Analysis of Leghemoglobin Present in Chipilin-Rhizobia Symbiosis

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Abstract

Chipilin, a legume, is a key ingredient in many customary dishes in Southern Mexico and Central America, where the plant thrives. Due to the influx of immigrants from this region to New England, there has been an increased desire for Chipilin to be available in local markets. Chipilin does not grow well in New England due its high nitrogen requirements, which it obtains from the rhizobia soil bacteria through a symbiotic process. Unfortunately, the strains of rhizobia that work best with Chipilin do not naturally occur in New England. The purpose if this project is to identify a strain of rhizobia bacteria that forms the best symbiotic relationship with Chipilin, in order to make Chipilin a more profitable crop to be grown in New England. The two strains of rhizobia bacteria tested were PNL0i-Brady and USDA 3384. 168 plants were grown from seeds and inoculated with either one of the two strains, a combination of the two strains and a control with no rhizobia. Immunoblots were performed on the leghemoglobin which resides in plant nodules, which were a result of the Chipilin-rhizobia symbiosis process. The rhizobia's effectiveness was quantified and evaluated.

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Introduction

The legume, Chipilin is a crop that originates in Southern Mexico and Central America, and is used as a key ingredient in many customary dishes. Due to the influx of immigrants from this region of the world to the Northeast, United States (US), there has been an increased desire for Chipilin because of this new local immigrant population. However, this need is met with some difficulty as the plant does not grow well here in local conditions. This is due to the high nitrogen requirements of the Chipilin plant(Eckert). The amount of nitrogen needed to grow Chipilin is similar to its cousin the soybean which requires around 220 N lbs/A, compare to wheat, which only requires105 Nlbs/A(Nachurs). This requirement is alleviated in its local environment by the mutually symbiotic relationship of legumes and rhizobia bacteria which can fix over 100 pounds of nitrogen per acre(Eckert). This is not the case in the Northeast, because the most beneficial rhizobia bacterium for Chipilin does not reside locally.

Legumes and Rhizobia bacteria have a close symbiotic relationship. Legumes provide carbohydrates to the Rhizobia in exchange for the Rhizobia fixing nitrogen into ammonia for the legume.(Hirsch) A vast majority of the legumes produce nodules through the symbiosis process, but each legume forms a relationship with each strain of Rhizobia with varying level of success.(Hirsch) This success is measured by the amount of nitrogen fixed for the Chipilin, the increase in the well-being of the Chipilin, as well as the increased benefit of the bacteria within the plant.

The purpose of this study is to find a strain of Rhizobia that can form a strong nitrogen fixing relationship with the legume Chipilin. This relationship would improve the yield of the Chipilin plant, and reduce the amount of nitrogen fertilizer that is used to

help the plant grow, thus improving the profitability of the crop. The strain of Rhizobia that fixes nitrogen naturally for Chipilin is unavailable because of legal constraints, in which permits are required for importation to the US.(USDA - APHIS - Plant Health, Plant Protection and Quarantine)

Chipilin Overview

Crotalaria longirostrata commonly known as “Chilipin” is a legumous plant that is natively grown in Southern Mexico and Central America. Specifically in Central America it is grown in El Salvador and Guatemala.(Crotalaraia Longirostrata) Figure 1 shows a map of this region. Chipilin can be characterized as a green leafy perennial plant that grows to approximately five feet in height and has yellow flowers.(Crotalaraia Longirostrata) In Southern Mexico and Central American Chipilin is readily available in the markets and is commonly used as an herb to add flavor in soups, tamales and tortillas.(Cooperative Extension) Chipilin is also very nutritious since its leaves are high in calcium, iron, thiamine, riboflavin, niacin, and ascorbic acid.(Morton) Figure 2 shows a picture of a Chipilin plant, and Figure 3 is a photograph of a soup made with Chipilin leaves.

Figure 1: Map of Central America where Chipilin is grown.(Atlas)

Figure 2: Chipilin Plant (Cooperative Extension)

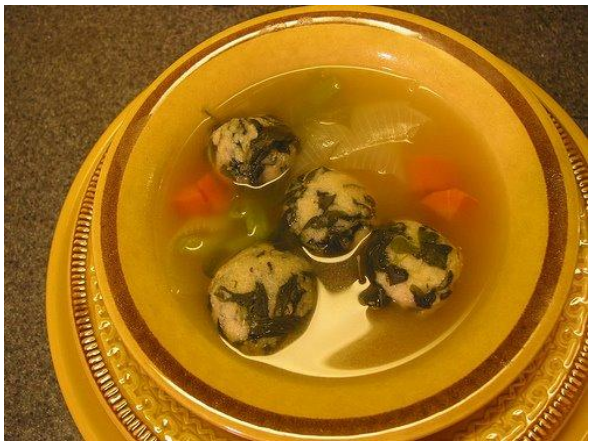

Figure 3: Chipilin Dumpling Soup (Sopa de bolita de chipilin)

Since Chipilin is a tropical plant it flourishes in Southern Mexico and Central America.(Crotalaraia Longirostrata) The climate in these two regions is stable and has an average temperature of 22ºC, throughout the year, providing an excellent environment for Chipilin to grow. In the Northeast, US the climate is dramatically different, with wide ranges of temperature from 0-24ºC throughout the year.(Countries of the World) However, it is not only the climate difference that diminishes the Chipilin's growth and profitability, but it is also the lack of the right type nitrogen fixing bacteria to help the plant flourish in the Northeast.

Despite Chipilin's popularity in Southern Mexican and Central American cuisine, it still remains relatively unknown as a food crop in the US. This is most likely due to the fact that it is considered to be noxious or a weedy and invasive plant in the US where it is able to grow, specifically Hawaii.(PLANTS) Also its popularity is limited because of the large region of the US where the Chipilin is unable grow. Chipilin also requires a large amount of nitrogen to grow in the Northeast. This large requirement makes it unprofitable for production and sale. This is because Chipilin in its natural environment receives approximately five times as much bacteria when forming a symbiotic relationship with the proper rhizobia than the Chipilin growing alone.(Eckert) Therefore, it is only rarely used in the US in ethnic foods. Because of this Chipilin is not common in American markets. However, with the growing population of Latin American immigrants in Northeastern, US, there has been a heightened demand for ingredients for their native foods such as Chipilin. If Chipilin becomes more popular and can be grown in a controlled environment, it could be seen for its culinary uses rather than its lack of growth.

Dr. Frank Mangan and his team of the UMASS Research Farm in Deerfield, MA, have recently been working to grow Chipilin in a profitable way in the Northeast for this influx of the immigrant population and as well for cash crop farmers. They are growing the perennial Chipilin as an annual because of the climate difference. As part of this program they sell the Chipilin in local Latino stores in Massachusetts with much success. However the growth of Chipilin in the Northeast is hindered by the potato leaf hopper, which eats the plants. Because of this they have been working on ways to stop the bug such as using a combination of row covers and insecticide.(Hazzard)

Rhizobia Infection

Legumes have a special symbiotic relationship with bacteria within the Rhizobia genus. Rhizobia bacteria fall within two main groups of proteobacteria, the alpha and beta (Weir). Rhizobia are soil bacteria, which fix nitrogen when they are inside the root nodules of a legume. Both the legume and the rhizobia benefit from this occurrence.(Jones) The way in which the rhizobia are able to invade a legume is a complex multi-step process, which will be explained in this section.

Legumes, such as Chipilin, are different than many plants in that they need more nitrogen than other plants to be at their best growth potential. Chipilin needs nitrogen to grow and flourish and the rhizobia are able to provide the legumes with this extra source of nitrogen. In return the rhizobia are provided nourishment and shelter by the legume. The first step in this symbiotic process is taken by the legume. The legume excretes a compound called flavonoid. A flavonoid is a plant pigment, with many roles such as attracting animals for pollination, signaling the rhizobia and helping them during the infection stage of the plant, and as an anti-fungus compound.(Galeotti). When the

flavonoids reach the soil they trigger the rhizobia to secrete Nod(Nodulation) factors, which are signals back for the rhizobia to the legume. When the legume senses the Nod factors, the root hairs of the legume in the soil begin to curl, and in doing so they trap the rhizobia inside the root hair. Once in the root hair the rhizobia cause an infection thread, which creates a path for the rhizobia to travel from the root hair tip to the internal legume. Simultaneously, the Chiplin's cortical cells begin a cell division process, which will allow the nodule to form later in the symbiosis process. Once fully inside the legume the rhizobia begin a cell division process around the infection thread, creating a nodule on the legume. In the nodule around the rhizobia will differentiate into nitrogen fixating bacteriods, and begin to fix nitrogen to the legume. The process of the nitrogen fixation will be discussed in greater detail in the next section. In return for fixing the nitrogen the rhizobia are provided with oxygen and carbohydrates.(Jones) This process is depicted in Figure 4.

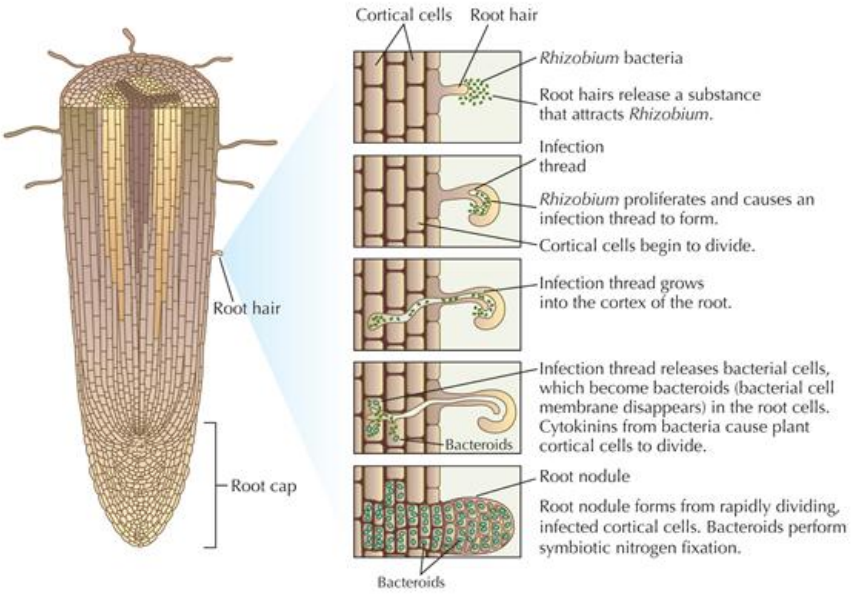

Figure 4: Symbiosis of Rhizobium bacteria with legumes (Symbiosis of Rhizobium Bacteria with Legumes)

Nitrogen Fixation and Leghemoglobin

Nitrogen fixation aids the legumes in development and allows them to compete with other plants. When the rhizobia fix nitrogen they take nitrogen from the atmosphere (N_2) and convert it into ammonium (NH_4^+), through a process called mineralization(Barbarick), which it provides to the plant.(Jones) If a legume is fully nodulated indicating that nitrogen fixation is occurring, then the legume will not be aided by any additional nitrogen sources such as fertilizer.(Barbarick)

Leghemoglobins are an important part of nitrogen fixation. Leghaemoglobins are plant proteins that bind oxygen and give active nodules a reddish color. It has recently been found that Leghemoglobins are required for nitrogen fixation because they provide the rhizobia with low free oxygen concentrations with a high binding affinity in the nodules and also provide high energy status. Leghemoglobin is only produced by the legume after symbiosis has taken place.(Ott)

Hypothesis

Our hypotheses were divided into several key points:

1. The amount of leghemoglobin in the nodules would increase over time.
2. The plants that received the rhizobia bacteria would have an increase in the number of nodules, as well as relative mass of leghemoglobin in their nodules.
3. The plants that received both of the two rhizobia bacteria would have an increase number of nodules, as well as relative mass of leghemoglobin in

their nodules compared to the plants that received only one kind of rhizobia bacteria.

Outcome

Over the course of 4.5 months 168 plants were germinated from seeds, and 126 were inoculated with either USDA 3384 strain, the PNL0i-Brady strain, or a combination of both of strains of rhizobia. The USDA 3384 strain was isolated in Porto Alegre Brazil and came from Patrick Elia (USDA ARS, Soybean Genomics and Improvement Laboratory, National Rhizobium Germplasm Resource, Maryland). The PNL0i-Brady strain came from Becker Underwood (ISO Rep Marita McCreary, QC Manager Padma Somasageran). After 12 weeks from initial planting the Chipilin was inoculated. At 6 and 10 weeks after plant nodules were inoculated, they were harvested and their degree of inoculation with the Rhizobia bacteria was analyzed. The nitrogen fixation relationship was analyzed through Immunoblots for leghemoglobin.

A total of 48 nodule samples were analyzed through Immunoblotting and only 10 of the samples were found to have leghemoglobin present. Two weeks after inoculation, the plants became infected with a foreign pathogen, which was believed to have skewed the results, since the plants began to stop growing earlier than expected. From the results obtained several conclusion were drawn about the experiment with respect to the validity of the hypothesis.

Methodology

Seed Sterilization and Growth

In order to reduce the chance of contamination in the plants from outside rhizobium, Chipilin seeds were sterilized with ethanol and bleach washes. Seeds were immersed in 70% ethanol for 2 minutes. Seeds were then soaked in 5% bleach solution for 5 minutes. Seeds were then rinsed 5 times with deionized water and left to air dry. Three Chipilin seeds were planted in each of 225 peat pots. Peat pots were kept in a controlled climate room at room temperature, (Goddard 206, WPI), under 12 hour lights at night. After 1.5 weeks only 75 of the 225 plants had sprouted. Three more unsterilized seeds were planted in the original 150 peat pots that had not sprouted. The seedlings were watered every 2-3 days as needed. After 4 weeks from the first planting, the peat pots were moved to the greenhouse. While in the greenhouse the light provided was controlled by the sun. The seedlings were left to grow until 7 weeks of age from planting before they were transplanted into 4-inch by 4-inch planting pots. The soil used for the transplanting was Sun Gro Metro-Mix 360 Growing Medium. This soil was sterilized before use in the autoclave on the fluid setting for 25 minutes. The plants were watered every 2-3 days as needed throughout the duration of the experiment. A total of 168 plants were transplanted to be used in the experiment.

Plant Organization

The plants were broken up into 4 groups.

1. Control: no plants were inoculated and the plants were left to grow by themselves.

2. Brady: plants inoculated with the PNL0i-Brady strain ($1.48*10^7$CFU).
3. 84: plants inoculated with the USDA 3384 strain ($2.76*10^7$CFU).
4. Both: plants inoculated with both Brady and 84 strains ($2.12*10^7$CFU).

Each group was comprised of 42 plants: 14 plants with sterilized seeds and 28 plants with unsterilized seeds.

Bacteria Growth

Brady and 84 rhizobia bacteria were grown on separate plates for 7 days. The plates consisted of a solid "Modified Arabinose Gluconate", MAG, growth medium. The formula and protocol for MAG can be found in Appendix A. The rhizobia were then transferred each into a 250mL of liquid MAG in a 500mL erlenmeyer flask. The flasks were placed in a 22ºC incubator shaker (Innova 4270 Refrigerated Incubator Shaker, New Brunswick Scientific). The Brady was incubated for 48 hours and the 84 for 96 hours. There was a difference in incubation times because the Brady rhizobia grew faster than the 84 rhizobia. These inoculated mediums were then used to inoculate the Chipilin plants.

Inoculation and Harvesting

At 12 weeks of age the plants were inoculated with their specific rhizobia strain(s) as noted above. 3.4ml of culture were placed at the bottom of the stem of each respective plant. In the case of the Both group 1.75ml of PNL0i-Brady and USDA3384 were each added, the CFU amount are given above in 'Plant Organization'. In the case on the Control group no rhizobia was added. Each plant was labeled with a number, type of inoculants and whether or not their seed had been serialized.

At 18 weeks of age and 6 weeks after inoculation the first set of plant roots were harvested. Six plants were selected from each of the 4 groups for a total of 24 plants. Of the 6 plants from each group 4 were from plants with unbleached seeds and 2 were from plants with bleached seeds. Plants roots were frozen at -80˚C for use at a later time. At 22 weeks of age plants were harvested again in the same manner.

Sample Preparation for Electrophoresis / Immunoblotting

The root nodules collected from each of the plants were later made into protein samples. The nodules were taken off the roots and crushed using liquid nitrogen in 1.5ml microfuge tubes with a small pestle. To release the protein from the crushed nodules 1ml of plant extraction buffer was added to each nodule sample. The protocol for the plant extraction buffer can be found in Appendix B. Samples were vortexed for 30 seconds each and then centrifuged for 20 minutes at top speed as indicated in protocol. Supernatant was removed and kept for later sampling.

A BSA standard curve was made to check the protein levels in each sample before the electrophoresis. The standard curve was made using a mixture of Pierce 660 reagent and 5 known concentrations of the BSA at an absorbance of 660nm. All sample values were plotted on the calibration curve to determine if they were in the correct range. Electrophoresis was performed twice on all samples. The protocol used was “One-Dimensional SDS Gel Electrophoresis of Proteins”, unit 10.2 from Current protocols in Molecular Biology, Volume 2. A 12% acrylamide gel was used and 50ul of protein was put in each well. The first set of samples was stained with GelCode Blue Stain Reagent to look for the protein bands. The protocol and reference for the Reagent can be found in Appendix C. The second set of samples was

Immunoblotted(Western Blot) to identify the presence of leghemoglobin in the protein. The protocol used was “Immunoblotting and immunodection”, unit 10.8 from Current protocols in Molecular Biology, Volume 2. An anti-leghemoglobin antibody was used to probe the samples. A goat anti-rabbit secondary antibody was used to visualize. A protein ladder was used as a marker. All data was normalized because of the different protein concentrations in each well. The normalization was done by setting the lowest protein concentration equal to one and then makes the other samples relative. ImageJ from the NIH was used to obtain the densities of the fluorescence on the immunoblots by calculating integrals. The same type of normalization was used to make the fluorescence density relative as well.

Results

Assays and Inoculation

The results from the Bradford assay and Fluorescence obtained from Immunoblotting can be found in Table 1. The absorbance of the Brady and 84 strains were 1.307 and 1.076 respectively. This information was used to generate a graph to easily compare the samples.

Table 1: Bradford assay protein concentrations

Sample Number	Absorbance	Normalized Protein	Normalized Fluorescence
5	0.8421	2.29772	2.209759
14	0.8937	2.09316	2.826885
16	0.7712	2.125733	2.82685
19	0.8579	1.824104	1
22	0.9265	2.27101	1.000009
25	1.2054	6.756478	16.07985
26	0.9346	4.071732	16.07978
27	0.7552	2.293138	16.07965
45	1.3492	8.182129	7.075753
46	1.1815	6.51953	7.075673

There was a difference in the CFUs applied for the Brady and 84 stains because the plants were inoculated with a specific volume of medium (3.4mL), and the CFU was calculated after the fact, in the interest of time. Table 2 shows the amount number of CFU each plant group was inoculated with.

Table 2: CFU in each inoculation group

CFU in Inoculation	
Control	0
Brady	$1.48*10^7$
84	$2.76*10^7$
Both	$2.12*10^7$

Immunoblotting

Out of the 48 root nodule samples analyzed, only ten samples were found to have leghemoglobin through the Immunoblotting experiment. The pictures of the gels can be found in Figures 5-9 and blots for can be found in Figures 10-14. Figure 15 depicts the relative leghemoglobin present in plant nodules to normalized protein concentration of each sample. Of the ten samples 4 were form the Control group (samples 5, 25, 26, 27), two were from the 84 group (samples 14 and 16), and four from the Both group (samples 19, 22, 45 and 46). Of these ten samples numbers 14, 19, 25, and, 26 were sterilized and 5, 16, 22, 27, 45 and 46 were unsterizalized. None of the sole Brady plants were found to have leghemoglobin. Some of the plants became infected at 14 weeks of age (2 weeks after inoculation) and the infection became widespread shortly afterward; the relevance of this will be elaborated on in the discussion.

1 2 3 4 5 6 7 8

Figure 5: 12% acrylamide gel run at 200V, denatured, 50ul of sample in each well, Lane 1=protein ladder, Lane 2=sample 1, Lane 3=sample 2, Lane 4=sample 3, Lane 5=Sample 4, Lane 6=sample 5, Lane 7=sample 6, Lane8=Sample 7

1 2 3 4 5 6 7 8 9

Figure 6: 12% acrylamide gel run at 200V, denatured, 50ul of sample in each well, Lane 1=protein ladder, Lane 2=sample 10, Lane 3=sample 11, Lane 4=sample 12, Lane 5=Sample 13, Lane 6=sample 14, Lane7=Sample 15. Lane 8=sample 16, Lane 9=sample 17

1 2 3 4 5 6 7 8 9

Figure 7: 12% acrylamide gel run at 200V, denatured, 50ul of sample in each well, Lane 1=protein ladder, Lane 2=sample 18, Lane 3=sample 19, Lane 4=sample 20, Lane 5=Sample 21, Lane 6=sample 22, Lane7=Sample 23. Lane 8=blank 24, Lane 9=blank

1 2 3 4 5 6 7 8

Figure 8: 12% acrylamide gel run at 200V, denatured, 50ul of sample in each well, Lane 1=protein ladder, Lane 2=sample 24, Lane 3=sample 25, Lane 4=sample 26, Lane 5=Sample 27, Lane 6=sample 28, Lane7=Sample 29. Lane 8=sample 30

1 2 3 4 5 6 7 8

Figure 9: 12% acrylamide gel run at 200V, denatured, 50ul of sample in each well, Lane 1=protein ladder, Lane 2=sample 43, Lane 3=sample 44, Lane 4=sample 45, Lane 5=Sample 46, Lane 6=sample 47, Lane7=Sample 48. Lane 8=blank

1 2 3 4 5 6 7 8

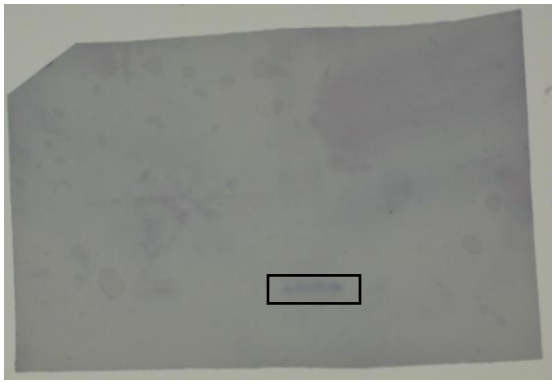

Figure 10: 12% acrylamide gel run at 100V, denatured, 50ul of sample in each well, antibody 1=anti-leghemoglobin, antibody 2=goat anti-rabbit, Lane 1=protein ladder, Lane 2=sample 1, Lane 3=sample 2, Lane 4=sample 3, Lane 5=Sample 4, Lane 6=sample 5, Lane7=Sample 6. Lane 8=sample 7,boxes indicate sample used

1 2 3 4 5 6 7 8 9

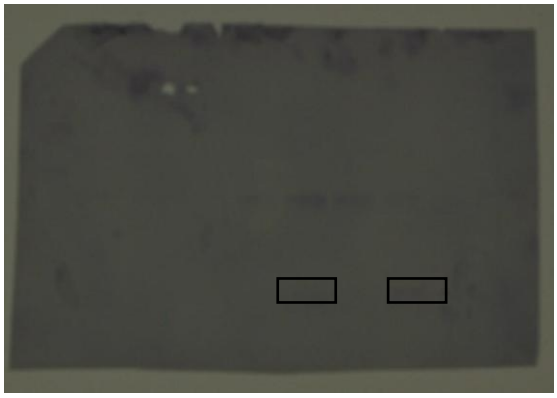

Figure 11: 12% acrylamide gel run at 100V, denatured, 50ul of sample in each well, antibody 1=anti-leghemoglobin, antibody 2=goat anti-rabbit, Lane 1=protein ladder, Lane 2=sample 10, Lane 3=sample 11, Lane 4=sample 12, Lane 5=Sample 13, Lane 6=sample 14, Lane7=Sample 15. Lane 8=sample 16, Lane 9=sample 17, boxes indicate sample used

1 2 3 4 5 6 7 8 9

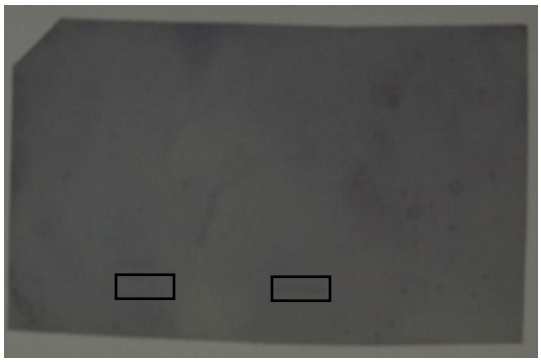

Figure 12: 12% acrylamide gel run at 100V, denatured, 50ul of sample in each well, antibody 1=anti-leghemoglobin, antibody 2=goat anti-rabbit, Lane 1=protein ladder, Lane 2=sample 18, Lane 3=sample 19, Lane 4=sample 20, Lane 5=Sample 21, Lane 6=sample 22, Lane7=Sample 23. Lane 8=blank, Lane 9= blank, boxes indicate sample used

1 2 3 4 5 6 7 8

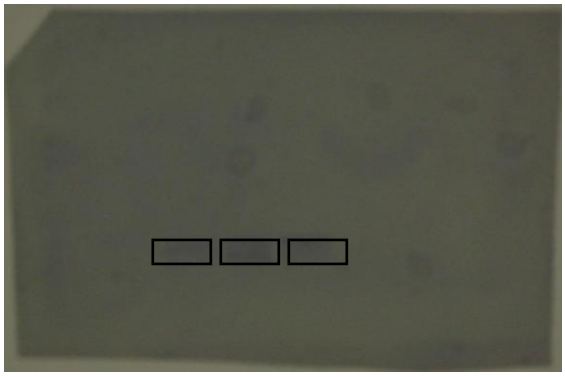

Figure 13: 12% acrylamide gel run at 100V, denatured, 50ul of sample in each well, antibody 1=anti-leghemoglobin, antibody 2=goat anti-rabbit, Lane 1=protein ladder, Lane 2=sample 24, Lane 3=sample 25, Lane 4=sample 26, Lane 5=Sample 27, Lane 6=sample 28, Lane7=Sample 29. Lane 8=sample 30, boxes indicate sample used

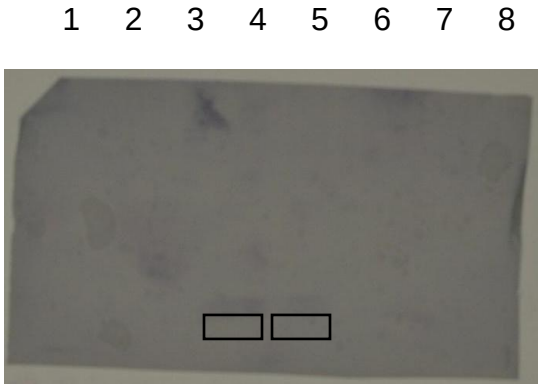

Figure 14: 12% acrylamide gel run at 100V, denatured, 50ul of sample in each well, antibody 1=anti-leghemoglobin, antibody 2=goat anti-rabbit, Lane 1=protein ladder, Lane 2=sample 43, Lane 3=sample 44, Lane 4=sample 45, Lane 5=Sample 46, Lane 6=sample 47, Lane7=Sample 48. Lane 8=sample blank, boxes indicate sample used

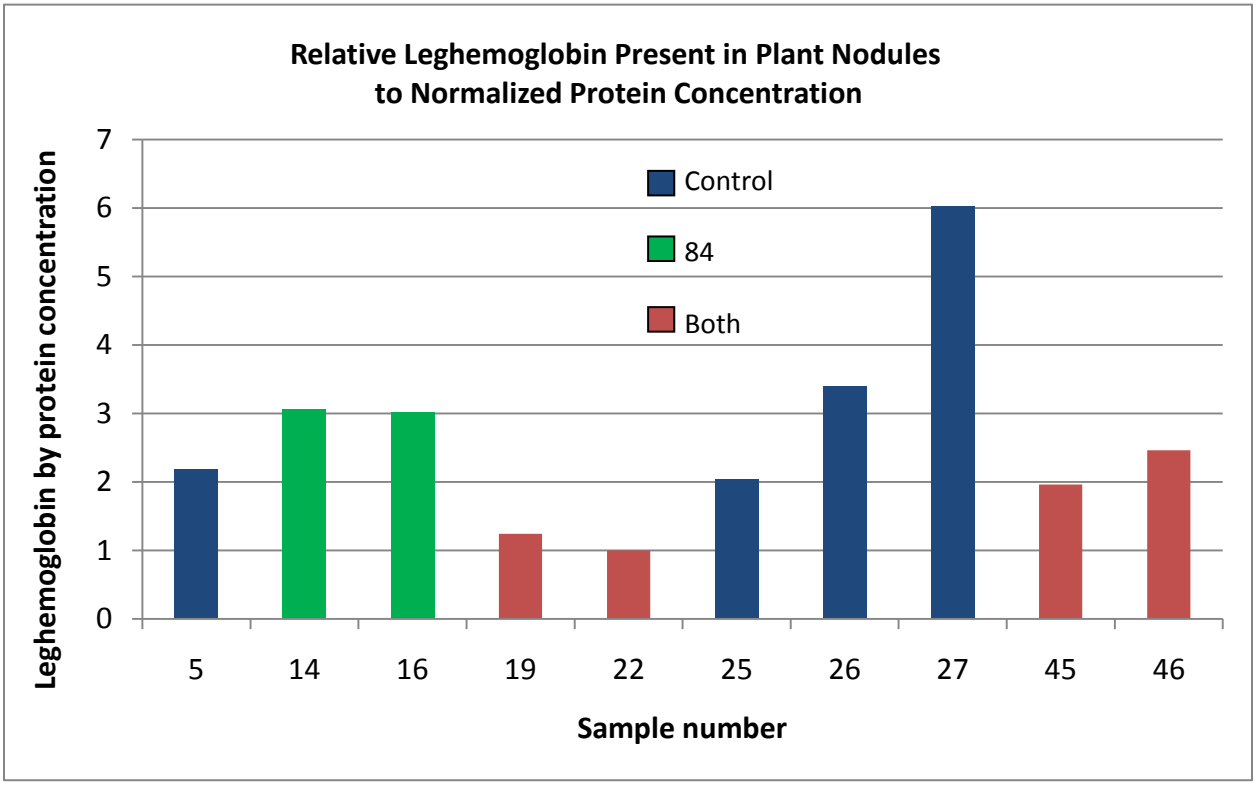

Figure 15: Relative Leghemoglobin Present in Plant Nodules to Normalized Protein Concentration. The sample numbers represent a specific plant. All samples are relative to sample 22 which is set equal to one.

Discussion

Analysis of our data reveals that our hypotheses were not supported by our results. For example, the prediction that the amount of leghemoglobin in the nodules would increase over time is not substantiated by the data, because it only had a confidence ratio of 85%, also known as a p value of 0.15. The data did not support the hypothesis that the relative mass of leghemoglobin in the samples infected with the rhizobia bacteria would increase. This hypothesis was rejected and there was found to be a decrease in the leghemoglobin in the nodules for the plants infected with rhizobia, with a p value of 0.2. The prediction that the samples infected with both types of rhizobia would have more nodules then the samples infected with Brady or 84 was found to be true, with p values 0.01 and 0.2 respectively, but was later rejected as the number of samples with leghemoglobin present was equivalent to the control.

Several conclusions were drawn from the results obtained. Even though sterilization of the seeds resulted in a low germination rate, there appears to between correlation between plant seed sterilization and a decrease in the appearance of leghemoglobin. The plants whose seeds were sterilized had leghemoglobin appear at the same rate as the plants whose seeds were not sterilized as was determined from the data in Table 1. It is possible that the health of the plants skewed the results, impairing the formation of the symbiotic bond between Chipilin and rhizobia. Some unknown pathogen infected and spread through all plants two weeks after inoculation. Since the pathogen appeared not long after the plants had been inoculated, it could have possibly come from the inoculums, however this is not certain. Other possible sources are from human contact as well as watering.

It is believed that the Brady rhizobia stock may have been contaminated with a faster growing bacterial species which may explain the plants inoculated solely with the Brady did not test positive for leghemoglobin. After completion of the project it was found that Bradyrhizobium are considered to be a slow growing rhizobium compared to other rhizobium(Somasegaran). When the Brady grown was in liquid culture it grew twice as fast as the 84 strain, leading to the belief that the Brady may have been contaminated.

Some recommendations can be made for future related projects. Better sterilization technique should be used after planting, such as sterilized pots and water, as well limited human contact to lower the chances of the plants becoming infected. The use of It is also recommended that standard be used on all immunoblots so that a comparisons can be more accurately made between different blots. PCR could be preformed on the nodule samples to more closely analyze the leghemoglobin. Further research could be done to see if there are any other proteins associated with symbiosis.

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Appendix

Appendix A

Opening and Reconstituting Lyophilized Culture Vials

NOTE: All transfer work should be performed under a Laminar Flow Hood.

1) Remove aluminum crimp cap being careful not to remove rubber stopper.
2) Using flame sterilized forceps, remove rubber stopper & place aside. Using sterilized forceps, flame sterilize the vial opening/neck.
3) Introduce 1-2ml of sterile liquid media to vial, pipetting to thoroughly mix liquid media and lyophilized skim milk/bacteria.
4) Remove mixture and pipet into sterile test tube with broth.
5) Place reconstituted cultures on Gyratory Shaker for growth; Set speed to ensure proper aeration. A temperature of 25°C is optimal for good growth. (Temp. higher than 30°C will inhibit growth.)
6) After 7 days, if sufficient growth is observed, plate on MAG from liquid growth for isolation.

Recovering Cultures from MAG stabs/slants.

After streaking out the culture from the MAG stab, incubate it at 25°C for optimal growth. Do not use TY or LB, Bradyrhizobium will not grow on these. (You must avoid temperatures at or above 30°C as this will inhibit growth.) Single colonies should be observed after 7 days.

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.

Appendix B

Plant Protein Isolation

Rapid isolation of protein for SDS-PAGE analysis
(Essentially the same protocol as that described for **GUS Assays**)
A. Method for ~1g or more of tissue.
1. Label all tubes. Prepare solutions and have ready at hand.
2. Remove the tissue from the –80^{o}C freezer and thaw on ice. If the tissue is fresh, keep on ice (or alternatively work in a cold room).
3. Place tissue in a mortar and pestle.
4. Add ~ 2ml of QB per ~1g tissue.
5. Grind tissue until no more chunks are visible.
6. Remove ~1ml of the liquid grindate into a microfuge tube.
7. Place on ice.
8. Rinse mortar and pestle (and any other paraphernalia that came into contact with the sample) to remove all traces of sample and proceed to the protein isolation of the next tissue sample.
9. Spin samples at top speed in the microfuge (4^{o}C for 15+ minutes).
10. Transfer the liquid supernatant into a second (new) microfuge tube.
11. Sometimes excess tissue is transferred over into the second microfuge tube. If this is the case, spin a second time for about 10 minutes and transfer this supernatant into a third microfuge tube.
12. Store samples in the –80^{o}C.

B. Alternative method for small (<1g) quantities of tissue.
1. Working in the fume hood, prepare a pestle by flaming the end of a blue pipette tip and sealing the end by gently smashing it into a microfuge tube. Prepare as many pestles as tissue samples to be isolated.
2. Using the newly created pestle, grind the tissue directly in a microfuge tube.
3. Add ~1ml of QB to the ground tissue, mix and transfer the supernatant to a second microfuge tube.
4. Follow the procedure in A above the rest of the way.

C. Solutions and stuff
A. Solutions
1. QB
Stock For 100ml Final []
2M KPO_4 (pH 7.8) 5ml 100mM
0.5M EDTA 200µL 1mM
Triton X-100 1ml 1%
80% Glycerol 12.5ml 10%
dH_2O 81.1ml
Store RT
DTT (1.0M) 100µL 1mM
(Alternatively directly add 15.4mg DTT per 100ml)
Add DTT immediately before using. Store QB w/DTT at –20^{o}C.
2. 2M KPO_4 (pH 7.8)
Stock For 200ml Final []
K_2HPO_4 63.2g
KH_2PO_4 5.0g
pH should be ~7.8
If not, adjust pH to 7.8
F, a/c

3. 0.5M EDTA (pH 8.0)
Stock For 250ml Final []
EDTA 46.52g 0.5M
H_2O to 250ml
pH w/ 10N NaOH to 8.0
(Alternatively use ~5 pellets of NaOH.)
f, a/c.
Store RT.
Note: EDTA will not completely go into solution until the pH approaches 8.0 and the H_2O is almost at final volume. Essentially, the pH needs to be continuously adjusted as the EDTA dissolves.
4. 10N NaOH
Stock For 250ml Final []
NaOH 100g 10N
dH_2O to 250ml
Store at RT in a PLASTIC bottle. (NaOH will react with glass.)
5. 80% Glycerol
Stock For 100ml Final []
100% Glycerol 80ml 80%
dH_2O 20ml
a/c
6. 1M DTT
Stock For 10ml Final []
DTT 1.545g 1M
0.01M NaOAc to 10ml
(pH 5.2)
Filter sterilize
Aliquot into 1ml portions
Store at -80^{o}C
0.01M NaOAc is 33µL of 3M NaOAc pH~5.2 in 9.67ml dH_2O.)

B. Stuff
1. Mortar and pestle and/or flame seal blue tips.
2. Microfuge tubes, pipette tips.
3. Test Tubes

VI. References

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Stockinger Lab; Version 09/10/01

Appendix C

INSTRUCTIONS

GelCode™ Blue Stain Reagent

Thermo SCIENTIFIC

24590 24592 0714.3

Number	Description
24590	**GelCode Blue Stain Reagent**, 500 ml, sufficient for 20 mini gels
24592	**GelCode Blue Stain Reagent**, 3.5 liters, sufficient for 175 mini gels

Note: A convenient dispenser pump (Product No. 72300) for the 3.5 liter container is available free, upon request, with the purchase of Product No. 24592.

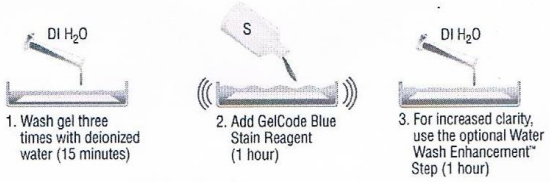
Storage: Upon receipt store product at 4°C. Product is stable up to 6 months at room temperature and longer than 1 year at 4°C. Product shipped at ambient temperature.

Introduction

GelCode Blue Stain Reagent uses the colloidal properties of coomassie G-250 dye for protein staining on polyacrylamide gels. This unique reagent stains only protein and allows bands to be viewed directly on the gel during the staining process. After staining, a water equilibration step (Water Wash Enhancement™ Step) further enhances staining sensitivity and yields a clear background.[1] With GelCode Blue Stain there is no need for multi-step destaining procedures typically associated with other gel staining systems.[2]

Procedure Summary

Total assay time:
1 hour 15 minutes



Reagent Preparation

Mix the GelCode Blue Stain Reagent solution immediately before use by gently inverting or tipping and swirling the bottle several times. Such mixing is especially important when using Product No. 24592 with a dispenser pump. Do not shake bottle to mix the solution.

Note: GelCode Blue Stain Reagent contains additives that help to slow down the formation of dye-dye and dye-protein aggregates, which form in all coomassie dye-based protein staining reagents. If left undisturbed, the reagent will form visible dye-dye aggregates that settle in the bottom of the bottle. Fortunately, gentle mixing completely disperses these aggregates. Therefore, it is good practice to mix the stain reagent before pouring or dispensing to ensure that a homogeneous sample of the reagent is used.

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