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SOP21:

Standard Operating Procedure (SOP) for Pollen Tube Growth and Monitoring (PTGM) in Plantain and Banana Breeding

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1 Introduction

Pollen tube growth monitoring is a crucial technique in plant reproductive biology, particularly in the breeding of plantain and banana. This procedure allows for assessing pollen viability, compatibility, and fertilization efficiency, essential for improving hybridization success and enhancing hybrid recovery in a breeding program. By tracking the elongation and development of pollen tubes, researchers can evaluate pollen-pistil interactions, identify factors affecting fertilization, and optimize cross-breeding techniques in *Musa* species. This SOP outlines the standardized methodology for monitoring pollen tube growth under controlled conditions to ensure the accuracy and reproducibility of results in plantain and banana breeding programs.

2 Purpose

The procedure aims to evaluate pollen viability and germination potential, assess compatibility between different plantain and banana genotypes, and identify factors influencing pollen tube elongation and fertilization success necessary for developing improved varieties.

3 Scope

This SOP outlines the procedures for monitoring pollen tube growth in plantain and banana breeding programs. It covers the preparation of plant material, required tools, and chemicals for assessing pollen tube elongation through the pistil. It also includes safety guidelines, hybridization techniques and methods for evaluating fertilization success. It is intended for the pollination team, research technicians and breeders working on Musa genetic improvement.

4 Materials and Methods

This section outlines the factors influencing the success of pollen tube growth monitoring (PTGM), equipment, tools, chemicals, and protocols used to monitor plantain and banana pollen tube growth.

Factors Influencing the Success of PTGM

- The quality and age of sampled florets determine pollen viability and stigma receptivity.

- Pollen quality is affected by improper storage, environmental stress, and mechanical damage, leading to artefacts in PTGM.
- Pistillate florets that are too old, dry, or excessively wet may hinder pollen adhesion and tube growth.
- Aged, over dried or damaged pollen loses viability and may not germinate effectively.
- Pollen stored for a long period undergoes structural degradation, reducing its ability to fertilize successfully.
- Humidity and light conditions influence pollen hydration and growth dynamics.
- Some genotypes may have better pollen germination on the stigma than others.
- The receptivity of the stigma is crucial for successful pollen tube growth and penetration.
- Poor staining (e.g., aniline blue staining for callose deposits) hinders visualization.
- Contaminants or microbial infections can hinder pollen tube growth.

5 Required Chemicals, Tools, and Equipment

Successful PTGM in plantain and banana breeding requires specific chemicals, tools, and equipment. This section outlines the essential materials needed to ensure accuracy, efficiency, and reproducibility of the procedure.

5.1. Chemicals

The following chemicals are required for the preparation and execution of PTGM:

S/N	Product	Quantity/Description
1	Tri-potassium phosphate trihydrate (Extra pure)	Pellets, 100 g or more
2	Silica gel (orange with humidity indicator)	Granulated, 1 kg
3	Tween-20	200 ml or more
4	Glycerol	500 ml or more
5	Aniline blue (diammonium salt)	25 g or more
7	Ethanol (96% absolute)	1 L or more
8	Acetic acid	As required
9	Chloroform (optional)	1 L

5.2. Equipment and Tools

The following equipment and tools are necessary for carrying out the procedure:

S/N	Item	Quantity/Description
1	Computer	1
2	Extension box	2 pcs
3	HDMI cord	2 pcs
4	Adapter	4 pcs
5	UV fluorescent microscope	1
6	Microscope slides	10 packs or more
7	Cover slips (22 × 22 mm)	1 pack or more
8	Cover slips (large, 24 × 50 mm)	2 packs (200 pcs) or more
9	Scalpel handles	1 pack or more
10	Scalpel blades (both large and small)	10 packs or more
11	Forceps (fine-point, Sigma-Aldrich No. Z168777-1EA)	As required
	Surgical scissors	4 pcs
12	Refrigerator (<10°C)	1 large unit
13	Gas cylinder	1
14	Water bath or cooking pot (large enough to hold a test tube rack)	2
15	10 cm Petri dishes or reusable sample vessels	A box (several 100 pcs)
16	22-mm glass test tubes (Pyrex, without rim)	As required
17	Test tube racks (for 22-mm tubes, metal or heat-resistant)	2 or more
18	Beaker glasses	50, 100, 250, 500, 1000 ml (2× each or more)
19	Schott bottles	1 L bottles, 10 pcs or more
20	1-way plastic pipettes (Dropper)	As required

21	Trays	5 pcs
22	Napkins and paper towels	As required
23	Permanent marker pens (assorted)	As required
24	Paper towels	Large quantity (1 box or more)
25	Paper tape	10 pcs
26	Field book	As required
27	Spray bottles	As required
29	Racks and shelves	As required
30	Well-equipped laboratory	

5.3. Personal Protective Equipment (PPE)

The following PPE items are required to maintain hygiene, prevent contamination and ensure the safety of personnel handling chemicals and biological samples during PTGM procedures.

S/N	Item	Quantity/Description
1	Rubber gloves	2 packs (large size) or more
2	Safety glasses (Anti-UV)	4 pairs or more
3	Lab coat	As required
4	Field coat	As required
5	Field boots	As required
6	Face masks	1 box (50 pcs)

6 Essential Safety Precautions

To ensure a safe working environment while monitoring pollen tube growth in plantain and banana breeding, the following safety precautions must be strictly adhered to:

General Laboratory Safety

- Keep all chemical containers properly labelled and securely closed when not in use.
- Avoid skin and eye contact with chemicals; always use appropriate personal protective equipment (PPE).
- Never eat, drink, or apply cosmetics in areas where hazardous chemicals are handled.
- Wash hands and any exposed skin thoroughly before leaving the laboratory.
- Secure long hair and loose clothing to prevent entanglement in equipment.
- Limit lab access to authorized personnel only.

Chemical Handling and Storage

- Assume all unknown chemicals are hazardous and handle them with caution.
- Use a fume hood when working with volatile or hazardous chemicals to minimize exposure.
- Follow the correct order when mixing reagents (e.g., always add acid to water).
- Do not taste, sniff, or mouth-pipette chemicals. Use pipetting aids instead.
- Store chemicals in designated, clearly labelled areas, separate from incompatible substances.
- Properly dispose of chemical waste following approved protocols; do not pour chemicals down the drain.

Equipment Use and Maintenance

- Use laboratory equipment only for its intended purpose and follow manufacturer guidelines for maintenance.
- Regularly inspect equipment for damage or wear and ensure it functions correctly before use.
- Maintain and document equipment certifications, repairs, and maintenance records.

Emergency Preparedness

- Be aware of emergency protocols and know how to respond to chemical spills, fires, and accidental exposure.
- Be aware of emergency exit routes.
- Report all accidents, spills, or safety concerns to the lab supervisor immediately.

- Post warning signs when hazardous materials or procedures are in use.

By adhering to these safety precautions, researchers can minimize risks, ensure a safe working environment, and maintain the integrity of the PTGM process.

7 Preparation of Reagents Used

The following reagents and their respective concentrations are used to monitor pollen tube growth in plantain and banana breeding.

7.1 Schreiter's Solution

This reagent serves as an integrated solution for fixing, macerating, staining, and clearing. It is specifically designed for cytological investigations of pollen and pollen tubes within pollinated flowers (in situ) (Trognitz, 2025). The principle is that pollen and pollen tubes contain a special component called callose, which takes up and retains aniline blue dye upon staining and clearing and fluorescence when excited by UV light.

The following quantities of each chemical should be dissolved in distilled water to prepare 1 L of solution:

S/N	Chemical	Quantity	Distilled Water	Final Concentration
1	$K_3PO_4.3H_2O$ (tripotassium phosphate)	41 g	542 ml	0.154M
2	NaOH (dehydrated)	7 g	160 ml	0.175M
3	Aniline blue	1.7 g	82 ml	0.17%
4	Tween-20 or high-surfactant detergent (colorant-free)	16 ml	150 ml	1.6%
5	**Final volume**		**1000 ml**	

Ensure that all chemicals are completely dissolved, and the solution is well-mixed and the volume adjusted to 1000 ml before use.

7.2 Glycerol-Water Mixture

Glycerol is used as a mounting media. It increases the refractive index of the media to achieve brighter and higher resolution images. A glycerol-water mixture was used in a 1:1 ratio (50 % glycerol and 50 % distilled water) to prepare the final reagent. This mixture enhances sample preservation, improves optical clarity, and facilitates proper visualization of pollen tubes during cytological analysis.

8 Steps Involved in the PTGM Process

This section outlines the step-by-step procedures involved in pollen tube growth monitoring, from floret sampling and pollination to microscopic analysis and scoring. Proper execution of these steps ensures accurate assessment of pollen tube development and fertilization efficiency

8.1 Floret Sampling and Pollination Procedures

These procedures are critical to ensuring successful PTGM. The process involves careful selection, preparation, and controlled pollination of florets to facilitate optimal pollen deposition on the stigma.

Collection and Preparation of Staminate Florets

- Collect open staminate florets the day before pollination.
- Excise the anthers using a surgical blade and place them on a clean, dry paper towel. Allow them to wilt for several hours or overnight under a 40-watt heat source (tungsten lamp or wardrobe dryer) to remove moisture and facilitate the opening of the pollen sacs.

Anther Processing and Storage of Anther Dices (Pollen-Anther-Stigma Technique)

- Using a sharp blade or scissors, cut the wilted anthers transversely into ~2-mm sections to expose the pollen attached to the sticky tapetum.
- Use the diced anthers immediately for pollination or store them at 8 °C in a refrigerator for up to one day**.**

Preparation of Pistillate Florets

- Emasculate pistillate florets (fingers) by removing all adhering sepals within the bunch, the female part of the banana inflorescence.

Stigma Preparation

- Clamp the stigma of the florets with strong forceps to separate the six stigmatic lobes, enhancing accessibility for pollen.
- Thoroughly clean the florets to remove nectar, plant sap, and water with a paper towel.

Pollination Process

- Immediately dip the stigma into the bulk of diced anthers.
- Using three latex-gloved fingers, press the diced anthers containing pollen firmly onto the stigma surfaces for at least 15 seconds.
- Natural plant fluids from the stigma help bind the anther fragments and pollen, ensuring effective contact.

Pollen Quantity

- To sufficiently pollinate a single stigma, use anther dices equivalent to approximately ten anthers or two staminate florets.

Protection of Pollinated Florets

- Ensure adequate foliar shading to protect the pollinated florets from direct sunlight.
- Trim any foliage that may damage the florets or exposed pistils due to strong wind or rain.

Hand Pollination Timing

- Perform hand pollination by brushing entire staminate florets onto pistillate florets.
- Conduct pollination during cooler periods, preferably in the evening, for optimal results.

8.2 Microscopic Examination

Microscopic examination is a crucial step in PTGM, allowing for the visualization and assessment of pollen tube penetration into the pistillate tissues.

This process involves the collection, fixation, staining, and imaging of pollinated florets to track pollen tube development.

Floret Collection

- Collect complete pistillate florets from the nursery (field) 48 to 96 hours post-pollination, allowing sufficient time for pollen tube growth into the ovarian cavity.
- Process the samples immediately or after overnight storage at 8^{o}C.

Sectioning and Fixation

- Remove the carpel's exocarp using a handheld scalpel, cut 1 mm thick longitudinal sections from the stigma through the pistil, nectary, and ovaries.
- Immediately immerse the sections in Schreiter's solution to enable fixation. If immediate processing is not possible, store the samples overnight at 8 °C.

Heat Treatment and Staining

- Treat the samples in Schreiter's solution at 100°C in a water bath for 15–25 minutes. During this step, aniline blue dye stains the callose in pollen and pollen tube walls while sodium hydroxide softens the pistillate tissues.

Mounting of Sample Slides

- Carefully place the samples in a 50% glycerol-water mixture between two microscope slides.
- Gently squash the samples to expose more cellular layers within the softened tissues.

Microscopic Analysis

- Examine the samples using a UV-optical digital microscope equipped with a ring-shaped UV light-emitting electrode set.

Image Capture and Processing

- Capture fluorescence images using a Tucsen True Chrome AF camera, connected via USB 2.0 and operated with Capture 2.4 software (the system operates at 30 frames per second, producing high-resolution images and videos of fluorescing pollen grains and tubes at 22× to 1000× magnification).

Pollen Tube Tracking

- Visually analyze the qualitative and quantitative presence of pollen grains on the stigmatic surfaces.
- Track the extension of pollen tubes from the grains into the floret's tissues across the sample sections.

9 Scoring Procedures

A combined quantitative and qualitative scoring system is used to characterize the development of pollen tube populations within individual pistillate carpels. This evaluation helps determine pollen viability, fertilization success, and crossing efficiency.

Pollen Grain Count

- Count the number of pollen grains present on each stigma and accurately record the data.

Pollen Tube Observation

- Count the number of pollen tubes that have successfully penetrated the female reproductive structures of the pollinated flower.

Pollen Tube Growth Measurement (PTGM Scale)

- Use the PTGM scale to assess the probability of pollen tubes reaching the ovules successfully.

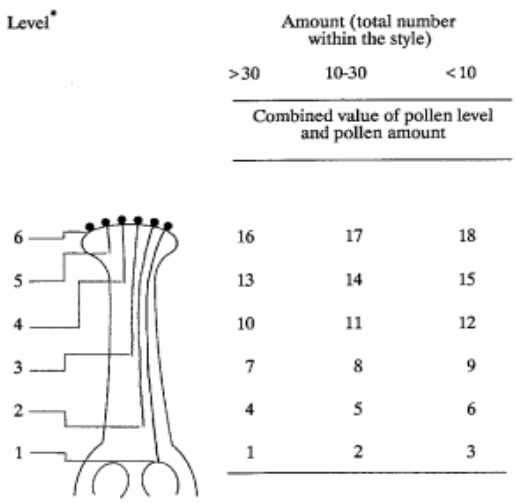

Fig. 1. Matrix of combined values for evaluation of (qualitative) level of pollen tube length and (quantitative) amount of pollen tubes. ** Levels of pollen tube length up to: 6- none; pollen tube not germinated; 5 - stigma; 4 - stigma-style; 3 - style; 2- style (full length); 1 – placenta* (Trognitz, 1991).

Microscopic Examination & Data Recording

- Analyze multiple samples under a UV-optical digital microscope.
- Grade each sample based on observed pollen tube penetration.
- Record all observations in an Excel spreadsheet for further statistical analysis and final report preparation.

Assessment of Pollen Viability & Crossing Efficiency

- Evaluate pollen viability based on germination success and pollen tube elongation.
- Assess the crossing efficiency by comparing successful pollen tube penetration across different parental lines.
- Properly document the pollen tube images using a standardized naming convention.

Tracking Pollen Tube Growth Pathway

- Observe the pollen tube's growth trajectory from the anther dices (after PAS pollination**)** through:

  a) The stigma

b) The style

c) The placenta within the ovarian cavity, approaching the ovules

Final Data Compilation

- Document and summarize key data, including pollen grain count, pollen tube penetration rate, and pollen tube growth scores for a comprehensive evaluation.

10 Conclusion

This Standard Operating Procedure (SOP) for Pollen Tube Growth Monitoring (PTGM) outlines a comprehensive workflow for assessing pollen viability, fertilization success, and crossing efficiency. By integrating floret sampling, pollination techniques, microscopic examination, and quantitative scoring, this SOP ensures standardized and reproducible results.

Additionally, detailed guidelines on the reagents, chemicals, tools, equipment, and safety precautions ensure that procedures are conducted with precision and adherence to laboratory best practices. The insights gained from PTGM are essential for optimizing breeding strategies, improving seed production efficiency, and advancing genetic research in *Musa* improvement programs.

11 References

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