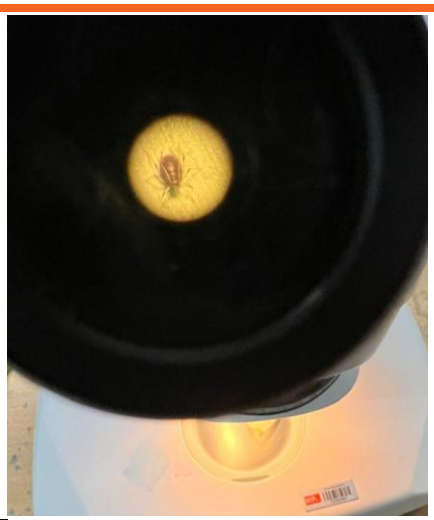
SOP23:

Standard Operating Procedure (SOP) for phenotyping for BBTV resistance and PCR confirmation



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Standard operating procedure for phenotyping for BBTV resistance and PCR confirmation

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1. Introduction

Banana bunchy top disease (BBTD) caused by banana bunchy top virus (BBTV) which is transmitted by the aphid *Pentalonia nigronervosa* (Magee, 1927) is one of the most devastating viral banana diseases. BBTV is a member of the *Nanoviridae* family, genus *Babuvirus*. The virus is composed of multi-component, single-stranded DNA genomes encapsulated in small isometric particles measuring 18 to 20 nm in diameter (Mansoor et al., 2005; Shiragi et al., 2010). Banana aphids are a common sight on banana plants and are generally harmless. However, their role as vectors of BBTV is highly destructive (Völkl et al., 1990). Once infected, aphids remain infective for life and continue to colonize host plants. Notably, adult aphids do not pass the virus on to their offspring; each new aphid must acquire the virus independently by feeding on an infected plant. Aphids typically form colonies between the sheath of the most recent leaf and the base of the pseudostem, as well as on the lower surface of leaves near the midrib. Winged forms of aphids (alatae) emerge after 7 to 10 generations of wingless apterae, often triggered by overcrowding or declining plant quality due to aging or yellowing (Mukwa, 2016).

BBTV is transmitted in a persistent mode (Mukwa, 2016) and is characterized by stunted growth with distorted leaves having a bunchy and erect appearance in young plants, while bunches of mature plants do not fill, as such fruits remain inedible (Magee, 1927; Niyongere et al., 2011). In addition to devastating yield losses and economic losses for farmers of up to 100% (Caruana-Iskra, 2003; Kumar et al., 2015; Niyongere et al., 2011), BBTV is a major constraint on the distribution of planting material due to the risk of transmission.

BBTV is transmitted through infected planting materials and the aphid vector *P. nigronervosa* (Magee, 1927). Disease spread is dependent on survival of infective aphids to spread the virus, susceptibility of host plants, and efficiency in detection of infected plants before they become a source of inoculum. In addition to crop management practices, multiple environmental factors including temperature, altitude, precipitation, wind speed, and abundance of the host influence the vulnerability of plantations to BBTD (Bouwmeester et al., 2023; Niyongere et al., 2013; Raymundo and Pangga, 2011). Management of BBTD focuses more on the seed systems, exclusion, virus diagnostics, digital and spatial mapping tools and most recently recovery of production through roguing. Vector management is a possible option in BBTD management. Studies showing virus and vector host behaviour and phenomics are important to support approaches to BBTD control.

This standard operating procedure focuses on screening banana breeding genotypes for resistance to BBTD.

2. Purpose

The purpose of this SOP is to provide guidance for the banana breeding program in phenotyping and indexing of banana genotypes for resistance to banana bunchy top virus disease using aphid populations as vectors to transmit BBTV in test genotypes under semi-controlled screenhouse conditions.

3. Scope

This document contains a comprehensive, step-by-step guide to assessing resistance of banana genotypes to BBTV through capturing, colony formation and characterization of infective aphid populations, data collection and analysis.

4. Definition of terms

Aphids: *Pentalonia nigronervosa* (family: Aphididea; order: Hemiptera), small, reddish-brown to almost black insects with an oval shape having two characteristic projections from the rear of its abdomen.

BBTD: Banana bunchy top disease caused by the banana bunchy top virus (BBTV).

Phenotyping: Process of intentionally exposing banana plants to BBTV-infected aphids, observing BBTV symptoms on the plants, and quantitatively documenting them in a designed trial.

Infective aphid population: A population of aphids that have acquired the virus and can transmit the virus.

Resistance: Ability of a banana genotype to restrict the growth and symptoms of BBTV as compared to a susceptible genotype.

PCR: Polymerase Chain Reaction.

5. Roles and responsibilities

The list of people responsible for each activity during phenotyping of banana genotypes for resistance to BBTV infection is presented below:

Tissue culture: Initiation and multiplication of banana genotypes.

Pathologists: Responsible for planning and supervising the experiments. Conduct data analysis and virus indexing.

Research assistant: Creating an experimental design using cycDesign, experimental setup, data collection and analysis.

Data manager: Consolidates data from pathologist and ensures it is uploaded and stored on MusaBase.

Breeder: Determines the breeding materials to be phenotyped. Also, uses the phenotyping results to make breeding decisions.

Molecular Breeder: Develops mapping populations

6. Procedure/Protocols

Activities include:

a) Generation of plant materials in tissue culture and raising them in the screenhouse for three months prior to phenotyping.
b) Development and maintenance of healthy and infective aphid populations.
c) Checking transmission efficiency for the infective aphid colonies.
d) Data collection through observation of BBTD symptoms on plants with the naked eye.

Equipment:

Infrastructure:

- Tissue culture lab - for initiation and multiplication of test genotypes.
- Glasshouse - area designated for introducing infective aphid populations to test plants.
- Screenhouse - area where infected plants are observed for symptom development.

Materials include:

- Petri dish
- Holoroft 240 trapping brush
- Fine mesh cloth
- Rubber band
- Microscope

- 50ml Beaker
- Scissors
- White newsprint
- Paper towel
- Piece of healthy banana leaf
- Notebook
- Pen
- Marker pen

6.1. Aphid capture and rearing

- Visibly healthy adult aphids are collected in the field using a trapping brush from healthy banana plants to limit the risk of virus contamination.
- Five Petri dishes are prepared by placing healthy sections of a banana leaf (upper side facing up) into each Petri dish containing moist tissue paper.
- One adult aphid is placed on the leaf into each Petri dish and observed for freshly hatched nymphs. Each aphid will generate one clonal population.

Note: Field aphids may carry fungal infections or may be parasitized by micro-Hymenoptera. Collecting visibly healthy aphids and starting each colony from freshly born nymphs minimizes such risks.

- For the next 2-3 days, aphids are observed frequently during the day to collect freshly born nymphs over 2-3 days, transferring all nymphs from an individual aphid to a healthy banana plant to start aphid colonies, i.e. 4-5 colonies.
- Colonies are maintained on banana plants in separate wire cages at temperatures in the range of 25°C. Each colony is clonal and should have consistent characteristics.
- Generation time for each colony is between 3 to 4 weeks.

Note: It is important to keep the plant and soil moist, however, care should be taken not to over water.

- Minimize contamination of colonies by mealybugs, mites and ants by keeping the plants in a contamination-free area.
- Avoid cross-contamination between colonies.
- A month after colony establishment, confirm the identity of the colony species using COI gene barcoding by PCR and sequencing of a sample from each of the colonies to ensure that you have *Pentalonia nigronervosa*, not *P. caladii*. Both species are vectors, but *P. nigronervosa* has banana as its preferred host and is apparently a more efficient vector of BBTV.
- Sequence the COI gene PCR amplicon generated using primers LepF and LepR (Folmer et al., 1994; Foottit et al., 2010).

Note: *P. nigronervosa* populations have transmission efficiency of 46-67%, so the efficiency of the colonies needs to be checked next (see below), and the best one retained, discarding the others.

- The colony conditions need to be monitored daily to ensure temperature and moisture conditions are appropriate for the aphids.

6.2. Checking the transmission efficiency of colonies

- Allow adult and late instar nymphs from each colony a 24-48 h acquisition access period (AAP) on the same BBTV-infected leaf piece. Late instar nymphs are the final developmental stage just before it becomes an adult aphid.
- From each colony, transfer 2 aphids to each of 10 caged healthy banana plantlets. Mark the youngest leaf at the time of inoculation (e.g. cut off tip) to allow monitoring of the rate of leaf production and symptom development.
- Allow a 24-48 h inoculation access period (IAP), then kill aphids with an insecticide spray.
- Record the number of infected plants after they have produced 5-6 new leaves.
- Identify and retain the most efficient vector colony.

Note: Bananas produce single new leaves sequentially and only leaves formed after infection will contain the virus. Generally, two or more new leaves are formed after inoculation before symptoms appear. The time taken for this depends on the growth rate of the plant rather than the absolute number of days.

Efficiency of transmission can be affected by:

- Transmission efficiency of the aphid vector.
- The number of individual aphids used for inoculation.
- Resistance/tolerance of the test plant.
- Residual insecticides on the test plant or acquisition source plant.
- The virus titre of the acquisition source plant.
- The AAP and IAP duration.
- Degree of care taken not to damage aphids during transfers, i.e. ensure the stylet is withdrawn from the plant before manipulation.
- Ambient temperature during transmission (~25°C is optimal).
- Aphids can have preferences on different cultivars, so they need to be restrained to the plant/cultivar during inoculations.

Notes:

a) There is always a chance of escapes in the inoculation experiments, so this needs to be taken into consideration.
b) Every experiment also needs constant susceptible control so that efficiency of inoculation can be monitored and so that successive experiments can be compared.

c) It is useful to have an infective colony, kept well away from the healthy aphid colony, to use for inoculations. This saves repeating the AAP step each time. Ideally the identity of the BBTV isolate should be confirmed by PCR and sequenced. This single isolate (colony) should be maintained for all subsequent screening activities.

6.2.1 Method

Plant materials

1) TC generated plant material including:

1) Test genotypes:
    a) Parents
    b) Hybrids
2) Land races controls
    a) TM-28 Obino lewai
    b) Mbwazirume
    c) Mchare

- Use 10 plants of the susceptible control (e.g. a Cavendish cv. or other known highly susceptible cultivar) and 10 plants of each test line, ideally small plants 100-150 cm tall.
- Allow aphids at least a 24-48 h AAP on a susceptible source plant or use infective colony.
- Place aphids on each test plant individually and cage plants separately if possible.
- Use a consistent number of aphids per test plant (e.g. 10) and allow at least 24-48 h IAP.
- Mark the youngest leaf at the time of inoculation (e.g. cut off tip).
- Spray aphids with insecticide after IAP and monitor for symptoms, preferably until 10 new leaves have been produced.
- Monitor and record symptoms for each plant.
- Infection should be confirmed by qPCR or ELISA if necessary.
- ELISA or qPCR are useful for monitoring relative virus concentration. The youngest leaf present at the time of sampling should be used.
- Symptomless plants should be indexed by ELISA or PCR, and if negative, should be reinoculated to assess whether they were simply escapes in the inoculation process.

Other materials

1. Sterile Forest soil/Loam soil
2. Sterile sand/saw dust
3. Aphid proof nets
4. 13-liter plastic pots
5. Watering cans
6. Aphids

Experimental design (Research Assistant)

A Randomised complete block design (RCBD) with three blocks will be adopted.

6.2.1.1 PCR conditions

Target	Primer name	Primer sequence	Ta (°C)	Product size (bp)	Reference
BBTV Rep gene	BBT1	CTCGTCATGTGCAAGGTTATGTCG	60	349	Thomson & Dietzgen, 1995
	BBT2	GAAGTTCTCCAGCTATTCATCGCC			
Aphid COI gene	LCO1490	GGTCAACAAATCATAAAGATATTGG	50	700	Folmer et al., 1994
	HC02198	TAAACTTCAGGGTGACCAAAAAATCA			

6.2.2 Data collection

For each plant, record:

- Time to symptom expression and virus titre (also a useful trait which would influence disease epidemiology).
- Number of leaves to emerge till the first symptoms.
- Type and severity of symptoms.
- Result of ELISA/PCR if conducted.

6.2.3 Symptom rating

Not all cultivars show the full range of possible symptoms and the severity of each symptom type can vary.

Symptoms include:

- Dark green streaks on midrib and petiole.
- Narrow leaves with chlorotic margins.
- Dark green dots and dashes on minor leaf veins when viewed from the underside with transmitted light.
- Dark streaks on the tips of bracts.
- Plant stunting.
- Increasingly smaller young leaves.
- Bunching aspect of leaves.

Note:

a) The first symptoms to appear are usually dark green streaks on midrib and petiole and narrower leaves with chlorotic margins. These symptoms usually occur in the second or later newly formed leaves after inoculation and can be very mild when first observed.
b) The most consistent symptoms across different cultivars are the narrow leaves with chlorotic margins. Not all cultivars express dark green dots and dashes on minor leaf veins, but in those that do, it is an unmistakable diagnostic symptom.

Symptoms are rated using the categories below:

Disease score	Symptom type
1	No symptoms
2	Dark green streaks on midrib and petiole and narrower leaves with chlorotic margins
3	Severe symptoms of narrow, chlorotic leaves and stunting.

Disease incidence is estimated as = $\frac{\sum symptomatic\ plants/genotype}{\sum total\ number\ of\ plants\ per\ genotype} x\ 100$

Notes:

These procedures provide a rigorous test for immunity or resistance. It should be noted, though, that some cultivars that can be shown to be susceptible in glasshouse inoculations but show apparent "resistance" in the field. This implies that some aspects of the aphid inoculation must differ. It is known that banana aphids have definite preferences when allowed a choice in feeding trials, but this is not necessarily linked to resistance e.g. BBTV-resistant *M. balbisiana* is a good host for rearing the aphids.

6.3. Confirmation of identity of colony species using qPCR

6.3.1 Reagents

Water: The water used for buffer preparation, as well as for sample preparation steps, must be of analytical quality to ensure that the performance criteria expected for the tests are met. Water used for PCR steps (mix preparation, amplification dilution) must be of sufficient quality for use in molecular biology (ultrapure water).

Molecular biology reagents:

- Ultrapure water
- plant DNA extraction kit
- Mastermix or commercial thermostable polymerase core kit
- dNTP, primers.

Composition and preparation:

The list of buffers required to implement the method is as follows:
-Grinding pad (type GEB)
-Extraction buffers (e.g. from Qiagen's DNeasy® Plant mini kit)
-Migration buffer (e.g. TAE or TBE 10X)
-Load buffer (if not integrated into master mix buffer)

Other reagents and consumables

Single-use consumables:

- Sterile filter microcones.

- Sterile microtubes.
- Sterile PCR microtubes, strips or plates in volumes appropriate for the thermocycler used.
- Ethanol 70° (and possibly a disinfectant detergent such as Aniospray): disinfection of work surfaces and equipment during the assay, grinding and isolation stages.
- DNA Away decontamination products: disinfection of work surfaces and equipment during molecular biology testing.

Controls and indicators: Reference samples must be included during the analytical process to validate the various stages of the method. In accordance with the requirements of MOA 022, these references consist of:
- a negative process control (NC): matrix not containing the target organism, processed under the same conditions as the samples to be analyzed and declared as uncontaminated at the end of handling (uninfected banana leaves).
- a positive process control (T+): matrix containing the target organism treated under the same conditions as the samples to be analyzed, declared contaminated at the end of the handling (infected banana leaves). At the very least, it provides assurance that the handling has been carried out correctly.

OPTIONAL internal amplification indicator (IAI): this indicator corresponds to a calibrated plasmid solution containing the target sequences of the 2 primers directed at BBTV (F and R BBTV Rep1). The amplicons obtained from this plasmid is of a different size to that obtained from the virus. The control is added to the reaction mixture and will therefore be present in each well. It is then amplified at the same time as the target pathogen, using the same primers as the target, in each well. The control is used to highlight samples that would not be usable by PCR due to the presence of inhibitors in the extract (thus avoiding false negatives).
- a negative PCR control (A- or T-water): this contains all the elements of the reaction mixture, but no DNA extract is added, only water; this verifies the absence of contamination during the PCR reaction.

6.3.2. Appliances and equipment

To implement this method, the laboratory will use the equipment described in the MOA 022 official method of analysis. Different systems may be used, depending on the equipment available in the laboratory. All methods are valid if the means are used to avoid the risk of cross-contamination.

Size	**MTE**
Volume	≤ 10 mL: MTE = ± 10% ≥ 10 mL: MTE = ± 5%
Mass	MTE = ± 10%
pH	MTE = ± 0.3 pH
Temperature	Incubator: MTE = ± 3ºC Refrigerator: 5ºC and MTE = ± 4ºC Freezer: ≤-18ºC Deep freezer: ≤-65ºC Thermocycler*: MTE accuracy = ± 1ºC

	MTE homogeneity: MTE = ± 2ºC
Time	MTE = ± 10%

The maximum tolerated errors (MTE) to be taken into consideration are given in the table above (otherwise, specifications are given in the text of the method).

a. Grinder

A ball mill (such as Bioreba's "SPEXSample P" model 6) with sample grinding in a plastic grinding tube with lead balls. The ground sample can then be collected directly. Any other grinding system may be used, provided it achieves equivalent grinding quality and limits the risk of cross-contamination.

b. **Thermal cycler for end-point PCR and AriaMax Real-time PCR system**

The protocol can be used on Applied Biosystems' VeritiTM Thermal Cycler and Applied Biosystems' GeneAmp® PCR System9700. Other thermal cyclers providing equivalent results may be used.

6.3.3. Samples

a) Sample acceptance conditions

Fresh samples: plant material must arrive at the laboratory fresh (leaves without yellowing, necrosis or signs of senescence). Each sample consists of a minimum of 0.5g of plant material.

Dehydrated samples: samples must be completely dehydrated using a desiccant such as $CaCl_2$. The test sample consists of at least 0.1g of dehydrated leaf.

b) Sample preservation before analysis

The time between receipt of the sample and the start of analysis should be as short as possible. For leaves collected under good conditions, the time between receipt of fresh samples and the start of analysis should preferably be less than 3 days and should not exceed 5 days. Pending analysis, samples should be stored at +5°C. If samples cannot be processed within this period, they should be frozen at a temperature of -18°C or below, pending processing (maximum 1 month). In this case, if possible, the test should be carried out before freezing.

c) Retention of samples or residues after analysis

Unless explicitly stated to the contrary, or unless technically impossible, laboratories must retain the relevant residues (nature, quantity and quality) of material submitted for analysis, in appropriate conditions guaranteeing their integrity, until at least the tenth working day following dispatch to the applicant of an analysis report concluding that the organism of interest has not been detected. This deadline is designed to give the person requesting the analysis time to contest the result with the laboratory (which prolongs the retention of the residue until the outcome of the dispute) and, if necessary, to request a contradictory analysis.

6.3.4. Method

6.3.4.1 Preparing samples for analysis

Various authors report that the distribution of BBTV in banana allows the pathogen to be detected on leaves (leaf blade and midrib), petioles, pseudostem, roots, rhizomes and meristems. However, according to Wu et al. (1992), infected plants contain the highest concentrations of BBTV in the leaves (limb and midrib), petioles and sheaths of the second leaf from the top.

It is therefore preferable to carry out the test on young leaves, with the last three leaves fully unfolded, including part of the midrib.

In cases where there are symptoms of BBTV (dark green streaks on the secondary veins with hook-shaped facies on the main vein, particularly visible when the underside is viewed against the light), the test should preferably be taken in these areas, always trying to include part of the central vein.

For test samples, the operator will take care to collect the best-preserved tissue (no sign of senescence). He will operate with a procedure adapted to his context (infrastructures, organization) which aims to avoid any risk of confusion between samples or contamination of one sample by another. Between each test, the operator must disinfect the equipment used for sampling. It may be useful to prepare the same sample two times in the event of a repeat test (for confirmation, for example).

6.3.4.2 Sample grinding

Grind the plant material in the grinding buffer at a weight/volume ratio of 1:10, i.e. 0.5g of fresh plant material per 4.5mL of buffer, using a ball mill, or any other grinding method that gives equivalent results.

For a test on dehydrated leaves, the recommended ratio is 0.1g of dehydrated material to 4.5mL of buffer.

The crushed material obtained should be stored at +5°C and used as quickly as possible (within the day at the latest).

Transfer the shredded material to Eppendorf tubes, leaving about 1 cm of empty space for freezing (e.g. 1.5mL of shredded material in a 2mL tube). The tubes are then centrifuged at high speed (e.g. 15 minutes at 13,000g) to pellet the virus particles (use of a refrigerated centrifuge is recommended). Remove the supernatant. This pellet can either be stored at +5°C (for a few hours), at -18°C or below (for a few days), pending extraction, or extracted directly.

6.3.4.3 DNA extraction

The kit initially validated for this method is Qiagen's DNeasy® Plant mini kit. Viral DNA is extracted and purified following the supplier's Plant Tissue extraction protocol, starting with the step described in the manual: "place 400µl of AP1 buffer and 4µl of RNase A in each tube, homogenize well". Then continue as described. DNA extracts can be stored for several weeks at -18°C or below, pending amplification.

6.3.4.4 qPCR Amplification

6.3.4.4.1 Preparing the reaction mixture

The size of the amplicons generated is 389 bp for the BBTV target and 254 bp for TIA (Refer to section 6.2.1.1)

Reagents	**Stock solution**	**Wells**	**Volume per well (µl)**
Ultra pure water			10.8
Buffer	5X	1 X	5
$MgCl_2$	25 mM	1.5 mM	1.5
dNTPs	10 mM	0.2 mM	0.5
Primer F	10 µM	0.40 µM	1
Primer R	10 µM	0.40 µM	1
Taq	5U/µl	1U/25µl	0.2
TIA (optional)	350 fg/µl (Dilution to 10-6 in our case)	28fg/µl	2 (in this case 700fg)

Notes - As TIA is DNA, it must be added to the reaction mixture at a different workstation from that at which the reaction mixture is prepared. The distribution of the reaction mixture containing TIA will therefore not be carried out in the usual workstation. If TIA is not used, add 2µl of water to the reaction mixture, giving a total of 12.8µl.

- The final volume is 25µl in each well, i.e. 22µl of reaction mixture and 3µl of DNA or water.

6.3.4.4.2 Amplification program

Temperature (°C)		**Duration**	**Cycles**
Initial denaturation	95	2 min	
Denaturation	95	45 sec	35
Hybridisation	58	30 sec	
Elongation	72	45 sec	
Final elongation	72	5 min	
Conservation	12	∞	

The times indicated depend on the Taq used, and it is necessary to follow the supplier's recommendations when using a Taq different from that used for method validation.

6.3.4.4.3. Results

a) Checking the validity of results

Checking that controls conform to expectations is a prerequisite for interpreting the results of samples submitted for analysis.
The analysis is validated if the following conditions are met.

Type of control	**Expected result**	
	Target	**TIA**
Negative process indicator (TS)	Negative	Positive
Negative amplification control (A- or T water)	Negative	Positive

Positive process control (T+)	Positive	Positive (or negative**)

*: Optional

**: If the positive control is very highly concentrated, there may be competition between the target and the TIA, which will then be barely visible. However, the target is amplified, validating the PCR.

b) Calculating and expressing results

Detection with TIA: The test is negative for samples showing no band at the expected target size and showing the TIA amplification band. The test is positive for samples showing a band at the expected size (with or without TIA).

If no fragment is visible (TIA and target), the sample is considered as not validated: re-test the sample in stock solution and with a 1/10 dilution of the DNA extract.

If amplification is not improved, the result will be expressed as "sample not analyzable, presence of inhibitors".

If the process controls are validated and a BBTV band of very low intensity is detected on the gel, i.e. hardly visible to the naked eye of an operator with visual acuity considered correct, the operator can repeat the detection test to confirm its interpretation.

The results can be interpreted as follows:

Analysis		**Result of target**	**Result of TIA**	**Formulation**
Shaft 1	**Shaft 2**			
+	+	Positive	Positive / negative	Positive result for BBTV detection
+	-	PCR to be repeated	Positive / negative	If at least 1 out of 2 is positive again, the result is interpreted as positive.
-	-	Negative	Positive	Negative result for BBTV detection

Detection without TIA: The test is negative for samples showing no band at the expected target size. The test is positive for samples with a band at the expected size.

If the process controls are validated and a very faint BBTV band is detected on gel, the decision to repeat the detection test is left to the discretion of the Technical Manager.

The results can be interpreted as follows:

Analysis		**Result of target**	**Formulation**
Shaft 1	**Shaft 2**		

+	+	Positive	Positive result for BBTV detection
+	-	PCR to be repeated.	If at least 1 out of 2 is positive again, the result is interpreted as positive
-	-	Negative	Negative result for BBTV detection

Data analysis

The model $Yij = \mu + \tau_i + \beta_j + \varepsilon_{ij}$

Where μ= overall mean; τ_i = effect of treatment; β_j = effect of block j; ε_{ij} = random error

To determine variability across experimental units, ANOVA will be carried out with blocks as factor to account for variability across experimental units. If treatment effect is significant, we shall compare treatment means using LSD, Tukey's HSD or Duncan's test to determine which treatments differ from each other.

Check for normality of residuals, homogeneity of variances and independence of errors.

Coefficient of variation (CV%) and goodness of fit expressed as coefficient of determination (R^2) will be reported to determine how much observed variation is explained by the model and reliability of the experiment.

7. Virus indexing

Virus indexing is carried out to detect viruses in plant tissues and is critical for plant propagation, especially in the context of micropropagation and the maintenance of mother plants (Selvarajan, et al., 2011; Singh et al., 2011). Comprehensive virus indexing in a banana tissue culture laboratory is primarily conducted on suckers collected from foundation stock for initial screening and again when plantlets reach the final nursery stage to ensure clean, disease-free material for release or further propagation (Prakash et al., 2010; Vijith et al., 2018). Virus indexing can be conducted at any subculture stage, requiring approximately 150-200 mg of the test sample (Thanuja, et al., 2025). But it is crucial for the initial foundation mother plants and the final plantlets before they leave the laboratory. Therefore, three key stages for virus indexing are:

1. **Foundation Stock Indexing:** The selection procedure of appropriate mother plants is a crucial step in initiating in vitro culture and propagation to ensure that the original mother plants (foundation stock) are virus-free before starting the tissue culture process (Cassells and Doyle, 2005; Suman, S. 2017). Healthy status confirmation for virus diagnosis is done by combining visual symptom inspection and molecular detection techniques (Viljoen et al., 2017). Sword suckers should be healthy and not less than 60-80 days of age while the growing meristem should be 1.0 cm^3 in size (Singh et al., 2011). One sucker, preferably Sword Suckers from each

foundation stock is collected and tested for viruses using techniques like ELISA, LAMP, RPA or TaqMan real-time qPCR (Purified extracts).

2. **Micropropagated Plantlet Indexing:** The detection and elimination of any viruses that might have infected the material during the tissue culture process must be done at 2nd and 3rd subcultures stages (Singh et al., 2011). But ideally the final plantlets before sending out for planting are a critical checkpoint (Thanuja, et al., 2025). Leaf samples are collected from the in vitro cultures and tested for viruses using techniques like ELISA, LAMP, RPA or TaqMan real-time qPCR (Purified extracts).
3. **Final Nursery Stage Indexing:** After the initial subculture, at 20-25 days post-initiation, cultures are meticulously examined for contamination. A final confirmation that plantlets are free of viruses before being moved from the tissue culture laboratory to the nursery for acclimatization and further growth. This is a critical final check after the plantlets have gone through the regeneration and initial growth phases. Pooled/single leaf samples are collected from the in vitro cultures and tested for viruses using techniques like ELISA, LAMP, RPA (Recombinase polymerase amplification) or TaqMan real-time qPCR for crude/purified extracts.

7.1 Virus indexing using ELISA : The Elisa test is the is a qualitative serological assay for the detection of *Banana bunchy top virus* (BBTV) in banana leaves. Upon successful completion of the test, samples containing the target analyte turn yellow, due to the alkaline phosphatase enzyme label, while negative samples remain colorless.

7.1.1. Materials (Kadam et al. in 2017; Sawardekar et al. in 2017; AGDIA instruction kit; Pietersen et al., 1996; Manchanda et al., 2010**)**

1) A Triple Antibody Sandwich (TAS) ELISA reagent set, with capture and detection antibodies (CPC 24700)
2) Alkaline phosphatase-conjugated antibodies
3) Positive/negative controls.
4) ELISA plates
5) Humid box for incubation
6) Sodium chloride
7) Sodium phosphate
8) Potassium chloride
9) Sodium azide
10) Etraction buffer (GEB2)
11) Wash buffer (PBST) for washing the plate between steps to remove unbound components
12) Substrate buffer used with the substrate to produce a color change
13) Plant tissues
14) PNP substrate tablets used to generate detectable signals
15) Powdered Egg Albumin
16) Tween-20
17) Diethanolamine

18) HCl for pH adjustment
19) 96-well microtiter plates

7.1.2. Methodology

1) To detect BBTV using ELISA, the sandwich ELISA protocol is followed in eight steps:
2) The ELISA plate is coated with 100µl of a capture specific antibody to BBTV antigen (Manual instructions supplied with the kit of AGDIA in 2025 and Manchanda et al. (2010)).
3) Cover the plate with a sealing film to prevent evaporation and contamination and incubate for 2-hours at 37°C.
4) Add the banana plant sample to bind with BBTV antigens incubate plate for 2 hours at 37°C.
5) Wash the plate 3-5 times with 300µl PBS containing 0.05–0.1% Tween 20.
6) Enzyme-labeled detection antibody is added to bind with the captured antigens.
7) Wash the plate 3-5 times with 300µl PBS containing 0.05–0.1% Tween 20.
8) 100µl of the substrate is added and plate is incubated in the dark for 1 hour. Color development happens in the presence of the enzyme.
9) Color development with the enzyme which converts the substrate into a colored product. The intensity of the color is directly proportional to the amount of BBTV antigen present in the original sample.
10) 50–100 µL of stop solution (e.g., 1 M H_2SO_4 for TMB) is added to each well.
11) The results are read with a microplate reader which measures the optical density (OD) of the color to determine the concentration of BBTV in the sample, often by comparing it to a standard curve (Pietersen et al., 1996; Manchanda et al., 2010; Djailo et al., 2015; Kadam et al., 2015; Kadam et al., 2017).

7.1.3. Interpretation of results

The specific interpretation can vary depending on the exact ELISA format and the kit being used. Always follow the manufacturer's instructions. Usually, the five steps below allow for a good interpretation of the analysis results as stated in the manual guide of AGRIA:

1. Wells are visually inspected to remove bubbles, if present. OD is measured with a spectrophotometer at 405 nm.
2. The test is valid if the positive and negative control OD results meet expected values.
3. Positive and negative thresholds are determined using é times the healthy average. Any samples with an OD value higher than é times the healthy average are positive, and samples with an OD value below 2 times the healthy average are negative.

Method 1	Healthy Avg	0.105	2x Healthy Avg.	0.210

	Sample 1	0.355 (Positive)	Sample 2	0.190 (Negative)

An alternative method for the threshold calculations is the healthy average plus 3 times the standard deviation of the healthy sample set.

Method 2	Healthy Avg	0.105	Std. Dev.	0.030	Healthy Avg. +3 x Std. Dev.	0.195
	Sample 1	0.355 (Positive)	Sample 2	0.190 (Negative)		

4. Positive OD values indicate the presence of BBTV.

7.2. Virus indexing using LAMP and RPA

7.2.1. Materials

1. LAMP instrument (Genie II/Genie III)
2. Sterile distilled water (SDW)
3. Alkaline poly-ethyl glycol (PEG) extraction buffer (pH 13.3)
4. LAMP tube strips (Optigene tube strips)
5. Maceration bags
6. 2.0 mL microcentrifuge tubes
7. Inoculation loops
8. Pipettes and pipette tips
9. Internal, external and loop primer pairs
10. LAMP master mix (isothermal master mix). This can be purchased in lyophilised format which is more suitable for outdoor use.

Note: Downstream, for RPA the reagents and consumables are exactly the ones used in the LAMP assay except for the primers, probe and isothermal mix.

7.2.2. Methodology for RPA

1) Grind approximately 100 mg of banana leaf tissue in 300µl of 0.5 M NaOH, followed by centrifugation at 12,000 rpm for 2 min.
2) Separate the supernatant from the plant debris and use as template in the RPA reaction.
3) The PCR mixture should include: 100 ng of template DNA (1µl), 0.25 lM of primer (each forward and reverse), 1.25 mM of MgCl2, 0.5 mM of dNTPs, 1X reaction buffer and 1 unit of DyNAzyme II DNA polymerase (Thermo Fisher Scientific, MA, USA), in a total volume of 25 µl.
4) The PCR conditions are: one cycle of initial denaturation at 94 ºC for 3 min, 30 cycles of denaturation at 94 ºC for 30 sec, annealing at 66ºC (F1/R1 & F1/R3) and 60 ºC (F2/R2) for 30 sec, extension at 72 ºC for 15 sec and one cycle of final extension at 72 ºC for 10 min.
5) Resulting PCR amplicons (10 µl) are electrophoresed on 1.8% agarose gel containing ethidium bromide and visualized under UV illumination.
6) RPA is performed using the Twist Amp Basic Kit (TwistDx, Cambridge, UK) in a total reaction volume of 25 µl, according to the manufacturer's instructions.

7) The reaction mixture, is prepared in a total volume of 50µl, mixed with the freezedried pellet and distributed in two reaction tubes.
8) Add the DNA template (100 ng) and 1.25µl of 280 mM magnesium acetate.
9) Incubate the reactions at 37 oC for 30 min using a dry bath.
10) RPA reactions using 1µl of crude leaf sap as template are carried out in a similar manner.
11) To visualize RPA amplicons, 10µl products are incubated at 65 oC for 10 min prior to loading on 1.8% agarose gel and mixed with 5% SDS while loading (Londono et al., 2016).
12) The RPA amplicons derived from all primer pairs are gel purified and cloned separately in pGEMT Easy vector (Promega, Madison, USA).
13) Each positive clone is sequenced in forward direction using the T7 universal primer.

The sequences obtained are analyzed by BLAST search in the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/blast).

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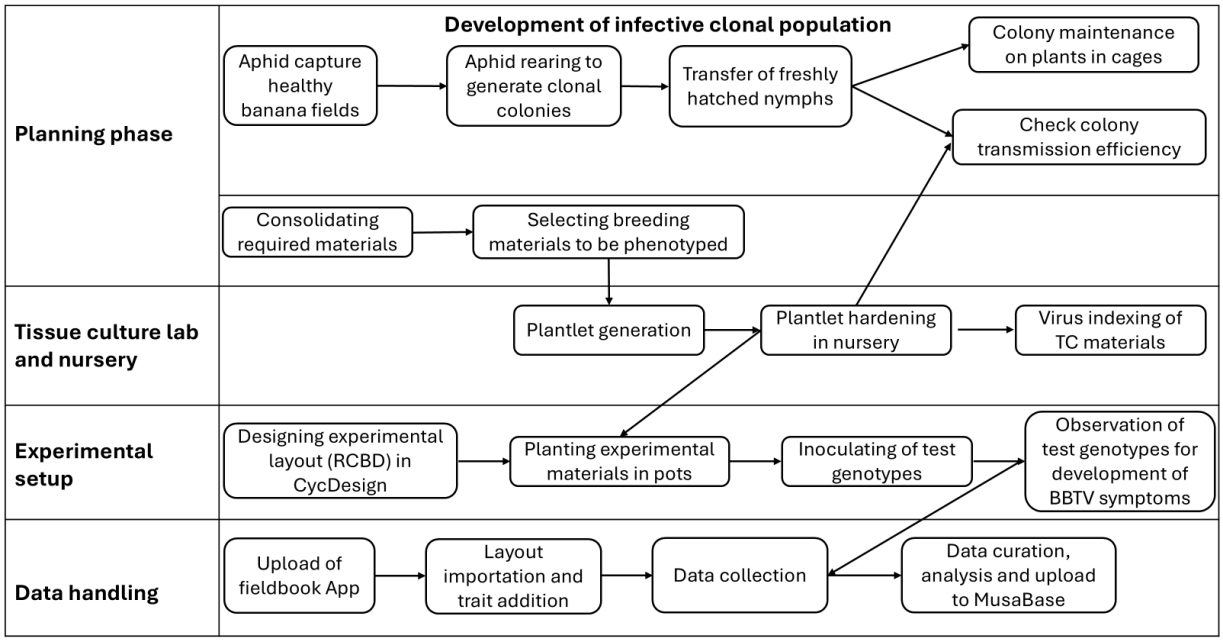
Flow chart for phenotyping for BBTV resistance in banana genotypes



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