Source: Pérez-Vicente, L., M.A. Dita, E. Martinez-de la Parte. 2014. Prevention and diagnostic of Fusarium Wilt (Panama disease) of banana caused by Fusarium oxysporum f. sp. cubense Tropical Race 4 (TR4). Technical manual prepared for the regional training workshop on the diagnosis of Fusarium wilt organized by the FAO Regional Office of the Caribbean and CARDI (Caribbean Agricultural Research and Development Institute) on 5-9 May 2014 in St. Augustine, Trinidad and Tobago. 74 pp. http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/caribbeantr4/13ManualFusarium.pdf

# PROTOCOL FOR THE ISOLATION OF *Fusarium oxysporum f. sp. cubense* FROM TISSUE SAMPLES OF FUSARIUM WILT OR PANAMA DISEASE AFFECTED BANANA PLANTS AND SOIL

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## ISOLATION OF THE FUNGUS FROM PLANT DISEASED COLLECTED MATERIAL.

### Fungal isolation from affected colored strands.

- The isolation can be attempted as soon as the strands with vessels are dry (possibly the day after the collection)
- Plate small sections (3-6 mm long) of the tissues with vascular vessels in Petri plates with ¼ strength potato dextrose agar (PDA) or water agar (WA) with an antibacterial agent (i.e. streptomycin sulfate1.2 mL / 240 mL of PDA).
- If *Fusarium* is present, it will grow out from the vessels in 2-4 days (see Figure 6).
- If the sample is contaminated with bacteria, the fungal growth could be masked. If this occurs, allow the sample dry more and increase the streptomycin sulfate in the media.
- From samples that have been prepared correctly, a high rate of *Fusarium* recovery is possible.
- Prepare single conidia cultures of each specimen.

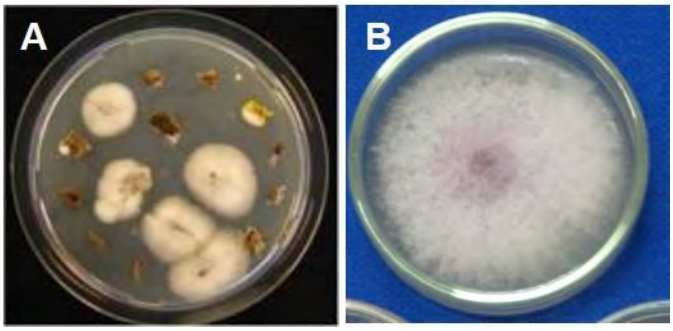


Figure 6. *Foc* growth in agar plates. A. *Foc* colonies from pseudostem discoloured strands in Water Agar. B. *Foc* single conidia culture in PDA plate.

### 1. Isolation from soil

- Collect a soil sample from the first 25 cm depth and store in a paper bag.
- Let samples air dry in the more aseptic conditions for 24-48 hours
- Grind the larger particles in a mortar

- Prepare a soil suspension in sterile water in a proportion of 1:50 soil weight / water volume (If the suspension is too concentrated because of high *Fusarium* population in the sample, a 1:100 proportion can be prepared). Shake the suspension for better release and distribution of soil particles and fungal structures.
- Dilute 1 mL suspension in 10 cm Petri plates with modified K2 media at close to melting temperature to achieve a good dispersion of the soil in the culture media.
- 1 mL of suspension can also be distributed on the surface of the plate with solidified K2 media. Agar should be allowed to dry for 3-4 days in the plates before plating the spore suspension so that it can absorb a higher amount of the spore suspension.
- Distribute the suspension as uniformly possible and allow it to stand for two minutes. Remove excess soil suspension from the plate and incubate it at 27°C upside down.
- Recovered colonies are transferred to other appropriate media to obtain single conidial isolates.

**2. Single spore isolations (single conidia).**

*Fusarium oxysporum* single spore isolations are obtained by the plate dilution method and streaking plates (showed ahead). For both methods:

- Collect a scrape of sporulating hyphae from cultures growing on PDA (¼ strength) and dissolve in 10 mL sterile distilled water in test tubes.
- From an initial suspension, a dilution serial can be prepared. Pipette or streak 1 mL of each of the dilutions on water agar.
- Incubate plates overnight at 25°C with caps in upside position
- Check the plates under a dissecting microscope the following day to localize germinated conidia and transfer with a sterile needle or scalpel single conidia isolated from the water agar to new 90 mm plates with ¼ strength PDA.
- Additionally, single-spore cultures can also be obtained by dissecting the tip of a single growing hypha of an old culture grown in carnation leaf agar (CLA).

## CULTURE MEDIA FOR THE ISOLATION AND CULTURE OF *F. oxysporum*

### 1. Potato dextrose agar ¼ strength (PDA ¼).

(Ainsworth, G.C., 1971. Ainsworth and Bisby's Dictionary of the Fungi. 6th. Ed. Commonwealth Mycological Institute, Kew Surrey, England, 663 pp).

Ingredients for a litre of distilled water**.**

| | |
|---|---|
| Peeled pieces of potatoes | 100 g |
| Dextrose | 10 g |
| Agar. | 20 g |

***Method***. Boil the potatoes in the distilled water for an hour and filter through eight cheese cloth layers. Discard the solid portion; then add dextrose and agar to the liquid portion, dissolve well and return to heat until the agar is fully dissolved (around 40-50 min). Withdraw the media from heat, dispense in flasks or bottles and autoclave immediately (humid cycle, 100 kPa at 121°C for 20 min.) When fresh, tighten the caps and mark the flasks or bottles with **PDA** and date.

**2. PDA supplemented with streptomycin.**

Proceed to melt the required number of 240 mL PDA bottles in a water bath. When media has melted, place the bottles in a water bath at 50°C for 20 min or until the media reaches 50°C. For each 240 mL of media, add 1.2 mL of streptomycin solution (1g of streptomycin sulfate powder to 100 mL distilled water) just before dispensing the media in the Petri plates.

**3. Carnation leaves agar (CLA).**

(Burguess, L.W., Liddell, C.M. and Summerell, B.A. 1988. Laboratory Manual for Fusarium Research, 2nd Edition, University of Sydney, Australia, 156 pp.)

***Method.*** Four to ten sterilized pieces of carnation leaves are placed on water agar surface before media hardens (solidifies). After the media has solidified, the plates with CLA are stored in a refrigerator at 4°C.

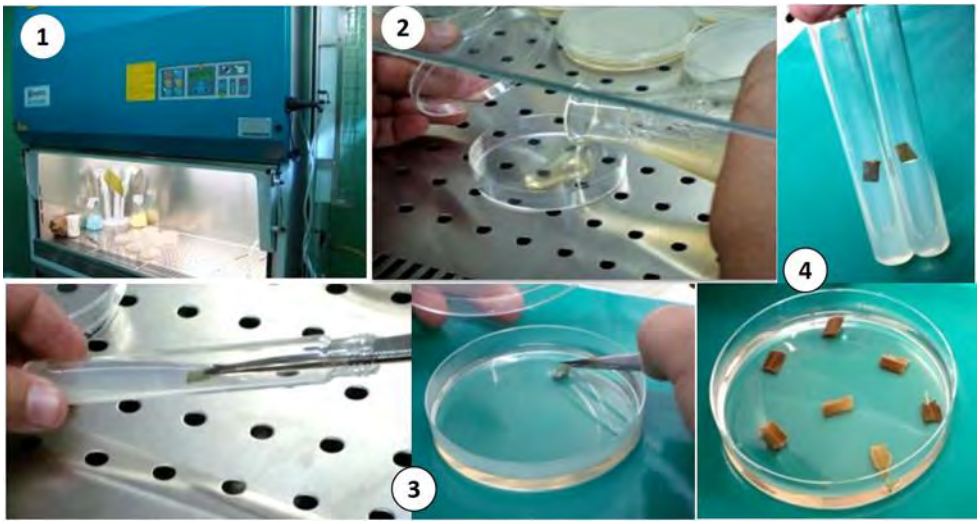


Figure 7. Preparation of Carnation Leaf Agar. 1. Biological safety cabinet. 2. Water Agar 2% poured in plates. 3. Sterilized Carnation leaf fragments placed in plates and tubes. 4. Plates and tubes with CLA ready for inoculation

***Preparation of carnation leaves*** (Figure 7)*:*

- Fresh carnation leaves not treated with agrochemicals, are cut in 8 x 3 mm pieces before being placed in an oven at 70°C to dry.
- When dry, place it in containers that are suitable to receive Gamma radiations (i.e. glass, polystyrene containers or Petri plates sealed with Parafilm). Note that after repeated exposure to Gamma radiations, plastic will degrade.

- Radiate containers in a Gamma cell for a total rate of 2.5 Mega Rad.
- Store sterile pieces in a refrigerator at 4 °C until use.

**4. Komada modified media (K2)**

(Sun, E.J., Su, H.J and Ko, W.H., 1978. Identification of *Fusarium oxysporum f. sp. cubense* race 4 from soil or host tissue by cultural characters. *Phytopathology* 68: 1672-1673).

Ingredients for 900 mL of distilled water:

| | |
|---|---|
| D-galactose | 10.0 g |
| L-Asparagine | 2.0 g |
| $KH_2PO_4$ | 1.0 g |
| KCl | 0.5 g |
| $MgSO_4 \cdot 7H_2O$ | 0.5 g |
| FeNa EDTA | 10.0 mg |
| Agar | 20.0 g |
| Distilled $H_2O$ | 900 mL |

- Sterilize at 120°C for 20 min.
- Adjust pH to 3.8 with 10% phosphoric acid.
- Add at a temperature close to 50°C, 100 mL of a solution is sterilized by filtration with:

| | |
|---|---|
| Streptomycin sulfate | 0.3 g |
| Oxgall | 0.5 g |
| $N_{a2}B_4O_{7.}$ | 0.5 g |
| PCNB (75% PH) | 0.9 g |

Inoculate plates with a 0.5 ml diluted suspension of soil in sterile water.

**5. Spezieller Nährstoffarmer agar (SNA).**

| | |
|---|---|
| $KH_2PO4$ | 1 g |
| $KNO_3$ | 1 g |
| $MgSO_4 \cdot 7H2O$ | 0.5 g |
| KCl | 0.5 g |
| Glucose | 0.2 g |
| Sucrose | 0.2 g |
| Distilled $H_2O$ | 1L |

Sporulation is stimulated if sterile filter paper Whatman # 1 pieces are included.

This media is appropriate for producing microconidia in a stable way. Suitable for chlamydospores detection.

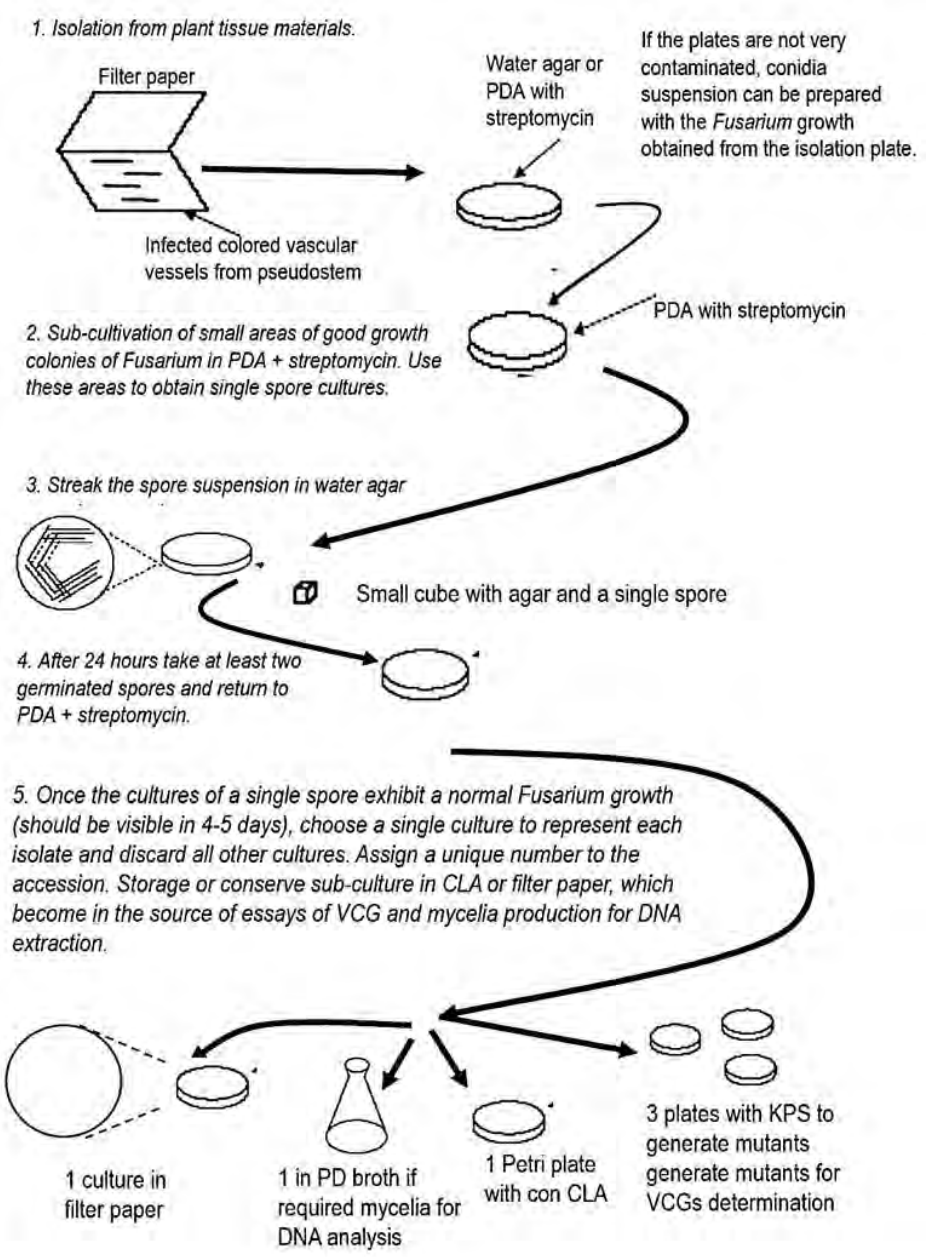


Figure 8. Scheme of the process of isolation, obtain single spore isolates and store for different purposes.