Standard Operating Procedure (SOP) for Phenotyping Banana for Resistance to the Root Burrowing Nematode (*Radopholus similis*)

Authors & Contributors

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1. Introduction

Plant-parasitic nematodes (PPNs) such as *Pratylenchus* spp., *Helicotylenchus* spp., and *Radopholus* spp. amongst others are a major constraint to sustainable bananas (Musa spp.) production, (Stover and Simmonds 1987) and have been identified as a major factor contributing to declining production (Speijer *et al.,* 1999) especially among small holder farmers. The root burrowing nematode, *Radopholus similis,* has been reported as the most destructive nematode parasite of banana in the tropics (Gowen, 1993). To avert the yield reducing constraints due to nematode infestations, timely management and control of these bio-constraints is warranted. Nematodes can be controlled with chemicals, however, most of these chemicals have adverse effects to the environment and human health (Nyczepir and Thomas 2009). Furthermore, chemical nematicides are too expensive for the small holder farmers (Savangikar 2004). Besides other nematode control and management strategies, development of nematode resistant banana genotypes through breeding is a promising strategy for the management of nematode infection in banana (Speijer and De Waele, 1997). This standard operating procedure focuses on screening banana breeding genotypes for resistance to *R. similis*.

2. Purpose

The purpose of this SOP is to provide guidance for the banana breeding program in assessment of genetic resistance to *R. similis*

3. Scope

This document contains the procedures required in the screening of banana genotypes for resistance to the banana nematode *R. similis*. It covers steps from extraction of *R. similis* from plant roots, culturing of *R. similis* in the laboratory, screening of parental genotypes in the screen house. It also covers the data collection procedures.

4. Definition of terms

- **Hybrids:** Varieties of banana plants generated after crossing two different varieties
- **Checks:** These are genotypes known to be susceptible or resistant to *R. similis* and are used as reference material in the experiment
- **Parental genotypes**: Varieties of banana plants to be screened for resistance to the five biotic constraints
- **Control**: These are banana genotypes with known response *to R. similis* and are used (by destructive sampling) to confirm nematode damage after inoculation of the experiment. The control is inoculated at the same time with the main experiment
- **Landrace**: Varieties of banana plants commonly cultivated for food in Uganda

5. Roles and Responsibilities

Research Technicians are responsible for Tissue Culture Lab plantlet generation, Inoculum preparation, Inoculation, Data collection, Data curation and Analysis.

Pathologists/Research Assistants is/are responsible for experiment Planning and Supervision.

Pathologist/Breeder are responsible for data analysis and publications.

Field Assistants are responsible for data collection.

6. Procedure/Protocols

Step 1: Experimental Planning (Nematologist/Pathologist/ Research Assistant)

This is the initial step for setting up a successful screening experiment. Planning involves:

1. Listing the genotypes and support materials required:

   Genotypes to be used include test genotypes i.e., Parental genotypes and newly developed Hybrids, Checks and Controls i.e., Resistant check (**SH3142** and **Calcutta 4**), Land races checks (**Mbwazirume**, **TM 28 Obino lewai** and **Mchare**), control (Valery)

- Materials include sand soil, 1-liter plastic pots, Watering cans, 840-micron sieves, CycDesigN Software, *R. similis* monoaxenic culture

2. Determining the experimental design to be used; the Partially Replicated (P-rep) design (refer to **Step 3)**

3. Selecting the experimental site. All experiments are conducted in the screen house

4. Step 2: Generating Plants for screening (Research Technician Tissue Culture Lab)

The genotypes to be screened are generated from Tissue Culture (TC), then transferred for weaning (refer to Weaning **annex 5)** in the humid chamber for 4 weeks, then hardened for 4 weeks under controlled environmental conditions (screen house) until ready for experimental establishment.

Step 3: Developing an experimental design and layout (Research Assistant)

NB: Any design like CRD, RCBD etc., can be applied depending on prevailing conditions

In this case, a partially replicated experimental design (P-Rep) is developed using updated CycDesigN Computer software. This design is very useful when running experiment in batches in cases where plant genotypes are many and cannot be evaluated at once, and availability of space is a limiting factor. It can allow running repeated experiment evaluations at different locations and time.

The design development depends on the number of test genotypes to be screened and checks to be included (varies per experiment).

3.1 CycDesigN (Illustration)

Below is a layout of a P-rep design for 51 parental genotypes including checks developed using CycDesigN software. The average efficiency is 0.99 indicating that the design is optimal. Numbers used in the blocks represent respective genotypes to be screened, and the red characters represent checks in each block

Table 1. P-rep lay out for banana nematode screening for the available 51 parental lines including checks

Block1	29	6	42	**S5**	24	47	27	28	17	11
	51	45	1	10	39	**S4**	38	41	19	15
	34	44	14	**S6**	**S2**	37	26	50	32	18
	43	48	4	49	33	**S1**	9	36	22	**S3**
Block2	39	**S4**	**S2**	28	45	23	19	24	35	**S6**
	7	20	44	3	16	48	1	2	26	31
	5	30	38	18	50	49	10	21	25	**S1**
	8	40	4	**S3**	46	12	47	**S5**	13	33
Block3	27	41	43	**S3**	**S6**	35	13	36	51	11
	S1	46	31	2	29	14	42	21	16	40
	30	**S5**	6	20	**S4**	34	25	22	32	37
	15	8	12	17	**S2**	9	3	23	7	5

Note: *Numbers used in the blocks represent respective genotypes to be screened, and the alphanumerical characters represent checks in each block. The experiment will comprise of 3 pseudo replicates per plot and 2 plots (hence 2 true replicates) in the entire experiment for each test parental banana genotype.*

Step 4: Setting up the experiment (Research technician, Research assistant and pathologist)

4.1. Multiplication, weaning, and transplanting of tissue culture plantlets

- Banana plantlets multiplied in tissue culture laboratory are weaned (annex 5) and maintained in a humid chamber for 4 weeks before transplanting into 1-liter plastic pots containing sand sieved through 840 microns.

- The plantlets are left to acclimatize in the screen house for 4 weeks prior to inoculation.
- To effect quality control of the substrate, the pre-sterilized substrate is examined for nematode contamination by subjecting it to nematode extraction and identification using the modified Baermann technique as described in Coyne *et al*. (2007) prior to establishment of the experiment.
- In case nematodes are present, sterilise the sand and wait for 1 week or at least 3 days before using this sand for the experiment.

4.2. Inoculum preparation and Inoculation

- After isolation and multiplication of *R. similis* (annexes 1, 2, & 3), nematodes are washed from carrot discs and petri dish inner surfaces and suspended in water in a 500ml conical flask to the 500ml mark to form a stock suspension
- Determine the concentration of the stock solution by taking and quantifying (using a compound microscope) three 2ml aliquots of nematode suspension from the stock suspension.
- Prepare a working nematode suspension by adjusting nematode concentration to 250 nematode/ml.
- Make 4 holes in the soil around the plant using a pencil.
- Inoculate each banana plant with 1000 *R. similis* nematodes. This is achieved by pipetting 1 ml (250 nematodes) of the working nematode suspension into each of the four holes made around each plant. Always homogenize the suspension by thorough agitation of the suspension container before pipetting to allow uniform distribution of nematodes in the suspension.
- Also include and inoculate at the same time on the side, a set of about 6-8 dummy plants of a susceptible genotype (Valery) that will be used for destructive sampling in determining when to terminate the experiment

- NOTE: Plants should be watered (from the bottom) approx. 2 hours before inoculation with nematodes and should not be watered for 48 hours (2days) after inoculation with nematodes. Bottom watering will be accomplished by pouring water in the lead on which the pot is resting
- Plants are drenched with soluble (for example NPK 19:19:19) following a 14 day interval at a rate of $2.5gL^{-1}$. The rate used may depend on manufacturer's specification
- Maintain plants for approx. 6 weeks after nematode inoculation, then check for nematode infection and multiplication by uprooting and observing the dummy susceptible check plants (Valery) for nematode multiplication and necrosis to inform termination of the main experiment. Give the experiment more time if susceptible check plants yield no or very low levels of infection.

Step 5. Data Collection

- Data are collected at termination.
- Download the Field Book App from Android play store and install it on any android device.
- Import the experimental layout into the field book App
- Add all the traits that are to be assessed
- Start collecting Data per genotype to include the traits above
- Immediately Save and Export the weekly data collected from the Field book App into a Gmail Account/Dropbox/OneDrive for back up purposes

NB: Ensure data are exported on the day of data collection to the backup drive immediately to prevent any loss of data.

The following parameters/traits are used to assess the reaction of banana genotypes to banana nematode *R. similis:* Total number of standing leaves (NSL), Total number of

functional leaves (NFL), Fresh root weight (FRW), Fresh shoot weight (FSW), Fresh corm weight (FCW), Root necrosis, Nematode population density. Apart from Root necrosis and nematode population density, data on the rest of the traits is collected twice in the course of the experiment, ie mid-way the course of the experiment and at termination.

Step 6. Data Curation and Analysis

6.1. Nematode damage assessment

- Individual plants in respective pots are removed after softening the soil by watering
- Wash plant roots under running tap water to free the plants of any debris
- Harvest and weigh all roots from the corm
- Randomly select five (5) roots for root necrosis assessment
- Each of the 5 roots is trimmed to a length of 10cm
- The 10cm root segments are dissected longitudinally to expose the cortical region
- For each root segment, one side of the exposed cortical regions is used to score for necrosis
- Each longitudinal root section is scored at scale of 0-20 for cortical necrosis and sum the score from the 5 roots to get the score of 100 for the plant expressed as a percentage

  Root necrosis data will be analysed using the generalized linear mixed models

6.2. Nematode extraction and counts

- After scoring for necrosis, all roots from an individual plant are chopped into about 0.5cm pieces including the strands used in scoring for necrosis

- The chopped pieces are homogenized and a 10g sample of the homogenate used in nematode extraction using the modified Baermann technique as described in Coyne *et al*. (2007).
- After extraction, the nematode suspension is decanted to 25 ml and nematodes quantified from three 2ml aliquots using a compound microscope to compute for nematode density.
- The nematode density of each plant is subjected to plant root weight adjustment to obtain a total nematode population for each plant root system.

7. References

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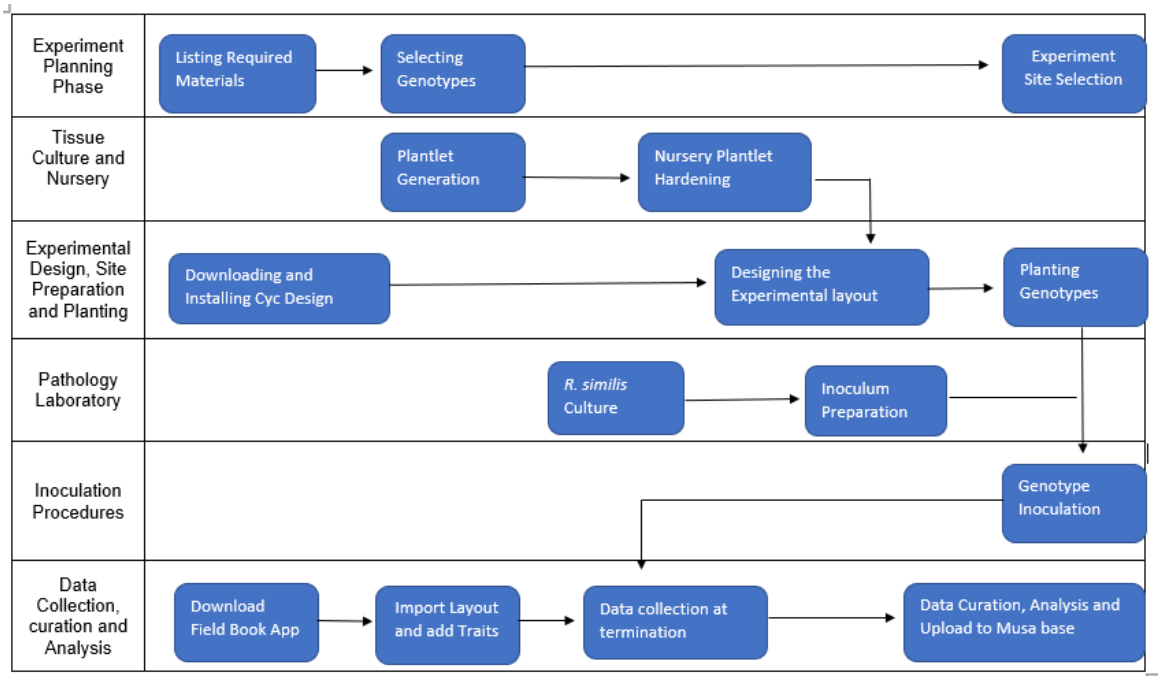
8. Annex: Forms/Templates to be used for monitoring and data collection

Annex 1: Flow Chart for the Screening Banana Genotypes against *R. similis*



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 ca. 1cm 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 25ml volume, 2ml 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 sulphate and transferred to readily prepared carrot discs in petri-dishes under a lamina flow hood.
- The nematodes are then left to multiply in an incubator with temperature set at 28 ^{o}C.

Annex 3: Sub-culturing of *R. similis* on carrot disks

- Select good discs containing nematodes.
- Wash nematodes from the petri dish (2 ml suspension) into sterile test tube.
- Each petri dish should be washed into an individual test tube.
- Prepare an ant-biotic solution by dissolving 0.06g of ant-biotic (streptomycin sulphate) in 10 ml of sterile and distilled water.
- Using a syringe, suck in the ant-biotic solution.
- Fit the micro filter on the syringe (2 µm pore size).

- Release the solution through the micro filter into a sterile test tube.
- Pipette 2.5 ml of the solution to each test tube.
- Add 3 ml to each test tube. The final volume will be 7.5 ml.
- Leave the samples to stand for about 2 hours.
- Wash sample by Pipetting/reducing the sample volume to a negligible volume.
- Add 5 ml of sterile and distilled water to each sample.
- Leave sample to stand for 1 hour.
- Again wash samples by pipetting/reducing sample volume to a negligible volume.
- Add 3 ml of sterile and distilled water.
- Leave the samples to stand for 30 s.
- Add to the sample little volume of water (sterile and distilled) enough to inoculate the previously prepared carrot discs.

Annex 4: Preparation of carrot discs for nematode culturing

- Select clean and sizeable carrots from market.
- Wash the carrot with tape water and rinse with distilled water.
- Dry the carrot by wiping them with a tissue paper.
- Under the aseptic hood, 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.
- Peel the carrot and the cut it into sizeable disks that in your petri dishes.
- Introduce the discs into petri-dishes.
- 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 container and place in an incubator at 28 °C.

Annex 5: Protocol for Weaning Banana Tissue Culture Plantlets

- In preparation for weaning of banana tissue culture plantlets, cocopeat is treated with calcium nitrate to raise its pH to between 5.6 – 6.5.
- $Ca(NO_3)_2 + Na_2CO_3 \rightarrow CaCO_3 + 2NaNO_3$.
- A 5kg block of coco peat is treated by breaking it apart and soaking it into water.
- Fill a trough with 150 liters of clean tap water.
- Add 15g of calcium nitrate and stir thoroughly.
- Break apart the 5kg block of calcium nitrate and immerse it in the above solution, ensuring the coco peat block gets thoroughly soaked.
- Let the contents stand for 24 hours.
- Rid the contents of excess solution and rinse the solid component with clean tap water for about 7 rinses.
- Free the solid cocopeat of excess water.
- Fill seedling trays with this moist cocopeat in preparation for weaning.
- Obtain plantlets ready for weaning from the tissue culture laboratory.
- Free the plantlets of agar by gently washing off the agar using clean tap water while maintain9ng their labelling.
- Transfer individual banana plantlets into seedling trays containing cocopeat and label accordingly to include the name of the banana genotype and weaning date.
- Transfer the plantlets in the seedling trays to the humid chamber (relative humidity > 80%, Temp = 25 ± 2 ℃).
- Regularly apply a nutrient boost (for example water soluble NPK 19:19:19) to boost plant growth and development.
- Maintain the set for 4 weeks before hardening them off.