Standard Operating Procedure (SOP) for *In vitro* high throughput (HTP) phenotyping of banana for nematode resistance

Authors & Contributors

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1. Introduction

Parasitic nematodes (PPNs) mainly *Radopholus* spp. also known as burrowing nematodes are a major biotic threat to banana production in most African banana-growing regions (Keshari, and Mallikarjun, 2022). They cause plant toppling because root damage weakens soil anchorage and disrupts mineral and water uptake from the soil resulting in poor yields (Avhad, 2023). Despite the adoption of various management options to avert the effects of nematode damage, cultivation of resistant genotypes developed through breeding, is the most effective and economical sustainable way (Menon, 2016).

Hybrids produced in the breeding program require evaluation for their response to nematode infection and the most used technique was to evaluate the genotypes in a controlled environment within the screenhouse (*in vivo*). The *in vivo* technique requires a considerable amount of space, time, and materials, hence is very costly and time-consuming. We have developed a high throughput (HTP) technique for rapid *in vitro* evaluation of large numbers of genotypes

2. Purpose

This SOP provides an alternative method to the *in vivo* system to screen banana for nematode resistance against *R. similis*. It is an *in vitro* high throughput technique which is faster and cheaper.

3. Scope

A comprehensive step-by-step guide for assessing the resistance of banana genotypes to *R. similis* using *in vitro* as well as for data collection and analysis.

4. Definition of terms

- **Hybrids:** Genotypes of banana generated after crossing two different banana genotypes.
- **Checks:** Genotypes with known resistance to nematode infestation used as resistant controls in experimental trials.
- **Parental genotypes**: Genotypes of banana commonly used in Mchare, Matooke and Plantain breeding.
- **Landraces**: Genotypes commonly cultivated and known to be susceptible to the target biotic constraints.

5. Roles and Responsibilities

Below is a list of individuals responsible for each activity when screening banana germplasm for resistance to *R. similis*:

Research Technicians: Multiplication of plants in TC (tissue culture or in vitro culture), inoculum preparation, inoculation, data collection and preliminary data analysis.

Pathologist: Generate experimental design using cycDesign (https://vsni.co.uk/software/cycdesign), data analysis and overall supervision.

6. Procedure/Protocols

Plant materials

All materials are generated in TC and are used when a rooting stage

- Genotypes to be screened (Parental genotypes and newly developed hybrids)
- Resistant controls: SH3142 and Calcutta 4
- Land race / susceptible checks: Mbwazirume (matooke), TM-28-OBINO L'EWAI (plantain) and Mchare mlelembo

Other materials:

- River sand
- 1 mm sieves

- Nematode cultures
- Baby jars
- Murashige and Skoog (MS) media
- 1ml pipette and tips
- Cling film
- Marker pen
- Laminar flow cabinet

Preparation of basal Murashige and Skoog (MS) media

- Sieve river sand through a 1mm sieve into a clean container.
- Wash the sand multiple times with running water until the rinse water is visually clear.
- Air dry the sand at room temperature and later transfer it into baby jars up to the 50 mL mark.
- Prepare liquid MS media supplemented with 30 g/l sugar at pH 5.8 (Annex 1 for MS media components). Exclude phytagel and plant growth hormones.
- Fill baby jars containing sand with liquid MS media to the level of the sand (50 mL mark) until the sand is completely soaked with the basal MS media.
- Gently invert the jars for 15 min to remove excess MS media while allowing the sand to fully absorb the MS media.
- Remove surplus unabsorbed MS media by placing a kitchen towel on top of the sand-MS media mixture so that the media is soaked but not dripping.
- Autoclave at 121 °C and 15 psi for 15 min to sterilize the sand-MS media mixture and leave to cool overnight.

- Plantlets at subculture 3 are prepared by removing roots aseptically, the corm cleaned, and leaves trimmed under the laminar flow cabinet.
- Each plantlet is carefully introduced onto the sand-MS media prepared above; thereafter, the jar is covered, labelled, and sealed with cling film to prevent any possible contamination from the growth room.
- The sealed jars are incubated in the growth room for 8 weeks at 25 °C with 16 and 8 hours of dark and light cycles respectively until there is optimal root growth.

Experimental design and layout

- A partially replicated (P-Rep) experimental design is adopted and developed using CycDesigN software (https://vsni.co.uk/software/cycdesign).
- Depending on the available number of test genotypes and checks, a p-Rep (https://cran.r-project.org/web/packages/FielDHub/vignettes/partially_replicated.html) consists of blocks with each test genotype duplicated across the available blocks while checks/controls are replicated in all blocks.
- Each plot consists of four plants per genotype in the experiment.

NB: p-rep designs are adopted when experimental materials/space are limiting, therefore allowing repeated trials at different times. They form designs and layouts with an average efficiency of 0.99.

Inoculum preparation and inoculation process

- Nematodes already characterized as *R. similis*, are multiplied on carrot discs to the required numbers (Annexes 2, 3 and 4).
- The nematodes are washed off the carrot discs and Petri-dish inner surfaces using sterile distilled water and introduced in a sterile 50 ml falcon tube to form a stock suspension. Ensure the carrot discs used are free from any contamination.
- Nematode concentration in the stock suspension is determined by getting the average of three counts of nematodes in 2 mL aliquots using a nematode counting slide under an X4 compound microscope objective.
- Working suspension is prepared by diluting the stock suspension to a known volume that is enough for a given number of plants within the experiment.
- Each plant receives approximately 50 nematodes per ml dispensed using a pipette calibrated to draw this concentration per inoculation aliquot.
- The inoculum is carefully introduced into the sand-MS media near the plant roots.
- The jars are sealed and arranged according to the p-rep layout developed using CycDesign above and left for 4 weeks under similar TC growth conditions (25^{o}C with 16 and 8 hours of dark and light cycles respectively).

Nematode damage assessment

- At one-month post-inoculation, the experiment is terminated. Nematode multiplication is assessed by counting the number of nematodes in the roots of each test plant inoculated.
- Each plant is removed from sand-MS media and the roots are washed thoroughly under running tap water to remove the sand-MS media debris.
- All roots are trimmed off the plant and chopped into small pieces using a blade and nematodes extracted from the chopped roots using the modified Baermann technique as described in Coyne et al. 2007.
- Nematode extraction takes three days and the filtrate is decanted into a baby jar labeled with the plant genotype identity to form a suspension.
- The suspension is left to stand for 1-2 hours for nematodes to settle at the bottom.
- Excess water is decanted off to 25 ml from which three 2 ml aliquots are pipetted to compute for nematode density.
- The average total number of nematodes per sample is computed across the three aliquots.
- The reproductive factor (RF) is calculated as the ratio of the final average nematodes to the initial inoculated nematode population

Data Analysis

- The data obtained is cleaned in Excel and analyzed using an updated GenStat Software and checked for normal distribution and if required transformed to normal distribution.
- Residual Variance is calculated to determine the outliers and then data re-organized.
- The analysis is performed using a mixed model residual maximum likelihood (REML) model, under multiple experiments using a linear mixed model fitted as

  response = μ + genotype + trial + trial.batch.block + error

- The significance of the difference between Controls and Genotypes is calculated using Fisher`s Protected LSD at a 5% level of significance.

7. References

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8. Annex: Forms/Templates to be used for monitoring and data collection

Annex 1: Basal Murashige and Skoog (MS) Media Components

Elements	Components	Concentration (g/l)

Sucrose		30.000
MS I – Macroelements	KNO_3	1.9000
	NH_4NO_3	1.6500
	$CaCl_3.2H_2O$	0.4400
	$MgSO_4.7H_2O$	0.3700
	KH_2PO_4	0.1700
MS II - Iron-EDTA Solution	$FeSO_4.7H_2O$	27.8
	$Na_2EDTA.2H_2O$	37.3
MS III - Micro Elements	$MnSO_4.H_2O$	16.9
	H_2BO_3	6.2
	$ZnSO_4.7H_2O$	8.6
	KI	0.83
	$NaMoO_4.2H_2O$	0.25
	$CuSO_4.5H_2O$	0.025
	$CoCl_2.6H_2O$	0.025
MS IV - Vitamins	Ascorbic Acid	20
	Thiamine-HCL	0.4
	Pyridoxine-HCL	0.5
	Nicotinic Acid	0.5
Glycine		2

Annex 2: Isolation and multiplication of *R. similis*

- Banana roots infected with nematodes are obtained from the field, cleaned with tap water to free them of soil debris.
- The roots are then subjected to nematode extraction by cutting them into 1 cm pieces, blended and subjected to nematode extraction using the modified Baermann technique as described in Coyne *et al*. (2007).
- The nematode suspension is then collected from the Modified Baermann tray set up into a baby jar.
- After decanting the nematodes to 25 ml volume, 2 ml aliquots are pipetted into a nematode counting slide and identified under a stereo microscope.
- Using morphological features, *R. similis* females and juveniles are picked from the suspension using a wire loop.

- The picked nematodes are then sterilized using streptomycin sulfate and transferred to readily prepared carrot discs in Petri dishes under a lamina flow cabinet.
- The nematodes are then left to multiply in an incubator with the temperature set at 28 °C.

Annex 3: Preparation of carrot discs for nematode culturing

- Select clean, undamaged carrots from the market. (Carrots with a considerable biomass)
- Wash the carrot with tape water and rinse with distilled water.
- Dry the carrots by wiping them with tissue paper.
- Under the aseptic laminar flow cabinet, hold the carrot with a pair of forceps.
- Spray the carrot with 96 % ethanol and flame until it is dry.
- Peel the carrot lightly with a potato peeler.
- Again spray ethanol on the peeled carrot and flame until it is dry.
- Cut the carrot into sizeable transverse discs.
- Introduce each disc into a separate sterile Petri dish.

Annex 4: Sub-culturing of *R. similis* on carrot discs

- Using a microscope, observe and select Petri dishes with carrot discs that contain live nematodes.
- Using sterile distilled water, wash off nematodes from the Petri dish walls (2 ml suspension) into a sterile test tube.
- Each Petri dish should be washed into a separate test tube and left to stand for 1 hour.
- Prepare an anti-biotic solution by dissolving 0.06-0.1g of streptomycin sulfate into 10 ml of sterile and distilled water.
- Using a syringe, suck in the antibiotic solution.
- Filter sterilize the antibiotic solution by fitting a 2 µm pore size micro filter onto the syringe
- Release the solution through the microfilter into a sterile test tube.
- Pipette off 3 ml from the nematode solution prepared in each test tube earlier.
- Add 3 ml of the filter-sterilized antibiotic solution to each test tube.
- Leave the samples to stand for about 2 hours.
- Reduce the volume again by pipetting off 3 ml from the solution and replenishing with sterile distilled water
- Leave the sample to stand for 1 hour.

- Reduce the solution again to a negligible volume and add an adequate volume of sterile water to inoculate the freshly prepared carrot disk.
- Inoculate 2-3 micro drops (50-100 nematodes/micro drop) of previously prepared nematode suspension per disc.
- Seal the Petri dishes with a parafilm.
- Put the sealed Petri dishes into a large sterile container and place them in an incubator at 28°C.

Annex 5: HPT Workflow Summary