Increasing soybean yield under drought through enhanced symbiotic nitrogen fixation (2024)
Principal Investigator: Carrie Miranda, PhD
Coprincipal Investigator Barney Geddes, PhD

Technical report

a. Title: Increasing soybean yield under drought through enhanced symbiotic nitrogen fixation (2024)
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b. Research Overview and Objectives:

Research Overview: Drought is a major cause of soybean yield loss. Western North Dakota is characterized by low water or drought-like conditions, with soybean yields suffering as a result. While there may be multiple mechanisms to enhance drought tolerance in soybean, many have a negative effect on agronomic performance and yield during well-watered conditions compared to drought susceptible varieties. Interestingly, it has been shown that a greater ability to sustain symbiotic nitrogen fixation (SNF) under drought conditions improves soybean yield by 85%. The ability to sustain SNF in soybeans has been shown to have a genetic basis, and this trait has been observed in U.S. germplasm collections and is used in some breeding programs. Our long-term aim is to incorporate sustained SNF into NDSU varieties to improve yield in drought conditions. To take the first steps towards this, we will first investigate the ability to sustain SNF under drought in existing advanced NDSU germplasm. In addition, we will begin incorporating well characterized germplasm that has been shown to have an elite ability to sustain SNF under drought into the NDSU breeding program.

Objectives:
- Objective 1: Screen for the ability to sustain SNF under drought in NDSU advanced breeding lines.
- Objective 2: Initiate breeding populations using known germplasm with high drought SNF.

c. Materials and Methods:

Objective 1: Conduct field and greenhouse screens of NDSU soybean breeding lines that are entered into 3rd and 4th year yield trials to find experimental lines that may have sustained symbiotic nitrogen fixation.

Field experiment:

In 2023, 3rd and 4th year yield trials were planted in dryland and irrigated locations at Nesson Valley, ND near to Williston, ND. Thirty experimental lines (genotypes) plus two positive controls and one negative control were planted in randomized complete block design with 3 reps each. The trials were planted May 13th, 2023. Plots were solid seeded, 7 rows per plot by 15 feet. Plants were maintained with normal field conditions for the yield trials. At growth stage R2-3, on July 18th, 2023, 10 plants from each plot were sampled from every rep in both dryland and irrigated treatments. The root systems from all plants were dug to ensure capturing as much root biomass as possible. Dirt clods were carefully shaken out from roots. Above ground biomass and the root system were separated with sheers where the dirt line ends on the stem. The root and shoots were bagged separately, in plastic and paper bags respectively. All samples were transported to Fargo, ND in a refrigerated truck for preservation. Upon arrival at the NDSU campus, shoot biomass was placed in driers for desiccation and the root systems were moved to a working room to begin sample processing. Phenotyping for the field study included:

1. Nodule counts, wet weight and dry weight- All nodules were removed from each plant, counted and immediately weighed to calculate nodule counts and wet weight. Removed nodules were

then placed in a dryer until weights plateaued to record dry weight. Results from 10 plants from each plot were averaged to give one measurement per genotype per plot per treatment.

2. 15N analysis- Shoot biomass was examined for recoding growth stage, dried, and ground using a Wiley mill grinder. Samples were prepared in the lab of Dr. Kelsey Greisheim and 15N analysis was performed at the University of Illinois. Results were reported in May 2024.

Greenhouse experiment: Based on the results of nodule counts from the field experiment, a subset of lines from the 2023 AYTs was selected based on highest and lowest values of nodule count/dry weight. This was due to space constraints in the greenhouse. Entries were:

Entry	Name	Purpose
1	R01-581F (no inoculant)	Negative control
2	ND Benson (no inoculant)	Negative control
3	R01-416F	Positive control
4	R01-581F	Positive control
5	ND Benson	Experimental (High nodule ratio)
6	ND14-6120(GT)	Experimental (Low nodule ratio)

Watering treatments were:

DT1	Well watered (80%)
DT2	Moderate drought (55%)
DT3	Heavy drought (30%)
DT4	No water

~650 lbs of sand were autoclaved to prevent contamination. 138 1-gallon buckets were weighed for an empty weight measurement. They were then prepared by being filled with autoclaved sand and then watered until sand was fully saturated, once the pots were no longer dripping water the pot was considered to be at 100% well watered conditions. This weight was recorded and used to determine the correct weight/ amount of water necessary to add for each water treatment. Pots were then labeled and 5 seeds of each entry were planted in a single pots per water treatment. At the time of planting, seeds were inoculated with *Rhizobium* inoculant provided by the Geddes lab except in the negative controls which had a non-inoculated treatment. One additional pot per entry/genotype, was grown in a well watered treatment with nitrogen. Five replications per genotype and treatment were planted for a total of 120 plots + 6 well watered with nitrogen controls. Pot layout in the greenhouse was in split plot design, where each watering treatment for each rep was a block and pots were randomized within the treatment block. All pots were maintained as well watered with a nutrient solution containing nitrogen for two weeks to allow for healthy plant development. After emergence, pots were thinned to 2 plants per pot to ensure uniformity. Once plants reached the V2 stage of development, the 120 experimental pots were watered according to their watering treatment with a nutrient solution with nitrogen. The 6 control pots were maintained as well watered with nitrogen nutrient solution. Pots were weighed daily and watered as necessary to maintain their water treatment for two weeks. At the end of two weeks, the experimental was taken down to start data analysis. Roots and shoots were separated as described above. The phenotyping was as follows:

1. Nodules counts and weights (dry/wet)- same as described above

2. Acetylene reduction assay (roots)- This analysis was performed in the Geddes lab using an established protocol to determine the amount of nitrogen fixation by measuring the reduction of acetylene to ethylene.
3. 15N analysis- due to the prohibitive cost of the analysis and the difficulty in sample preparation, it was decided to postpone this analysis until a small, refined sample set was ready for nitrogen fixation validation

Objective 2: Two experimental lines were acquired from University of Arkansas with known sustained symbiotic nitrogen fixation capabilities and high yield potential: R01-581F and R01-416F. These lines are maturity group V and crossing to ND lines must be conducted in growth chambers to control daylength and allow synchronization of flowering times for pollination. Four high yield North Dakota lines with disease resistance were selected as parents. These lines were grown in a growth chamber with 16 hour daylength. The two Arkansas lines were grown in a growth chamber with 13 hour daylength. For the first generation of this population development, we successfully synced pollination for 3 cross combinations: ND21-16321 x R01-581F, ND20-14884 x R01-581F, and ND20-14884 x R01-416F. Crosses were initiated in April 2023, F_1 seed harvested and replanted in July 2023, and F_2 seed was harvested in October 2023. In November 2023, F_2 seed was shipped to Costa Rica Seeds in Upala, Costa Rica to expediate generation advancement. The F_2 seed was planted and harvested as F_3 seed and replanted in February 2024. F_4 seed was harvested in May 2024 and replanted as F_5 seed in June 2024. These plants will be single plant threshed and sent back to North Dakota for greenhouse genotyping and phenotyping in winter 2025.

d. Research Results

Objective 1:
Nodule data: It was determined with literature research, Barney Geddes's experience, and consultant advice from Dr. Felix Fritschi from University of Missouri that the nodule dry weight data and dry weight/nodule number were important metrics for analyzing nodule data. Figure 1a. shows a histogram of nodule dry weight data from the Nesson Valley dryland field 2023 data set with positive (R01-581F Arkansas line) and negative controls (non nodulating dicot weed) and the Benson line with the highest nodule weight. Figure 1b. shows dry weight/nodule number with the positive and negative controls (same as Figure 1a.) the ND18-22422(GT) line with the highest ratio. Benson also is in a higher category in the nodule ratio data analysis and that was labeled too.

Figure 1a.

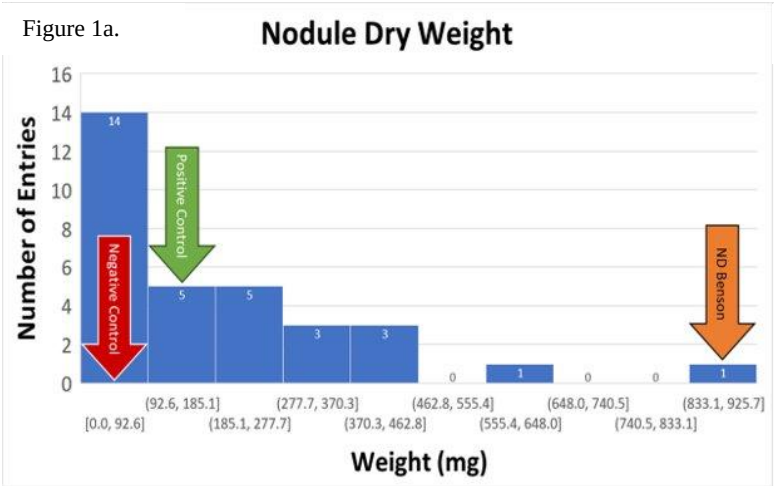

Figure 1b.

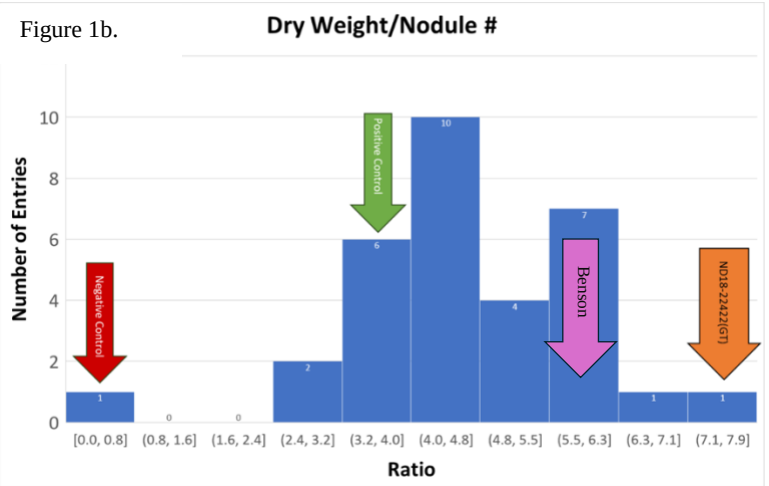

Figure 1: Nodule data collected from Nesson Valley dryland field trials in 2023. Figure 1a: Histogram of nodule dry weight data. Means of nodule dry weight data for 32 entries shown on the x axis as ranges of means. Y axis shows number of individuals in each mean weight range. Negative control (non nodulating dicot weed) and positive control (R01-581F) and highest weighing line (Benson) are highlighted with arrows. Figure 1b. Histogram of nodule dry weight/nodule number. Means of the ratio are shown on the x axis as ranges and number of individuals in each mean range are shown on the y axis. The line with the highest ratio (ND18-22422GT) is highlighted along with controls and Benson.

15N data for the shoot biomass from the Nesson Valley 2023 field trials were received March-May 2023. The dried biomass of 10 plants per plot were bulked, ground together and analyzed as two subsamples per plot/rep. One of the important results from the 15N analysis is the Delta 15N value, where a lower value correlates to nitrogen fixation from atmospheric nitrogen which suggests fixation due to symbiosis. This analysis is very sensitive when done without N isotope supplementation, which is very difficult to complete in a field setting and we opted out of supplementation for these first field trials. Filtering for data quality with CV%s less than 30% resulted in 32 lines of useful data out of 96 potential plots of data for the dryland field site. The irrigated field site had 86 useful lines of data out of 96. Means across reps were generated for filtered data, CV% were generated across reps and data was discarded that was above 30% which left 16 entries was useable data plus the two controls. Due to the unbalanced data, statistical analysis of variance was not completed. Results are in Figure 2. Figure 2a is Nesson Valley 2023 dryland data and Figure 2b is Nesson Valley 2023 irrigated data.

Figure 2a.

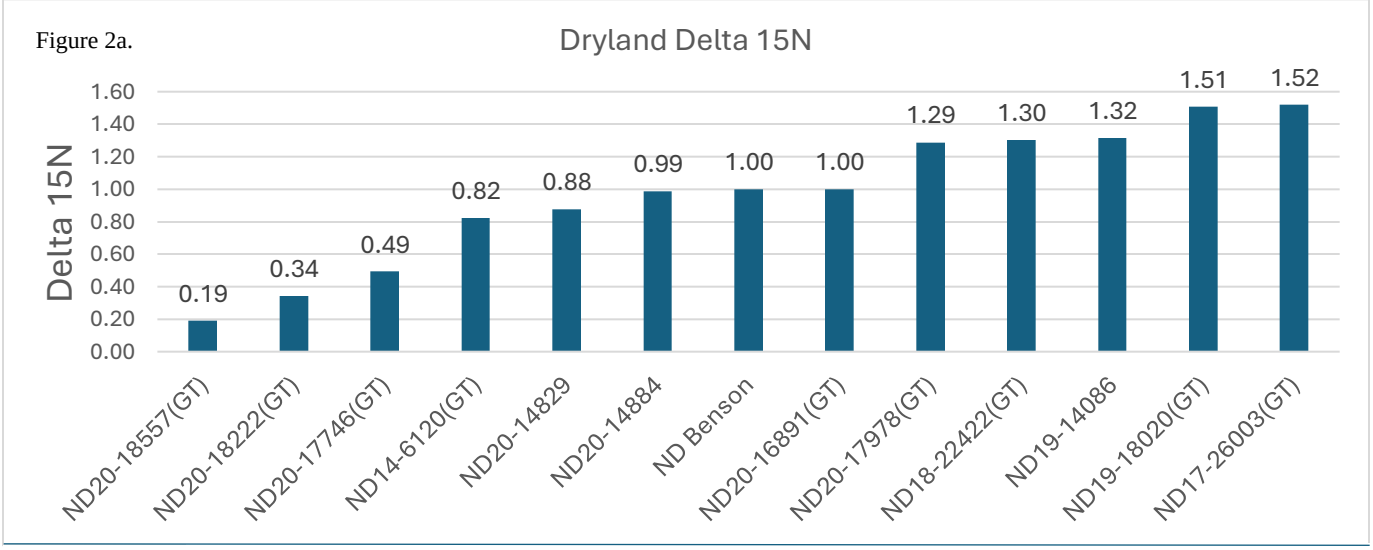

Figure 2b.

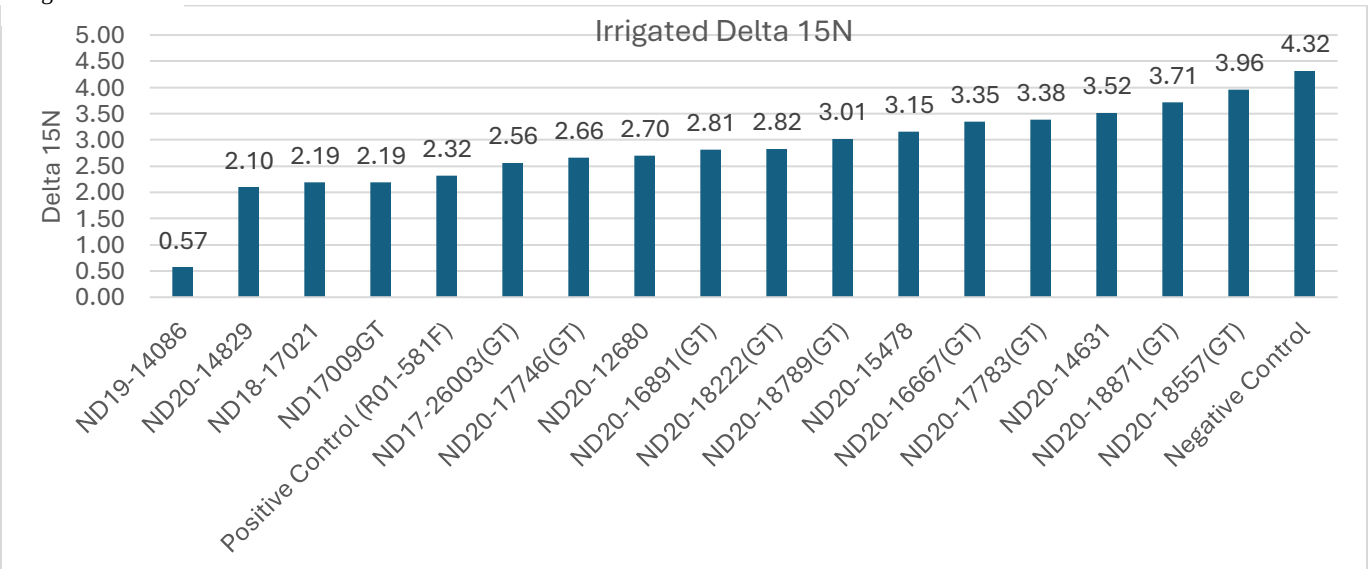

Figure 2. Delta15N analysis of shoot biomass from Nesson Valley trials in 2023. Figure 2a. Dryland trial delta15N. Figure 2b. Irrigated trial delta N15 data.

Greenhouse results

Greenhouse trials were evaluated in April 2024 for nodule count and weight and acetylene reduction. Nodule counts were averaged and are shown in Figure 3.

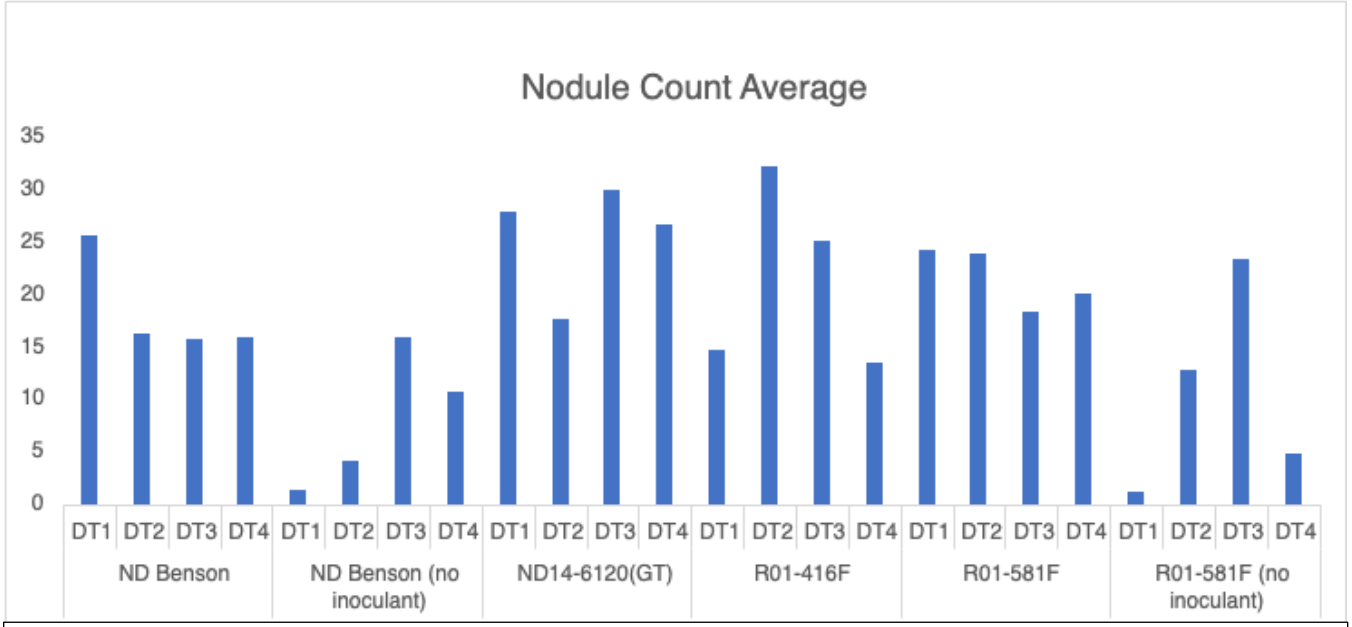

Figure 3. Mean nodule counts for genotypes under various water treatments. DT1= well-water 80%, DT2= moderate drought 55%, DT3= heavy drought 30%, DT4 = no water

Acetylene reduction assay measures the reduction of acetylene to ethylene and are shown as the sum of the ethylene area. This is typical assay used for determining nitrogen fixation due to symbiosis. Results are found in Figure 4.

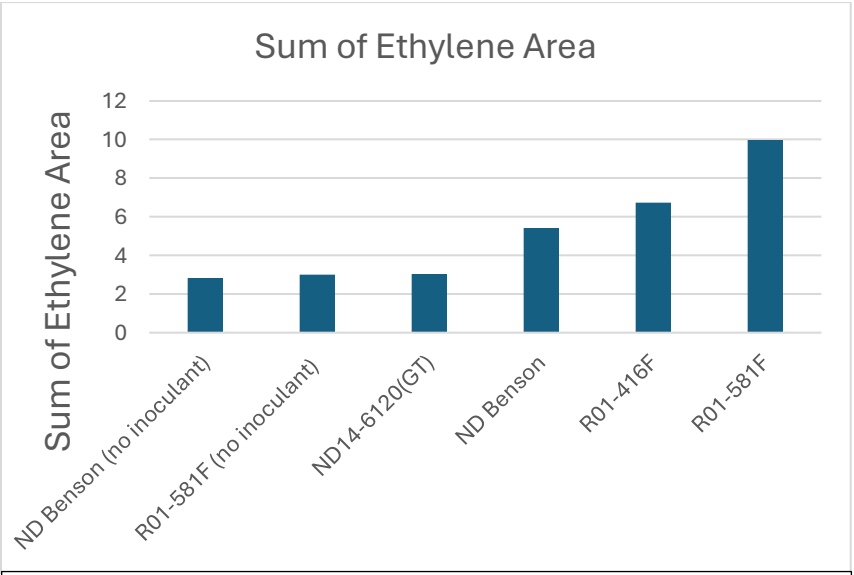

Figure 4. Sum of ethylene area after acetylene reduction assay.

e. Listings of inventions or variety releases

None

f. Discussion:

Breeding for drought resilient soybean, or any crop, is one of the greatest challenges in modern breeding. However, it is a very desirable trait for western North Dakota that is characterized by low rainfall. Sustained symbiotic nitrogen fixation (SNF) is one mechanism that can be used to create drought resiliency. Symbiotic nitrogen fixation is one of the first traits lost in drought conditions. Varieties that have been bred to possess the sustained SNF trait have less yield loss than those without. Further research has shown that there is no yield drag associated to this trait either. Breeding efforts have been started to introgress the sustained SNF trait into NDSU germplasm. Several challenges arise however, 1) this trait is primarily studied in Arkansas and southern breeding program which are around Maturity Group V) there is not a well developed phenotyping protocol to accurately and easily detect SNF in field conditions 3) the genetic mechanisms controlling the SNF trait is unknown, so markers do not exist for detection. The goal of this project is to start the trait introgression process while also developing phenotyping protocols for accurate detection of SNF in both a greenhouse and field setting.

Due to the timing of funding initiation, we started phenotyping the field trials first in summer 2023. There are a limited number of phenotyping methods available for SNF in the field so we analyzed nodule count and the nodule dry weight/nodule count ratio. This method is labor intensive but relatively easy with little chance for error. Our results of our nodule analysis suggest that there are potential experimental lines within the NDSU that may have an ability to sustain SNF in low water conditions as they outperformed the positive control from the Arkansas breeding program that was released as a variety known to have the SNF trait. However it is unclear how closely nodule data results correlate to SNF, and further analysis is needed.

15N analysis is also used to detect the amount of nitrogen fixation derived from atmospheric versus soil nitrogen. Although sample collection and preparation are easier than nodule collection, there is a lot of room for error which made the data more difficult to interpret. In addition, the cost for this analysis can be prohibitively expensive in the early stages of screening for SNF. We had to discard a large percentage of our collected 15N field data due to statistical irregularities of the delta 15N which make the resulting useful data difficult to interpret. For example, the two most successful lines from the nodule analysis were excluded from the final data results of the 15N analysis due to bad data. It was decided to postpone this analysis until breeding lines that we believe have the sustained SNF trait need to be validated for potential variety release.

In the greenhouse, we are able to control our environment and apply specific watering treatments to understand the amount of drought severity the sustained SNF is capable of overcoming. It also gives us more opportunity to optimize our protocols for detecting the SNF trait. Interestingly, nodule count data results were not consistent with field results. Benson was selected for greenhouse analysis because of its high nodule count in the field, which is still true here, but ND14-6120GT was selected because of its low nodule count in the field but in this experiment, the nodule counts are higher than Bensons.

In addition, we are able to use another typical assay for SNF detection, an acetylene reduction assay. This method is how the sustained SNF trait was researched historically. However, since the method can only be used in a controlled environment, such as a greenhouse, there exists skepticism how the sustained SNF trait performs in a field when grown in an uncontrollable environment such as a

typical farmer's field. However, this assay could be used to confirm the usefulness of an assay that can be used in a field. This assay requires use of a gas chromatograph which can be a finicky instrument. We had issues with the instrument not recording data, and lost about 1/3 of our replicated samples, making statistical analysis difficult. The resulting data confirm our hypothesis however, that the negative controls will have the lowest SNF, followed by ND14-6120 GT which was selected due to its low nodule count in the field, then Benson, and finally the lines with the highest amount of nitrogen fixation were the positive controls. These results are across all watering treatments.

Trait introgression of the sustained SNF trait from Arkansas cultivars is one the correct timeline as proposed. Three populations have been successfully initiated and F5 seed will be received in North Dakota by winter 2024 to begin greenhouse phenotyping and genotyping of these lines for sustained SNF phenotyping, and marker development for future breeding efforts. More populations are being initiated and more successful crosses are possible as the growth chamber pollination pipeline becomes further optimized.

g. Conclusion

Drought research is not an easy feat; however we have made significant progress this past funding year both with initiating new breeding populations and optimizing phenotyping protocols for the sustained symbiotic nitrogen fixation trait. Dr. Geddes and I feel confident that in the following year of this project, we will complete troubleshooting the phenotyping component of this project so that we can successfully identify the sustained SNF trait in our experimental breeding lines in winter 2024/25. This will allow us to start field trials in 2025. Once these breeding lines are confirmed for correct maturity to North Dakota and possessing the sustained SNF trait, yield testing and the discussion for variety release is around the corner. This research could be hugely impactful for growers in western North Dakota to give a yield boost in non irrigated soybean production areas.