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Evaluation of *Glycine max* genotypes (maturity groups IV and V) for resistance to *Meloidogyne enterolobii* via phenotypic and GWAS assays

Research Article

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Abstract: The production of soybean is a vital industry within North Carolina (NC) and the United States, accounting for millions of dollars in annual revenue, yet is currently under threat due to the presence of *Meloidogyne enterolobii*. Plant resistance is a crucial management technique for this nematode, but *M. enterolobii* is known to overcome resistant cultivars that have suppressed infection by other root-knot nematodes such as *M. incognita*. Therefore, identifying novel resistance genes that work antagonistically to *M. enterolobii* is paramount. In this study, 198 lines of *Glycine max* were assessed for resistance to *M. enterolobii* through a greenhouse assay. During initial screening, soybean lines were inoculated with a NC isolate of *M. enterolobii* and assessed after 60 days for resistance using three metrics, including root galling severity, reproduction factor, and eggs per gram of root. The ten most resistant soybean lines were then tested in a confirmation screening. Three soybean lines: PI 548555, PI 639740, and PI 556852, were confirmed to be resistant with all three lines resulting in resistance metrics under the defined resistance threshold. The use of these lines could provide much needed support to soybean growers in NC and allow for novel resistance loci or genes to be bred into existing cultivars. A Genome-wide association study analysis was performed using publicly available marker data for resistance traits, but no significant associations were detected in the current analysis.

Keywords: *Bioassay • Genome-wide association study • Guava root-knot nematode • Resistant cultivar • Soybean*

Soybean (*Glycine max*) is a vital crop in North Carolina (NC) and national agriculture. Soybean is planted on over 1.6 million acres in NC, which generates up to 600 million USD annually (USDA-NASS, 2025). In the US, soybeans were planted over 87.2 million acres in 2021, accounting for 31% of soybean production globally (American Soybean Association, 2022). US soybean production is under threat from an economically devastating root-knot nematode, *Meloidogyne enterolobii* (guava root-knot nematode).

Meloidogyne enterolobii is a known pathogen of soybean and is an obligate parasite that enters the host plant roots to feed (Philbrick et al., 2020). The nematode uses a needle-like mouthpart named a stylet to probe and release cellulolytic and proteolytic enzymes that allow for the nematode to move toward the vascular cylinder of the host (Philbrick et al., 2020). Once it has reached the host's vascular system, the nematode becomes sedentary and uses its stylet to create a feeding site composed of "giant cells." These giant cells are 5–7 plant cells that surround the head of the nematode and are formed through the deregulation of the plant cell's cycle (Favery et al., 2016). The giant cells are multinucleated, enlarged cells that have undergone nuclear division, but not cytokinesis. The juvenile nematodes undergo a series of molts while at the feeding site, reaching the mature adult stage after the final molt. Once the adult females are mature, they become swollen with eggs, which are then laid on the outside of the root in a gelatinous matrix. In a full lifecycle of 30–35 days, one *M. enterolobii* can typically produce 500–1,000 eggs via parthenogenesis (Philbrick et al., 2020). The giant cells stimulate the surrounding plant cells to divide rapidly creating large galls on the plant roots, which are detrimental to the overall root architecture and therefore the life of the plant (Favery et al., 2016). Symptoms produced by

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this nematode can include chlorosis, stunting, and most importantly for growers, a reduction in yield.

Meloidogyne enterolobii is currently found in 16 counties in NC (Bonyak et al., 2024). The first report of a field infestation of *M. enterolobii* in NC was on soybean and cotton plants in two counties in 2011, which shows the ability of *M. enterolobii* to parasitize and reproduce on soybean as well as its ability to rapidly spread (Ye et al., 2013). The geographic distribution of this nematode in the central Coastal Plains region of NC is a concern, as the population increases rapidly through quick reproduction and is easily spread through soil attached to machines, tools, even footwear (Philbrick et al., 2020). *Meloidogyne enterolobii* has a very broad range of host plants, including sweet potato, which is often rotated with soybean in this region (Gorny et al., 2023).

There are a number of management techniques that can mitigate the effects and population growth of *Meloidogyne enterolobii* in agricultural fields. Types of disease management for root-knot nematodes includes cultural, biocontrol, chemical, and plant resistance (Azlay et al., 2023). Plant host resistance is one of the most effective management strategies for root-knot nematodes while also being environmentally sustainable (Schwarz and Gorny, 2024). Resistance genes within the host plant can initiate a hypersensitive response (cell death) in the cells of feeding sites, decrease reproduction, or inhibit effectors released by the root-knot nematode (Saucet et al., 2016; Sato et al., 2019; Bali and Gleason, 2024). Studying host resistance is critically important for agricultural stability and proper management of *M. enterolobii*.

Once a number of plants from a resistance assay have been identified, genomic commonalities can be determined through analyses such as a genome-wide association study (GWAS). A GWAS examines plant cultivar genomes for genomic variants that could be potentially associated with phenotypic traits such as resistance to *M. enterolobii*. Identification of a genetic marker facilitates the transfer of resistance to agronomically valuable progeny (Balding et al., 2019). In 1994, a single gene *Rmi1* was found to confer partial resistance in soybean to *Meloidogyne incognita,* but when tested against isolates of *M. enterolobii,* the gene was overcome and the resistance was not maintained (Luzzi et al., 1994; Schwarz and Gorny, 2024). Plant resistance is a key tool for regulating *M. enterolobii* and research that pursues new sources of resistance are vital for the continued management of this pathogen.

A study conducted by Schwarz and Gorny (2024) evaluated *Glycine max* and *Glycine soja* genotypes for novel sources of resistance to *M. enterolobii*. *Glycine max* is the preferred soybean species for commercial breeding programs, and has been cultivated to promote favorable phenotypic traits for growers, such as higher yield and vigor (Schwarz and Gorny, 2024). Wild soybean, *Glycine soja*, although not possessing the phenotypic traits required by growers, is an important pool of genetic diversity and may be a source of resistance genes to regulate nematode infections (Schwarz and Gorny, 2024). Of the 72 *Glycine soja* and 44 *Glycine max* lines tested by Schwarz and Gorny (2024), only five of the *G. soja* were found to be resistant and none of the *G. max* showed resistance to *M. enterolobii*. While this study was successful in analyzing the host status of these soybean lines, it was not able to find any resistance to *M. enterolobii* in the commercially viable *G. max* lines. By shifting the focus toward *Glycine max* genotypes and increasing the total number of soybean lines evaluated, there is potential to find resistance. The objective of this greenhouse screening trial was to assess diverse soybean genotypes (*Glycine max*) for resistance to *M. enterolobii*.

1. Materials and methods

1.1 Planting materials

The evaluation of 198 unique lines of *Glycine max* for resistance to *M. enterolobii* was conducted at NC State University Method Road Greenhouse facilities. Soybean seed was obtained from the USDA-ARS Germplasm Resources Information Network (GRIN). While the selection of lines for inclusion in the study was ultimately random, the criteria for selection of lines included those in the maturity groups IV or V (ideal for growing in the South and Mid-Atlantic regions) and coming from diverse geographical locations (to capture potential diverse sources of resistance).

1.2 Experimental design and inoculation

The lines were randomized and divided into four rounds (R1, R2, R3, and R4), where each line was tested twice in an initial screening experiment. Each round included five tomato plants (“Rutgers”), which are susceptible to *M. enterolobii*, as positive inoculation controls. Also included in each round of the initial screen were five *Glycine max* lines that were among the least susceptible genotypes identified in a previous study (Schwarz and Gorny 2024) (PI 646156, PI 614732, PI 562611, PI 527702, and PI 629013) to act as susceptible controls. Three seeds of each line were sown into a 1:1 mix of steam sterilized sand and soil in a 10.16 cm diameter plastic pot, then thinned to one plant

per pot after emergence. Plants were fertilized once at planting using Osmocote Smart-Release Plant Food (The Scotts Company, Marysville, OH).

The plants were grown for 20 days in a greenhouse with temperatures maintained at 27–28°C. At 20 days after seeding, each plant was inoculated with 3,000 *M. enterolobii* eggs. The inoculum was produced from a NC isolate (Schwarz et al. 2020) maintained on tomato ("Rutgers") and bell pepper ("California Wonder") in the greenhouse. The egg inoculum was collected from the culture plants using the NaOCl extraction method of Hussey and Barker (1973). The roots of culture plants were removed from the pots, rinsed in water to remove excess soil, then massaged by hand in a 10% bleach solution (Clorox, The Clorox Company, Oakland, CA) to release the eggs from the roots. The egg and bleach solution was then poured over a set of stacked sieves (from bottom to top, 25, 75, and 250 µm) allowing for the collection of clean eggs on the bottom sieve and thorough washing of any remaining bleach solution. The eggs were decanted from the bottom sieve into a clean 50 mL Falcon tube along with 35 mL of water. A sucrose solution (70% w/v sugar) was added to the Falcon tube to bring the total volume up to 50 mL. The Falcon tube was centrifuged at 800 ***g*** for 5 min to allow the eggs to float in the sucrose solution while remaining soil sank to the bottom. The contents of the Falcon tube were poured over the 25 µm sieve, avoiding the debris pellet, and the eggs were rinsed to remove any remaining sucrose. The Falcon tube was rinsed and used to collect the cleaned eggs off the sieve. The eggs were tallied by taking three 100 µL aliquots from the total sample and counted using an inverted Nikon TMS microscope (Nikon Instruments, Melville, NY). The three counts were averaged and multiplied by 10 to determine the number of eggs per milliliter. The calculations were completed to determine the number of milliliters needed to inoculate the soybean lines with 3,000 eggs each.

Plants were inoculated by creating a small, shallow hole (1 cm × 2.5 cm) in the soil near the base of the plant, pipetting the inoculum solution into the hole, and then closing the hole. The plants were maintained through daily watering, increasing to twice daily watering in the summer, in the greenhouse for 60 days post inoculation. The initial screening experiments were carried out from May 2024 to November 2024.

1.3 Data collection

At 60 days post inoculation, the plants were cut at the soil line to remove foliage. The foliage for each plant was weighed. The root systems were removed from the pot and cleaned thoroughly to remove the soil. The gall severity ratings were determined by examining the clean root architecture and assigning a percentage using the modified rating scale of Bridge and Page (1980). The gall severity rating was used as one metric to quantify resistance; soybean lines with less than or equal to 10% galling were identified as possibly resistant and were included in the confirmation screening test. The roots of all plants were weighed, then underwent the NaOCl extraction method as described above to extract *M. enterolobii* eggs, resulting in a 50 mL egg solution from each plant. Three, 100 µL aliquots were taken from the egg solution and eggs were counted. The counts for the three aliquots were averaged, then multiplied by 10 to estimate the eggs present in 1 mL. This value was then multiplied by 50 to determine the eggs within the total 50 mL egg solution and estimate the total number of *M. enterolobii* eggs per root system. The eggs per gram of root for each plant was calculated by taking the total number of eggs and dividing by the root weight. Soybean lines with less than or equal to 500 eggs per gram of root tissue were identified as possibly resistant. The reproductive factor (RF) was used to quantify the ability of *M. enterolobii* to reproduce successfully on the host plant root system. To calculate this, the final population (total number of eggs extracted at the termination of the experiment) was divided by the initial population (number of eggs used for inoculation, herein 3,000). This ratio was utilized to define the host status of the soybean line, where an RF value <1 indicates host resistance and an RF value >1 indicates host susceptibility.

1.4 Confirmation screening experiment

Once all 198 lines were assessed twice for resistance status using the defined resistance metrics of eggs per gram of root (<500 eggs/g root), gall severity rating (<10%), and RF values (<1), the ten soybean lines exhibiting the greatest degree of resistance to *M. enterolobii* using these metrics were selected for a confirmation screening experiment. This experiment was performed as described above for the initial screening rounds, except soybean lines were tested in high replicate numbers (ten replicates per line). This round also included soybean lines PI 556852 and PI 548559; these lines had an average gall rating of 0% while still having other metrics within the susceptible range. The "Rutgers" tomato plants were included as positive controls in the confirmation screening. Two soybean lines identified as susceptible from a previous study (Schwarz and Gorny 2024), PI 562611 and PI 527702, and two soybean

lines (PI 548330 and PI 548386) tested in the initial screening that had extremely high RF values were included as susceptible controls. The confirmation screening was conducted using the same protocols as above. An additional metric, the reproductive index (RI), was calculated to allow for a standardized value to be compared across trials and locations. The RI was calculated by taking the RF for a soybean line and dividing it by the average RF of the tomato controls (herein, RF = 11.45) and then multiplying by 100.

1.5 Analysis of greenhouse data

The statistical analysis was completed in RStudio (version 4.4.1; R Core Team, 2024). The data from the initial screening were not statistically analyzed and instead were observed for evidence of potentially phenotypic resistant lines for the confirmation screening. The data from the confirmation screening were assessed and found to have a non-normal distribution for the following variables: RF, root galling severity, and eggs per gram of root. Therefore, non-parametric methods were used to assess the effect of soybean genotype on these response variables. The effect of soybean line on RF, root galling severity, and eggs per gram of root were evaluated using the Kruskal–Wallis rank sum test. A significant result was found at the $\alpha = 0.05$ level for the Kruskal–Wallis test for each of these response variables. To separate the means, a Dunn's Test was completed using the Holm correction. The Dunn's Test computed the multiple comparisons between soybean and control lines and an adjusted p-value of 0.05 was used to determine significance between the pairwise comparisons. The RI for each soybean line was transformed ($\log10(x + 1)$) to fit assumptions of normality and an ANOVA test indicated that the soybean line had a significant effect on RI at the $\alpha = 0.05$ level. To separate mean values, Fisher's least significant difference (LSD) test was applied to identify which soybean lines were significantly different at the $\alpha = 0.05$ level.

1.6 GWAS

The USDA Soybean Germplasm collection has previously been genotyped with the SoySNP50K beadchip, and the marker data from this study are publicly available on soybase (Grant et al., 2009; Song et al., 2013). While these public data had high density SNP data for 152 of the soybean lines used in this study, they did not have marker data for 46 lines, for which we had the phenotypic data. To obtain genotypic data for the remaining 46 lines, 25 seeds of each line were germinated on germination paper for 5 days after which sprouted root tips were harvested and frozen in liquid nitrogen. Frozen root tissue was then lyophilized for 2 days in a VirTis Benchtop Pro freeze dryer, and DNA was then extracted from the lyophilized tissue using a Qiagen DNEasy Plant Mini DNA extraction kit (Qiagen Sciences Inc., Germantown, MD) following the manufacturers protocol. Extracted DNA was then genotyped with the BARCSoySNP6K genotyping assay at the Soybean Genomics and Improvement Lab in Beltsville, MD, USA (Song et al., 2014). Lower density marker data from these 46 lines were then imputed to the higher density marker set in the publicly available data using the AlphaPlantImpute2 software (Niehoff et al., 2022).

The rTASSEL software was used to perform GWAS analysis in R version 4.5 (Monier et al., 2022). Principal components were calculated with the pcaMethods package, and the first three principal components were used in the analysis to control for population structure (Stacklies et al., 2007). The presence of population structure was checked using quantile-quantile plots with rTASSEL. Total egg count, eggs per gram of root, gall rating, and reproduction factor phenotypes were analyzed in the GWAS analysis and all were log-transformed prior to analysis to normalize their distributions. Marker quality control was performed with rTASSEL. Markers with a minor allele frequency above 0.05, a maximum heterozygosity of 0.2, and a missing frequency below 0.15 were retained in the analysis. The centered IBS method was used to calculate a kinship matrix, and the mixed linear model method was used to perform the GWAS analysis. A Bonferroni correction was utilized to account for multiple testing.

2. Results

2.1 Initial screening host status

In the initial screening experiments (R1 through R4), eight soybean genotypes were found to be resistant to *M. enterolobii* (Tables 1–4). In In R1, 26 the soybean lines were foundto be resistant (RF < 1) including PI 548682, PI 548555, PI 639740, PI 548518, PI 548339, PI 548401, PI 553052, PI 556912, PI 548548, PI 548627, PI 595362, PI 619232, PI 559931, PI 548546, PI 506417, PI 548392, PI 548431, PI 598358, PI 548619, PI 612146, PI 548422, PI 556860, PI 559934, PI 548602, PI 595626, and PI 548466) and three of the susceptible control lines were found to

Table 1. Response of soybean lines to infection by *Meloidogyne enterolobii* in an initial greenhouse screening experiment, round 1. [a]All soybean lines, including the susceptible controls, were planted once (n = 1) and the positive inoculation controls, tomato, had five replicates (n = 5). Plants were inoculated with 3,000 eggs of *M. enterolobii* and evaluated at 60 days post inoculation. The table includes the root galling severity as a percentage, eggs per gram of root tissue (Eggs g^{-1} of root), and RF for each line tested. Lines were scored as susceptible (S, RF > 1) or resistant (R, RF < 1)

Soybean line	Root gall severity (0–100%)	RF	Eggs g^{-1} of root	Susceptible or resistant
PI 548682	0	0.28	54	R
PI 548555	1	0.11	16	R
PI 639740	1	0.17	59	R
PI 548518	1	0.17	41	R
PI 548339	1	0.06	16	R
PI 548401	0	0	0	R
PI 553052	0	0.44	145	R
PI 556912	0	0.17	55	R
PI 548548	0	0.17	45	R
PI 548627	2	0.22	106	R
PI 595362	1	0.67	131	R
PI 619232	1	0.56	105	R
PI 559931	0	0.44	59	R
PI 553048	10	3.33	714	S
PI 508269	4	12.91	3,073	S
PI 670461	5	10.33	1,512	S
PI 556546	7	6.50	1,204	S
PI 548546	3	0.78	182	R
PI 597384	3	7.94	973	S
PI 543793	1	16.00	1,644	S
PI 597388	5	4.56	607	S
PI 632418	10	7.00	1,250	S
PI 556913	2	8.78	1,017	S
PI 543794	7	3.33	503	S
PI 548413	0	1.56	188	S
PI 548390	2	3.22	486	S
PI 506417	0	0.16	31	R
PI 548392	1	0.17	38	R
PI 590932	3	2.00	216	S
PI 667740	5	6.39	668	S
PI 544354	10	7.06	1,126	S
PI 556876	30	18.50	2,382	S
PI 548475	50	16.00	1,420	S
PI 548458	60	10.50	1,221	S
PI 548696	40	10.83	778	S
PI 548977	90	15.39	2,308	S
PI 533605	25	16.83	1,048	S
PI 548678	3	3.72	1,074	S
PI 355067	35	4.33	508	S
PI 665036	80	14.11	1,647	S
PI 548342	20	23.00	2,091	S
PI 548633	80	5.28	701	S
PI 548364	2	4.83	558	S
PI 548343	10	29.50	4,116	S
PI 614155	80	5.78	610	S
PI 556576	75	11.17	753	S
PI 593653	5	4.28	3,774	S
PI 548431	0	0.06	208	R
PI 548430	55	4.22	379	S
PI 548415	50	5.00	593	S
PI 664026	3	3.72	221	S
PI 510670	60	13.72	1,486	S
PI 540884	60	8.11	891	S
PI 548671	2	1.06	434	S
PI 561218	75	9.78	1,281	S
PI 548645	50	11.56	1,981	S
PI 561219	80	7.39	972	S
PI 548563	60	23.72	1,888	S
PI 540555	30	9.61	1,153	S
PI 607380	40	15.44	2,032	S
PI 598358	0	0.11	14	R
PI 548619	1	0.78	84	R
PI 548613	70	16.17	2,354	S
PI 612146	1	0.83	102	R
PI 548626	20	5.22	933	S
PI 518668	7	4.17	510	S
PI 556846	10	4.61	699	S
PI 664027	5	3.67	595	S
PI 548344	50	1.39	152	S
PI 620883	2	6.17	826	S
PI 576440	1	3.83	485	S
PI 548422	2	0.94	140	R
PI 556860	1	0.44	150	R
PI 559934	0	0.50	167	R

(Continued)

Table 1: *Continued*

Soybean line	Root gall severity (0–100%)	RF	Eggs g^{-1} of root	Susceptible or resistant
PI 548547	1	1.67	255	S
PI 561191	5	8.94	1,491	S
PI 548386	50	40.61	10,413	S
PI 597382	6	4.00	510	S
PI 548532	7	1.72	270	S
PI 508268	5	5.44	770	S
PI 548350	10	11.33	2,099	S
PI 553051	10	9.28	1,435	S
PI 560207	0	4.94	693	S
PI 548602	1	0.06	325	R
PI 595626	3	0.56	85	R
PI 556871	2	2.28	419	S
PI 548466	1	0.72	141	R
PI 593654	1	5.83	946	S
PI 559370	1	8.67	1,444	S
PI 646156 (Control)	0	0.11	21	R
PI 614732 (Control)	5	3.94	497	S
PI 562611 (Control)	0	0.17	38	R
PI 629013 (Control)	0	0.17	93	R
Tomato Control	12	0.37	221	S

[a]Lines from round 1 that either did not germinate or died shortly after inoculation include: PI 556826, PI 561596, PI 518664 (seed no longer available at GRIN), PI 556820, PI 601983, PI 548464, PI 577798, PI 561599, PI 556930, PI 527702 (Control), Tomato 1.

be resistant (RF < 1, PI 646156, PI 562611, and PI 629013) (Table 1). In R2, 17 soybean lines were found to be resistant (RF < 1, PI 593256, PI 548667, PI 355070, PI 548598, PI 548679, PI 556906, PI 604100, PI 548559, PI 631122, PI 512039, PI 556852, PI 606748, PI 548309, PI 598222, PI 548622, PI 548359, and PI 543856) and one susceptible control line was found to be resistant (RF < 1, PI 527702) (Table 2). In R3, 11 soybean lines were determined to be resistant (RF < 1, PI 639740, PI 619232, PI 670461, PI 556546, PI 548392, PI 665036, PI 561218, PI 540555, PI 548532, PI 556871, and PI 548466) and one susceptible control line was found to be

Table 2. Response of soybean lines to infection by *Meloidogyne enterolobii* in an initial greenhouse screening experiment, round 2. [b]All soybean lines, including the susceptible controls, were planted once (n = 1) and the positive inoculation controls, tomato, had five replicates (n = 5). Plants were inoculated with 3,000 eggs of M. *enterolobii* and evaluated at 60 days post inoculation. The table includes the root galling severity as a percentage, eggs per gram of root tissue (Eggs g^{-1} of root), and RF for each line tested. Lines were scored as susceptible (S, RF > 1) or resistant (R, RF < 1)

Soybean line	Root gall severity (0–100%)	RF	Eggs g^{-1} of root	Susceptible or resistant
PI 593256	0	0.15	44	R
PI 548991	20	4.11	512	S
PI 548346	20	11.22	2371	S
PI 595363	10	3.41	451	S
PI 606749	2.5	3.86	566	S
PI 548667	3	0.67	107	R
PI 355070	0	0.08	19	R
PI 518673	25	5.03	1376	S
PI 548402	20	2.72	287	S
PI 633970	50	7.89	568	S
PI 548598	0	0.06	15	R
PI 548679	1	0.39	82	R
PI 556635	30	1.33	88	S
PI 548654	3.5	3.41	629	S
PI 534646	50	10.31	1844	S
PI 548987	10	1.44	156	S
PI 556906	0	0.17	23	R
PI 604100	1	0.56	157	R
PI 548559	0	0.06	13	R
PI 548410	5	2.11	603	S
PI 548428	60	14.67	1588	S
PI 564525	70	9.11	882	S
PI 548549	7	1.56	328	S
PI 548439	7	6.06	804	S
PI 548441	1	6.00	1104	S
PI 550734	10	9.22	1390	S
PI 595765	5	5.72	777	S
PI 556841	33	5.42	714	S
PI 631122	5	0.56	38	R
PI 596540	35	6.00	684	S
PI 561400	25	3.78	281	S
PI 560206	1	2.74	596	S
PI 512039	0	0.17	30	R
PI 556852	0	0.06	5	R
PI 611112	7	8.38	1497	S

(Continued)

Table 2: *Continued*

Soybean line	Root gall severity (0–100%)	RF	Eggs g^{-1} of root	Susceptible or resistant
PI 606748	0	0.28	45	R
PI 548309	0	0.36	61	R
PI 550731	20	4.00	508	S
PI 553049	25	12.28	1835	S
PI 598222	0	0.06	9	R
PI 556711	30	3.78	290	S
PI 615582	20	9.60	1051	S
PI 548517	10	5.89	1077	S
PI 548622	0.33	0.57	105	R
PI 614806	5	3.78	640	S
PI 553043	5	2.94	24	S
PI 548586	50	5.11	518	S
PI 548606	10	6.44	1510	S
PI 553038	2	11.89	1518	S
PI 548330	55	17.89	2736	S
PI 612608	2	12.33	3190	S
PI 572240	7.5	4.19	713	S
PI 548514	15	3.78	389	S
PI 548685	0	1.10	198	S
PI 572239	50	7.89	947	S
PI 583366	25	2.50	199	S
PI 548359	3.5	0.64	141	R
PI 602496	20	2.83	659	S
PI 543856	0	0.33	66	R
PI 548976	60	12.56	1235	S
PI 586981	1	1.67	177	S
PI 525454	80	12.00	1040	S
PI 646156 (Control)	1	2.83	500	S
PI 614732 (Control)	5	3.83	383	S
PI 562611 (Control)	20	6.78	992	S
PI 527702 (Control)	10	0.56	83	R
PI 629013 (Control)	15	1.33	165	S
Tomato 1	24	2.97	688	S

[b]Lines from round 2 that either died or did not germinate include: PI 630984, PI 355068, PI 561597, PI 548674, PI 618809, PI 533654, PI 548460, PI 533654, PI 548603, PI 556773, PI 564849, PI 583288, PI 556834, PI 636463, PI 633567, PI 550732, PI 548541, PI 665035, PI 548429, PI 560307, PI 556506, PI 553049, PI 548669, PI 548301, PI 550733, PI 564082, PI 527701, PI 596414, PI 54853.

resistant (RF < 1, PI 629013) (Table 3). In R4, three soybean lines were found to be resistant (RF < 1, PI 548392, PI 548654, and PI 525454) (Table 4).

2.2 Selection of soybean lines for confirmation screening

A total of ten suspected resistant lines were included in the confirmation screen. All but two lines were under the threshold of resistance for the following metrics: galling rating, RF value, and eggs per gram of root (PI 548682, PI 639740, PI 619232, PI 559931, PI 548392, PI 595626, PI 548466, and PI 561576). Two other soybean lines had RF values slightly above 1.0 but had indications of resistance to *M. enterolobii* for their gall ratings and eggs per gram of root values (PI 548555 and PI 559934).

2.3 Root galling severity

The Kruskal–Wallis test found that at least one soybean genotype had a significantly different root gall severity rating ($P < 0.05$). The Dunn Test indicated that soybean lines PI 548682, PI 639740, PI 559931, PI 548466, PI 548555, PI 556852, and PI 548386 were significantly different than the tomato control ($P < 0.0001$, Table 5) (Figs 1 and 2).

2.4 RF

The Kruskal–Wallis test found that at least one soybean (*Glycine max*) genotype had a significantly different RF ($P < 0.05$). The Dunn Test indicated that soybean lines PI 548682, PI 639740, PI 559931, PI 559934, PI 548555, and PI 556852 were significantly different than the tomato control ($P < 0.0001$, Table 5).

2.5 Eggs per gram of root

The Kruskal–Wallis test found at least one soybean genotype to have a significantly different number of eggs per gram of root ($P < 0.05$). The Dunn test indicated that soybean lines PI 548682, PI 639740, PI 559931, PI 559934, PI 548555, PI 556852, and PI 562611 (control line) were significantly different than the tomato control ($P < 0.0001$, Table 5).

Table 3. Response of soybean lines to infection by *Meloidogyne enterolobii* in an initial greenhouse screening experiment, round 3. [c]All soybean lines, including the susceptible controls, were planted once (n = 1) and the positive inoculation controls, tomato, had five replicates (n = 5). Plants were inoculated with 3,000 eggs of M. *enterolobii* and evaluated at 60 days post inoculation. The table includes the root galling severity as a percentage, eggs/g root tissue (Eggs g^{-1} of root), and RF for each line tested. Lines were scored as susceptible (S, RF > 1) or resistant (R, RF < 1)

Soybean line	Root gall severity (0–100%)	RF	Eggs g^{-1} of root	Susceptible or resistant
PI 548682	12.5	0.81	132	S
PI 548555	5	2.22	179	S
PI 639740	10	0.56	42	R
PI 548401	34	2.57	387	S
PI 548548	60	4.61	498	S
PI 619232	0	0.11	15	R
PI 559931	15	1.44	175	S
PI 553048	10	2.28	594	S
PI 508269	5	8.94	1,214	S
PI 670461	10	1.00	91	R
PI 556546	5	0.44	33	R
PI 548546	50	7.72	1,114	S
PI 543793	15	1.78	237	S
PI 597388	30	1.72	146	S
PI 632418	20	0.72	42	S
PI 556913	10	2.83	478	S
PI 543794	20	2.94	429	S
PI 548413	5	4.94	1,514	S
PI 548390	60	8.28	1,364	S
PI 506417	30	17.17	1,565	S
PI 548392	0	0.89	202	R
PI 561596	32.5	3.61	808	S
PI 667740	10	1.11	154	S
PI 556876	25	9.17	986	S
PI 548475	25	1.62	103	S
PI 548458	20	2.33	149	S
PI 548696	10	3.89	525	S
PI 533605	25	3.56	544	S
PI 548678	60	10.89	981	S
PI 355067	10	3.17	642	S
PI 665036	1	1.06	110	R
PI 548342	10	1.83	224	S
PI 548633	25	11.22	1,433	S
PI 548364	40	2.06	339	S
PI 548343	5	5.56	947	S
PI 614155	15	2.33	236	S
PI 556576	20	2.06	147	S
PI 593653	10	3.28	692	S
PI 548431	15	9.56	1,129	S
PI 548430	60	2.72	391	S
PI 548415	25	5.83	931	S
PI 664026	10	3.44	517	S
PI 510670	1	2.33	761	S
PI 540884	50	3.72	395	S
PI 548671	60	5.28	851	S
PI 561218	10	0.61	50	R
PI 548645	1	3.33	758	S
PI 561219	60	9.61	1,872	S
PI 548563	50	9.28	910	S
PI 540555	0	0.50	183	R
PI 607380	70	5.61	399	S
PI 598358	60	9.61	2,827	S
PI 548619	10	2.83	368	S
PI 548613	40	11.83	1,320	S
PI 612146	17.5	4.14	443	S
PI 556820	10.5	5.94	828	S
PI 548626	50	3.33	488	S
PI 518668	5	2.39	326	S
PI 556846	20	2.44	346	S
PI 664027	20	4.50	431	S
PI 601983	8.3	1.20	332	S
PI 548344	60	12.44	821	S
PI 620883	60	4.44	430	S
PI 576440	10	1.75	169	S
PI 548422	20	10.61	2,166	S
PI 556860	40	3.39	319	S
PI 559934	1	1.61	250	S
PI 548547	5	2.56	782	S
PI 561191	22.5	3.44	474	S
PI 548464	1	5.28	1,667	S
PI 548386	45	7.08	1,079	S
PI 597382	20	5.89	849	S
PI 548532	10	0.89	139	R
PI 508268	5	2.17	363	S

(Continued)

Table 3: *Continued*

Soybean line	Root gall severity (0–100%)	RF	Eggs g^{-1} of root	Susceptible or resistant
PI 548350	3.7	1.61	143	S
PI 548602	30	7.78	558	S
PI 577798	25	3.58	378	S
PI 595626	3.7	1.02	146	S
PI 556871	0	0.17	69	R
PI 561599	45	2.80	509	S
PI 548466	0	0.55	18	R
PI 593654	15	2.94	517	S
PI 556930	6	1.36	153	S
PI 559370	60	29.06	2,602	S
PI 646156 (Control)	0	2.33	222	S
PI 562611 (Control)	12.5	3.03	576	S
PI 629013 (Control)	0	0.28	80	R
Tomato	90	3.39	1,640	S

[c]Lines from round 3 that either died or did not germinate include: PI 556826, PI 548518, PI 548339, PI 553052, PI 556912, PI 548627, PI 595362, PI 597384, PI 590932, PI 518664 (seed not available from GRIN), PI 544354, PI 548977, PI 553051, PI 560207, PI 614732 (Control), PI 527702 (Control).

2.6 RI

An ANOVA test found that at least one soybean genotype had a significantly different RI than the others ($P < 0.05$). The LSD test found soybean lines PI 548386, PI 595626, PI 548559, PI 561576, PI 619232, PI 548466, PI 559934, PI 559931, PI 548682, PI 639740, PI 556852, PI 548555, PI 527702, and PI 562611 to be significantly different than the tomato control ($P = 0.025$).

2.7 GWAS

A total of 32,674 single nucleotide polymorphism (SNP) markers were used in the GWAS analysis following quality control filtering. A Bonferroni corrected threshold ($\alpha = 0.05$) was used to determine if an association signal was significant. Following the application of these criteria, no significant associations were detected in the current data set for any of the phenotypes analyzed.

Table 4. Response of soybean lines to infection by *Meloidogyne enterolobii* in an initial greenhouse screening experiment, round 4. [d]All soybean lines, including the susceptible controls, were planted once ($n = 1$) and the positive inoculation controls, tomato, had 5 replicates ($n = 5$). Plants were inoculated with 3,000 eggs of M. enterolobii and evaluated at 60 days post inoculation. The table includes the root galling severity as a percentage, eggs/g root tissue (Eggs g^{-1} of root), and reproductive factor (RF) for each line tested. Lines were scored as susceptible (S, RF > 1) or resistant (R, RF < 1)

Soybean line	Root gall severity (0–100%)	RF	Eggs g^{-1} of root	Susceptible or resistant
PI 548518	0	3.11	915	S
PI 548339	20	4.44	725	S
PI 548401	20	43.72	7,287	S
PI 556912	10	5.56	,1042	S
PI 548627	20	6.17	1,555	S
PI 595362	5	3.33	667	S
PI 597384	30	14.61	2,884	S
PI 548392	0	0.89	202	R
PI 590932	15	8.44	1,189	S
PI 544354	15	3.17	270	S
PI 556930	20	2.28	240	S
PI 593256	1	9.17	1,536	S
PI 630984	13.3	15.33	1,622	S
PI 548991	15	4.67	1,157	S
PI 548346	25	12.67	3,455	S
PI 595363	40	21.17	2,318	S
PI 355068	12.5	3.11	377	S
PI 606749	20	10.00	1,685	S
PI 548667	15	7.78	791	S
PI 355070	15	5.39	624	S
PI 518673	60	6.94	1,532	S
PI 561597	11.7	3.39	407	S
PI 548402	0	6.00	687	S
PI 633970	20	5.78	623	S
PI 548674	60	13.61	6,282	S
PI 618809	5	4.72	1,889	S
PI 548598	5	3.67	542	S
PI 548679	25	10.33	925	S
PI 556635	15	5.28	519	S
PI 548654	1	0.61	80	R
PI 534646	40	12.17	2,897	S
PI 548987	20	9.00	2,213	S
PI 556906	0	9.39	2,965	S

(Continued)

Table 4: *Continued*

Soybean line	Root gall severity (0–100%)	RF	Eggs g^{-1} of root	Susceptible or resistant
PI 604100	1	3.72	1,283	S
PI 548460	3	7.50	2,073	S
PI 548559	0	2.67	559	S
PI 533654	3	7.61	1,177	S
PI 548603	30	15.67	1,787	S
PI 556773	50	18.89	4,077	S
PI 548410	20	8.72	1,118	S
PI 564849	1	8.61	1,625	S
PI 583288	5	12.61	1,532	S
PI 556834	3	5.72	698	S
PI 633567	40	30.94	7,253	S
PI 548428	10	10.39	849	S
PI 550732	10	9.72	1,611	S
PI 548549	5	3.61	686	S
PI 553044	3	5.11	1,357	S
PI 548541	5	2.50	305	S
PI 548439	5	9.67	1,306	S
PI 665035	7	10.28	1,799	S
PI 548441	5	17.89	3,292	S
PI 548429	10	12.50	2,072	S
PI 550734	10	30.67	4,107	S
PI 595765	0	5.11	431	S
PI 560307	20	9.83	1,085	S
PI 631122	10	3.11	316	S
PI 596540	0	7.17	881	S
PI 561400	0	4.44	620	S
PI 556506	1	1.44	175	S
PI 560206	5	7.50	771	S
PI 512039	1	16.17	1,474	S
PI 556852	1	2.17	323	S
PI 561576	10	2.39	456	S
PI 611112	10	10.39	1,430	S
PI 606748	1	2.89	392	S
PI 548309	50	8.78	1,125	S
PI 550731	5	1.44	476	S
PI 553049	25	7.06	3,159	S
PI 556697	15	9.67	1,330	S
PI 598222	1	9.06	1,269	S
PI 556711	1	1.06	132	S

Table 4: *Continued*

Soybean line	Root gall severity (0–100%)	RF	Eggs g^{-1} of root	Susceptible or resistant
PI 615582	0	7.50	1,071	S
PI 548517	5	3.17	519	S
PI 614808	5	14.17	3,512	S
PI 548622	5	6.61	1,349	S
PI 614806	5	8.50	1,244	S
PI 548586	15	4.06	503	S
PI 556851	10	5.94	529	S
PI 548669	27	18.24	3,017	S
PI 548301	3	3.55	269	S
PI 548606	10	3.89	637	S
PI 553038	5	23.11	4,561	S
PI 548330	30	22.78	2,680	S
PI 550733	5	6.56	1,009	S
PI 612608	15	3.50	477	S
PI 572240	2	2.83	379	S
PI 548514	30	9.11	952	S
PI 548685	5	1.39	105	S
PI 564082	5	2.67	303	S
PI 572239	25	4.22	370	S
PI 548359	10	40.22	4,588	S
PI 561578	30	4.44	460	S
PI 602496	10	6.44	770	S
PI 543856	1	3.61	1,354	S
PI 548976	20	8.78	823	S
PI 596414	60	5.89	448	S
PI 586981	1	2.28	304	S
PI 525454	0	0.78	121	R
PI 548538	10	3.00	209	S
PI 562611	0	11.89	1,690	S
(Control)				
PI 527702	10	8.33	812	S
(Control)				
PI 629013	0	5.33	590	S
(Control)				
Tomato	30	4.24	1,487	S

[d]Lines from round 4 that either died or did not germinate include: PI 636463, PI 564525, PI 556841, PI 553043, PI 527701, and PI 583366.

Table 5. Response of soybean lines to infection by *Meloidogyne enterolobii* in a confirmation screening greenhouse experiment. [e]All soybean lines, including the susceptible controls, and the positive inoculation controls, tomato, had ten replicates (n = 10). Plants were inoculated with 3,000 eggs of *M. enterolobii* and evaluated at 60 days post inoculation. The table includes the root galling severity as a percentage, eggs/g root tissue (Eggs g^{-1} of root), and RF for each line tested. Values in the table are the average of ten replicates. Lines were scored as susceptible (S, RF > 1) or resistant (R, RF < 1)

Soybean line	Root gall severity (0–100%)	RF	Eggs g^{-1} of root	Susceptible or resistant
PI 548682	11 abc	0.85 ab	108 ab	S
PI 639740	8 abc	0.79 ab	115 ab	R
PI 619232	36 abd	1.81 abcd	215 abcde	S
PI 559931	7 ac	1.18 abc	216 abcd	S
PI 548392	45 bd	8.14 cd	1,339 cde	S
PI 559934	23 abcd	1.13 abc	170 abd	S
PI 595626	35 abd	3.39 bcd	492 bcde	S
PI 548466	6 ac	1.84 abcd	598 abcde	S
PI 561576	27 abcd	2.16 abcd	321 abcde	S
PI 548555	4 c	0.62 a	82 a	R
PI 556852	5 ac	0.78 ab	126 ab	R
PI 548559	40 abd	2.23 abcd	497 abcde	S
PI 548330 (Control)	46 bd	7.85 cd	1,682 ce	S
PI 548386 (Control)	10 abc	4.55 bcd	1,862 e	S
PI 527702 (Control)	55 abcd	1.56 abcd	186 abcde	S
PI 562611 (Control)	31 abd	1.86 abcd	208 abcd	S
Tomato	71 d	11.45 d	3,177 e	S

[e]Lines from confirmation screening that either died or did not germinate include: PI 548466, PI 561576, PI 548555, PI 556852, PI 548559, PI 548330, PI 548386, and PI 527702 (Control).

3. Discussion

In this study, the three resistant soybean lines that were under the resistant threshold values for all three-resistance metrics (RF, eggs per gram of root, and root gall severity) were PI 548555, PI 639740, and PI 556852 (Table 5). The soybean line PI 548555 is within the maturity group IV and was developed in Kansas, United States. The

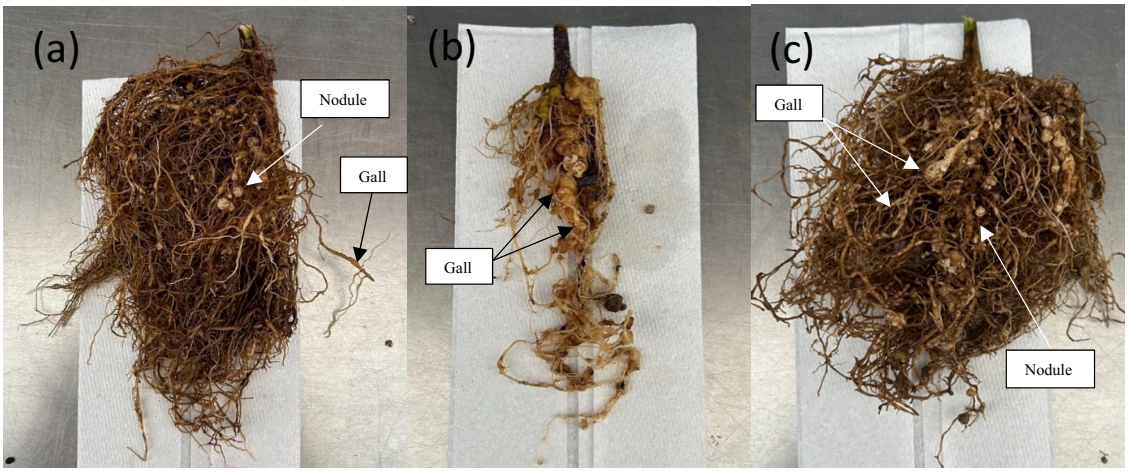

Figure 1. (a) Resistant *Glycine max*, PI 639740, inoculated with *Meloidogyne enterolobii*. Small, infrequent galls and nitrogen-fixing nodules are present. (b) Susceptible tomato ("Rutgers") root system inoculated with *Meloidogyne enterolobii*. Large galls and nitrogen-fixing nodules are present. (c) Susceptible soybean control, PI 527702, root system inoculated with *Meloidogyne enterolobii*. Large galls and nitrogen-fixing nodules are present. All images are root systems photographed at 60 days post inoculation.

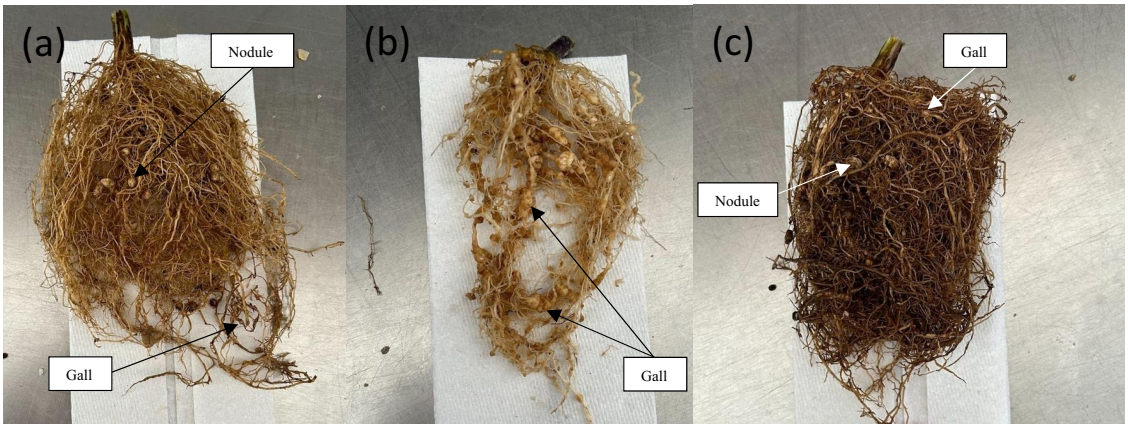

Figure 2. (a) Resistant *Glycine max*, PI 548555, inoculated with *Meloidogyne enterolobii*. Very small galls and nitrogen-fixing nodules are present. (b) Susceptible tomato ("Rutgers") root system inoculated with *Meloidogyne enterolobii*. Large galls are present. (c) Resistant *Glycine max*, PI 556852, inoculated with *Meloidogyne enterolobii*. Very small galls and nitrogen-fixing nodules are present. All images are root systems photographed at 60 days post inoculation.

soybean line PI 639740 is also within the maturity group IV and was developed in Illinois, United States. The soybean line PI 556852 met these resistance requirements and was included in the confirmation screening due to low galling percentages, despite an elevated RF value in R4 of the initial screening. The soybean line PI 556852 is within the maturity group IV and was developed in the United States. The two soybean lines with high RF values in the initial screening, PI 548330 and PI 548386, that were included in the confirmation screening as susceptible controls were confirmed to be susceptible to *M. enterolobii* in the confirmation screening.

The five *Glycine max* lines that were among the least susceptible genotypes identified in a previous study (Schwarz and Gorny, 2024; PI 646156, PI 614732, PI 562611, PI 527702, and PI 629013) were included as susceptible controls in the initial screening trial. While the majority were found to be susceptible, a few were classified as resistant within the four rounds of the initial screen. The previous study by Schwarz and Gorny (2024) for which these results were compared to, was conducted differently. In this study, the soybean lines were planted into 10.16 cm diameter pots, whereas Schwarz and Gorny (2024) used cone containers that were only 3.8 cm in diameter. These cone containers may have restricted the growth of the soybean roots and allowed soil to dry out more rapidly when compared to the large diameter pots (Poorter et al., 2012). The restricted root growth and drought stress potentially put the plant into distress and may have allowed for *M. enterolobii* to reproduce more quickly than when compared to this study. Additionally, there was a noted decrease in non-germinating or dead plants as the initial screening progressed through the four rounds. The first three rounds were conducted over the warmest months for summer in NC (May through early September) and the soybean plants are particularly sensitive to heat, as they wilt and can enter distress very quickly. To prevent heat stress, watering was increased to twice a day, but the average air temperature during summer of 2024 was high, leading to multiple soybean plants entering distress. The final round of the initial screening was completed from the very end of August through November when the temperature was cooling off.

The confirmation screening used two of the five susceptible controls that were previously tested, PI 562611 and PI 527702. These controls had resistance metrics that identified them as susceptible to *M. enterolobii* in the confirmation screening, which is consistent with the results from the study by Schwarz and Gorny (2024). In the confirmation screening, these lines had a higher rate of galling (31 and 55%, respectively) when compared to the Schwarz and Gorny (2024) study (15.5 and 11.5%, respectively). Since these lines were grown in cone containers in the Schwarz and Gorny (2024) study, the restricted root architecture could explain the lack of galling (Poorter et al., 2012). This previous study also had higher RF values for these soybean controls (14.86 and 17.41, respectively) when compared to the present study's confirmation screening (1.86 and 1.55, respectively). As addressed earlier, the increased stress on the plant could have allowed the nematodes to reproduce more successfully than in the present study (Poorter et al., 2012). Here, these soybean lines were tested in replicates of ten in the confirmation screening and in the Schwarz and Gorny (2024) study, only five replicates were used, which could also impact data analysis.

The GWAS indicated that there were no genomic variations that could be attributed to the phenotypic resistance to *M. enterolobii* seen for the three resistant lines PI 548555, PI 639740 and PI 556852, but this could be due to a number of reasons. The process of assessing plant cultivars for phenotypic resistance, that could indicate potential genotypic resistance, can be a lengthy experiment due to the large number of plants that must be tested. GWAS often include hundreds more cultivars than were used in this study, for logistical reasons involved with the processing of plants infected with *M. enterolobii* this would take several years to complete. When determining resistance to *M. enterolobii*, as completed in this study, it can be quite laborious particularly due to the pathosystem involving a nematode. Each plant must be manually processed in its entirety including the root system to properly quantify resistance, this can add countless hours of physical work. Due to the time constraints of this study, the total number of soybean cultivars was limited to 198 lines. While this is still a large number of lines to examine, it does not necessarily provide the power needed to distinguish between alleles that might be conferring resistance to *M. enterolobii*. Another potential reason for the lack of results from the GWAS could be explained by the low number of resistant lines found in this study. Out of the 198 lines, only 3 were found to be resistant, which is a small percentage of the genetic material analyzed for variations. Other candidate explanations could lie in the as yet unknown genetic control of this resistance as this specific analysis would be unlikely to uncover causal alleles if the resistance is controlled by many alleles with small effects. There is also no precedent for genetic material of soybean that is resistant to *M. enterolobii* that the genomes can be compared to or analyzed with as these are the first resistant *Glycine max* lines to *M. enterolobii*.

The aim of this screening study was to investigate *Glycine max* lines for resistance to *M. enterolobii,* and PI 548555, PI 639740, and PI 556852 were found to have resistance. While these lines did present phenotypic resistance, it is vital that these lines be further tested, especially tested out in fields, to ensure that resistance is consistent. In order to mitigate loss of soybean production due to *M. enterolobii* in NC and other soybean producing states, the development of resistant varieties is essential. While the GWAS for this specific study found no common genetic variation that could be associated with the resistance documented, there is still a future for these *Glycine max* lines. Resistant progeny are in development through crosses with these three resistant lines, PI 548555, PI 639740, and PI 556852. Once more genetic materials are available for *Glycine max* lines that are resistant to *M. enterolobii,* there is potential for future studies such as a GWAS, to investigate if a single gene or loci is regulating the documented resistance. The challenge of *M. enterolobii* is still present but the use of future resistant cultivars bred from those found in this screening study especially in tandem with other management methods such as the use of crop rotation with non-host crops and chemical management allow growers to get ahead of this nematode pathogen.

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Conflict of interest statement

Authors state no conflict of interest.

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