Research Project Title: Potential for combatting iron deficiency chlorosis with the soybean microbiome FY24.

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Research Overview:

Iron deficiency chlorosis (IDC) is a wide-spread problem strongly affecting soybean production in North Dakota. The characteristic yellowing of plant leaves suffering from IDC is caused by a lack of chlorophyll formation due to poor function of iron-requiring enzymes involved in chlorophyl biosynthesis. North Dakota soils normally contain more than enough iron for plant function, however much of the iron is not in soluble form needed by the plant. A reduction in iron solubility at high soil pHs caused by high levels of $CaCO_3$ (lime) in the top-soil is the main cause of IDC; white the iron is there, it isn't available to the plant. High lime soil is common in North Dakota, and IDC is exacerbated by salinity which is also becoming more and more common.

In this study we aim to build on previous work to assess the potential of the soybean microbiome as a new tool to combat IDC. In FY22, in a study that analyzed four fields in Eastern ND with varying levels of IDC, we observed a significant correlation in the structure of the soybean root and rhizosphere microbiome with the IDC level of the soil (see FY22 report). We hypothesize that unique groups of microbes that are enriched under IDC conditions could help alleviate IDC in soybeans when cultured and used as inoculants along with root nodule forming rhizobia. We have already optimized a greenhouse assay that will be suitable for measuring growth potential of microbiome members, and a plate screening assay that can identify siderophore producers (microbe-produced iron solubilizing molecules that function like Fe-chelating fertilizers). With this study we aim to utilize these resources to attempt to identify individual microbes or groups of microbes, cultures from the IDC soybean microbiome that could have a beneficial effect to soybean plants grown under IDC conditions.

Objectives:

Objective 1) Culture a community of North Dakota microbes from the soybean microbiome.

Objective 2) Siderophore production screen from members of soybean microbial community.

Objective 3) Evaluate reduction of IDC from microbial inoculants with an optimized "Goos" Greenhouse assay.

Materials and Methods:

Objective 1) To address objective 1, we adapted a high throughput culturomics pipeline developed by J. Zhang et al., 2021, to isolate the members of the soybean microbiome. We cultured bacteria from freshly harvested soybean roots using dilution-enrichment culturing

techniques. We used a concentration of diluted root homogenates that allowed for approximately 40% of the wells to exhibit bacterial growth originating from a single bacterial cell. Wells with bacterial growth were split for 16S rRNA sequencing and high-quality glycerol stocks. We created our own bioinformatic pipeline to identify matched amplicon sequence variants to those tracked in microbiome data from the field. According to the analysis, the wells with a purity greater than 95% were selected from the preserved glycerol stocks and streaked repeatedly on agar plates. Finally, these isolates were validated by full-length 16S rRNA gene Sanger sequencing before being stocked and stored at the temperature of -80°C.

Objective 2) We have optimized a Chrome Azurol S colorimetric assay (Louden et al., 2011) to identify the siderophore-producing microbes based on orange color formation in the CAS medium. This assay utilizes Chrome Azurol S (CAS) substrate and hexadecyltrimethylammonium bromide (HDTMA) as indicators. The CAS/HDTMA agar media is prepared by mixing it with $FeCl_3$, which serves as a blue dye. We assessed the siderophore-producing ability of each of the 64 soybean bacterial isolates cultured from the high-IDC (Leonard) and no-IDC (Casselton) sites. We cultured these individual microbes on TSOY agar media from their respective freezer glycerol stocks. Following a 72-hour incubation at 28°C, the culture plates were overlayed with CAS/HDTMA-agar media. Siderophore molecules secreted by candidate microbes chelated iron from the CAS/HDTMA complex after 4-6 hours incubation period and transformed color from blue to orange, indicating the potential presence of siderophore-producing microbes.

Objective 3) We optimized a greenhouse assay originally developed by Dr. Jay Goos to evaluate the capacity of our taxonomically diverse SynCom members to promote plant growth under iron-deficient conditions by enhancing access to unavailable iron. The experiment was conducted using a sterile IDC soil/sand potting mixture where 10 mM Nitrate/5 mM Bicarbonate was supplemented to induce iron-deficient conditions as found in calcareous soils. For iron-rich medium, soil/sand mixture was treated with the 10 mM Nitrate/5 mM Bicarbonate and iron-chelating fertilizer Soygreen, an efficient and highly soluble iron source. Two pregerminated soybean seedlings were planted per pot under available and unavailable iron sources with SynCom inoculation and a control group without SynCom inoculation. We included 20 replicated pots per condition. The plants were cultivated on iron-rich or iron-deficient medium for three to four weeks while exposing them to live or no SynCom members.

Research Results and Outcomes:

Objective 1) As a result of our cultivation efforts we successfully cultivated 64 unique isolates from the no-IDC and high-IDC sites (Table 1). This enabled us to selectively choose microbes from our culture collection that demonstrated high levels of colonization of the soybean root and rhizosphere and displayed sequence identity match with the field microbiome data. This broader culture collection serves as a valuable community resource, enabling us to create a well-defined synthetic bacterial community (SynCom) to explore the potential of enriched or depleted microbes during IDC to produce siderophores and/or alleviate IDC stress in soybeans

Table 1. Microbes in the process of culturing for Objective 1.

ASV number	Genus	Location	Year	Siderophore production
9	Variovorax robiniae	Casselton (No IDC)	2022	−
10	Pseudomonas silesiensis	Casselton (No IDC)	2022	+

13	Pseudomonas cerasi	Casselton (No IDC)	2022	−
15	Pseudomonas koreensis	Casselton (No IDC)	2022	+
24	Pseudarthrobacter sulfonivorans	Casselton (No IDC)	2022	−
34	Phyllobacterium ifriqiyense	Casselton (No IDC)	2022	−
37	Pseudomonas oryzihabitans	Casselton (No IDC)	2022	+
55	Lysobacter antibioticus	Casselton (No IDC)	2022	−
61	Pantoea agglomerans	Casselton (No IDC)	2022	−
68	Chryseobacterium gregarium	Casselton (No IDC)	2022	−
71	Paeniglutamicibacter sulfureus	Casselton (No IDC)	2022	−
75	Paenarthrobacter nitroguajacolicus	Casselton (No IDC)	2022	−
84	Cellulomonas cellasea	Casselton (No IDC)	2022	−
87	Variovorax paradoxus	Casselton (No IDC)	2022	−
88	Aeromicrobium ginsengisoli	Casselton (No IDC)	2022	−
89	Aeromicrobium ginsengisoli	Casselton (No IDC)	2022	−
102	Variovorax paradoxus	Casselton (No IDC)	2022	−
105	Variovorax paradoxus	Casselton (No IDC)	2022	+
110	Variovorax ureilyticus	Casselton (No IDC)	2022	−
120	Massilia agri	Casselton (No IDC)	2022	−
126	Rhodococcus qingshengii	Casselton (No IDC)	2022	−
129	Curtobacterium pusillum	Casselton (No IDC)	2022	−
130	Pseudorhodoferax soli strain TBEA3	Casselton (No IDC)	2022	−
131	Hydrogenophaga intermedia	Casselton (No IDC)	2022	−
132	Polaromonas eurypsychrophila	Casselton (No IDC)	2022	−
136	Bacillus proteolyticus	Casselton (No IDC)	2022	−
23a	Pseudomonas brassicacearum	Leonard (High IDC)	2022	−
28a	Ensifer adhaerens	Leonard (High IDC)	2022	−
43	Pseudoxanthomonas japonensis	Leonard (High IDC)	2022	−
64	Cellvibrio ostraviensis	Leonard (High IDC)	2022	−
72	Pseudomonas brassicacearum	Leonard (High IDC)	2022	+
3	Ferrovibrio	Leonard (High IDC)	2023	−
4	Ferrovibrio	Leonard (High IDC)	2023	−
6	Pseudomonas	Leonard (High IDC)	2023	+
7	Bosea	Leonard (High IDC)	2023	−
10	Asticcacaulis	Leonard (High IDC)	2023	−
11	Ensifer	Leonard (High IDC)	2023	−
13	Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium	Leonard (High IDC)	2023	−
16	Pseudoxanthomonas	Leonard (High IDC)	2023	−
20	Pseudoxanthomonas	Leonard (High IDC)	2023	−
22	Pseudoxanthomonas	Leonard (High IDC)	2023	−
23	Lysobacter	Leonard (High IDC)	2023	−
24	Lysobacter	Leonard (High IDC)	2023	−
25	Lysobacter	Leonard (High IDC)	2023	−
27	Sphingopyxis	Leonard (High IDC)	2023	−
28	Novosphingobium	Leonard (High IDC)	2023	−
33	Chitinophaga	Leonard (High IDC)	2023	−
35	Taibaiella	Leonard (High IDC)	2023	−
36	Chryseobacterium	Leonard (High IDC)	2023	+
41	Dyadobacter	Leonard (High IDC)	2023	−
42	Microbacterium	Leonard (High IDC)	2023	−
43	Pseudarthrobacter	Leonard (High IDC)	2023	−
44	Microbacterium	Leonard (High IDC)	2023	−
54	Aeromicrobium	Leonard (High IDC)	2023	−
60	Methylibium	Leonard (High IDC)	2023	−
61	Variovorax	Leonard (High IDC)	2023	−
62	Variovorax	Leonard (High IDC)	2023	+
65	Ramlibacter	Leonard (High IDC)	2023	−
66	Variovorax	Leonard (High IDC)	2023	−

68	Xylophilus	Leonard (High IDC)	2023	–
69	Limnohabitans	Leonard (High IDC)	2023	–
70	Paucibacter	Leonard (High IDC)	2023	–
71	Acidovorax	Leonard (High IDC)	2023	–
85	Pseudorhodoferax	Leonard (High IDC)	2023	–

Objective 2) Using the optimized CAS assay we evaluated siderophore production in each of the cultured ND soybean microbes. The resulting data is summarized as a column in Table 1 and example photos in Figure 1. Overall, 8/64 microbes showed potential for siderophore production.

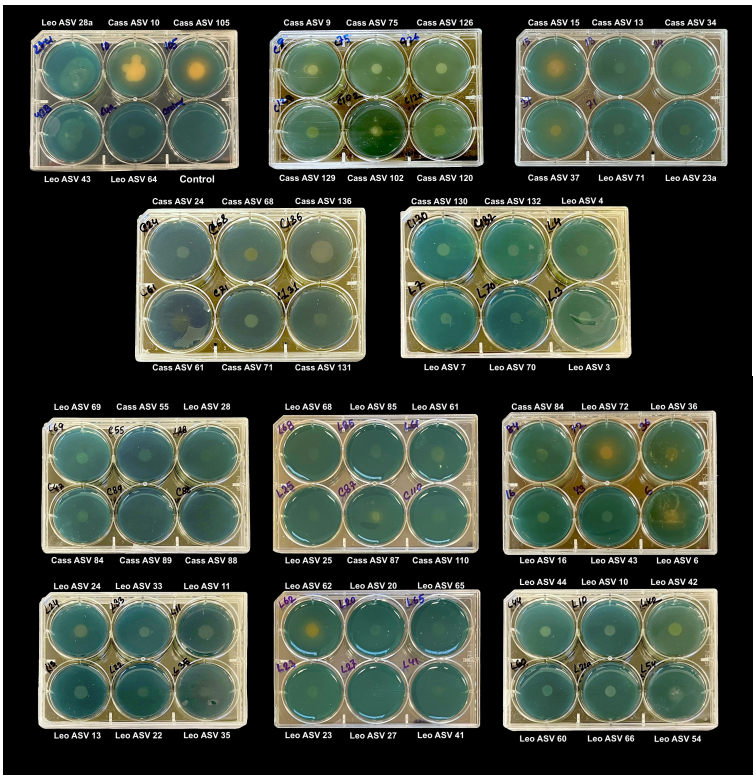

Figure 1. CAS assay results for soybean isolates. A positive result is visualized as an orange halo surounding the spot of microbial inoculant.

Objective 3) We successfully optimized an assay for evaluating IDC reduction by microbial inoculants. The resulting experiment involved using a sterile IDC soil/sand potting mixture where 10 mM Nitrate/5 mM Bicarbonate was supplemented to induce iron-deficient conditions as found in calcareous soils. Microbial inoculants were added to pre-germinated soybean roots before planting, and an iron-chelating fertilizer Soygreen was added as a positive control to uninoculated pots. To test the assay, we created a microbial consortium that included 11 members from our soybean isolate collection that either showed significant enrichment or depletion in response to IDC based on previous years microbial community data. The experiment successfully induced IDC in soybeans that were untreated (Figure 3 and 6)). Remarkably, the microbial treatment showed significant alleviation of IDC symptems to levels (Figure 4 and 6) comparable to adding the soygreen fertilizer (Figure 5 and 6).

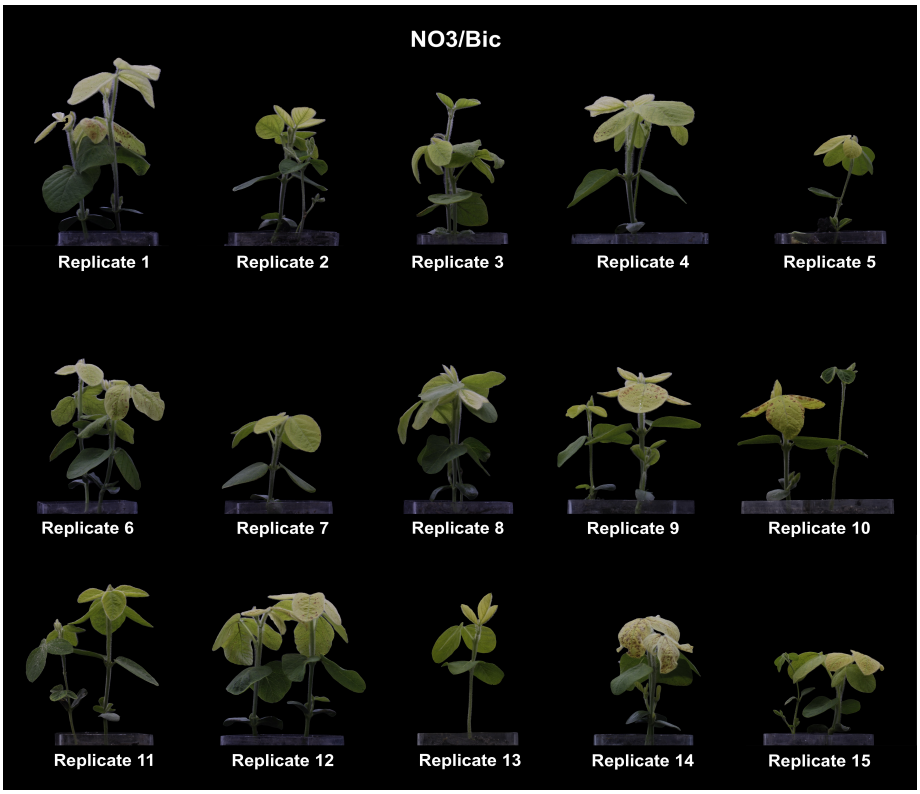

Figure 3. Images of IDC symptoms induced in the greenhouse by the newly optimized assay.

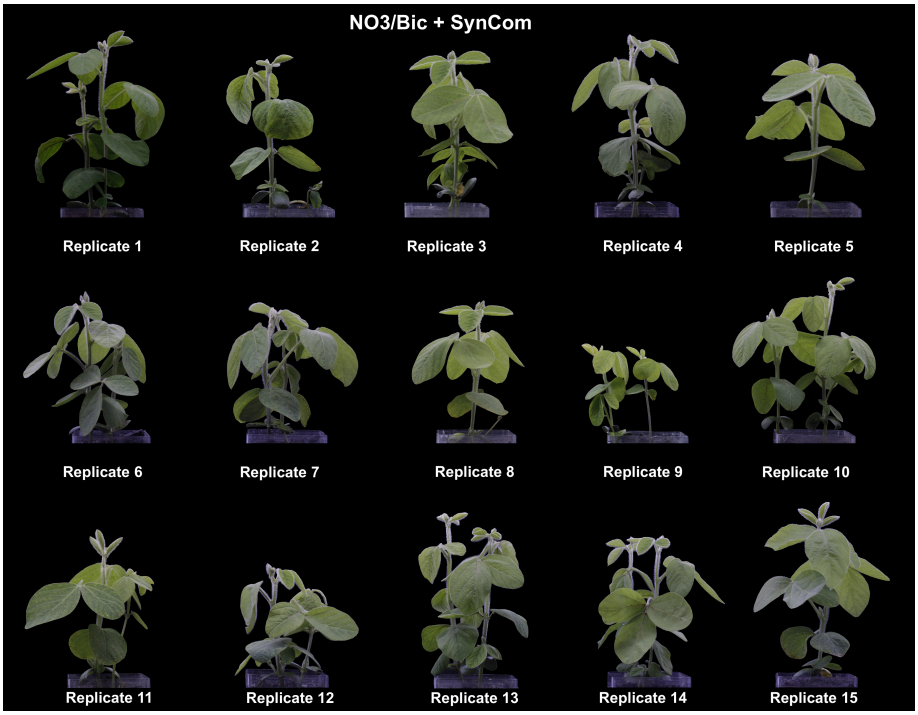

Figure 4. Images of microbe-treated soybeans grown in the same IDC symptom producing conditions as Figure 3.

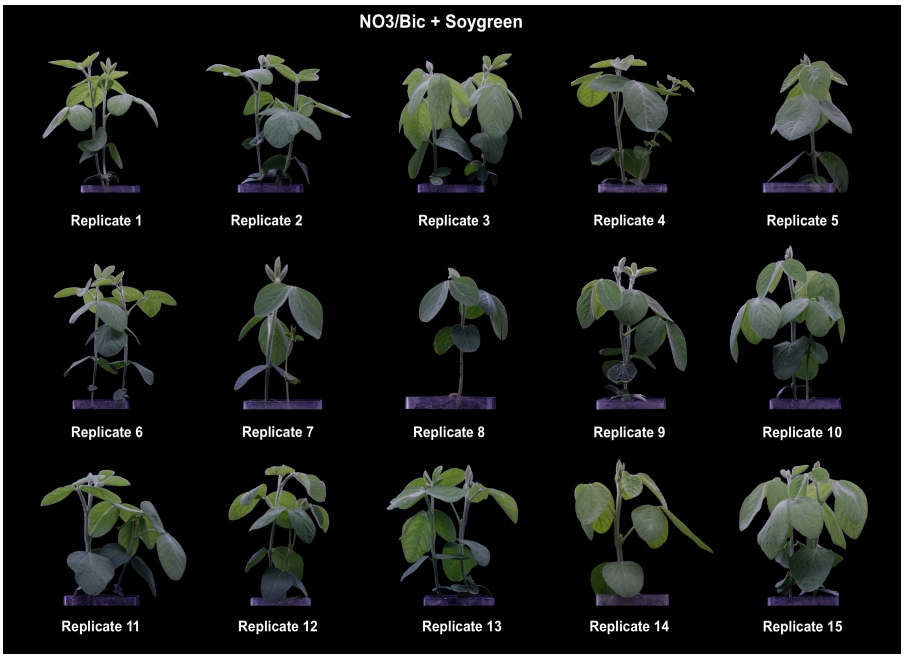

Figure 5. Images of Soygreen-treated soybeans grown in same IDC symptom producing conditions as Figure 3.

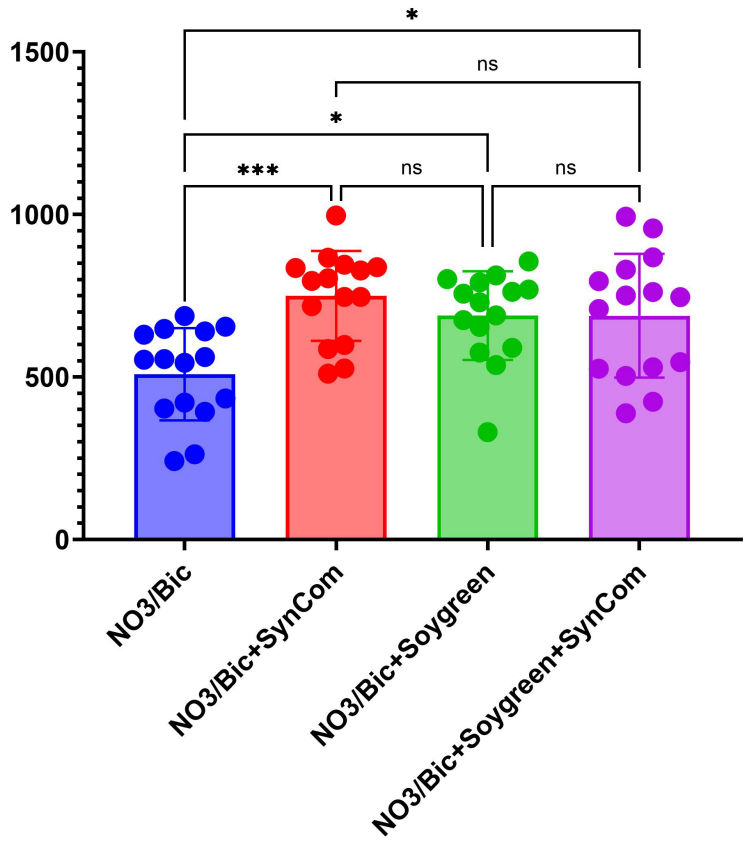

Figure 6. Recovery of shoot dry weight from microbial treatment (SynCom) and Soygreen treatment in newly developed assay.

Discussion:

Overall, we have developed and optimized several elements of a comprehensive pipeline for isolation and characterization of microbes with the potential to reduce iron deficiency chlorosis in soybean. Our preliminary data from testing the system indicate that there is significant potential for microbial inoculants to reduce IDC and we have effectively developed an experimental procedure that can screen for and measure reduction of IDC from microbial treatments. Going forward, utilizing this system to detect and characterize individual microbes with the potential to reduce IDC will prove a powerful approach to provide sustainable management solutions to this

Conclusion/Benefit to Soybean Farmers:

IDC remains an important agronomic issue without perfect solutions. The microbiome offers a solution that can supplement genetics for IDC resistance which would be more cost effective than iron fertilizers and could be applied concurrently with rhizobium inoculants. In this project we successfully developed a pipeline for the isolation of potential IDC-reducing microbes and characterization of their potential for IDC reduction. Early results indicate microbial applications have the capacity to reduce IDC at a similar effectiveness to Soygreen fertilizer, the current best option.

References:

1. Louden, B. C., Haarmann, D., & Lynne, A. M. (2011). Use of blue agar CAS assay for siderophore detection. *Journal of Microbiology & Biology Education*, *12*(1), 51–53.
2. Zhang, J., Liu, Y.-X., Guo, X., Qin, Y., Garrido-Oter, R., Schulze-Lefert, P., & Bai, Y. (2021). High-throughput cultivation and identification of bacteria from the plant root microbiota. *Nature Protocols*, *16*(2), 988–1012.