SOP22:

Standard Operating Procedure (SOP)

for phenotyping plantain and banana genotypes for resistance to nematodes (Radopholus similis and Meloidogyne sp.) using macropropagated plantlets

IITA Transforming African Agriculture CGIAR	**Crop: Banana** **Function:** Phenotyping plantain genotypes for resistance to nematode (Radopholus similis and Meloidogyne sp.) using macropropagated plantlets	**SOP #**	IITA-BP-SOP22
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Standard Operating Procedure (SOP) for phenotyping plantain and banana genotypes for resistance to nematodes (Radopholus similis and Meloidogyne sp.) using macropropagated plantlets

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1. Introduction

Plant-parasitic nematodes (PPNs), such as the burrowing nematode (*Radopholus similis*), root-knot nematodes (*Meloidogyne* spp.), root-lesion nematodes (*Pratylenchus* spp.), etc., are a major threat in plantain and banana production (Speijer et al. 2001; Olajide et al. 2023a, b) and are a major factor contributing to declining production (Sikora et al. 2018). The burrowing nematode, *R. similis,* has been reported as the most destructive nematode parasite of banana in the tropics (Gowen 1993). To overcome the yield-limiting impact of nematode infestations in plantain and banana cultivation, a combination of integrated nematode management (INM) strategies is recommended. This strategy includes (1) clean planting material, (2) crop rotation and intercropping, (3) biological control, and (4) chemical control. However, the use of resistant or tolerant cultivars is the most sustainable approach.

This standard operating procedure focuses on phenotyping plantain and banana genotypes for resistance to nematodes using macropropagated plantlets.

2. Purpose

The purpose of this SOP is to provide guidance for the plantain and banana breeding program in the assessment of resistance to nematodes (*R. similis* and *Meloidogyne* spp.) using macropropagated plantlets.

3. Scope

This document contains the procedures required in the screening of plantain and banana genotypes for resistance to the burrowing nematode (*R. similis*) and root-knot nematodes (*Meloidogyne* spp.). It covers steps from extraction of nematodes from plant roots, culturing of nematodes, macropropagation, and screening of parental genotypes in the screenhouse. It also covers the data collection procedures.

4. Definition of terms

- **Hybrids:** Cultivars of plantain and banana plants generated from crossing two different varieties
- **Checks:** Cultivars known to be susceptible or resistant to *R. similis* and *Meloidogyne* spp. and used as reference material
- **Parental genotypes**: Cultivars of plantain and banana plants screened for resistance to nematodes
- **Control**: These are plantain and banana cultivars with known response to *R. similis* and *Meloidogyne* spp. 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 and set to establish the most appropriate time for the end trial evaluation
- **Macropropagation:** Macropropagation is a plant propagation technique that uses whole suckers or large pieces of the corm to produce plantlets for use as planting material
- **Landrace**: Cultivars of plantain and banana plants commonly cultivated for food in a particular community

5. Roles and Responsibilities

Research Technicians are responsible for macropropagation, inoculum preparation, inoculation, data collection, data curation and analysis

Pathologists/Research Assistants is/are responsible for 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 (SH 3142, Calcutta 4 and Pisang Jari Buaya), control Agbagba and Obino L'ewai

- Materials include sand and absorbent polymer, corms, 2-litre plastic pots, watering bottle, 840-micron sieves, CycDesigN Software, *R. similis* and *Meloidogyne* spp. monoaxenic cultures

2. Experimental design: Completely Randomized Design (CRD) or Partially Replicated (P-rep) design (see **Step 3)**

3. All experiments are conducted in the screenhouse

4. Step 2: Generating plants for screening (field technician /macrochamber)

The genotypes to be screened are generated from the macropropagation chamber after collecting sword suckers from a labelled field. After a period of three to five weeks, plantlets with three well developed leaves will undergo a secondary decapitation or are directly transferred into an acclimatizing pot

Step 3: Experimental design and layout (Research assistant)

Any design such as CRD or a partially replicated experimental design (P-Rep) etc., can be applied depending on prevailing conditions**.** A partially replicated experimental design (P-Rep) is developed using updated CycDesigN Computer software. This design is very useful when running experiments in batches in cases where plant genotypes are many and cannot be evaluated at once, and availability of space is a limiting factor. It will enable running repeated experimental evaluations at different locations and time (Refer to SOP1)

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

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

4.1. Transplanting of macropropagated plantlets

- The secondary buds sprouting from the corm after three to five weeks are transplanted into 2-litre plastic nursery bags containing specific substrates, and acclimatized for four weeks (Annex 1**)** in the screenhouse until ready for experimental setup
- The plantlets are then transferred into 2-litre plastic pots containing sand and absorbent polymer (SAP) substrate and allowed to acclimatize in the screenhouse for four weeks prior to inoculation

4.2. Inoculum preparation and inoculation

- Isolate and multiply *R. similis* and *Meloidogyne* spp. (Annex 1-4), then wash *R. similis* from carrot discs or extract *Meloidogyne* spp. From infected tomato roots (Coyne et al. 2014, 2018) and Petri-dish inner surfaces and suspend in water in a 500 ml conical flask to the 500 ml mark to form a stock suspension
- Determine the concentration of the stock solution by taking and quantifying (using a compound microscope) 3 X 2 ml aliquots of nematode suspension from the stock suspension
- Prepare a working nematode suspension by adjusting nematode concentration to a given concentration (e.g. 500 nematode/ml)
- Make a ring in the soil around the plant
- Inoculate each banana plant with 2,000 nematodes (mixed life-stages (no eggs) of *R. similis* or with 2,000 second stage juveniles (J2s) of *Meloidogyne* spp.). This is achieved by pipetting 4 X 1 ml (500 nematodes) of the working nematode suspension into the ring 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
- Include and inoculate at the same time, a set of about 6-8 dummy plants of a susceptible genotype (Agbagba, set as control) that will be used for destructive sampling to determine 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 after inoculation with nematodes to prevent nematodes being washed out. Bottom watering will be accomplished by pouring water into the plate/dish 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 2.5 gL^{-1}
- Maintain plants for approx. 60 days after inoculation with *R. similis* and 90 days after inoculation with *Meloidogyne* spp., then check for nematode infection and multiplication by uprooting and observing the dummy/control susceptible check plants (Agbagba)
- List of checks are provided in Annex 5

Step 5. Data Collection

- Data are collected at termination which coincides with plant uprooting
- 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 above traits
- Save and export the weekly data collected from the Field book App into a Gmail Account/Dropbox/OneDrive for backup purposes

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

The following parameters/traits are to be collected: Total number of standing leaves (NSL), Total number of functional leaves (NFL), Fresh root weight (FRW), Fresh shoot weight (FSW), Root necrosis, Nematode final population density. Apart from root necrosis and nematode population density, other data is collected twice, i.e. mid-way through the course of the experiment and at termination

- ***Nematode damage assessment***

- Individual plants are removed from pots after softening the soil by watering
- Wash plant roots under running tap water to rinse the roots free of debris
- Harvest and weigh all roots
- Randomly select five (5) roots for necrosis assessment
- Trim the five roots to a length of 10 cm
- The 10 cm root segments are dissected lengthwise to expose the cortical region
- One side of the exposed cortical regions is used to score for necrosis
- Each longitudinal root section is scored at a scale of 0-20 for cortical necrosis and galling
- Sum the score from the 5 roots to achieve a score of 100 which will provide a percentage root damage score (Coyne et al. 2014, 2018).

  Analyze root necrosis and galling data using the generalized linear mixed models

❖ ***Evaluation at nematode extraction and counts***

- After scoring for necrosis, all roots from an individual plant, including those used for damage scoring, are chopped into ~0.5 cm pieces
- Homogenate and mix the chopped pieces and remove a 10 g sample for nematode extraction using the modified Baermann technique (Kolombia et al. 2017a, b)
- After extraction, decant the nematode suspension into a beaker and quantify from 3 X 2 ml aliquots using a compound microscope to compute nematode density. Calculate the nematode density of each plant by taking into account the plant root weight and volume of nematode suspension to obtain a total nematode population for each plant root system

Step 6. Data Curation and Analysis

Statistical analyses can be performed using any statistical analysis software. Data transformations are performed whenever required. Log-transformed ($\log_{10}\backslash[x + 1]$) and arcsine transformation ($arsin(\sqrt{x})$) are often applied for the nematode count and root necrosis, respectively, to comply data to normality. Statistical significance among the root damage and the nematode reproduction are determined at $P \leq 0.05$

7. References

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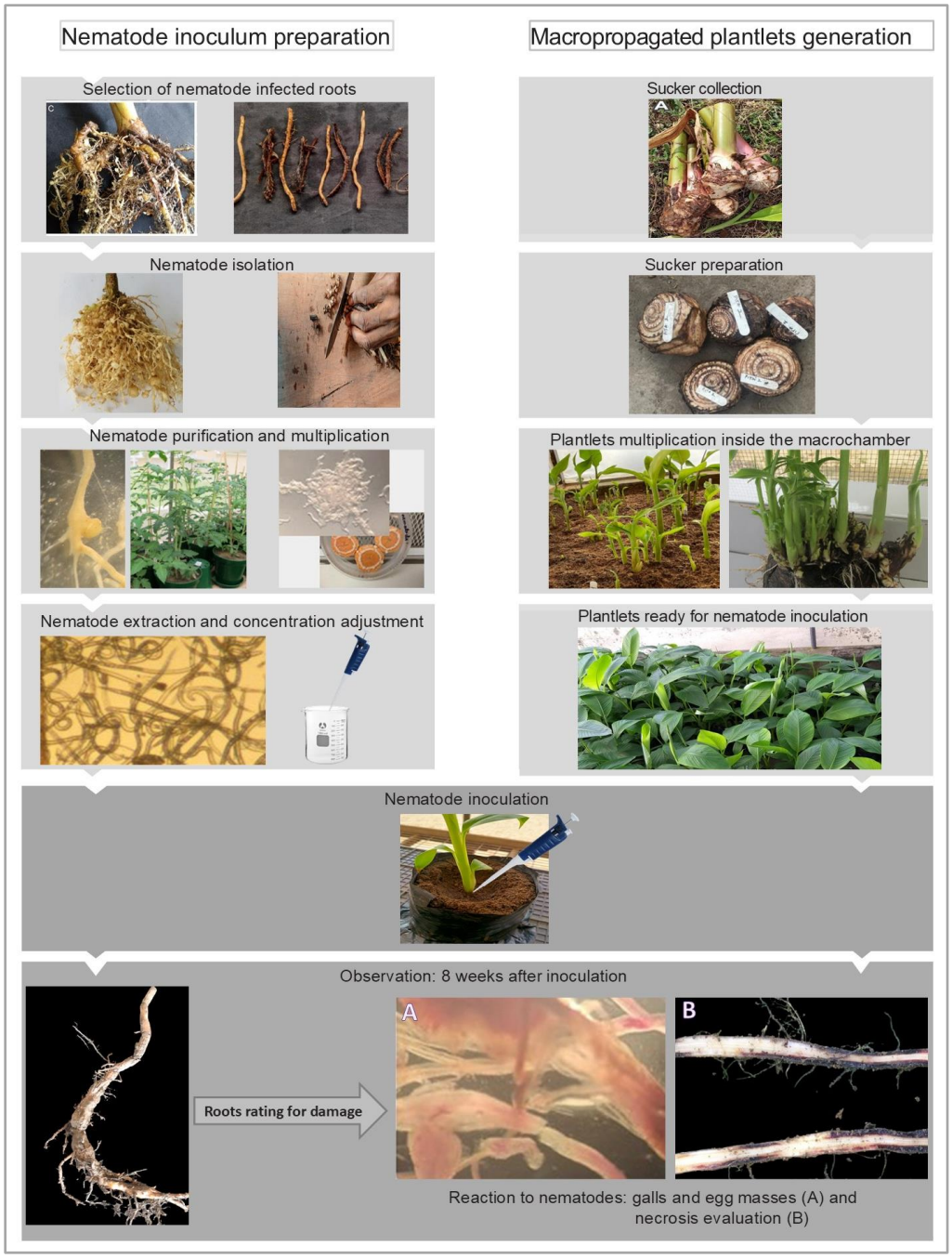
8. Annex/Appendix

Annex 1: Flow chart for screening plantain and banana genotypes against *R. similis* and *Meloidogyne* spp.



Annex 2: Isolation and multiplication of *Radopholus similis* and *Meloidogyne* spp.

- Plantain and banana roots infected with nematodes are obtained from the field, and soil debris washed off
- Roots are cut into ca. 1 cm pieces, blended and subjected to nematode extraction by using the modified Baermann technique (Kolombia et al. 2017a, b)

- The nematode suspension is 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 nematodes identified under a stereo microscope
- *R. similis* females and juveniles are picked from the suspension with a wire loop
- The picked nematodes are sterilized with 5% streptomycin sulphate and transferred to prepared carrot discs in Petri-dishes under a laminar flow hood
- The nematodes are then left to multiply in an incubator at 28°C

Annex 3: Sub-culturing of *Radopholus similis* on carrot discs

- 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 antibiotic solution by dissolving 0.06 g of antibiotic (streptomycin sulphate) in 10 ml of sterile and distilled water
- Using a syringe, suck in the antibiotic 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 barely measurable 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 barely measurable volume
- Add 3 ml of sterile and distilled water
- Leave the samples to stand for 30 s
- Add to the sample little volume water (sterile and distilled) enough to inoculate the previously prepared carrot discs

Annex 4: Preparation of carrot discs for nematode culturing (Coyne et al. 2018)

- Select clean and sizeable carrots from market
- Wash the carrot with tape water and rinse with distilled waters
- Dry the carrot by wiping 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 discs (0.5 cm thick sections of 3-4 cm diameter)
- Discs must not be less than 2 cm diameter
- 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: Reference materials for plantain resistance evaluation to *Radopholus similis* and *Meloidogyne* pp**.**

SN	Category	Genotype	Status to *R. similis*	Status to *Meloidogyne* spp.
1		SH 3142	Resistant	?
2		Pisang Jari Buaya	Resistant	Resistant
	Checks	Calcutta 4	Resistant	Resistant
3		Obino L'ewai	Susceptible	Susceptible
4		Agbagba	Susceptible	Susceptible
		Grande naine	Susceptible	Susceptible
5	Control	Agbagba	Susceptible	Susceptible

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