Final report for North Dakota Soybean Council (July 1, 2023 to June 30, 2024)

a. Research Project Title, Principal and Co-Investigators

Title: Understanding How Fusarium Affects Soybean in North Dakota and Development of Disease Management Strategies

Principal Investigator: Febina Mathew (North Dakota State University, Department of Plant Pathology)

Co-Investigators: Richard Webster, Samuel Markell, Carrie Miranda, Guiping Yan, Thomas Baldwin, and Joao Paulo Flores

b. Research Overview and Objectives

Seedling diseases, caused by *Fusarium*, can be a major problem in U.S. soybean production. In North Dakota, sudden death syndrome (SDS), which is caused by *Fusarium virguliforme*, was confirmed in 2020, and two other species of *Fusarium*, *F. solani,* and *F. tricinctum*, have been implicated in causing root rot. The ND soybean farmers have limited options to manage *Fusarium* diseases, e.g. varieties with tolerance to *F. virguliforme*. Currently, the distribution of *Fusarium* species (including SDS) and other seedling organisms such as species of *Rhizoctonia* and *Pythium* in ND is not understood. Additionally, Fusarium diseases may be showing up in fields where soybean cyst nematode (SCN) may be present. Thus, in the proposed study, we developed these objectives: (1) Characterize the species distribution of *Fusarium* associated with soybean; (2) Characterize the pathogenicity of *Fusarium* species; (3) Determine how the presence of SCN can affect the development of SDS; and (4) Determine the impact of seed treatment on *Fusarium*. The information obtained from this study will complement NDSU's efforts to help and educate farmers to manage SCN, SDS, and other *Fusarium* diseases with effective disease management strategies.

c. Materials and Methods

Objective 1. Characterize the species distribution of *Fusarium* associated with soybean (Mathew, Webster)

A soybean disease survey was conducted across 30 counties (3-5 fields per county, Fig. 1) at the vegetative (May – June) or reproductive growth stages (August – September) of soybeans to collect soil samples and infected plant samples. For fungal isolation, about 20 grams of homogenized soil from each field was moistened to 60 to 70 percent and baited with a single 7-day-old Barnes seedling. For each field, six replications were maintained. After 10 days of incubation, the infected roots were surface sterilized in 0.5 % bleach solution and 70 % ethanol for 30 sec, rinsed 2 times in sterile distilled water, and blotted dry. Root pieces were placed in antibiotic-amended (0.06% streptomycin sulfate) half-strength potato dextrose agar (PDA) media at 24± 2°C for 14 days under diffused light conditions. The fungal isolates were purified

by hyphal tipping and morphological characterization was done based on colony appearance and spore production on PDA. Based on morphology, isolates were grouped, and representative samples were subjected to molecular characterization and identification using Internal Transcribed Spacer (ITS) gene sequencing for the *Rhizoctonia* or *Pythium* genus and Translation elongation factor 1 (EF1) gene sequencing for the *Fusarium* genus and the identity of the fungal organisms was confirmed using the type sequences available in the Mycobank database and Fusarium ID. About 600 isolates of seedling pathogens were recovered from 100 fields where *Fusarium* sp. and *Rhizoctonia* sp. added up to 44% of the total pathogen population, whereas the frequency of Pythium sp. was close to 15% (Fig. 2). At least nine species of Fusarium (accounting for 38% of the total pathogens) were obtained, which include *F. acuminatum (*syn. *F. tricinctum* species complex), *F. armeniacum* (syn. *F. sambucinum* species complex), *F. clavum* (syn. *F. incarnatum-equiseti* species complex), *F. oxysporum* (syn. *F. oxysporum* species complex), *F. sambucinum* (syn. *F. sambucinum* species complex), *F. scirpi* (syn. *F. incarnatum-equiseti* species complex), *F. solani* (syn. *F. solani* species complex), *F. sporotrichioides* (syn. *F. sambucinum* species complex) and *F. vanettenii* (syn. *F. solani* species complex). In addition, a difference in species distribution was observed across counties.

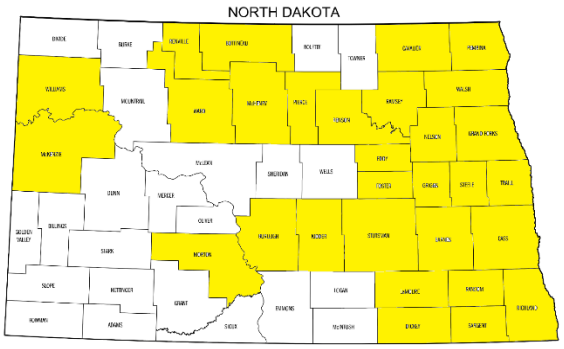


Fig. 1. North Dakota counties surveyed in 2023.

Objective 2. Characterize the pathogenicity of *Fusarium* species (Mathew, Baldwin, Miranda).

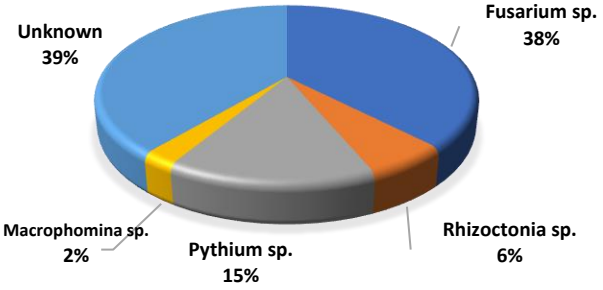
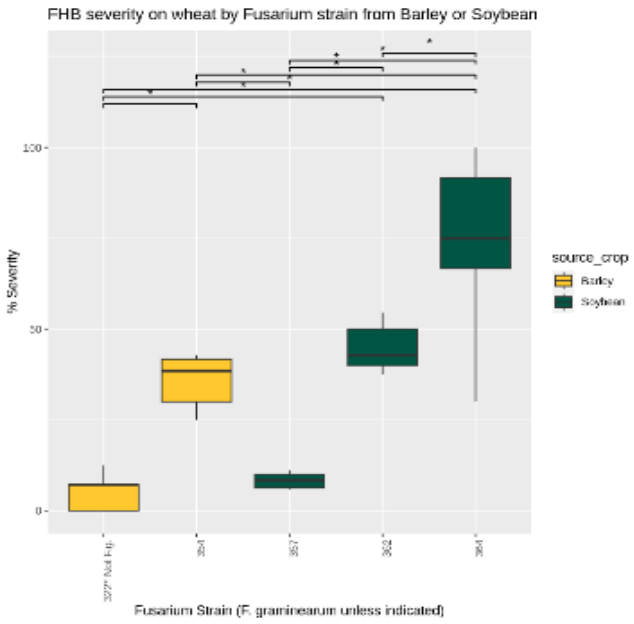


Fig. 2. Seedling pathogens characterized from the 2023 survey (*n*= 100 fields).

This objective was split into two sub-objectives: (1) Screen soybean accessions for root infection of *F. proliferatum* using an inoculum layer technique, and **(2)** Study cross-pathogenicity of isolates of *Fusarium graminearum* from soybean and barley on both crops in the greenhouse using the layer (soybean) and point inoculation (barley) techniques.

Sub-objective (1): For the screening study, seeds of 53 accessions were obtained from Dr. Carrie Miranda's breeding program at NDSU. One *F. proliferatum* isolate FUS026 (McCook County, SD) was arbitrarily selected from 2 isolates that showed the same median disease severity of 4 (= germination and greater than 75% of the roots having lesions) in a study by Okello et al. (2020) on the susceptible check 'Asgrow 1835'. The fungus was grown on potato dextrose agar (PDA; Oxoid, Basingstoke, UK) amended with streptomycin sulfate (0.3 g/L) to prevent bacterial contamination. The Petri plates were then incubated at 23±2°C for 14 days

under a 12-hour light/dark cycle. To prepare the inoculum, a sand and corn meal mixture (3:1 ratio) was autoclaved twice and inoculated with five mycelial plugs of 15 mm square size were added. The inoculated mixture was incubated at 23±2°C for 14 days and thoroughly mixed. At planting, 40 grams of potting mix was added to 475 ml transparent plastic cups, followed by adding 20 grams of inoculum and 20 grams of potting mix. Over this layer, three 3-day pre-germinated seeds were planted and covered with 20 grams of potting mix. The experiment was performed in the greenhouse (24 ±2°C; 12-hour light and dark conditions) and laid out in a completely randomized design. Six replicates were maintained for each accession. The plants were watered at 80 % of the water-holding capacity every other day. The experiment was performed twice. The plants were pulled out 21 days after planting and gently cleaned under running tap water. The root rot severity was determined immediately after washing and rated on a disease scale of 1-5 (Acharya et al.2015, Okello et al. 2020) where, 1 = seedlings germinated with no visible root discoloration, 2 = seedlings germinated with1 to 19% root lesions, 3 = seedlings germinated with 20 to 74% root lesions, 4 = seedlings germinated with roots having 75% or greater percentage lesions; and 5 = No seeds germination and complete colonization by the fungus. Using non-parametric statistics (Shah and Madden 2004), a significant effect of the RTEs caused by *F. proliferatum* isolate FUS026 was observed on the accessions (ATS = 6.81; df = 5.87; *P* = <0.0001). Based on 95 % confidence intervals, none of the accessions tested were significantly less susceptible to the fungus compared to the control 'RG200RR'.



Sub-objective (2): Two barley isolates (322, 354) and three soybean isolates (357, 362, 364) were grown in PDA for seven days, and DNA was extracted using a modified CTAB method. Isolates were grown in mungbean agar for seven days before harvesting. Spores were standardized to 500,000 per ml, and 10 ul was inoculated to 2nd or 3rd full kernel of the wheat head. After point inoculation, the whole head was covered with small plastic bags. After 72 hours, the plastic bags were replaced with glassine bags. Heads were evaluated after 14 days for present disease severity calculating infected/total kernel*100. Among the isolates, only isolate 322 was not *Fusarium graminearum*. The disease severity rating showed that this caused minimal damage to the barley heads, giving statistically comparable results to isolate 357. Both of these isolates had <25% severity. Isolate 354 and 362 have statistically similar severity (>25%, <50%). Among the isolates, 364 obtained the highest severity (>50%, <100%). It is also worth noting that only isolate 322 did not spread in the entire barley heads. Overall, the *F. graminearum* isolates from soybeans exhibited a wide range of virulence on wheat, including an isolate that was highly virulent and an isolate that did not spread through the rachis and might be incapable of producing DON.

Objective 3. Determine how the presence of soybean cyst nematode can affect the development of root rot caused by *Fusarium graminearum* (Mathew, Yan)

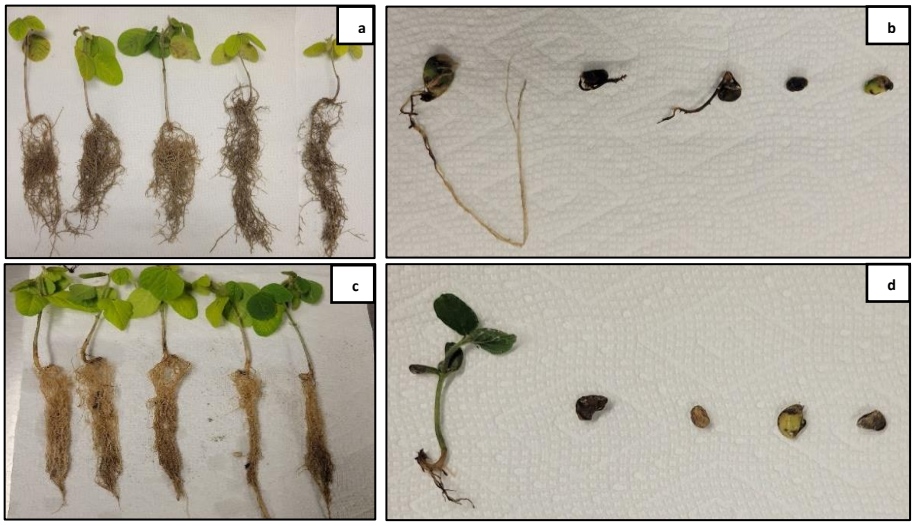
The study was conducted in a growth chamber using the SCN/*F. graminearum*-susceptible soybean cultivar 'Williams 82'. The *F. graminearum* isolate FUS052 used in this study was recovered from diseased soybean roots sampled from McCook County in South Dakota in 2014. To prepare the inoculum, the fungus was grown on potato dextrose agar (PDA) and a sand-cornmeal mixture was inoculated as described previously. For SCN inoculum, an HG type 2.5.7 isolate of SCN provided by Dr. Guiping Yan's lab was used. To prepare the inoculum, nematode cysts were collected from the soil using a decanting and sieving protocol (Krusberg et al. 1994). A 100-cc soil sample with SCN was collected in a bucket filled three-fourths with water. The soil was mixed thoroughly by stirring to suspend it in water. The suspension was then poured through a 710-µm-pore sieve (No. 25) placed above a 250-µm-pore sieve (No. 60). The SCN cysts collected on the No. 60 sieve were washed into a beaker using water gently sprayed from a spray bottle to avoid harming the SCN cysts. The suspension was brought to 50 ml, and the eggs and juveniles were extracted from the cysts by rupturing them with a rotating rubber stopper (Faghihi and Ferris 2000). The eggs and juveniles were collected in a 75-µm-pore sieve (No. 200) placed above a 20-µm-pore sieve (No. 635). The SCN eggs were collected and suspended in 100 ml of distilled water. The number of eggs present per ml of suspension was counted under a dissecting microscope at 40X magnification using a nematode counting slide. For inoculation, the total number of nematode eggs was adjusted to approximately 600 eggs (600 ± 100) per ml.

The experiment was performed in a growth chamber, and the treatments were *F. graminearum* only, SCN only, *F. graminearum* + SCN, and control. Each treatment had five replications, and plastic cone-tainers filled with river sand with or without inocula and one 4-day pregerminated seed served as a replication. The cones were filled with river sand following the inoculum layer inoculation method modified by Bilgi et al. (2008). For *F. graminearum* only treatment, each cone was filled initially with 60 grams of pasteurized river sand, and then 20 grams of fungal inoculum was added, followed by another 20 grams of river sand. A pre-germinated seed of 'Williams 82' was planted on top of this layer and covered with 20 grams of river sand. For *F. graminearum* + SCN treatment, a small hole was made using a pipette tip in the river sand layer above the fungal inoculum layer, and a 3.1 ml suspension of approximately 600 eggs/ml was added to the hole using a pipette. The pre-germinated seed was sown near this hole and covered with river sand. For SCN-only treatments, the cones were filled with pasteurized river sand and inoculated as described for the *F. graminearum* + SCN treatment. For all treatments containing SCN, efforts were made to ensure that the primary roots (radicle) of the seedlings were in contact with the nematode egg suspension. The cones for the non-inoculated control treatment were filled with 100 grams of river sand, and a pre-germinated seed was sown on top, which was then covered with 20 grams of river sand. To prevent soil loss while watering and to restrict the roots within the cone-tainers, the undersides of the cones were covered with a black cloth held with rubber bands. Each cone was watered every other day with 7-8 ml of water, approximately 70% of the water holding capacity. The temperature in the growth chamber was maintained at 23±2°C with a relative humidity of 70-80% and 12-hour light and

dark conditions. The experiment was performed three times. After 42 days of inoculation, each experiment was terminated, and the roots were gently pulled out from each cone and washed under running tap water. The shoot length and root length (tap root) of each plant were measured using a ruler. The washed roots were rated for root rot severity on a disease scale of 1-5 (Okello et al. 2020). To count the nematode cysts, the plants in the cone-tainers were gently removed and placed in a beaker along with the infested soil. Using the decanting and sieving protocol (Krusberg et al. 1994), SCN cysts were collected, and the eggs were extracted and counted following the protocol described by Faghihi and Ferris (2000). The total number of SCN eggs per ml was calculated from all the cones inoculated with the nematode. The non-inoculated control and the *F. graminearum*-only treatments were also examined for the presence of nematodes. Since the disease scoring data was ordinal and the data for root length, shoot length, and SCN egg count, non-parametric statistics (Shah and Madden 2004) was used. After 42 days of inoculation, approximately 90% of the plants inoculated with *F. graminearum* and a combination of *F. graminearum* and SCN showed complete seed infection. A significant effect of treatments was observed on the root rot severity (ATS = 230.16; df = 1.72; P = 1.60 x 10^-87) at 95% confidence intervals (below Table).

Treatment	Relative Treatment effect (RTE) Root rot severity	Relative Treatment effect (RTE) Root length	Relative Treatment effect (RTE) Shoot length	Relative Treatment effect (RTE) SCN egg count
FG only	0.7244* (0.6960, 0.7494)	0.2494* (0.2197, 0.2863)	0.2906* (0.2501, 0.3396)	0.3583 (0.3270, 0.3922)
SCN only	0.2494 (0.2273, 0.2753)	0.6989* (0.6560, 0.7356)	0.6528* (0.6036, 0.6963)	0.8461* (0.7212, 0.8704)
FG + SCN	0.7750* (0.7458, 0.7984)	0.2611* (0.2219, 0.3117)	0.2250* (0.2016, 0.2542)	0.4372 (0.3823, 0.4950)
Control	0.2511 (0.2288, 0.2771)	0.7906 (0.7409, 0.8234)	0.8317 (0.7607, 0.8596)	0.3583 (0.3270, 0.3922)

Fig 5: (a) 'Williams 82' treated with SCN HG type 2.5.7 only; (b) with *F. graminearum* isolate FUS052 + SCN HG type 2.5.7;(c) non-inoculated control plants of cv. 'Williams 82'; (d) with *F. graminearum* isolate FUS052.



A significant effect of RTE caused by *F. graminearum* was observed for root length (ATS = 60.68; df = 2.05; $P = 9.68 \times 10^{-28}$) shoot length (ATS = 111.52; df = 2.48; $P = 2.18 \times 10^{-60}$) and SCN egg counts (ATS = 66.16; df = 1.82; $P = 4.14 \times 10^{-27}$) at 42 days after inoculation. The presence of SCN didn't significantly increase the severity of root rot caused by the fungus (Fig 5). A significant reduction in the root length and shoot length was observed for plants with *F. graminearum* only and *F. graminearum* + SCN treatments compared to the control. Additionally, the root and shoot lengths of plants inoculated only with SCN were significantly less compared to the control (Fig. 5). The SCN-only treatment showed a significant increase in the SCN egg count compared to the control. The SCN egg count was reduced in the treatment that had a combination of *F. graminearum* and SCN compared to the initial inoculum amount. The results from this study show that the presence of SCN didn't increase the severity of root rot caused by *F. graminearum*.

Objective 4. Determine the impact of seed treatment on *Fusarium*. (Mathew, Markell, Webster, Flores)

The trial was planted at the NDSU's Main Research Station in Fargo, ND by Dr. Markell's crew and Dr. Webster. The trial was established on a randomized complete block design with seven treatments (Base, Base + ILEVO, Base + Saltro, Base + TBZ + Headsup + Biost + SAR, Base + Saltro + Ataplan, Base + CeraMax, Base + ILEVO + CeraMax, and a non-treated control) on a susceptible soybean variety and there were four replications per seed treatment. Each plot (replication) was about 20 ft. long, and 10 ft wide (>100,000 plants/A). The trial was not inoculated. Plant emergence was evaluated three weeks after planting, and the number of emerged plants was statistically compared with the non-treated control plots. We did not observe any significant differences in the number of plants per acre as indicated in Table 1. We used drone sensors (cameras available at NDSU) in collaboration with Flores's lab to assess crop response to seed treatments (Fig. 6) and calculated normalized difference vegetation index (NDVI) from the sensor data to assess the density of vegetation (Table 2). We took the drone readings three times during the season but did not observe significant differences in NDVI among treatments. However, we noticed that the vegetation was denser in August when compared to July (NDVI values close to 1 indicate dense vegetation) and certain products such as ILEVO, Saltro, and CeraMax provided greater NDVI values when compared to the non-treated control plots. Yield data was obtained at the end of the growing season (October), and we observed greater yield with products such as ILEVO, Saltro, and CeraMax (11 to 17% yield increase) when compared to non-treated control plots (Table 2).

Table 2. Effect of fungicide seed treatments on soybean yield in Fargo, ND

Treatments	Plant per acre	NDVI (07/16/2023)	NDVI (08/06/2023)	NDVI (08/26/2023)	Yield (bu/A)
Non-Treated	69696.0	0.4	0.8	0.7	23.5
Base	71874.0	0.5	0.7	0.7	23.6
Base + ILEVO	70567.2	0.5	0.8	0.8	26.7
Base + Saltro	65557.8	0.4	0.8	0.8	27.3
Base + TBZ + Headsup + Biost + SAR	71874.0	0.5	0.8	0.8	30.1
Base + Saltro + Ataplan	68607.0	0.4	0.7	0.7	21.3

Treatments	Plant per acre	NDVI (07/16/2023)	NDVI (08/06/2023)	NDVI (08/26/2023)	Yield (bu/A)
Base + CeraMax	72309.6	0.5	0.8	0.8	28.5
Base + ILEVO + CeraMax	67953.6	0.5	0.8	0.8	26.2
CV	**13.8**	**9.0**	**5.4**	**5.9**	**21.5**
***P*-value**	**0.9**	**0.4**	**0.2**	**0.2**	**0.4**

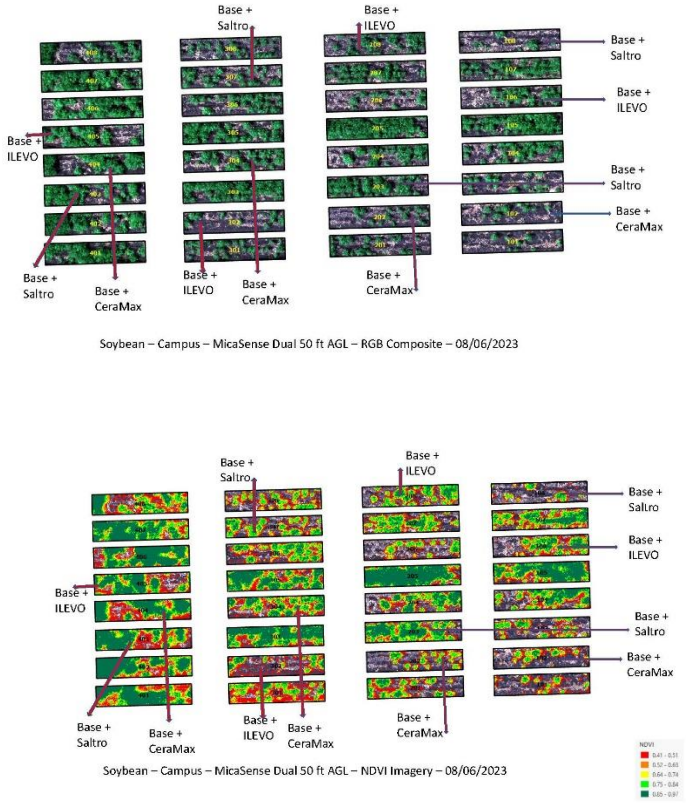


Fig. 6. Drone images when the soybean plants in the field trial were in the flowering growth stage (R1 growth stage).

d. Research results/outcomes

- Nine species of *Fusarium* have been isolated from soil samples collected from 100 fields in addition to *Rhizoctonia* and *Pythium* spp. Among the species of *Fusarium, F. clavum, F. scirpi,* and *F. vanettenii* have not been reported on soybeans previously in the U.S.
- Preliminary results suggested that the presence of SCN didn't increase the severity of root rot caused by *F. graminearum*.
- Preliminary results also suggest that *F. graminearum* isolates from soybeans exhibited a wide range of virulence, including an isolate that was highly virulent and an isolate that did not spread through the wheat rachis and might be incapable of producing DON.

- Greenhouse screening of 53 soybean lines from Dr. Miranda's breeding program at NDSU for resistance *to F. proliferatum* showed that none of the accessions had lesser disease severity compared to the susceptible check 'RG200RR'.
- Results from the seed treatment trials show greater yield with products such as ILEVO, Saltro, and CeraMax (11 to 17% yield increase) when compared to non-treated control.
- One manuscript published from Fusarium research in 2024 (Rafi et al. 2024 - https://doi.org/10.1094/PDIS-02-24-0477-RE) in a peer-reviewed journal, *Plant Disease*

e. Listings of any disclosures of inventions or plant varieties

None

f. Discussion

In North Dakota, a survey conducted in 2023 across 100 fields in 30 counties, with funding from the North Dakota Soybean Council, revealed that the major seedling pathogens are Fusarium, Rhizoctonia, Pythium, and possibly *Macrophomina*. Among the Fusarium species identified were *F. solani*, *F. acuminatum*, *F. caucasicum*, *F. oxysporum*, *F. curvatum*, *F. serpentinum*, *F. clavu*s, and *F. communae*. Furthermore, three species of *Fusarium*, namely *F. caucasicum*, *F. curvatum*, and *F. clavus*, were previously unreported in the United States. Considering the limited management options available to farmers, the survey evaluated fungicide seed treatments. The results demonstrated that products such as ILEVO, Saltro, and CeraMax led to higher soybean yields (11 to 17% increase) compared to the non-treated control. Moreover, our study highlighted that certain *Fusarium* species, such as *F. graminearum*, known to be pathogenic to rotational crops like wheat, also pose a threat to soybeans. This emphasizes the importance of developing resistant soybean cultivars to combat Fusarium infections and reduce inoculum for crop rotation.

g. Conclusion/Benefits to the North Dakota Soybean Farmers and the Industry

Our study highlights the importance of investigating seedling pathogens, such as *Fusarium*, *Rhizoctonia*, *Pythium*, and others, in North Dakota. These organisms are known to cause a yield loss of $9.84 per acre in soybean production regions throughout the United States. We propose that factors such as heavy rainfall (above-normal precipitation) in North Dakota in 2023, tillage practices, and limited options for host resistance may have contributed to the widespread occurrence of seedling diseases. Specifically, our findings emphasize the role of Fusarium in causing soybean seedling disease and root rot in North Dakota. The data gathered from our seed treatment trials will be used to develop and refine disease management programs that target *Fusarium* specifically. Additionally, we will continue collaborating with Dr. Miranda's breeding program to screen different varieties for resistance to *Fusarium* species, which can then be adopted by farmers in the future. Furthermore, our study on the *Fusarium*-SCN experiment highlights the importance of screening cultivars for resistance to both pathogens, ultimately leading to more effective disease control.