Nondestructive Imaging and Characterization of Cotton / Reniform Nematode Interactions

Phenotype Screening Corporation

January 2013

Introduction

Our focus in 2012 was on furthering our soft-tissue X-ray based method, software and protocols for reniform nematode, *Rotylenchulus reniformis*, susceptibility assessment on cotton plants.

The salient features of our method are:

i. It is based upon soft-tissue X-ray imaging. (Soft-tissue X-ray imaging uses the low-energy portion of the X-ray spectrum where X-ray photons are partially attenuated by the tissue of plant organs such as leaves, stems and roots.)
ii. The entire root system (root volume) of the cotton plant under study is captured in an X-ray image.
iii. The resolution of the X-ray imaging system allows for reniform nematode egg mass site identification and counting
iv. Egg mass location can be tied to specific attributes of the plant's root system.

Reniform Nematode Susceptibility Screening Demonstration

In late 2011 we demonstrated that reniform nematode egg masses had a distinctive appearance in X-ray images and could be differentiated from soil particles and other root structures in X-ray images. We also demonstrated that harvested cotton roots could be carefully washed without removing egg masses from the roots.

Figure 1. Microscope images of reniform (Rotylenchulus reniformis) egg masses on Auburn Line B124 cotton roots (left and center) after root washing, right most photograph includes mature females on Auburn Line B227 cotton roots.

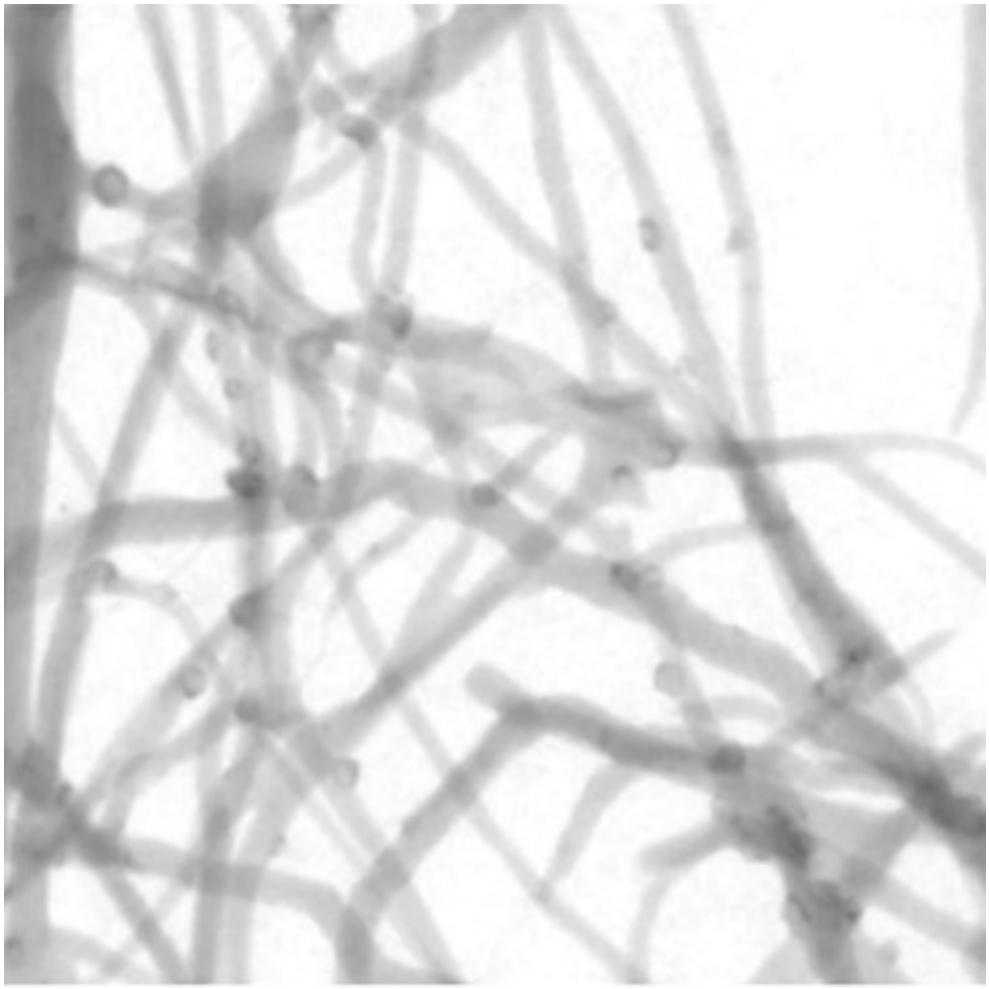


Figure 2. X-ray Image of Reniform Egg Masses on Cotton Roots of Auburn Breeding Line A209

In 2012 we built up our population of reniform nematodes using cantaloupe and sunflower as host plants. The original reniform population was extracted from loessy soil we acquired from infected cotton fields in West Tennessee. The original West Tennessee infected reniform soil was placed in 27 pots (12 planted with cantaloupe and 15 planted with sunflower), grown for 50 days to increase inoculum, then the soil from the 27 pots was stored for about 1 month in large buckets with lids. A sample of this soil was assayed to determine total number of vermiform reniform nematodes. We measured an average of 2,668 reniform nematodes per 100 cubic cm of soil.

We tested a method to manually count and log egg mass locations distributed along the root system. We developed software to graphically represent egg mass counts as a function of root system depth. We also developed software to integrate root architecture information (root diameter distributions, root density by depth, etc.) with egg mass count distributions.

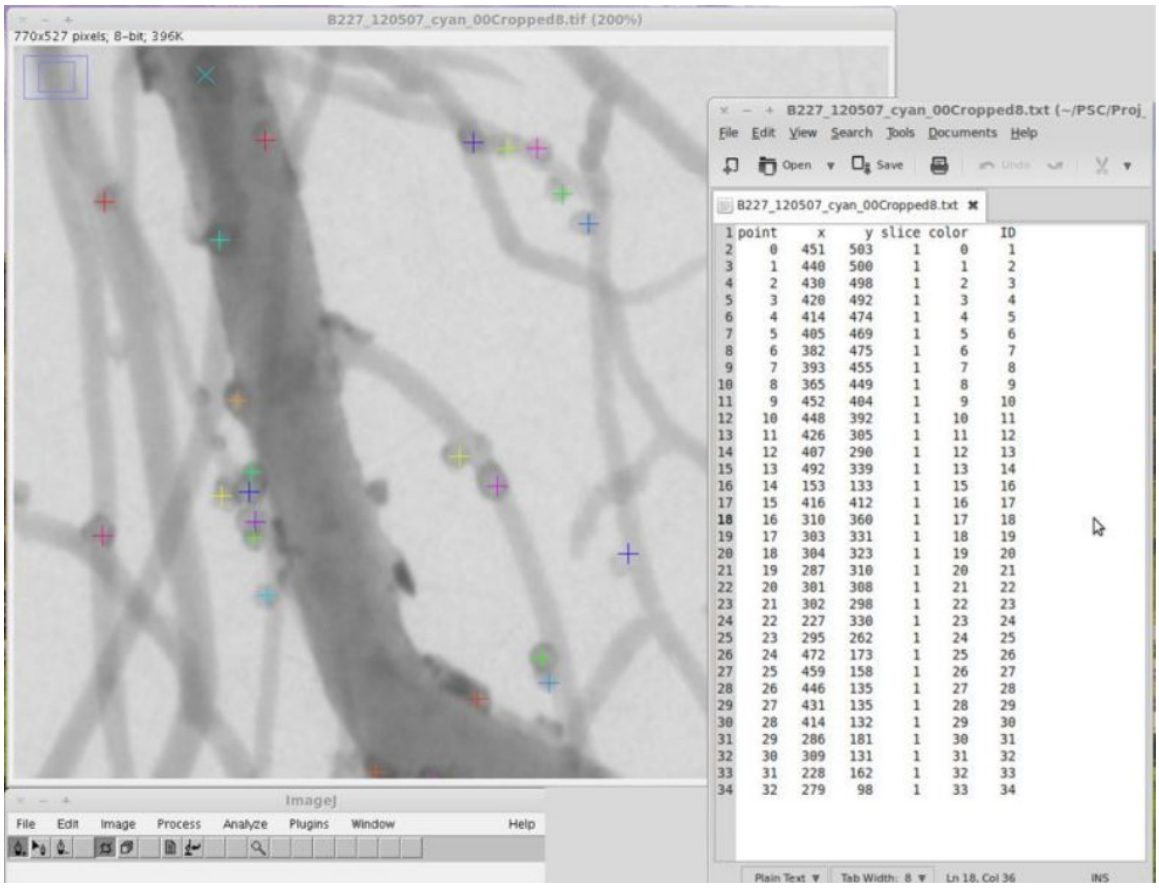
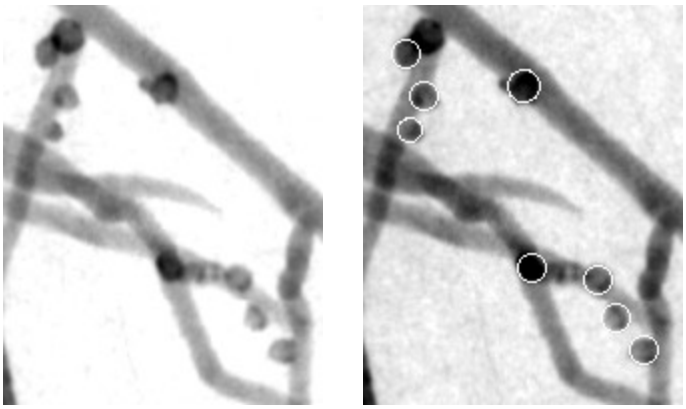


Figure 3. Manual Nematode Egg Mass Counting

We developed a proof of concept application that demonstrates that automated counting of reniform egg mass counting is possible. Figure 4.(a) shows a small portion of an x-ray image containing reniform egg masses on roots. Figure 4.(b) shows the locations of egg masses determined automatically and Figure 5 shows the output file format with x,y location and radius in pixels of each egg mass detected. Further work will be required in order to improve sensitivity and minimize false positives.



(a) (b)

Figure 4. Automated Egg Mass Counting

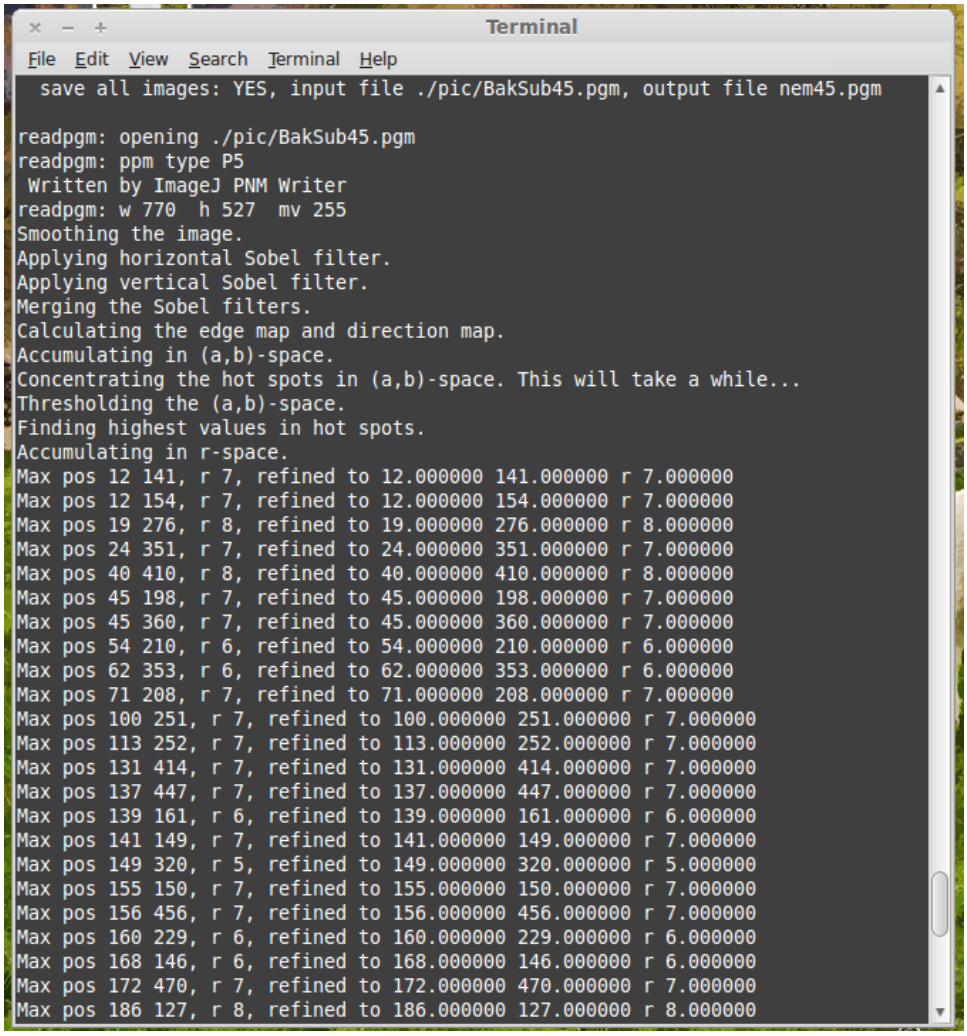


Figure 5. Results Table

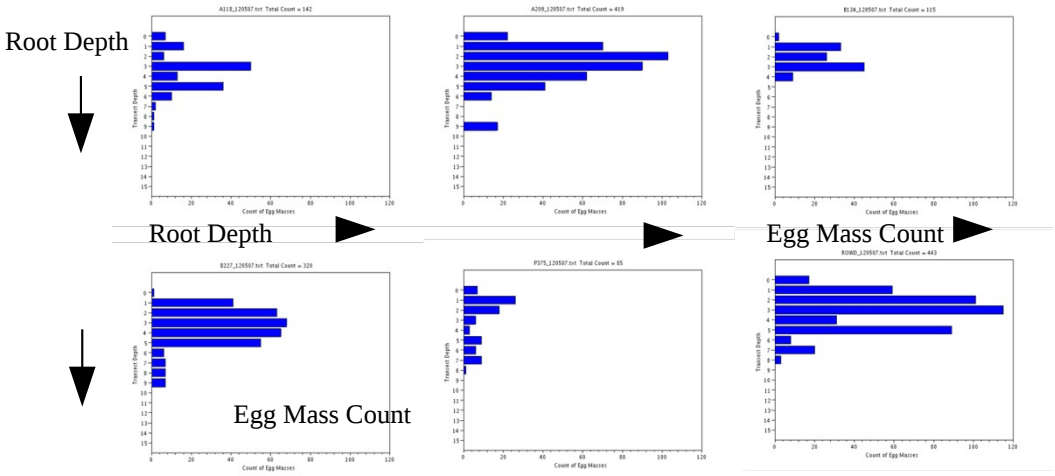


Figure 6. Reniform Nematode Egg Mass Counts As a Function of Root Depth for Six Cotton Plants

We developed special preprocessing protocols to surface sterilize linted cotton seeds and assure no cross contamination. In the end, all seed were treated with 72% sulfuric acid (Fluka) for varying times, depending on the thickness of lint on the seed.

During the last quarter of the year we completed an end-to-end demonstration of our method with a reniform nematode susceptibility growth trial using samples from the Cotton Incorporated sponsored resistance breeding program at Auburn University. The results from this trial were presented at the 2013 Beltwide Cotton Conference in San Antonio, TX. The presentation, "Nematode Susceptibility Rankings from Soft-Tissue X-ray Imaging" was given during the Cotton Disease Council meeting on January 9th.

The trial began on September 19, 2012 and the plants were harvested on November 9, 51 days after start of germination. Six cotton lines from the Auburn breeding program and two USDA germplasm lines were evaluated. The susceptible Auburn lines A118, A209, B124 and B227 were F2:6 seed of the cross LONREN-1 x FM966. The resistant Auburn lines A107 and B103 were F2:6 seed of the cross LONREN-2 x FM966. The resistant USDA lines were LONREN-2, a germplasm line based on *G. longicalyx* and BARBREN-713, a germplasm line based on GB713. All seeds were provided by Dr. David Weaver at Auburn University.

All seed were treated with the 72% sulfuric acid (Fluka) for the times indicated below.

Green linted seeds	**Naked seed**	**White linted seeds**
LONREN-2 (10 min)*	GB713 (1 min)	A118 (25 min)*
LONREN-1 (10 min)	BARBREN-713 (1 min)*	A209 (25 min)*
B103 (20 min)*		B227 (25 min)*
A107 (10 min)*		B124 (20 min)*

*Indicates seeds used in the trial.

The treated seeds were then pre-germinated on filter paper. We achieved 100% germination of the seeds with this protocol. We selected viable seedlings of similar development and transplanted the seedlings to cone-tainers with infected soil.

The soil previously infested with reniform nematodes was mixed with some fine sand (5 parts reniform soil, 2 parts sand) and then used in the cone-tainers. This dilution gave 1,500 reniform nematodes per 100cm^3. Each cone-tainer received 500 cm^3 of this mix.

We began growing eight reps of each of eight varieties in the infected soil. Two reps of each variety were planted in sanitized West Tennessee cotton field soil mixed with sand as controls. The soil of the control plants was a bit different. Stored autoclaved soil from a previous supply was mixed with sand in the same proportion as above, then placed in the cone-tainers. This mix is slightly better draining than the reniform mix. As previously mentioned each plant was grown for 51 days after germination.

Figure 7. Infected and Control Cotton Plants at The University of Tennessee Greenhouse at Harvest

The cotton plants were then harvested and the root systems were carefully washed and prepared for X-ray imaging. Above-ground plant data were not taken for this experiment

Figure 8. Removing Soil from Harvested Roots

Figure 9. Final Rinse of Harvested Roots

Figure 10. Washed Roots Were Then Prepared for X-ray Imaging

Figure 11. X-ray Images of Cotton Root Systems Organized by Line

A cursory examination of the X-ray images revealed that approximately 13% of the cotton plants in this trial demonstrated low root vigor. This was surprising as all of our plants were preselected for uniform apparent health and vigor at the seedling stage prior to planting. This root stunting may be a reaction of some of the plants to nematode feeding. Why some infected plants within a line exhibit stunting and others do not is not yet understood. Unless otherwise noted all data presented in this report included the stunted root systems in the analysis.

All of the plants were analyzed using our RhizoTraits automated root characterization software. RhizoTraits was enhanced in 2011, under Cotton Incorporated funding, to included cotton root system parameters as a pull down default option in its graphical user interface. RhizoTraits software generates root system "signatures" and "distributions."

RhizoTraits root system signatures are traits presented as a function of root diameter ranges. We break down the root system into five overlapping size classes. We then measure three traits across each size class. This generates a five element vector (signature) for each trait. The three traits are Projected Root Area, Total Root Length and Total Number of Root Transect Crossings.

In this experiment the cotton plants were only grown for seven weeks. Their roots did not grow thicker than 3 mm during the experiment. Therefore only three size classes were necessary:

- Size Class 3: root diameters of 1.3 mm to 2.9 mm
- Size Class 4: root diameters of 0.7 mm to 1.5 mm
- Size Class 5: root diameters of 0.3 mm to 0.7 mm

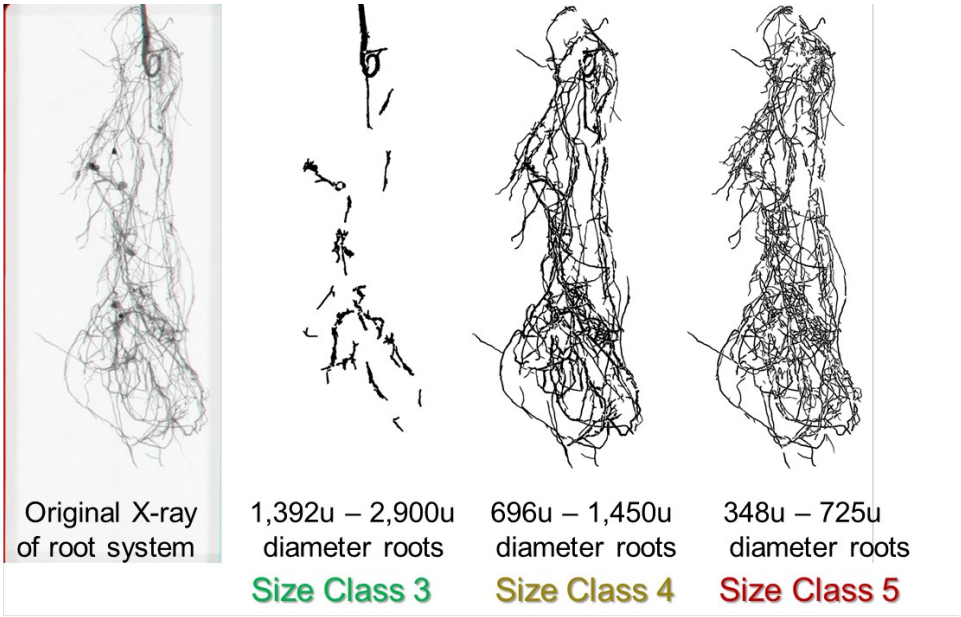


Figure 12. Analyzing X-ray Images by Size Class

RhizoTraits root system distributions are root characteristics presented as a function of root depth. Root diameter histograms, root counts, root density and root width, all as a function of root depth are examples of root system distributions. The root depths that measurements occur are set by the analyst. Typically the first measurement is made 50 mm below the soil line and at 25 mm increments below that. Distributions are generated for each size class.

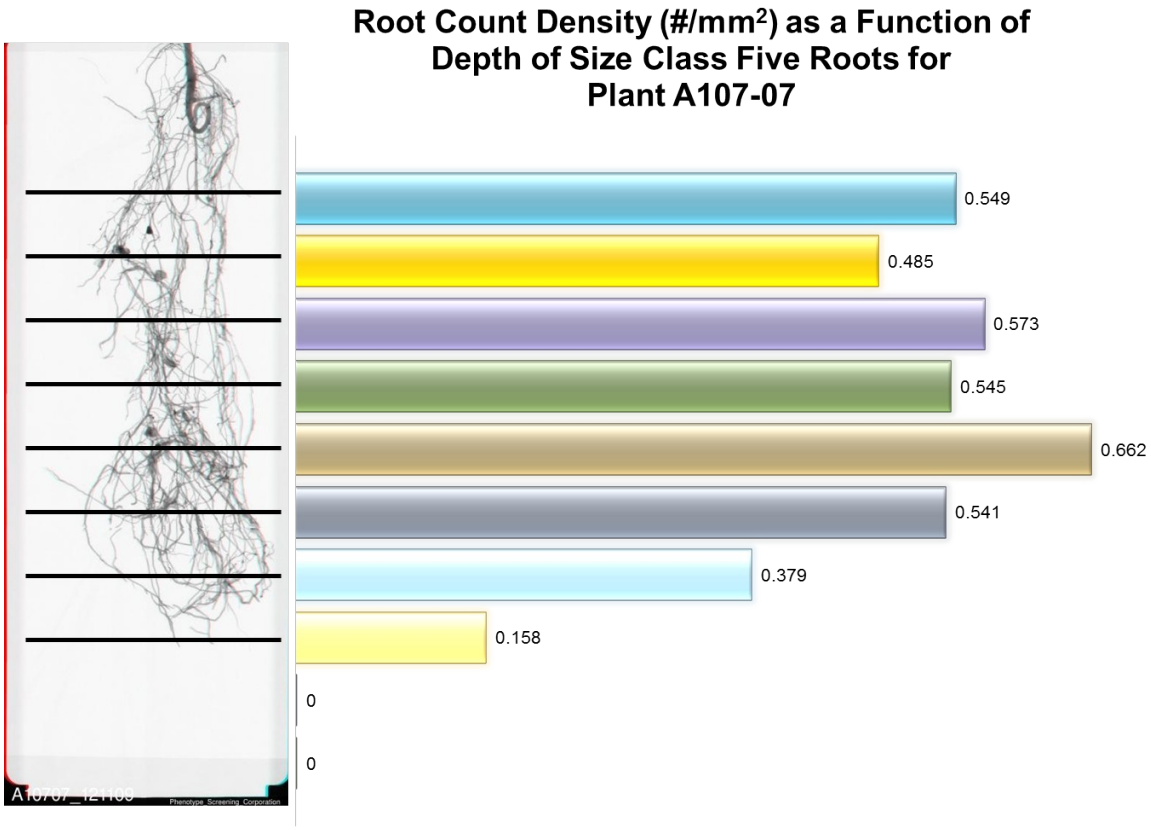


Figure 13. Root Density Distribution of a Cotton Plant in 25 mm Increments

In the series of panels below the X-ray image of each plant is presented along with the number of egg masses counted on that plant's root system and the total root lengthsignature of that plant's root system. In each panel a red box around an X-ray image indicates a low vigor root system and a gold box indicates an uninfected control plant.

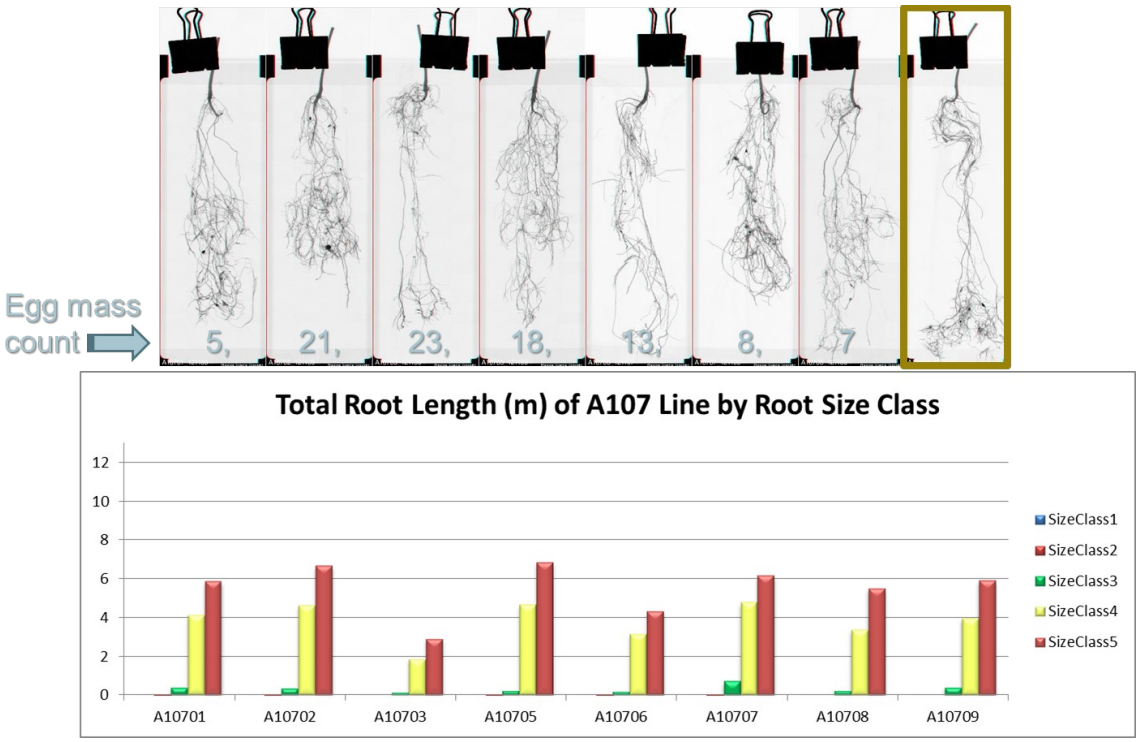


Figure 14. Auburn Resistant Line A107

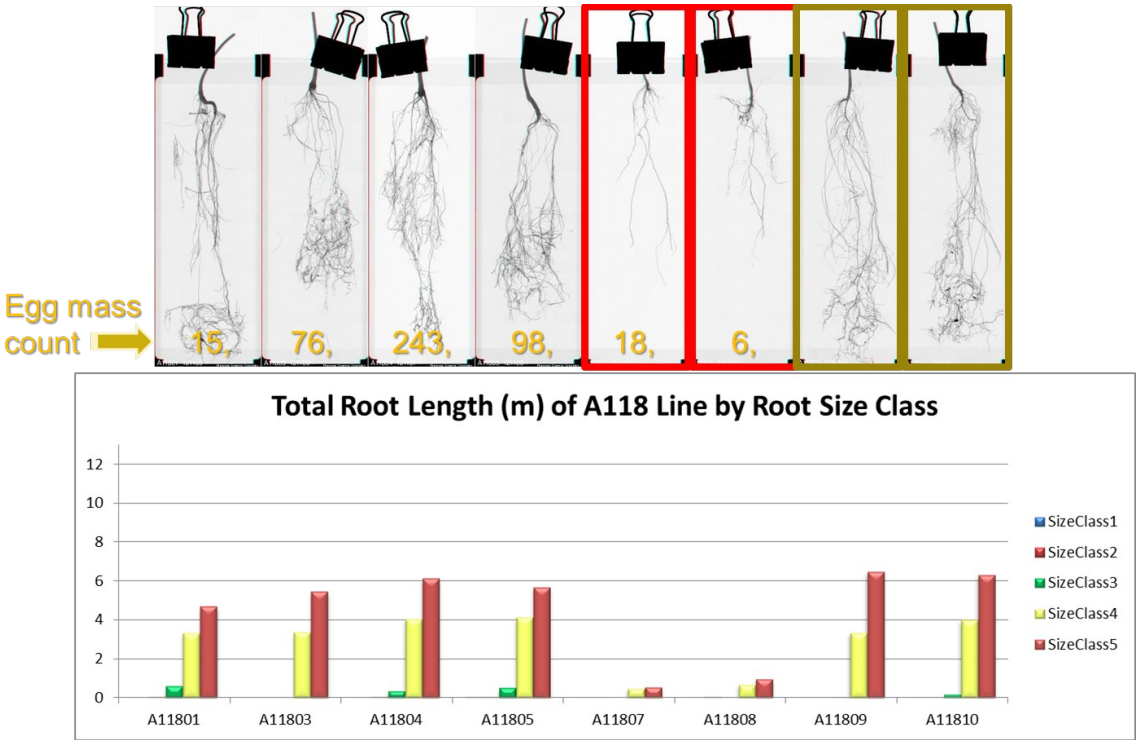


Figure 15. Auburn Susceptible Line A118

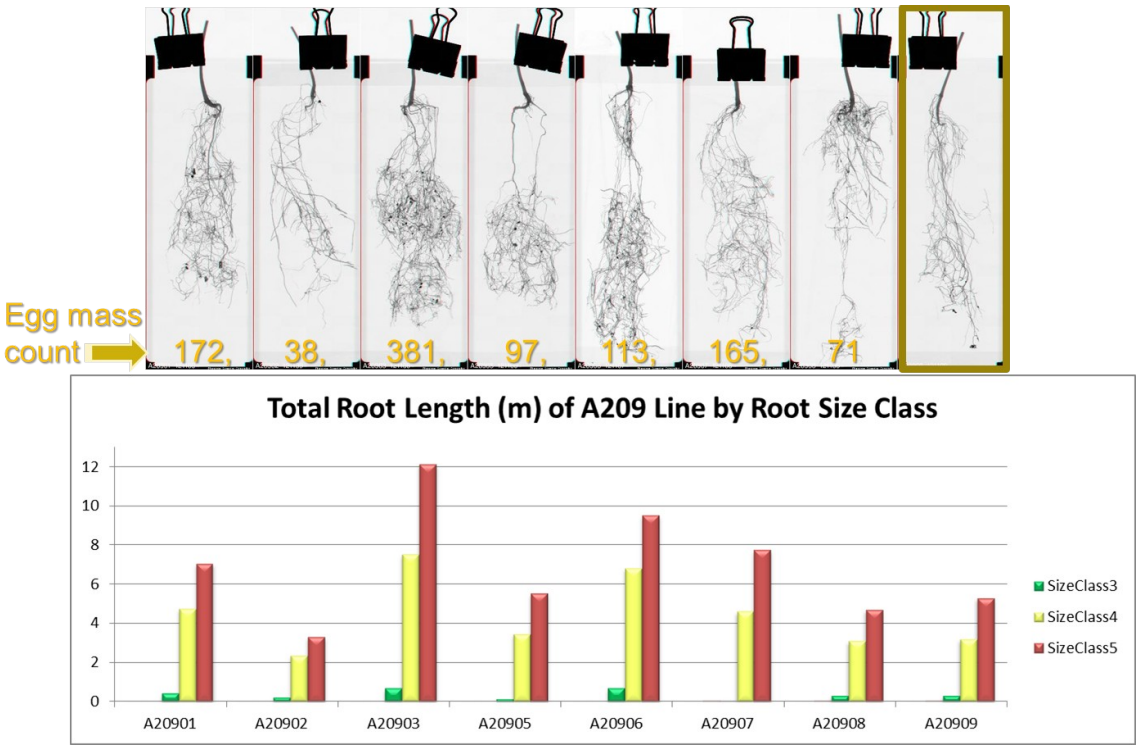


Figure 16. Auburn Susceptible Line A209

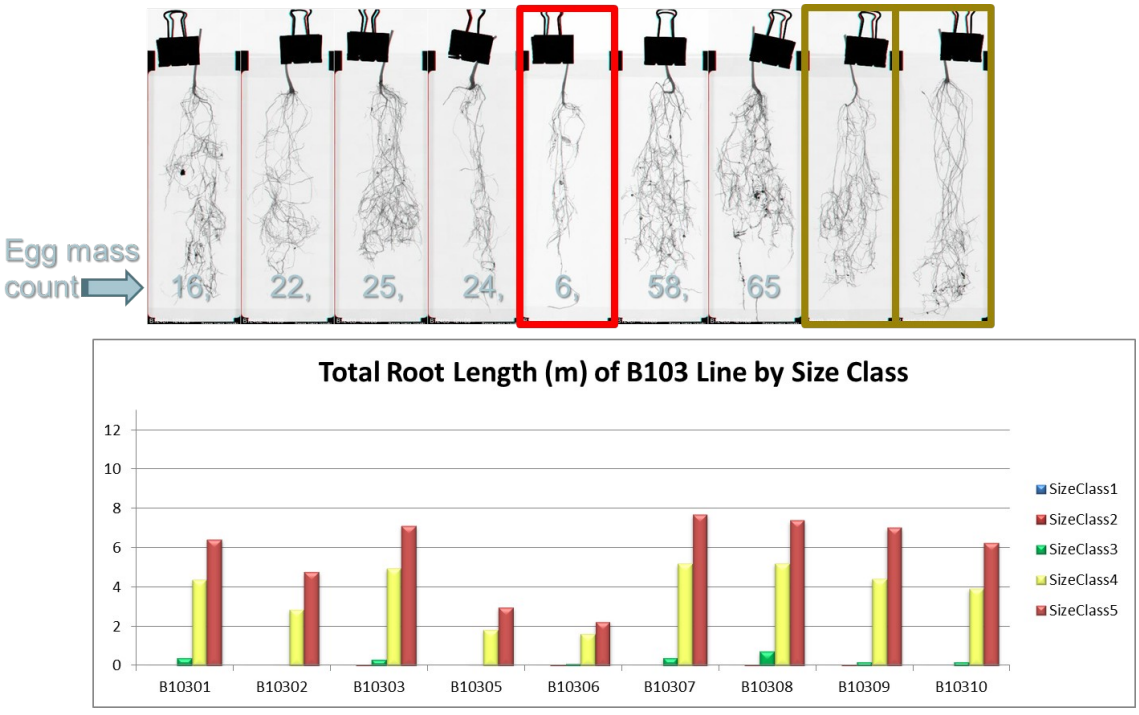


Figure 17. Auburn Resistant Line B103

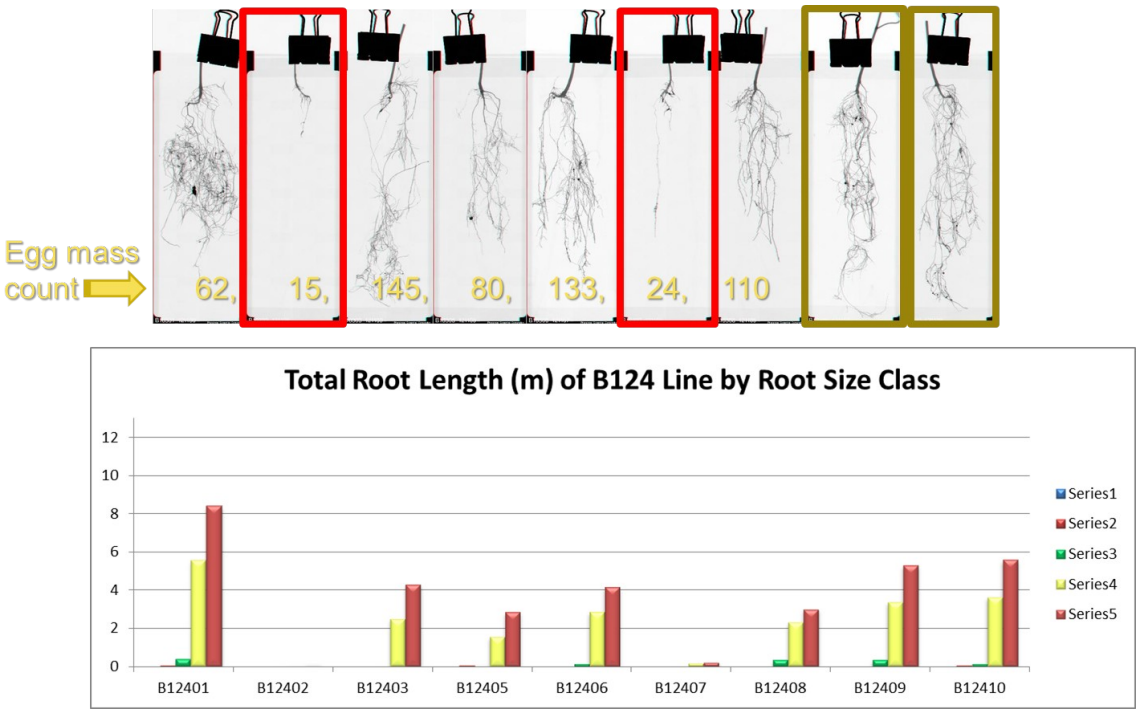


Figure 18. Auburn Susceptible Line B124

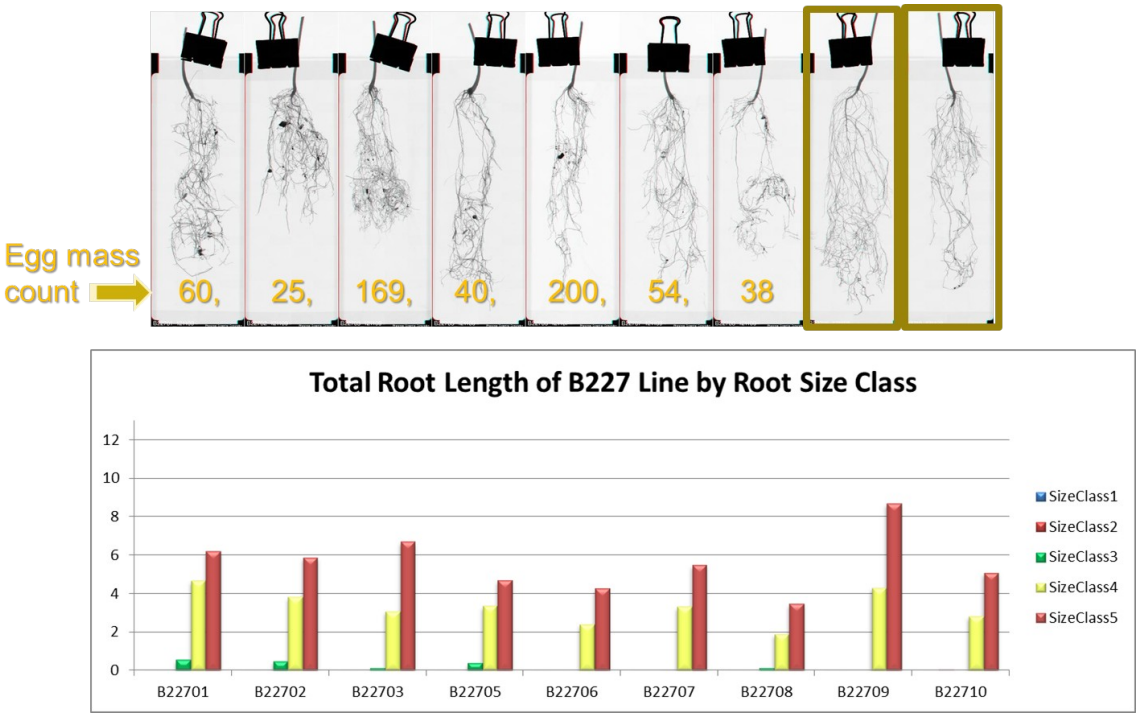


Figure 19. Auburn Susceptible Line B227

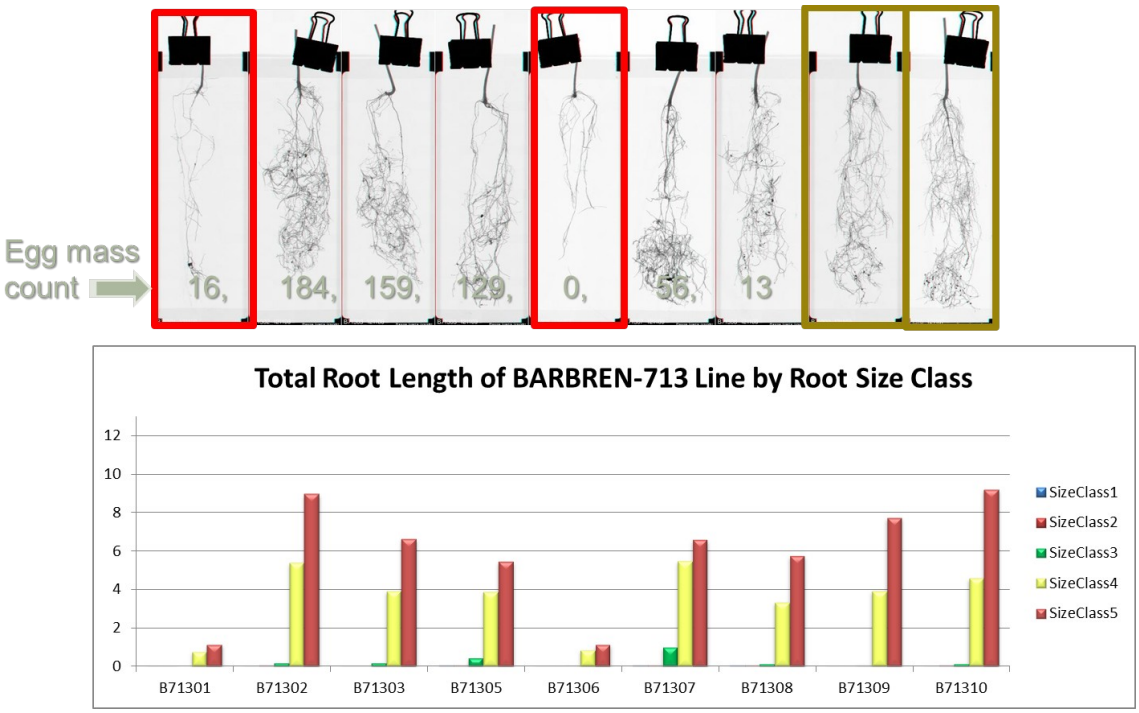


Figure 20. USDA Resistant Line BARBREN-713

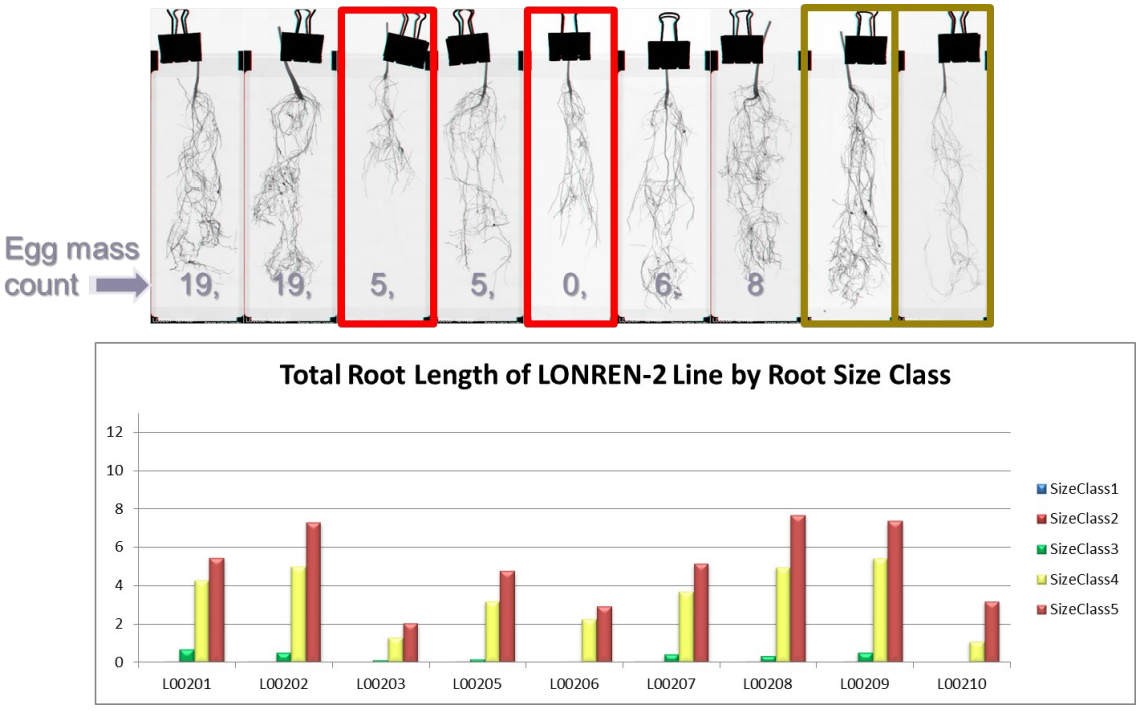


Figure 21. USDA LONREN-2 Line

From the panels above we can see that low root vigor is often accompanied by a very low egg mass count. This suggests a hyper-sensitive response in some plants to initial nematode feeding, i.e., sacrificing plant tissue to kill feeding nematodes.

We examined the relationship between total root length and egg mass counts to see if the total root length was correlated with feeding site counts. In the data sets below, all low root vigor plants were removed.

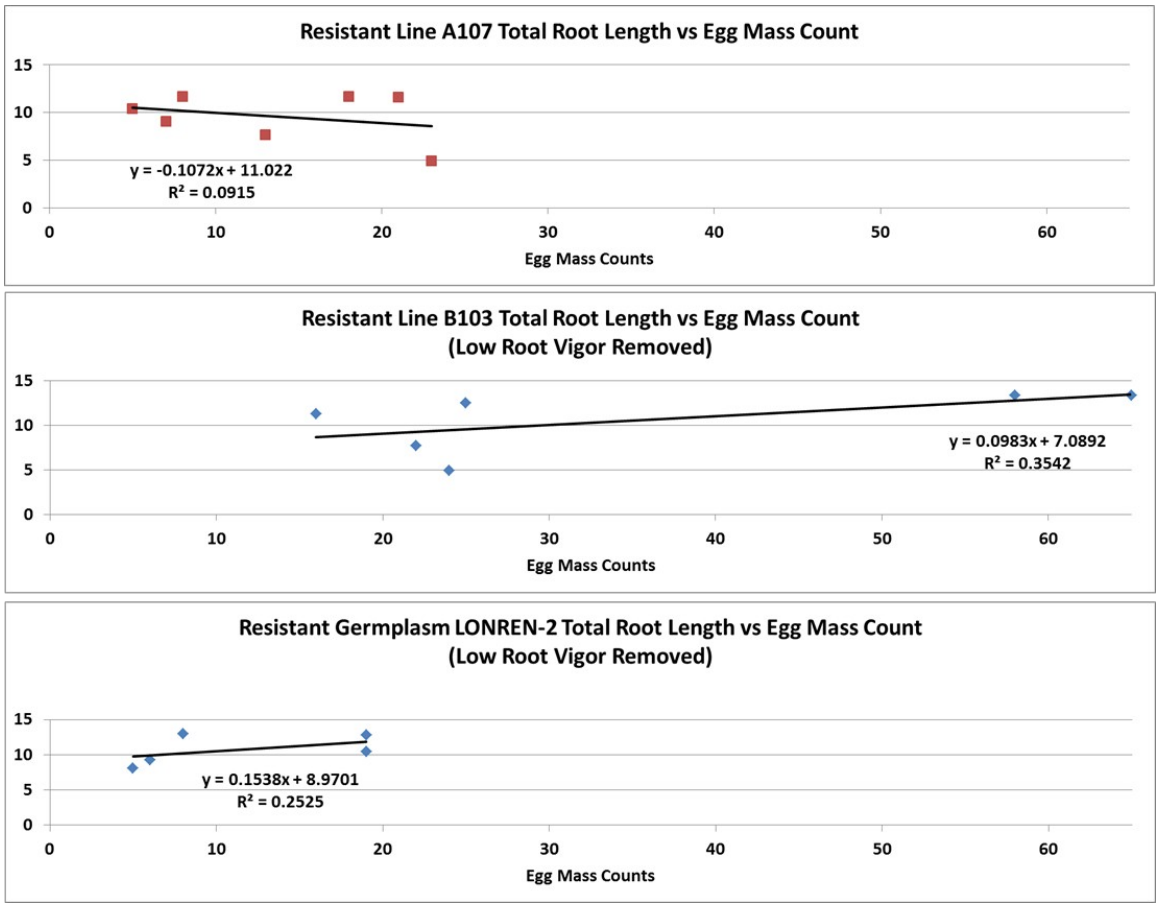


Figure 22. The Total Root Length of Resistant Lines Reveal Different Relationships to Increasing Egg Mass Counts

The Auburn resistant line A107 had a similar range of nematode egg mass counts but was oppositely correlated with the USDA germplasm line LONREN2. The Auburn resistant line B103 supported many more egg masses and had a larger total root length at higher egg mass counts.

The susceptible varieties are shown below and reveal that two of the Auburn susceptible varieties A118 and B227 have root systems that appear not affected by increasing egg mass counts. While one Auburn susceptible variety A209 shows an increasing total root length at higher egg masses and another Auburn susceptible variety B124 suggests shorter total root lengths at higher egg mass counts.

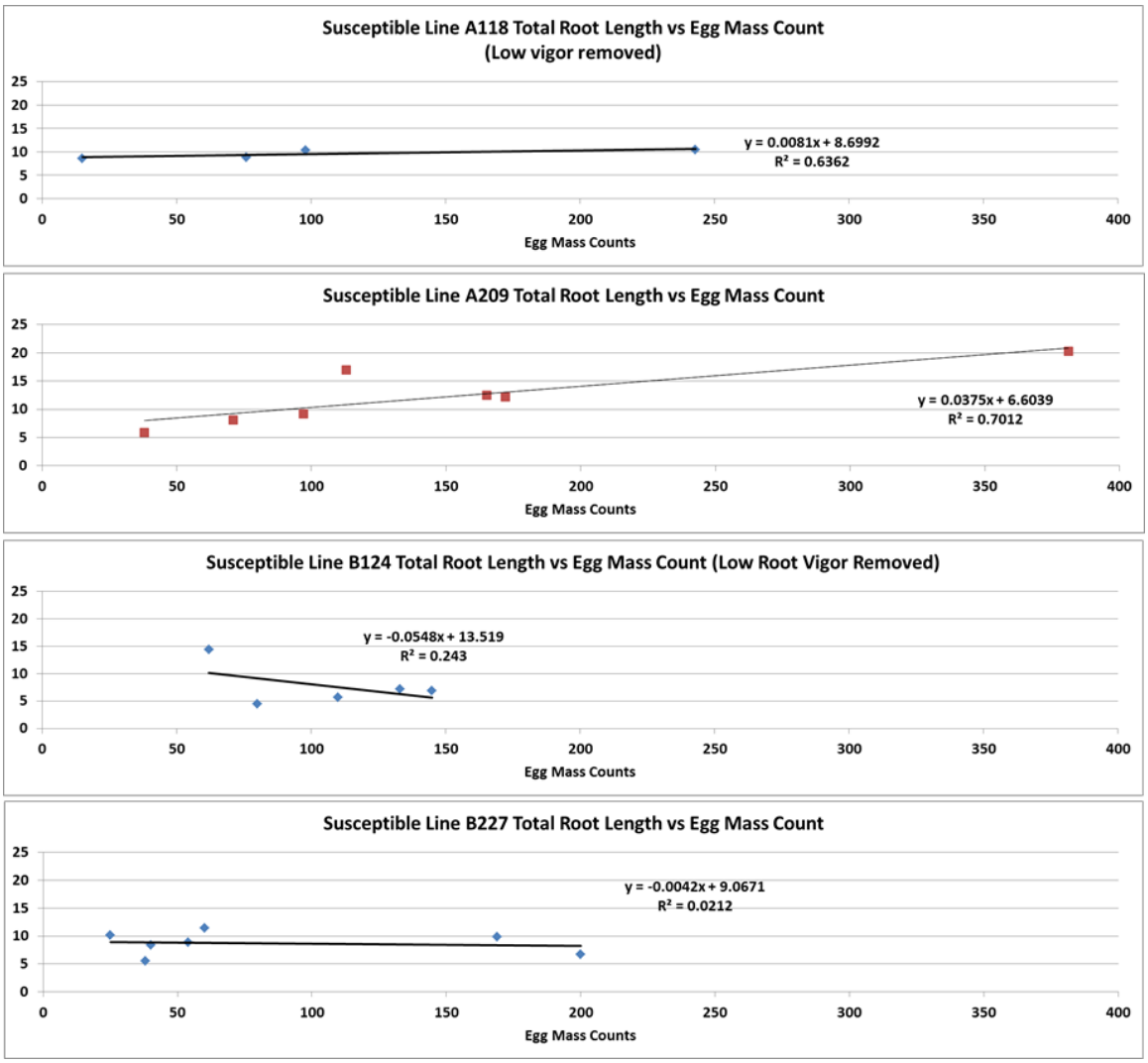


Figure 23. The Total Root Length of Susceptible Lines Reveal Different Relationships to Increasing Egg Mass Counts

The mean total root length signatures for each line were calculated from the individual plant data. The mean egg mass counts for each line were also calculated. All infected plants were included in the mean calculations below.

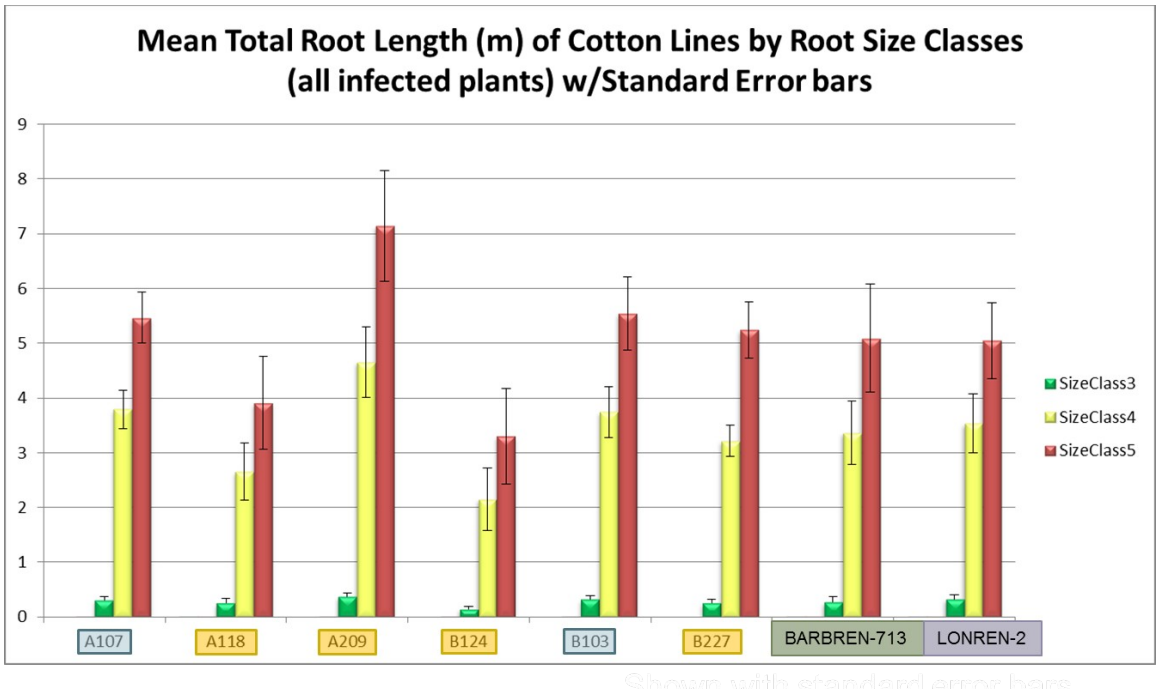


Figure 24. Mean Total Root Length of Each Line

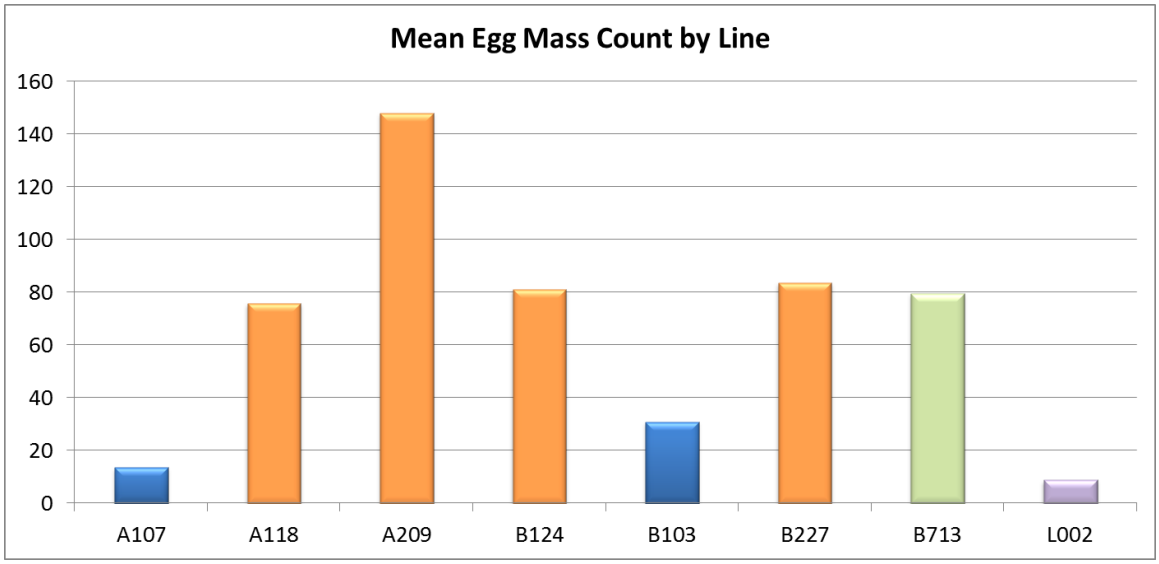


Figure 25. Mean Reniform Egg Mass Count of Each Line

The mean egg mass counts can be normalized by various root system signatures to investigate different representations of nematode "susceptibility." For instance normalizing egg mass counts by total root length gives a measure of potential fractional damage.

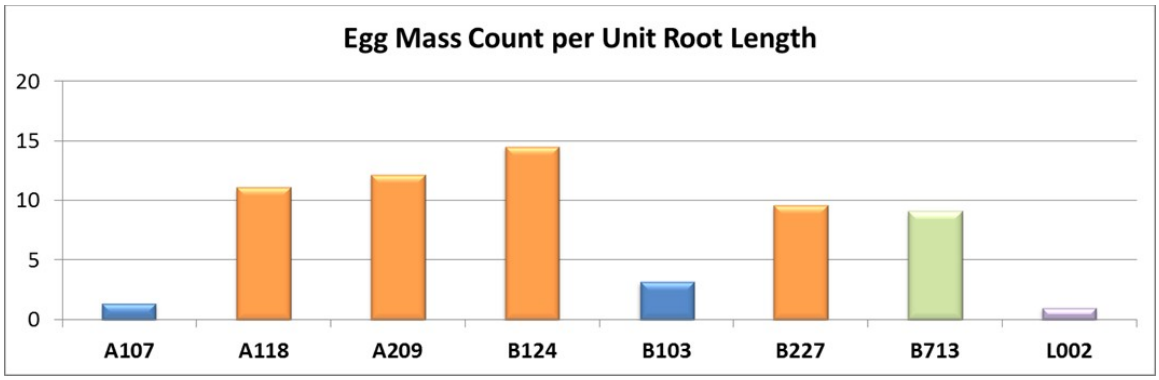


Figure 26. Normalized Egg Mass Counts: Mean Egg Mass Count per Mean Total Root Length

When normalized in this way B124 appears to be the most "susceptible" line. It also tends to have smaller root systems at higher feeding site counts. These observations may suggest that B124 would not be a good yielding line; however it ranked number 1 and number 2 of these susceptible lines in the Auburn field trials over the past two years in lint yield. Clearly, the relationship between nematode susceptibility and yield is not yet understood.

From the last two figures it appears that the USDA line BARBREN-713 supports relatively high nematode reproduction. This result is not consistent with the results of several field trials with this line. Dr. Weaver has indicated that his lab also measures higher than anticipated nematode reproduction in his greenhouse experiments. We received our seed from Dr. Weaver. We may have a bad seed source or there may be some segregation of the resistance gene from some seed multipliers. This issue has not yet been definitively resolved.

We have also analyzed the differences in root density distribution as a function of root depth and egg mass count distributions as a function of depth for each line. In our experiment the nematode infested soil was mixed with sand in a manner to approximate uniform nematode density throughout the soil depth profile. We expected uniform egg mass distribution along the roots. From the figure below we see that the mean root density distribution varied by line. Some lines have higher root densities at shallow depths and some have higher root densities at deeper depths.

Nematode feeding sites are also uniquely distributed by variety. Some varieties support a nearly uniform distribution of egg masses by depth (LONREN-2, B103, and A107.) Some varieties support more egg masses at shallower sites along the root system (B227 and A118.) Some support more egg masses at deeper depths (BARBREN and B124) and some support a normal like distribution along the root system depth (A209.)

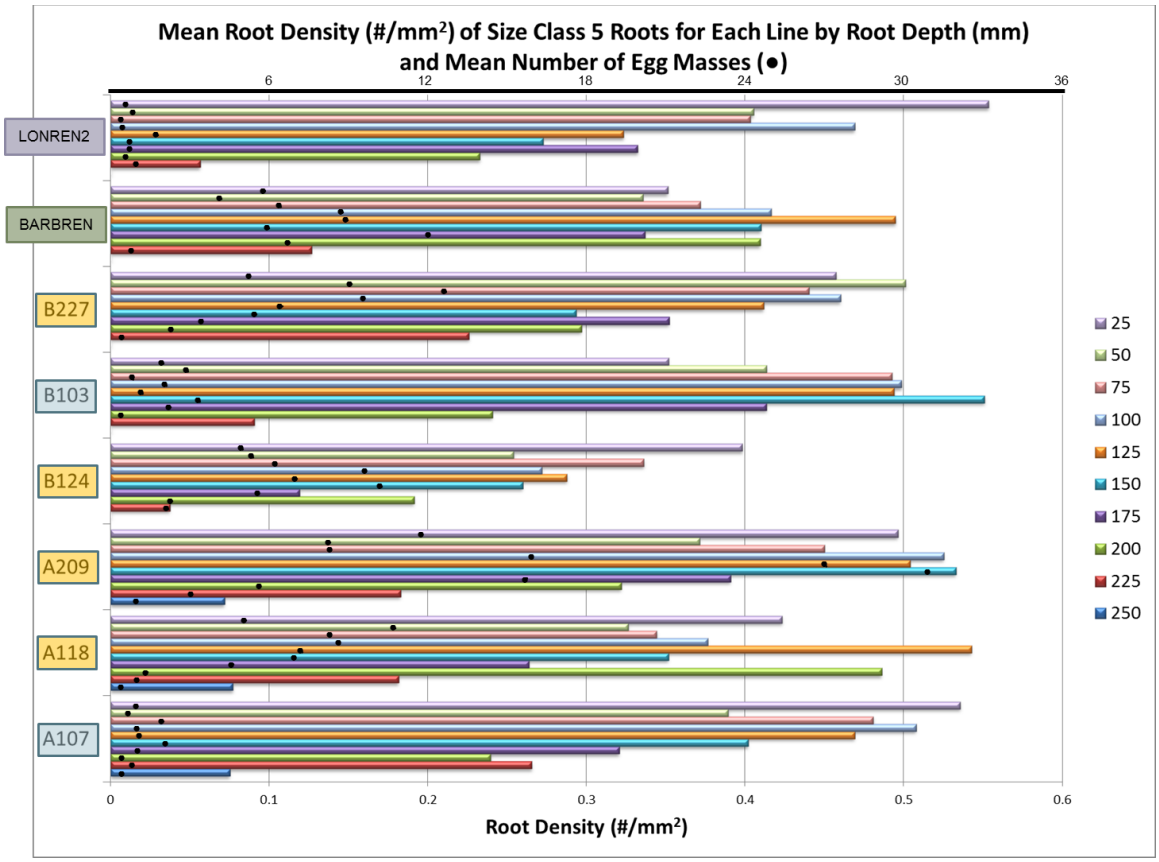


Figure 27. Integrating Root Density Distribution with Egg Mass Distribution Data. Color Coded Bar at Left Is Key for Root Depth in mm. Black Dots Indicate Number Of Egg Masses At A Given Depth.

The significance of these differences to breeding program objectives is not yet understood.

Conclusions

Our conclusions from the demonstration trial is that our method:

- ✓ Detects root system stunting as a response to reniform nematode feeding,
- ✓ Identifies differences in reniform nematode reproduction among the lines under test,
- ✓ Differentiates reniform nematode resistant from susceptible lines,
- ✓ Quantifies differences in the root systems of each line,
- ✓ Quantifies differences in reniform nematode feeding site count and distribution for each line,
- ✓ Quantifies relationships between the size of root systems and the number of reniform nematode feeding sites for each line.
- ✓ Has the potential for automated counting of egg masses.