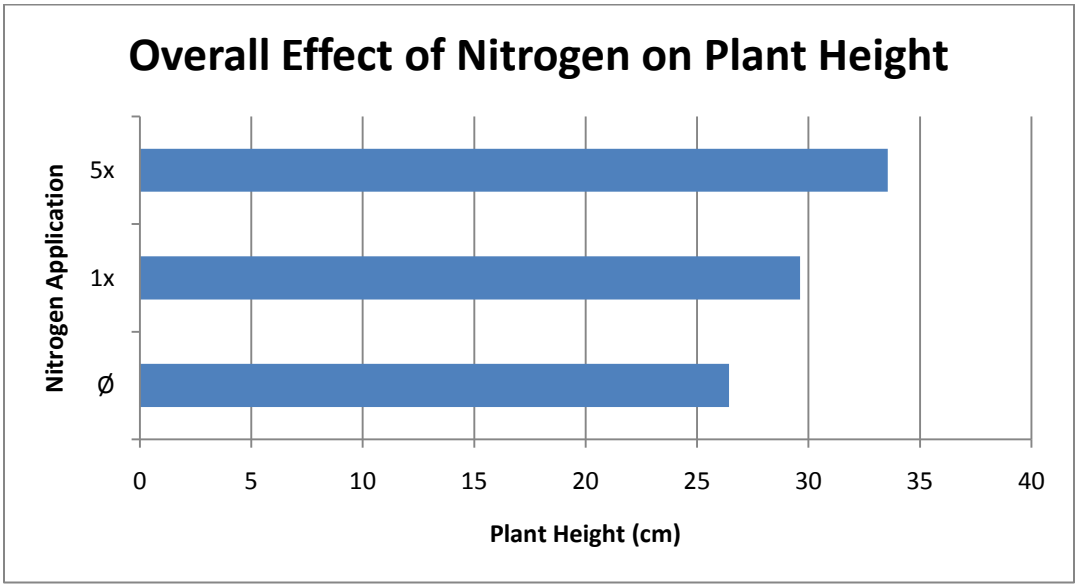


Figure 4.206: Overall effect of nitrogen on plant height

4.207 Competition Study Nodule Fresh Weights

The average nodule fresh weights for each group of inoculated plants in the competition study are plotted based on the nitrogen treatment they received. Brady had a significantly higher average nodule weight than most other plant inoculant groups in the nitrogen free treatment, but not in the 1x or 5x nitrogen treatments. This suggests that the nitrogen already present in the Deerfield in combination with the nitrogen applied in the 1x and 5x subsets might have inhibited nodulation in the plant groups inoculated with Brady. A similar trend was observed in plants inoculated with USDA 3456.However, increasing the concentration of nitrogen application did not appear to affect the weight of nodules in any other groups of plants.

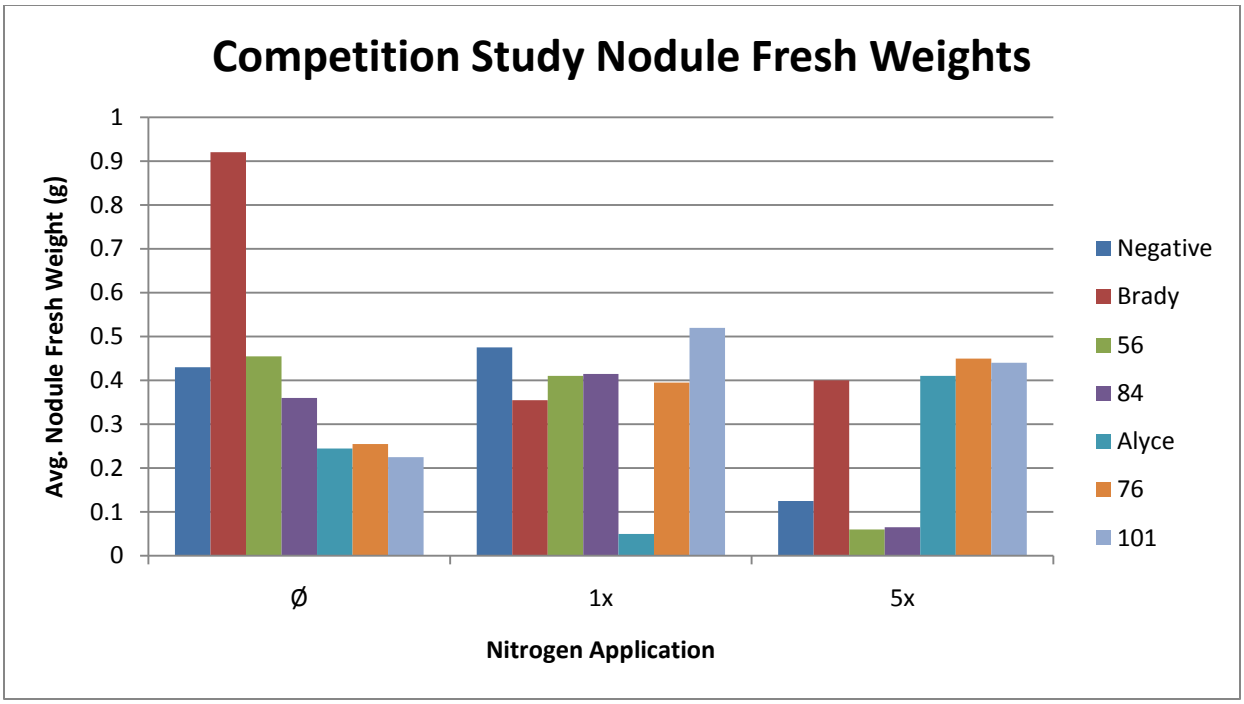


Figure 4.207: Competition study fresh nodule weights at harvest

4.208 The Effect of Fertilization on the Competition Study

The average fresh nodule weights across all inoculant subsets are separated into three groups based on nitrogen application. As nitrogen application was increased, the average nodule fresh weights seemed to decrease. Thus, this suggests that the increase in nitrogen might have inhibited nodulation to some degree across all plant groups.

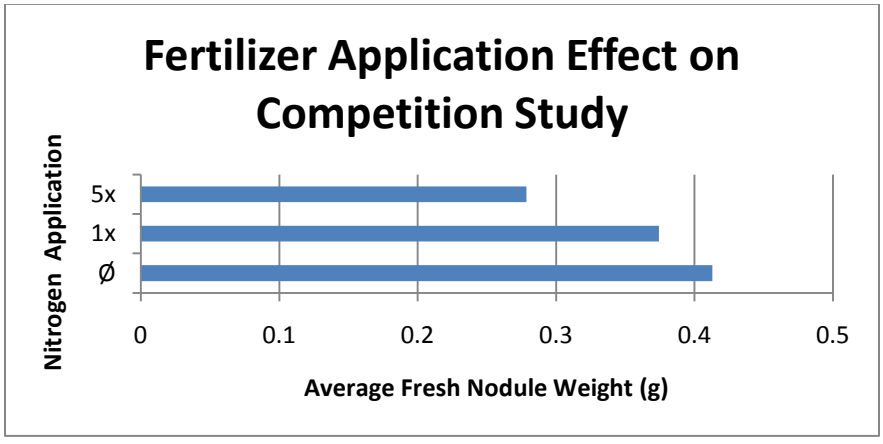


Figure 4.208: Fertilizer application effect on completion study nodule weight

4.209 The Effect of Nitrogen Application on Plant Height during the Competition Study

The average nodule fresh weights for each group of inoculated plants in the competition study are plotted based on the nitrogen treatment they received. Plant heights seem to increase as nitrogen application increases for plants inoculated with USDA 2376 and USDA 101, suggesting that the increase in nitrogen was not enough to inhibit nodulation, but was effectively taken up by the plant and encouraged plant growth. However, increasing the concentration of nitrogen application did not appear to affect the weight of nodules in any other groups of plants in the competition study.

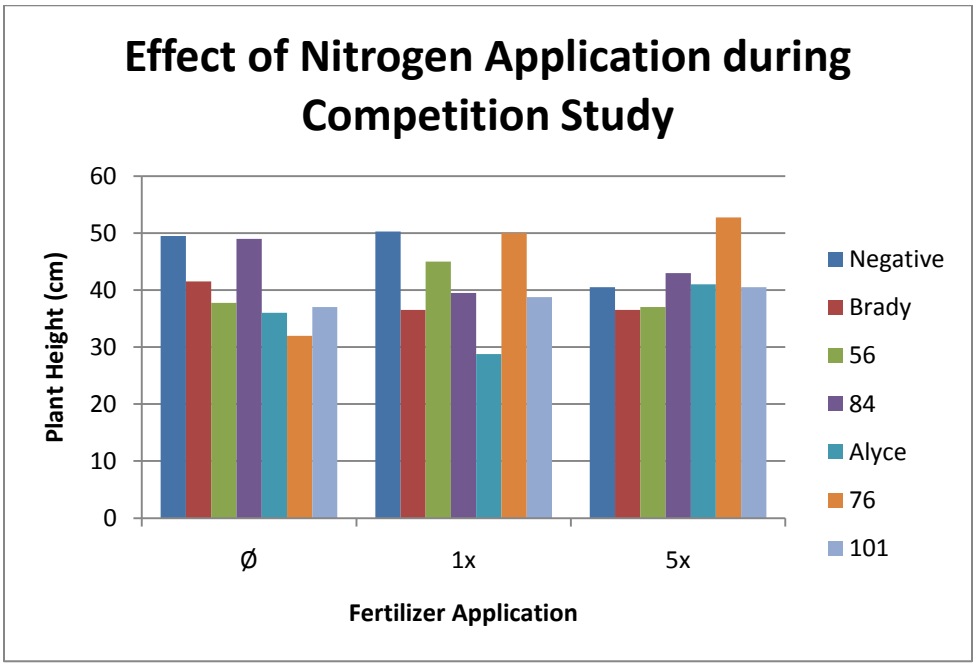


Figure 4.209: The effect of nitrogen application during the competition study

4.210 The Overall Effect of Nitrogen Application on Plant Height during the Competition Study

The average plant heights across all inoculant subsets in the competition study are separated into three groups based on nitrogen application. As nitrogen application was increased, the average nodule plant heights seemed to increase. Thus, this suggests that the increase in

nitrogen was not enough to inhibit nodulation, but was effectively taken up by the plant and encouraged plant growth.

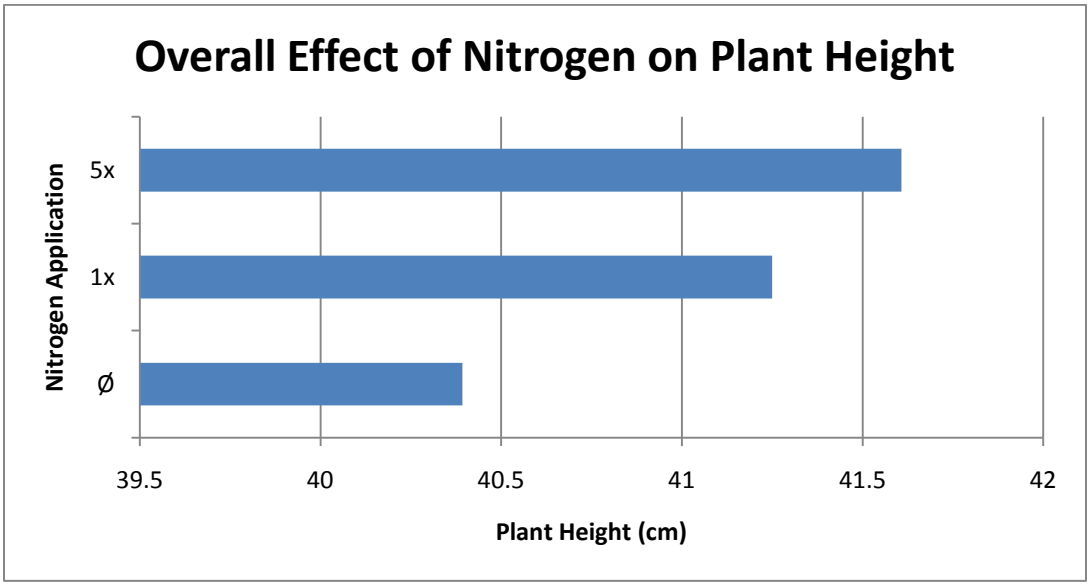


Figure 4.210: Overall effect of variant nitrogen application on plant height

4.211 Globular Nodule Morphology

The first and second images shows globular-type nodules as seen when attached to the root. The third image shows a globular-type nodule that is split in half and attached to a root. This nodule morphology was observed across all strains, but had only a minimal presence on all negative controls and on all plants in the competition study.

Figure 4.211: Globular nodule morphology. Pictures on bottom taken by Dr. Dan Gibson.

4.212 Coralloid Nodule Morphology

The first image shows the coralloid shape of nodules attached to plant root hairs, as found on many *Crotalaria* species. The second image shows an intact coralloid nodule as seen under a light microscope. The third image shows cross-sections of the same coralloid nodule in the second image. The pink color of nodule in each image is typical of healthy nodules that are effectively fixing nitrogen, as the hemoglobin protein component is generally this color.

Figure 4.212: Coralloid nodule morphology. Pictures on bottom taken by Dr. Dan Gibson.

4.3 Microscopy

4.301 Possible Infection Thread

Cross-sections of a USDA 3456 plant nodule were suspended in resin and osmium and then viewed under a light microscope. This image suggests the presence of an infection root as indicated by the arrow.

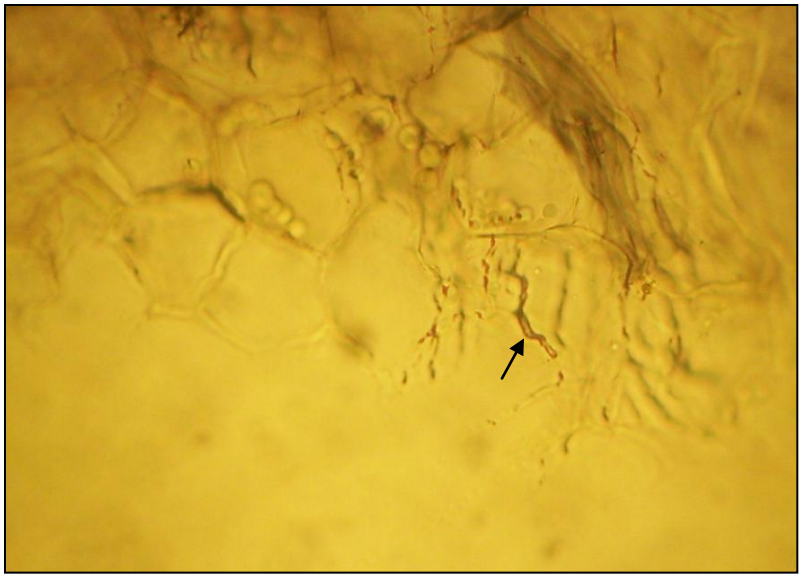


Figure 4.301: Possible infection thread. Picture by Dr. Dan Gibson.

4.302 Toluene Blue Stain

Bacterial infection may be indicated as absence of color in cells stained with toluene blue. The enhanced image to the right points at an elongated rod shape (as one example) that may be bacterial in origin. It may also be possible that bacteroid infected cells turn blue and uninfected cells remain unstained, as can be seen in the right upper corner, where the nucleus is clear, and no bacterial morphology may be seen.

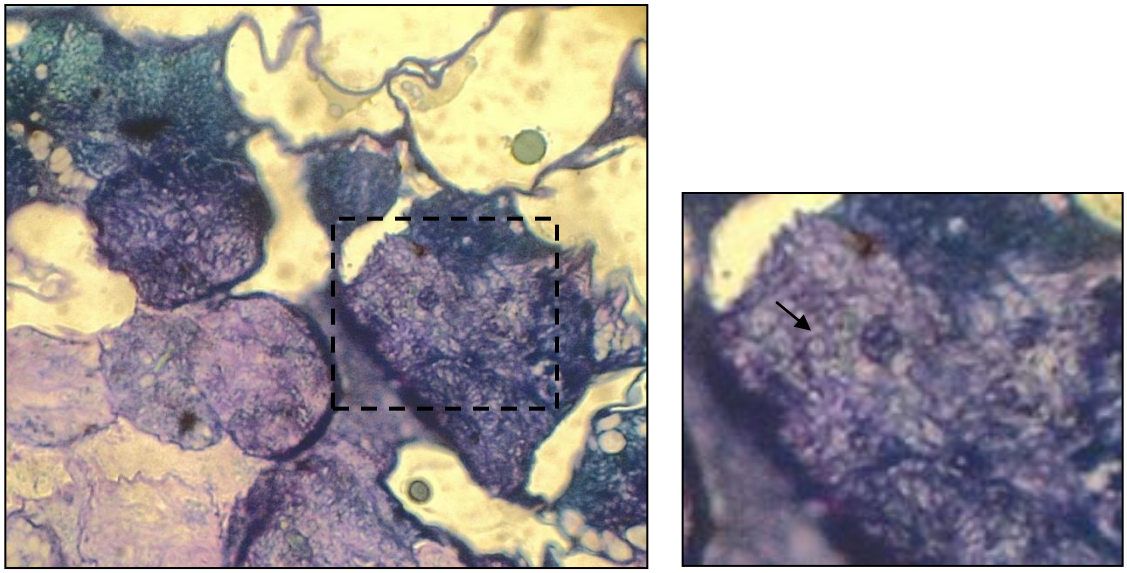


Figure 4.302: Toluene Blue stain indicating possible bacterial infection

4.303 Toluene Blue and Basic Fuchsin Stain

The following image was taken after staining with both toluene blue and basic fuchsin stains. A bacterial infection may be indicated as the dark specks within the pink colored cells.

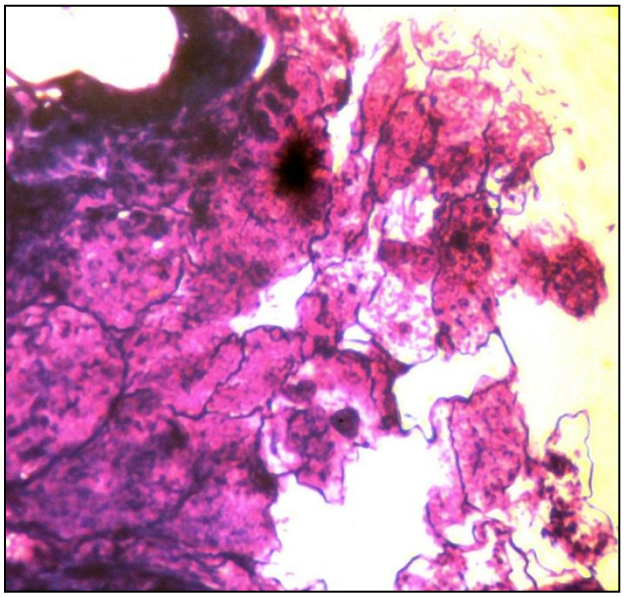


Figure 4.303: Toluene blue and basic fuchsin stains indicating infection

4.304 Nodule Preserved without Osmium

Infection may also be seen in a nodule sample not stained with osmium during preservation as dark spots seen under higher magnification.

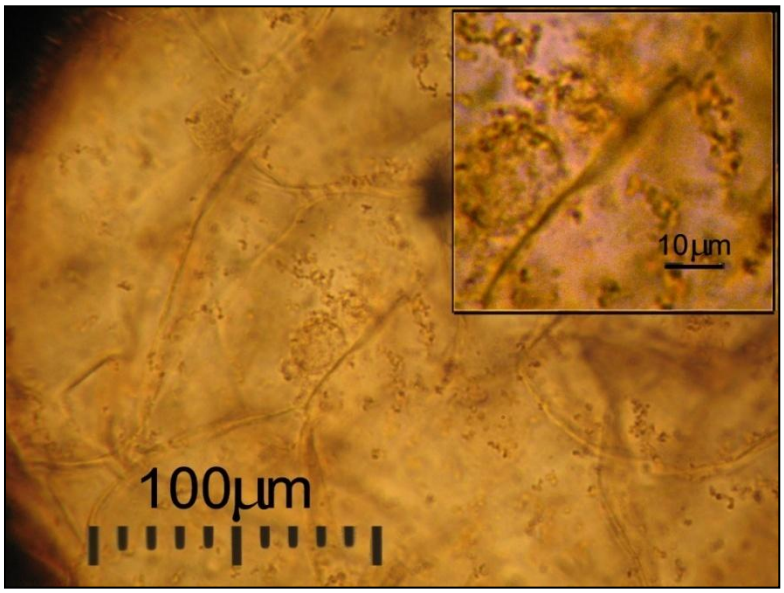


Figure 4.304: Bacterial infection indicated as morphologically indicated shapes under high magnification

4.4 Protein Extraction

4.41 BSA Standard Curve

The BSA Standard curve was created using 7 different known concentrations of BSA mixed with Pierce 660 reagent. Concentration values were plotted against absorption at A660 to create a calibration curve.

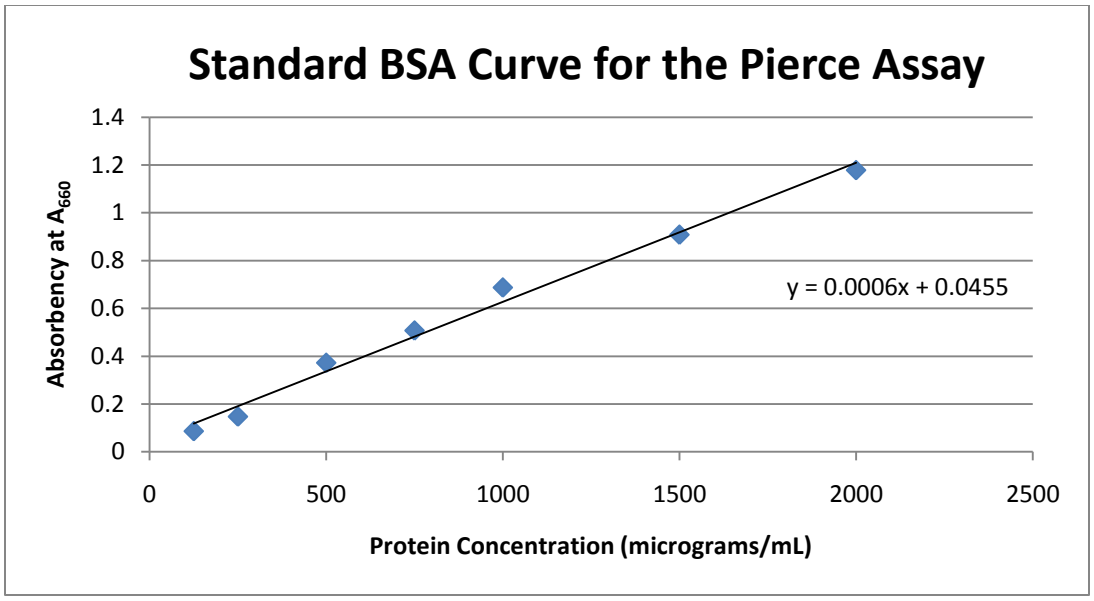


Figure 4.41: BSA Standard Curve

4.42 Extracted Protein Concentrations for Nodules of the Main Set

The extracted protein concentration for the main set were determined through the Peirce 660 Protein Quantification Assay and use of the standard curve in Figure 4.41. The values are plotted below. There was no trend for protein concentration across groups.

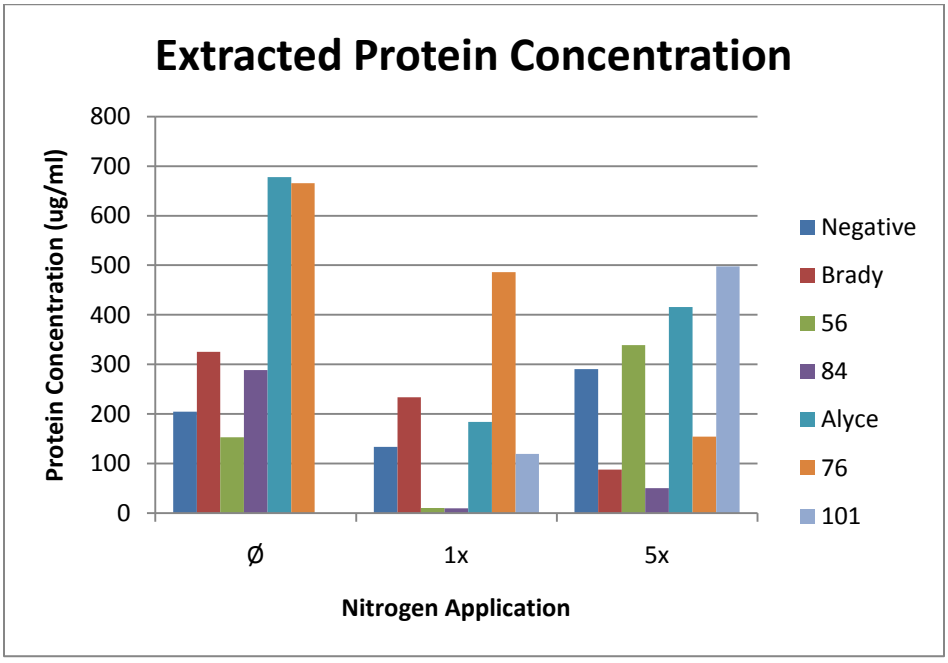


Figure 4.42 Extracted protein concentration for nodules of the main set

4.43 Extracted Protein Concentrations for the Nodules of the Competition Study

The extracted protein concentration for the competition set were determined through the Peirce 660 Protein Quantification Assay and use of the standard curve in Figure 4.41. The values are plotted below. There was no trend for protein concentration across groups. Raw data may be found in Appendix L.

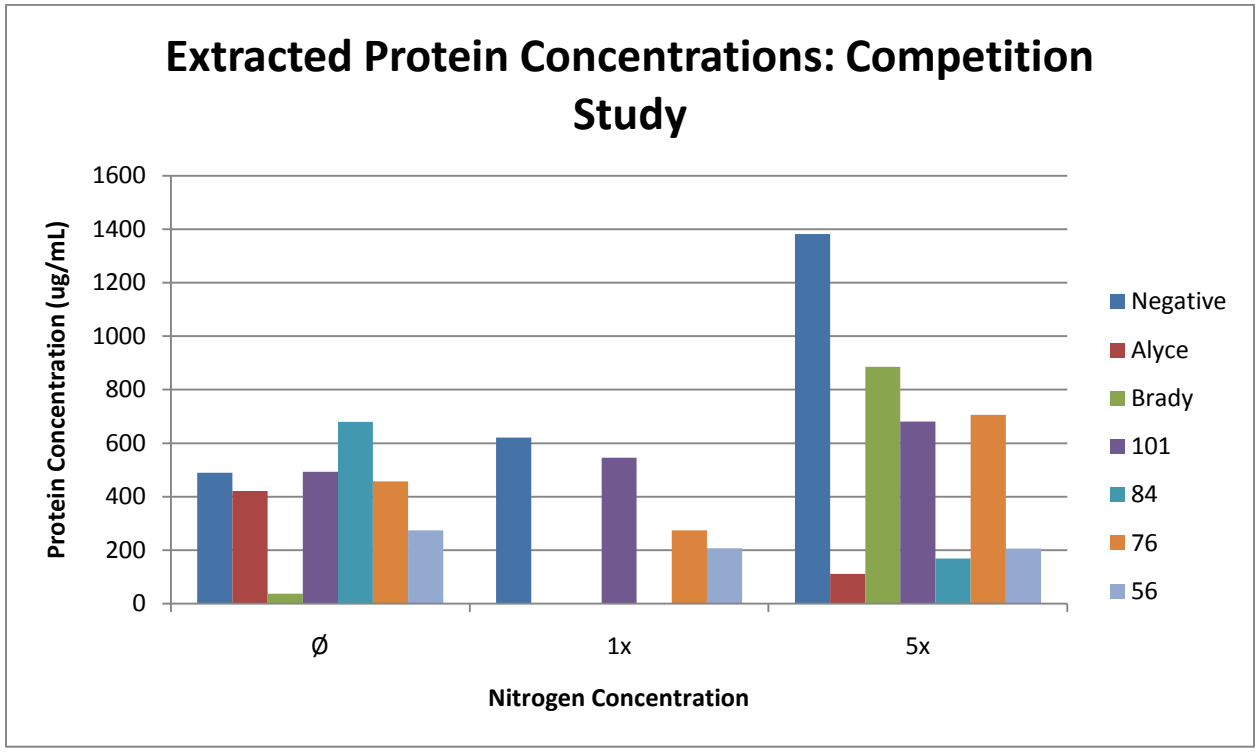


Figure 4.43: Extracted protein for the nodules harvested from the competition set

4.5 Western Blots

4.51 Western Blot, Run 1

Protein samples from the main set of plants, which included Media, Brady, USDA 3456, and USDA 3384 inoculated plants exposed to each fertilization level were run in Western blot Set 1 along with a negative control that contained leaf extract and a protein ladder. Each sample was probed using an anti-leghemoglobin antibody and then visualized using a goat anti-rabbit

secondary antibody. Bands were observed at approximately 30 kDa, when leghemoglobin usually produces a band at 16 kDa. Possible reasons for this discrepancy are considered in the discussion.

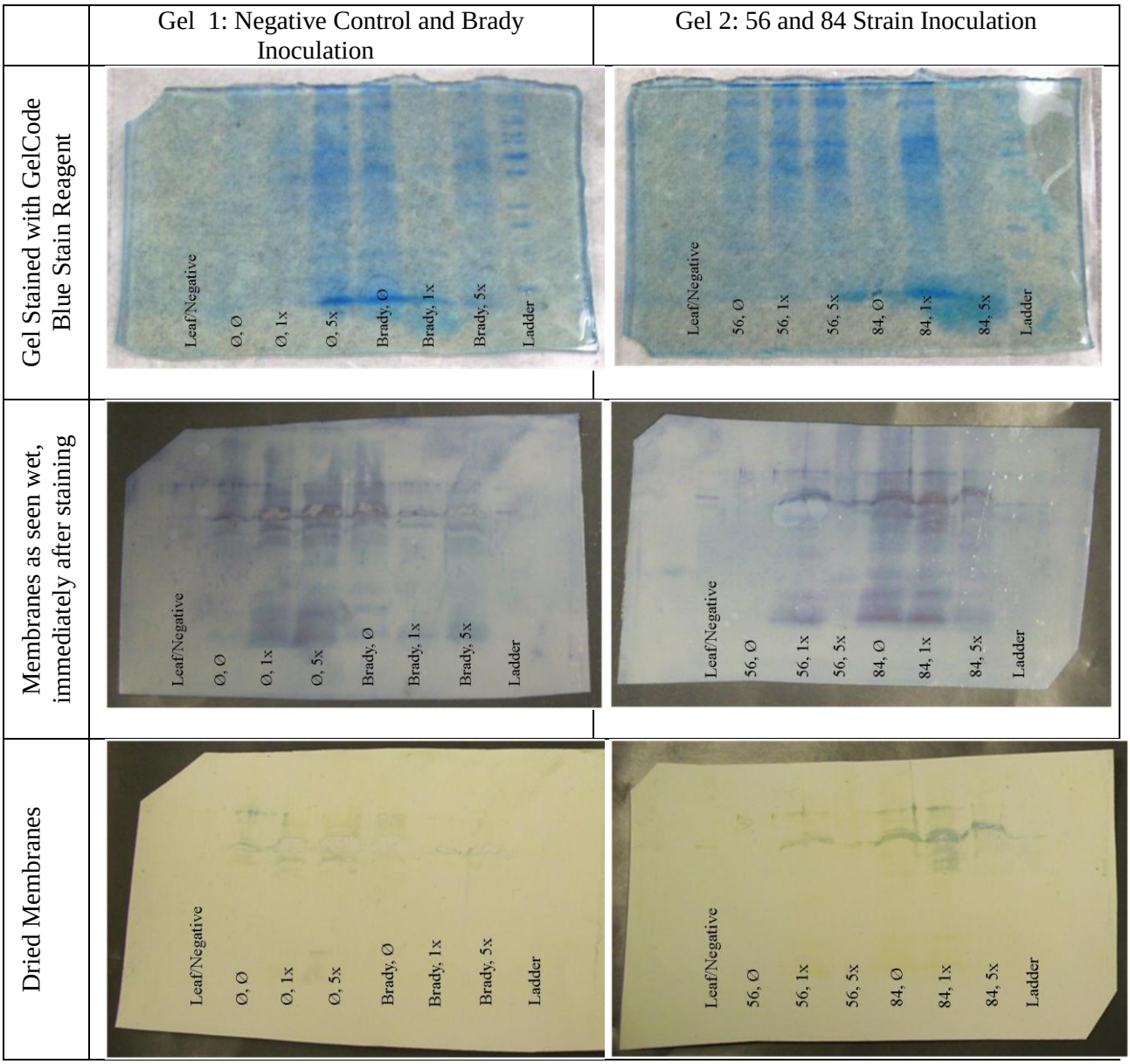


Figure 4.51: Western Blot Set 1

4.52 Western Blot , Run 2

Protein samples from the main set of plants, which included Media, Brady, USDA 3456, and USDA 3384 inoculated plants exposed to each fertilization level were run in Western blot Set 2 along with a negative control that contained leaf extract and a protein ladder. Each sample was probed using an anti-leghemoglobin antibody and then visualized using a goat anti-rabbit secondary antibody. Bands were observed at approximately 30 kDa, when leghemoglobin usually produces a band at 16 kDa (Ganter et. al). Possible reasons for this discrepancy are considered in the discussion.

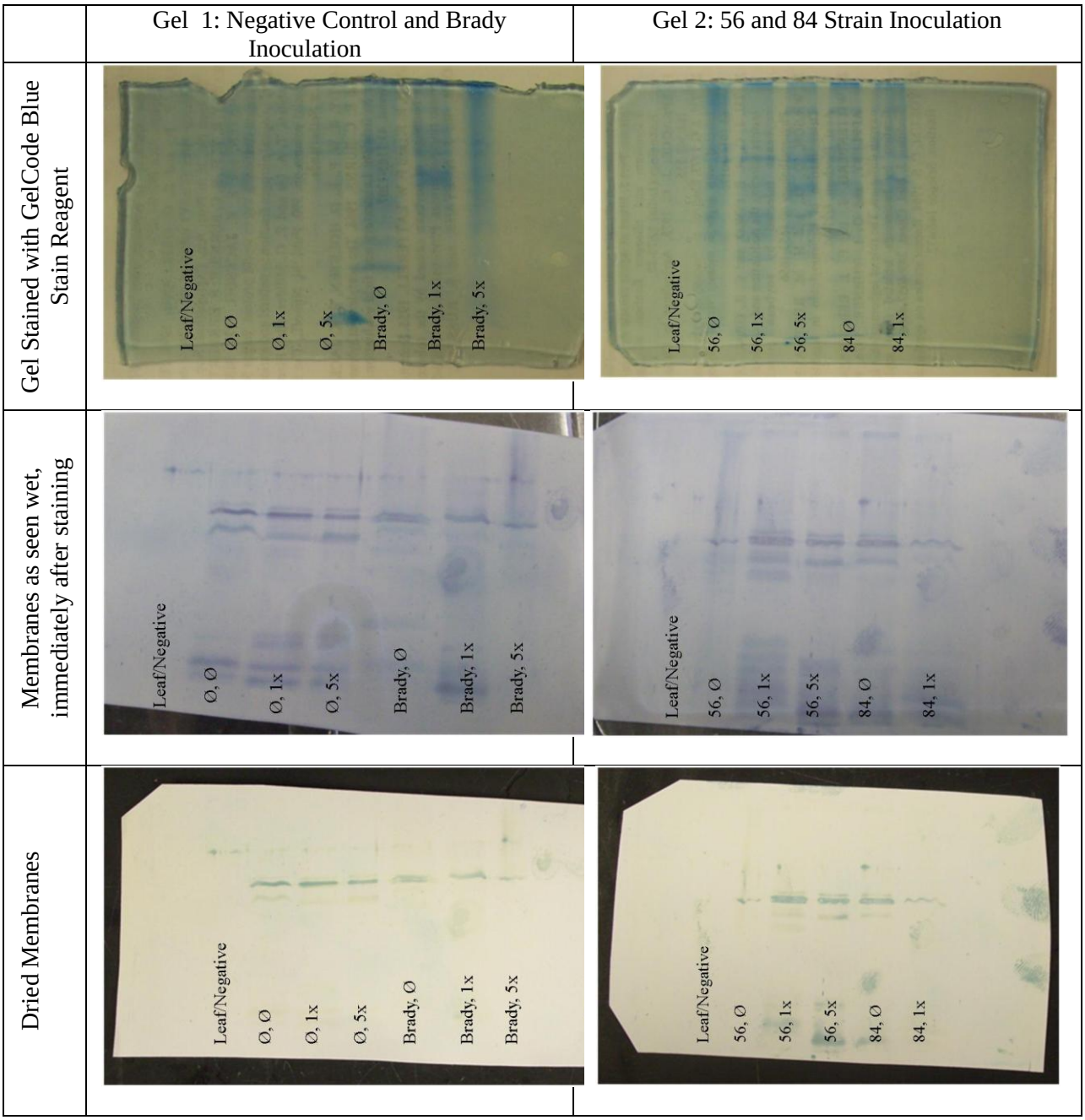


Figure 4.54: Western Blot Set 1

4.53 Western Blot: Set 2 and Competition Study

Nodule samples from the second set of plants, which included USDA 2376, USDA 101, and Alyce inoculate plants exposed to each fertilization level were run in Western Blot Set 3 Gel 1 along with a negative control that contained leaf extract and a protein ladder. Each sample was

probed using an anti-leghemoglobin antibody and then visualized using a goat anti-rabbit secondary antibody. Bands were observed at approximately 30 kDa, when leghemoglobin usually produces a band at 16 kDa. Possible reasons for the discrepancy are considered in the discussion.

Nodule samples with high protein content from the Deerfield competition were run in Western Blot Set 3 Gel 2 along with a negative control that contained leaf extract and a protein ladder. Each sample was probed using an anti-leghemoglobin antibody and then visualized using a goat anti-rabbit secondary antibody. Bands were observed at approximately 30 kDa, when leghemoglobin usually produces a band at 16 kDa. Possible reasons for the discrepancy are considered in the discussion.

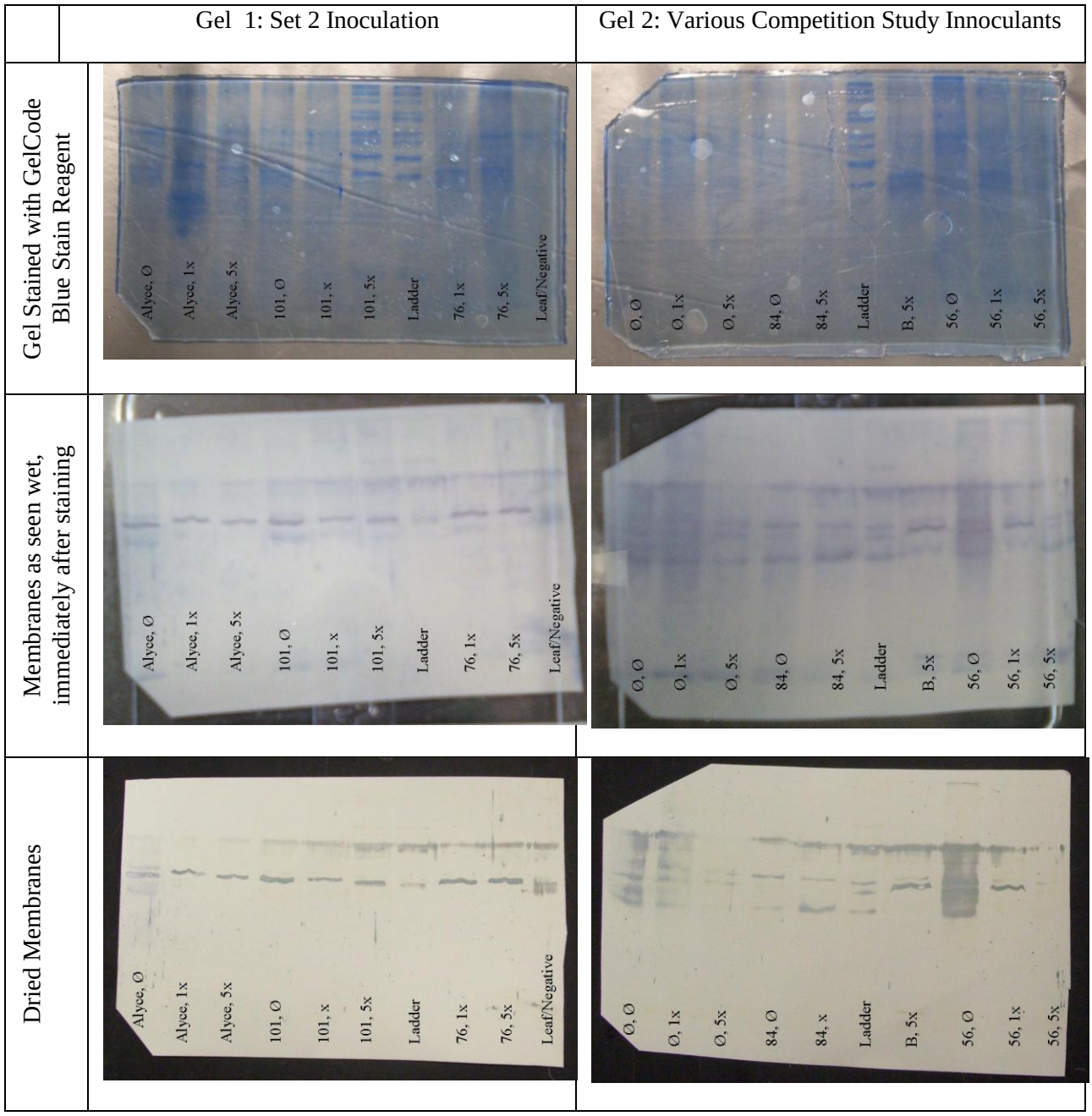


Figure 4.53: Western blot of Set 2 and the competition study

4.6 Plasmid Profiles

The plasmid profile was run 3 times. The last run was both visualized with cybergreen and Ethidium bromide using appropriate light sources. The last trial revealed several inconclusive bands and smears indicated by arrows that suggest plasmid existence. Further studies are required to adapt the procedure from full size to mini gels in order to obtain accurate plasmid profiles of the strains.

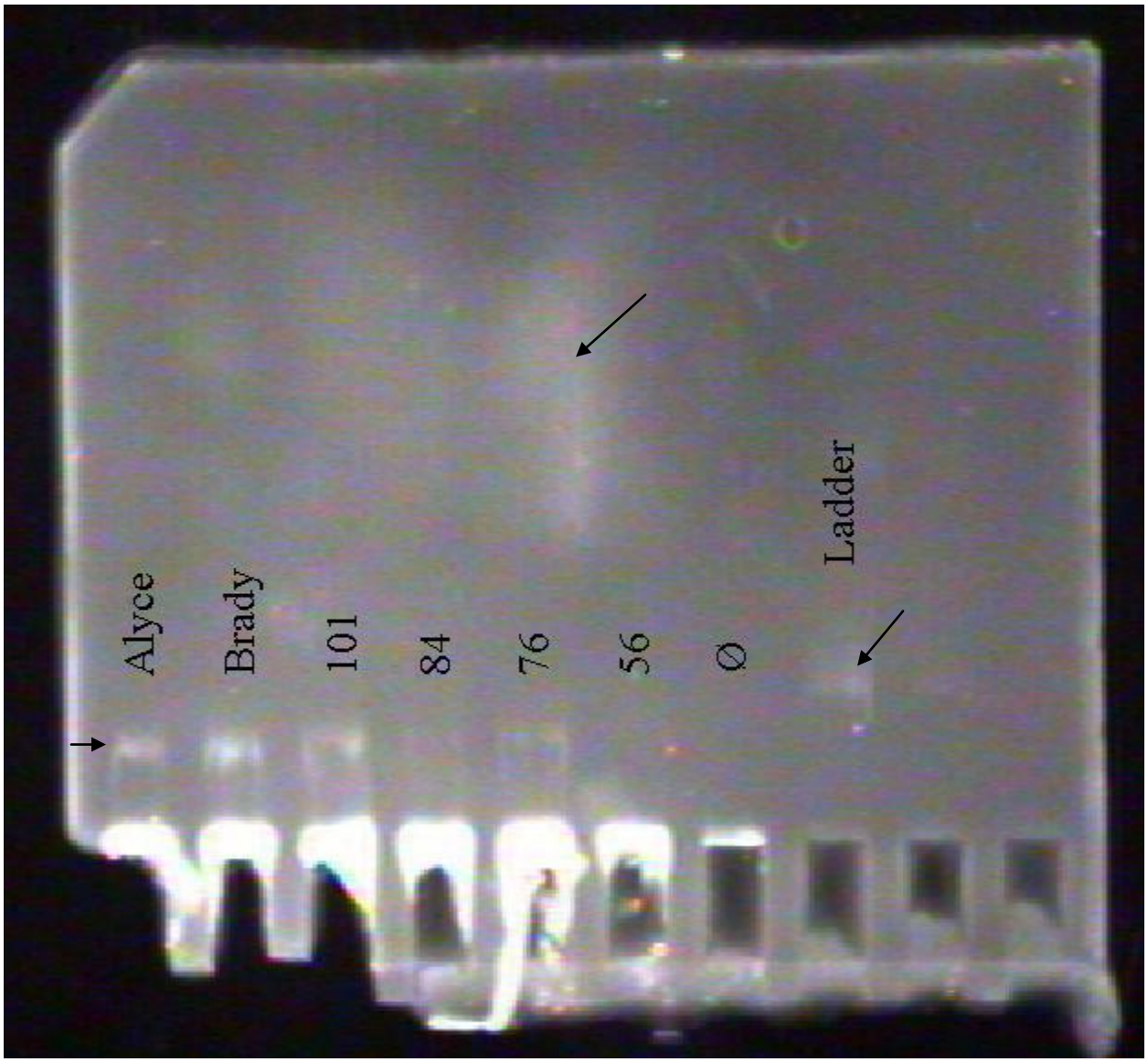


Figure 4.6: Plasmid profile trial 3

4.7 Cultured/Plated from Plants

4.71 Standards

The standard strains were cultured and plated to observe standard morphology of the strains.

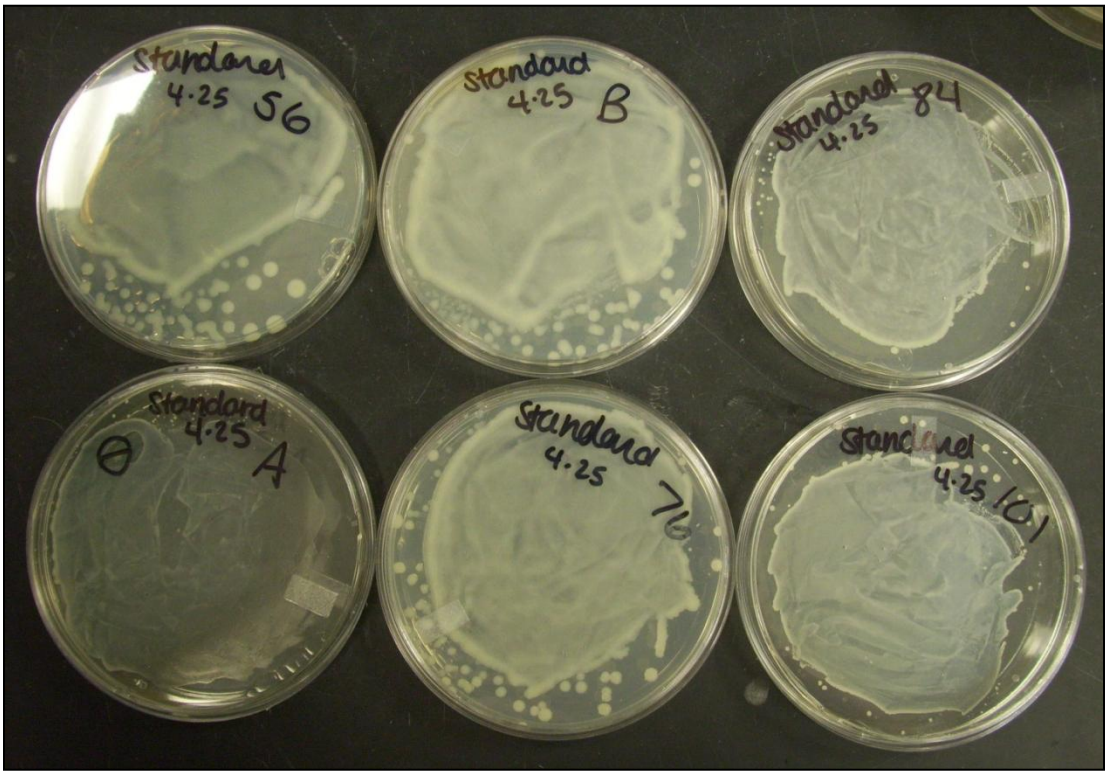


Figure 4.71: Standard strain morphology

4.72 Seed Cultured Growth

The plates were streaked with culture obtained directly from the seeds. The morphology of this culture included being beige in color, rapidly growing, and a minimal amount of exopolysaccharides.

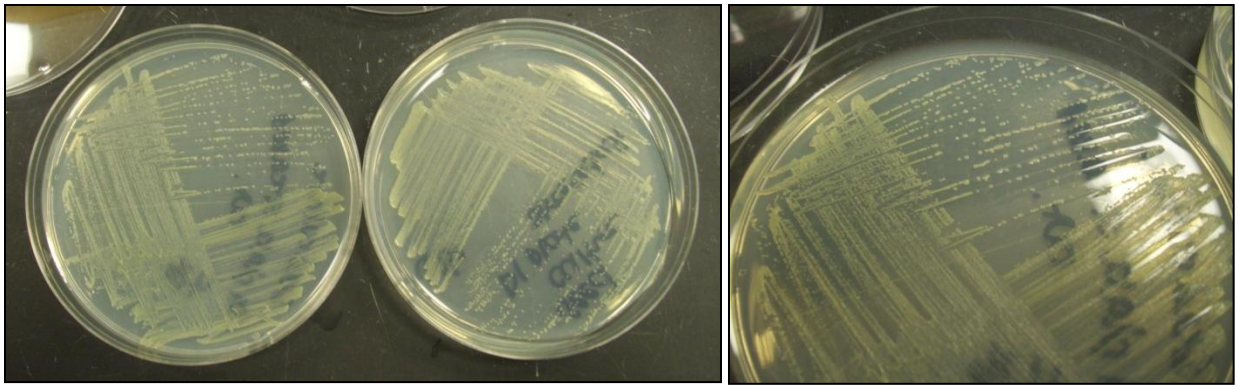


Figure 4.72: Seed culture morphology

4.73 Cultured Sample Nodules from Set 1

The set of negative controls that showed nodule formation in Set 1 was cultured to observe the morphology of the encroaching strain. All show similarities to the strain of bacteria cultured from the seeds. The color of the first two nitrogen applications especially suggest that the same strain that nodulated the plants was also found on the seeds.

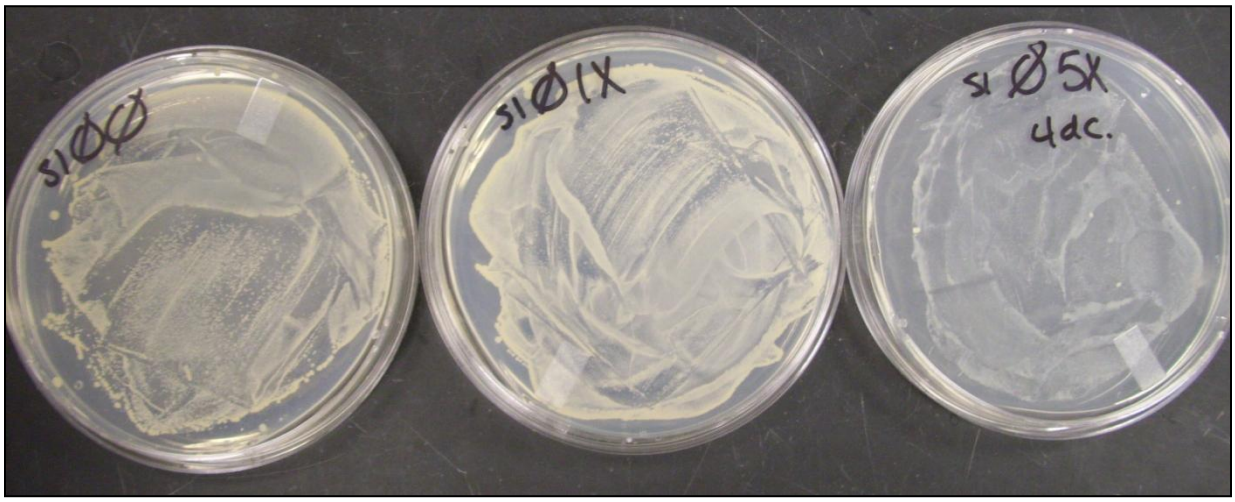


Figure 4.73a: Negative control nodules harvested and cultured

The Brady set of cultures also showed similarities to the cultures grown from the seeds verses the cultures grown from the Brady strain. The standard Brady strain showed a white color and much excreted polysaccharides, whereas the cultures grown from the nodulated plants

showed characteristics such as beige color, and less (external name). This suggests that the bacteria found on the seeds may have assisted the Brady rhizobia in nodulating the plants.

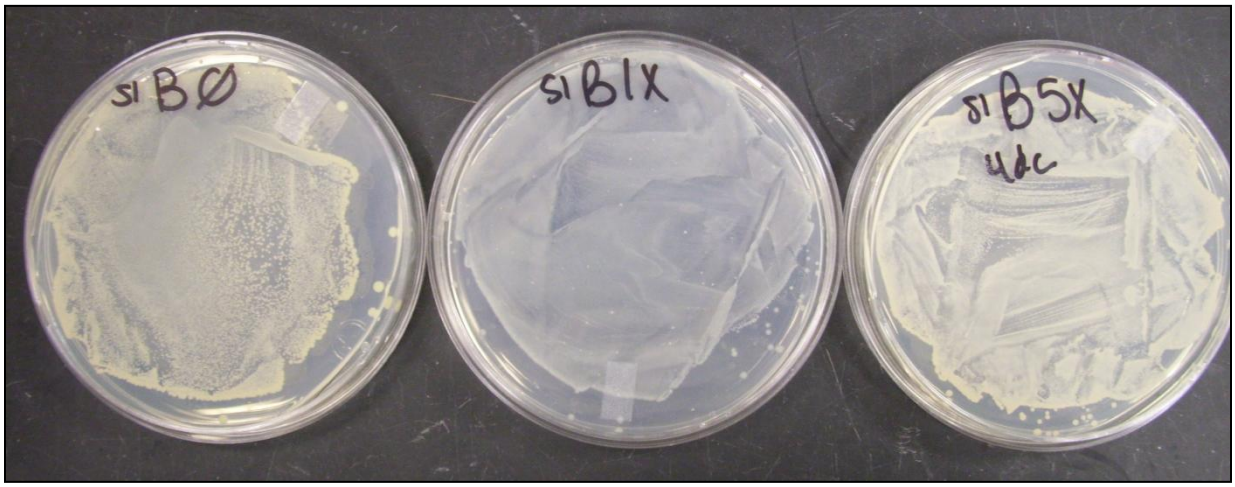


Figure 4.73b: Brady nodules harvested and cultured

The 84 plant nodule cultures also show similarities to the morphology of the bacteria from the seeds in both color and morphology.

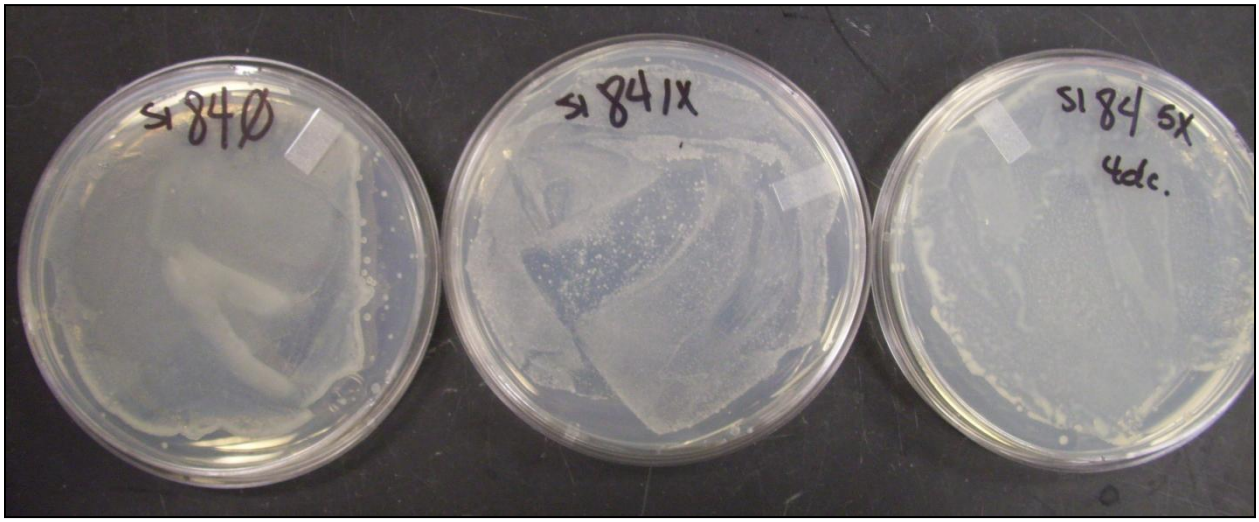


Figure 4.73c: Strain 84 nodules harvested and cultured

The 56 strain shows the least contamination of the seed culture in terms of morphology observations. This includes the color and morphology. This seems rational as the 56 strain had many globular nodules that weren't seen on the negative control, suggesting that the 56 strain may have been able to nodulate soundly in spite of competition from the seed contamination.

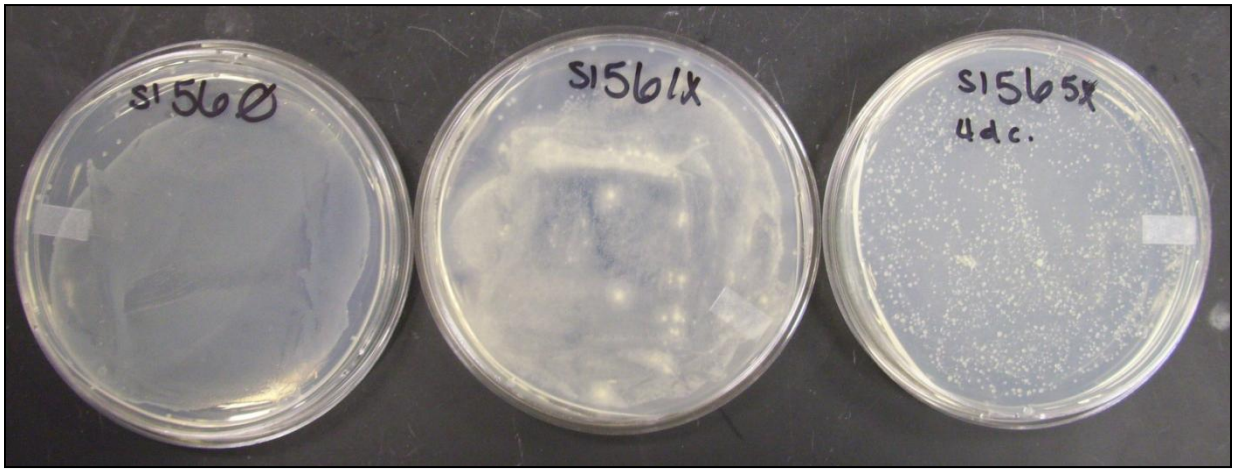


Figure 4.73d: Strain 56 nodules harvested and cultured

Chapter 5 Conclusions and Recommendations

The raw harvest data from this study confirms that the *Crotalaria longirostrata* can form a symbiosis, to some extent, with each rhizobia strain tested. As Figure 4.203 shows, USDA 3456 averaged the highest nodule weight over all fertilization groups, followed by PNL0i-Brady, USDA 3384 and the negative control group. Although this would suggest that strain USDA 3456 was the most effective inoculant, the morphology of the nodules observed in each group make this difficult to prove.

Most of the USDA 3456 plants formed several globular white nodules (See Figure 4.211), but only a few pink coralloid nodules. Since healthy nitrogen-fixing nodules contain the symbiotic protein leghemoglobin, they are usually pink. Therefore, the pink coralloid nodules (See Figure 4.212) are more likely to have provided the plants with an efficient source of nitrogen. Additionally, species of *Crotalaria* are known to produce nodules of this morphology. USDA 3384 and PNL0i-Brady produced many more coralloid nodules than those inoculated with USDA 3456; this suggests that although USDA 3456 produced more nodules overall, the nodules produced by USDA 3384 and PNL0i-Brady were able to provide the plants with more nitrogen.

This hypothesis was further confirmed through light microscopy of cross-sections from a USDA 3456 globular nodule and a PNL0i-Brady coralloid nodule. USDA 3456 plant cells stained with toluene blue (See Figure 4.302), stained purple if they were successfully infected with rhizobia and the bacteria cells within the stained plant cells stained as white blotches. Further trials also revealed the presence of a possible infection thread (See Figure 4.301) and further bacteria colonization (See Figure 4.303). Live cross-sections of the PNL0i-Brady nodule stained with Gram's iodine (not pictured), showed several starch granules and a "soup" of

bacteria suggesting that these coralloid nodule cross-sections contained many more bacteria cells than the USDA 3456 globular nodule cross-sections.

Although microscopy could not be performed on all plants, or even on a sample from each strain, the presence of bacteria in samples of each morphology suggested that each plant likely had some bacteria-infected nodules. In order to investigate this theory and determine whether these bacteria might be fixing nitrogen, two trials of Western Blots staining for the leghemoglobin protein were performed on each group. The first Western blot, seen in Figure 4.51, produced bands at approximately 30 kDa, around twice the normal band size produced by the leghemoglobin protein. Experimental error was ruled out as a cause of this discrepancy when a subsequent trial of the Western blot (as seen in Figure 4.52) produced bands similar to the first. The third Western Blot (Figure 4.53) confirmed the presence of leghemoglobin in plants of the Deerfield Competition Study and plants inoculated with Alyce, 76, and 101 (Set 2). Thus, it is more likely that this specific strain of leghemoglobin is either prone to dimerization or the protein extraction buffer used produces leghemoglobin radicals that cause the protein to dimerize (Moreau et. al). It is also possible that the concentration of the SDS-PAGE buffer was too low to effectively break the disulfide bonds in the leghemoglobin protein which might have caused the protein to produce bands much larger than 16 kDa. Future studies might use a different buffer to extract protein from plant nodules and investigate whether similarly-sized bands are produced. Nonetheless, the results of the Western blot suggest the presence of leghemoglobin within each group of plants and support the results of the nodule harvest and microscopy. Future studies might also consider running a leghemoglobin assay to further quantify the presence of the protein within nodules. Since a random assortment of nodules from each group were tested, it is possible

that the coralloid nodules present in each group were mainly responsible for the nitrogen fixation, but only future studies could confirm this theory.

Unfortunately, it is impossible to determine which strain produced the coralloid nodules because they were also observed on the negative control, which was thought to contain no inoculant. Therefore, it is possible that the nodules formed were not a result of the inoculated strains that were supplied, but a result of outside contamination to which each group was equally exposed. It is believed that the source of contamination might be from the Chipilín seeds because they were assumed to have been sterilized by the distributor, and were never properly sterilized in this study. Further studies could run plasmid analysis tests to confirm whether the coralloid nodules resulted from an applied strain or an outside source. If these results were inconclusive, bacteria from a coralloid nodule could be cultured and then sent to the USDA rhizobia bank for identification.

Regrettably, the results of the fertilization study revealed no significant difference in nodule formation between fertilizer applications. Although nitrogen application is known to inhibit nodulation, it is likely that a two-week interval between nitrogen applications was not enough to cause any significant effects. Future studies might not only consider increasing the frequency of nitrogen application, but also increasing the frequency of basic nutrient applications as many of the plants failed to develop strong stalks or began to yellow. Plants grown in Deerfield soil were naturally exposed to a greater supply of nutrients and also grew better than the main set of plants, which further suggests that this withering of plants might be due to a lack of nutrients.

Each group of plants in the competition study only formed coralloid nodules, suggesting an inability for strains that form globular nodules to compete with the other rhizobial strains naturally found in the Deerfield research farm soil. However, Dr. Frank Mangan never observed coralloid nodules on the Chipilín plants he grew in Deerfield, but it is possible that superfluous nitrogen application inhibited nodule formation. Further studies with this particular coralloid strain might prove promising.

Resources

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Appendix A: Fertilizer Calculations

Geoff Wells

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Company: General Hydroponics (manufactures 0-10-10 Li

Phone number: 707 824 9376, ext 117

Email address: gwells@genhydro.com

RE: Kabloom 0-10-10/micronutrients geow4gh@sonic.net [geow4gh@sonic.net]
Sent: Wednesday, January 27, 2010 5:56 PM
To: Mason, Tamara

Hi Tamara
Sorry I missed your email yesterday-

ppm is the same as mg/L
1% is equal to 10,000 ppm => 100% * 10,000ppm= 1,000,000 ppm which is equal to 1

there are 3785 ml per gallon. 1 tsp is approximately 5 ml (actually it is 4,8 ml)
so 1 tsp in a gallon gets diluted by 3785/5= 757
so if you have 1% of an element this is equal to 10,000 ppm of the element
if you dilute 1 tsp per Gallon 10,000 ppm of the element / 757 = 13.2 ppm of the element

Fertilizer N P K is expressed in the oxides for P and K which is P2O5 and K2O
Hydroponic profiles are expressed ass elemental P and elemental K.
to convert P2O5 to P divide by 2.29
to convert K2O to K divide by 1.2

2.5 ml of Liquid Kool Bloom per Gallon (1/2 tsp per Gallon) gives approximately 29 ppm elemental P and 55 elemental K. If you need an exact number I can get a more accurate calculation.
When you add a micronutrient blend I would get enough iron to get between 2 and 3 ppm, and hopefully all the other micronutrients in the blend will be balanced. If your element is 1%, then if you add 1 tsp per gallon you will get about 13 ppm of the element and if you add 1/2 tsp you will get about 6.6 ppm
In your experiment you still need to get calcium magnesium and sulphur. If you don't your plants will very likely not grow in hydroponic culture. If you grow in a potting soil these elements may be present, but you dont know what else will also be present and you may have little control over this.

You could probably use calcium sulphate and magnesium sulphate to get these elements. Calcium sulphate is not very soluble in water, so sometimes people will use calcium carbonate. I can help you calculate this, but I am currently running out of time.
Go study Hoagland nutrient profile and you will get a better understanding of nutrient dynamics. Hopefully this makes sense.
If you this is confusing you can call me at 800 374 9376 We are in California. I may not be back in the office until Monday.
Geoff

Fertilizer Calculations for Greenhouse Crops

$$\text{Amount of fertilizer to make 1 volume of stock solution} = \frac{\text{Desired concentration in parts per million} \times \text{Dilution factor}}{\text{\% of element in fertilizer} \times C}$$

where the dilution factor is the larger number of the fertilizer injector ratio and the conversion constant C is determined by the units desired:

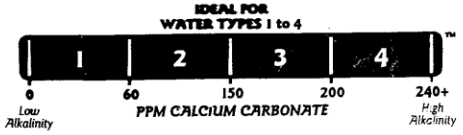
Unit	Conversion constant
Ounces per U.S. gallon	75
Pounds per U.S. gallon	1200
Grams per liter	10

(Boyle)

PETERS EXCEL® WATER SOLUBLE FERTILIZER

Customizing Component

10-0-0 MAGNITRATE SPECIAL



GUARANTEED ANALYSIS **F 1143**
For Continuous Liquid Feed Programs)

Total nitrogen (N)	10%
10% nitrate nitrogen	
Magnesium (Mg) (Total)	9%
9.0% water soluble magnesium (Mg)	
Boron (B)	0.0125%
Copper (Cu)	0.0125%
0.0125% water soluble copper (Cu)	
Iron (Fe)	0.0500%
0.0500% chelated iron (Fe)	
Manganese (Mn)	0.0250%
0.0250% water soluble manganese (Mn)	
Molybdenum (Mo)	0.0050%
Zinc (Zn)	0.0250%
0.0250% water soluble zinc (Zn)	

Derived from: magnesium nitrate, boric acid, copper sulfate, iron EDTA, manganese sulfate, ammonium molybdate, zinc sulfate.

Information regarding the contents and levels of metals in this product is available on the internet at **http://www.regulatory-info-sc.com**.

WARNING: This fertilizer contains more than .001% molybdenum (Mo). The application of fertilizing materials containing molybdenum (Mo) may result in forage crops containing levels of molybdenum (Mo) which are toxic to ruminant animals.

Product Properties		
Potential Basicity	**Conductivity of 100 ppm**	**Maximum Solubility**
357 lbs. calcium carbonate equivalent per ton	0.70	5 lbs/gal

TABLE 2 Weight (In Ounces) of Product Needed To Mix One Gallon of Concentrate

Target Fertilizer Concentration (ppm N) After Dilution	Injector Ratios 1:15	1:100	1:200	EC mmhos/cm of Target Feed Rate After Dilution
50	1	6.8	13.5	0.35
100	2	13.5	27	0.7
200	4.1	27	54	1.4
300	6.1	40.5	***	2.10

TABLE 3 Gallons of Water Required to Dissolve One 25 Lb. Bag of Fertilizer

Target Fertilizer Concentration (ppm N) After Dilution	Injector Ratios 1:100	1:200
50	59.3	29.6
100	29.6	14.8
200	14.8	7.4
300	9.9	***

***Exceeds maximum solubility.

FOR PROFESSIONAL USE ONLY

0 32247 99160 5

99160

042107

Liquid Koolbloom 0-10-10 Fertilizer

Murashige and Skoog basal salt micronutrient solution:

Media Component	Murashige and Skoog basal salt micronutrient solution mg/L
Boric acid	6.2
Cobalt chloride • $6H_2O$	0.025
Cupric sulfate • $5H_2O$	0.025
Na_2-EDTA	37.3
Ferrous sulfate • $7H_2O$	27.8
Manganese sulfate • H_2O	16.9
Molybdic acid (sodium salt) • $2H_2O$	0.25
Potassium iodide	0.83
Zinc sulfate • $7H_2O$	8.6

(Murashige & Skoog Media)

Appendix C: Media Formulations

Yeast Mannitol Broth M716

HiMedia Laboratories Technical Data

Yeast Mannitol Broth M716

Yeast Mannitol Broth is used for cultivation of *Rhizobium* species.

Composition***

Ingredients Gms / Litre
Yeast extract 1.000
Mannitol 10.000
Dipotassium phosphate 0.500
Magnesium sulphate 0.200
Sodium chloride 0.100
Calcium carbonate 1.000
Final pH (at 25°C) 6.8±0.2
**Formula adjusted, standardized to suit performance parameters

Directions

Suspend 12.8 grams in 1000 ml distilled water. Heat just to boiling. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well and dispense into sterile test tubes.

Principle And Interpretation

Beijerinck was first to isolate and cultivate an aerobic gram negative rod-shaped microorganism from the nodules of legume. He named it *Bacillus radicicola* , which was subsequently placed under the genus Rhizobium. Bacteria belonging to the genus *Rhizobium* live freely in soil and in the root region of both leguminous and non-leguminous plants. However they can enter into symbiosis only with leguminous plants by infecting their roots and forming nodules on them. *Rhizobium* present in these root nodules fixes atmospheric nitrogen i.e. gaseous nitrogen from air to organic nitrogen compounds, which is absorbed by plants. Thus role of *Rhizobium* is noteworthy for their major contributions to soil fertility. Yeast Mannitol Broth is used for the cultivation of the symbiotic nitrogen fixing organisms viz. *Rhizobium* species (1).

Yeast extract serves as a good source of readily available amino acids, contain vitamin B complex and accessory growth factors for *Rhizobia* . It also poises oxidation - reduction potential of medium in the range favorable for *Rhizobia* and serves as hydrogen donor in respiratory process (2). Mannitol is the fermentable sugar alcohol source. Calcium and magnesium provide cations essential for the growth of *Rhizobia* @.

Quality Control

Appearance

White to cream homogeneous free flowing powder

Colour and Clarity of prepared medium

Whitish buff coloured opalescent solution in tubes.

Reaction

Reaction of 1.28% w/v aqueous solution at 25°C. pH : 6.8±0.2

Cultural Response

M716: Cultural characteristics observed after an incubation at 30°C for upto 5 days.

Organism Growth

Rhizobium leguminosarum
ATCC 10004
luxuriant
Rhizobium meliloti ATCC
9930
luxuriant

Reference

1. Subba Rao N.S., 1977, Soil Microorganisms and Plant Growth, Oxford and IBG Publishing Company.
2. Allen. E.K. and Allen. O.N., 1950, Bacteriol. Rev., 14:273.

Storage and Shelf Life

Store below 30°C and the prepared medium at 2- 8°C. Use before expiry date on the label.

(CITE FORMULATION DATA- WHERE DID WE GET THIS? AB)

Modified Arabinose Gluconate [MAG]

[Quantities are per liter of medium]

HEPES		1.3g
MES		1.1g
Yeast Extract		1.0g
Arabinose		1.0g
Gluconic Acid		1.0g
KH_2PO_4	0.22g	
Na_2SO_4		0.25g

Stock Solutions (solution concentrations)

NH_4Cl	(16g/100ml)	2.0ml
$FeCl_3$	(0.67g/100ml)	1.0ml
$CaCl_2$	(1.5g/100ml)	1.0ml
$MgSO_4$	(18g/100ml)	1.0ml

Adjust to pH 6.6 w/KOH. Autoclave 20-30 minutes at 120C
*Add 18g Bacto-Agar per liter for solid media**

Some labs use YM, it is a poor growth medium because YM has poor buffering capacity. You will get 5 to 10 fold less cells/ml if you grow bradyrhizobium in YM. Yeast is inhibitory to the growth of bradyrhizobia, so its use should be limited. Phosphate buffer is the most optimal at keeping the media at pH 6.6 – 6.8.

Source: Patrick Elia

Appendix D: Growth Curve Raw Counts

Trial 1

Trial 1, Day 0: 12/15, counted 12/22						
Strain	**Dilution**	**TM**	**AB**	**Avg**	**CFU/ml**	**Avg CFU/ml***
Brady	-2	0	0	0		0
	-3	0	0	0		
	-4	0	0	0		
3384	-2	17	19	18	1.80E+04	2.85E+01
	-3	35	38	37	3.70E+05	
	-4	0	1	1	1.00E+05	
2376	-2	0	0	0		2.00E+00
	-3	3	3	3	3.00E+04	
	-4	1	1	1	1.00E+05	
3456	-2	0	0	0		4.00E+00
	-3	4	4	4	4.00E+04	
	-4	0	0	0		

Trial 1, Day 1: 12/16, counted 12/22						
Strain	**Dilution**	**TM**	**AB**	**Avg**	**CFU/ml**	**CFU/ml Avg**
Brady	-2		104	104	1.04E+05	2.14E+06
	-3		111	111	1.11E+06	
	-4		52	52	5.20E+06	
3384	-2	310		310	3.10E+05	2.00E+06
	-3	79		79	7.90E+05	
	-4	49		49	4.90E+06	
2376	-2		lawn			
	-3		lawn			
	-4		lawn			
3456	-2		68	68	6.80E+04	2.30E+06
	-3		64	64	6.40E+05	
	-4		62	62	6.20E+06	

Trial 1, Day 2: 12/17, counted 12/22						
Strain	**Dilution**	**TM**	**AB**	**Avg**	**CFU/ml**	**Average CFU/ml**
Brady	-3		3	3	3.00E+04	4.43E+05
	-4		3	3	3.00E+05	
	-5		1	1	1.00E+06	
3384	-3		lawn			1.69E+07
	-4		177	177	1.77E+07	
	-5		16	16	1.60E+07	
2376	-3	lawn				4.21E+08
	-4	lawn				
	-5	421		421	4.21E+08	
3456	-3		lawn			9.75E+06
	-4		115	115	1.15E+07	
	-5	8		8	8.00E+06	

Trial 1, Day 3: 12/18, counted 12/22						
Strain	**Dilution**	**TM**	**AB**	**Avg**	**CFU/ml**	**CFU/ml Avg**
Brady	-3		23	23	2.30E+05	7.77E+05
	-4		1	1	1.00E+05	
	-5		2	2	2.00E+06	
3384	-3		lawn			1.07E+08
	-4		lawn			
	-5		107	107	1.07E+08	
2376	-3		183	183	1.83E+06	2.38E+06
	-4		23	23	2.30E+06	
	-5		3	3	3.00E+06	
3456	-3		62	62	6.20E+05	2.23E+07
	-4		94	94	9.40E+06	
	-5		57	57	5.70E+07	

Trial 1, Day 4: 12/19, counted 1/7/10						
Strain	**Dilution**	**TM**	**AB**	**Avg**	**CFU/ml**	**CFU/ml Avg**
Brady	-7	0		0		1.00E+10
	-8	0		0		
	-9	1		1	1.00E+10	
3384	-7	0		0		0
	-8	0		0		
	-9	0		0		
2376	-7	1		1	1.00E+08	1.05E+09
	-8	2		2	2.00E+09	
	-9	0		0		
3456	-7	0		0		0
	-8	0		0		
	-9	0		0		

Trial 1, Day 5: 12/20, counted 1/7/10						
Strain	**Dilution**	**TM**	**AB**	**Avg**	**CFU/ml**	**CFU/ml Avg**
Brady	-7	0		0		2.05E+10
	-8	1		1	1.00E+09	
	-9	4		4	4.00E+10	
3384	-7	16		16	1.60E+09	4.53E+09
	-8	2		2	2.00E+09	
	-9	1		1	1.00E+10	
2376	-7	30		30	3.00E+09	5.83E+10
	-8	12		12	1.20E+10	
	-9	16		16	1.60E+11	
3456	-7	26		26	2.60E+09	7.62E+10
	-8	16		16	1.60E+10	
	-9	21		21	2.10E+11	

Trial 1, Day 6: 12/21, counted 1/7/10						
Strain	**Dilution**	**TM**	**AB**	**Avg**	**CFU/ml**	**CFU/ml Avg**
Brady	-7	3		3	3.00E+08	3.00E+08
	-8	0		0		
	-9	0		0		
3384	-7	0		0		4.00E+10
	-8	0		0		
	-9	4		4	4.00E+10	
2376	-7	9		9	9.00E+08	7.30E+09
	-8	1		1	1.00E+09	
	-9	2		2	2.00E+10	
3456	-7	0		0		0
	-8	0		0		
	-9	0		0		

Trial 1, Day 7: 12/22, counted 1/7/10						
Strain	**Dilution**	**TM**	**AB**	**Avg**	**CFU/ml**	**CFU/ml Avg**
Brady	-7	3		3	3.00E+08	3.00E+08
	-8	0		0		
	-9	0		0		
3384	-7	lawn				
	-8	lawn				
	-9	lawn				
2376	-7	12		12	1.20E+09	4.24E+10
	-8	6		6	6.00E+09	
	-9	12		12	1.20E+11	
3456	-7	11		11	1.10E+09	2.27E+10
	-8	7		7	7.00E+09	
	-9	6		6	6.00E+10	

Trial 2

Trial 2, Day 1: 1/8/10, counted 1/13/10, 12pm					
Strain	**Dilution**	**TM**		**CFU/ml**	**Avg CFU/ml**
Brady	-2	lawn			
	-3	lawn			
	-4	lawn			
3384	-2	lawn			3.27E+07
	-3	1030*	Q1: 207, Q2:308	1.03E+07	
	-4	550		5.50E+07	
2376	-2	lawn			
	-3	lawn			
	-4	lawn			
3456	-2	lawn			1.69E+07
	-3	860*	Q1:215	8.60E+06	
	-4	251		2.51E+07	

Trial 2, Day 2: 1/9/10, counted 1/14/10, 12pm					
Strain	**Dilution**	**TM**		**CFU/ml**	**Avg CFU/ml**
Brady	-3	lawn			5.36E+08
	-4	lawn			
	-5	536		5.36E+08	
3384	-4	398		3.98E+07	6.36E+07
	-5	71		7.10E+07	
	-6	8		8.00E+07	
2376	-4	620*	Q1: 155	6.20E+07	3.18E+08
	-5	252		2.52E+08	
	-6	64		6.40E+08	
3456	-3	60		6.00E+05	6.00E+05
	-4	0		0.00E+00	
	-5	0		0.00E+00	

Trial 2, Day 3: 1/10/10, counted 1/15/10, 2pm				
Strain	**Dilution**	**TM**	**CFU/ml**	**Avg CFU/ml**
Brady	-3	lawn		
	-4	lawn		
	-5	lawn		
3384	-4	532	5.32E+07	1.24E+09
	-5	294	2.94E+08	
	-6	89	8.90E+08	
2376	-4	7	7.00E+05	8.22E+07
	-5	6	6.00E+06	
	-6	24	2.40E+08	
3456	-3	67	6.70E+05	3.10E+07
	-4	14	1.40E+06	
	-5	91	9.10E+07	

Trial 2, Day 4: 1/11/10, counted 1/16/10, 2:15pm					
Strain	**Dilution**	**TM**		**CFU/ml**	**Avg CFU/ml**
Brady	-4	lawn			6.75E+08
	-5	675		6.75E+08	
	-6	lawn			
3384	-5	0		0.00E+00	9.60E+08
	-6	2		2.00E+07	
	-7	19		1.90E+09	
2376	-5	604*	Q1: 151	6.04E+08	4.72E+09
	-6	195		1.95E+09	
	-7	116		1.16E+10	
3456	-4	77		7.70E+06	3.02E+07
	-5	13		1.30E+07	
	-6	7		7.00E+07	

Trial 2, Day 5: 1/12/10, counted 1/17/10, 1:45pm					
Strain	**Dilution**	**TM**		**CFU/ml**	**Avg CFU/ml**
Brady	-4	lawn			2.48E+09
	-5	854*	H1: 427	8.54E+08	
	-6	411		4.11E+09	
3384	-5	87	11	8.70E+07	2.82E+08
	-6	26	5	2.60E+08	
	-7	5	0	5.00E+08	
2376	-5	694		6.94E+08	1.58E+09
	-6	126		1.26E+09	
	-7	28		2.80E+09	
3456	-4	305		3.05E+07	1.23E+08
	-5	119		1.19E+08	
	-6	22		2.20E+08	

Trial 2, Day 6: 1/13/10, counted 1/17/10, 2:15pm					
Strain	**Dilution**	**TM**		**CFU/ml**	**Avg CFU/ml**
Brady	-5	lawn			
	-6	lawn			
	-7	lawn			
3384	-6	24		2.40E+08	3.88E+09
	-7	14		1.40E+09	
	-8	10		1.00E+10	
2376	-6	804		8.04E+09	1.22E+11
	-7	458		4.58E+10	
	-8	313		3.13E+11	
3456	-5	tntc			5.75E+10
	-6	1212*	Q1: 303	1.21E+10	
	-7	1028*	Q1: 257	1.03E+11	

Trial 2, Day 7: 1/14/10, counted 1/19/10, 1pm					
Strain	**Dilution**	**TM**		**CFU/ml**	**Avg CFU/ml**
Brady	-6	lawn			
	-7	lawn			
	-8	lawn			
3384	-7	76		7.60E+09	8.95E+10
	-8	41		4.10E+10	
	-9	22		2.20E+11	
2376	-7	1208*	Q1: 302	1.21E+11	4.44E+11
	-8	675		6.75E+11	
	-9	535		5.35E+11	
3456	-6	tntc			
	-7	tntc			
	-8	tntc			

Trial 3

Trial 3, Day 1: 1/28/10, counted 2/2/10					
Strain	**Dilution**	**TM**		**CFU/ml**	**Avg**
Alyce	-3	67		6.70E+05	3.36E+07
	-4	81		8.10E+06	
	-5	92		9.20E+07	
USDA 101	-3	tntc			5.23E+08
	-4	1140	Q1: 285	1.14E+08	
	-5	932	Q2: 233	9.32E+08	

Trial 3, Day 2: 1/29/10, counted 2/3/10					
Strain	**Dilution**	**TM**		**CFU/ml**	**Avg**
Alyce	-3	tntc			5.61E+08
	-4	1372	Q1: 343	1.37E+08	
	-5	984	Q1: 246,	9.84E+08	
USDA 101	-5	2		2.00E+06	8.01E+08
	-6	0		0.00E+00	
	-7	16		1.60E+09	

Trial 3, Day 3: 1/30/10, counted 2/4/10					
Strain	**Dilution**	**TM**		**CFU/ml**	**Avg**
Alyce	-5	2		2.00E+06	5.10E+07
	-6	0			
	-7	1		1.00E+08	
USDA 101	-4	tntc			8.73E+08
	-5	476	H1: 238	4.76E+08	
	-6	127		1.27E+09	

Trial 3, Day 4: 1/31/10, counted 2/5/10					
Strain	**Dilution**	**TM**		**CFU/ml**	**Avg**
Alyce	-5	778	H1: 389	7.78E+08	5.43E+09
	-6	320		3.20E+09	
	-7	123		1.23E+10	
USDA 101	-6	7		7.00E+07	2.00E+10
	-7	19		1.90E+09	
	-8	58		5.80E+10	

Trial 3, Day 5: 2/1/10, counted 2/6/10					
Strain	**Dilution**	**TM**		**CFU/ml**	**Avg**
Alyce	-4	tntc			2.69E+09
	-5	666	Q1: 126, Q2: 207	6.66E+08	
	-6	472	H: 236	4.72E+09	
USDA 101	-6	94		9.40E+08	4.68E+11
	-8	104		1.04E+11	
	-9	130		1.30E+12	

*H- ½ of the plate was counted, Q- ¼ of the plate was counted

Appendix E: Concentrations of Cultured Strains Over Time as Calculated from Plates Counted

Brady:			
Day	T1 CFU/ml	T2 CFU/ml	Avg CFU/ml
1	2.14E+06	tntc	2.14E+06
2	4.43E+05	5.36E+08	2.68E+08
3	7.77E+05	tntc	7.77E+05
4	1.00E+10	6.75E+08	5.34E+09
5	2.05E+10	2.48E+09	1.15E+10
6	3.00E+08	tntc	3.00E+08
7	3.00E+08	tntc	3.00E+08

3384			
Day	T1 CFU/ml	T2 CFU/ml	Avg CFU/ml
1	2.00E+06	3.27E+07	1.73E+07
2	1.69E+07	6.36E+07	4.02E+07
3	1.07E+08	1.24E+09	6.72E+08
4	Ø	9.60E+08	9.60E+08
5	4.53E+09	2.82E+08	2.41E+09
6	4.00E+10	3.88E+09	2.19E+10
7	tntc	8.95E+10	8.95E+10

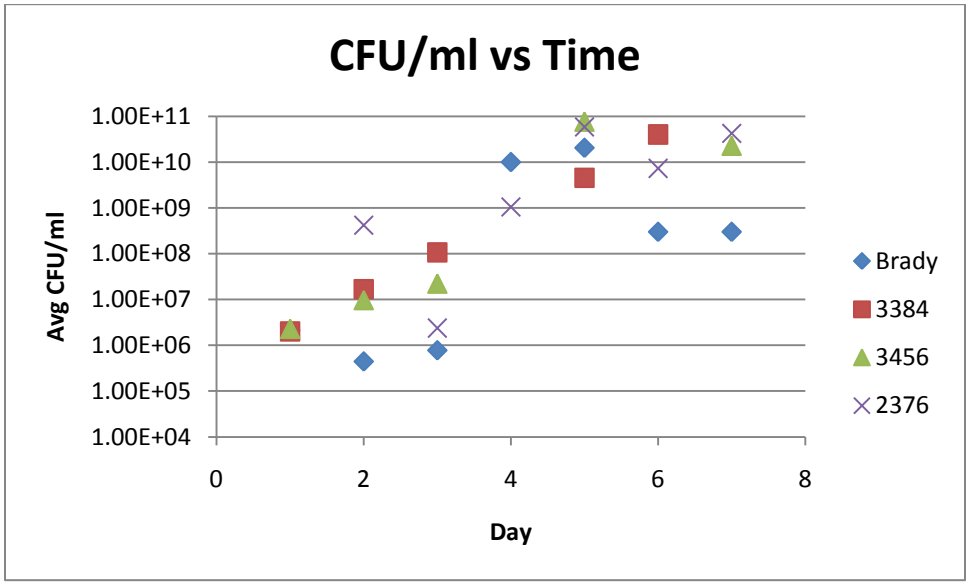
2376			
Day	T1 CFU/ml	T2 CFU/ml	Avg CFU/ml
1	tntc	tntc	
2	4.21E+08	3.18E+08	3.70E+08
3	2.38E+06	8.22E+07	4.23E+07
4	1.05E+09	4.72E+09	2.88E+09
5	5.83E+10	1.58E+09	3.00E+10
6	7.30E+09	1.22E+11	6.48E+10
7	4.24E+10	4.44E+11	2.43E+11

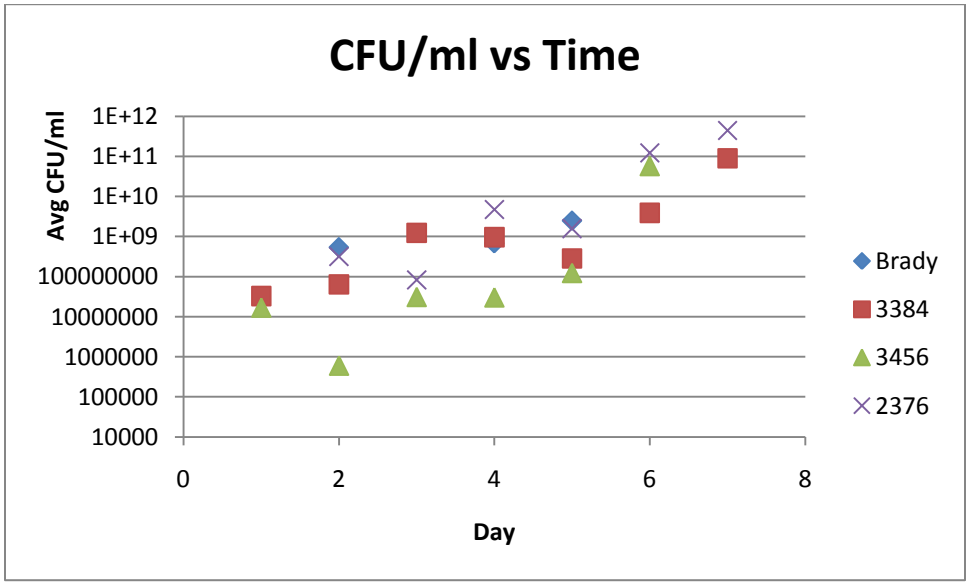
3456			
Day	T1 CFU/ml	T2 CFU/ml	Avg CFU/ml
1	2.30E+06	1.69E+07	9.58E+06
2	9.75E+06	6.00E+05	5.18E+06
3	2.23E+07	3.10E+07	2.67E+07
4	0.00E+00	3.02E+07	1.51E+07
5	7.62E+10	1.23E+08	3.82E+10
6	tntc	5.75E+10	5.75E+10
7	2.27E+10	tntc	2.27E+10

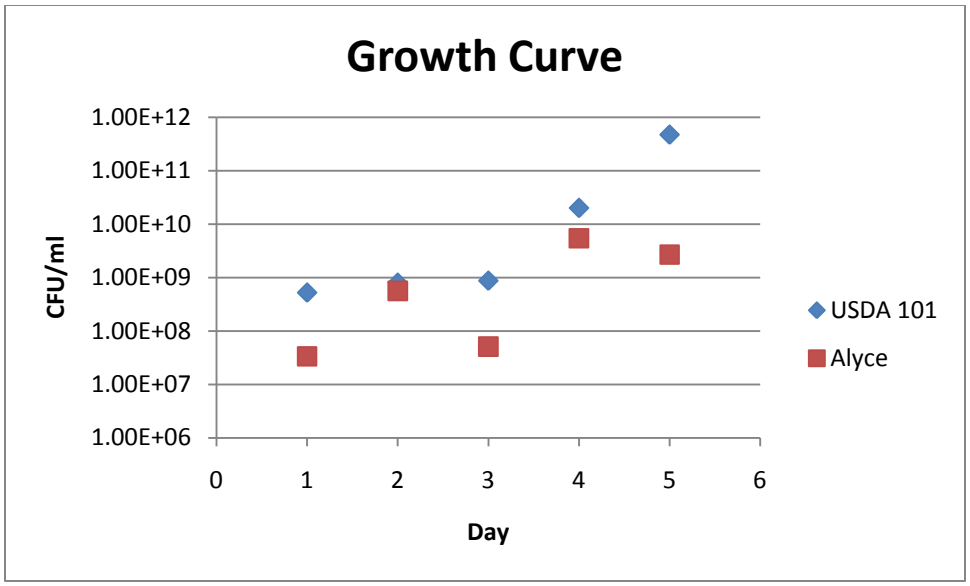
UDSA 101:	
Day	Avg CFU/ml
1	5.23E+08
2	8.01E+08
3	8.73E+08
4	2.00E+10
5	4.68E+11

Alyce:	
Day	Avg CFU/ml
1	3.36E+07
2	5.61E+08
3	5.10E+07
4	5.43E+09
5	2.69E+09

Appendix F: Growth Curves Produced for Individual Trials






Growth Curve
1.00E+12
1.00E+11
1.00E+10
1.00E+09
1.00E+08
1.00E+07
1.00E+06
CFU/ml
0
1
2
3
4
5
6
Day
USDA 101
Alyce

Appendix G: Calculated concentrations of cultures that inoculated plants

Overall CFU/ml that Inoculated our Trials:		
Strain	*CFU/ml*	*Date Inoculated*
Alyce	5.49E+10	2/1/2010
B	4.94E+11	1/14/2010
56	7.94E+11	1/14/2010
76	1.06E+11	2/1/2010
84	7.95E+11	1/14/2010
101	5.05E+10	2/1/2010

Appendix H: Individual Plant Harvest Data

Harvest						Nodules		Plants	
Set	Strain	Fert	ID	Harvest Date	Date from Inoc.	Wet Weight (g)	Obsv.	Height (cm)	Wet Weight (g)
1	Ø	Ø	a	3/31/2010	76	0.02		26	0.56
1	Ø	Ø	b	3/31/2010	76	0.2		16	1.59
1	Ø	Ø	c	3/31/2010	76	1.18		27.4	1.18
1	Ø	Ø	d	4/1/2010	77	0.16		25.5	1.03
1	Ø	Ø	e	4/1/2010	77	0.36		33.5	1.79
1	Ø	Ø	f	4/1/2010	77	0.28		26	2.07
1	Ø	Ø	g	4/1/2010	77	0.11		22.5	1
1	Ø	Ø	h	4/1/2010	77	0.22		38.5	1.94
1	Ø	Ø	i	4/1/2010	77	0.14		19.5	0.73
1	Ø	Ø	j	4/1/2010	77	0.01		23	0.47
1	Ø	1x	a	4/6/2010	82	0.08		17	0.58
1	Ø	1x	b	4/6/2010	82	0.12		21	0.39
1	Ø	1x	c	4/6/2010	82	0.17		42	2.72
1	Ø	1x	d	4/6/2010	82	0.1		26	0.88
1	Ø	1x	e	4/6/2010	82	0.19		36	2.01
1	Ø	1x	f	4/6/2010	82	0.06		24.5	0.97
1	Ø	1x	g	4/6/2010	82	0.04		17	0.57
1	Ø	1x	h	4/6/2010	82	0.1		18.5	0.68
1	Ø	1x	i	4/6/2010	82	0.08		17.5	0.57
1	Ø	1x	j	4/6/2010	82	0.14		32.5	1.8
1	Ø	5x	a	4/7/2010	83	0.12		29.5	2.44
1	Ø	5x	b	4/7/2010	83	0.06		45.5	2.5
1	Ø	5x	c	4/7/2010	83	0.06		35.5	1.8
1	Ø	5x	d	4/7/2010	83	0.06		37.5	1.97
1	Ø	5x	e	4/7/2010	83	0.03		28	1.28
1	Ø	5x	f	4/7/2010	83	-		30	1.22
1	Ø	5x	g	4/7/2010	83	0.25		42.5	2.63
1	Ø	5x	h	4/7/2010	83	0.24		48	2.93
1	Ø	5x	i	4/7/2010	83	0.27		51	4.01
1	Ø	5x	j	4/7/2010	83	0.17		53.5	2.57
1	84	Ø	a	3/31/2010	76	0.2		30.5	1.82
1	84	Ø	b	3/31/2010	76	0.48		39.5	2.27

1	84	Ø	c	3/31/2010	76	0.22		18	0.3
1	84	Ø	d	3/31/2010	76	0.14		40	2.35
1	84	Ø	e	3/31/2010	76	0.25		21	0.72
1	84	Ø	f	3/31/2010	76	0.17		28.5	1.43
1	84	Ø	g	3/31/2010	76	0.12		41.5	2.29
1	84	Ø	h	3/31/2010	76	0.36		38	2.18
1	84	Ø	i	3/31/2010	76	0.19		24.5	0.86
1	84	Ø	j	3/31/2010	76	0.26		30.5	1.52
1	84	1x	a	4/5/2010	81	0.99		32.5	1.46
1	84	1x	b	4/5/2010	81	0.66		33.5	1.07
1	84	1x	c	4/5/2010	81	0.11		30	2.01
1	84	1x	d	4/5/2010	81	0.13		30	1.76
1	84	1x	e	4/5/2010	81	0.24		38	2.43
1	84	1x	f	*					
1	84	1x	g	4/5/2010	81	0.25		30.5	0.93
1	84	1x	h	4/5/2010	81	0.27	Fl. pods	23	0.47
1	84	1x	i	4/5/2010	81	0.2		28.5	1.6
1	84	1x	j	4/5/2010	81	0.23	Flower+	44	2.73
1	84	5x	a	4/6/2010	82	0.08		39	2.38
1	84	5x	b	4/6/2010	82	0.75		38	1.46
1	84	5x	c	4/6/2010	82	0.57		42	1.56
1	84	5x	d	4/6/2010	82	0.58		29	1.27
1	84	5x	e	4/6/2010	82	0.22		30.5	0.83
1	84	5x	f	4/6/2010	82	0.43		41	2.16
1	84	5x	g	4/6/2010	82	0.08		37	2.06
1	84	5x	h	4/6/2010	82	0.12		45	2.6
1	84	5x	i	4/6/2010	82	0.35		22.5	0.7
1	84	5x	j	4/6/2010	82	0.24		34.5	1.7
1	56	Ø	a	3/31/2010	76	0.53		26	0.7
1	56	Ø	b	3/31/2010	76	0.19		17.5	0.62
1	56	Ø	c	3/31/2010	76	0.23		14.5	0.39
1	56	Ø	d	3/31/2010	76	0.08		27	0.65
1	56	Ø	e	3/31/2010	76	0.4		18.5	0.58
1	56	Ø	f	3/31/2010	76	0.16	2 stalks	16.5	0.42
1	56	Ø	g	3/31/2010	76	0.28		42	2.86
1	56	Ø	h	3/31/2010	76	0.54		23.5	1.65
1	56	Ø	i	3/31/2010	76	0.26		33	0.61
1	56	Ø	j	3/31/2010	76	0.7	Sideplant (sm.)	39	2.94

1	56	1x	a	4/5/2010	81	0.26		29	0.86
1	56	1x	b	4/5/2010	81	0.26	Sideplant (sm.)	41	1.44
1	56	1x	c	4/5/2010	81	0.34		29.5	1.18
1	56	1x	d	4/5/2010	81	0.92		30	1.28
1	56	1x	e	4/5/2010	81	0.55		21.5	0.64
1	56	1x	f	*					
1	56	1x	g	4/5/2010	81	0.28		34.5	1.29
1	56	1x	h	4/5/2010	81	0.34		27	0.79
1	56	1x	i	4/5/2010	81	0.31		43.5	2.99
1	56	1x	j	4/5/2010	81	0.47		39	2.72
1	56	5x	a	4/7/2010	83	0.57		45	3.36
1	56	5x	b	4/7/2010	83	0.64		40.5	3.18
1	56	5x	c	4/7/2010	83	0.58		42	1.95
1	56	5x	d	4/7/2010	83	0.15		35.5	1.54
1	56	5x	e	4/7/2010	83	0.5		29	1.19
1	56	5x	f	4/7/2010	83	0.38		46	3.3
1	56	5x	g	4/7/2010	83	0.17		44	2.28
1	56	5x	h	4/7/2010	83	0.15		39	1.6
1	56	5x	i	4/7/2010	83	0.48		41.5	2.28
1	56	5x	j	4/7/2010	83	0.75	flower	43.5	1.71
1	B	Ø	a	3/30/2010	75	0.5		31	1.74
1	B	Ø	b	3/30/2010	75	0.12		16.5	0.56
1	B	Ø	c	3/30/2010	75	0.36		32.5	1.32
1	B	Ø	d	3/30/2010	75	0.75		40.5	1.74
1	B	Ø	e	3/30/2010	75	0.54	2 plants	29	1.77
1	B	Ø	f	3/30/2010	75	0.26		38.5	3.73
1	B	Ø	g	3/30/2010	75	0.25		41.5	2.43
1	B	Ø	h	3/30/2010	75	0.5		23	1.21
1	B	Ø	i	3/30/2010	75	0.22		21	0.73
1	B	Ø	j	3/30/2010	75	0.5		31	1.66
1	B	1x	a	4/1/2010	77	0.28		16	0.54
1	B	1x	b	4/1/2010	77	0.47		48	2.6
1	B	1x	c	4/1/2010	77	0.43		23	0.83
1	B	1x	d	4/1/2010	77	0.25		68.5	5.31
1	B	1x	e	4/1/2010	77	0.32		27	1.11
1	B	1x	f	*					
1	B	1x	g	4/1/2010	77	0.28		32.5	1.77
1	B	1x	h	4/1/2010	77	0.25		21.5	2.14

1	B	1x	i	4/1/2010	77	0.23		25	0.67
1	B	1x	j	4/1/2010	77	0.28		37	2.44
1	B	5x	a	4/6/2010	82	0.44		55	3.67
1	B	5x	b	4/6/2010	82	0.26		28	0.98
1	B	5x	c	4/6/2010	82	0.04		30.5	0.82
1	B	5x	d	4/6/2010	82	0.3		18	1.24
1	B	5x	e	4/6/2010	82	0.22		35.5	1.38
1	B	5x	f	4/6/2010	82	0.33		31.5	1.54
1	B	5x	g	4/6/2010	82	0.17		24.5	0.49
1	B	5x	h	4/6/2010	82	0.03		50.5	3.51
1	B	5x	i	4/6/2010	82	0.15		23	1.02
1	B	5x	j	4/6/2010	82	0.42		37	1.79
2	101	Ø	a	4/19/2010	77	0.18	most bigger pink	20.5	0.96
2	101	Ø	b	4/19/2010	77	0.18	All pink	27	1.04
2	101	Ø	c	4/19/2010	77	0.21	lots pink	37	2.33
2	101	1x	a	4/19/2010	77	0.13		18.5	0.76
2	101	1x	b	4/19/2010	77	0.16	all pink	34	1.5
2	101	1x	c	4/19/2010	77	0.46		36	1.87
2	101	5x	a	4/19/2010	77	0.15	many small, not many fans	22.5	0.37
2	101	5x	b	4/19/2010	77	0.06	broken one, 3 total	11.5	0.9
2	101	5x	c	4/19/2010	77	0.18	2 plants	34.5	1.57
2	A	Ø	a	4/18/2010	76	0.05	Few. Several coraloid	24	0.93
2	A	Ø	b	4/18/2010	76	0.34	Many/all fans, pink	26	1.62
2	A	Ø	c	4/18/2010	76	0.18	All elongated/fans, pink	24.5	1.16
2	A	1x	a	4/18/2010	76	0.17	All fan, no small	23.5	1.03
2	A	1x	b	4/18/2010	76	0	1 total. Small.	18	0.44
2	A	1x	c	4/18/2010	76	0.28	Many fans, lg red	37.5	2.53
2	A	5x	a	4/18/2010	76	0.14	1 pink/lg, 3 total	29.5	1.35
2	A	5x	b	4/18/2010	76	0.21	Many lg pink/elongated	30	1.55

2	A	5x	c	4/18/2010	76	0.09	Crooked plant growth. Only a few nodules.	33	1.03
2	76	Ø	a	4/18/2020	76	0.02		17.5	0.35
2	76	Ø	b	4/18/2010	76	0.01		18	0.3
2	76	Ø	c	4/18/2010	76	0		21	0.52
2	76	1x	a	4/18/2010	76	0.15	Mostly only fan	25	0.49
2	76	1x	b	4/18/2010	76	0	1, not coraloid	19.5	0.97
2	76	1x	c	4/18/2010	76	0.28	Globular, all, large, dark colored	40	1.36
2	76	5x	a	4/18/2010	76	0.07	4 total. Half fan, half small	24.5	1.19
2	76	5x	b	4/18/2010	76	0.17	5 total, only fan. Flower	33.5	1.78
2	76	5x	c	4/18/2010	76	0.26	Many fan, all pink	36	2.58
DF1	Ø	Ø	a	4/19/2010	94	0.5		51	3.48
DF1	Ø	Ø	b	4/19/2010	94	0.36		48	2.97
DF1	Ø	1x	a	4/19/2010	94	0.28		38	2.06
DF1	Ø	1x	b	4/19/2010	94	0.67		62.5	5.43
DF1	Ø	5x	a	4/19/2010	94	0.15		48.5	3.7
DF1	Ø	5x	b	4/19/2010	94	0.1		32.5	1.3
DF1	56	Ø	a	4/19/2010	94	0.39		39	1.63
DF1	56	Ø	b	4/19/2010	94	0.52		36.5	2.31
DF1	56	1x	a	4/19/2010	94	0.58		53.5	4.89
DF1	56	1x	b	4/19/2010	94	0.24		36.5	1.46
DF1	56	5x	a	4/19/2010	94	0.1		42	2.34
DF1	56	5x	b	4/19/2010	94	0.02	2 total	32	0.92
DF1	84	Ø	a	4/19/2010	94	0.27		42.5	3.41
DF1	84	Ø	b	4/19/2010	94	0.45	lots, small	55.5	4.56
DF1	84	1x	a	4/19/2010	94	0.31		36	2.13
DF1	84	1x	b	4/19/2010	94	0.52	flowers	43	2.43
DF1	84	5x	a	4/19/2010	94	0.04		52.5	3.79
DF1	84	5x	b	4/19/2010	94	0.09		33.5	1.5
DF1	B	Ø	a	4/19/2010	94	1.4		41	2.4
DF1	B	Ø	b	4/19/2010	94	0.44		42	1.81
DF1	B	1x	a	4/19/2010	94	0.6		44	2.22
DF1	B	1x	b	4/19/2010	94	0.11	Only small	29	1.15

DF1	B	5x	a	4/19/2010	94	0.48	Mostly only pink	41	3.49
DF1	B	5x	b	4/19/2010	94	0.32	2 plants	32	2.75
DF2	76	Ø	a	4/19/2010	77	0.16		30	1.58
DF2	76	Ø	b	4/19/2010	77	0.35		34	1.93
DF2	76	1x	a	4/19/2010	77	0.64		60	5.3
DF2	76	1x	b	4/19/2010	77	0.15		40	1.76
DF2	76	5x	a	4/19/2010	77	0.29		43.5	4.12
DF2	76	5x	b	4/19/2010	77	0.61		62	5.35
DF2	101	Ø	a	4/19/2010	77	0.02		31.5	1.52
DF2	101	Ø	b	4/19/2010	77	0.43		42.5	3.34
DF2	101	1x	a	4/19/2010	77	0.82	lots of big, pink	47	3.66
DF2	101	1x	b	4/19/2010	77	0.22		30.5	1.69
DF2	101	5x	a	4/19/2010	77	0.44		46.5	3.7
DF2	101	5x	b	4/19/2010	77	0	1 (small)	34.5	0.98
DF2	A	Ø	a	4/19/2010	77	0.15	3 (1 lg, 1sm, 1med)	34.5	2.23
DF2	A	Ø	b	4/19/2010	77	0.34	only big (lots)	37.5	2.53
DF2	A	1x	a	4/19/2010	77	0.07	sev. Pink ones (1 big, others small)	31	1.76
DF2	A	1x	b	4/19/2010	77	0.03	1, very small amt of roots	26.5	1.04
DF2	A	5x	a	4/19/2010	77	0.7		47	4.92
DF2	A	5x	b	4/19/2010	77	0.12	3 total: big, pink	35	1.78

Notes:

- * Plants harvested one week prior to overall harvest date due to use in microscopy methodology
- Set 1, DF1 inoculated 18 days prior to Set 2, DF2
- DF1/DF2 are the Competition Study Trials

Appendix I: Nodules Taken for Microscopy Studies

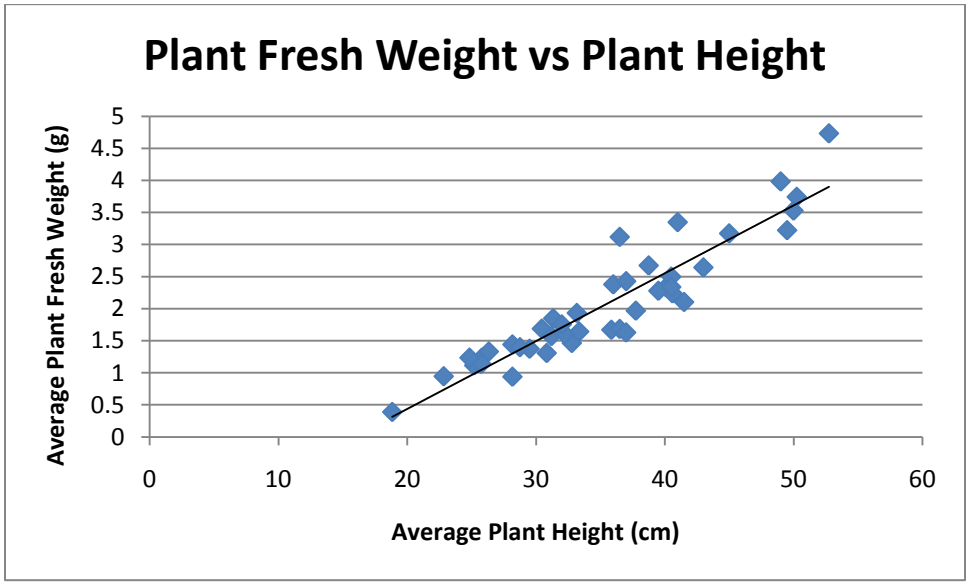
Nodules Taken Done for Microscopy with Dr. Dan Gibson			
3.19.10			
Plant ID #	Nodule/Plant Notes	Nodule Weight (g)	Plant Weight with Roots (g)
B1XF	Some leaf death seen	0.6	3.27
561XF	Some leaf death seen	0.14	1.47
3.23.10			
Plant ID #	Nodule/Plant Notes	Nodule Weight (g)	Plant Weight with Roots (g)
8f41XF	Some yellow leaves	0.17	

Appendix J: Average Harvest Data

Harvest						Nodules		Plants		
Set	Strain	Fert	N=	Harvest Date	Date from Inoc.	Avg. Wet Weight (g)	Total Dry Weight (g)	Height (cm)	Avg. Wet Weight (g)	Total Dry Weight (g)
1	Negative	Ø	10	3/30/2010 4/1/2010	77	0.268	0.2	25.79	1.236	3.2
1	Negative	1x	10	4/6/2010	82	0.108	0.14	25.2	1.117	3.3
1	Negative	5x	10	4/7/2010	83	0.14	0.19	40.1	2.335	7.2
1	Brady	Ø	10	3/30/2010	75	0.4	0.48	30.45	1.689	4.43
1	Brady	1x	9	4/1/2010	77	0.31	0.3	33.167	1.934	4.95
1	Brady	5x	10	4/6/2010	82	0.236	0.35	33.35	1.644	n/a
1	56	Ø	10	3/31/2010	76	0.337	0.07	25.75	1.142	2.8
1	56	1x	9	4/5/2010	81	0.414	0.61	32.778	1.4655	3.8
1	56	5x	10	4/7/2010	83	0.437	0.64	40.6	2.239	7.1
1	84	Ø	10	3/31/2010	76	0.239	0.29	31.2	1.574	2.29
1	84	1x	9	4/5/2010	81	0.342	0.606	32.22	1.607	4.3
1	84	5x	10	4/6/2010	82	0.342	0.44	35.85	1.672	n/a
2	Alyce	Ø	3	4/18/2010	76	0.19	n/a	24.833	1.23667	1
2	Alyce	1x	3	4/18/2010	76	0.15	n/a	26.333	1.333	1.1
2	Alyce	5x	3	4/18/2010	76	0.1467	n/a	30.83	1.31	0.9
2	76	Ø	3	4/18/2010	76	0.01	n/a	18.833	0.39	0.2
2	76	1x	3	4/18/2010	76	0.1433	n/a	28.167	0.94	0.5
2	76	5x	3	4/18/2010	76	0.1667	n/a	31.333	1.85	1.4
2	101	Ø	3	4/19/2010	77	0.19	n/a	28.167	1.443	0.9
2	101	1x	3	4/19/2010	77	0.25	n/a	29.5	1.376	1.3
2	101	5x	3	4/19/2010	77	0.13	n/a	22.83	0.9467	1
DF1	Negative	Ø	2	4/19/2010	94	0.43	n/a	49.5	3.225	1.7
DF1	Negative	1x	2	4/19/2010	94	0.475	n/a	50.25	3.745	1.8
DF1	Negative	5x	2	4/19/2010	94	0.125	n/a	40.5	2.5	1.2
DF1	Brady	Ø	2	4/19/2010	94	0.92	n/a	41.5	2.105	1.2
DF1	Brady	1x	2	4/19/2010	94	0.355	n/a	36.5	1.685	0.6
DF1	Brady	5x	2	4/19/2010	94	0.4	n/a	36.5	3.12	1.1
DF1	56	Ø	2	4/19/2010	94	0.455	n/a	37.75	1.97	0.8
DF1	56	1x	2	4/19/2010	94	0.41	n/a	45	3.175	1.6
DF1	56	5x	2	4/19/2010	94	0.06	n/a	37	1.63	0.8
DF1	84	Ø	2	4/19/2010	94	0.36	n/a	49	3.985	1.8

DF1	84	1x	2	4/19/2010	94	0.415	n/a	39.5	2.28	0.8
DF1	84	5x	2	4/19/2010	94	0.065	n/a	43	2.645	1.5
DF2	Alyce	Ø	2	4/19/2010	77	0.245	n/a	36	2.38	1.2
DF2	Alyce	1x	2	4/19/2010	77	0.05	n/a	28.75	1.4	0.3
DF2	Alyce	5x	2	4/19/2010	77	0.41	n/a	41	3.35	1.6
DF2	76	Ø	2	4/19/2010	77	0.255	n/a	32	1.755	1
DF2	76	1x	2	4/19/2010	77	0.395	n/a	50	3.53	1.8
DF2	76	5x	2	4/19/2010	77	0.45	n/a	52.75	4.735	2
DF2	101	Ø	2	4/19/2010	77	0.225	n/a	37	2.43	1
DF2	101	1x	2	4/19/2010	77	0.52	n/a	38.75	2.675	1.3
DF2	101	5x	2	4/19/2010	77	0.44	n/a	40.5	2.34	0.8

Appendix K: Correlation between Fresh Weight and Plant



Appendix L: Protein Extraction

BSA Standard Curve Raw Data

ug/ml	A_{660}
2000	1.178
1500	0.908
1000	0.687
750	0.507
500	0.372
250	0.147
125	0.086

Set 1 Raw Protein Extraction Data: Individual Extraction Trial Data

			Trial 1		Trial 2		Trial 3		Average
Strain	N_2	Dry Weight of Nodule Sample (g)	*A660*	*Protein Concentration (ug/ml)*	A660	Protein Concentration (ug/ml), (accounting for 1:2 Dilution)	A660	Protein Concentration (ug/ml)	Protein Concentration (ug/ml)
Ø	Ø	0.02	*0.04*	*-9.167*	0.138	308.334	0.229	100.83	204.582
Ø	1x	0.02	*0.012*	*-55.833*	0.138	308.334	0.056	-40.83	133.752
Ø	5x	0.02	*0.027*	*-30.833*	0.14	315	0.235	265.833	290.4165
Brady	Ø	0.02	*0.065*	*32.5*	0.014	-105	0.217	650.83	325.415
Brady	1x	0.02	*0.018*	*-45.833*	0.159	378.334	0.057	89.167	233.7505
Brady	5x	0.02	*-0.01*	*-83.33*	0.006	-131.666	0.106	175.833	87.9165
56	Ø	0.02	*0.064*	*30.83*	0.017	-95	0.106	305.83	152.915
56	1x	0.02	*0.054*	*14.167*	0.01	-118.334	0.021	20.83	10.415
56	5x	0.02	*0.037*	*-14.167*	0.154	361.666	0.205	315.83	338.748
84	Ø	0.02	*0.012*	*-55.833*	0.133	291.666	0.436	285.83	288.748
84	1x	0.02	*0.003*	*-70.833*	0.014	-105	0.099	19.167	9.5835
84	5x	0.02	*0.037*	*-1.167*	0.038	-25	0.151	100.83	50.415
Leaf/Neg			*-0.01*	*-67.5*	0.002	-145	0.033	-20.833	0

Blank: Protein Extraction Buffer
Protein concentration found using the equation formulated from BSA standard curve. Equation used X= y-0.0455/0.0006
Dry weight of Trial 3 was approximately 0.02.
All Negative Numbers Assumed to be 0.
Trial 1 was discounted due to unimpressive protein extraction most likely due to short reconstitution period (several hours verses overnight).

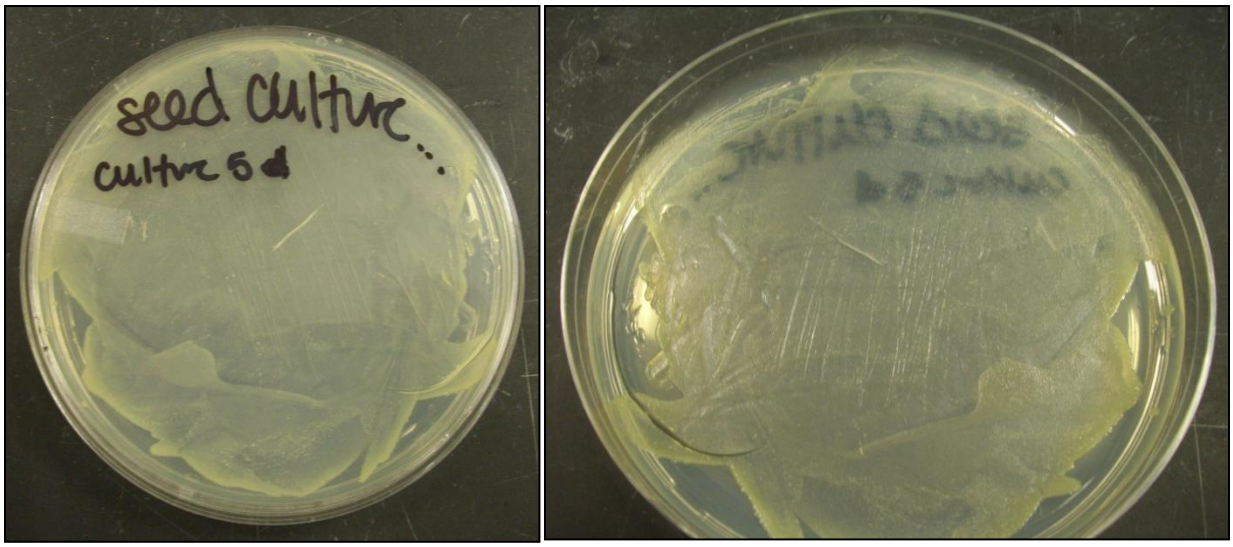
Raw Protein Extraction Data for Set 2

Strain	Fertilizer	Dry Weight of Nodules	A660	Protein Concentration (ug/ml)
Alyce	Ø	0.026	0.452	677.5
Alyce	1x	0.0328	0.156	184.167
Alyce	5x	0.0422	0.295	415.83
76	Ø	0.0055	0.445	665.83
76	1x	0.0358	0.337	485.83
76	5x	0.0335	0.138	154.167
101	Ø	0.0382	0.016	-49.167
101	1x	0.033	0.117	119.167
101	5x	0.0313	0.344	497.5
L			0.033	-20.83

Raw Protein Extraction Data for Competition Study

Strain	Fertilizer	Dry Weight of Nodules	A660	Protein Concentration (ug/ml)
Negative	Ø	0.0496	0.339	489.167
Negative	1x	0.0507	0.418	620.833
Negative	5x	0.0288	0.875	1382.5
Alyce	Ø	0.0271	0.298	420.83
Alyce	1x	0.0163	0.025	-34.167
Alyce	5x	0.0344	0.112	110.83
Brady	Ø	0.0424	0.068	37.5
Brady	1x	0.033	0.008	-62.5
Brady	5x	0.0469	0.577	885.833
101	Ø	0.0509	0.341	492.5
101	1x	0.0728	0.373	545.833
101	5x	0.0436	0.454	680.83
84	Ø	0.0304	0.453	679.167
84	1x	0.0399	0.011	-57.5
84	5x	0.0155	0.147	169.167
76	Ø	0.0454	0.32	457.5
76	1x	0.0608	0.21	274.167
76	5x	0.0473	0.469	705.83
56	Ø	0.0332	0.21	274.167
56	1x	0.0397	0.17	207.5
56	5x	0.0164	0.169	205.83
Leaf/Neg		0.02	0.033	-20.83

Appendix M: Seed cultured growth



Pictures of the direct bacteria cultures grown from the seeds. These pictures were taken two days after the plates were cultured.