Geddes lab min-term progress report to North Dakota Soybean Council: A tool for Cheap and Rapid Tracking of Soybean Inoculant Populations in Field Soil

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 (see preliminary results).

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, and therefore as an outcome from this work we recommend transition to a qPCR-based assay for further development and testing (see preliminary results)

Although not initially proposed in the grant, we further 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 that our assay needs to exceed in order to make a reliable recommendation to farmers for when they should see a positive response to inoculation.

c. Preliminary Results

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

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°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°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°C annealing temperature but continued to show poor specificity (high non-specific signal) with the dPCR technology (data not shown).

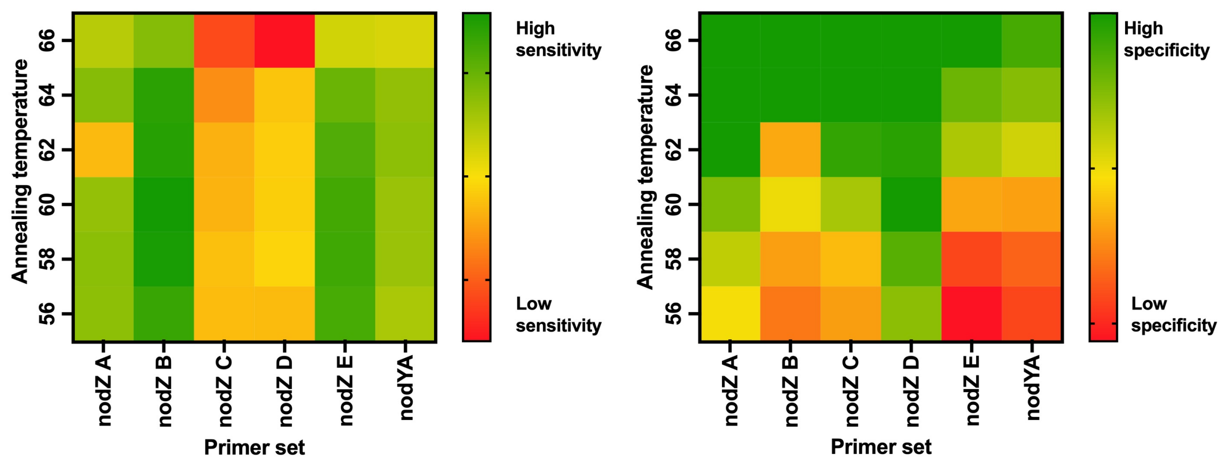

Figure 1. Sensitivity and specificity of tested primer sets.

Calibrating qPCR result 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 2). 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.

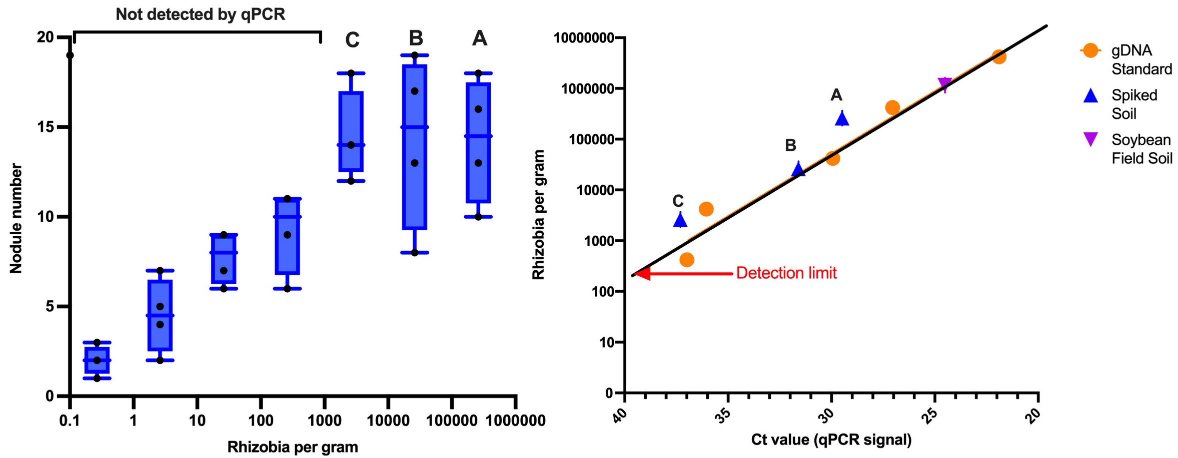

Figure 2. Spiked soil assay for soybean nodulation and detection by qPCR assay.

d. Work to be Completed

Remaining to be completed is Objective 3. In the spring, we will put the current iteration of the assay to the test by evaluating inoculant populations from Soybean fields from Western ND. Since qPCR has proven a more reliable technology for the assay than dPCR thus far, we plan to carry out this objective using qPCR rather than dPCR as originally intended.