FINAL REPORT 2019/401

A NEW HIGH THROUGHPUT METHOD FOR SCREENING FOR ROOT-KNOT AND ROOT-LESION NEMATODE RESISTANCE IN SUGARCANE

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ABSTRACT

Root-lesion nematodes (*Pratylenchus zeae*) and root-knot nematodes (*Meloidogyne javanica*) are the two most important nematode pathogens of sugarcane in Australia. They cause loses of over $82 million to the Australian sugar industry annually (Stirling et al. 2011). Sugarcane varieties have some resistance to root-knot nematodes, but no resistance is present in sugarcane varieties to root lesion nematodes. The most effective way for farmers to maintain high yields on soil that contains nematodes is to develop resistant varieties. The current screening method that is used to identify germplasm that contains resistance takes up to 16 weeks to obtain a result. A new screening method was developed in this project which takes 3 weeks to determine if a clone is resistant to or susceptible to root-knot and root lesion nematodes. This new method has the potential to reduce the cost of screening for nematode resistance and to increase the number of clones that can be screened in a year. This has an impact on the sugarcane breeding program as the more clones that are screened the faster a new resistant variety can be produced. The project also identified resistance in new wild germplasm, *Saccharum spontaneum* and *Erianthus arundinaceus*. These clones have the potential to be used in an introgression breeding program to introgress resistance from the wild germplasm into sugarcane varieties. The new screening method developed in this project could potentially speed up the introgression process by enabling larger numbers of clones to be screened thus increasing the chance of success.

EXECUTIVE SUMMARY

Root-Knot and Root-lesion nematodes cost the Australian sugarcane industry over $82 million a year due to yield loss caused by infestations of these nematodes in sugarcane fields. The only economically viable way to overcome this yield loss is to develop resistant varieties that can produce high yields in the presence of these nematodes. The current method for screening for nematode resistance is costly and takes up to 16 weeks to identify resistant germplasm. This project was a collaboration between CSIRO and SRA Woodford. SRA staff at Woodford currently screen all sugarcane germplasm for resistance to nematodes which depending on the number of clones to screen takes up to 16 weeks to complete. This project combined the expertise in screening for nematode resistance and growth of nematodes for inoculation from the SRA Woodford staff with the CSIRO expertise on sugarcane genetics. The projects goal was to develop a new method to screen for these nematodes that can provide information on resistance within one week of inoculation. The method uses germinated setts that are maintained in sterile plastic pots and inoculated with the nematodes. Once the roots are inoculated, they are kept in the dark to allow the symptoms to develop. As early as four days after inoculation the roots can be sampled to determine the presence of nematodes or nematode eggs. As the roots are not in soil, they are easier to sample and symptoms can be clearly identified.

The outputs from this project includes information on the level of resistance that *Saccharum spontaneum* and *Erianthus arundinaceus* have to root-knot and root lesion nematodes. This project identified three *S. spontaneum* clones and one *E. arundinaceus* clone that are resistance to root lesion nematodes. This is important information as no resistance has been found in sugarcane varieties. This project also identified four *S. spontaneum* clones that are highly resistant to root knot nematodes and one *E. arundinaceus* clone. The project increased knowledge of nematode screening in sugarcane and provided training for one industrial trainee at CSIRO.

This project has produced a new screening method that can be used for screening for resistance to root knot and root lesion nematode in sugarcane. The new method can identify resistant and susceptible clones much faster than the current method which has implications for the sugarcane breeding program. An ability to screen large numbers of clones quickly means that new varieties can be developed that are resistant to nematodes faster. The faster screen can also be used to introgress resistance from wild germplasm in an introgression breeding program. Introgression breeding is a long process and the number of clones screened is limited using the current method of screening for nematode resistance. This new method will allow many more clones to be screened over a year which will increase the chance of successful introgress of resistance genes from wild germplasm.

This project screened the same germplasm using both the existing nematode screen and the new method and was able to show that both methods gave the same results. Further work on the new method is required to develop an optimised rating system but the new method has the potential to speed up the production of new varieties resistant to both root-knot and root-lesion nematodes.

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1. BACKGROUND

1.1 Breeding for resistance to nematodes

Root-lesion nematodes (*Pratylenchus zeae*) and root-knot nematodes (*Meloidogyne javanica*) are the two most important nematode pathogens of sugarcane in Australia. They cause loses of in excess of $82 million to the Australian sugar industry annually (Stirling et al. 2011). While no commercial sugarcane varieties are resistant to these nematodes some varieties are classed as tolerant. More recently work has identified resistance in the related wild germplasm *Saccharum spontaneum* and *Erianthus arundinaceus* (Stirling et al. 2011; Bhuiyan et al. 2014, 2016, 2019). These studies demonstrated that wild accessions of *S. spontaneum* had significantly reduced the reproduction ability of both nematodes compared with commercial cultivars. This study demonstrated that *S. spontaneum* lines possessed resistance to these two nematode species. Targeted crossing of selected *S. spontaneum* accessions with commercial hybrid parental lines could be used to introduce root-knot and root-lesion nematode resistance into sugarcane cultivars for the Australian sugar industry.

An introgression breeding program using *S. spontaneum* was established in 1961 in Australia to increase ratooning ability, yield and disease resistance, the main difficulty was in recovering commercial clones with acceptable sucrose levels. Up to four generations were required to reach acceptable sucrose levels (Berding and Roach 1987). To use introgression to introduce nematode resistance would require the same process and would mean a very large number of plants would have to be screened at each backcross cycle to recover the nematode resistance phenotype.

The current method for screening for either root-knot or root-lesion nematode resistance is very expensive and time consuming and is not feasible to use for introgression breeding for these traits. The method used for screening for root-lesion nematode resistance requires single eye setts to be grown in 1 L pots of pasteurised sand/peat for 4 weeks before being inoculated with the appropriate nematodes. The plants are then grown in an air-conditioned bench with the temperature within the pots maintained at 26 +2^{o}C for the duration of the trial. After inoculation the plants are grown for a further 11 weeks before being harvested and scored. The process for screening for root-knot nematode resistance is similar although the one-eyed setts are grown in sand. For both methods the plants are harvested 11 weeks after inoculation and both the shoot and root biomass are measured. The nematodes and eggs were extracted and counted to obtain the reproduction factor. At time of harvest the roots are also given a visual score. Bhuiyan et al. (2019) determined that a few *S. spontaneum* lines were resistant to both types of nematode which is highly unusual but would be very useful for breeding for both root-knot and root-lesion nematode resistance.

This project proposes to test a new high throughput method for screening for nematode resistance that was developed for grapevine and used to identify and map a resistance gene for root knot nematode (Harley et al. 2018). This method uses in vitro grown roots, is high throughput and takes less than a week from inoculation to scoring the data. This project will adapt this method from grapevine to sugarcane and determine by comparison with existing disease ratings for nematode resistance carried out using the current method whether similar results are obtained. If a high throughput method for screening for root-knot and root-lesion nematodes can be developed it will allow many more sugarcane clones to be screened cost effectively. This will benefit the sugarcane breeding program by increasing the number of clones screened to allow the selection of more tolerant varieties within the existing germplasm. In addition, it will allow the cost-effective introgression of novel genes for resistance from *S. spontaneum* and potentially *E. arundinaceous* accessions into the breeding program.

2. PROJECT OBJECTIVES

2.1 Research proposal objectives

(a) Testing and optimising for sugarcane a new high throughput method for screening plant roots for resistance to root-knot and root-lesion nematodes.
(b) Developing a rating method for disease resistance using the digital image of infected roots that is less subjective than the current screening method.
(c) Determine if the new method which is faster and cheaper than the current method gives the same or improved results.

3. OUTPUTS, OUTCOMES AND IMPLICATIONS

3.1 Outputs

The following outputs were generated from this project:

(a) A new high throughput screening method for sugarcane to identify clones with root-knot and root-lesion nematode resistance.

A new method was developed in this project which would allow high throughput screening of large amounts of clones for resistance to root knot nematodes (RKN) and root lesion nematodes (RLN). Although the same results were obtained using the new method as had previously been found with the old method further work is required to develop a more refined rating method for this screen and more clones need to be screened to help with this.

(b) Data that provides information on how the new method compares to the existing method for screening for resistance to root-knot and root-lesion nematodes.

We compared the old method of screening RKN and RLN with this new method for two clones and found that the resistant clone remained resistant with the new method and the susceptible clone was susceptible at 4 days post inoculation. The new method takes around 3 weeks to complete compared to 16 weeks for the old method.

(c) Information on the interaction between *S. spontaneum* and *E. arundinaceus* roots and both root-knot and root-lesion nematodes.

Data is available on the interaction between six *S. spontaneum* and two *Erianthus arundinaceus* clones and RKN and RLN resistance.

(d) Development of an introgression plan for incorporating novel resistance genes to root-knot and root-lesion nematodes from *S. spontaneum* into commercial varieties.

The new method could be used to introgress the resistance from *S. spontaneum* and *Erianthus arundinaceus* into sugarcane germplasm as large numbers can be screened at a time and the screen takes one week to complete compared to the current 16-week timeframe.

3.2 Outcomes and Implications

This project has developed a new faster screen for resistance to nematodes. The use of this new method has the potential to reduce the cost of developing new sugarcane varieties that are resistant to both root-knot and root-lesion nematodes. Having varieties that are resistant to these nematodes could save the industry over $80 million a year. The new method can save time in the screening process as it takes up to 3 weeks to complete compared to the current screening method which takes up to 16 weeks. This means that in one year more germplasm can be screened for resistance to these nematodes. Increasing the number of clones that can screened means more breeding material can be assessed for resistance which should increase the speed of development of new varieties that are resistant to root knot and root lesion nematodes. This also has implications in introgression breeding where large numbers of progeny are screened to identify the germplasm that contains the genes for resistance. The decrease in time required for screening with this new method means that resistant varieties will be generated faster and sugarcane farmers will be able to grow varieties that are resistant to nematodes and obtain higher yields on soils that contain root knot and root lesion nematodes.

4. INDUSTRY COMMUNICATION AND ENGAGEMENT

4.1 Industry engagement during course of project

This project is a collaboration between CSIRO and SRA Woodford and was carried out jointly. The new method has been developed with Shamsul Bhuiyan and his colleagues at SRA. The key results have been communicated to the team who routinely screen sugarcane clones for resistance to RKN and RLN using the current method.

Further work will be required before this method can be used in a routine screening procedure, but it may be useful for early screening of germplasm which will allow highly susceptible germplasm to be discarded. The new method can be carried out in a much shorter timeframe which will allow larger numbers of germplasm to be screened.

4.2 Industry communication messages

Key communication points are as follows:

- The new screening method for resistance to root-knot and root lesion nematodes developed in this project has the potential to screen sugarcane germplasm at a much faster rate than the current method used.
- This faster method will allow a much higher throughput of germplasm for resistance screening which will reduce the time to develop varieties with resistance to these two types of nematodes.

- This project has also delivered new information on wild species that have resistance to root-knot and root-lesion nematodes.
- This method could be used to introgress genes for resistance from wild germplasm into current sugarcane varieties.

5. METHODOLOGY

5.1 Glasshouse experiments

(a) Screening germplasm- Experiment 1-2020

A set of germplasm was identified containing wild species that could be used to assess the range of resistance to root knot and root lesion nematodes present in the germplasm (Table 1).

Table 1. Germplasm used to identify novel resistance to RKN and RLN

Germplasm	Species	RKN rating	RLN rating
SES208a	*Saccharum spontaneum*	unknown	unknown
Coimbatore	*Saccharum spontaneum*	unknown	unknown
Mandalay	*Saccharum spontaneum*	unknown	unknown
Okinawa	*Saccharum spontaneum*	unknown	unknown
UM69-4	*Saccharum spontaneum*	unknown	unknown
SES-196	*Saccharum spontaneum*	unknown	unknown
YN02-356	BC1 *Saccharum spontaneum*	unknown	unknown
IJ76-333	*Erianthus arundinaceus*	unknown	unknown
IJ76-370	*Erianthus arundinaceus*	unknown	unknown
Q208	Variety	Susceptible	susceptible
KQ228	Variety	Resistant	tolerant
ROC25	Foreign variety and parent of BC1 population	Unknown	unknown
Q135	Variety	susceptible	susceptible
Q231	Variety	Resistant	tolerant

The initial experiment used the conventional method to screen for nematode resistance to establish the results that we would expect to get from a faster high throughput method. The screen was set up as in Bhuiyan et al. (2016) as follows:

5.1.a.i.1 Screen for resistance to RLN

Setts from all accessions were germinated in March 2020 in vermiculite at 32^0C for two weeks. The germinated setts were then planted into 1 L plastic pots (one sett per pot) in potting mix made up of 33/33/33 mix of river sand/potting mix and peat moss. Each batch is mixed for 20 pots together with Ridomil 0.56g/500ml water plus 20g of Osmocote. Pots were maintained on a bench in the glasshouse at Sugar Research Australia in Woodford and watered when required. Approximately four weeks after planting into pots the plants were inoculated with 1997 nematodes per pot. Where possible 3 replicates were inoculated per genotype and three uninoculated pots. Nematodes were prepared as below and added into 5 ml deionised water which was injected towards the base of the plant in two holes using a pipette.

5.1.a.i.1.1 Preparation of RLN

Nematodes (P. zeae) for inoculation were grown on sterile carrots in clear plastic bottles using methods by (Kagoda et al. 2010; Moody et 1973). Nematodes were collected when they were visible in the water inside the bottles and stored in deionised water before inoculation.

5.1.a.i.1.1.1 Trial design and assessment

Each experiment was set out on a glasshouse bench using a split plot design where the inoculated and uninoculated blocks were arranged as main plots and test clones comprised the subplots. Approximately 10 weeks after inoculation the experiment was harvested. For shoot mass the shoots were cut near the base of the plant and placed into paper bags. The root biomass of uninoculated plants the whole root system was placed into a paper bag. For the inoculated plants a portion of the roots from the root system was collected and weighted. All plant material was dried in a drying cabinet at 65^{0}C for 2 weeks. The final weight of the root samples was measured and then the biomass of the whole root system was estimated for the inoculated plants. The remainder of the root system for the inoculated plants were used for nematode extraction.

Nematodes were extracted from the mixture of root and potting mix using the method from Bhuiyan et al. 2016 which was based on the Whitehead tray method (Whitehead and Hemming 1965). Approximately 250g of root and potting mix was placed on double layered tissue paper on a steel mesh set in a flat tray. The soil and roots were almost covered in water and left for 48hr at 25^{0}C. Nematodes were collected on a 38um sieve and the extract then poured into a 30 ml plastic vial. The extracted nematodes and egg samples were stored at 6^{0}C until counting. The nematodes were counted using a compound microscope (10x-40x) using a Hawksley slide counting chamber of 1ml capacity.

5.1.a.i.2 Screen for RKN

The procedure for plant growth and inoculation was the same for RKN as for RLN. Apart from the potting mix used for the RKN screen was 70% river sand, 15% potting mix and 15% peat moss.

5.1.a.i.2.1 Preparation of RKN

Nematodes (M. javanica) were reared in the roots of highly susceptible tomato plants in a glasshouse as described by Van den Bergh et (2002). Nematode eggs were extracted from the tomato plants using the bleach method as in Stirling et al. (2011). Approximately 2 months after inoculation the roots of tomato plants were washed free of sand cut into 2 cm pieces and soaked in 0.5% of sodium hypochloride (NaOCl) for 5 min. Egg suspensions were passed through a 150 um sieve to remove debris and finally retrieved on a 38 um sieve. Each plant was inoculated with 4784 eggs per pot.

5.1.a.i.2.1.1 Trial design and assessment

The trial was designed the same as for the RLN experiment and approximately 10 weeks after inoculation the trial was assessed for shoot and root biomass using the same method as for the RLN experiment.

For assessment of resistance to RKN the roots were washed free of the potting mix and visually rated for galling using a previously described method (Shepherd 1979) with modifications by Bhuiyan et al. (2016) on a scale 0 to 5 where 0=no galls, 1=<2% of roots galled, 2 = 2-25% of roots galled, 3 = 25-50% of roots galled, 4 = 51-75% of roots galled, 5 = >75% of roots galled. For the egg extraction the roots were washed free of the potting mix and submerged in1% bleach (NaOCl) and agitated for 5 min. The bleach solution was then poured through two sieves (a 150um sieve over a 38um sieve), and the RKN eggs were collected in the smaller sieve then washed into a collection vial. Extracted eggs were stored at 6^{0}C until counting then counted as for RLN.

1.1.1. Data collection and statistical analysis

The number of nematodes (RLN) or eggs (RKN) per pot or plant was calculated and the reproduction factor (RF) was determined as RF = pf/pi, where pf is the final nematode population or eggs after extraction and pi is the initial nematode or egg population used for inoculation. Number of nematodes or eggs per g of dry root was estimated form the total number of nematodes or eggs present in the total amount of root biomass. An analysis of the data was carried out as in Bhuiyan et al. (2016) using PROC MIXED in SAS version 9.4 and fitting a linear mixed model where clone, inoculation status and their interaction effects were treated as fixed effects and the block and interaction between block and inoculation status and the error term were treated as random effects. Values were log-transformed (in(x+1) before analysis to normalise the data including the root and shoot biomass.

Estimated log-transformed values were then back transformed for presentation of the results. For means comparisons all possible pairwise differences of the means were tested at alpha = 0.05, using fisher's protected LSD test.

(b) Experiment 2- 2021

This experiment was designed to repeat the previous experiments for two genotypes that were extreme in their reaction to RKN and RLN. Having only two genotypes allowed an increase in replication and the ability to use the same stalk material for both the conventional screening method and the new method so the results could be directly compared.

(I) EXPERIMENTAL SET-UP

Four genotypes were sourced from SRA Woodford, SES208A which is resistant to both RKN and RLN and Q208 which is susceptible to both nematodes as well as two Erianthus clones. The experiment was set up in the glasshouse at SRA Woodford in March 2021 in the same way as for the first experiment. The only difference was that for the RLN experiment 2109 nematodes were inoculated into each pot and 245 juveniles (j2's) were inoculated into each pot for the RKN experiment.

5.2 Microscopy of roots from glasshouse experiment 2020

Roots from experiment 1 were sampled by cutting 2cm from the tips of roots from each plant and fixing in 70% ethanol. These roots were then embedded in 5% low melting agarose for sectioning. The sections were cut with a vibratome set at 15-20 um per section. The sections were mounted on slides in 50% glycerol and photographed on a compound microscope and the sections compared between the different species.

5.3 Control environment experiments

The rational for these experiments was that screening for RKN and RLN resistance takes a total of 16 weeks from germination of the setts to harvesting the experiment. If a new faster method could be developed it would reduce the cost of screening for nematode resistance which would have a significant impact on the development of new resistant varieties. The experiments were set up as below using sterile nematodes as follows:

(a) Sterilisation of nematodes

(I) RLN

Once nematodes are extracted (see 5.1.a.i.1.1) a solution of Streptomycin is made by dissolving 0.06g in 10 ml of water. Add 1ml of Streptomycin sulphate to 2ml of containing nematodes and leave for 24 hours at room temp. Then the supernatant was removed and sterile water added then time was taken to wait for the nematodes to settle to the bottom then the process was repeated twice more. Then nematodes were resuspended in the required volume.

(II) RKN

Once nematodes are extracted (see 5.1.a.i.2.1) they are washed in 0.5% chlorine then rinsed 3 x in a 10um sieve to remove chlorine then hatched in sterile water.

(b) Experiment 1

From the pot experiment four genotypes were selected, two highly resistant (SES208A, IJ76-333) and two highly susceptible (Q208 and Q135). These clones were sourced from SRA Meringa and Mackay as stalks. The stalks were cut into one eye setts, and hot water treated at 50^0C for 30 min then surface sterilised with 1% sodium hypochlorite (Na OCl) then rinsed three times in sterile water. The setts were left to dry in the laminar flow cabinet then placed on wet filter paper in plastic pots at 32^0C in high humidity in the dark to allow the roots to germinate. Once the roots had germinated, they were inoculated directly with 25 juvenile RLN and RKN. Once inoculated they were moved to 24^0C and the roots observed for a response to the nematodes.

(c) Experiment 2

The first experiment had to be modified as the sett in direct contact with the wet filter paper resulted in the juice leaking on to the filter paper and causing fungal growth which contaminated the experiment. Two modifications were made. The ends of the filter paper were sealed with melted wax and the setts were placed above the filter paper on lids so that the sett did not touch the paper.

(d) Experiment 3

From the previous experiments it was clear that the roots for most genotypes, (Q208 being the exception) took a long time to germinate which allowed bacterial contaminates and fungus to grow and affect the experiment. This was probably due in some cases to the health of the plants that the stalks were harvested from. The other

problem was that often the roots did not reach the paper and thus ended up dried which was not a good source of food for the nematodes. For these reasons it was decided to set up an experiment where the setts were germinated in sterile vermiculite to grow the roots to a length that would allow them to sit on the wet filter paper for the inoculation phase.

Two genotypes were selected for the experiment Q208 (susceptible) and SES208A (resistant) then 94 single eye setts of SES208A and 90 setts of Q208 were hot water treated then planted in vermiculite and left to germinate at 32^{0}C under lights for 18 days. Once the roots had grown, they were removed from the vermiculite and the ends of the setts coated with melted wax using a paint brush. They were then placed in sterile clear plastic pots containing two Whatman 70 mm filter paper discs and 10ml of sterile water was added to each pot. For each genotype 5 replicates were used for control, RKN and RLN. The roots were sampled at 4, 7- and 21-days post inoculation. Water was added to the pots when required.

Once the pots were set up five replicates for each time point were inoculated directly onto the roots with 279 RKNs and 267 RLN per pot. The pots were then covered in black plastic to keep the nematodes in the dark and placed at 24^{o}C. Five pots were sampled at 4, 7- and 21-days post inoculation

5.4 Staining for nematodes

In order to determine if the nematodes had infected the roots of the control environment experiments the roots were stained to visualise the nematodes and eggs as follows:

The roots were cut from the setts and placed in a 1.5% sodium hypochlorite solution (NaOCl) for 5 minutes, stirring occasionally. The bleach solution was then removed, and the roots were submerged in a 1% acetic acid solution for 15 minutes. The Acid was drained and the roots place in 30 ml of distilled water with 1 ml of stain (0.35g acid fuchsin, 25 ml acetic acid, 75 ml water). The solution was boiled for 30 seconds and left to cool for 15 minutes. The stain was removed, and the roots rinsed in water. The roots were then placed in de-stain (2ml HCl, 300ml water, 700mlof glycerol). The de-stain was boiled then the flask placed in water to cool rapidly and left for 15 minutes. The roots were drained and mounted in de-stain on slides. The slides were viewed on a compound microscope and photographed. The number of nematodes or eggs was assessed.

6. RESULTS AND DISCUSSION

6.1 Glasshouse Experiment_1 (2020)

(a) Identification of resistance germplasm

This experiment was harvested in July 2020 and analysis was carried out on all plants for both RKN and RLN (Figure 1).

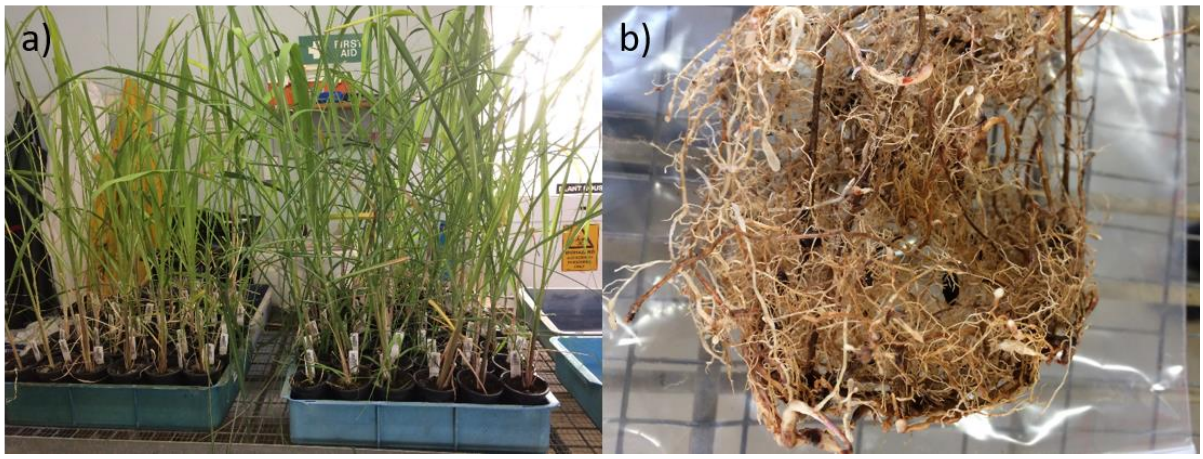

Figure 1. a) showing plants from Experiment 1 and b) Q208 infected with RKN showing galls on roots

(I) RLN

While there is some resistance or tolerance to RKN in the current sugarcane breeding germplasm there is no resistance to RLN. In this experiment 14 genotypes were screened for resistance, the same genotypes as for

RKN with the addition of SES-196 a *S. spontaneum*. In this experiment Q231 and KQ228 were classed as intermediate between susceptible and resistant and Q208 and Q135 were susceptible. There were only four genotypes that were considered resistant and all were wild germplasm and included SES208A, Mandalay and SES-196 which are all *S. spontaneum* clones and IJ76-370 an *E. arundinaceous*. Interestingly two of the *S. spontaneum* clones were susceptible to RLN indicating that there is variation for resistance within the *S. spontaneum* species (Table 2).

Table 2. Measures of resistance to RLN including number of nematodes per gram of roots relative to the susceptible standards (RGRT)

clone	Shoot biomass	Root biomass	Nematode/plant	Nematode per g root	RGRT	Resistance category
SES-208A	8.7 a*	4.8 bcdef	11041 abcde	121 g	7 f	R**
YN02-356	6.9 ab	3.3 ef	24218 abc	1879 abc	108.3 abc	S
Q208	6.7 ab	9.8 b	52260 a	1759 ab	101.2 ab	S
KQ228	6.5 abc	16.6 a	35252 ab	508 cefg	29.4 def	I
ROC-25	5.7 bcd	4.4 def	38333 ab	4595 a	264.2 a	S
IJ76-370	5.3 bcd	9.1 bc	9501 bcd	239 fg	13.8 ef	R
Q135	4.7 d	6.3 bcde	20708 abc	1559 abd	89.6 abc	S
Q231	4.7 d	6.2 bcde	12322 bcd	639 bcefg	37 bcdef	I
Coimbatore	4.6 d	3.4 ef	11492 bcd	765 bcef	44.1 bcde	I
IJ76-333	4.3 d	8.7 bcd	7228 cde	484 efg	28 def	I
Mandalay	4d e	4.6 cdef	3055 de	260 fg	15.3 ef	R
Okinawa	3.9 cde	3 cdef	13364 abcde	2793 ab	160.5 ab	S
UM69-4	2.5 e	1.3 f	2205 e	1214 bce	70.5 bcd	S
SES-196	1.6 e	1.7 ef	1047 e	307 cdefg	17.6 cdef	R

*Data in a column followed by the same letter(s) are not significantly different using Fisher's protected LSD test (p=0.05)

** Resistance category- R resistant, I intermediate, S susceptible.

Number of nematodes per plant varied from Q208 which had an average of 52,260 to SES-196 which had 1047 indicating that within SES-196 the nematodes were not able to multiply as well as within Q208. Measuring the shoot and root growth can be used to assist in comparing clones. Both RLN and RKN are obligate parasites of plants and their reproductive capacity is limited by the availability of roots. If clones with similar root biomass have different numbers of nematodes, then the difference is most likely due to resistance. If a clone has a smaller root system like SES-196 the lower number of nematodes could be due to the limitations on the root biomass available as a food source. The reproduction factor for SES-196 is very low (figure 2) but when you adjust this measure to include the root biomass by using number of nematodes per gram of roots relative to the susceptible standards (RGRT) the lowest number is for SES-208A (Table 2, Figure 3). This indicates that the low number of nematodes in SES208A is more likely to be due to resistance than to a lack of available food for the nematodes.

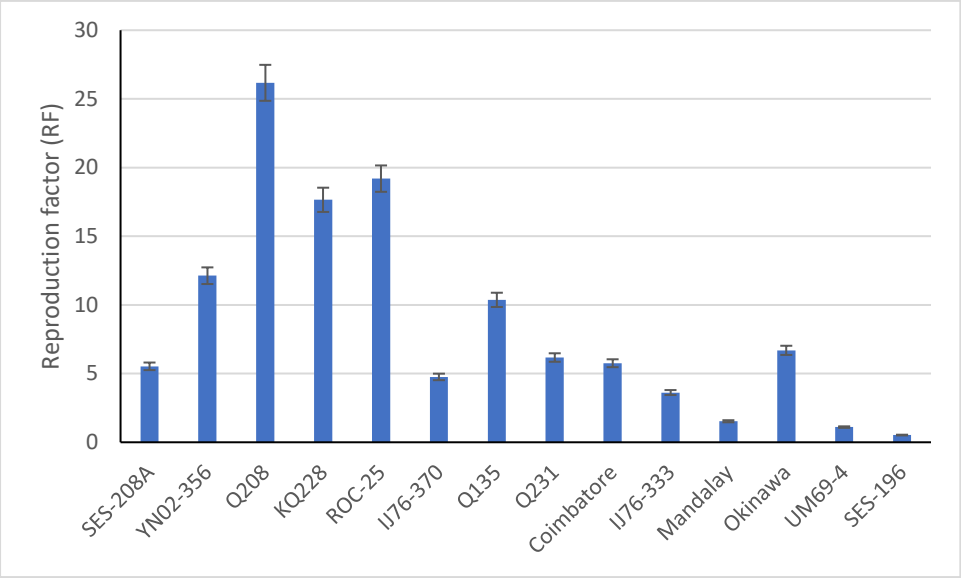

Figure 2. Reproduction factor for RLN

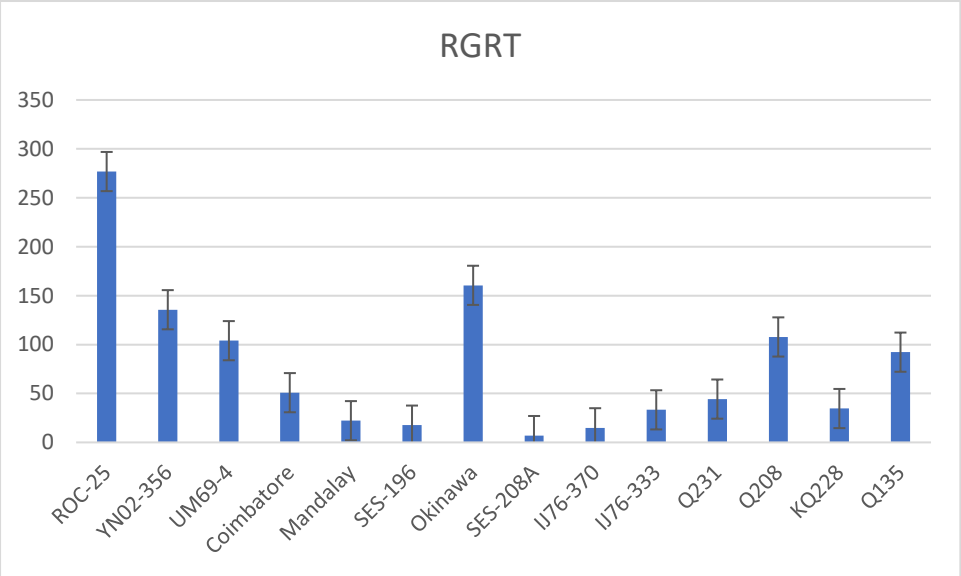

Figure 3. The number of RLN per gram of roots relative to the susceptible standards (RGRT)

(II) RKN

In total 13 clones were screened for RKN resistance in this first experiment to identify potential resistant germplasm to test with the new method. Of these 13 clones four varieties were used as controls. Q208 and Q135 are known to be susceptible to RKN and this was confirmed in this experiment with both varieties having the highest number of eggs per plant and per gram of root at the end of the experiment (Table 3).

Table 3. Measures of resistance to RKN including number of nematodes per gram of roots relative to the susceptible standards (RGRT)

clone	Shoot biomass	Root biomass	Egg/plant	Egg/ g root	Visual rating	RGRT	Resistance category
SES-208A	6.5 a*	1.94 ab	957 de	511 f	1.0 abc	1.3 ef	HR**

KQ228	4.4 b	1.69 ab	7199 bc	4295 cd	1.7 ab	10.9 bc	R
YN02-356	4.1 bcd	1.73 abc	12784 abc	7424 abcd	3.0 a	20.5 abc	R
ROC-25	4.0 bc	0.95 e	6292 bc	6977 cd	1.6 ab	20.5 b	R
Q208	3.7 cd	1.13 ced	33546 a	31763 a	2.7 a	80.0 a	S
Q135	3.0 de	1.42 bcd	32156 a	22712 ab	2.7 a	57.0 a	S
Q231	3.0 de	1.86 a	15751 ab	8584 bc	2.2 a	21.6 b	R
Coimbatore	3.0 de	0.88 e	3703 cd	4441 cd	1.0 bc	11.2 bc	R
UM69-4	3.0 de	1.30 bcde	1100 de	850 ef	0.4 cd	2.5 def	HR
IJ76-370	2.6 ef	0.94 de	3406 cd	3648 cde	0.4 cd	9.2 bcd	R
Okinawa	2.2 ef	0.82 de	471 e	620 ef	1.0 abc	1.6 def	HR
IJ76-333	2.0 f	0.91 e	2602 cd	2918 de	0.7 c	7.3 cde	HR
Mandalay	1.7 f	0.74 e	50 f	71 g	0.0 d	0.2 f	HR

*Data in a column followed by the same letter(s) are not significantly different using Fisher's protected LSD test (p=0.05)

** Resistance category HR highly resistant, R resistant, S susceptible.

The varieties KQ228 and Q231 known to have some resistance to RKN were also seen as being resistant (Table 3). Interestingly all the wild germplasm was either resistant or highly resistant confirming the results seen in Bhuiyan et al. (2016, 2019). Although all the wild germplasm had a rating score of either resistant or highly resistant based on the RGRT value they did not all produce the same root biomass (Figure 4, Table 3).

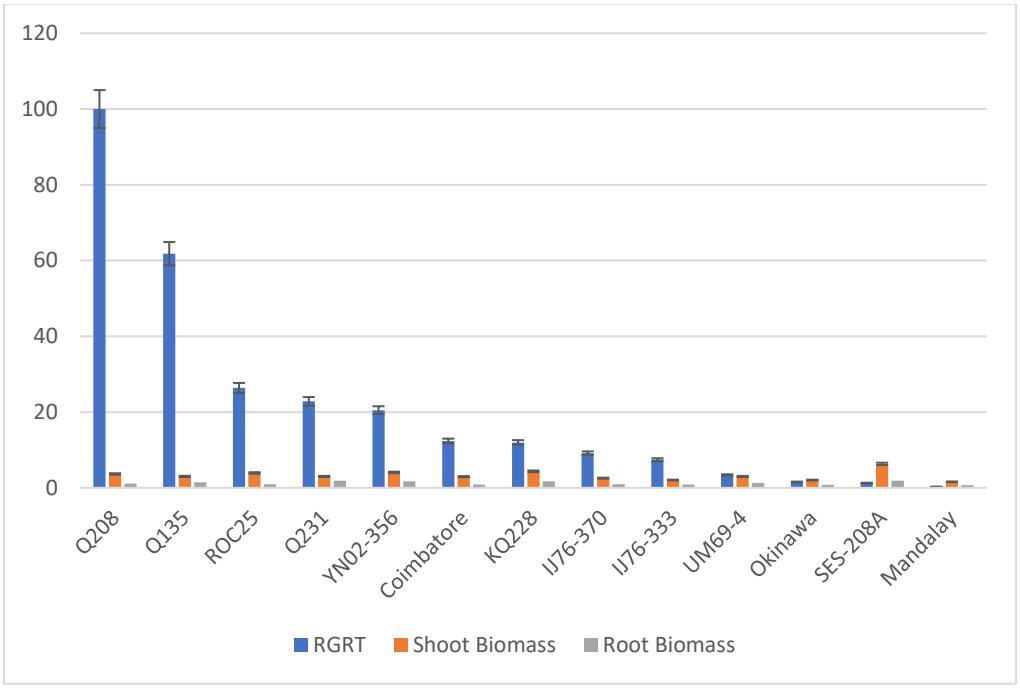

Figure 4. The number of RKN per gram of roots relative to the susceptible standards (RGRT), shoot biomass and root biomass of clones

These results indicate that amongst the wild germplasm SES-208A is one of the most resistant to RKN but also maintains a higher shoot and root biomass compared to the other accessions and therefore is the most likely to have less impact on biomass if used in an introgression breeding program. There was a small correlation

between shoot and root biomass (Figure 5) indicating that good above ground biomass does not always indicate good root growth.

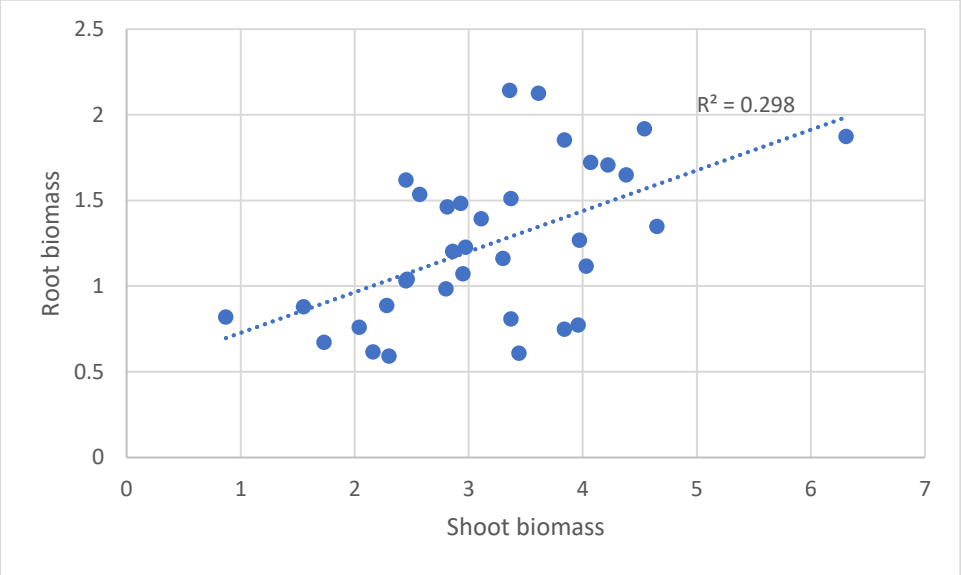

Figure 5. Correlation between shoot and root biomass of all the clones

A visual score is recorded for RKN resistance and this experiments indicates that the visual score is correlated to number of nematode eggs per gram of root tissue (Figure 6).

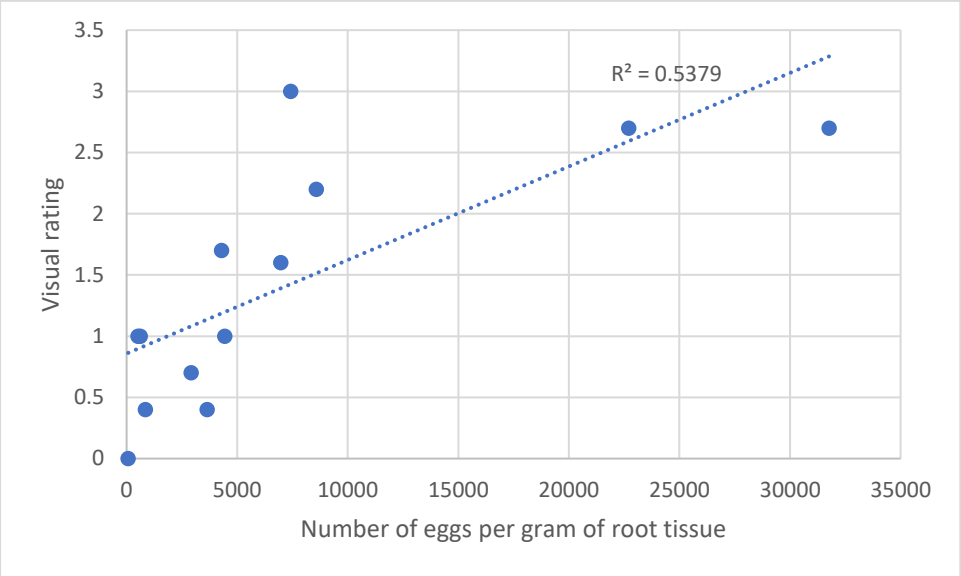

Figure 6. Correlation between number of nematode eggs per gram of root tissue and visual score of galls.

This experiment was able to confirm the resistance of the wild germplasm to RKN and when the reproduction factor was calculated all the *S. spontaneum* and *E. arundinaceous* clones were below 1 (Figure 7).

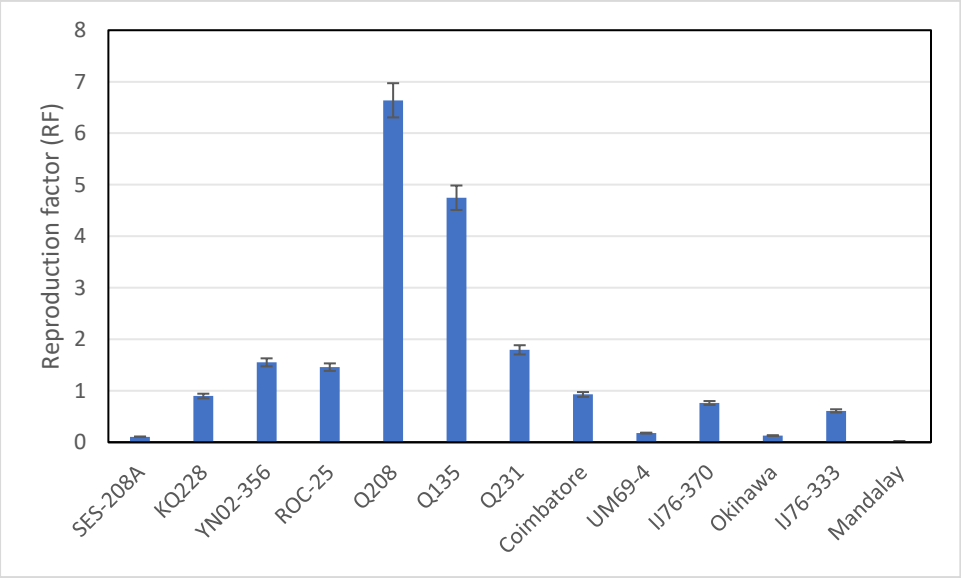

Figure 7. Reproduction factor for RKN experiment for all germplasm

(III)

(b) Experiment 2

As germination of the wild germplasm was poor for experiment 1 there were only enough plants to have two replicates each for inoculation of the RKN and the RLN. As resistance to nematodes is highly environmentally variable, we decided to repeat the experiment with 3 replicates and use only two genotypes. We chose SES208A as the resistant genotype as it was highly resistant to RKN in the previous experiment and resistant to RLN with the lowest value for RGRT. The most susceptible genotype was Q208 which had the highest reproduction factor for both RKN and RLN. The same conditions were used as for experiment 1. The plants were harvested 9 weeks after inoculation for RKN and 12 weeks after inoculation with RLN (Figure 8)

Figure 8. a) Q208 inoculated with RKN showing galls b) SES208A inoculated with RKN showing no visible symptoms

Q208 had an average visual rating for RKN of 2.8 which indicates susceptibility as expected which SES208A had an average visual rating of 1 indicating resistance. These results were similar to experiment 1 verifying the results.

Figure 9. a) Q208 inoculated with RLN b) SES208A inoculated with RLN

Both clones showed good growth when inoculated with RLN (Figure 9). Q208 did show some brown lesions when inoculated with RLN (Figure 10) but the symptoms were less obvious than for RKN.

Figure 10. A brown lesion from inoculation with RLN on Q208

The extraction of eggs and nematodes from the clones provided the expected results with very high numbers of nematodes and eggs for Q208 and low numbers for SES208A.

Table 4. Results for experiment 2 showing measures of resistance to RKN and RLN.

Clone	Replicate	Nematode Type	Nematode count per plant (pF)	Total Egg per fresh root (pF)	Nematode/eggs per gram of dry root	Nematode Inocula pI	pF/pI
Q208	1	RLN	36924		257	2109	18
Q208	2	RLN	27860		197	2109	13
Q208	3	RLN	36350		311	2109	17
Q208	1	RKN		4120	930	245	17
Q208	2	RKN		12126	2990	245	49
Q208	3	RKN		10620	2288	245	43
SES208A	1	RLN	10793		169	2109	5
SES208A	2	RLN	12536		89	2109	6
SES208A	3	RLN	4162		108	2109	2
SES208A	1	RKN		20	5	245	0
SES208A	2	RKN		123	31	245	1
SES208A	3	RKN		402	68	245	2

This second experiment verified the results from the first experiment with SES208A being more resistant to both RLN and RKN than Q208 (Table 4).

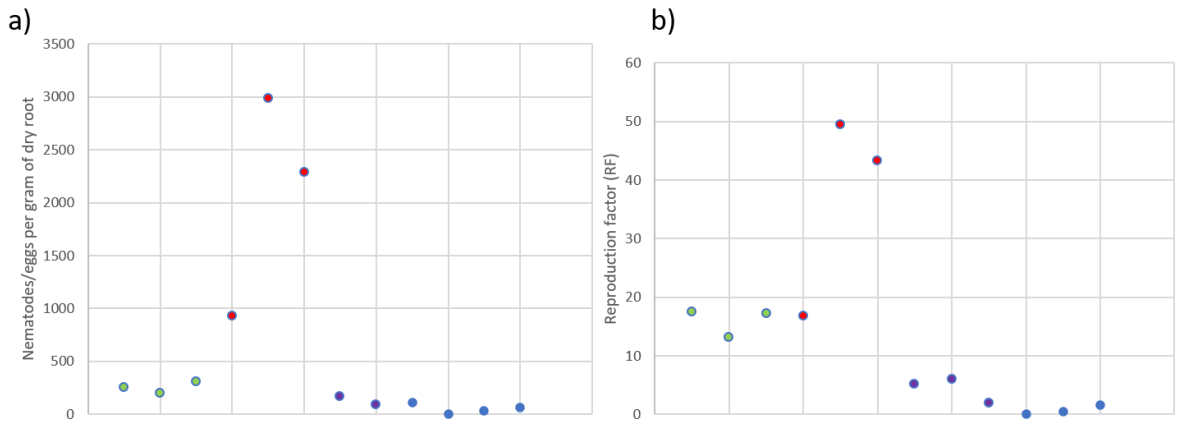

Figure 11. a) The number of nematodes/eggs per gram of dry root and b) the reproduction factor, green circles Q208 RLN, red Q208 RKN, purple SES20aA RLN and blue SES208A RKN

The data showed good separation for both number of nematodes (RLN) and eggs (RKN) per gram of dry roots and RF between the two genotypes (Figure 11).

These results provided us with the data to compare the results from the new method of screening for RLN and RKN.

6.2 Microscopy of roots

From the microscopy of the roots taken from the first glasshouse experiment, it is clear that there is a large amount of variation in the root phenotype even within one genotype (Figure 12). The phenotype of the root changes with distance from the root tip with increased aerenchyma forming the further away from the root tip the sample is taken. This was noted by Grandis et al. (2019) in their work on root phenotypes of the sugarcane clone SP80-3280. There was no consistent change in root phenotype between the species tested although the

Erianthus clones had a larger root stele area compared to the other species (Figure 12). There was no change in the root phenotype with the inoculation of either RKN or RLN (see appendix 2-4).

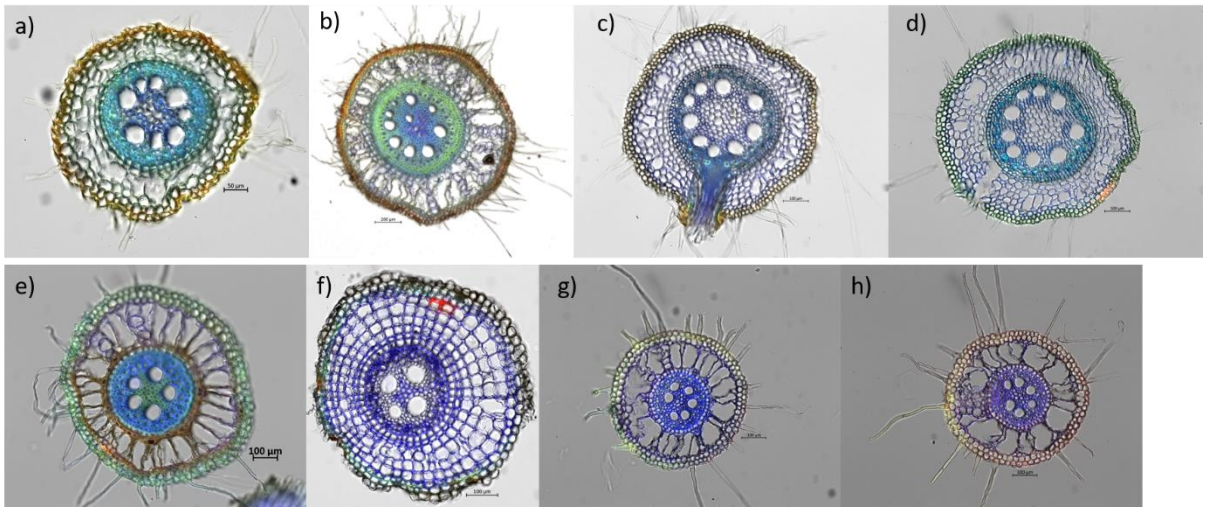

Figure 12. Cross-sections of root segments showing the difference in relative size of the central section of the root containing the phloem and xylem vessels, a and b - IJ76-333, c and d- IJ76-360, e and f – Q135, g and h – Q208.

6.3 Controlled environment experiments

(a) First experiments

The goal of these experiments was to develop a new screening method for assessing clones for resistance to RKN and RLN. This method would be used to screen a large number of clones and would potentially replace the current screening method which takes around 16 weeks.

Initially the idea was to get the roots growing fast so that they could be inoculated with the nematodes quickly and assessed within a week. For the first experiment from the result of our glasshouse experiment we selected three genotypes. Q208, which is susceptible to both RKN and RLN and is known to grow roots quickly. We also selected two *Erianthus* clones, IJ76-370 which is resistant to both RKN and RLN and IJ76-333 which is resistant to RKN and intermediate to RLN. These clones were cut into one eye setts placed in sterile containers at 32^{o}C in the dark to germinate roots. Once the roots had grown, they were inoculated with nematodes at 7 days (Figure 13).

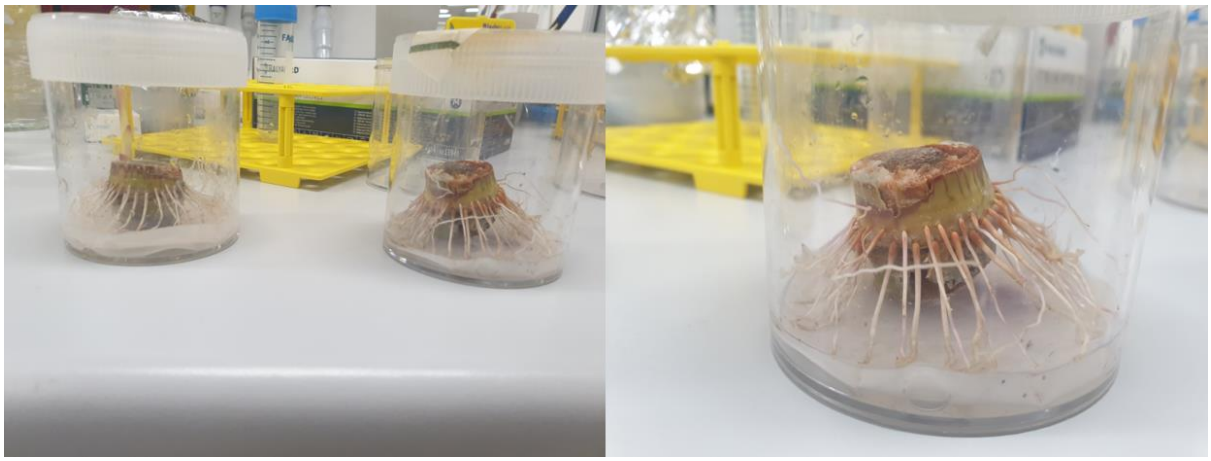

Figure 13. Q208 showing good root growth at 7 days

The roots of Q208 were healthy with low contamination at this stage (Figure 14).

Figure 14. Q208 showing healthy roots at 7 days.

Once the roots had grown far enough to reach the paper the nematodes were inoculated directly onto the roots. The roots were then assessed every day for a visual response (Figure 14).

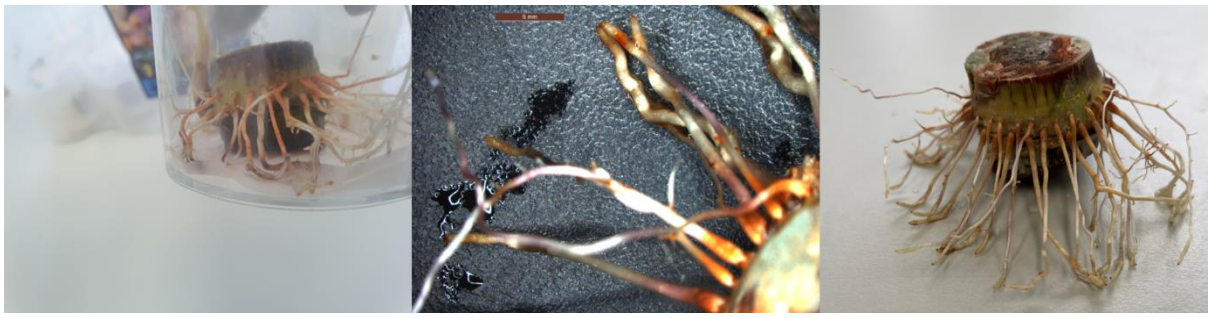

Figure 15. Q208 inoculated with RLN

Four days after inoculation the roots were showing signs of lesions for RLN. The major problem was the contamination of the setts with bacterial and fungal growth which made it difficult to determine what was causing the symptoms (Figure 15).

It was clear that for Q208 there was a response to inoculation with RKN (Figure 16).

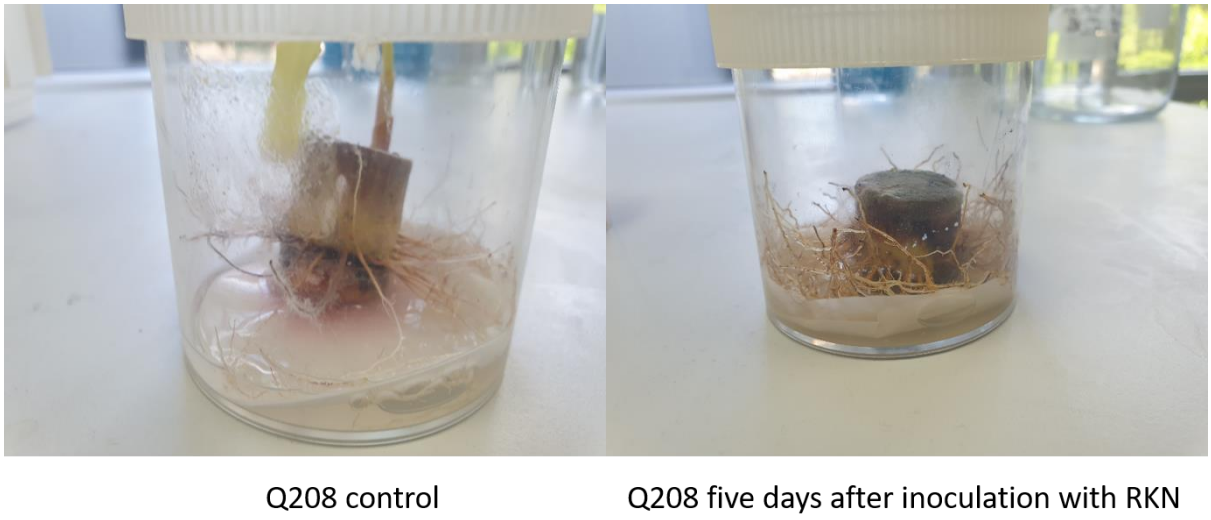

Figure 16. Q208 control and five days after inoculation with RKN

Figure 17. Q208 showing extensive contamination of bacteria and fungus.

This contamination was occurring even with the setts being pre-treated with 1% bleach and a hot water treatment to kill any bacteria and autoclaving the plastic containers, paper and water (Figure 17).

Another problem was that the *Erianthus* clones did not grow roots very fast and the setts would get contaminated before the roots were long enough to reach the water-soaked paper (Figure 18). As the nematodes where inoculated onto the roots directly the roots needed to have reached the paper.

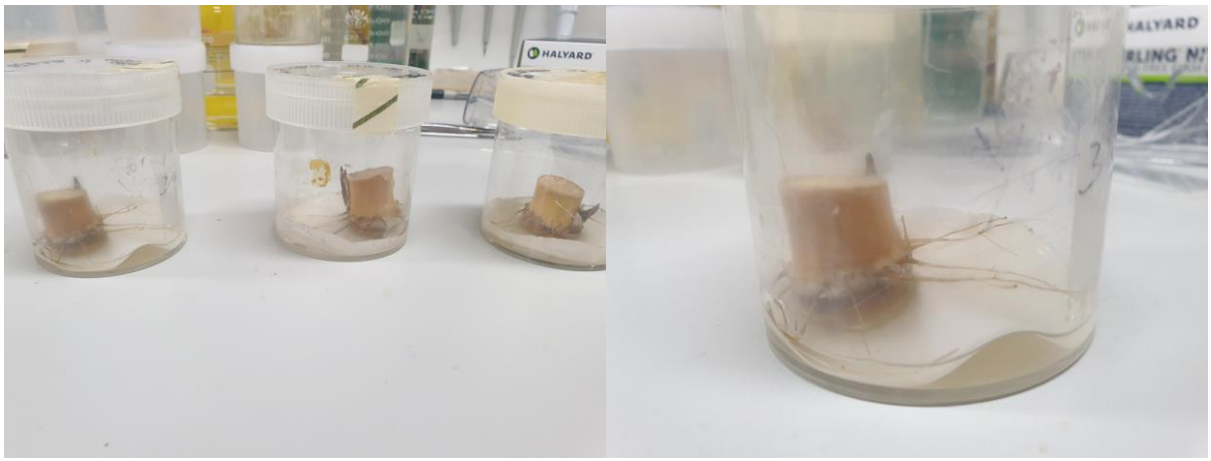

Figure 18. IJ76-370 with poor root growth and inoculated with RLN

Further modifications were needed to increase the amount of root growth and reduce contamination. The juice leaking from the setts appeared to be increasing the contamination of bacteria and sealing the ends of the setts with wax did help reduce this problem. There was still a problem with slow root growth and to improve this the move was made in the last experiment to germinate the setts in sterile vermiculite then transfer them to clear pots for the inoculation.

(b) Final modified method

Two genotypes were selected Q208 as the susceptible genotype to both RKN and RLN and SES208A which is resistant to both RKN and RLN. These were germinated for 18 days to produce god root growth (Figure 19).

Figure 19. Germination of Q208 and SES208A

The setts were then removed from the vermiculite and the ends of the setts painted with melted wax (Figure 20).

Figure 20. Painting the end of the setts with wax to seal the sett

The setts were then transferred to plastic pots but were placed above the wet paper to reduce the chance of contamination letting the roots rest on the paper for inoculation (Figure 21).

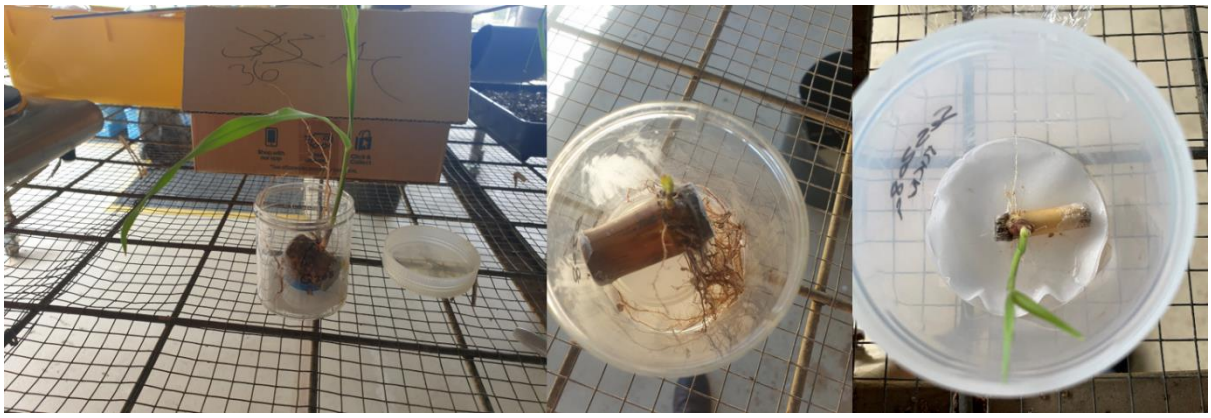

Figure 21. Q208 and SES208A placed in plastic resting on a small container allowing the roots to touch the wet paper but not the sett

Once the setts were placed in the containers sterile water was added to the paper and the nematodes were inoculated directly onto the roots of the setts. The pots were then covered with plastic wrap to reduce evaporation then all pots were covered in black plastic to keep the nematodes in the dark (Figure 22).

Figure 22. Inoculated pots wrapped in plastic and covered with black plastic to keep the nematodes in the dark

The pots were then placed at 27^{O}C until sampled. The pots were sampled at 4, 7- and 21-days post inoculation. Lesions from the RLN could be seen on the roots of Q208 at 7 days post inoculation and the roots became highly branched after inoculation of RKN (Figure 23).

Figure 23. Q208 7 days after inoculation with RKN and RLN

No symptoms could be seen on SES208A even after 21 days (Figure 24).

Figure 24. SES208A at 21 days post inoculation

Root growth of SES208A was still very slow but roots were still healthy at 21 days post inoculation.

To determine if nematodes were infecting the roots staining of the roots for nematodes was carried out using acid fuchsin. At 4 days post inoculation RKN and eggs could be seen in the roots of Q208 (Figure 25), no nematodes were seen in SES208A.

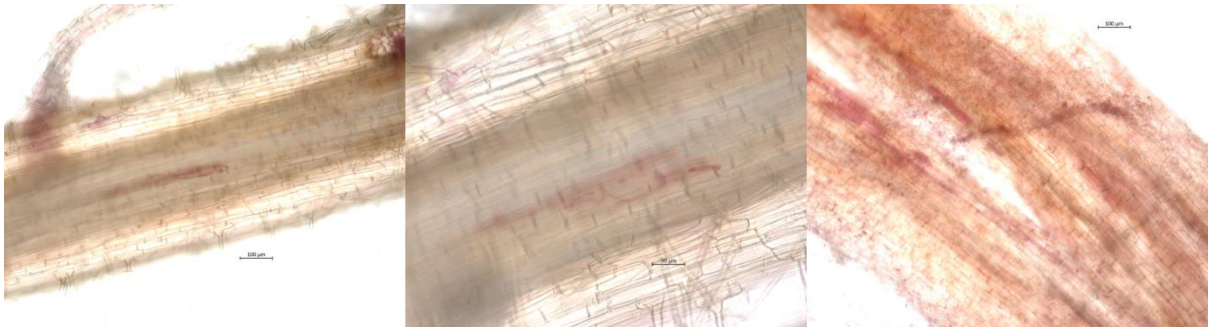

Figure 25. Stained roots of Q208 4 days post inoculation showing RKN inside the roots

These results indicate that it is possible to determine if a clone is resistant or susceptible to RKN and RLN within 22 days from starting the setts germinating using root staining to detect nematodes or eggs. This compares to the 112 days that the current method of screening for RKN and RLN resistance takes to determine a resistant genotype. The results obtained for the two clones Q208 and SES208A were consistent between the current nematode screening method and the new method.

Further work is required to test more genotypes and develop a rating method for both the RKN and the RLN new screen.

7. CONCLUSIONS

The idea for a fast-high throughput screen for RKN and RLN resistance was based on work by Smith et al. (2018) who developed a high throughput screen for grape. Their work was based around nodal cuttings from grape which were sterilised and grown on media which initiated root growth. As sugarcane is generally grown from setts this project concentrated on using the sett roots for the screen. The development of the screen was hindered by the setts having a high sugar content which encourage bacterial and fungal contamination. After a number of iterations, the project developed a screen that can be used for both root knot and root lesion nematodes. The screen takes less than 3 weeks to complete as opposed to around 16 weeks for the current method.

This project also provided information on new germplasm that is resistant to RKN and RLN, this germplasm could be used in an introgression breeding program to transfer the resistant genes into sugarcane varieties. The new screening method would allow large numbers of clones to be screened increasing the chances of success.

Further work is required to optimise the new screening method and to develop a rating method. Currently only a highly resistant clone and a highly susceptible clone have been processed through both the current screen and the new method developed in this project. The results were the same for both the methods but more testing of a set of germplasm with a range of ratings is required.

This new screening method has the potential to reduce screening costs for RKN and RLN and increase the efficiency of the resistance breeding process. It could contribute to the development of new varieties resistant to both nematodes faster.

8. RECOMMENDATIONS FOR FURTHER RD&A

The new screening method has been developed using two extreme genotypes, one that is highly resistant to both root knot nematodes and root lesion nematodes and one that is susceptible to both nematodes. This project has demonstrated that the new method gave the same result as the current method in a much shorter timeframe. Further work is required using a range of germplasm that is known to have a range of resistant to susceptible responses to the nematodes. This will allow the development of a rating method that can be standardised for the new screening method. Currently the new method has used staining of nematodes and eggs to determine if the clones are resistant or susceptible but potentially collection and counting of the nematodes or eggs could provide a similar rating method to the current screening method.

9. PUBLICATIONS

10. ACKNOWLEDGEMENTS

We wish to thank all members of the project team especially Jingchuan Li from CSIRO for her help in setting up the root microscopy experiments and help with the RKN and RLN screens. We would also like to thank Jack Cobon from CSIRO for his work on the development of the new high throughput screen. We would like to thank Kylie Garlick and her colleagues at SRA Woodford for all their help with the RKN and RLN screens to identify the resistant wild germplasm. We would especially like to thank Kylie Garlick for the generation of RKN and RLN for the many experiments we ran to develop the new screen. We also acknowledge the support of other SRA and CSIRO colleagues such as Annelie Marquardt who provided the root microscopy method to look at variation in root phenotypes.

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12. APPENDIX

12.1 Appendix 1 METADATA DISCLOSURE

TABLE 5 METADATA DISCLOSURE 1

Data	Image files for root microscopy and root stained for nematode detection
Stored Location	CSIRO Bowen storage server (secure)
Access	Restricted
Contact	Karen Aitken, Principal Research Scientist

TABLE 6 METADATA DISCLOSURE 2

Data	Data from RKN and RLN experiments
Stored Location	SRA
Access	Restricted
Contact	Shamsul Bhuiyan, Plant Pathologist

12.2 Appendix 2. Cross sections of roots from control uninoculated plants

Appendix 2.pptx

12.3 Appendix 3. Cross sections of roots inoculated with root-knot nematodes

Appendix 3.pptx

12.4 Appendix 4. Cross sections of roots inoculated with root-lesion nematodes

Appendix 4.pptx