Resistance of Soybean Varieties to *Pratylenchus dakotaensis*, a New Root-Lesion Nematode Species Infecting Soybean

TECHNICAL REPORT
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a. Background Information

Root-lesion nematodes (*Pratylenchus* spp.) pose a significant threat to soybean production because they can cause yield losses by infecting and damaging plant roots. These are endo-migratory nematodes that infect soybean roots and above ground symptoms are only visible after severe damage, making them difficult to manage. During a soil survey in 2017, a new root-lesion nematode species was reported in North Dakota with population densities 125-2000 per kg soil. It was different from other reported *Pratylenchus* species based on both morphology and sequencing of the genomic regions.

When the infested soil was planted with a cultivar of soybean (Barnes) in a greenhouse bioassay, a reproductive factor as high as 5.04 was observed. Hence, soybean was a good host to this nematode and it can be a potential threat to ND soybean production. Effective management strategies must be deployed in time before it causes significant yield losses. For this, timely identification and quantification of the nematode species *P. dakotaensis* is necessary. However, the traditional methods of identification and quantification require a lot of time and expertise in nematology. Therefore, there is a necessity to develop an efficient detection assay that can rapidly identify *P. dakotaensis* from the infected soybean roots as more than 50% of the nematode population was found to reside in the root versus soil.

b. Research Objectives

- Evaluate ten additional soybean varieties to determine the levels of resistance to the new root-lesion nematode, *P. dakotaensis* detected in North Dakota.
- Establish a system to culture and increase pure population of *P. dakotaensis* for further investigating the nematode impact on soybean.
- Develop a real-time PCR assay to detect and quantify *P. dakotaensis* directly in DNA extracts from soybean plant roots.

c. Materials and Methods

To meet the objectives, soil samples were collected from a soybean field in Richland County during the summer 2023 where *P. dakotaensis* was first reported. These samples were properly homogenized into a composite sample to assure uniform distribution of nematodes. The initial nematode population density was then determined from three subsamples and the species was confirmed through PCR. For the resistance screening, ten varieties grown in the region, namely NS 2031NR2, NS 60743NXR2, NS 0571NLL, NS 0773NLL, P04A98E, P06A38E, P11T36E, P17A87E, Ashtabula, and JF30-93N were selected. Furthermore, Barnes was used as a positive

control and an unplanted control was used as a negative control. In addition to this, the cultivar having highest mean population density was used as a susceptible check in each trial.

The greenhouse trial consisted of twelve treatments and five replications in a completely randomized design. The large cone-type containers were filled with equal amount of the well mixed infested soil which was 500 g in Trial 1. Pre-germinated seeds were sown in the containers. The plants were subsequently grown in a controlled greenhouse environment for 15 weeks at 22 °C with a photoperiod of 16 hours. The experiment was repeated with 700 g of soil in each cone-type containers and same greenhouse conditions. Trial 2 was harvested 12 weeks after planting as the plants started turning yellow.

The aerial portion of each plant was removed at harvest, and soil with roots were taken for nematode extraction. The plant roots were separated from soil and nematodes were extracted from soil by Sugar Floatation method. The roots were washed under continuous stream of water to make them free from soil particles and blotted dry. Then the roots were weighed and cut into 1 to 2-cm pieces, mixed thoroughly and nematodes were extracted from roots using the Whitehead tray nematode extraction method. The extracted nematodes were collected in a nematode suspension vial through a 20 µm-aperture sieve. The root-lesion nematodes were identified to genus level and quantified under an inverted light microscope. The final population densities of root-lesion nematodes from both roots and soil were then averaged across the five replicates of each variety. The reproductive factor was determined by dividing the final mean population density by the initial population density (Table 1). In the second trial, 0.6 to 0.8 g of roots from each plant in one of the replications were separated for DNA extraction.

The varieties were classified into four different categories based on ratios of final mean population densities: Resistant (R), Moderately Resistant (MR), Moderately Susceptible (MS) and Susceptible (S) using the rating scale published (Chowdhury et al. 2022). The ratio of final population densities was cucullated by dividing the final mean population density in a test variety by the final density in the susceptible check. The variety having highest final mean population density among all varieties was used as the susceptible check. In this ranking, ratios were expressed in percentage, with less than or equal to 25%: R; 26% to 50 %: MR; 51 to 75%: MS; and greater than or equal to 76%: S.

In order to develop the pure culture of *P. dakotaensis* in lab, Gamborg's B5 medium along with the corn explant was used. The corn variety used was found to be the host of this nematode. Corn seeds were kept in agar medium for germination. After the roots were 1.5-inch, they were cut and allowed to grow in Gamborg's B5 medium. Once the root explant covered the whole petri-dish, they were inoculated with different numbers (10, 40, and 80) of nematodes (Table 3). Additionally, two other root-lesion nematode species, namely *P. penetrans* and *P. scribneri* were kept for pure culture as controls following the same procedures. The plates were incubated at 25˚C for 5 months, and their final number and reproductive factor were calculated. Moreover, a susceptible cultivar (Barnes) was grown in the greenhouse with *P. dakotaensis*-infested soil to increase the population which was used for obtaining pure population from plant roots.

To develop the real-time PCR (qPCR) assay for detecting and quantifying this new species directly in DNA extracts from infected roots, the primer set (IC-ITS1F/IC-ITS1R) was tested for its specificity with *P. dakotaensis*. The specificity test was conducted with other root-lesion nematode species (*P. scribneri, P. neglectus* and *P. penetrans*) reported in the region and other

common plant-parasitic nematodes. A two-fold serial dilution using DNA extracted from the roots inoculated with a single nematode was prepared to determine the detection sensitivity. 0.5 µl of PCR enhancer (Bovine Serum Albumin: BSA) was added to the reaction mixture and the DNA was extracted in triplicate and qPCR was performed in duplicate for each dilution factor. A standard curve was generated through sequential three-fold dilution of DNA extracted by inoculating 32 *P. dakotaensis* individuals in 0.2g of non-infected soybean roots. DNA extraction was done using the FastDNA Spin Kit and qPCR reaction was performed for each level of dilution. Melting curve analysis was done to monitor the specificity of the assay.

To validate the assay, varying number of *P. dakotaensis* (1, 5, 10, 15, and 20) were picked and added to 0.2 g of non-infected roots. DNA was extracted and qPCR was run for each level of inoculation. The numbers assayed by qPCR was plotted with the inoculated numbers of *P. dakotaensis*. In addition, nematodes from 11 soybean varieties' infected roots from the greenhouse bioassay were manually extracted and counted thrice under a microscope. DNA was extracted from each root sample in triplicate and assayed by the qPCR and standard curve. Correlation analysis was conducted to determine relationship between the nematode numbers determined by the qPCR assay and traditional microscopic method.

d. Research Results and Discussion

The first and second trials yielded similar resistance rating results with some differences in mean final population densities and reproductive factors. In Trial 1, the mean final population density and reproductive factor values of *P. dakotaensis* for the varieties ranged from 1,520 to 3,163 nematodes per container from both soil and roots and from 1.07 to 2.23, respectively. In Trial 2, these two parameters varied from 2,881 to 6,239 nematodes per container and from 1.52 to 3.28, respectively. The variability could be linked to the variation in initial soil quantity. The second trial used more amount of soil and expectedly had a greater number of initial inoculum than the first trial. Therefore, all of the varieties in Trial 2 had higher final population densities and reproductive factors compared to Trial 1.

In Trial 1, NS 2031NR2, NS 60743NXR2, NS 0571NLL, NS 0773NLL, P06A38E, P11T36E, and Ashtabula were moderately susceptible, P04A98E was moderately resistant, P17A87E and JF30-93N were susceptible. In Trial 2, NS 2031NR2, NS 60743NXR2, NS 0773NLL, P06A38E, P11T36E, P17A87E, and JF30-93N were moderately susceptible, NS 0571NLL was moderately resistant, P06A38E and Ashtabula were susceptible. The varieties (P04A98E and NS 0571NLL) that showed moderate resistance in one of the trials were only 2% or 4% less than 50% being classified as moderately susceptible. The variation in ratings could be due to minor difference in environment conditions (e.g. soil water content) and nematode populations (e.g. penetration ability). Combined results of the two trials revealed that one variety Ashtabula (76%) was susceptible and the remaining varieties were moderately susceptible to *P. dakotaensis* (Figure 1). The positive control Barnes (90%) was rated as a susceptible cultivar, supporting our previous results. None of the screened varieties were found to be resistant or moderately resistant.

A new system to culture and increase the population of *P. dakotaensis* was adopted using Gamborg's (GB5) media and corn explant in our lab. It was successful to increase the population of *P. dakotaensis* up to 10.42 times (Table 3). In the greenhouse bioassays, soybean (Barnes) had the average reproductive factor of 2.44.

The developed qPCR assay was specific to the target nematode *P. dakotaensis*. Inoculated soybean roots produced an average Cq (quantification cycle) value of 25.97 and a single peak at 81.5˚ C in melting curve analysis (Figure 2). Other three root-lesion species (*P. scribneri*, *P. penetrans* and *P. neglectus*), common plant-parasitic nematodes (*Paratylenchus* sp., *Paratrichodorous* sp., *Helicotylenchus* sp., *Xiphinema* sp., *Heterodera glycines*), and uninfected soybean roots were not amplified by the primers. The qPCR assay could detect up to $1/128^{th}$ of a single *P. dakotaensis* individual per 0.2 g soybean roots. The generated standard curve ($y = -3.3674x + 26.946$) showed a high amplification efficiency (E = 98.1%) and had a strong correlation between the Cq values and log number of nematodes added to uninfected roots ($R^2 = 0.9738$) (Figure 3). The standard curve was validated by a strong, positive correlation between the numbers of *P. dakotaensis* determined by qPCR and numbers of *P. dakotaensis* (1, 5, 10, 15, and 20) added to 0.2 g of uninfected soybean roots ($y = 0.6439x + 1.14$, $R^2 = 0.8407$) (Figure 4). Moreover, a positive correlation was found between qPCR estimates and manual counts of the root samples from infected roots of 11 soybean varieties including Barnes from the greenhouse bioassay.

e. Benefits to ND Soybean Farmers and Industry

The research successfully evaluated ten soybean varieties for resistance reactions to this newly identified root-lesion nematode species in ND. In addition to this, a new method for culturing and increasing the pure population of *P. dakotaensis* was developed, which will facilitate future investigations in its impacts on soybean growth and yield. Moreover, a specific and sensitive qPCR assay was developed, enabling the direct identification and quantification of *P. dakotaensis* in DNA extracts from soybean plant roots. These findings will help growers to choose soybean varieties rationally and also provide a crucial tool for early detection as well as quantification of this species which will play an important role in effective management of the nematode disease. Further research should focus on identifying resistance levels of more soybean varieties and identifying new sources of resistance enhancing overall crop performance.

Table 1. Mean final population density and reproductive factor of the new root-lesion nematode species (*P. dakotaensis*) on each of the soybean varieties and controls in two trials conducted.

		Mean final population density*		Reproductive factor	
Variety ID	Maturity Group	Trial 1	Trial 2	Trial 1	Trial 2
NS 2031NR2	2.0	1,826	3,626	1.29	1.91
NS 60743NXR2	0.7	1,752	4,106	1.24	2.16
NS 0571NLL	0.5	1,905	2,881	1.34	1.52
NS 0773NLL	0.7	1,751	3,789	1.24	1.99
P04A98E	0.4	1,520	4,182	1.07	2.20
P06A38E	1.4	1,893	5,302	1.34	2.79
P11T36 E	1.1	1,817	4,090	1.28	2.15
P17A87E	1.7	2,504	3,829	1.77	2.02
Ashtabula	0.4	1,659	6,239	1.17	3.28
Barnes	0.3	3,163	5,014	2.23	2.64
JF30-93N	-	2,912	3,214	2.06	1.69
Non-planted control	-	130	1,252	0.09	0.66

*Mean final population density was determined by averaging five replicates of each of the varieties planted in large cone-type containers with 500g soil in Trial 1 and 700 g soil in Trial 2. Reproductive factor was determined by dividing the average mean final population density by the initial population density.

Table 2. Resistance responses of the soybean varieties tested to the new root-lesion nematode species (*Pratylenchus dakotaensis*).

		Trial 1		Trial 2	
Variety ID	Maturity Group	Ratio* (%)	Resistance rating	Ratio* (%)	Resistance rating
NS 2031NR2	2.0	57.7	MS	58.1	MS
NS 60743NXR2	0.7	55.4	MS	65.8	MS
NS 0571NLL	0.5	60.2	MS	46.2	MR
NS 0773NLL	0.7	55.4	MS	60.7	MS
P04A98E	0.4	48.1	MR	67.0	MS
P06A38E	1.4	59.9	MS	85.0	S
P11T36 E	1.1	57.5	MS	65.6	MS
P17A87E	1.7	79.2	S	61.4	MS
Ashtabula	0.4	52.5	MS	100.0	S
Barnes	0.3	100.0	S	80.4	S
JF30-93N	-	92.1	S	51.5	MS
Non-planted control	-	4.1	_	20.1	-

* Ratio of final population densities (%) = (final population density in a test line/ final population density of the susceptible check) x 100. The ratio for each variety and the non-planted control was then averaged across the five replicates. The rating was categorized based on the ratio: Resistant = R (postharvest population density ≤ 25 % of the susceptible check), Moderately Resistant = MR (26-50 %), Moderately Susceptible = MS (51-75 %), and Susceptible = S (≥ 76 %). Barnes was selected as the susceptible check in Trial 1 as it showed the highest reproduction of *P. dakotaensis*. Ashtabula was selected as the susceptible check in Trial 2 as it showed the highest reproduction of this nematode.

Table 3. Reproduction of *Pratylenchus dakotaensis* in Gamborg's B5 (GB5) media with corn explants as compared to two other root-lesion nematode species (*P. penetrans* and *P. scribneri*).

Species	Explant variety	No. of nematodes inoculated	Months incubated	Final population	RF*
P. dakotaensis	IO Chief	10	5	104.17	10.42
P. dakotaensis	IO Chief	40	5	26.04	0.65
P. dakotaensis	IO Chief	80	5	546.88	6.84

P. penetrans	X5B-8801	10	5	85.23	8.52
P. penetrans	IO Chief	40	5	580.36	14.51
P. penetrans	IO Chief	80	5	363.78	4.55
P. scribneri	X5B-8801	10	5	966.72	96.67
P. scribneri	IO Chief	40	4	1,206.60	30.16
P. scribneri	IO Chief	80	4	207.22	2.59

* RF= Reproductive Factor which is the ratio of final nematode number divided by the initial nematode number inoculated into each plate with the media. The plate having highest RF among three replications was represented in the table.

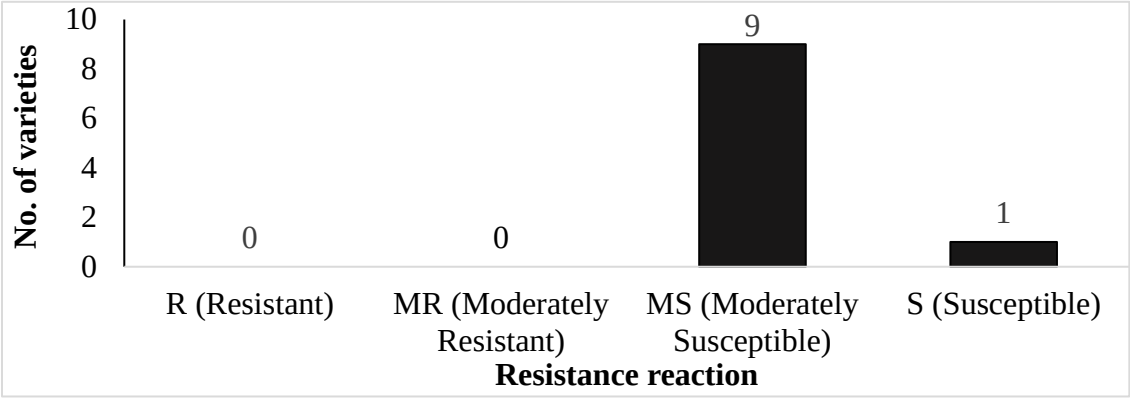

Figure 1. Classification of the resistance responses of ten soybean varieties to the new root-lesion nematode species (*Pratylenchus dakotaensis*) based on the combined data of the two trials.

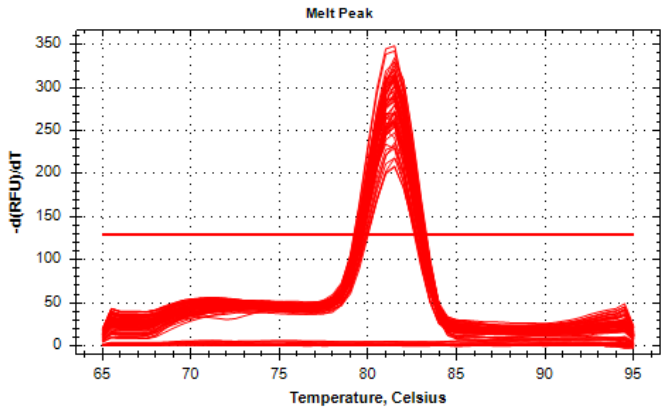

Figure 2. *Pratylenchus dakotaensis* melting curve profile for the qPCR assay. A single melting peak was observed at 81.5˚C. No amplification was observed for negative controls.

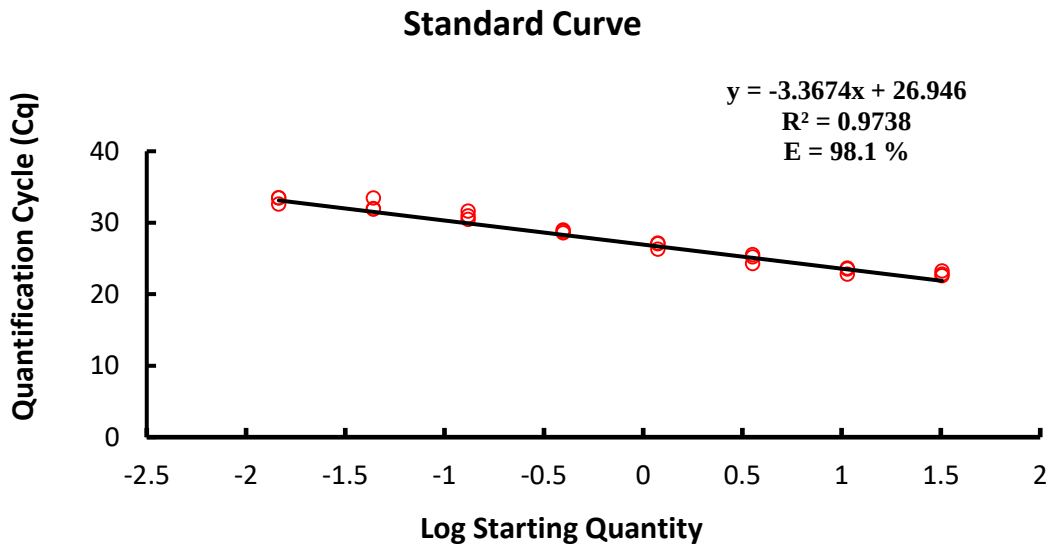

Figure 3. The standard curve with regression equation and co-efficient of determination (R^2) for the qPCR assay developed for detection and quantification of *P. dakotaensis* in soybean roots. Quantification cycle (Cq) was plotted against the log of the number of *P. dakotaensis* by sequential three-fold dilutions of DNA from 32 nematodes added to 0.2 g of uninfected roots. Each red dot represents an average of three biological replicates for each dilution run in duplicate. Amplification efficiency (E) = $10^{(1/-m)} - 1$, where m is the slope of the regression equation.

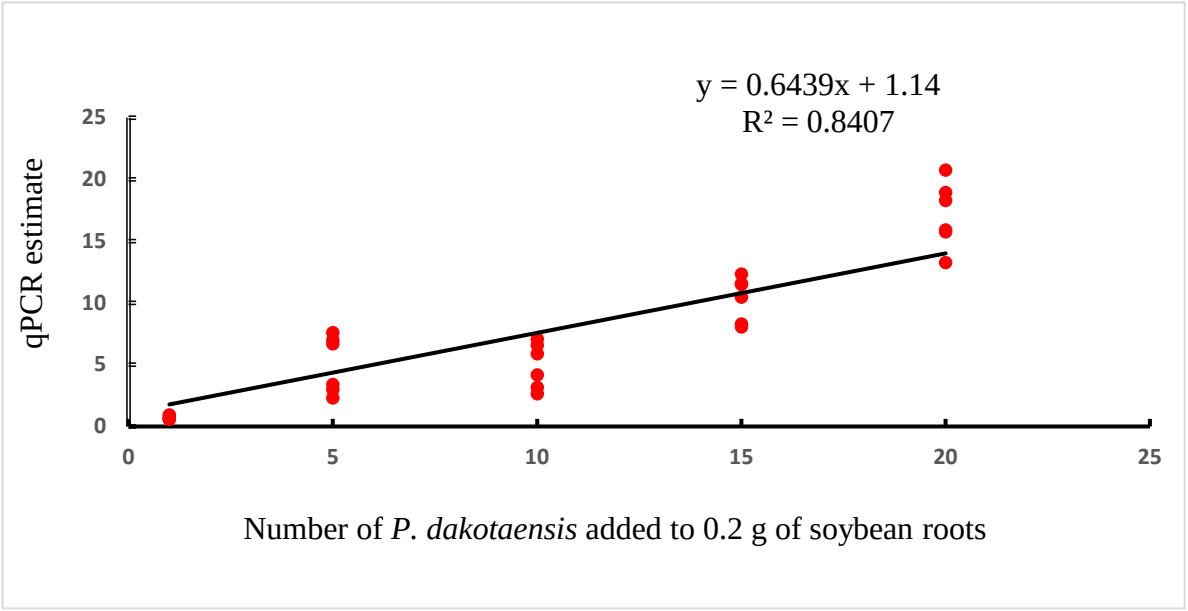

Figure 4. Correlation analysis between the numbers of *Pratylenchus dakotaensis* determined by the qPCR assay and the numbers of *P. dakotaensis* (1, 5, 10, 15, and 20) added to 0.2 g of uninfected soybean roots. DNA was extracted in duplicate and the qPCR was run in triplicate.