Geddes lab annual progress report to North Dakota Soybean Council: A tool for Cheap and Rapid Tracking of Soybean Inoculant Populations in Field Soil (Renewed 2023)

a. Objectives of the Research

Objective 1: Design and test a digital PCR primer set to identify soybean inoculant strains and discriminate them from other soil microbes

Objective 2: Validate digital PCR as a tool for absolute quantification of *B. japonicum* numbers from soil and translate population size estimates to predictions of successful or unsuccessful soybean nodulation

Objective 3: Use the new digital PCR assay to address inoculant strain survival in in soils with challenging conditions and previous soybean planting and inoculation history in Western North Dakota

b. Completed Work

Objective 1: We designed five primer sets and tested them in combination with one primer set from the literature. The primer sets were successfully evaluated for sensitivity and specificity and optimized with different cycle parameters.

Objective 2: The validated primer sets were tested with digital PCR as well as qPCR with both a genomic DNA standard curve and a "spike-in" soil assay. Overall, in our hands qPCR showed superior performance than dPCR. Based on these data from Objective 1 and 2 we finalized a prototype version of the assay for quantifying rhizobia in ND field soil (NDSoy1.0).
We used a greenhouse assay to establish the nodulation response of soybean to different levels of rhizobia in the soil. This allowed us to determine a sensitivity threshold of NDSoy1.0 which was determined to be ~1000 rhizobia per gram of soil.

Objective 3: We sampled 13 fields from growers and Research Extension Centers from Western North Dakota with varied previous planting/inoculation histories in Spring 2022 and employed the current assay (NDSoy1.0) to predict the residual rhizobia present in these fields after different numbers of years post soybean planting and inoculation.

c. Results so far

Evaluation of primer sets in prototype dPCR and qPCR assays

In molecular quantitation approaches such as dPCR and qPCR, primer sets are short DNA sequences that are used to target a specific molecular signature for detection. To start out we designed 5 primer sets, and identified one from the literature to target the *nod* genes (*nodZ* and *nodYA*) of *Bradyrhizobium japonicum*. These genes are present only in symbiotic

Bradyrhizobium, thus ensuring we only quantify the microbes with capacity to form symbiosis with soybeans (Table 1).

Table 1. Primer sets tested for qPCR and dPCR assay

Primer set	Forward primer sequence	Reverse primer sequence
nodZ A	GGTTTGGCGACTGTCTGTGGTC	TTCCACCATGTTGGAAAGAATGGTCC
nodZ B	GGTTGAAGACATTGGCGGAG	CGCGTTCCCTGAAAATCTGC
nodZ C	CGCGATTCCAAAGCAGTTCC	CAGCGGGCAAGGAGATACAT
nodZ D	GGTTGAAGACATTGGCGGAG	TTCCACCATGTTGGAAAGAATGGTCC
nodZ E	GGTTTGGCGACTGTCTGTGGTC	AGACTGGAAAGGCATTGGTG
nodYA	GCATCTCAGCATTCATCGGC	GGGGAGACGGCAATGTTCAT

For evaluation of primer sets we used both the new-to-market technology digital PCR (dPCR) and the more traditional approach that has been more routinely successfully employed, quantitative PCR (qPCR). Both approaches utilize the same design principles and parameters for DNA amplification, and thus we were able to test all the primer sets using both technologies. Sensitivity was evaluated based on the lowest concentration *Bradyrhizobium japonicum* genomic DNA able to be detected (based on a 10 fold dilution standard curve). Initial tests indicated a similar sensitivity, able to detect the equivalent of ~1000 rhizobia/gram of soil. Specificity was evaluated by comparing the "positive" signal in a soil sample that contained high amounts of *Bradyrhizobium* (Spring 2021 collection from field planted to soybean and inoculated in the previous year) to the "negative" signal in a soil sample expected to contain low to no *Bradyrhizobium* (Collected from National Grasslands in South Dakota, at least 30 years without farming). Specificity evaluation suggested a good ability to differentiate high from low populations of *Bradyrhizobia* in soils via qPCR, but a poor ability in digital PCR due to a high non-specific signal from the no *Bradyrhizobium* control (data not shown).

Optimization of specificity and sensitivity in qPCR and establishment of NDSoy1.0 Assay

The five primer sets were optimized in an effort to maximize sensitivity and specificity by altering the anneal temperature parameter of the PCR reaction, and contrasted with one another for sensitivity and specificity across annealing temperatures in qPCR (from 56 to 66^{o}C). Sensitivity was defined by the amplification of the target at an earlier cycle threshold (Ct), and specificity was defined based on the absence of amplification in the no *Bradyrhizobium* control soil sample, and a melting curve from the high *Bradyrhizobium* soil sample that matched the genomic DNA (gDNA) standard curve (Figure 1). A reaction condition which rendered all primer sets highly specific in qPCR (based on no amplification of the no *Bradyrhizobia* control microbiome sample) was identified (66^{o}C annealing temperature), therefore the primer set with the greatest sensitivity (nodZ B) was selected to proceed utilizing these reaction conditions. The nodZ B primer set was tested with dPCR using the 66^{o}C annealing temperature but continued to show poor specificity (high non-specific signal) with the dPCR technology (data not shown).Thus the qPCR assay utilizing nodZ B at 66^{o}C annealing was selected as the final conditions for the first version of our assay which we named NDSoy1.0 (Figure 2).

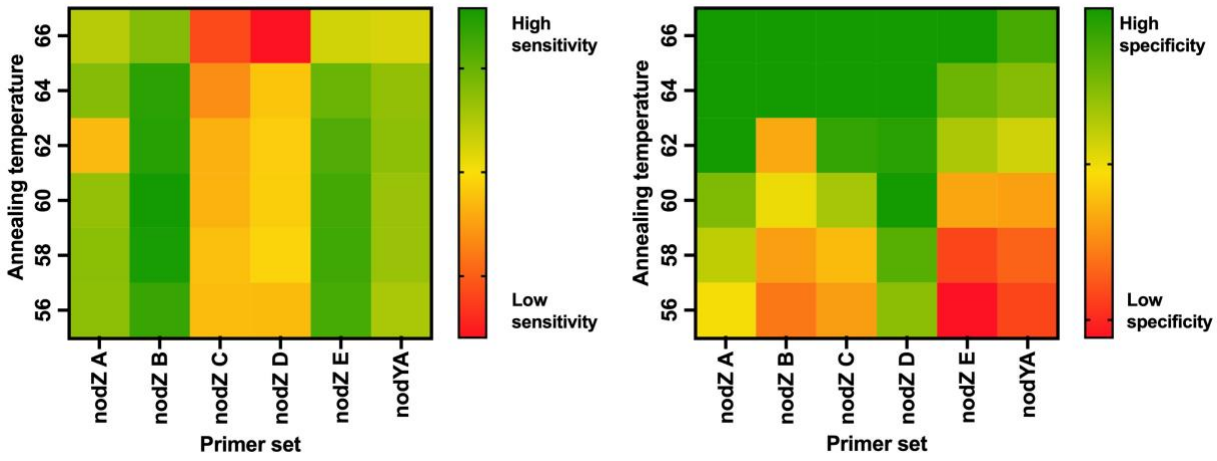

Figure 1. Sensitivity and specificity of tested primer sets.

NDSoy1.0 Assay Procedure

Reaction setup

1. Thaw the QIAcuity EG PCR master mix, template DNA or cDNA, primers, and RNase-free water. Mix the individual solutions.
2. Prepare a reaction mix according to Table 2. Due to the hot-start polymerase, it is not necessary to keep samples on ice during reaction setup or while programming the instrument.
3. Vortex the reaction mix.
4. Dispense appropriate volumes of the reaction mix, which contains all components except the template, into the wells of a standard PCR plate. Then, add template DNA or cDNA into each well that contains the reaction mix.
5. Run the reaction in a qPCR machine with the appropriate cycle parameters.

MM:

1 reaction	75 reactions	
6.65 µl	498.75 µl	EG MM (QIAcuity EG PCR Kit Cat. No/ID: 250111)
1 µl	75 µl	Forward Primer (nodZ-B F)
1 µl	75 µl	Reverse Primer (nodZ-B R)
6.35 µl	476.25 µl	H_2O

15 µl MM added to each well
5 µl DNA template from 250 mg field soil DNA extraction

qPCR Cycle Parameters:

Cycles	Temperature	Time
1x	95°C	2 minutes
40x	95°C	15 seconds
	66°C	15 seconds
	-Plate Read-	
	72°C	15 seconds

Figure 2. Final assay conditions of NDSoy1.0

Calibrating NDSoy1.0 to optimal nodulation of soybean

With an optimal primer set selected, we next set out to calibrate *Bradyrhizobium* detection with the amount of *Bradyrhizobium* that need to be present in the soil for optimal nodulation in a greenhouse assay. To perform this assay we spiked *Bradyrhizobium*-free soil with known concentrations of *Bradyrhizobia* (from 0 to 1,000,000 cells). The spiked soil was then used directly for DNA extraction and qPCR assay, and for planting of soybean plants. After 4 weeks the soybean plants were removed from the pots and the nodulation was assessed by counting nodules, with optimal nodulation defined as a concentration of *Bradyrhizobia* after which no increased nodulation was achieved. Optimal nodulation was observed at concentrations greater than 1,000 cells per gram. When the qPCR assay using the nodZ B primer set was done with the spiked soil, results correlated nicely with the estimated rhizobia number from a gDNA standard curve. The current assay was able to detect *Bradyrhizobia* at concentrations greater than ~1,000 cells per gram (Figure 3). Therefore, we are already capable of detecting if sufficient *Bradyrhizobia* are present for optimal nodulation based on the greenhouse assay, though we believe the reliability of the assay would be improved by enhancing the detectable limits to low, non-optimal numbers of *Bradyrhizobia* in field soil.

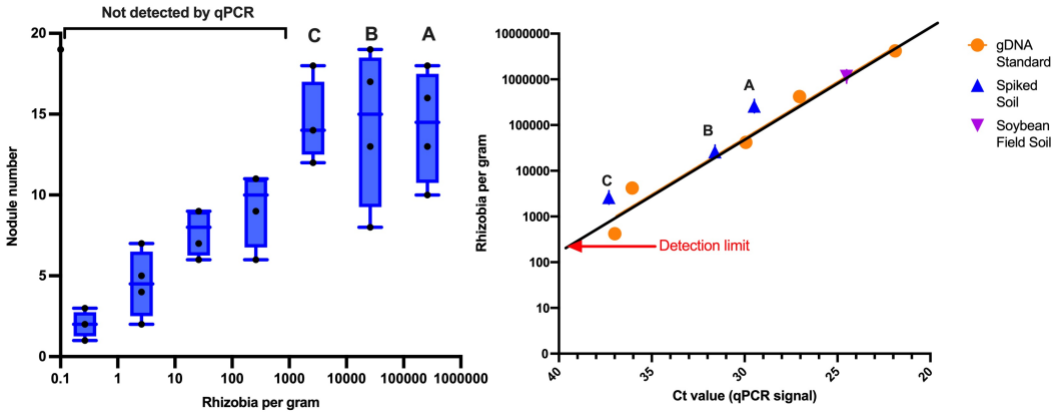

Figure 3. Spiked soil assay for soybean nodulation and detection by qPCR assay.

Application of NDSoy1.0 assay to fields from Western ND

We set out to pilot deployment of NDSoy1.0 using field soil samples from Western ND collected in Spring 2022. Sites were selected from Western ND with varied years since planting soybeans and used to estimate the populations of Bradyrhizobium in the soil. As an example for future implementation, we proposed a tentative inoculation recommendation based on our preliminary greenhouse data that <1000 rhizobia per gram resulted in sub-optimal nodulation. Rhizobia populations of <10,000 rhizobia per gram were assigned a recommendation to inoculate, between 10,000 and 100,000 rhizobia per gram suggested to inoculate if soybeans were grown the following year, and rhizobia >100,000 per gram were recommended not to inoculate. Overall Western ND soils (S103-S114) showed lower estimated rhizobia per gram than eastern ND controls (S100/101). We reached an inoculation recommendation threshold with 5/12 fields tested, ranging from 2-6 years since the last soybean crop. By sampling inside or outside the irrigated zones of fields we also investigated the effect of irrigation at two sites that were continuously irrigated and had soybeans either 1 (S109/S110) or 5 years ago

(S111/S112). In both cases the non-irrigated parts of the field had less rhizobia, and in the case of the 5 year previous field, the population was either robust or nearing levels that inoculation would be suggested. These results demonstrate the importance and impact of field conditions on the rhizobia populations.

Table 2. Rhizobia levels in Western ND soils estimated by NDSoy1.0

Sample	Location	Region	Years Since Soybeans	Irrigation	Average Rhizobia per	Overall Rhizobia	Tentative Inoculate Recommendation
S100	Carrington	Eastern ND	1	No	1,208,939	High	**No**
S101	Medina	Eastern ND	3	No	695,195	High	**No**
S103	Hettinger	Western ND	2	No	Not Detected	Low	**Yes**
S104	Hettinger	Western ND	3	No	16,777	Medium	**Next Year**
S105	Hettinger	Western ND	6	No	3,901	Low	**Yes**
S106	Hettinger	Western ND	4	No	4,937	Low	**Yes**
S107	Madison	Western ND	2	No	19,406	Medium	**Next Year**
S108	Dickinson	Western ND	4	No	Not Detected	Low	**Yes**
S109	Alexander	Western ND	1	Yes	1,176,471	High	**No**
S110	Alexander	Western ND	1	No	581,132	High	**No**
S111	Alexander	Western ND	5	Yes	411,419	High	**No**
S112	Alexander	Western ND	5	No	12,593	Medium	**Next Year**
S113	Alexander	Western ND	2	No	4,769	Low	**Yes**
S114	Williston	Western ND	2	No	20,738	High	**No**

It is important to note that more work is needed to validate critical rhizobia populations at which nodulation, symbiosis and ultimately yield are not optimal before reliable recommendations can be made to farmers. These values may change under different weather conditions, for eg. drought, where poor nitrogen fixation has been correlated with yield loss in drought. In FY24 we hope to collaborate with RECs and to utilize the updated version of the tool finalized in FY223 to investigate these questions.

d. Work to be Completed

With good success from FY22 and establishment and employment of NDSoy1.0, we are poised to further improve the assay in FY23. The objectives for the coming year include:

Objective 1: Evaluate improvement of sensitivity with TaqMan probes and finalize technology Platform

Objective 2: Establish reliability using different soil types and sampling procedures, and optimize as necessary

Objective 3: Test finalized assay using farmer's field soil, with a focus on inoculant survival in acidic soils from Western ND