12-1-2015

DEVELOPING CHEMICALLY MUTAGENIZED EMS FORREST SOYBEAN POPULATION FOR HIGH OIL PROFILE

Joshua William Gunther
Southern Illinois University Carbondale, jgunther@siu.edu

DEVELOPING CHEMICALLY MUTAGENIZED EMS FORREST SOYBEAN POPULATION FOR HIGH OIL PROFILE

By

JOSHUA GUNTHER

B.S., Southern Illinois University Carbondale, 2014

A Thesis

Submitted in Partial Fulfillment of the Requirements for the

Masters of Science

Department of Plant, Soil, and Agricultural Systems

In the Graduate School

Southern Illinois University Carbondale

December 2015

THESIS APPROVED

DEVELOPING CHEMICALLY MUTAGENIZED EMS FORREST SOYBEAN POPULATION FOR HIGH OIL PROFILE

By

JOSHUA GUNTHER

Supervised by

Dr. Khalid Meksem

Dr. Naoufal Lakhssassi

A Thesis Submitted in Partial

Fulfillment of the Requirements

For the Degree of

Masters of Science

In the field of Plant, Soil, and Agricultural Systems

Approved by:

Dr. Khalid Meksem, Chair

Dr. Alan Walters

Dr. Stella Kantartzi

Graduate School

Southern Illinois University Carbondale

November 2nd, 2015

AN ABSTRACT OF THE THESIS OF

JOSHUA GUNTHER, for the Masters of Science degree in Plant, Soil and Agricultural Systems, presented on November 2nd, 2015, at Southern Illinois University Carbondale.

TITLE: DEVELOPING CHEMICALLY MUTAGENIZED EMS FORREST SOYBEAN POPULATION FOR HIGH OIL PROFILE.

MAJOR PROFESSOR: Dr. Khalid Meksem

SUPERVISOR: Dr. Naoufal Lakhssassi

Soybeans (Glycine max (L.) Merr.) are the most important crop that provides a sustainable source of oil and protein worldwide. Five major fatty acids are known, Palmetic, Stearic, Oleic, Linoleic and Linolenic acid, and each is essential for both nutrition and biodiesel. Oil demand for biodiesel production is constantly on the rise both because of high crude oil prices and because of the search for a sustainable fuel source. In biodiesel production a high level of Oleic Acid is ideal. Commodity soybean oil usually contains around 20% oleic acid. The objective of this research is to increase the Oleic acid content to increase the quantity of biodiesel that can be produced from one bushel of soybeans. With the process of chemical mutagenesis using Ethyl Methanesulfonate (EMS), soybean populations can be produced with varying fatty acid levels. Once these populations are isolated and tested they can be bred into a new cultivar with higher percentages of Oleic acid and grown on a large scale for biodiesel production. From the 'Forrest' cultivar that was used in the chemical mutagenesis process, there was one mutant line (FM3 2014-2031) that produced 43.26% Oleic Acid. This was almost twice the concentration of the Forrest wild type that was used as a control which had an Oleic Acid concentration of 23.17%.

DEDICATION

I dedicate this work to my loving wife Brittany Gunther who has been by my side throughout my graduate school experience at Southern Illinois University Carbondale. I know that I would not be where I am today without her love and support.

ACKNLOWLEDGMENTS

I would like to start off by thanking God for good health and all the great possibilities that he has put into my life. I have been very fortunate in my life and been allowed the opportunity for higher education.

I would also like to thank my wonderful wife, Brittany Gunther. She has always seemed to see more potential in myself than I have. She has always pushed me to accomplish more than I ever thought I would be able to. I know I would not be where I am at today without her love and support.

Another great thanks goes out to my parents, John and Debbie Gunther who raised me into the young man I am today. I would not have been able to accomplish so much in my undergraduate studies without their love, support, and guidance.

A big thanks goes out to my Major Professor, Dr. Khalid Meksem. Thank you for having the faith in myself and giving me the opportunity to study under your guidance during the duration of my Master's program.

I would also like to give acknowledgements to Dr. Naoufal Lakhssassi who was my co-supervisor during my research. The day to day operations during my research would have been nearly impossible without the guidance of Dr. Lakhssassi, he was by my side every step of the way and provided countless suggestions on how to better my studies. I am very thankful for your time, help and support throughout my graduate studies at Southern Illinois University Carbondale.

I would like to take a moment to acknowledge my thesis committee members Dr. Khalid Meksem, Dr. Alan Walters, and Dr. Stella Kantartzi for their guidance and feedback during my

academic research. I wouldn't have been able to make it through this Master's Project without all of their suggestions and feedback.

Another thanks goes out to the rest of Dr. Meksem's research team, Dr. Shimming Liu and Zhou Zhou. They were crucial elements to the success of the entire research team. They were always there to bounce ideas off of and give a helping hand wherever they could. The lab would have been a lonely place without these two.

One of the most helpful people throughout my academic research was John Miller the Manager for the Horticulture Research Center at Southern Illinois University. Whether it was through the acquisition of materials or basic greenhouse procedures, John was always there to oversee my study. Another great thanks goes to Eric Larson who worked at the Horticulture Research Center during my studies. His efforts went a long way to the overall success of my project. I would not have been able to have success with the field experiments without his aid. He took care of all of the field preparation before transplanting took place and helped maintain an adequate growing environment.

I would also like to acknowledge Theodore B. Ballard the Senior Agriculture Research Technician and the rest of the staff at the Agriculture Research Center at Southern Illinois University in Carbondale. Everyone there was more than willing to help my with my research in any way they could.

Lastly, I would like to thank my undergraduate advisor at Southern Illinois University Carbondale, Dr. Paul Henry. Dr. Henry provided me guidance and acted as a sounding board throughout both my undergraduate and graduate experience.

TABLE OF CONTENTS

LIST OF TABLES

TABLE PAGE

LIST OF FIGURES

CHAPTER 1

LITERATURE REVIEW

1.1 Importance of Soybean in the World

Soybean (*Glycine max (L.) Merrill*) is one of the most important agronomic crops not only in the United States, but in the entire world. In 2014 there were 11.5 billion bushels produced in the world (www.soystats.com/international-world-soybean-production/). The largest soybean producing countries are the United States, Brazil, and Argentina, which make up 82% of the soybean production by bushels produced (Fig. 1). The demand for soybeans for both oil production and protien content will be on the rise as the worlds population is increasing. "United Nations Secretary-General Ban Ki-moon called for a 50% increase in global food production by 2030 in order to meet the increasing demand of a growing world population" (Ainsworth et al., 2012). In order to meet this 50% increase there are going to need to be some advancements in the yeilding potential of soybeans and how efficently the amount of land that is currently in production is being used.

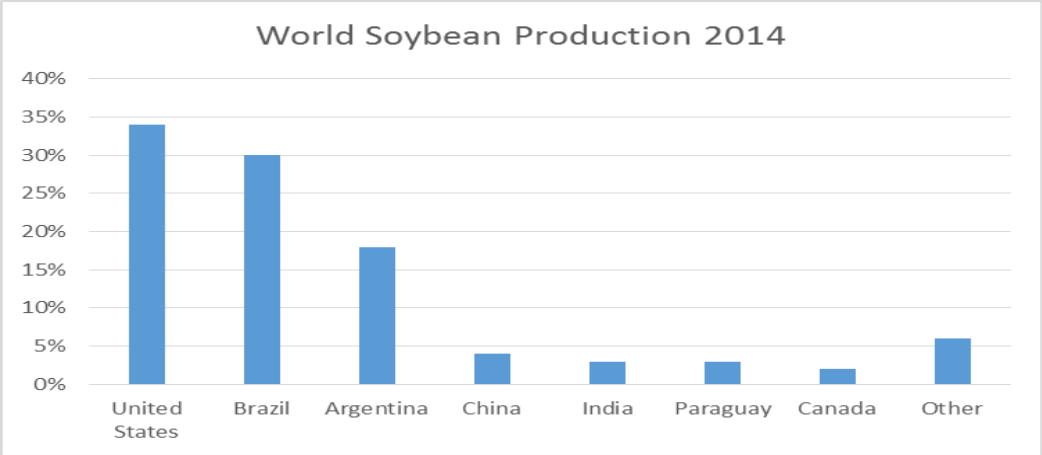

Fig. 1. World Soybean Production 2014 broken down by percent each country produced (www.soystats.com/international-world-soybean-production/)

1.2 Soybean Production in the US

Soybeans (*Glycine max (L.) Merrill*) is the second most important crop in the United States when compared on an economic basis. In 2014 soybean production in the United States was valued at $40.2 billion when measured in dollars and corn in 2014 valued $53.4 billion dollars (www.nass.usda.gov). Soybeans are also behind corn when you look at total crop area planted in the United States. 34% of the crop area is planted in corn while 31% are planted to soybeans in 2014(Fig. 2). Even though in 2014 there were fewer acres planted with soybeans than corn, the number of acres planted in soybeans have been steadily increasing over the past 25 years. In 1989 there were 60.8 million acres planted in soybeans, and when compared to 2014 there were 83.7 million acres planted in soybeans (www.soystats.com/u-s-yield-production-history/). This is a 37.7% increase in the number of acres planted in soybeans over the past 25 years. This shows that soybeans are starting to control more of the marketplace in agriculture and the demand for them is on the rise.

Along with the number of acres planted, there have also been many advances in the way that farmers are raising their soybeans. The average bushels per acre have also dramatically increased over the past 25 years. From 1989 to 2014 the average yield in the United States has increased from 32.3 bushels per acre to 47.8 bushels per acre. This is a 48% increase in the past 25 years (www.soystats.com/u-s-yield-production/). These advancements are due to many different things from, the advancement of farm machinery, the use of precision planting equipment, the kinds of fertilizers being used and the genetics that are being used in the soybean seed itself.

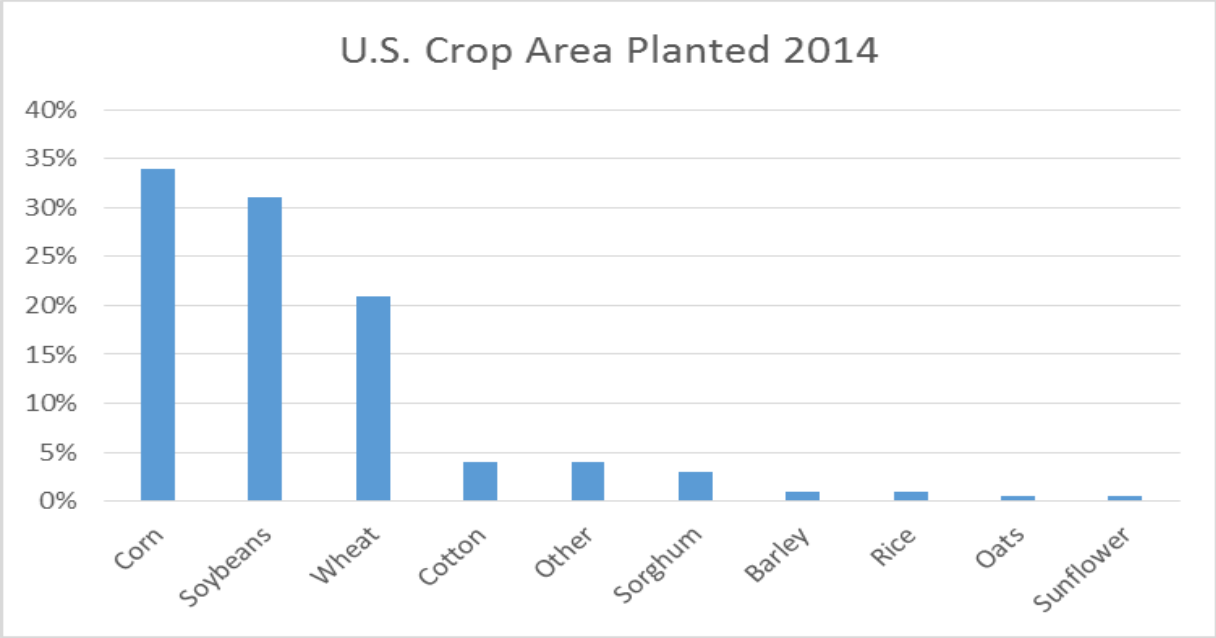

Fig. 2. U.S. Crop Area Planted 2014 broken down by percentage of acres for each crop (www.soystats.com/u-s-yield-production/)

1.3 Soybeans Used as Biofuel Source

In the United States over 50% of the vegetable oil that is used comes from soybeans (www.soystats.com/soybean-oil-u-s-vegetable-oils-consumption/). With soybeans being the largest oil producing crop in the United States it is obvious why it was one of the largest crops used in the biofuel industry. There are currently three primary markets that use biodiesel, mass transit, marine industry, and on farm (Kinney, 2005). These three markets make up a large portion of the demand for diesel fuel. The amount of Biodiesel Production has skyrocketed in the past decade. In 2003 there were only 20 million gallons of biodiesel produced compared to 1,800 million gallons produced in 2013 (Table 1) and (Fig. 3). That is an 8900% increase in one decade. The largest jump from year to year in that decade was from 2010 to 2011, where the

production of biodiesel increased by 785 million gallons (www.soystats.com/biodiesel-u-s-production-history/).

One of the largest problems that biofuel engineers have faced with the wide scale production of biodiesel is fuel oxidation. "Fuel oxidation is usually manifested as the formation of gums and sediment in the fuel which in turn can clog the fuel filter" (Kinney 2005). The high oxidative instability is due to polyunsaturated fatty acid content of soybean oil. Reducing the amount of linoleic and linoleic acids in the oil has had a great impact on lowering the oxidative reactivity of the biodiesel being produced (Kinney 2005). "To maximize the fuel characteristics of a biodiesel, (Duffield *et al.* 1998) suggested the development of an oil high in oleic acid and low in saturated fatty acids, thereby simultaneously improving oxidative stability whilst augmenting cold flow" (Graef et al. 2009).

Table 1. Amount of U.S. Biodiesel Produced from 1999-2014 (www.soystats.com)

Year	Million Gallons	Million Liters
1999	0.5	1.9
2000	2	7.6
2001	5	18.9
2002	15	56.7
2003	20	75.6
2004	25	94.5
2005	112	423
2006	250	945
2007	450	1701
2008	700	2646
2009	545	2060
2010	315	1191
2011	1100	4158
2012	1100	4158
2013	1800	6804
2014	1750	6615

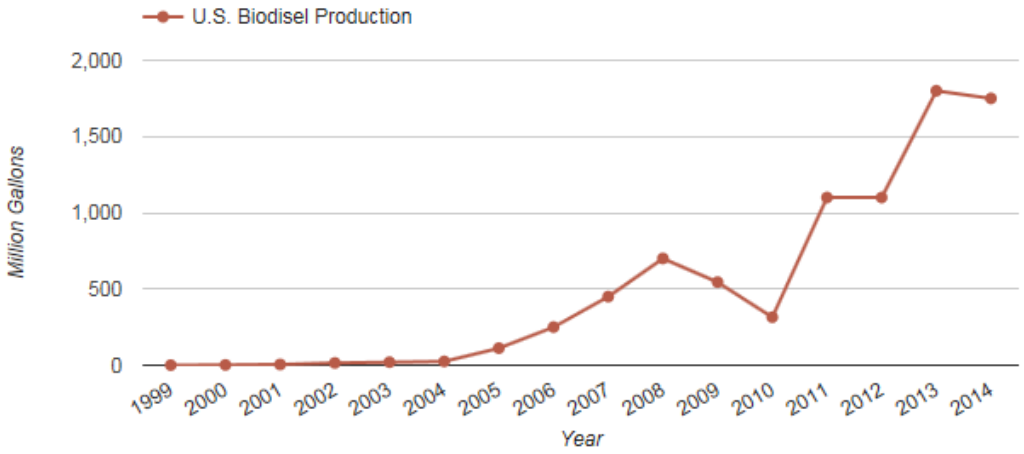

Fig. 3. Amount of U.S. Biodiesel Produced from 1999-2014 (www.soystats.com)

1.4 Importance of Soybean Cyst Nematode

Soybean cyst nematode (*Heterodera glycines*) is the most devastating soybean pathogen in the world. "Soybean (*Glycine max* (L.) Merr.) disease loss estimates were compiled for the 1998 harvested crop from the top 10 soybean-producing countries in the world. These 10 countries (United States, Brazil, China, Argentina, India, Canada, Paraguay, Indonesia, Italy, and Bolivia) produced 97.6% of the world's total soybean crop in 1998. Total yield losses caused by soybean cyst [Heterodera glycines Ichinohe] in these 10 countries were greater than those caused by any other disease" (Wrather et al., 2001).

Yield suppression from SCN (Soybean Cyst Nematode) was estimated at 2.9 million t (106.6 million bushels) in the United States during 2003 (Wrather et at., 2006). This amount of yield loss adds up to 782.4 million dollars of economic loss for America's farmers based on the average soybean price paid to farmers in 2003 of $7.34/bushel (www.soystats.com/value-prices-paid-to-farmers-history/).

When a soybean cyst nematode infects a root it can also cause many other agronomic problems. The worst of which being a soil borne pathogen *Fusarium virguliforme* sp. Which causes Sudden Death Syndrome (SDS). In the United States SDS can be found throughout most of the soybean producing region (Swoboda, 2010). During 2002 in the United States it was estimated that 728,838 metric tons (26.8 million bushels) of soybeans were lost due to SDS (Wrather et al., 2003)(Fig. 4). While this may not seem like a large percentage of the total soybean crop, it ends up being a very significant economic loss. This was an economic loss of 148.2 million dollars to America's farmers based on the average price of soybeans in 2002 of $5.53/bushel (www.soystats.com/value-prices-paid-to-farmers-history/).

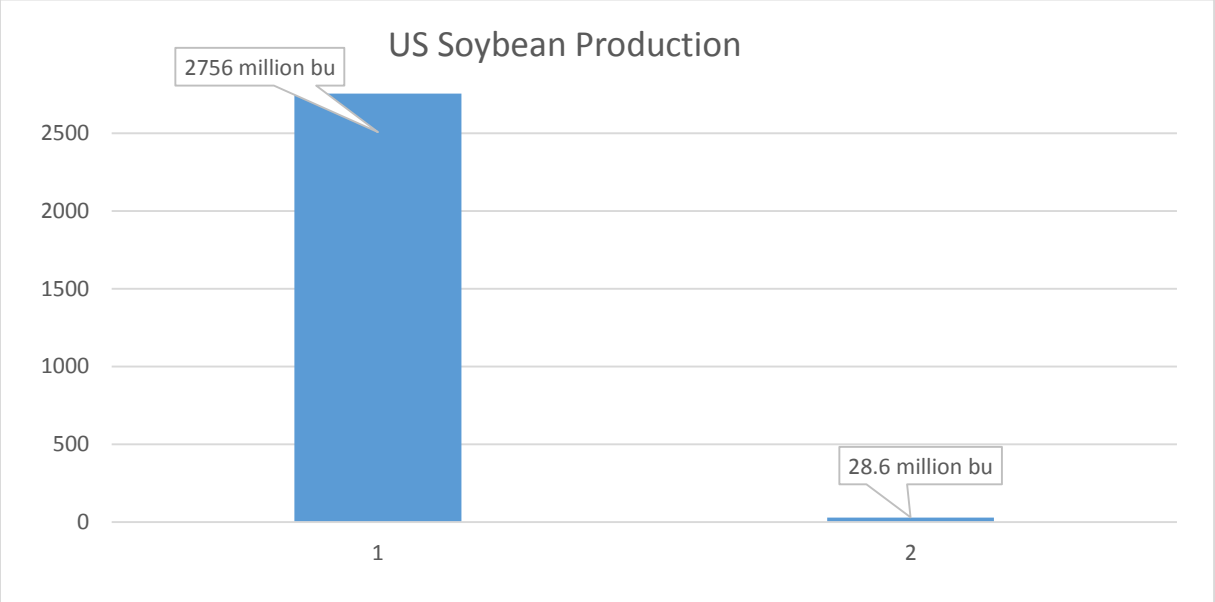

Fig. 4. U.S. soybean loss due to SDS estimated from 2002 U.S. harvest data

1.5 Chemical Mutagenesis

Chemical mutagenesis is one of the most useful technologies in soybean production. Mutations can be artificially induced in plants by chemical mutagens to produce new superior

lines from traditional varieties (Heffner et al., 2009). Unlike transgenic breeding the resulting lines used to make cultivars can be considered Non-GMO's which gives it an upper hand in today's marketplace. Selection of effective and efficient mutagens is the key for receiving a larger concentration of mutants with desirable traits (Kavithamani, 2010). One of the most common chemical mutagens is Ethyl Methanesulfonate (EMS) because it results in a higher frequency or irreversible mutations (Van et al., 2000). In mutation breeding the chemical mutagen is used to alter the DNA of the plant to cause random genetic mutations. Because these mutations are random, the larger the subject group is for the mutations the greater the chances will be that the end result will produce the desired traits.

Using chemical mutagenesis in the search for elevated fatty acid profiles in the soybean seed is on the rise with the demand for soybean oil continuing to increase. In soybean breeding, genetic modification to the concentration of the 5 major fatty acids is an important goal (Huang 2009). With the goal of producing a new cultivar that produces and elevated amount of any of the 5 major fatty acids, chemical mutagenesis is one of the best breeding methods to exercise.

1.6 Research Objective

The main objective of this research is to obtain a mutant line that produces elevated amounts of oleic acid for biodiesel production. Most cultivars produce around 20% oleic acid, in the mutant line that is desired it would be beneficial to have at least 40% oleic acid. Another minor objective for this research would be to isolate high yielding mutant lines to cross with a high oleic acid content line to develop a cultivar that produces both high yields and an elevated amount of oleic acid.

CHAPTER 2

MATERIALS AND METHODS

2.1 EMS Mutagenesis

The full scale mutagenesis was carried out with both 'Forrest' and PI88788 soybeans (*Glycine max*). The first step was to first identify the EMS rate to be used, by testing the wild type seed to determine the correct concentration of EMS (ethyl methanesulfonate) needed for each. The concentrations of EMS in the test ranged from 0% to 1% (v/v). The wild type seeds were treated in groups of 100 (estimated by weight) at a time in 500 ml bottles.

For Forrest 10 different EMS concentrations were used (0%, 0.3%, 0.4%, 0.5%, 0.55%, 0.6%, 0.65%, 0.7%, 0.8%, and 1%). These seeds were soaked in 100 ml of the EMS solution for 15 hours in the fume hood (Fig. 5). After the 15 hours the seeds were gently washed 3 times using 300 ml of water for each wash to remove any trace amounts of excess EMS from the seed coat. This rinse water was then neutralized with a 10% (w/v) sodium thiosulfate solution. The seeds where then immediately taken to the Horticulture Research Center at Southern Illinois University of Carbondale and planted in 48 cell trays filled with ProMix BX. For each concentration 96 seeds were planted from the 100 to fit into two trays.

For PI88788 6 different treatments were used (0%, 0.5%, 0.6%, 0.7%, 0.8% and 1%). These seeds were treated the same using 100 ml of the EMS Solution but were placed under slight agitation on a New Brunswick Scientific Platform Shaker (Edison, NJ) during the 15 hour soaking period (Fig. 6). This agitation helps the EMS penetrate through the thicker seed coat of the PI88788. After the 15 hours of agitation the seeds were rinse three times with 300 ml of

water for each rinse. This rinse water was also neutralized with a 10% (w/v) sodium thiosulfate solution, and the seeds were immediately taken to the Horticulture Research Center at Carbondale, IL and planted in the same way.

Once all of the seeds were planted and the trays labeled with the corresponding line and treatment concentration, they were placed in the greenhouse for 10 days to germinate. The trays were watered twice per day during this period to be certain they had adequate moisture to germinate. The greenhouse was kept at an average temperature of 30°C and a photoperiod of 16 light hours to 8 dark hours.

After 10 days the two trays from each concentration were examined for germination. The plants that germinated were counted. The ideal germination rate for EMS mutagenesis is 50% (Meksem et al., 2008). Once each concentration is counted the correct concentration of EMS can be figured for both Forrest and PI88788.

Fig. 5. EMS treatment bottle used in the EMS mutagenesis (Zhou 2013)

Fig. 6. Automated Shaker for EMS Treatment

2.2 Developing Mutant Soybean Population

When developing a mutant soybean population using chemical EMS (ethyl methanesulfonate) mutagenesis, the first step is to determine what percentage of EMS needs to be used. The percentage should be calculated to find a rate that produces a 50% germination rate (Meksem et al., 2008). Once the correct EMS percentage is established, then it is necessary to determine how many mutant plants to develop. For this research there were 4000 Forrest mutants and 4000 PI88788 mutants developed. To do so 8000 Forrest wild type seeds with an EMS solution of 0.64% (v/v) were treated, and 8000 PI88788 wild type seeds with an EMS solution of 0.70% (v/v) were treated.

After all of the wild type seeds are treated with the correct percentages of EMS, they were taken to the Horticultural Research Center at Southern Illinois University of Carbondale, and planted them in 48 cell trays ordered from Hummert International. The growing medium that was used was ProMix BX (Fig. 7) also ordered from Hummert International. Each tray was labeled with either Forrest or PI88788.

They trays were placed in greenhouse 2 at the Horticultural Research Center at Southern Illinois University of Carbondale (Fig. 8). The greenhouse was kept at an average daytime temperature of 30°C with a photoperiod of 16 hours light with 8 hours of dark. These trays were watered twice a day for the first four weeks. The seedlings were fertilized once with a liquid fertilizer and Marathon 1% Granular® (Fig. 9) was applied to control greenhouse pests. After four weeks the seedlings reached the M1 growth stage, they were then transplanted to the field.

The field was prepared by tilling and then laying down black plastic rows with drip tape irrigation (Fig. 10 & 11). The black plastic rows were used for weed suppression and the drip tape was used for irrigation. Once the field was prepared the M1 growth stage mutants were transplanted into the black plastic rows.

After all of the mutants were transplanted the seedling were water with the drip tape irrigation for 4 hours a day on days it was not raining. Every two to three weeks during the growing season the spaces between the plastic rows were sprayed with Firestorm® applied with a shrouded backpack sprayer for weed control. Once over the growing season the mutants were fertilized with osmocote slow release fertilizer. Over the growing season the mutants were constantly scouted for pest and disease pressure.

Fig. 7. Pro-Mix BX used as media plant soybeans in greenhouse

Fig. 8. Greenhouse 2 at the Horticulture Research Center

Fig. 9. Marathon 1% insecticide used on the soybeans

Fig. 10. Field with drip tape (Alaswad 2014)

Fig. 11. Field with plastic rows (Zhou 2013)

2.3 Fatty Acid Analysis

The levels of fatty acid were measured in the $FM_2$2014 populations by randomly selection 96 mutants to screen for fatty acid content. Along with these 96 mutants there were also 8 Forrest wild type seeds screened as a control. The method that was used to measure the fatty acid content is a two-step methylation procedure.

The first phase in the two-step methylation procedure is to mix all of the solutions that were used. There are three different solutions that need to be mixed before the procedure can be conducted.

The first solution is sodium methoxide at 0.5 M in methanol. The sodium methoxide that was used is bottled at 5.4 M. 500 ml of this solution was made at a time. To reduce the sodium methoxide from 5.4 M to 0.5 M, 46 ml of 5.4 M sodium methoxide was mixed with 454 ml of

methanol to make 500 ml of 0.5 M sodium methoxide. Once this solution was prepared it was stored it in a refrigerator at 4 ° C

The second solution is 5% methanolic HCl. This solution can cause an explosive reaction if mixed improperly. 660 ml of this solution was made. To start 600 ml of cold methanol in an ice bath was placed on an automatic stirrer (Fig. 12). Then 60 ml of acetyl chloride was added drop by drop to the cold methanol to avoid an explosive reaction. Once this solution was prepared it was stored it in a refrigerator at 4 ° C.

The last solution that needs to be prepared for the two-step methylation procedure is 6% potassium carbonate (K_2CO_3). For this solution 1 L of distilled water was mixed with 60 g of K_2CO_3.

The first two solutions (0.5 M sodium methoxide and 5% methanolic HCl) should be stored in a refrigerator at 4 ° C when not being used. Warm temperatures and light breakdown the solutions quicker. These two solutions only have a shelf life of 7 days before they need to be discarded and remixed.

Once all of the solutions are mixed the second phase can be started. The second phase begins with crushing the selected soybeans. Gloves should be worn during this step and forward to avoid oils from your hands skewing the results. To do this a pair of pliers were used to crush the individual soybean. Once the soybean is crushed all pieces of it are placed in a correspondingly labeled glass vial. The pliers used to crush the soybean should be cleaned with alcohol after each soybean is crushed to prevent any cross contamination of oils. The threads on the vials should then be wrapped in Teflon® tape to prevent any evaporation in later steps. After

this step is done the vials with the soybean can be stored at -20 ° C if the samples are not going to be ran immediately.

The next phase in the two-step methylation procedure is to add 2 ml of the 0.5 M sodium methoxide solution and cap each vial tightly, then vortex each vial for about 5 seconds to mix the contents. After mixing the vials should be placed in a hot water bath at 50 ° C for 10 min. After the samples are removed from the 50 ° C hot water bath they were let cool for 5 min. Once cooled the caps should be removed and placed aside in order because each cap should be replaced with the same corresponding vial later. Once uncapped, 3 ml of 5% methanolic HCl were added to each sample. Next each sample was checked to see that the Teflon tape was still intact and if not more Teflon tape was added at this point. The samples were then recapped and mixed well (about 10 seconds) with a vortex machine. After mixing the samples were then placed in an 80 ° C hot water bath for 10 min. and allowed 7 min. to cool afterwards.

After the 80 ° C hot water bath, each sample was examined to make sure that no evaporation of the solution has taken place. Once examined, there were 7.5 ml of 6% K_2CO_3 and 2 ml of hexane added to each sample. After this addition each sample was recapped with the original cap and inverted 20 times to mix properly. Once this is done the samples were placed into the centrifuge (Fig. 13) and centrifuged them at 1190 rpm for 6 min. This centrifuging separates the samples into different layers. After this step is done the samples needed to be carefully removed from the centrifuge being careful not to agitate the samples at all and placed them back under the fume hood.

Once under the fume hood each sample was opened and a glass Pasteur pipet was used to remove the top layer of liquid that separated in the sample and carefully place it in a correspondingly labeled chromatography vial. This top layer contains all of the fatty acids.

Once all of the samples top layers are transferred to chromatography vials caps are tightly fastened and placed in a labeled box. This box is then placed in a -20 ° C freezer until it can be ran through the gas chromatography machine (Fig. 14).

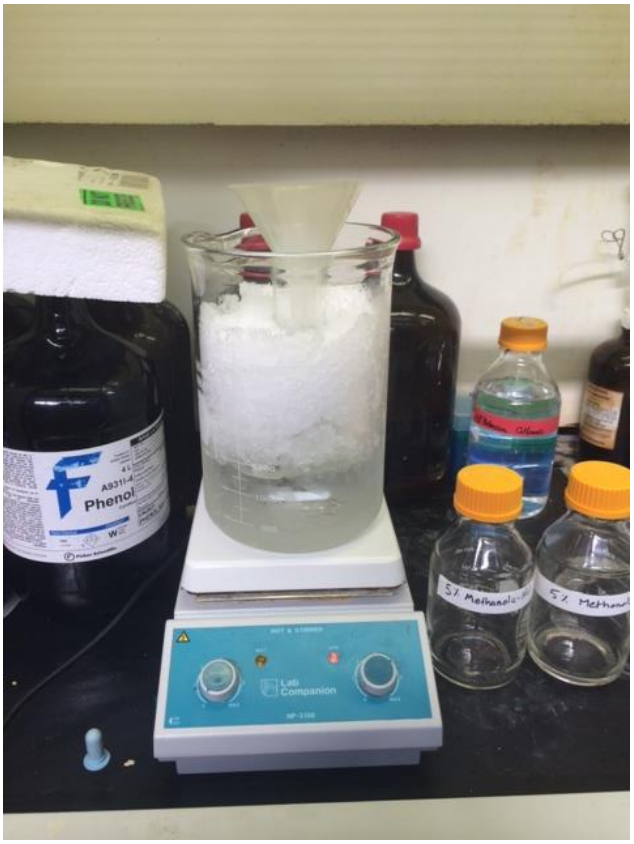

Fig. 12. Automatic Stirrer used in two-step methylation procedure

Fig. 13. Centrifuge us in two-step methylation procedure

Fig. 14. Gas Chromatography Machine used to acquire fatty acid analysis (Alaswad 2014)

CHAPTER 3

RESULTS

3.1 Development of EMS mutated soybeans

The initial experiment of ethyl methanesulfonate (EMS) was conducted on both Forrest and PI88788. This experiment was designed to test out the different percentage rates of EMS in the solution that the soybeans were soaked in for 15 hours. The objective was to find a percentage of EMS that produces approximately 50% germination of the seed. The EMS percentages and germination rates are as follows.

Table 2. Germination results from Forrest EMS Experiment #1 used to determine the concertation of EMS that should be used as the mutagen

Forrest EMS Experiment #1 May 20th 2014	
EMS Solutions (v/v)	Germination Rate
0.00	0.66
0.50	0.51
0.60	0.44
0.70	0.26
0.80	0.20
1.00	0.16

Table 3. Germination results from PI88788 EMS Experiment #1 used to determine the concertation of EMS that should be used as the mutagen

PI88788 EMS Experiment #1 May 20th 2014	
EMS Solutions (v/v)	Germination Rate
0.00	0.74
0.50	0.56
0.60	0.47
0.70	0.32
0.80	0.27
1.00	0.24

The EMS experiment on Forrest was repeated a second time due to low germination rate to ensure accuracy. The second EMS experiment percentages and germination rates are as follows.

Table 4. Germination results from Forrest EMS Experiment # used to determine the concertation of EMS that should be used as the mutagen

Forrest EMS Experiment #2 May 30th 2014	
EMS Solutions (v/v)	Germination Rate
0.00	0.83
0.30	0.74
0.40	0.70
0.50	0.57
0.55	0.63
0.60	0.55
0.65	0.39
0.70	0.32
0.80	0.21
1.00	0.11

In the second EMS experiment for Forrest there were ten different rates used instead of six in the first EMS experiment to better determine the exact percentage that would give approximately 50% germination to the seeds. Once all of this data was collected it was used to make a chart using JMP 12 to figure out what percent of EMS would provide the closest to 50% germination. The charts are pictured below (Fig. 15 & 16).

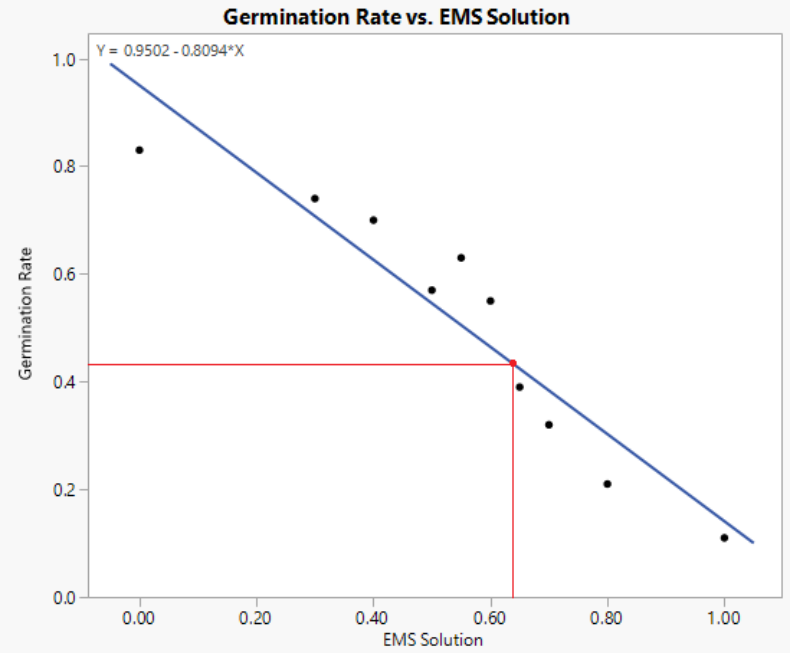

Fig. 15. Forrest germination rate (Y-axis) by the percent EMS solution used (X-axis) used to find what percentage of EMS resulted in 41.5% germination

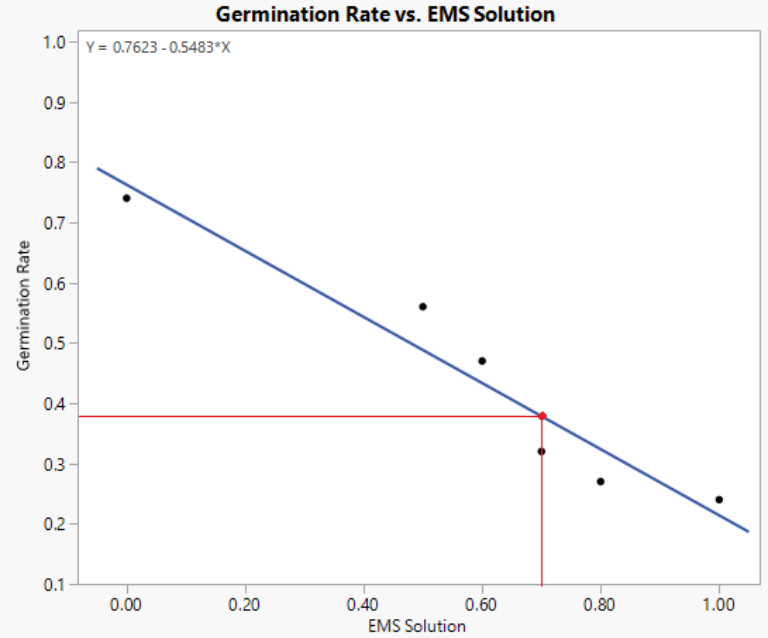

Fig. 16. PI88788 germination rate (Y-axis) by the percent EMS solution used (X-axis) used to find what percentage of EMS resulted in 37% germination

With the germination of the Wild Type seed or the 0% EMS being 83% for Forrest and 74% for PI88788, an EMS solution that would produce 41.5% and 37% germination of the seeds respectively was needed. The EMS solution that was used for Forrest 0.64% EMS (v/v) diluted with water. The solution used for PI88788 was 0.70% (v/v).

Once these percentage were calculated 8000 Wild Type seeds of both Forrest and PI88788 were treated. These seeds were planted in the greenhouse in 48 cell trays until they reached the VC through V1 stage of growth, at this growth stage they were transplanted into the field. These plants were then grown to maturity and harvested. The result was a population of second generation mutant Forrest soybeans of 2366 lines.

From this M1 generation there were 726 of the 2366 mutants that showed phenotypical mutations. That is 30.68% of the M1 generation mutants showing phenotypical mutations.

3.2 Yield Data

When doing anything in plant breeding one of the main questions is “does it yield well?” When looking at the yield data from the FM2 population there were 2366 mutants that produced viable seed. These seeds were threshed and then packaged keeping each mutant’s seed separate. These seeds were then weighed and counted. If there were fewer than 100 seeds the seeds were counted by hand or with counting boards in increments of 40, 60 or 100 whichever was most appropriate. If there were greater than 100 seeds the seed count was averaged from the weight of 100 seeds. It was decided to do the averages off of the weight of 100 seeds instead of 10 seeds as some researchers have done in the past to have a more accurate estimation on the seed count.

20 Forrest wild type plants were used as a control. These plants were grown side by side with the mutants getting the same treatment through the growing season. The average weight of wild type seeds produced was 8.627 grams, where the average seed weight from the FM2 population was 9.273 grams, which is a 7.5% increase in yield after the mutation process. However there was some variation in the Forrest wild type population, and even more in the FM2 population. The greatest weight that was recorded in the Forrest wild type population was FWT-15 it produced 22.025 grams. The greatest weight recorded from the FM2 population was FM2-363 and it produced 83.129 grams. That is a 277.4% increase when compared to the highest yielding Forrest wild type.

Another aspect that was looked at when comparing yield data was seed count or how many seeds were produced. The Forrest wild type that produced the most seeds was FWT-12.

FWT-12 produced 151 seeds. The mutant that produced the most seeds was once again FM2-363 and it produced 496 seeds. This is a 228.5% increase in the number of seeds produced.

The last characteristic that was looked at when comparing yields is seed size and this was covered by figuring the average weight of one seed. To figure the average weight of one seed, the weight of all of the seeds produced by that mutant or wild type was divided by the number of seeds that it produced. The Forrest wild type that produced the largest seed was FWT-15 and on average one seed weighed 0.164 grams. The mutant that produced the largest seeds was FM2-481. FM2-481's average seed weight was 0.317 grams, which is a 93.3% increase from the Forrest wild type.

Below there are histograms that have been produced showing the yield data using JMP 12 Software. The histograms illustrate the breakout of the average weight of the seeds (Fig. 17), seed count (Fig. 18), and average weight of 1 seed (Fig. 19).

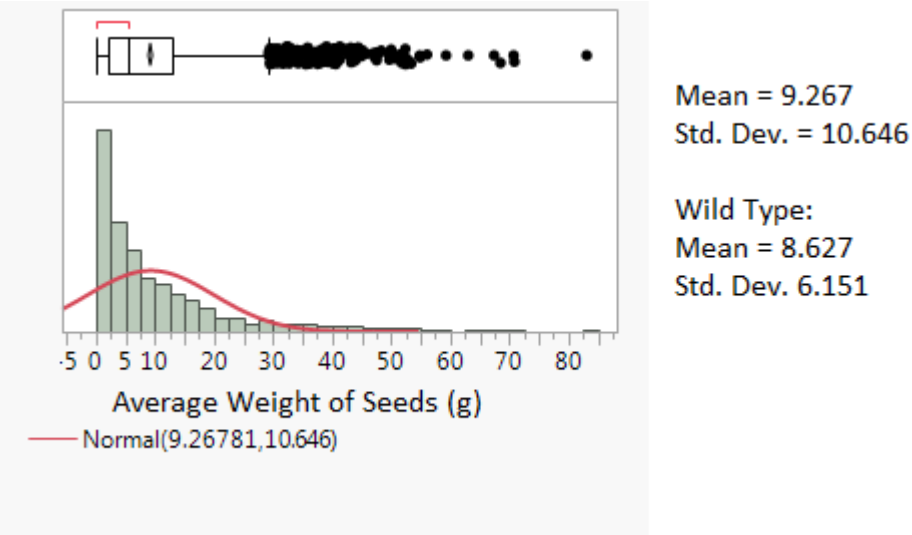

Fig. 17. Histogram of Average Weight of Seeds from Whole Plant from the FM2 generation

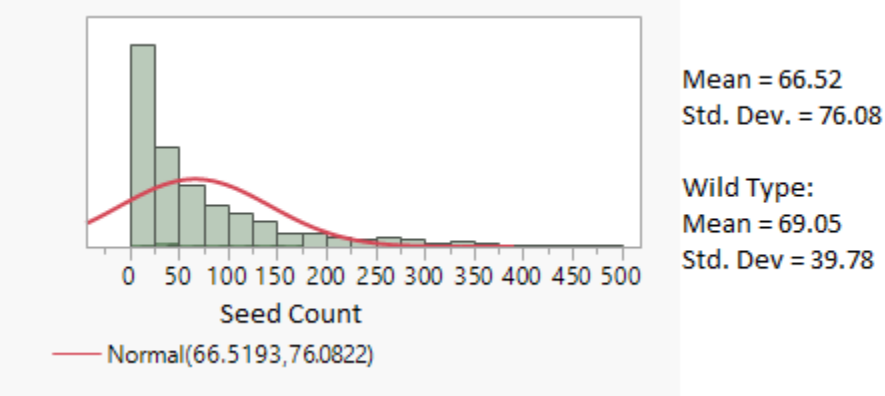

Fig. 18. Histogram of Seed Count from Whole Plant from the FM2 generation

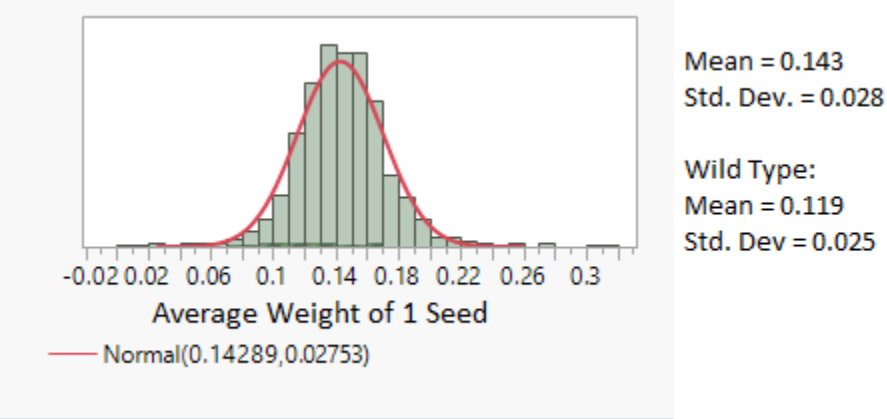

Fig. 19. Histogram of Average Weight of 1 Seed from the FM2 generation

3.3 Phenotypical Mutations

After the chemical mutagenesis treatment using Ethyl Methanesulfonate (EMS) many different mutations can happen to the following generations. Some of these mutations can be seen phenotypically and others cannot be seen with the naked eye, such as elevated oil levels in the soybean. In the FM3 generation there could be 12 different phenotypical traits that could be distinguished, that were cause by the mutation. They included: Lateral Branching, Chlorotic Leaves, Compact Plant, Dead Plant, Dwarfism, Early Maturity, Leaf Color, Leaf Shape, Leaf Texture, Lesions on the Leaves, No Pods, and Vine like. From the FM3 generation that was produced in the greenhouse there were 539 mutants that showed a phenotypical mutation out of the 1302 that were raised in the greenhouse. That is 41.4% showing phenotypical mutations which is a very high percentage for phenotypical mutations.

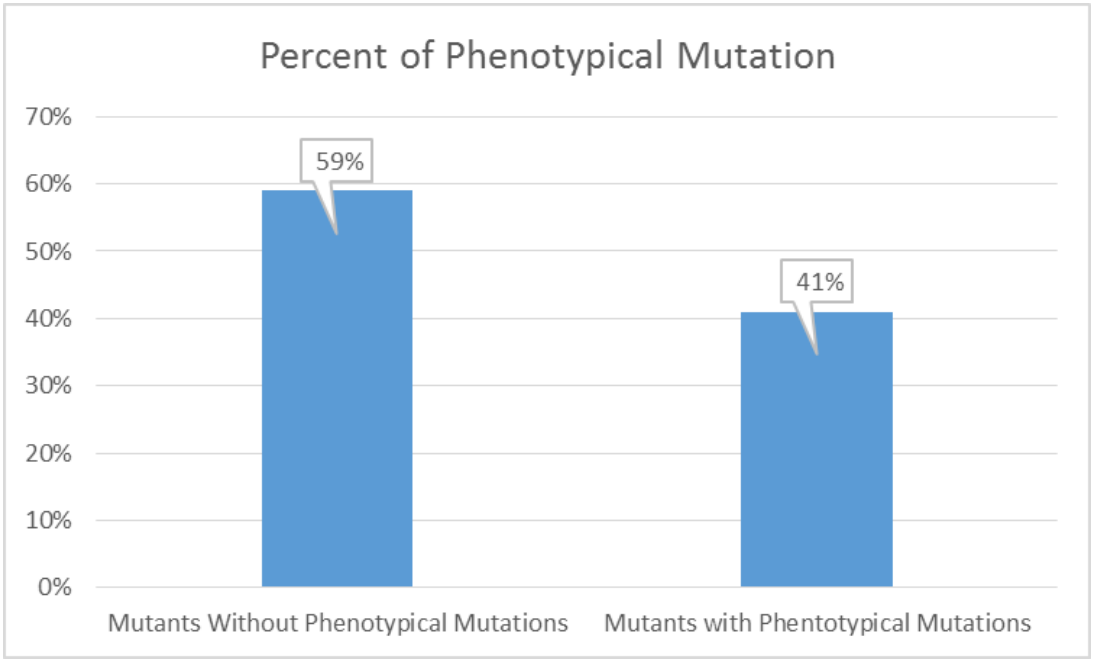

Fig. 20. Percent of phenotypical mutation from the FM3 generation consisting of 1302 mutant lines

Lateral Branching

The wild type of Forrest soybeans have one vertical stem that contains all of the reproductive parts of the plant. Once the flowers on the stem are pollinated the pods will be formed and attached to the main stem. With the phenotype of lateral branching there are lateral branches that contain reproductive parts of the plant that are not directly on the main stem. Notice in the picture that the main stem splits and there are bud produced on multiple branches. Lateral branching was observed in 6 out of the 539 mutants that showed phenotypical mutations.

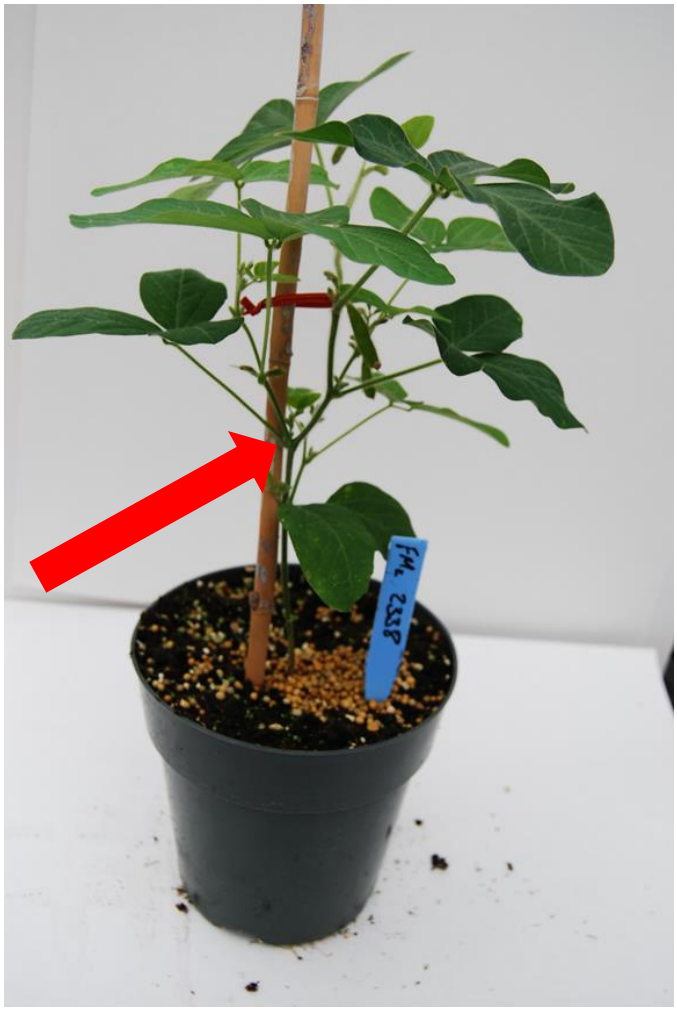

Fig. 21. Lateral Branching

Chlorotic Leaves

Chlorosis is defined as an abnormally yellow color of plant tissue. These chlorotic leaves can easily be seen in this mutant from the FM3 population. There were 13 out of the 539 mutants that were observed to have shown phenotypical mutations to have chlorotic leaves.

Fig. 22. Chlorotic Leaves

Compact Plant

I defined a compact plant by having the majority of its axillary buds compacted down within a few centimeters of each other. There were 58 mutants that showed this phenotypical mutation.

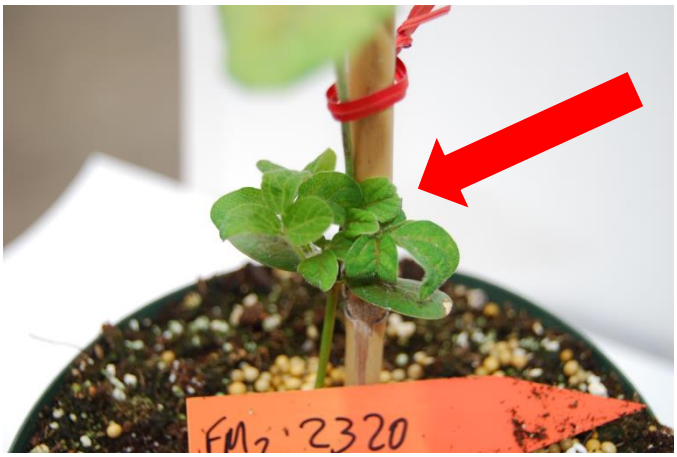

Fig. 23. Compact Plant

Dead Plant

The dead plant phenotype are plants that have completely died before physiological maturity due to the mutation process. There were 32 plants that showed this phenotypical mutation. Due to this mutation these lines were never advanced to the FM4 stage.

Fig. 24. Dead Plant

Dwarfism

Dwarfism is difined as plant much smaller than the average of its kind or species. These small plants reach physilogical maturity but usually only produce a few seeds apeice. At physilogical maturity these mutants usually one reach a hieght between 100 and 150 cm. There were 47 mutants that showed this phenotypic mutation out of the 539 mutants that showed phenotypic mutations.

Fig. 25. Dwarfism

Early Maturity

The phenotypical muation that was classified as early maturity flowered, set pods, and reached physilogical maturity before the Forrest wild type. The Forrest wily type reached full physisilogical maturity in about 128 days. When this picture was taken the Forrest wild type had not even started flowing yet. There were 33 out of the 539 mutants that showed phenotypical muations which showed signs of early maturity.

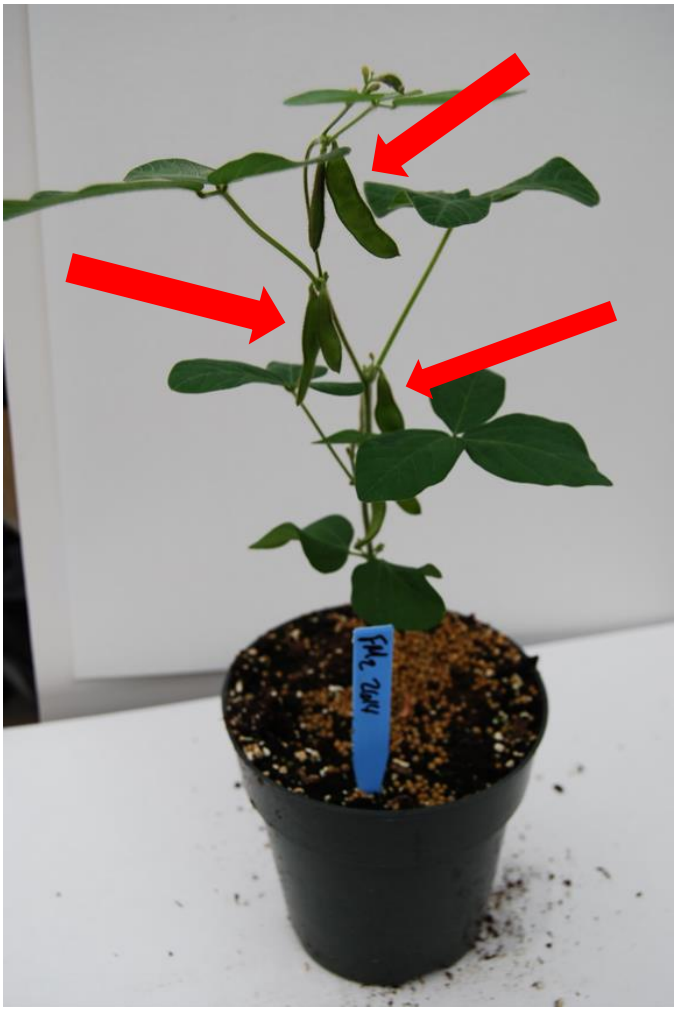

Fig. 26. Early Maturity

Leaf Color

The phenotypical mutations that were grouped together as leaf color includes all plants that showed strange leaf color that deviated from the normal dark green excluding and shade of yellow. Yellow leaves would fall under the chlorotic category. This category could has been referred to as chlorophyll deficiency or albino plants. The most common symptom that is seen in this category are white or partially white leaves. In these leaves the mutation has inhibited the development of some of the chlorophyll that makes the green color in the leaves. Where this chlorophyll is inhibited the white color on the leaves can be seen. "(EMS) was the most efficient mutagen for generating chlorophyll-deficient mutants" (Carroll et al., 1986). From the 539 mutants that showed phenotypical mutations there were 55 that had a leaf color mutation in them.

Fig. 27. Leaf Color (a)

Fig. 28. Leaf Color (b)

Leaf Shape

Mutants that fall into the leaf shape category have abnormally shaped leaves that deviate from the Forrest wild type leaf shape that is round to and broad oval. The most common shape that was recorded was a thin oval shape on either one leaf from each trifoliate or all of the leaves on the mutant. There were 38 mutants that showed this phenotype out of the 539 mutants that showed a phenotypical mutation.

Fig. 29. Leaf Shape (a)

Fig. 30. Leaf Shape (b)

Leaf Texture

The mutants that showed the phenotypical trait that was classified as leaf texture had a rough looking leaf surface. On the Forrest wild type plant the leaves are smooth and lay flat. In the phenotypical mutations the leaves are rougher and often more wrinkled and appear wavy. There were 68 mutants that feel into the leaf texture category out of the 539 total mutants that showed phenotypical mutations.

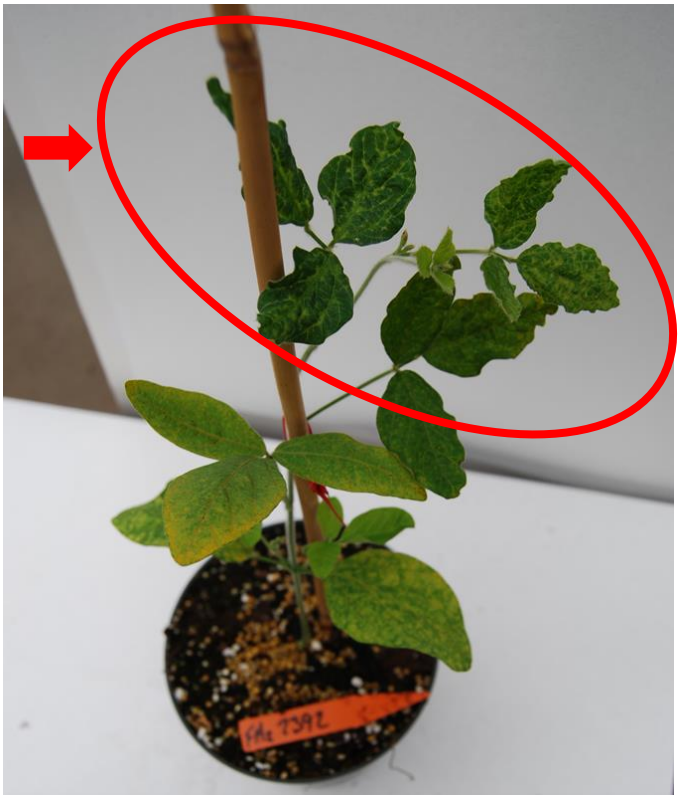

Fig. 31. Leaf Texture

Lesions on the Leaves

The mutants that showed a phenotypical trait that caused lesions on the leaves were grouped in the lesions category. These lesions often started as yellow splotches that would eventually start to desiccate in the center and turn brown. There were 55 mutants that showed the phenotypical trait for having lesions.

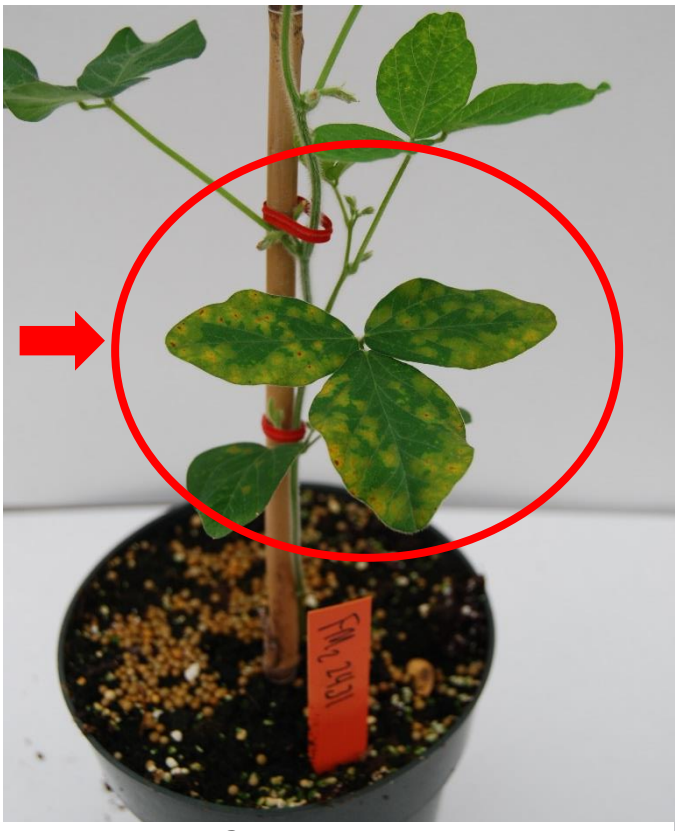

Fig. 32. Lesions on Leaves

No Pods

The mutants that fit into the phenotypical category of no pods grew to maturity but never developed pods. Because they never developed pods or seeds, they were not advanced to the FM4 population. This was the largest category with 126 mutants that did not produce pods due to the mutation process.

Fig. 33. No Pods

Vine Like

The mutants that were categorized in the vine like group of phenotypes did not grow upright without support as the Forrest wild type does. These mutants were given a stake in each pot to grow up so they did not grow across the bench and over to the next plant to grow up. There were 8 mutants from the FM3 population that showed this phenotype.


Fig. 34. Vine Like

Table 5. Percentages of phenotypical mutations observed in the FM3 population consisting of 1302 mutant lines

Phenotypical Trait	Number Observed	Total Mutant Population	Percentage
Lateral Branching	6	1302	0.46%
Chlorotic Leaves	13	1302	1.00%
Compact Plant	58	1302	4.45%
Dead Plant	32	1302	2.46%
Dwarfism	47	1302	3.61%
Early Maturity	33	1302	2.53%
Leaf Color	55	1302	4.22%
Leaf Shape	38	1302	2.92%
Leaf Texture	68	1302	5.22%
Lesions	55	1302	4.22%
No Pods	126	1302	9.68%
Vine Like	8	1302	0.61%

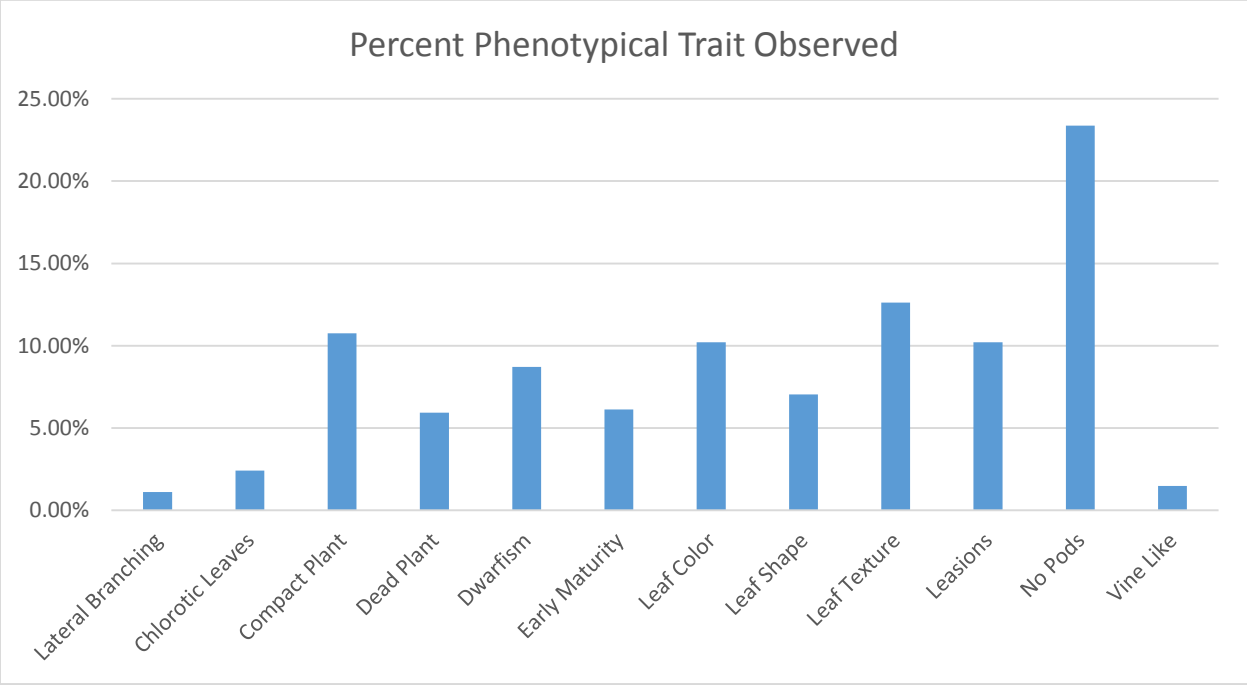

Fig. 35. Percent phenotypical trait observed from the FM3 population from the 539 observed phenotypical mutations

3.4 Fatty Acid analysis

With the population of the world on the rise the demand is becoming greater for oil for both nutrition and use as a power source. Production of Biodiesel has been a commonly discussed topic in the news recently. People are getting on board with it because it is an effective way to produce sustainable energy. One of the most crucial elements needed when making biodiesel is fatty acids extruded from soybeans, specifically Oleic Acid.

With the EMS Mutagenesis that was preformed, the percentages that the main 5 Fatty Acids are produced in were altered. Commodity soybean oil typically contains 11% palmitic acid (16:0), 4% stearic acid (18:0), 25% oleic acid (18:1), 52% linoleic acid (18:2), and 8% linolenic acid (18:3) (Fehr, 2007). From the FM3 generation that was developed, there were 96 mutants that were chosen at random to check the percent of each fatty acid that was produced. Along with these 96 samples, 8 Forrest Wild Type seeds were also tested, that were grown side by side with the mutants as a control.

From the fatty acid analysis on the Forrest Wild Type seeds they were averaged to find that on average they contained 8.8% palmitic acid (16:0), 1.5% stearic acid (18:0), 23.2% oleic acid (18:1), 65.0% linoleic acid (18:2), and 2.9% linolenic acid (18:3). These percentages of fatty acids from the Forrest Wild Type were used as a baseline to see how much the percentages of fatty acids produced in the FM3 line were altered. As an average, the FM3 line averaged 10.6% palmitic acid (16:0), 1.9% stearic acid (18:0), 13.9% oleic acid (18:1), 65.5% linoleic acid (18:2), and 6.1% linolenic acid (18:3).

On the average the percent of palmitic acid (16:0) produced was raised by 1.8% from 8.8% (FWT) to 10.6% (FM3), in the mutants when compared to the Forrest Wild Type line.

When looking at individual mutants however, there is one that produced over 30% palmitic acid. FM3 2014-2034 produced 37.14% palmitic acid. FM3 2014-2034 at 37.14% palmitic acid is a 322% increase over the average of the Forrest Wild Type.

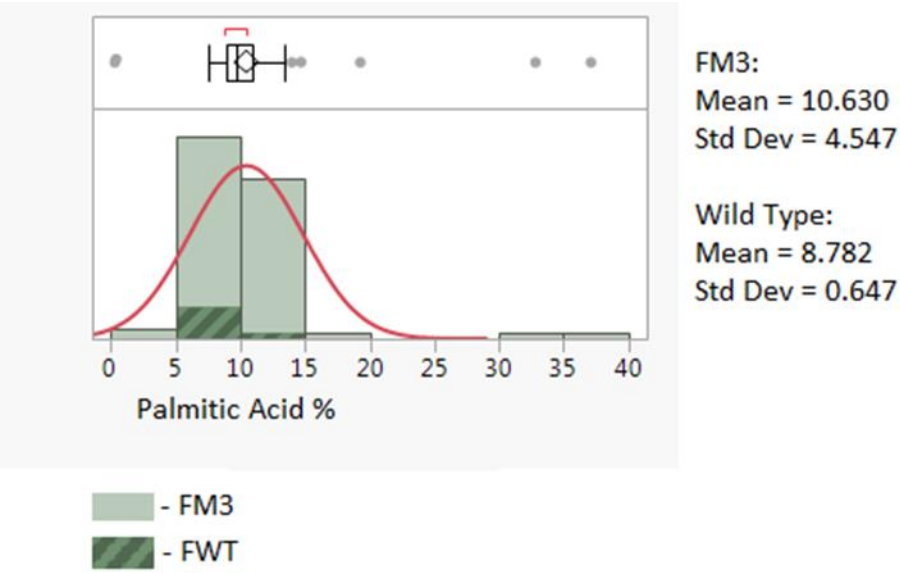

Fig. 36. Palmitic Acid Histogram from the 96 FM3 and 8 FWT samples ran for fatty acid analysis

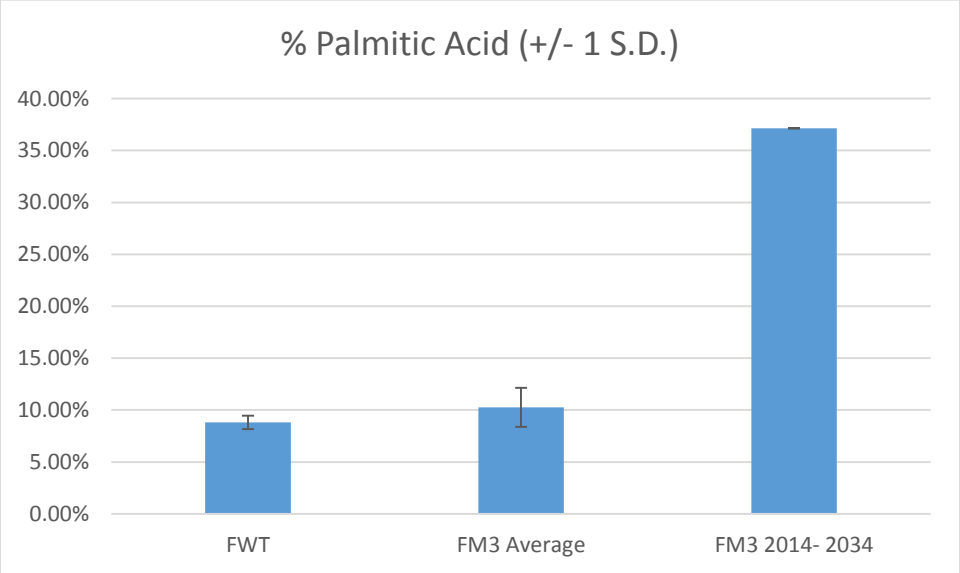

Fig. 37. Palmitic Acid bar chart showing the average percent palmitic acid from FWT and the FM3 generation, and showing the mutants with the highest percent palmitic acid from the FM3 generation

When looking at stearic acid (18:0) the FM3 line average only produced 0.4% more stearic acid than the Forrest Wild Type line. When looking at individual mutants again, FM3 2014-2034 also produced 11.0% stearic acid as well. This is a 624% increase over the Forrest Wild Type average.

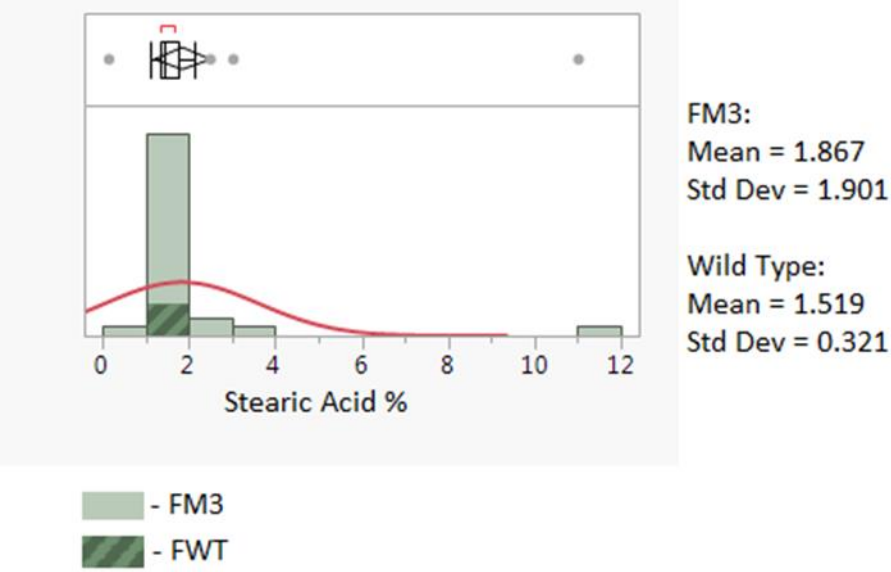

Fig. 38. Stearic Acid Histogram from the 96 FM3 and 8 FWT samples ran for fatty acid analysis

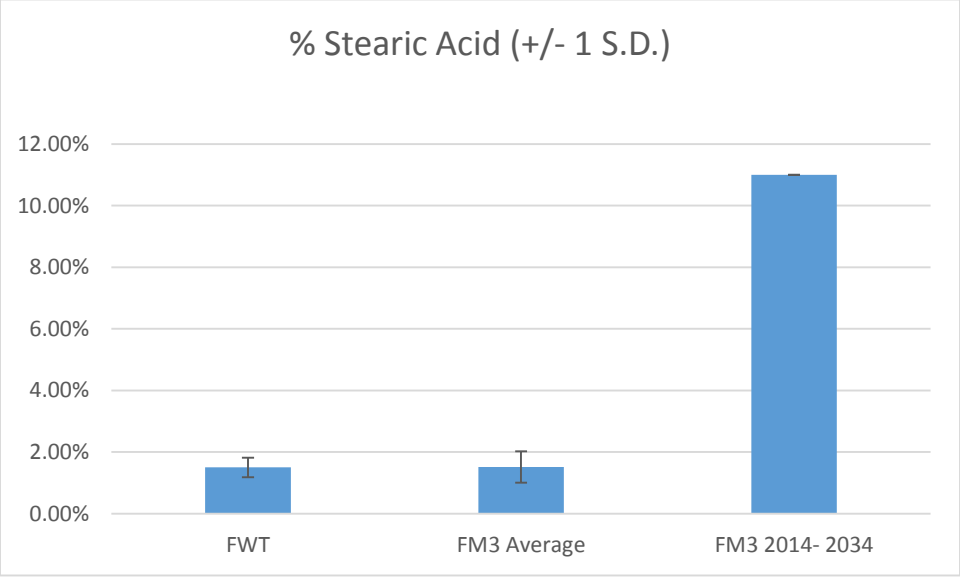

Fig. 39. Stearic Acid bar Chart showing the average percent palmitic acid from FWT and the FM3 generation, and showing the mutants with the highest percent stearic acid from the FM3 generation

For the oleic acid (18:1) which is the most important for biodiesel production the average of the FM3 population was actually 9.3% lower than the average of the Forrest Wild Type average. As individual mutants FM3 2014-2031 produced the highest percent of oleic acid at 43.26%. This is an 86% increase over the Forrest Wild Type Average.

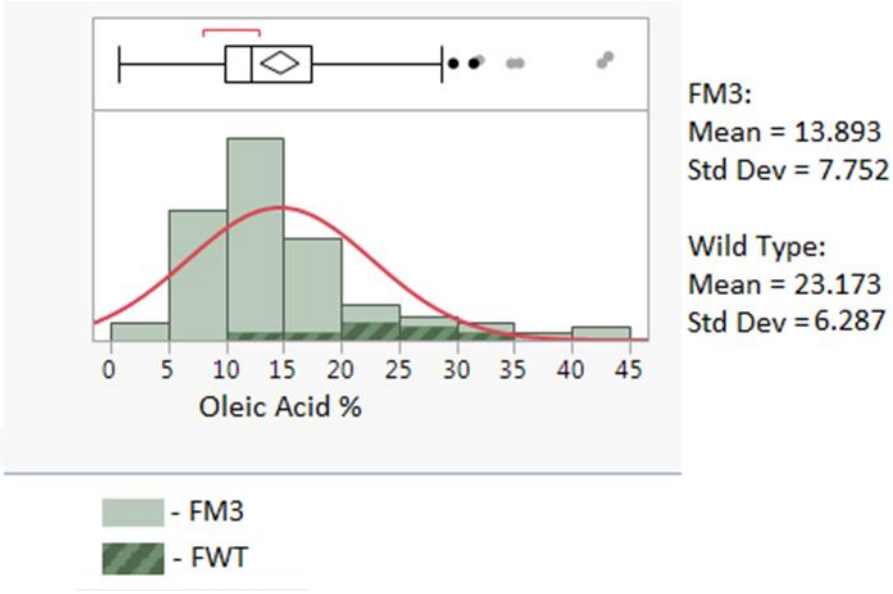

Fig. 40. Oleic Acid Histogram from the 96 FM3 and 8 FWT samples ran for fatty acid analysis

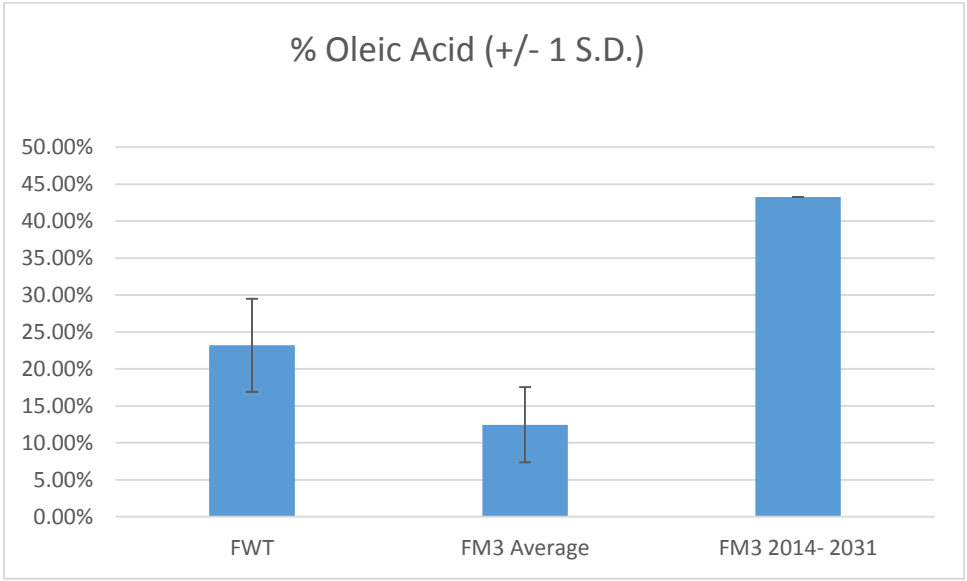

Fig. 41. Oleic Acid Bar Chart showing the average percent palmitic acid from FWT and the FM3 generation, and showing the mutants with the highest percent oleic acid from the FM3 generation

When it comes to linoleic acid (18:2) the average of the FM3 population was only 0.5% higher than the Forrest Wild Type population. There were three mutants however that produced over 80% linoleic acid. The highest of these was FM3 2014-1832 which produced 85.3% linoleic acid. This is a 31% increase over the Forrest Wild Type average.

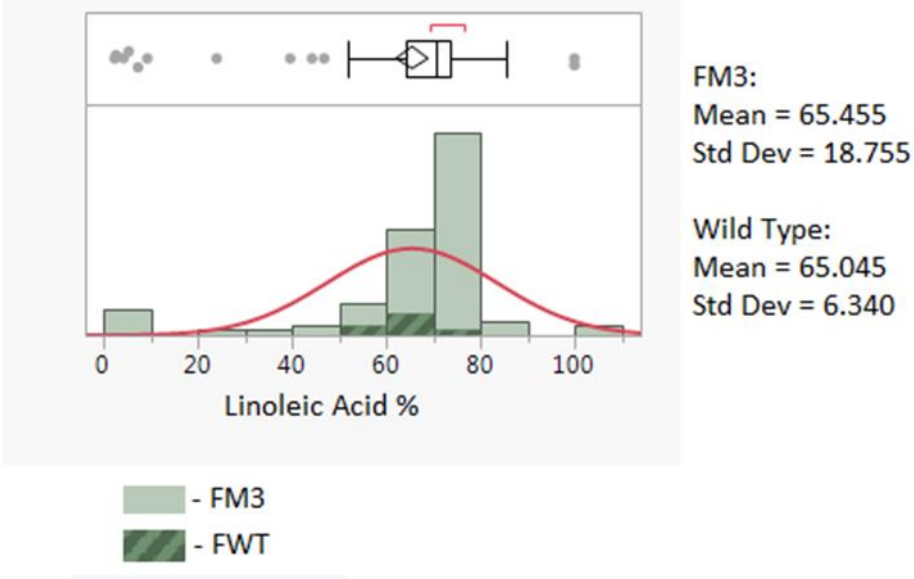

Fig. 42. Linoleic Acid Histogram from the 96 FM3 and 8 FWT samples ran for fatty acid analysis

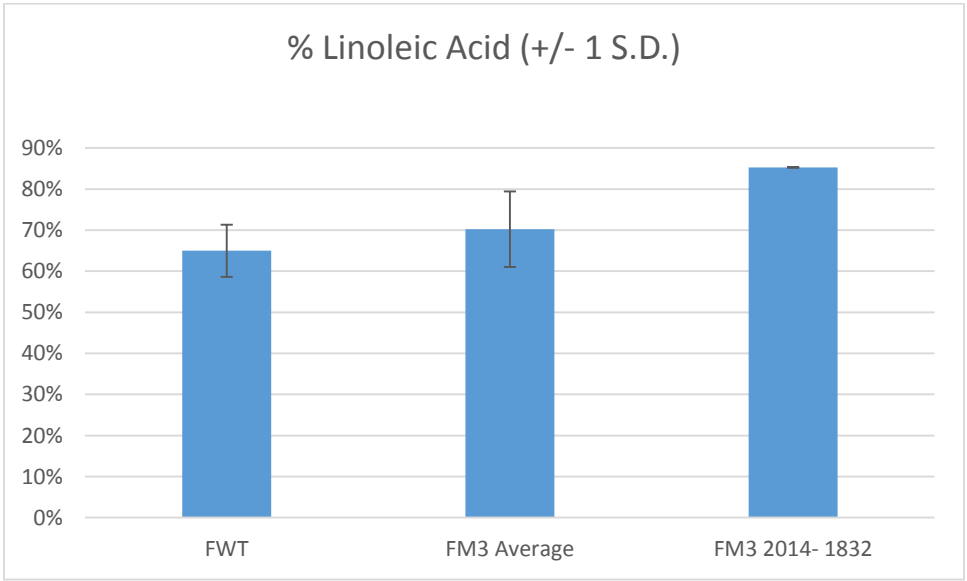

Fig. 43. Linoleic Acid Bar Chart showing the average percent palmitic acid from FWT and the FM3 generation, and showing the mutants with the highest percent linoleic acid from the FM3 generation

Lastly when comparing the amount of linolenic acid (18:3) produced in the FM3 population compared to the Forrest Wild Type population it was found that there was a 3.2% increase in the FM3 population. The mutant that produced the highest percent of linolenic acid was FM3 2014-2070 which produced 8.97% linolenic acid. This is a 204% increase when compared to the Forrest Wild Type average.

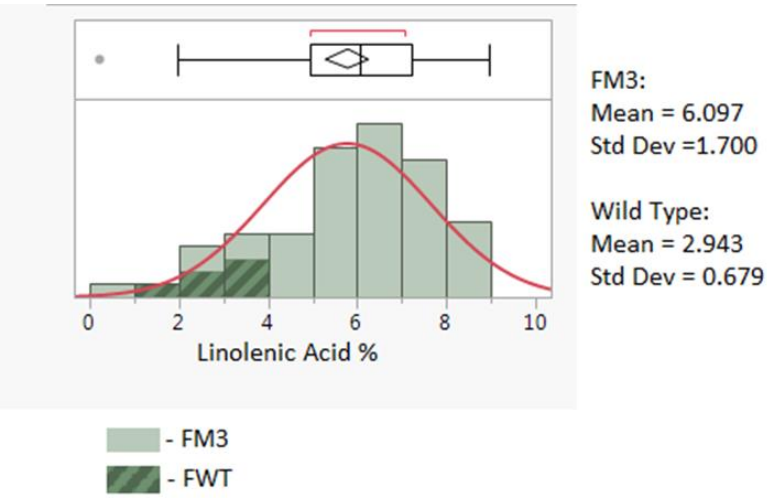

Fig. 44. Linolenic Acid Histogram from the 96 FM3 and 8 FWT samples ran for fatty acid analysis

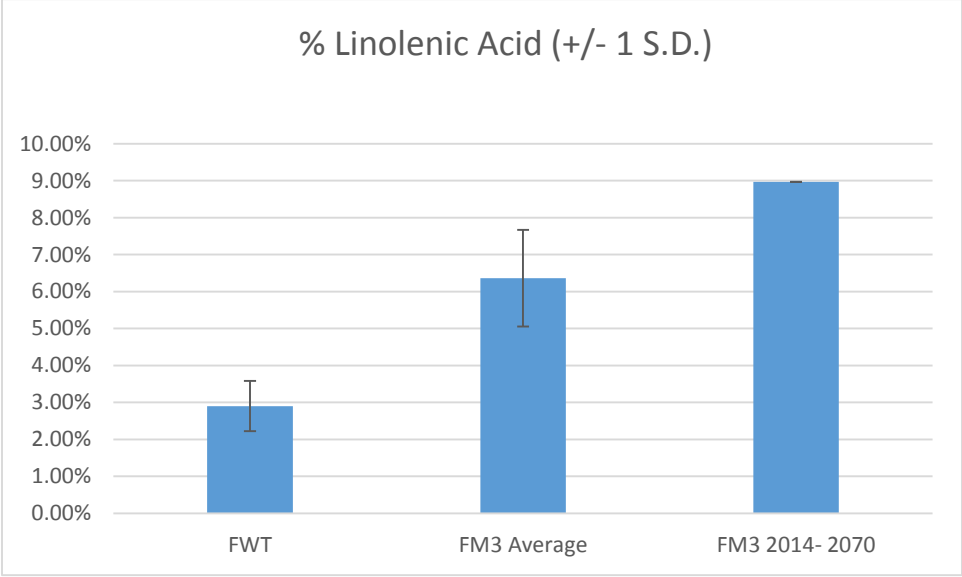

Fig. 45. Linolenic Acid Bar Chart showing the average percent palmitic acid from FWT and the FM3 generation, and showing the mutants with the highest percent linolenic acid from the FM3 generation

As a whole there are a few mutants in the FM3 population that show significant signs of increasing the percent of any of the five major fatty acids.

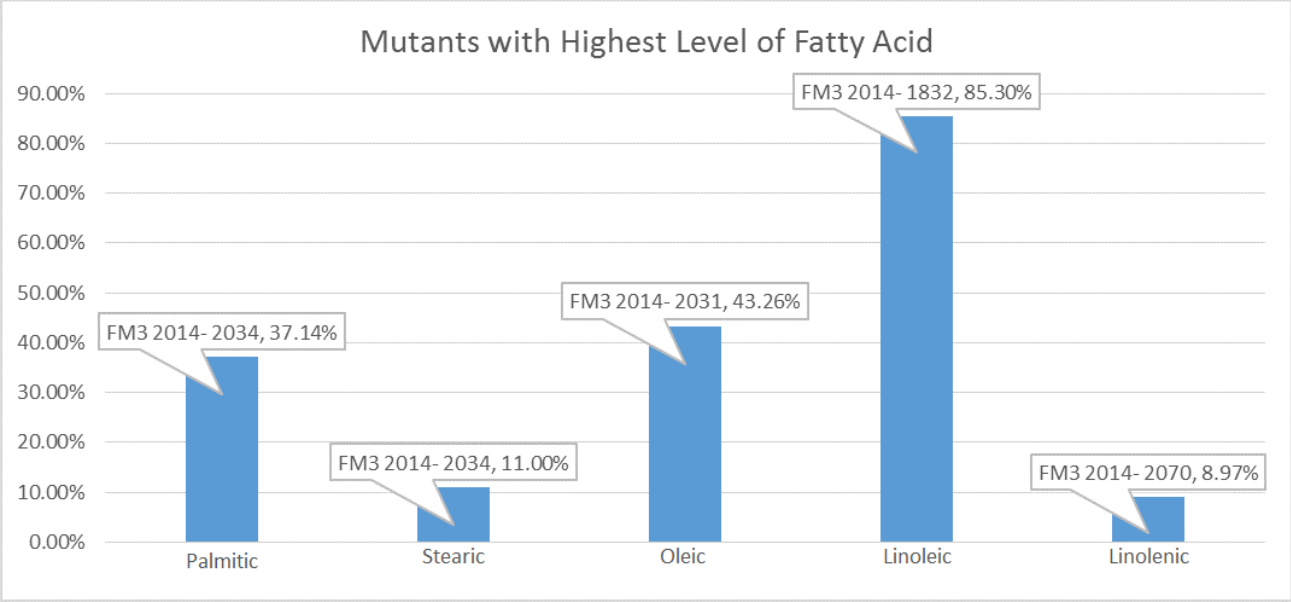

Fig. 46. Mutants with Highest Level of Fatty Acid from the 96 samples processed for fatty acid analysis from the FM3 generation

CHAPTER 4

DISCUSSION

4.1 Discussion

From the results of this current study it has been demonstrated that chemical mutagenesis using ethyl methanesulfonate (EMS) can be very useful in developing cultivars with many different agronomic traits. By using EMS mutagenesis on a large scale one can easily find benefits not only in yield but also in fatty acid content. Another benefit of using chemical mutagenesis is finding other phenotypes that have scientific interest, such as lateral branching and leaf color. All of this data demonstrates that a very high amount of genetic diversity can be produced using chemical mutagenesis.

This genetic diversity can easily be seen in even in the M1 generation. In our M1 generation there were 30.68% of the plants that showed phenotypical mutations. This is a very high percent considering there were a considerable amount that did not show phenotypical changes that were still changed genetically. This was proven both by yield data and the fatty acid analysis.

In the yield data alone there were various examples of the genetic diversity that was caused by EMS mutagenesis. The total weight of seeds produced by the highest producing Forrest Wild Type was 22.025 grams. There was one mutant that produced 83.129 grams of seed. That was a 277.4% increase over the highest producing wild type. That same mutant produced 496 seeds which was a 228.4% increase over the Forrest wild type plant that produced the highest number of seeds which produced 151 seeds. Lastly when looking at yield potential the last piece of the puzzle is seed size. When considering the weight of one seed the largest

seed size produced by Forrest Wild Type was 0.164 grams and the mutant that produced the largest weight of one seed weighed 0.317 grams. That was a 93.3% increase over the Forrest Wild Type.

Of course not all mutations are beneficial ones. There were plenty of mutants that produced significantly less yields than the Forrest Wild Type. This is one of the many reasons why it is a necessity to start with as large of a population as possible when using chemical mutagenesis. The larger the population that is stated, the greater the odds are that the specific mutation that is desired will be found

When looking at the phenotypical traits that were observed during this study it is obvious that the chemical mutagenesis had a significant effect. From the F2 generation there were 12 different phenotypical traits that could be observed. The recurrence of phenotypical traits also got more concentrated in this generation as well. There were 41.5% of these plants that showed signs of phenotypical traits.

Just like the yield data not all of the mutants showed good phenotypical traits. When dealing with chemical mutagenesis, the bad has to be taken along with the good. Some of the bad phenotypical traits included, lesions, dead plants, or sterile plants that produced no pods. But on the positive side, there were some phenotypical traits that can be very beneficial in certain instances, such as early maturity and lateral branching.

The experiment that showed the most genetic diversity from chemical mutagenesis in this study was the fatty acid analysis. The fatty acid analysis showed subtle variations in the mutant population. A plant that did not show any yield difference or phenotypical trait could still show some very significant differences when it came to the amount of different amounts of

fatty acids produced. There were quite a few mutants that showed some great potential for high oil production. The most important fatty acid that was observed in this study was Oleic acid which is crucial for biodiesel production. When looking at strictly oleic acid concentrations in the soybean the chemical mutagenesis increased one mutants by 86% over the Forrest Wild Type average. There were some fatty acids that were increased by much more. For instance, there was one mutants that increased its Stearic acid contents by 624% over the Forrest Wild Type average.

From these examples it has been demonstrated how effective chemical mutagenesis is when done on a population starting from 8000 seeds. The genetic diversity that can be obtained in a few growing seasons is one of the largest benefits of chemical mutagenesis.

4.2 Summary

- It has been demonstrated that chemical mutagenesis using ethyl methansulfonate as a mutagen can be very useful in developing cultivars with many different agronomic traits.
- It can be seen through yield data, phenotypical traits, and the fatty acid analysis that was conducted.
- The yield data results could be of interest to researchers' looking to make higher yielding soybeans.
- Some beneficial phonotypical mutations that were observed could be of scientific interest, such as lateral branching and early maturity.
- Lastly, the results from the fatty acid analysis showed that any of the 5 major fatty acids could be raised through chemical mutagenesis.
- The objective of this research was to isolate a mutated line of Forrest soybeans with elevated Oleic Acid content
    - With the mutant line FM3 2014-2031 producing 43.26% Oleic Acid, the concentration has almost doubled from the Forrest wild type of 23.17%

4.3 Future Work

- The first experiment that would be done if this project were to go further would be to run a SCN screening on all of the promising mutant lines to insure that the SCN resistance from the Forrest line was not altered.
- The next experiment that would be conducted would be to cross FM3 2014-2031 with a high yielding line to make a new variety that is high yielding and produces an elevated about of Oleic Acid.
- The last experiment that would be conducted would be to cross the high yielding mutant FM2-363 with a high yielding line or another SCN resistant variety to make a new cultivar that is high yielding with great SCN resistance.

REFERENCES

Ainsworth, Elizabeth A., et al. "Accelerating Yield Potential in Soybean: Potential Targets for Biotechnological Improvement." Plant, Cell & Environment 35.1: (2012): 38-52.

Alawad, Alaa. "Development of Chemically Mutagenized Soybean Populations for Improving Soybean Seed Oil Content and Forward and Reverse Genetics Screening": (2014): 1-72. MS Thesis, Southern Illinois University Carbondale.Print

Carroll, B.J., McNeil, D.L. & Gresshoff, P.M. "Mutagenesis of soybean (*Glycine max* (L.) Merr.) and the isolation of non-nodulating mutants": (1986): 109-114.

Fehr, W. R. "Breeding for modified fatty acid composition in soybean". Crop Science, (2007): S-72.

Graef, George, Bradley J. Lavallee, Patrick Tenopir, Mustafa Tat, Bruce Schweiger, Anthony J. Kinney, Jon H. Van Gerpen, and Tom E. Clemente. "A High-oleic-acid and Low-palmitic-acid Soybean: Agronomic Performance and Evaluation as a Feedstock for Biodiesel." *Plant Biotechnology Journal*: (2009): 411-21. Print.

Heffner, E. L., Sorrells, M. E., & Jannink, J. L. "Genomic selection for crop improvement". Crop Science: (2009): 49(1), 1-12.

Huang, E. "Development of Chemically Mutagenized Soybean Populations for Forward and Reverse Genetics Analyses": (2009): MS Thesis, Southern Illinois University Carbondale. Print.

Kavithamani, D., Kalamani, A., Vanniarajan, C., & Uma, D. "Development of new vegetable soybean (Glycine max L. Merill) mutants with high protein and less fibre content". Electronic Journal of Plant Breeding: (2010): 1060-1065.

Kinney, A.j., and T.e. Clemente. "Modifying Soybean Oil for Enhanced Performance in Biodiesel Blends." *Fuel Processing Technology*: (2005):1137-147. Print.

Meksem, K., Liu, S., Liu, X., Jamai, A., Mitchum, M.G., Bendahmane, A., et al. (2008). "TILLING: A Reverse Genetics and a Functional Genomics Tool in Soybean". In Guenter Kahl & Khalid Meksem (Eds.), The Handbook of Plant Functional Genomics: Concepts and Protocols: (2008): 251-266.

National Agriculture Statistics Service <www.nass.usda.gov> Web. 29 May 2015.

SoyStats®. “SoyStats”. <http://soystats.com/>. Web. 29 May 2015.

SoyStats®. “Biodiesel: U.S. Production History”. <http://soystats.com/biodiesel-u-s-production-history/>. Web. 29 May 2015.

SoyStats®. “International: World Soybean Production”. <http://soystats.com/international-world-soybean-production>. Web. 29 May 2015.

SoyStats®. “Soybean Oil: U.S. Vegetable Oils Consumption”. <http://soystats.com/soybean-oil-u-s-vegetable-oils-consumption/>. Web. 29 May 2015.

SoyStats®. ”U.S. Yield & Production: Yield History”. <www.soystats.com/u-s-yield-production-history/>. Web. 29 May 2015.

SoyStats®. “Value – Prices paid to Farmers History”. <http://soystats.com/value-prices-paid-to-farmers-history/>. Web. 29 May 2015.

Swoboda, Catherine Margaret, "Effect of agronomic practices on sudden death syndrome of soybean in Iowa": (2010): Graduate Theses and Dissertations. Paper 11559.

United Soybean Board. <http://www.soybeanunited.org>. Web. 16 May 2015.

Van, K., Kim, M. Y., Jun, T. H., & Lee, S. H. “Analysis of EMS Mutagenized Soybean by Combination of DOP-PCR and GS-FLX”: (2000): 453.

Wrather JA and SR Koenning “Estimates of disease effects on soybean yields in the United States 2003 to 2005”: (2006): 173–180.

Wrather, J.A., S.R. Koenning, and T.R. Anderson. “Effect of diseases on soybean yields in the United States and Ontario (1999-2002)”. Plant Health Progress: (2003).

Wrather JA, TR Anderson, DM Arsyad, Y Tan, LD Ploper, A Porta-Puglia, HH Ram, and JT Yorinori “Soybean disease loss estimates for the top ten soybean producing countries in 1998”: (2001): 115–121.

Zhou, Zhou. "Developing Chemically Mutagenized Soybean Populations for Functional Gene Analysis at the RHG1 Locus": (2013): 1-77. MS Thesis, Southern Illinois University Carbondale Print.

VITA

Graduate School
Southern Illinois University

Joshua W. Gunther

jgunther2015@gmail.com

Southern Illinois University Carbondale
Bachelor of Science, Crop Science & Environmental Management, May 2014

Thesis:

DEVELOPING CHEMICALLY MUTAGENIZED EMS FORREST SOYBEAN POPULATION FOR HIGH OIL PROFILE

Major Professor: Dr. Khalid Meksem