Standard Operating Procedure (SOP) for Banana Bacterial Wilt Resistance Screening

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1. Introduction

Banana is greatly affected by a bacterial pathogen *Xanthomonas vasicola* pv. *musacearum* (Xvm), that causes banana bacterial wilt.[1] It is reported to be the most devastating disease in East and Central Africa causing 100% yield loss.[2] The infection results into wilting and yellowing of youngest leaves or the male buds, premature ripening of fruits, internal vascular bundle discoloration mainly in the pseudo stem producing a yellow discharge "ooze", preventing fruit formation and resulting into complete death for susceptible banana cultivars.[3] The banana breeding program not only aims at developing hybrid banana varieties with higher Yields but also with more resistance to pests and diseases. Hence, screening for Xvm resistance among banana genotypes is of paramount importance to develop a resistant germplasm that can be used in breeding program to transfer resistance into new Banana varieties.

2. Purpose

This SOP document serves a purpose of guiding Banana Plant breeders, Phytopathologists, Technicians, and Data analysts during the screening of banana genotypes for resistance to banana bacterial wilt. It is designed to ensure consistence and minimization of errors.

3. Scope

This document clearly describes procedures and practices involved in screening banana genotypes for Resistance to Banana Bacterial wilt. It outlines, planning for the experiment, Genotype selection including checks and controls, Experimental design and layout, Inoculum preparation and infection, Data collection and Analysis.

NB: This SOP is strictly designed for work that involves screening for resistance to Banana Bacterial wilt among banana genotypes, it may not apply to other crops.

4. Definition of terms

- **Hybrids:** Varieties of banana plants generated after crossing two different varieties.
- **Checks: Known** Varieties susceptible or resistant to the constraint used as controls in experimental trials.
- **Control**: The Controls are Land races (varieties of banana plants commonly cultivated for food).
- **Xvm**: *Xanthomonas vasicola* pv. *Musacearum*

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 (Pathologist/ Research Assistant)

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

Planning involves;

- Listing the genotypes and support materials required,
    - Test genotypes
    - Checks and Controls i.e.,
        - Resistant and Susceptible
        - Tolerant Check
            i. Monyet
        - Land races controls
            i. TM-28 OBINO LEWAI
            ii. Mbwazirume
            iii. Mchare
    - Other materials
        1. Sterile loam soil
        2. Sterile sand
        3. 13-liter plastic pots/poly-planters

4. Watering cans
5. Xvm inoculum/Cultures
6. Polysheets
7. CycDesign Software or any other experiment design software

- Determining the Experimental design to be used
    - For example, a Partially Replicated (P-rep) design refer to **Step 3**
- Selecting the experimental site,
    - A screenhouse is preferred for potted trials however, establishing the experiment in an open space with uniform environmental control but minimal management practices like watering during dry spells can be considered.

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

The genotypes to be screened are generated from Tissue culture (TC), then transferred to the humid chamber for weaning for 4 weeks, then to the nursery to harden for 4 weeks under controlled environmental conditions in the screen house until ready for open space planting, (refer to Weaning **SOP IITA-BP-SOP06-06)**

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

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

In this case, a Partially replicated experimental design (P-Rep) is developed using updated Cyc Design Computer software. This design is very useful when running experiment in batches in cases where plant genotypes are many and can`t be evaluated at

once, and the availability of space is a limiting factor. It can allow running repeated trail evaluations at different locations and time.

Step 3.1 Cyc Design (Illustration)

For example, for 51 parental genotypes and 4 checks, three blocks are adopted with each parental/test genotype occurring in duplicate while the checks in triplicate for the entire experimental set up. Each plot constitutes four plants per genotype.

51 parental genotypes including checks is shown in Figure 1 below.

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

Step 4: Setting up the experiment (Field Assistants)

4.1 The Selected experimental site (**Refer to Step 1**) is prepared, to include cleaning it and disinfection if necessary. Ensure uniform environment conditions, preferably a screen house or an open space that can accommodate the number of genotypes to be included in the experiment. In case of open space, a polysheet is laid on the ground to prevent weed germination

4.2 Planting bags (Poly bags) containing sterilized mixture of loam soil, Manure and sawdust mixed in a ratio of 3:1:1 respectively is placed in each position as indicated on the design layout (**refer step 3.1**)

4.3 Carefully transplant the weaned plants into planting bags (Polybags) following the designed experiment layout.

4.4 The plants are left to fully grow for 3 months with regular watering and monitoring until inoculation.

Step 5: Xvm Culture isolation/maintenance (Research Technician)

Xvm cultures are always maintained on YPGA media, (Yeast 5g/l, Peptone 5g/l, Glucose 10g/l and Agar 15g/l). **For maintaining Xvm cultures refer to Annex 2**

NB: In case of no Xvm cultures available, field isolation and molecular confirmation (**Refer to Annex 1)** is performed before experimental inoculation.

Step 5.2 Inoculum preparation (Research Technician)

The total volume of inoculum to be prepared is dependent on the number of genotypes to be inoculated.

5.2.1 Aseptically introduce an Xvm colony in a known volume of YPG broth and incubated at 25^{o}C - 28^{o}C for 48hours with shaking, to optimize cell growth.

5.2.2 Determine and record the culture cell concentration (Absorbance/Optical Density) using a spectrophotometer with a light wavelength of 600 ($O.D_{600}$).

5.2.3 Using the formula $C_1V_1 = C_2V_2$, adjust the culture cell concentration using YPG broth to a desired concentration of 10^8 Colony Forming Units (CFU) per ml which is approximately 0.5 OD_{600}

Step 5.2 Experiment inoculation (**Research Technician)** (Nakato *et al*., 2018)

This step is performed 3 months after the experimental set up i.e., Plants are well established in the experiment with enough number of leaves.

5.2.1 From the 10^8 CFU/ml cell solution prepared in **Step 5.2.3**, using a 1unit (1ml) insulin needle pick the equivalent volume and inject the content into the youngest open leaf via the petiole/midrib, for all genotypes in the experiment.

5.2.2 Tag the inoculated leaf with cello tape (White water resistant).

5.2.3 Record the date on which inoculation has been completed.

Step 6 Data Collection, (Research Technician) (Nakato *et al*., 2019)

Data collection begins 7 days after inoculation for 16 weeks.

Data is collected on the following traits:

a) Number of functional leaves
b) Number of chlorotic leaves (leaf wilting)
c) Death of entire plant (if any) per genotype are recorded
d) Senescence leaves
e) Date of data collection

NB:

Tag every new symptomatic leaf with a white-water resistant tape to differentiate senescence leaves from chlorotic leaves.

Immediately (on the day of data collection) Save and Export the weekly data collected from the Field book App into a Gmail Account/Dropbox/OneDrive or any other data backup provisions**.**

Step 7 Data Curation and Analysis

Extract the weekly data records backed up on the Gmail/Dropbox/OneDrive and merge them into one excel sheet to calculate:

a) Days Post Inoculation (DPI)
b) Disease index (DI)
c) Area Under Disease Progression Curve (AUDPC)

Disease Index (DI), Days of Post inoculation (DPI) and Area Under Disease Progression Curve (AUDPC) are calculated using the formulae below:

1. Disease Index (DI) = (((1*A) + (2*B) + (3*C))/Number of Plants) x100

Where:

A = Number of plants with inoculated leaf showing symptoms

B = Number of plants with uninoculated leaves showing symptoms

C = Number of Plants wilted/dead

Table 1: Disease classification based on DI

Disease Classification	
Resistant(R)	No plants wilted
Tolerant (T)	<30% of plants wilted
Moderately Susceptible (MS)	>30% and <50% of plants wilted
Highly Susceptible (HS)	>50% of plants wilted

2. Days of Post Inoculation (DPI) = Time interval (Counting number of Days from inoculation to symptom development and complete wilting).

3. Area Under Disease Progression Curve (AUDPC):

$$\text{AUDPC} = \sum_{i=1}^{N_i - 1} \left(\frac{Yi + Yi + 1}{2}\right)(ti + 1 - ti)$$

Where:

t = time in weeks of each Reading

Y = Percentage of affected plants at each Reading

N = Number of Readings

i = Reading

7. References

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8. Appendix

Annex 1. Isolation of *Xanthomonas vasicola* pv. *musacearum* (Xvm)

Xvm isolation media (Mwangi *et al*., 2007)

i. Wilbrink media
ii. YPGA (5g/l Yeast, 5g/l Peptone, 10g/l Glucose and 15g/l Agar)
iii. YDCA (10g/l Yeast, 20g/l $CaCO_3$ 20g/l Dextrose and 15g/l Agar)
iv. Cellobiose-cepholexin Agar (CCA)

Materials:

- ✓ Well labeled Plant Samples (preferably pseudo stem)
- ✓ Laminar flow hood
- ✓ Toothpicks
- ✓ Cling Film

- ✓ Wilbrink Isolation media plates or YPGA media plates (Yeast 5g/l, Peptone 5g/l, Glucose 10g/l and Bacteriological Agar 15g/l)
- ✓ Sterile Toothpicks
- ✓ 5-fluorouracil for eliminating fluorescent Pseudomonads (Nakato V *et al*., 2018)
- ✓ Cycloheximide for Fungal growth inhibition

Procedure:

1. Media is sterilized by autoclaving at 121 Ibs pressure for 15 minutes and dispensed 20mls poured into petri dishes after cooling to 30^{o}C.
2. The sample initials are recorded in the Laboratory sample book.
3. Using a sterile knife, the two outer most sheaths are peeled off and discarded from the 30-cm portion of the pseudo-stem.
4. The remaining exposed outer surface is sterilized with 70% ethanol in a laminar flow hood and placed on absorbent tissue paper to dry.
5. Aseptically cut the sterilized stems into two cross-section portions and keep slightly slanting for 15-10 minutes until Bacterial Ooze is discharged.
6. The Ooze is picked using a sterile toothpick, and directly streaked on the media plate (Wilbrink or YPGA).
7. The plate is sealed using a cling film (to prevent contamination) and incubated at 25^{o}C for 3 days.
8. Yellow mucoid convex shaped colonies are picked from media plate and re-cultured on YPGA media to obtain pure colonies.
9. Confirmation can be done using Xvm specific markers (Nakato *et al*., 2019)
10. Pure colonies are stored on 20% glycerol 50% YPG broth for long term storage at -80^{o}C

1. Wilbrink media Preparation, (Wang *et al*., 2018)

Wilbrink Media:	1000ml	500ml	200ml
Bacto Peptone	5g	2.5g	1g
Sucrose	10g	5g	2g
K2HPO4	0.5g	0.25g	0.1g
MgSO4·7H2O	2.5g	1.25g	0.5g
Na2SO3	0.25g	0.125g	0.05g
Agar	15g	7.5g	3g
YPGA Media:			
Yeast	5g	2.5g	1g
Peptone	5g	2.5g	1g
Glucose	10g	5g	2g
Agar	15g	7.5g	3g

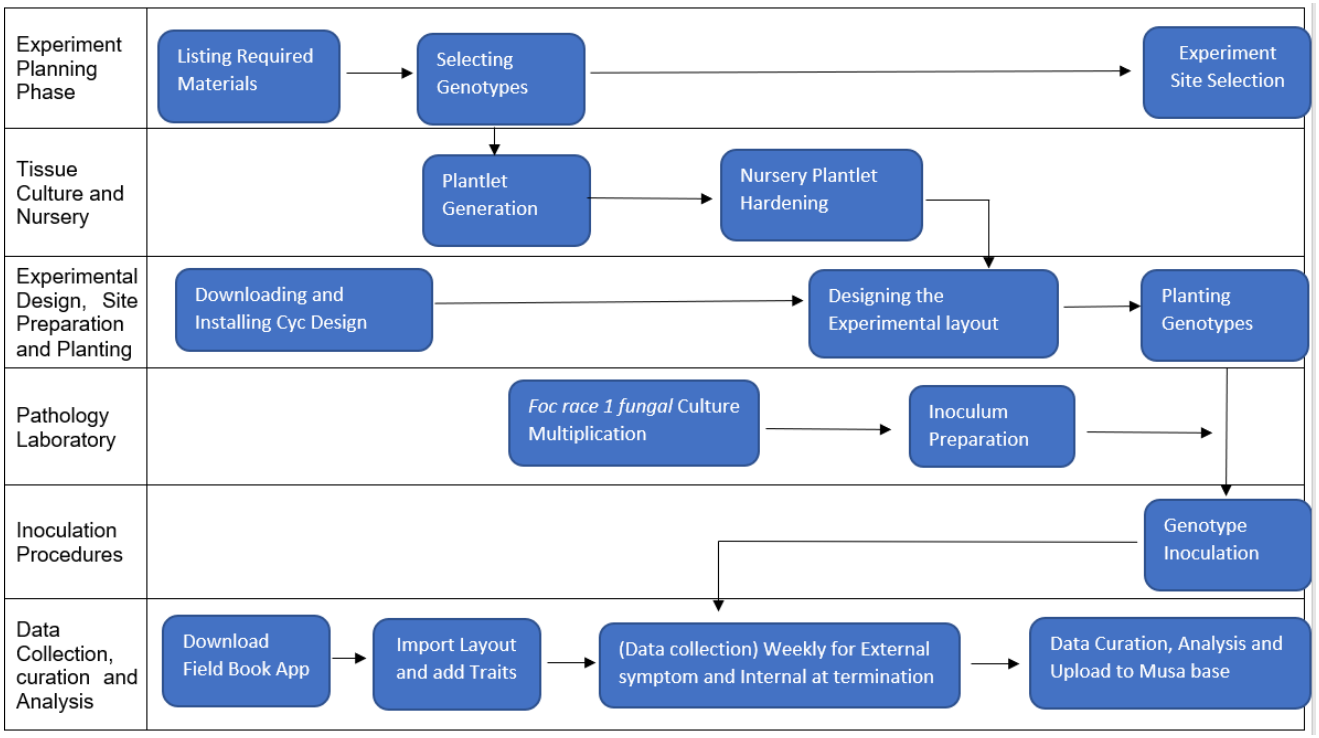
Table 2: Media for Xvm isolation

Annex 2: Maintaining Xvm Cultures in the Laboratory

Isolated/retrieved Xvm cultures are maintained weekly through subculturing on freshly prepared YPGA media.

1. Prepare YPGA media as described in Annex 1 above.
2. Using a Bunsen flame, sterilize a wire loop and tap the edges of a fully grown Xvm bacteria on an old YPGA media plate.
3. Transfer by tapping the tip of the wire loop onto fresh YPGA media.
4. Seal the plate using a cling or parafilm and store the plate at 28^0C – 30^0C
5. Monitor the plate to ensure no fungal or other bacterial contamination.

***Annex 3.* Flow Chart for the Screening of Banana genotypes against Banana Bacterial Wilt**


Experiment Planning Phase
Listing Required Materials
Selecting Genotypes
Experiment Site Selection
Tissue Culture and Nursery
Plantlet Generation
Nursery Plantlet Hardening
Experimental Design, Site Preparation and Planting
Downloading and Installing Cyc Design
Designing the Experimental layout
Planting Genotypes
Pathology Laboratory
Foc race 1 fungal Culture Multiplication
Inoculum Preparation
Inoculation Procedures
Genotype Inoculation
Data Collection, curation and Analysis
Download Field Book App
Import Layout and add Traits
(Data collection) Weekly for External symptom and Internal at termination
Data Curation, Analysis and Upload to Musa base