ABSTRACT

Isolation of Juniper Mitochondria

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The aim of the research was to conduct an experiment separating the organelles of the plant, *Juniperus virginiana,* in a gradient in order to isolate the mitochondria from chloroplasts. Purifying the mitochondria would allow sequencing of the mitochondrial genome of Juniper and learning more about the plant and its history. The challenge of the experiment was to see if the mitochondria and the chloroplast could be separated into different layers in a gradient in order to extract simply the mitochondria. To analyze the organelles found in each layer and locate specific organelles, gel electrophoresis and a western blot with antibodies for mitochondria and chloroplast were used. Several trials with different gradients were conducted including a sucrose gradient, a Percoll gradient and a combined sucrose and Percoll gradient. Each trial alters the procedure in an attempt to further isolate the mitochondria.

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ISOLATION OF JUNIPER MITOCHONDRIA

A Thesis Submitted to the Faculty of

Baylor University

In Partial Fulfillment of the Requirements for the

Honors Program

By

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DEDICATION

To Faiz Hussain for his constant love and support

CHAPTER 1

Introduction

Plant Information

Juniperus virginiana is a Juniper native to North America ranging from Eastern United States to the Gulf of Mexico to Eastern Canada. Also known as the Eastern Red-cedar, the plant is most known for its aromatic characteristic. The plant is a gymnosperm and a member of the Cupressaceae family. It is an evergreen tree with a trunk angled at the base and a narrow, compact crown. The tree is pyramidal when young but the grown form is quite variable. It usually grows to about 30-40 ft., however it has been known to reach up to 90 ft. in height. The tree has foliage that can be coarse or fine-cut. Juniper is the most widely distributed eastern conifer (cone bearing seed plant). It is native in 37 states, including Texas. In addition, it has resistance to weather extremes including heat and cold ("Juniperus virginiana (Eastern red cedar) | NPIN," n.d.).

The Eastern Red-cedar is dioecious, meaning that individual trees are either male or female. It is estimated that the Eastern Red-cedar becomes sexually mature at around 10 years old. Eastern red-cedar reproduction starts with male and female conelets developing in the late summer and fall on the trees. Male conelets develop pollen grains and turn a yellowish brown, maturing during the winter.

Now a very common tree, Juniper was once limited to rocky bluffs, deep canyons, and other areas where fire historically did not occur. The Eastern Red-cedar and other juniper trees are known to be fire intolerant. Wildfires had kept the now invasive tress in check. Since North America was settled by the Europeans fire has been suppressed,

enabling the Eastern Red-cedar to spread beyond its usual territories. Due to the reduced risk of fire it has spread across most of Oklahoma and Texas, forming dense populations in many locations. Unmanaged Eastern Red-cedar often dominate the landscape, creating monoculture-like plant communities with little plant and wildlife diversity.

There are five species of Junipers in the South. They include the:

- One-seeded juniper (*J. monosperma*)
- Red berry juniper (*J. pinchotii*)
- Rocky Mountain juniper (*J. scopulorum*)
- Ashe juniper (*J. ashei*)
- Eastern Red-cedar (*J. virginiana*)

The Ashe Juniper and Eastern Red-cedar are the two most common species of Junipers in the east three quarters of Oklahoma and northeast quarter of Texas.

Juniper in Texas

The three major species of Juniper in Texas include Ashe juniper (*Juniperus ashei*), Redberry juniper (*Juniperus pinchotii*) and Eastern Red-cedar (*Juniperus virginiana*) ("Juniper ecology « Texas Natural Resources Server," n.d.). Juniper has been a part of the Texas landscape for thousands of years; however, it has spread into other habitats and has increased in abundance in modern times. The increasing density of Juniper has created effects on vegetation, livestock, watersheds, and rainfall.

Vegetation/Livestock

Junipers can impact grassland plants they invade. According to a study near San Angelo, Texas, annual herbage production on a low stony hill range site supporting 117 mature Redberry junipers/acre was about 40% lower than the potential production of the site in the absence of mature junipers (1,156 lb/ac to 1,909 lb/acre) (Kenneth, Dye, Ueckert, & Whisenant, 1995). This finding is significant because the effect on grassland plant communities also affects livestock capacities. Without mature junipers, this site could support approximately 1 animal unit/20 acres. This is compared to the 1 animal unit/135 acres when the site changes to a canopy filled with Junipers ("Juniper ecology « Texas Natural Resources Server," n.d.).

Precipitation

Juniper increase has been reported to reduce the amount of rainfall that reaches the soil surface. A study showed an interception loss of 25.9% and 36.7% of precipitation for Redberry and Ashe juniper, respectively. Ashe juniper had the higher interception loss due to its denser canopy, which results in more surface area to catch rainfall ("Juniper ecology « Texas Natural Resources Server," n.d.). In comparison, canopies of live oaks intercept approximately 25.4% of gross rainfall. In addition, the rainfall also has to pass through the layer under the plant before it reaches soil. As a consequence of interception loss via the canopy and litter, only about 20.3 and 34% of the annual rainfall actually reaches the mineral soil surface under the canopies of the Ashe and Redberry junipers, respectively. This is a stark contrast to 82% and 89% of annual precipitation, which will

reach the soil under bunchgrass and short grass cover ("Juniper ecology « Texas Natural Resources Server," n.d.).

Wildlife

Unlike livestock and precipitation, Juniper has a positive effect on wildlife. Juniper is commonly found in deer diets. Redberry juniper comprised over 50% of the diets of deer in Terrell County through the months of January, February, October and December (Ortega, Soltero-Gardea, Drawe, & Bryant, 1997). In addition, the berries are common in diets of other animals such as fox, raccoon, and some birds. Studies identified 19 species of birds and 9 species of mammals that consumed juniper berries at the Sonora Experiment Station ("Juniper ecology « Texas Natural Resources Server," n.d.). Juniper plants are also known to provide wildlife shelter. The cover is way for animals to survive weather changes. They provide thermal covers in the winter. However, very dense juniper is not beneficial to the wildlife.

The balanced amount of juniper is based on management by a landowner. This can be done using fire, chemicals or biological means. Long-term planning is essential. When it comes specifically to the species of Juniper that is being studied (*Juniper virginiana*), other factors are also considered. This species of Juniper has been blamed for the change in habitat for small animals in multiple communities. The expansion of this species is changing the landscape and as a result altering the vegetation and habitats for small animals. In an experiment, the effects of the growth of the Eastern Red-cedar were examined in three plant communities These include tall grass prairie, old fields, and cross timbers forest ("Juniper ecology « Texas Natural Resources Server," n.d.). In two of

the locations, the number of mammals decreased with an increase in red cedar. There was also a loss in diversity due to the invasion of the red cedar.

Through all the positives and negatives of juniper, there are a lot of current changes that are taking place due to its increase in the Southern United States. Learning more about juniper will be helpful in understanding the changes its growth is bringing.

Uses of Juniperus virginiana

The soft brownish red wood is light but durable. The wood is valuable because of its great durability. The wood has rot resistance and can be used to make fences. It is used to manufacture wood of pencils and some various household structures. Boxes made of the wood are used to store furs due to the aromatic oil it contains. The wood is avoided by moths due to its fragrance allowing it to be a used in chests and closets for clothing storage. In United States, it is commonly used for cabinet making ("*Juniperus virginiana* (Eastern red cedar) | NPIN," n.d.).

Juniperus virginiana is an ornamental tree in Britain as it grows to 40 to 50 feet high. Even in some states of the United States it is used as a Christmas tree. A few cultivars have been used for garden planting including *Juniperus virginiana* Skyrocket. Farmers also use them as wind breaks to shelter other plants because these trees are weather tolerant. The oil of the plant is a commerce product with about 15,000 lb. of oil being produced in the United States. It is then used to make insecticides and soaps. Sometimes, it is used as a general perfume.

The berries are eaten by many kinds of insects and animals. They are a staple for many birds and small mammals as the tree is a shelter. On the other hand, it is also

known to be injurious to apple orchards because it is a host for the cedar apple rust (a fungal disease). This has brought about the need to keep *Juniperus virginana* away from apple orchards ("A Modern Herbal | Juniper Berries," n.d.). The berries are also used in the same way as those of other Junipers' such as Common Juniper. They have diuretic and stimulating properties that can be used in medicinal ways.

Juniper Medicinal Qualities

The interest in Juniper can be attributed to all of its uses and potential benefits. One of the uses of Juniper that has been proven through studies is the use of its berries for medicinal uses. Juniper berries have been known to have stimulating and disinfecting effects. For example, they have been used in cases of urinary tract infections. Juniper stimulates the urinary passages so the kidneys move fluids faster. This can help if urine is not flowing freely. Juniper, however, is not used for long-term kidney disease and should be used in small doses. It is often used with older people with chronic disease. It is useful in situations with loss of tone in tissue and organs ("Medicinal Benefits of Juniper Berries," n.d.).

Juniper berries are also used for joint pain, rheumatoid arthritis, and some muscle and nerve disorders. Juniper's oils have been used for coughs and lung congestion. It is valuable for respiratory infections because it opens bronchial passages. In France, the berries have been used to help chest pain. It has also been used to treat skin conditions. The oil of Juniper is a stimulant used by herbalists to improve late or irregular menstrual periods. Oil is also given as a diuretic for indigestion and flatulence. It relieves gas in the

digestive system and increases hydrochloric acid to help digestion ("Medicinal Benefits of Juniper Berries," n.d.).

Such medicinal uses and benefits of Juniper have yet to be further explored. To understand all the ways Juniper can help as a stimulant, including urinary, respiratory and digestive problems, more information is necessary. Sequencing the genome of Juniper could lead to more research of these medicinal uses. Knowing more about the plant and the species could contribute to the knowledge of Juniper and plants alike.

Sequencing Mitochondrial Genomes

DNA is packed in chromosomes in the nucleus. Mitochondria are organelles within the cells that create energy for the cell using oxidative phosphorylation. In addition, mitochondria have their own DNA called mitochondrial DNA. The mitochondrial genome is very variable. Compared to animal and fungi mitochondrial genomes, plants species have quite large genomes (200-2,400 Kb, 1-2 orders of magnitude larger than most mtDNAs,). In addition to being large and variable, plant genomes have non-conserved reading frames with unknown function "Our Projects - DOE Joint Genome Institute," n.d.).

Plant mitochondrial genomes are the last frontier for comparative sequencing. Comparative sequencing is important because it is a field of biological research in which the genomic features of different organisms are compared. Our knowledge of these genomes comes from mapping studies, Southern blot surveys, and limited sequencing. In addition, they have been hardly used in re-making plant phylogeny ("Why Sequence Seed Plant Mitochondria?," n.d.).

The initial generation of plant genome sequencing has passed, and we are now starting a new point in plant genomics research. The candidates for genome sequencing which are usually model species with small genomes or species of economic importance have either already been completed or are under review. Research states that the next round of choices should be made as part of a strategy based on a mixture of scientific and economic needs and should recognize the value of including phylogenetic position as a selection criterion.

Plant mitochondrial DNAs have been known to possess many unusual properties. These properties include the lowest known rates of synonymous substitution (50-100 times lower than that observed in animal mitochondrial genomes) and high rates of inversion and other internal rearrangements ("Why Sequence Seed Plant Mitochondria?," n.d.). Studies to compare the mitochondrial genomes of rapeseed and Arabidopsis show that plant mitochondria are conservative in terms of coding sequences but non-coding parts are dynamic in terms of sequence and structural changes.

Material lineages are identified through evolutionary studies, which make use of mitochondrial genomes. The lack of genomic data among plants has prevented researchers from mapping patterns of genome evolution (Richardson, Rice, Young, Alverson, & Palmer, 2013). However, some sequencing has brought about useful information that can be used for lineage studies and understanding mitochondrial genomes. Mitochondrial genome sequencing of many plants and animals has been performed. Their result has been beneficial in gaining more information about the specific species as well as mitochondrial genomes.

Mitochondrial Genome sequencing of the tulip tree (*Liriodendron tulipifera*).

Researchers from Indiana University and University of Arkansas sequenced the mitochondrial genome of *L. tulipifera.* They found that its mitochondrial genome has one of the slowest silent mutation rates compared to any known genome. In comparison to humans, the rate is 2000 X slower. For example, the amount of genomic change in one human generation would take 50,000 years for the tulip tree. In addition, for magnolia trees the rate is even slower; it would take 130,000 years for the same amount of mitochondrial genomic change (Richardson et al., 2013).

The mitochondrial genome of the white Leghorn Chicken has been sequenced containing 16, 775 bp. The genome was found to have the same set of genes as other vertebrate mitochondrial DNAs. The genes are similar to the chicken's mammalian counterparts; however, there are some distinct differences. A conserved sequence found in all vertebrate mitochondrial genomes that have been sequenced so far, is not present in the chicken genome. Such information shows that mitochondrial genomes characteristics that are present in animals such as chicken separated from mammal and amphibian genomes during evolution. The research shows that such characteristics are useful markers to understand phylogenetic relationships of taxonomy. Mitochondrial genomes allow researches to find more linkages in linages and changes in evolution (Desjardins & Morais, 1990).

The mitochondrial genome of Oryza sativa L. (rice) was sequenced and is substantially larger and more complex than vertebrate mitochondrial genomes. The genome is made up of 490,520 bp with many rRNA and tRNA sequences identified. The genome allowed identification of fragments that were similar among monocots like rice

but different from dicots. The research shows that there had been frequent gene flow from the genome during evolution of flowering plants, stating that this is what led to genetic variation between mitochondrial genomes of plants (Notsu et al., 2002).

Through these examples, it is evident that sequencing mitochondrial genomes has allowed a better understanding of evolutionary changes. The information gathered from the genomes of rice as well as the chicken have lead to "markers" that can help understand relationships, branching and changes in evolution. If the mitochondria of *Juniperus virginiana* was isolated and used for DNA sequencing, then more information about the plant itself and its similarity with other gymnosperms as well as other members of the Cupressaceae family could be determined.

CHAPTER 2

Methods and Material for Mitochondria Extraction from Juniper leaves

Introduction to the Procedure

The aim of this experiment was to separate the cell organelles of the Juniper plant in order to separate the mitochondria from chloroplast. Separating and eventually extracting the mitochondrial DNA would allow sequencing of the mitochondrial genome of Juniper. Leaves from *Juniperus virginiana*, a species of Juniper native to the eastern North America, were used for the experiment. The tree is currently located outside the Baylor Science Building $31^0$32'52"N $97^0$6'41"W. A challenge of the experiment was to see if the mitochondria and the chloroplast could be separated into different layers for this would be essential to extract only the mitochondrial DNA. For this separation between chloroplast and mitochondria, the protocol used was adapted from a procedure to separate mitochondria and chloroplasts from moss (Lang et al., 2011). The method uses a Percoll gradient to ensure collection of intact mitochondria. To analyze the fractions found in each layer and locate specific organelles, gel electrophoresis and a western blot with antibodies for mitochondria and chloroplast were used.

Isolation of mitochondria from Juniper

Around 30g of Juniper leaves were collected from the tree. The leaves were washed thoroughly with water. On wet ice (at 4°C), 21g of fresh juniper leaves were measured. 60ml of organelle isolation buffer [1% (w/v) polyvinylpolypyrrolidone (PVPP), 300 mM d-sorbitol, 50 mM HEPES, 2 mM Na-EDTA, 1 mM MgCl2 and 0.1%

BSA] with 0.1% protease inhibitor were added to the leaves. The mixture was chopped using a homogenizer for 20 min. After crushing and grinding into a thick liquid, the mix was filtered through three layer of Miracloth into two 50ml centrifuge tubes. The tubes were centrifuged (Beckman Coulter Avanti Centrifuge J-20) at 1,500xg for 10 min to pellet the chloroplasts. The supernatant was transferred to new centrifuge tubes for mitochondria isolation. The chloroplast pellet was discarded (Lang et al., 2011).

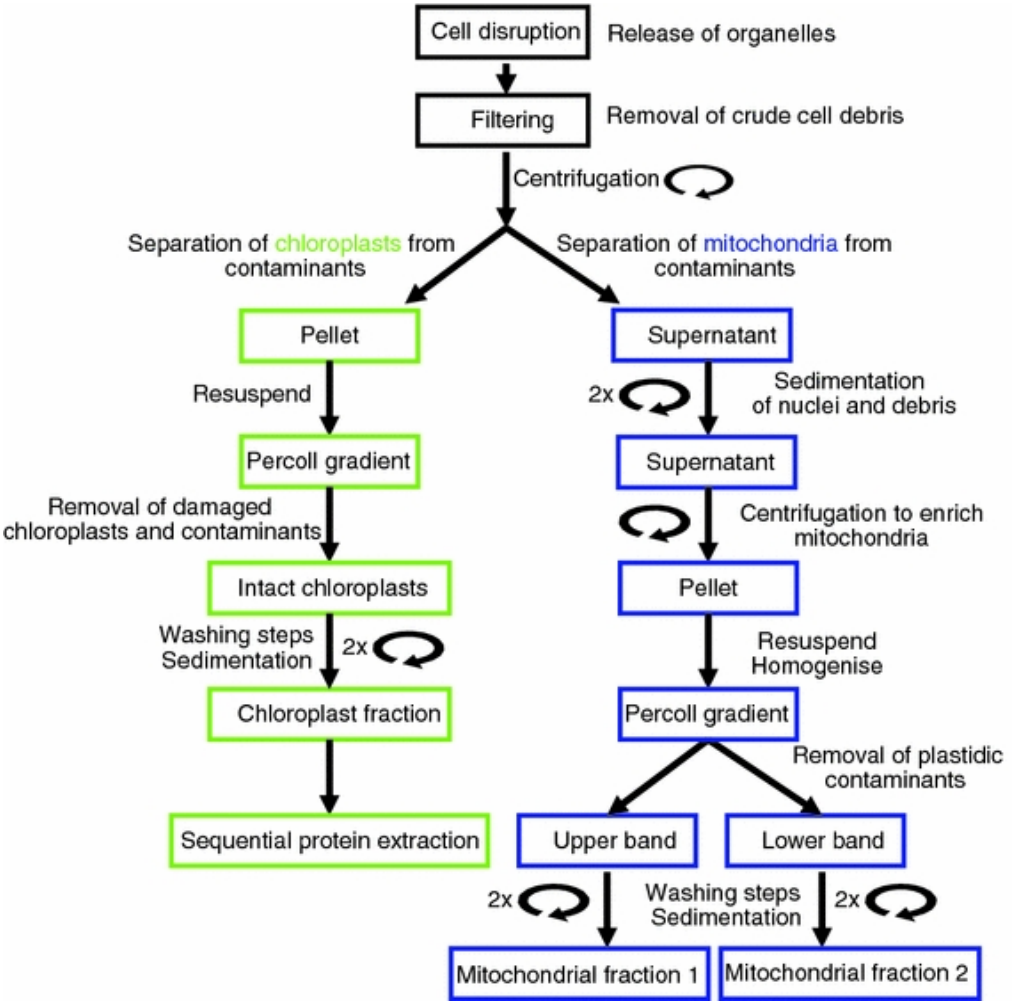

Figure 1. Flowchart of the isolation of mitochondria shows the different steps during the process of isolating both mitochondria and chloroplasts. However, the chloroplasts pellet was discarded due to the need of only mitochondria for this experiment. (Lang et al., 2011)

The supernatant was used to purify mitochondria using a Percoll density gradient. In order to pellet debris, the supernatant was centrifuged at 3,000×g for 5 min followed by 6,000×g for 5 min in the same tube. The new supernatant was transferred to a new tube and centrifuged at 18,000 for 20 min in order to pellet mitochondria.

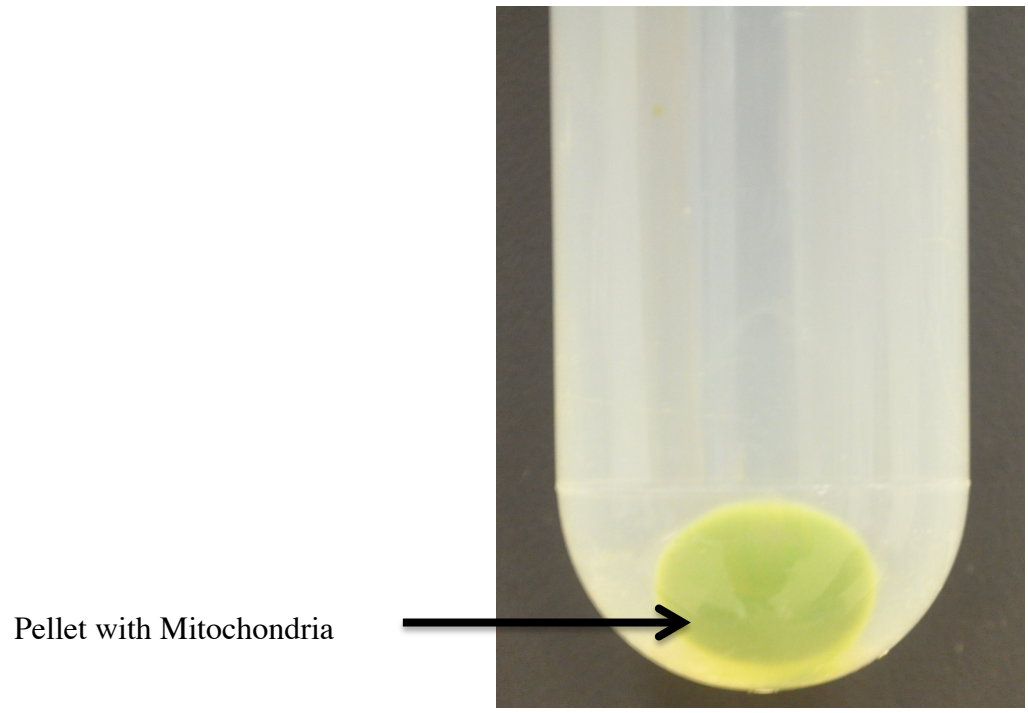

Figure 2. Final pellet rich with mitochondria applied to the Percoll gradient

The pellet was re-suspended in 1 ml of washing buffer and with a final volume of 3 ml placed in a Potter homogenizer and homogenized with 10 strokes. Then 0.6 ml of 100% Percoll was added to create a sample with about 20% Percoll. The gradient was created using 10 ml of 80% Percoll in washing buffer and 10 ml of 33% Percoll in washing buffer. The homogenized sample with 20% Percoll was placed on top of the gradient. The gradient with 80%, 33% and 20% (with sample) was centrifuged at 18,000xg for 1 hour (Lang et al., 2011).

A pale band was extracted at 33% - 80% interface. This is referred to as M2 in the figure. Using a pipette, the layer of mitochondria was extracted. 30 ml of washing buffer was added to the mitochondria in order to free it from Percoll. The mixture was

centrifuged at 18,000xg for 20 min with slow deceleration. The washing buffer and centrifugation was repeated. This yielded a greenish mitochondria pellet.

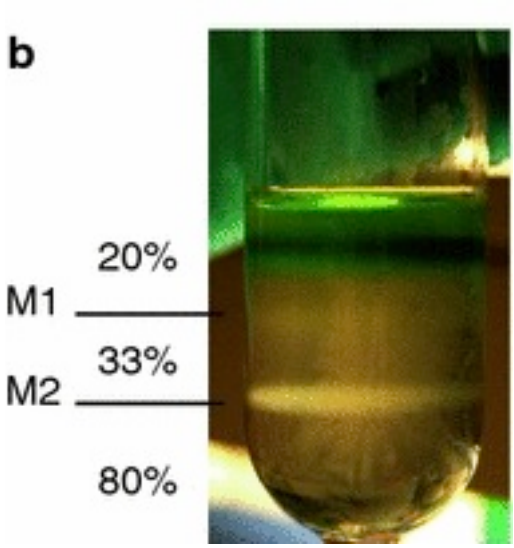

Figure 3. Figure 2 from protocol showing the two whitish layers containing intact mitochondria M1 and M2 (Lang et al., 2011).

Protein Extraction

The procedure for protein extraction was adapted from a Borate extraction method (Wallace, J. C., et al., 1990). Protein was extracted using Borate extraction buffer [12.5mM Sodium Borate, 1% SDS, 2% beta-mercaptoethanol (pH 10)] For extraction, 1 ml of borate buffer was added to the frozen green pellet to resuspend it. The solution was then centrifuged for 15 min at high speed at room temperature. The supernatant was transferred to new tube and used to run the gels.

Gel Electrophoresis

Electrophoresis refers to the migration of a charged particle in an electric field. The amount a molecule will move through the gel is proportional to the strength of the field, the charge, size and viscosity of the gel. When there is no difference in gel viscosity or electric field, the migration on the gel reflects their molecular weight. Larger proteins move more slowly than smaller ones. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). A gel is cast in the appropriate buffers: a separating gel (lower gel) and a short staking gel (upper gel). They are opposite in pH allowing proteins to alter their mobility.

Two SDS-PAGE gels were made, one for Coomassie Blue staining and the other for a western blot. A lower gel was created first and then an upper gel. A comb was used to create wells in the gel in order to load the samples. 7 microliters of blue ladder marker was loaded to the first well. This was followed by the loading of four wells with 16 microliters of sample mixed with 5 microliters of marker in each well. The gel was run for 1h and 30 min.

The western blot is an analytical technique used to detect proteins in a sample. It uses gel electrophoresis to separate proteins and then the proteins are transferred to a membrane on which they can be stained with antibodies to target specific proteins. The method used for transferring the proteins is called electroblotting and uses an electric current to pull proteins from the gel into the nitrocellulose membrane. The proteins move from the gel onto the membrane and maintaining the organization they had within the gel. Western blotting is a powerful and popular technique to visualize and identify proteins. Western blotting has the resolution of gel electrophoresis along with the use of

immunoassays. This allows individual specific proteins in mixtures to be identified and analyzed. Protein binding is based upon hydrophobic interactions, as well as charged interactions between the membrane and protein.

Preparing and Running the Western Blot

The upper gel was cut off, leaving only the lower gel to use for the western blot. Water was used to wet the sponge that was to hold the filter paper, membrane and gel. Under tris transfer buffer, the layers for the blot were created staring with one sponge, filter paper, gel, membrane, filter paper, and sponge again. There set was then placed inside the cathode and anode bracket and set to transfer for 1 h. The gel was then removed from the western blot transfer and placed in red marker buffer, washed with water, and placed in TBST. The TBST solution was then drained in order to add a protein blocker. 30 ml of TBS with 1.3g of non-fat dry milk was mixed and added to the membrane.

Antibody Markers

The purpose of the western blots for this experiment was the need to identity where the mitochondria and the chloroplasts are located. For these two organelles, the antibodies used were Cox II and Rubisco respectively. After obtaining a western blot, the gel was washed with TBS and farther blocked using milk. Then the primary antibody for the mitochondrial marker Cox II was added (Rabbit 1:1000). After an hour the gel was washed with TBST 3 times for 10 minutes each. Then, the secondary antibody (Goat Anti-Rabbit 1:30,000) was added for another hour. The gel was washed with TBST 3

times for 10 minutes. Using chemiluminescense solutions, the gel was imaged. Using glycine-stripping buffer, the gel was stripped and then re-blocked with milk in TBS. After washing with TBST, the procedure for an antibody was repeated with Rubisco to identify the chloroplast containing fractions. Using the concentration of 1:10,000 for primary and 1:30,000 for secondary antibody, the gel was imaged.

CHAPTER 3

Trials for Isolation of Juniper Mitochondria

Introduction

Five trials were conducted to separate the organelles and isolate mitochondria. For this separation between chloroplast and mitochondria, the protocol used was adapted from a procedure to separate mitochondria and chloroplasts from moss (Lang et al., 2011). The pictures of pellets and the density gradient were recorded for the trials. To analyze the fractions found in each layer and locate specific organelles, gel electrophoresis and a western blot with antibodies for mitochondria and chloroplast were used. The first trial conducted used a sucrose gradient that separated the organelles by density. The second, third, and fourth trials used a Percoll gradient to ensure separation of mitochondria and chloroplast. The fifth trial applied a mixed Percoll and sucrose gradient.

Trial One

To separate the organelles by density, sucrose solutions of different concentration were created. These were created to allow the organelles to settle in different layers. Usually the less dense proteins settle towards the top while the heavier organelles settle towards the denser concentrations at the bottom. The four 50ml concentrations created were 2 M, 1.5 M, 1.0 M, and 0.5 M. 8.75 g of juniper leaves were homogenized with 50ml of Buffer A and sand. Buffer A was created using 10mM of HEPES, 3mM of EDTA, 10mM of KCl and .2M of sucrose. After crushing and grinding into a thick liquid,

the mix was filtered through one layer of Miracloth into two 50ml centrifuge tubes. The tubes were centrifuged for 1 min at maximum speed in a clinical centrifuge. Each of the sucrose solutions was layered carefully into 2 ultracentrifuge tubes. 3ml of tissue extract was added to the top of the sucrose gradient. After checking weights and balancing tubes, the centrifuge was set at 28,000 rpm in a SW-41 rotor for 3 hours. The 3 fractions plus the pellet were removed with a pipette and placed in micro centrifuge tubes. Four micro-centrifuge tubes for each of the original tube allowed a total of 8 tubes. For analysis of the fractions, gel electrophoresis was run using Protocol 9 SDS-PAGE Analysis of Proteins. These gels were run with a solution made of 80 microliters of the sample with 20 microliters of running buffer. 15 microliters of the solution was loaded onto the gel in each column.

The gel obtained did not show any conclusive results, so the protocol was repeated with modifications in the concentration of the sucrose solutions. Using only the lowest and highest concentration of the sucrose solutions, layers were created through the same centrifuge procedure. Another difference was the use of a syringe to puncture the centrifuge tube and remove the three different layers directly from the tube through a pipe connected to the syringe itself. This would allow for more distinct bands without losing the distinctiveness that was lost using a pipette to transfer the different layers. Through these adjustments another set of gels were run.

With an improvement in the gels, the next step was to run a western blot using Protocol 10: Western Immunoblotting. The purpose of the Western for this experiment was the need to identity where the mitochondria and the chloroplasts are located. For these two organelles, the antibodies used were Cox II and Rubisco respectively. After

obtaining a western blot, the membrane was washed with TBS and blocked using milk. Then the primary antibody for mitochondria was added (Rabbit 1:1000). After an hour the gel was washed with TBST 3 times for 10 minutes each. Then, the secondary antibody (Goat Anti-Rabbit 1:30,000) was added for another hour. The gel was washed with TBST 3 times for 10 minutes. Using chemiluminescense solutions, the gel was imaged.

Using a glycine-stripping buffer, the gel was wiped out and was re-blocked with milk. After washing with TBST, the procedure for an antibody was repeated with Rubisco to identify the chloroplast. Using the concentration of 1:10,000 for primary and 1:30,000 for secondary antibody, the gel was imaged. However, no image was seen on the gel. The antibodies were repeated with a higher concentration of antibody to TBST (1:3,000 and 1:1,000). The gel was imaged once again.

Results

The images of the gels include gel showing the presence of mitochondria (light and without light) and gel showing the presence of chloroplast (light and without light).

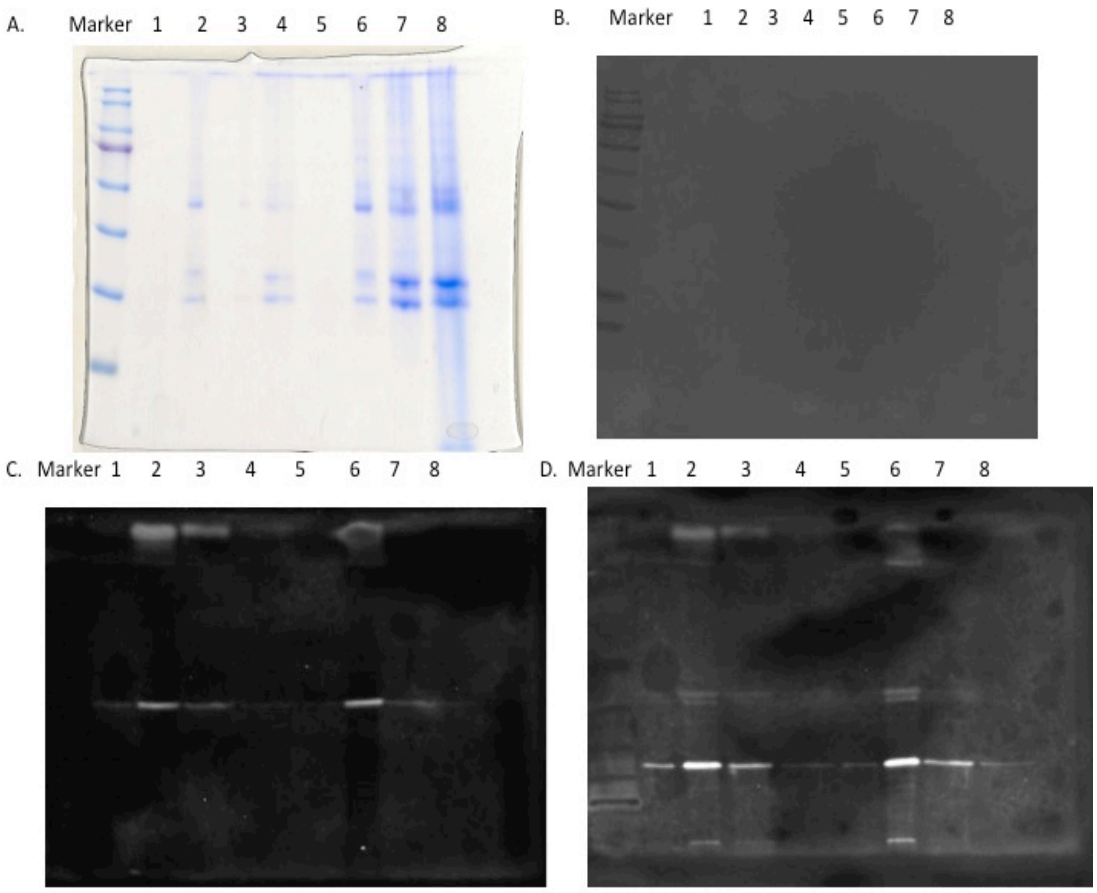

Figure 4. A. Coomassie gel created using the sucrose gradient. B. Visible light image using the sucrose gradient and syringe to collect samples. Since two tubes were used, the first four lanes are Tube 1 and the last four are from Tube 2. C. Western with anti-Cox II (Primary Rabbit 1:1000 Secondary Goat Anti-Rabbit 1:30,000) D. Western with anti-Rubisco (Primary Rabbit 1:1000 Secondary Goat Anti-Rabbit 1:30,000). Both chloroplast and mitochondria were present in all the samples. The antibody was brightest in samples 1, 2 and 6, 7. In all the lanes that Cox II was bright, Rubisco was highly bright as well.

Trial 2

Juniper leaves were collected from the tree. The leaves were washed thoroughly with water. 21g of fresh juniper leaves were measured on wet ice (at 4°C). 60ml of organelle isolation buffer [1% (w/v) polyvinylpolypyrrolidone (PVPP), 300 mM d-sorbitol, 50 mM HEPES, 2 mM Na-EDTA, 1 mM MgCl2 and 0.1% BSA] with 0.1% protease inhibitor (60ml) were added to the leaves. The mixture was chopped using a homogenizer for 20 min. After crushing and grinding into a thick liquid, the mix was filtered through three layers of Miracloth into two 50ml centrifuge tubes. The tubes were centrifuged (Beckman Coulter Avanti Centrifuge J-20) at 1,500xg for 10 min to pellet the chloroplasts. The supernatant was transferred to new centrifuge tubes for mitochondria isolation. The chloroplast pellet was discarded.

The supernatant was used to purify mitochondria using a Percoll density gradient. In order to pellet debris, the supernatant was centrifuged at 3,000×*g* for 5 min followed by 6,000×*g* for 5 min in the same tube. The new supernatant was transferred to a new tube and centrifuged at 18,000 for 20 min in order to pellet mitochondria.

The pellet was re-suspended in 1 ml of washing buffer and with a final volume of 3 ml placed in a Potter homogenizer and homogenized with 10 strokes. Then 0.6 ml of 100% Percoll was added to create a sample with about 20% Percoll. The gradient was created using 5 ml of 80% Percoll in washing buffer and 5 ml of 33% Percoll in washing buffer. The homogenized sample with 20% Percoll was placed on top of the gradient. The gradient with 80%, 33% and 20% (with sample) was centrifuged at 18,000xg for 1 hour.

Figure 5. First Percoll Gradient after Centrifugation. There is no clear separation within the gradient. Different levels of the gradient are not visible and there was no way to extract a sample. Compare to the figure 3.

Experimental Problem Trial 2

After the gradient containing the sample was taken out of the centrifuge, the results were not similar to the picture as reported by Lang et al. (2011) (Figure 3). The test tube did not have the expected separation and was green throughout. The error was found to be the separation of the initial pellet from the supernatant. A big part of the pellet was mixed with the supernatant and that is what caused the gradient to not separate accordingly. Changes were made to fix the problem of the pellet and supernatant mixing in the next trial. Changes in the next trial are listed.

Trial 3

When separating pellet from supernatant, it was crucial to watch for loose pellet. Before creating the gradient, the supernatant was transferred to a new centrifuge tube for mitochondria isolation very carefully making sure no part of the loose pellet was transferred with the supernatant.

The pellet was re-suspended in 1 ml of washing buffer and with a final volume of 2 ml placed in a Potter homogenizer and homogenized with 10 strokes. .3 ml of 100% Percoll was added to create a sample with about 20% Percoll. The gradient was created using 5 ml of 80% Percoll in washing buffer and 5 ml of 33% Percoll in washing buffer. The homogenized sample with 20% Percoll was placed on top of the gradient. The gradient with 80%, 33% and 20% (with sample) was centrifuged at 18,000xg for 1 hour.

Experimental Problem Trial 3

After the gradient containing the sample was taken out of the centrifuge, the results were similar to the as reported by Lang et al. (2011) (Figure 3) The test tube did have separation as seen by the arrows. However, the same interfaces from figure 3 were not present. M1 and M2 were not distinct layer, however, there was a whitish layer between the 33% and 80% interface. Samples were taken from this layer for the imaging process.

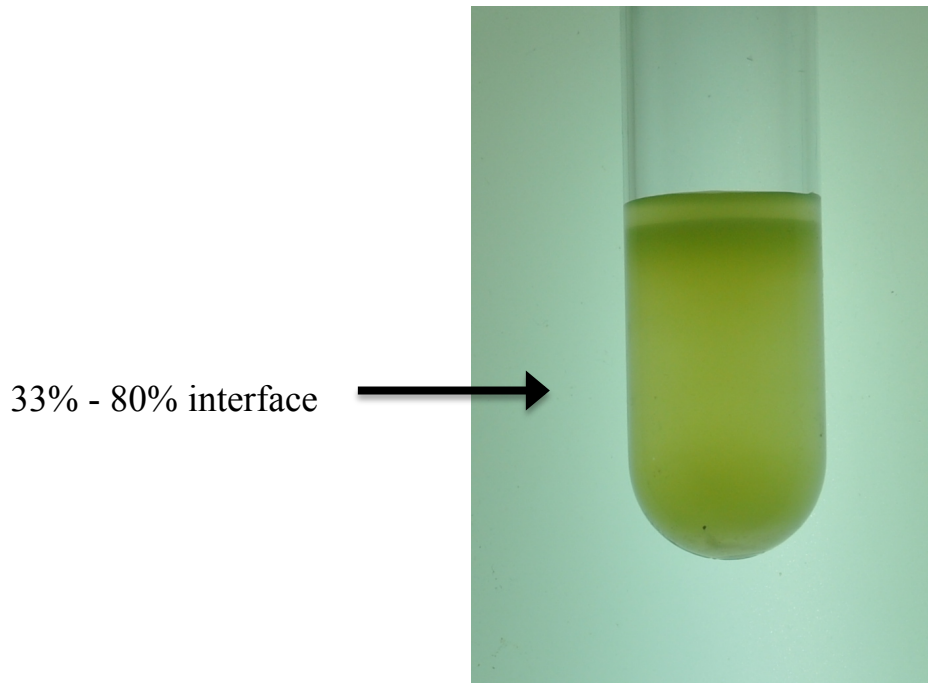

Figure 6. Percoll Gradient. The arrow indicated the 33% - 80% whitish layer that was extracted for imaging.

A pale band was seen at 33% - 80% interface. Using a pipette, the layer of mitochondria was extracted. 30 ml of washing buffer was added to the mitochondria in order to free it from Percoll. The mixture was centrifuged at 18,000xg for 20 min with slow deceleration. The washing buffer and centrifugation was repeated. This yielded a greenish mitochondria pellet.

Protein Extraction

The procedure for protein extraction was adapted from Borate extraction method (Wallace, J. C., et al., 1990). Protein was extracted using Borate extraction buffer [12.5mM Sodium Borate, 1% SDS, 2% beta-mercaptoethanol (pH 10)] For extraction, 1 ml of borate buffer was added to the frozen green pellet to re-suspend it. The solution

was then centrifuged for 15 at high speed at room temperature. The supernatant was transferred to new tube and used to run the gels.

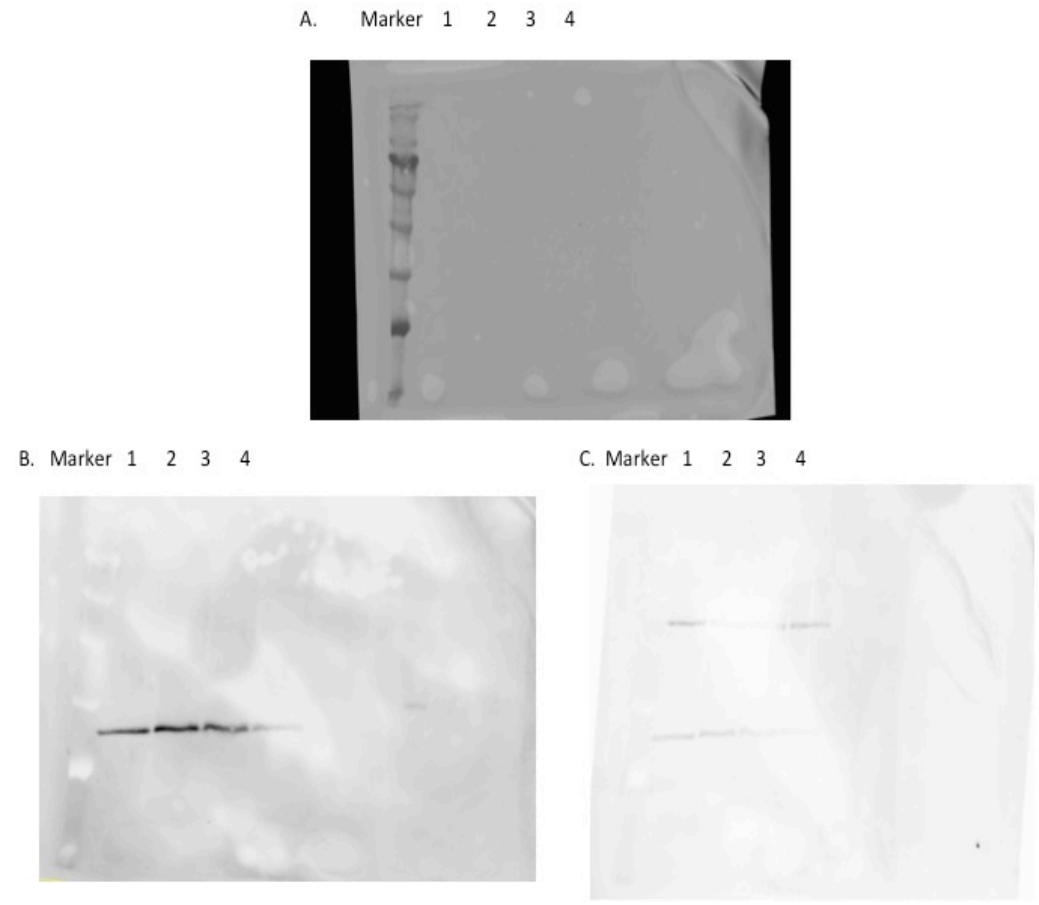

Figure 7. A. Visible light image. B. Western with anti-Cox II (Primary Rabbit 1:1000 Secondary Goat Anti-Rabbit 1:30,000) C. Western with anti-Rubisco (Primary Rabbit 1:3000 Secondary Goat Anti-Rabbit 1:10,000). The four lanes were loaded from sample from the 33% to 80% layers in the gradient. Lane one sample is from the lower layer of the gradient (80%) while lane 4 is closer to the top of the gradient (33%). The Cox II was bound most heavily in lanes 1, 2, and 3. Rubisco was bound most heavily in lanes 1 and 4. Mitochondria and Chloroplast was present in lanes 1 and 4. Lanes 2 and 3 show the presence of mitochondria with almost no chloroplast. These lanes show the most purification of mitochondria, however, there is still chloroplast present in the sample.

Trial 4

Similar to the change made in trial 3, the supernatant was transferred to new centrifuge tubes for mitochondria isolation very carefully making sure no part of the loose pellet was transferred with the supernatant. The chloroplast pellet was discarded.

To improve the separation of the gradient and create the M1 and M2 layers distinctly, the individual Percoll layers were increased from 5ml to 8ml. A larger gradient allows for more distinct layers in test tube so a third level was also added using 8 ml of 50% Percoll. The gradient was created using 8 ml of 80% Percoll in washing buffer, 8 ml of 50% Percoll in washing buffer, and 8 ml of 33% Percoll in washing buffer. This addition of a third level within the gradient was to help create a better separation allowing extraction of the exact interface, which contains the mitochondria. The homogenized sample with 20% Percoll was placed on top of the gradient. The gradient with 80%, 50%, 33% and 20% (with sample) was centrifuged at 18,000xg for 1 hour.

Figure 8. The gradient seen after the centrifugation showed no separation of the three levels within the gradients created. There was no way to extract from the 33% - 50% interface since no difference between the layers could be seen.

Experimental Problem with Trial 4

From the gradient that resulted, the addition of another level within the gradient (50%) did not help the separation. Increasing each layer from 5ml to 8ml could help create more clear layers, however, it did not occur in the gradient in trial 3. It is probable that the problem was due to the Percoll. It was unable to keep the 80%, 50%, 33% layered. All the different concentrations were mixing in the gradient. A more dense liquid was needed to keep the density layers in order.

Trial 5

Similar to previous trials, the supernatant was transferred to new centrifuge tubes for mitochondria isolation very carefully making sure no part of the loose pellet was transferred with the supernatant. The chloroplast pellet was discarded.

In order to attempt to fix the problem present in trial 3 and 4, the gradient was created using both Percoll and sucrose. 4 ml of 80% Percoll in washing buffer in 1.6 ml of 2 M sucrose, .2 ml of 50% Percoll in washing buffer in .8 ml of 2 M sucrose, and .1 ml of 33% Percoll in washing buffer in 0.4 ml of 2 M sucrose. This addition of sucrose within the gradient was to help create better density separation. Additional separation within the gradient was predicted to help create the distinct interfaces. The homogenized sample with 20% Percoll was placed on top of the gradient. The gradient with 80%, 50%, 33% and 20% (with sample) was centrifuged at 18,000xg for 1 hour.

After the gradient containing the sample was taken out of the centrifuge, the results were similar to the picture as reported by Lang et al. (2011) (Figure 3). Figure 9 shows the separation (as seen by the arrows) created from the mixed gradient.

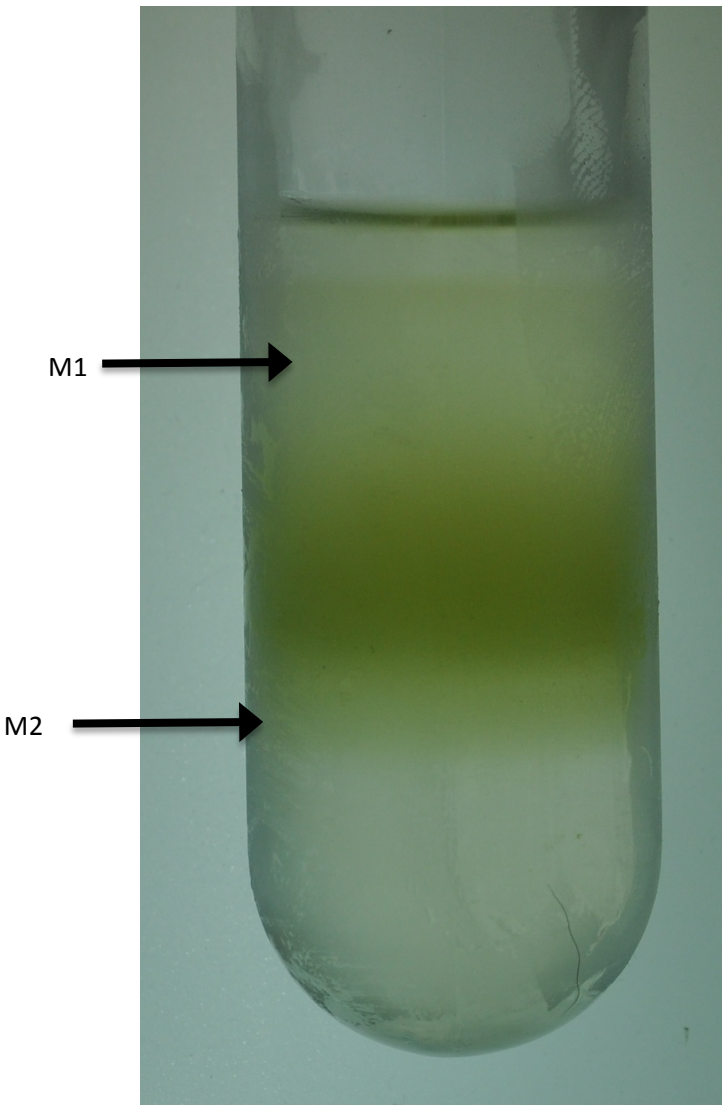

Figure 9. Mixed Percoll and Sucrose Gradient. The gradient shows clear separation of the density layers. The whitish mitochondrial layers are shown similar to the figure in provided in the protocol. Sample was extracted from both M1 and M2 layers.

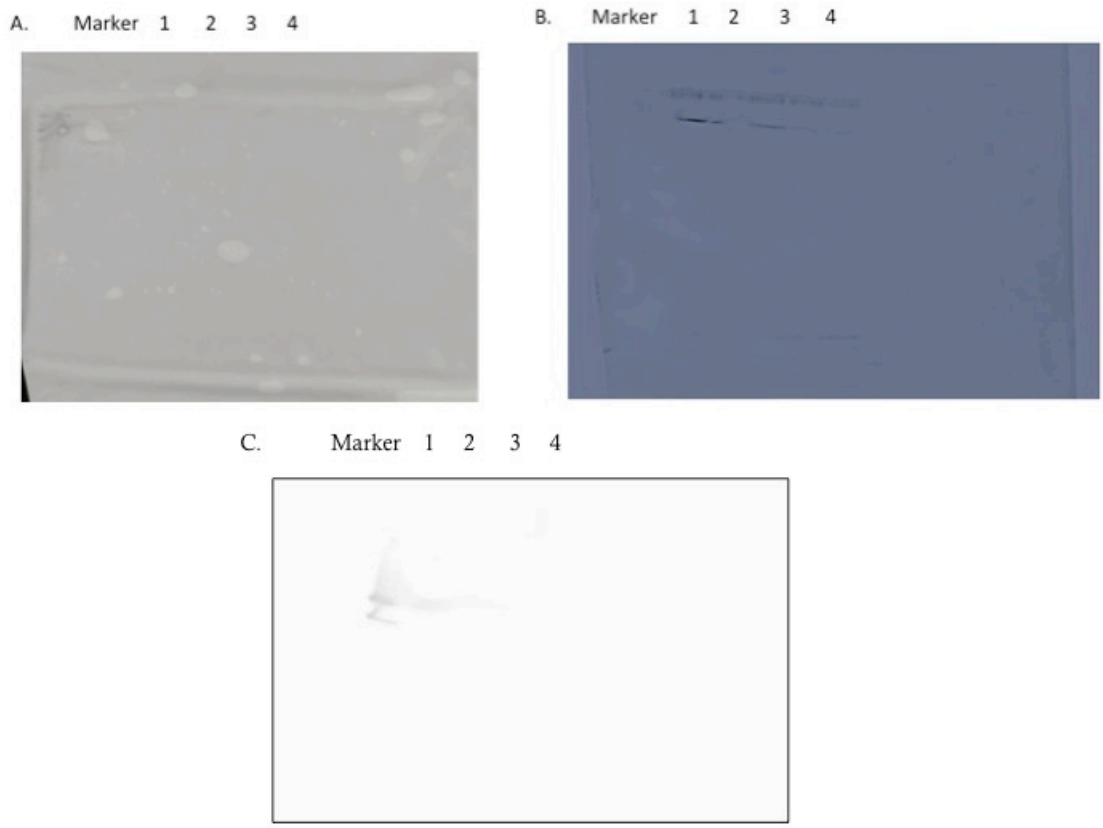

Figure 10. A. Visible light image. B. Western with anti-Rubisco (Primary Rabbit 1:3000 Secondary Goat Anti-Rabbit 1:10000). The first two lanes are from the lower band of mitochondria (M2) and the second two lanes are from the upper band (M1). Figure 10 shows that the lanes 2 and 3 show little to no presence of chloroplast in the sample. Lanes 1 and 4 show presence of chloroplast. Rubisco bound heavily to lanes 1 and 4; however lanes 2 and 3 show purification of mitochondria from chloroplast. C. The anti-CoxII shows non-specific binding to the molecular weight marker. Due to the inconclusive results of the anti-CoxII western, it does not indicate whether mitochondria were present or not.

CHAPTER 4

Conclusion

Discussion of Results

The goal of the experiment was to separate the cell organelles of the Juniper plant in order to isolate the mitochondria from chloroplast. All five trials show the use of a gradient to separate the organelles by density. The challenge was isolating the mitochondria from chloroplast, which are similar in density. Through the trials, multiple changes were made to create a more specific gradient. As more separation was achieved, the presence of chloroplast in the protein sample decreased as seen through imaging using antibodies.

Trial 1

The first trial is completely unique from the other trials. It used a sucrose gradient in which four 50ml concentrations created were 2M, 1.5M, 1.0M, and 0.5M. These were created to allow the organelles to settle in different layers. As discussed earlier, the use of a syringe to puncture the centrifuge tube and remove the three different layers directly from the tube through a pipe connected to the syringe itself was more successful. This was different from the use of a pipette that gave inconclusive results. 2 different test tubes were used and 8 samples were run.

Figure 4 shows the imaging for the gel and western for both the antibodies bound to the protein. Both chloroplast and mitochondria were present in all the samples. The

antibody was brightest in samples 1, 2 and 6, 7. In all the lanes that Cox II was bright, Rubisco was highly bright as well. While the western shows that mitochondria are heavier than the chloroplast, it also shows that the sample contains both. The images show that more separation is needed to isolate mitochondria. The sample had both organelles and a new procedure was needed to completely separate the two. This lead to the use of a procedure that was adapted from a procedure to separate mitochondria and chloroplasts from moss (Lang et al., 2011).

Trial 2

Trial 2 was the first trial using the Percoll gradient a procedure that was adapted from a procedure to separate mitochondria and chloroplasts from moss (Lang et al., 2011). The image of the final gradient (Figure 5) proves that more separation is needed within the gradient. The error was found to be the separation of the initial pellet from the supernatant. A big part of the pellet was mixed with the supernatant and that is what caused the gradient to not separate accordingly. Changes were made to fix the problem of the pellet and supernatant mixing in the next trial.

Trial 3

Figure 7 shows the visible light image and western blots from Trial 3. The imaging shows the presence of mitochondria and the presence of chloroplast. The Cox II was bound most heavily in lanes 1, 2, and 3. Rubisco was bound most heavily in lanes 1 and 4. Mitochondria and chloroplast was present in lanes 1 and 4. Lanes 2 and 3 show the presence of mitochondria with almost no chloroplast. Sample was taken from the 33% to

80% layers of the gradient. Lanes 2 and 3 have the most purification. Since these lanes were taken from the middle to of the 33% to 80% layers, the 50% layer could potentially contain the most purified sample. These lanes show the most purification of mitochondria, however, there is still chloroplast present in the sample. The experiment illustrated that both the mitochondria and the chloroplasts were present in the same fraction despite the separation techniques. Since both the antibodies were bound to proteins located in the exact same area, the Percoll gradient did not separate those organelles completely.

Trial 4

Trial 4 proves that more separation is needed within the gradient. Figure 8 shows there is no visible separation of the level in the gradient. Even adding the extra 50% interval did not lead to a strict gradient. The Percoll did not seem dense enough to cause the organelles to fall into different levels of the gradient. In addition, the Percoll levels do not hold well even when creating the gradient. It is nearly impossible to keep the 80% from mixing with the level that is pipetted on top of it. The imaging of these gradient illustrates that another way of separating should be used for the experiment. A better way to separate the organelles could be conducted through the combination of different gradients. Once the two organelles are in different columns of the gel, the mitochondria can be extracted without the interference of the chloroplast. There is no visible separation of the levels in the gradient in Figure 8. The Percoll does not seem heavy enough to cause the organelles to fall into different levels of the gradient. In addition, the Percoll levels do not hold well even when creating the gradient.

Trial 5

From figure 9 showing the gradient created during this trial, it is clear to see that the combined sucrose and Percoll gradient was most successful in creating the mitochondrial layers present in procedure to separate mitochondria and chloroplasts from moss (Lang et al., 2011). Comparing figure 9 to figure 2, the layer with intact mitochondria M1 and M2 can be seen.

Figure 10 shows the trial 5 images including the visible light image and western. The western with Rubisco antibody shows very little presence of chloroplast. There were four different samples present. The first two lanes are from the lower band of mitochondria (M2) and the second two lanes are from the upper band (M1). Figure 10 shows that lanes 2 and 3 show little to no presence of chloroplast in the sample. Lanes 1 and 4 show presence of chloroplast. Rubisco bound heavily to lanes 1 and 4; however lanes 2 and 3 show purification of mitochondria from chloroplast. If the experiment was to be continued, trial 5 should be repeated. The little presence of chloroplast in two lanes shows that the method of using both protocols and creating a gradient with both Percoll and sucrose is effective. If the approach is repeated, then isolation of mitochondria is possible.

Comparison of the Two Procedures

In the trial, two trials were used to achieve isolation of mitochondria. By trial 5, a combination of the two procedures was used to create a gradient. The procedure using sucrose was effective in making a gradient in which organelles could be separated by

density. The levels of sucrose concentrations remained distinct. The second procedure created used multiple rounds of centrifugation to first separate most of the chloroplasts before even creating a gradient. It was aimed at tackling the problem that was present through the first procedure. This procedure, however, used a Percoll gradient. Percoll is tool for efficient density separation used to isolate organelles or even cells. Although it is used in gradients, the Percoll did not seem to create as strong of a gradient. The different concentrations of Percoll would easily mix leading to inconsistency. The effectiveness of both the types of gradients was seen through the images of the gradients taken after centrifugation. Both the gradients had their advantages and disadvantage that could be combined to make a better gradient overall.

Incomplete Isolation

The experiment conducted to isolate mitochondria involved 5 trials. As the trials progressed, more isolation was seen. There is more separation as improvements were made to the overall procedure. By trial 5, there was little to no chloroplast present in the sample. Although the trials show progress towards the overall goal, complete isolation did not occur in any of the trials. While the procedure was not completely successful, it does show what methods are more successful than others. The trials show what works in helping isolation and also what hinders it.

Further Research

Isolation of Juniper mitochondria if successful can be a possible way to extract DNA and sequence the mitochondrial genome. Not only will the sequence be beneficial

for study of mapping and history of Juniper, the experimental procedure will be helpful for further extractions and isolation of mitochondria. The procedure using the sucrose and Percoll gradient which was most successful in creating a separating the two organelles mitochondria and chloroplast could be used to conduct experiments to isolate mitochondria and/or chloroplasts for other plants similar to Juniper. The produce if successful can be applied to all other species of Juniper and also to other gymnosperm. Other members of the Cupressaceae family could be the next plants for which the mitochondrial genomes could be sequenced. With the same procedure, a number of similar plants can have their mitochondrial genomes sequenced and then compared. The background of the different species of Juniper and their change in respect to each other could be studied through the genomes. The original procedure used for the experiment was for moss, something completely different from Juniper. With a way to isolate mitochondria for plants such as Juniper, a gateway to sequencing so many other plants similar to Juniper opens.

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