Guidelines for isolation and identification of *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 from banana

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KNOWLEDGE FOR LIFE

Contents

Acknowledgements 2
Abbreviations and acronyms 2
1. Introduction 3
2. Current distribution of Foc TR4 3
2.1 Causal agent and its hosts 4
3. Epidemiology and symptoms 5
3.1 Disease lifecycle 5
3.2 Movement and dispersal 6
3.3 Symptoms on banana 6
3.4 Similarity of Foc with other diseases 10
4. Diagnosis of Foc TR4 11
4.1 Surveillance 11
4.2 Collection and preparation of diseased plant tissue" 12
4.3 Isolation from plant material 13
4.4 Purification of Foc 14
4.5 Morphological characteristics used to identify Foc 16
4.6 Storage and preservation of Foc cultures 17
4.7 Molecular diagnosis 18
5. Other useful resources 20
6. Annexes 21
Annex I. Media for isolation of Foc 21
Annex II. Growth media for identification of Foc 22
Annex III. Teaching key to common species of *Fusarium* 24
Annex IV. DNA extraction from banana plant material and *Fusarium*. 26
7. References 27

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Abbreviations and acronyms

AAA	Triploid group cultivars of *Musa acuminata*
AAAA	Tetraploid group cultivars of *Musa acuminata*
AAB	Triploid cultivars of plantain
ABB	Triploid cultivars of cooking banana
CTAB	Cetyltrimethyl Ammonium Bromide
DNA	Deoxyribonucleic Acid
FAO	Food and Agriculture Organization of the United Nations
Foc	*Fusarium oxysporum* f.sp. *cubense*
FOSC	*Fusarium oxysporum* Species Complex
IPPC	International Plant Protection Convention
f. sp.	Formae speciales
LAMP	Loop-Mediated Isothermal Amplification
NPPO	National Plant Protection Organization
PCR	Polymerase Chain Reaction
qPCR	Real Time PCR
PDA	Potato Dextrose Agar
PSA	Potato Sucrose Agar
TR4	Tropical Race 4
TWA	Tap Water Agar
SNA	Spezieller Nährstoffarmer Agar
VCG	Vegetative Compatibility Group

1. Introduction

Banana production holds great significance in the Caribbean, playing a pivotal role in the region's economy, agriculture, and livelihoods. The tropical climate of the Caribbean islands provides ideal conditions for growing bananas, making it a major agricultural commodity. The banana industry contributes significantly to employment, offering jobs to numerous local farmers and laborers, thereby enhancing the region's economic stability. Additionally, bananas serve as a crucial export product, generating substantial revenue for Caribbean countries. Bananas are a staple food in the Caribbean diet, providing vital nutrients and sustenance to the local population. Furthermore, the cultivation of bananas promotes environmental sustainability by preserving natural habitats, as many Caribbean countries have adopted eco-friendly farming practices underlining its pivotal importance to the region.

Fusarium wilt of banana, caused by the highly virulent Tropical Race 4 (TR4) strain, poses a severe threat to the Caribbean's banana industry. TR4, a soil-borne fungus, infects banana plants, leading to their wilting and eventual death. This devastating disease can persist in the soil for years, making it highly challenging to control and eradicate. Given the Caribbean's heavy reliance on banana cultivation for economic stability and local consumption, the spread of Fusarium wilt TR4 could have catastrophic consequences, leading to significant economic losses, unemployment, and food insecurity. Efforts to prevent Fusarium wilt TR4 entering the Caribbean region are crucial, necessitating strict quarantine measures, to prevent entry and a comprehensive surveillance programme to contain the disease if it does gain entry.

Currently, TR4 is confirmed to be present in Colombia and Venezuela neighbouring South American countries. There is no effective commercial control strategies to manage this disease. These guidelines have been produced to support the National Plant Protection Agencies of Caribbean countries to support their regional surveillance programmes. The guidelines aim to provide information to support staff with early disease symptom recognition and protocols for field sampling, isolation and identification of the fungal causal agent. The guidelines also aim to provide a summary of useful resources available to plant protection agencies and research organisations in the region.

2. Current distribution of Foc TR4

Panama disease or Fusarium wilt of banana is one of the most damaging diseases of *Musa* species, caused by the fungal pathogen *Fusarium oxysporum* f. sp. *cubense*. The disease is believed to be native to Southeast Asia, originating in Malaysia or Indonesia. It was first recognised outside of Southeast Asia in Australia in 1876, then quickly reported in Central America, Costa Rica and Panama in 1890[1] - hence the name ‘Panama Disease’. The disease is now present in almost all banana growing areas of Asia, Africa, Latin America and the Caribbean.

It wasn’t until the mid-part of the 20th century that the disease became a major concern for the banana export industry when large plantations in Central America were devastated. The most commonly grown cultivar of banana for export to Europe and the USA at the time was Gros Michel and due to its susceptibility to Fusarium wilt, commercial producers were forced to find more resistant varieties and turned to production to Cavendish cultivars such as Dwarf Cavendish, Grande Naine, Valery and Williams[2].

Although there were early reports of Fusarium wilt symptoms on Cavendish varieties in the subtropics of the eastern hemisphere it wasn’t until the 1970’s that symptoms were reported on Cavendish clones in Taiwan in 1967 and Indonesia/Malaysia in the 1990s, this new strain was to be known as *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4 (Foc TR4). Since then, the new strain affecting Cavendish varieties has spread from Southeast Asia to India,

Pakistan and the Middle East and later detected in Africa in 2013. In 2019, Foc TR4 was reported on Cavendish varieties in the Americas for the first time where it was identified in Colombia[3]. Since then, Foc TR4 has been reported in banana producing regions of Peru and most recently Venezuela in 2023, threatening the future of commercial and small-scale banana production in the rest of the Americas and the Caribbean. Figure 1 below summarises the spread and timeline of the disease from Southeast Asia to Africa and into the Americas.

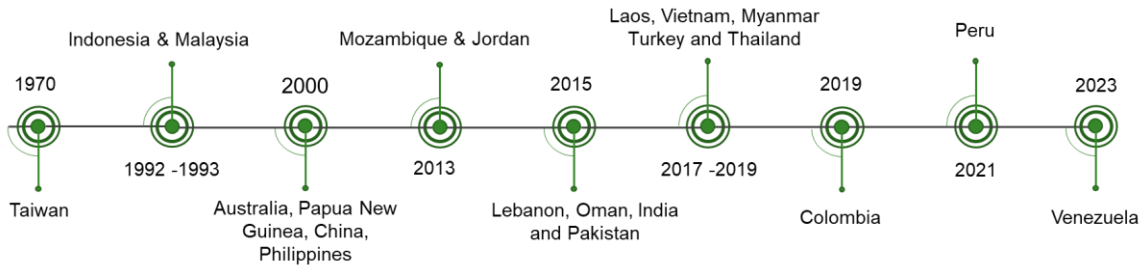


Figure 1. Geographical distribution of Foc TR4 and timeline of spread (Infographic: R. Reeder, CABI)

2.1 Causal agent and its hosts

The causal agent of Fusarium wilt of banana, *Fusarium oxysporum* f.sp. *cubense* (Foc), is a soil-borne fungus that belongs to the *F. oxysporum* species complex (FOSC).

Formae speciales: Strains of *F. oxysporum* that cause disease in plants have conventionally been placed into groups known as formae speciales (special forms) on the basis of the host species they affect. It has been estimated there are approximately 100 formae speciales of *F. oxysporum* that cause vascular wilts in plants such as cotton, tomato, oil palm and in this case, of banana. Fusarium wilt of banana (*Musa* spp.) and heliconia spp. has been associated with the formae specialis (f.sp.) '*cubense*'[4]. As well as causing disease in banana, Foc has been isolated from several weed species in banana growing areas which may provide alternative hosts for the pathogen[4].

Races: The formae speciales groupings for Foc can be further subdivided into different pathotypes commonly called 'races', in which pathogenic variations exist to different cultivars of the same host plant species. There are 3 know races affecting banana and 1 race affecting heliconia (Table 1).

Table 1. Races of Foc and susceptibility of different cultivars[5,6]

Cultivar	Race 1	Race 2	Race 3	Race 4
Gros Michel (AAA)	Susceptible	Resistant	Resistant	Susceptible
Cooking bananas/Bluggoe (ABB)	Resistant	Susceptible	Resistant	No data
Cavendish	Resistant	Resistant	Resistant	Susceptible
Heliconia spp.	Resistant	Resistant	Susceptible	No data

Foc Race 4 is subdivided into two groups: subtropical strains (STR4) and tropical strains (TR4). STR4 have been found to be pathogenic to the Cavendish group in subtropical regions but usually where they are predisposed to adverse environmental factors such as low temperatures and drought but is not known to cause disease to Cavendish in the tropics. TR4 is a much more aggressive strain and has the widest host range that includes Cavendish varieties previously resistant to the other races[4].

Note: in 2019, it was proposed by Maryani et al.[7] that FOC TR4 was a distinct species, *Fusarium odoratissum*, however, the new name has not yet been readily adopted and FOC TR4 is still used in much of the published literature.

Vegetative compatibility groups: The use of 'race' to distinguish the different pathotypes of Foc has limitations as exceptions are known and other races may exist. Different isolates of Foc have been characterised using their vegetative compatibility groups (VCGs). The VCG technique has been used to provide more information on the evolution and distribution of Foc[2]. TR4 belongs to VCGs 01213 and 01216, as these two VCGs are genetically very similar they are grouped under the code VCG 01213/16[4].

3. Epidemiology and symptoms

3.1 Disease lifecycle

Foc is a soilborne fungus which attacks the vascular system of the banana. There is no known sexual stage of the fungus, so it reproduces asexually. It produces three types of asexual spores: macroconidia, microconidia and chlamydospores.

Chlamydospores present in the soil and other infected plant debris are the main source of infection (Figure 2). Once stimulated to germinate in the presence of host root exudates the chlamydospores produce mycelium which colonise the small roots. Initial growth through the root tissue is relatively slow until the pathogen reaches the vascular tissues of the root. Once establish the vascular system of a susceptible cultivar the infection moves through the vascular system of the corm, pseudostem and fruit stalk. In resistant cultivars the fungus becomes blocked by substances (gels and tyloses) produced by the host as a defence mechanism and cannot move into the corm.

In susceptible hosts the disease progresses causing necrosis in the xylem tissue and the plant eventually dies. Once the plant is dead the pathogen moves out of the xylem and into the surrounding tissues.

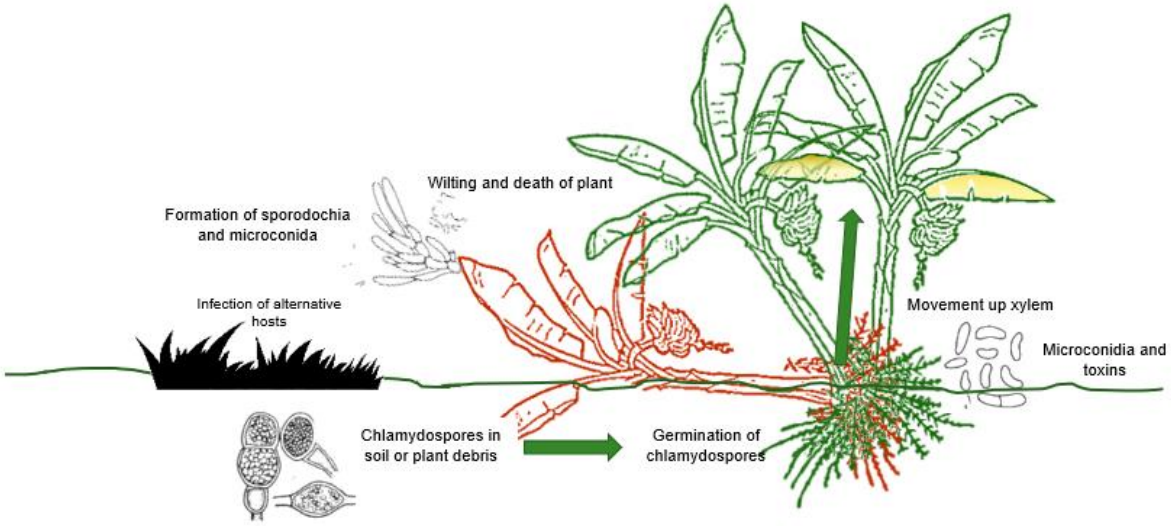


Figure 2. Foc life cycle and disease process (Infographic: R. Reeder, CABI)

The chlamydospores act as survival structures in the soil and diseased host debris and can survive for up 30 years[8]. Once in the soil the pathogen is impossible to eradicate. Foc also has the ability to colonise other grass and weed species where it can remain without causing symptoms ready to infect another host[9].

3.2 Movement and dispersal

Foc is spread mainly through the movement of infected plant material for propagation (suckers). The suckers appear to be symptomless (asymptomatic) and have been identified as agents in the unintentional spread of the disease locally, nationally and internationally. The pathogen can also be easily spread by the movement of contaminated soil, this can be on propagation materials, tools, vehicles and machinery. Foc is also easily spread through irrigation water, surface drainage waters and in rivers and streams that flow between infected and disease-free areas.

The presence of fruiting structures (known as sporodochia) and mycelium growing on the surface of infected plant tissue suggests that Foc might be spread aerially. Moving diseased pseudostem tissue and leaves of infected plants may also be means of dispersal – this is important as banana leaves are often used for wrapping or packing materials during transport. Foc has not been found on the skin or fruit pulp of banana fruits and does not produce any symptoms on or in the fruits. Foc infected plants often occur in clusters in the field, suggesting that plant-to-plant spread of the diseases takes place and insect vectors are thought to disseminate the disease, especially the banana weevil.

3.3 Symptoms on banana

The time between infection of bananas by Foc and observing symptoms can be as short as a few weeks or take up to several years. The appearance of symptoms depends on the level of inoculum, the susceptibility of the host and external environmental factors but the various races of Foc **cannot** be differentiated by their symptoms[10]. A description of the most important and recognisable symptoms to look for in the field are given below and corresponding images presented in Table 2.

As Cavendish varieties are resistant to Foc races 1 and 2 but susceptible to TR4 if any disease symptoms on Cavendish are observed in the field immediate action must be taken to identify the pathogen. However, definitive diagnosis of Foc TR4 can only be confirmed by molecular means or VCG testing.

Symptoms[1,2,4]

- The first detectable symptom seen on bananas is yellowing of the margins (edges) of the oldest and lowest leaves which starts at the bottom of the petiole (leaf stem).
- As the disease progresses the yellowing spreads to the younger leaves until only the recently unfurled leaves in the centre remain upright and green.
- The affected leaves can remain upright for a few weeks but eventually start to wilt and collapse at the petiole, become dry and hang down against the pseudostem and form a ‘skirt’.
- A few of the youngest leaves remain green and upright but the plant eventually dies.
- In some cultivars the yellowing does not occur, but the petioles bend and collapse beginning with the oldest leaves.
- Bulging and splitting of the pseudostem can occur and necrosis of the emerging heart or cigar leaf.
- Internal symptoms in the pseudostem begin with characteristic vascular discolouration.
- Initially vascular symptoms are a pale or yellow discolouration of one or two vascular strands in the early stages and as the disease advances the discolouration becomes darker turning from pale yellow to dark red, dark brown to almost black.
- The decolouration spreads from the corm through the pseudostem and can enter the bunch stalk however no symptoms appear in the bunch.
- The discolouration in the corm tissue is most distinct and can pass from the corm into the suckers through the stele but suckers may not show any signs of the disease but will not grow and mature.

- Above and below ground parts of affected plants eventually rot and die but only above ground parts of the plant fall when they die.

Table 2. Images of common symptoms caused by Foc in banana.

	**First detectable symptoms of Foc in the field is yellowing of the lowest, oldest leaves.** Image: @DR Jones
	**Advanced symptoms showing dried older leaves collapsed at the petiole (leaf stalk) and hang around the pseudostem forming a 'skirt'.** Image: Scot Nelson/via Flickr (CC0 1.0)
	**Early-stage vascular symptoms with limited yellow/light brown discolouration of the vascular tissue in the pseudostem.** Image: Scot Nelson/via Flickr (CC0 1.0)
	**Early-stage vascular symptoms, yellow/brown discolouration in the pseudostem.** Image: Scot Nelson/via Flickr (CC0 1.0)

Vascular staining seen in a longitudinal section through the pseudostem.

Image: Scot Nelson/via Flickr (CC0 1.0)

More advanced infection showing extensive dark brown discolouration of the vascular tissue in the pseudostem.

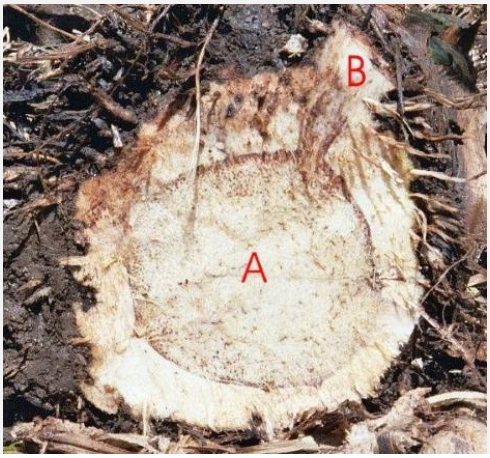
Image: Scot Nelson/via Flickr (CC0 1.0)

Advanced symptoms in the vascular tissue with staining seen in a longitudinal section through the pseudostem.

Image: Scot Nelson/via Flickr (CC0 1.0)

Swelling and splitting of the lower portion of the pseudostem at ground level.

Image: @DR Jones



Vascular staining present in the corm/rhizome of the mother plant (A) spreading into the attached sucker (B).

Image: @M Rutherford, CABI

The illustration below (Figure 3) gives a useful visual representation comparing the external symptoms of the banana plant and the internal disease process. Note that during the initial stages (Germination and Penetration) the banana plant exhibits no external symptoms (yellowing of the leaves or wilting) its only once colonization of the vascular tissue occurs that the oldest (lowest) leaves begin to show signs of yellowing. As the disease progresses and more and more of the vascular tissue becomes obstructed the external leaf yellowing and wilt becomes more advanced.

Figure 3. Representation of the life cycle of Foc with corresponding external symptoms (Source: IPPC Secretariat[11])

3.4 Similarity of Foc with other diseases

Whilst describing the symptoms of Fusarium wilt is it also important to mention that there are several other diseases of banana that produce similar symptoms and may cause confusion on initial observation and field diagnosis. The symptoms of three bacterial diseases have been compared to those of Foc in Table 3 below: Moko disease (*Ralstonia solanacearum* race 2), Banana Blood disease (*Ralstonia syzygii* subsp. *celebesensis*) and Banana Xanthomonas Wilt (BXW) caused by *Xanthomonas campestris* pv. *musacearum*. The main differences between Foc and Moko, Blood and BXM is that Foc infection does not produce symptoms on the flower buds or fruit bunches. Of the three bacterial diseases, only Moko disease is currently present in Latin America and the Caribbean.

Table 3. Differences between symptoms produced by Fusarium Wilt Disease, Banana Blood Disease, Moko Disease and Banana Xanthomonas Wilt (BXM)

Fusarium wilt	Moko[12,13]	Blood[14]	BXM[15]
Present in most banana growing regions	Some parts of Africa, South and Southeast Asia, the Americas and parts of the Caribbean	Recorded in Indonesia and Malaysia	East and central Africa
Symptoms progress from oldest leaves to youngest	Yellowing and wilting of the older leaves which dry and collapse	Yellowing and wilting of leaves. All mature leaves can yellow and collapse	Wilting of the emerging leaf or yellowing of the youngest leaves

Internal discolouration from pale yellow to red-brown to black. No bacterial ooze on the cut surfaces	Internal discolouration from pale yellow turning brown to black. Cut stems will exude bacterial ooze	Red-brown discolouration inside the pseudostem, when cut show droplets of bacterial ooze which can vary in colour from white, yellow, red/brown, black	Cream, yellow of pinkish bacterial ooze in the pseudostem
No symptoms in the bud or flower	Flower buds blacken and shrivel	Blackening and shrivelling of the male flower	Blackening and shrivelling of the male flower
No symptoms in the fruit	Premature ripening and splitting of fruit. Grey or brown rot of internal part of fruits	May appear externally healthy but internally there is a red/brown dry rot	Premature ripening and internal brown discolouration of immature fruits

4. Diagnosis of Foc TR4

If during surveillance activities suspicious symptoms are observed there are several steps to go through before an official diagnosis for Foc TR4 can be confirmed:

- Collection and preparation of diseased plant material
- Isolation of the fungus
- Morphological characterisation of the fungus
- Purification of the fungus
- Molecular diagnosis
- VGC analysis or whole genome sequencing
- Pathogenicity testing (performing Koch's postulates)

Whilst the first steps of collecting samples, fungal isolation and a preliminary molecular diagnosis can be carried out relatively quickly (usually in a matter of a few weeks), confirmation via pathogenicity testing can take several months. However, if preliminary molecular diagnosis is positive for Foc TR4 a precautionary response should be implemented to contain the spread of the disease[11].

4.1 Surveillance

In banana producing countries where Foc TR4 has not yet been reported, routine surveillance and detection surveys should be carried out regularly. Early detection is difficult as there is no way of detecting infected plants until the symptoms are visible and the disease, at this point, may have already been present for some time. Another compounding factor is that the symptoms of Foc TR4 cannot be differentiated from those caused by other races of Foc. Surveillance activities are usually organised by the NPPO, and the following points should be taken into consideration when planning and implementing detection surveys[11]:

- Officers carrying out the surveys should be well trained in the recognition of the external and internal symptoms of Fusarium banana wilt.
- Officers should also be aware of other diseases that cause similar symptoms in banana (Moko disease, Blood disease and BXM) and be able to differentiate between them and Fusarium banana wilt.
- Prioritisation of areas growing Cavendish varieties as these are resistant to Foc races 1 and 2, detection of symptoms may indicate the presence of infection by Foc TR4.
- Prioritize regions or areas in-country that are considered to be of greater risk to incursion of pests and diseases.
- Surveillance protocols need to include procedures to avoid potential spread of the disease during the survey activity and transportation of samples.

- Plants with suspected symptoms of Foc TR4 should be marked (location coordinates recorded), sampled and transported to the laboratory for analysis.
- The NPPO should inform the farm owner and initiate protocols to contain the possible spread of disease while the samples are processed.

4.2 Collection and preparation of diseased plant tissue[6,11,16]

Where a banana plant is suspected of being affected by Foc based on external symptoms a section of the pseudostem should be removed to observe the internal symptoms (Table 2; Figure 4):

- Suitable clothing should be worn when collecting samples: disposable protective suits, rubber boots and disposable gloves. All outer clothing and equipment (knives, cutting tools) should be disinfected or replaced between sites. Disposable gloves should be replaced, knives and boots disinfected between sampling plants. Suitable disinfectants include sodium hypochlorite (bleach) and quaternary ammonium compounds.
- Samples from the pseudostem should be removed at about 50-60 cm from the base of the plant. Cut a square approximately 5 x 8 cm in the centre of the stem.
- The sample should be removed as close to the centre of the pseudostem as possible and not from the outer layers of the leaves. The incisions should be deep enough to cut through several layers of the stem.
- Sections cut from the pseudostem showing internal staining can be placed individually in thick paper bags or wrapped in newspaper and placed in paper envelopes for transport back to the laboratory. Banana tissue is very wet and deteriorates quickly especially in hot and humid conditions, because of this the risk of bacterial contamination is high.
- Alternatively, from the section removed the out 2-3 layers can be removed, and the diseased section cut into smaller strips and placed in between dry pieces of filter paper then placed in a paper envelope.
- In addition to stem tissue, samples can also be taken from the corm if the disease symptoms are not too advanced. Small cubes (5cm x 5cm) can be removed from the corm and wrapped in paper and put into a separate envelope.
- Do not use plastic bags to store samples as this will cause the sample to sweat and encourages bacterial growth.
- Samples should be transported to the laboratory for further processing as quickly as possible because as the sample deteriorates the chance of recovering Foc decreases. If available a cool box or polystyrene box with ice packs should be used to keep samples cool whilst transporting them back to the lab.
- Place adhesive tape over the cut area on the stem to cover the wound.
- All samples should be labelled clearly, trees where the samples were taken marked and accurate notes recorded:
    - Date
    - Sample number
    - Cultivar (including local names)
    - Age of plants
    - Location and GPS coordinates if possible
    - Description of where the plant is growing (plantation, farm, garden etc.)
    - Number of other infected plants
    - Other information about the location is useful (near a busy road, amount of foot traffic through the site etc.)
    - Collector’s name

- Any other useful information or observations (other plants growing in the location, soil conditions etc.)

Figure 4. Field sampling and dissection of vascular tissue from the pseudostem of an infected banana plant: A: Banana showing external yellowing symptoms **B:** Section being removed from the pseudostem **C:** Disinfecting rubber boots after samples have been taken **D:** Section of vascular tissue removed from the stem showing red-brown discolouration **E:** Sections of vascular tissue (vascular strands) removed and dry on absorbent paper towel before being transported back to the lab (Images: ©FA Garcia-Bastidas)

4.3 Isolation from plant material[6]

Once transported back to the laboratory the discoloured vascular strands should be dissected from the pseudostem sample. This should be done as soon after collection as possible or at least the same day if possible. Work should be carried out in a laminar flow cabinet ideally to reduce contamination. If not available work on a clean bench next to a Bunsen burner:

- If the vascular strands were not dissected from the tissue sample in the field surface sterilise the cut section of pseudostem by wiping or submerging in 70% alcohol and placed on an individual piece of sterile filter paper or blotting paper.
- Using aseptic technique dissect the discoloured vascular strands from the first sample and place on a sheet of sterile blotting or filter paper. Use a scalpel to dissect the strands and flame in a Bunsen burner between each section of vascular tissue.
- When the vascular strands from the first sample have been dissected cover with another piece of sterile blotting/filter paper labelled.
- Set up three sterile Petri dishes:
    - 1. Containing 3% solution of sodium hypochlorite (NaOCl)
    - 2. Sterile distilled water
    - 3. Sterile filter paper

Note: Lab grade NaOCl usually contains 12-13% available chlorine and 30ml diluted in 70ml water would give a concentration of approximately 3%. Household bleach can be used but usually contains a much lower concentration of chlorine (% is usually on the label) so this will need to be taken into account when making your solution.

- Place the vascular strands from sample 1 in the 3% sodium hypochlorite for 1 min to surface sterilise (Figure 5).
- After 1 min with sterile forceps flamed in alcohol place the vascular strands in the sterile distilled water for 1 min to remove the sodium hypochlorite then flaming the forceps again place the strand in the Petri dish with the sterile filter paper.
- With a sterile scalpel cut the strands into sections 3-5 mm long and leave on the filter paper for a few minutes until dry (Figure 5).
- Once dry, with sterile forceps place 5 or 6 sections of tissue onto a plate of isolation medium. Flame the forceps between placing each piece on the agar surface and pressing down gently. 2% Tap Water Agar (TWA) or ¼ Potato Dextrose Agar (PDA) can be used for this purpose (recipes in Annex I). Antibiotics can also be added to growth media to reduce the chance of bacterial contamination (Annex I).
- Place the lid on the Petri dish and invert so the lid is on the bottom. Repeat the process for each sample another 4 time this will result in 5 replicate isolation plates for each sample.
- Incubate the plates at 25-28ºC for 3-5 days, observing each day. Fungal growth should start to emerge from the vascular tissue samples after 2-5 days (Figure 5).
- To subculture the emerging fungal growth from one of the samples cut a small block of agar at the edge of the fungal growth and place it surface down on a culture medium suitable for Fusarium growth such as SNA, PSA or PDA (see Annex II for recipes). Invert the plates and incubate at 25-28ºC until the fungus begins to grow.
- If the isolation plates are heavily contaminated with bacteria the fungi may not grow. If this is the case, repeat the isolation procedure when the vascular strands are drier or add antibiotics to the isolation medium.

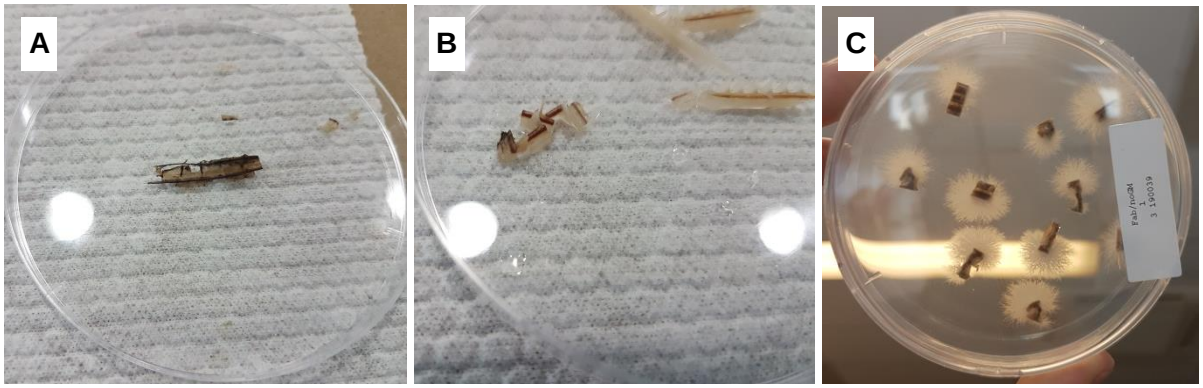


Figure 5. Preparation of vascular tissue for fungal isolation. A: Section a vascular strand. **B**: section of vascular tissue cut into smaller segments after surface sterilisation. **C:** Foc growing from small sections of vascular tissue after several days (Images: ©FA Garcia-Bastidas)

4.4 Purification of Foc

For pathological and molecular work Fusarium isolates should always be purified by single spore isolation. There are several methods available for preparing single spore cultures below is an outline of the Streaking Method[17] which is a relatively simple and quick method.

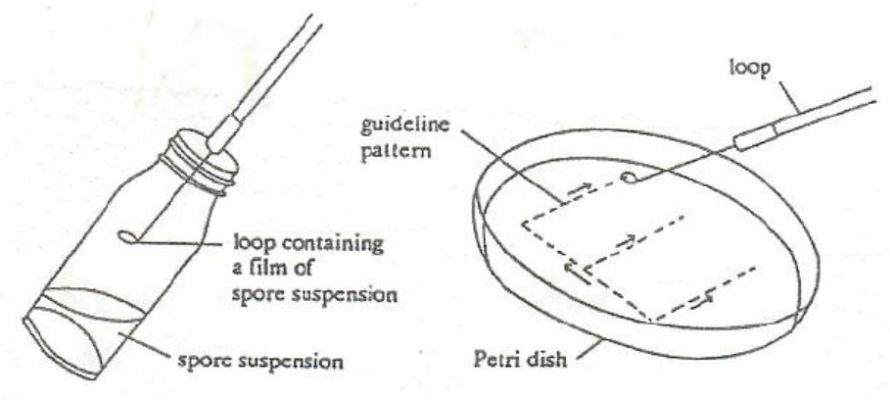
Preparation of a spore suspension

Draw the shape of an 'E' on the bottom of a thinly poured plate of 2% TWA or ¼ PDA. Take a small quantity of material from a sporulating culture with a sterile loop. Transfer the material on the loop into 5-10 ml of sterile distilled water and agitate to form a spore suspension. Alternatively Sterilise a microscope slide by passing it through the flame of a Bunsen burner, place a drop of sterile distilled water on the slide then using a sterile loop

transfer some material from a sporulating culture and mix with the water to form a suspension on the slide.

Streaking out the spore suspension

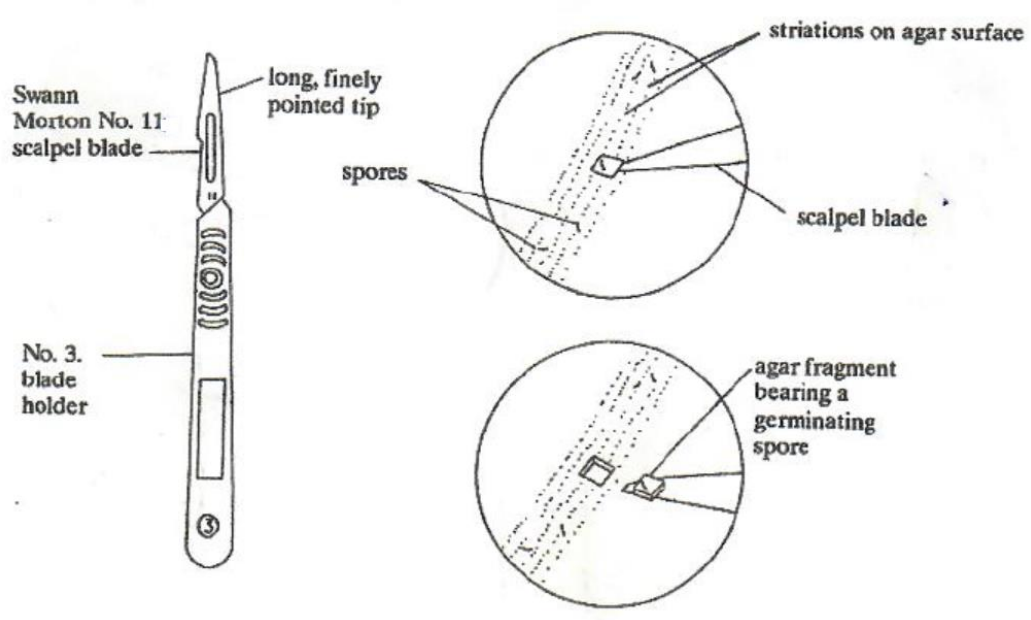
Once the spore suspension has been prepared, pick up a loopful of the suspension using a cooled sterilised loop and streak along one side of the agar plate above the longest line of the ‘E’. Re-sterilise the wire loop and make streaks from the longest line (the first streak) along each smaller line of the ‘E’. This will separate out the conidia. Incubate the plates for 18-24 h at 25^{o}C to allow the conidia to start to germinate.



Isolating the germinated spores

After incubation, examine the plates using a microscope. Where conidia have begun to germinate a germ tube can be seen to be growing, mark the location with a pen and pick of the germinating conidium with a sterile needed or cut a small agar block out of the agar with a sterile scalpel containing the germinating conidium.

The single conidium can then be transferred to a plate of suitable culture medium (such as SNA or PDA) and incubated at 25^{o}C for the isolate to grow. This process should be repeated for each isolate 4 or 5 times and transferred to a new plate of medium each time to ensure a pure single spore culture for each isolate is obtained.



If the culture is not producing spores (although Foc should produce large numbers of microconidia on agar plates) under a dissecting microscope a single hyphal tip of the fungus can be cut from the edge of a sparsely growing colony of 2% TWA or ¼ PDA and transferred to a new agar plate.

4.5 Morphological characteristics used to identify Foc

Morphological characteristics are often used as the initial method to identify fungi and many manuals and keys published to support this. Caution should however be exercised as morphological descriptions are dependent on the conditions used to grow the isolates such as media, light and temperature. When comparing morphological descriptions to those already published it's important to replicate the conditions used by the Author/s.

This is especially important with Fusaria as their morphology can be quite variable on some media even between isolates of the same species. Much of the information in this section has drawn from descriptions and illustrations produced by Leslie & Summerell (2006) in The Fusarium Laboratory Manual[19] (Figure 6) and Booth, 1970[18] (Figure 7).

Fungi are often identified based on their conidia (spores) and how they are produced. Foc produces three types of asexual spores: macroconidia, microconidia and chlamydospores (thick-walled resting spores that can survive in soil and tissue for longer time periods).

Conidia are produced on specialised hyphal strands or 'stalks' known as conidiophores. The part of the conidiophore at the top of the 'stalk' that produces the conidium is known as the phialide (Figure 7).

Colony morphology

Mycelium colour white to pale orange but usually with a purple tinge. Mycelium texture can be sparse or abundant then floccose becoming felted and sometimes wrinkled in older cultures.

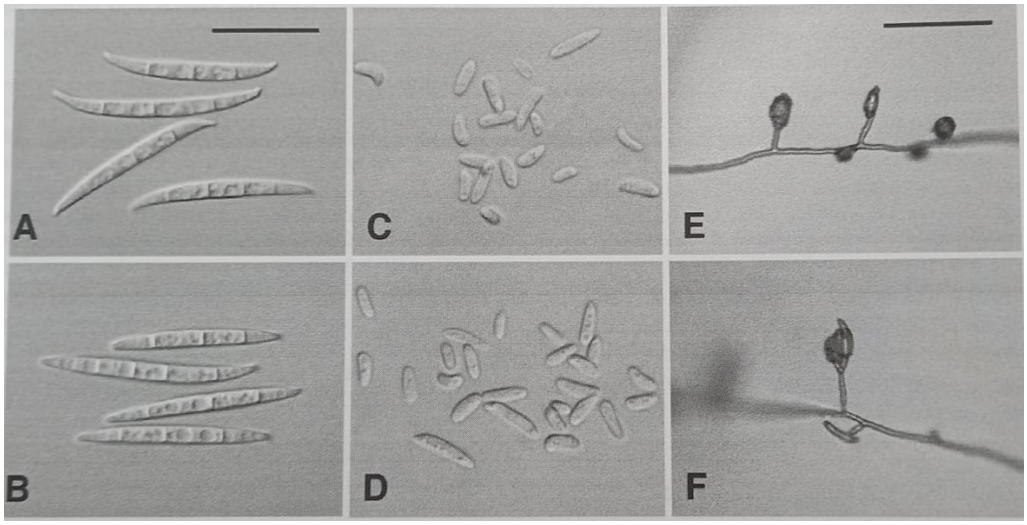


Figure 6. *F. oxysporum* A-B macroconidia; C-D microconidia; E-F microconidia grown on carnation leaf agar; A-D scale bar 25µm; E-F scale bar 50 µm[19]

Macroconidia (large)

- Production can be sparse in some strains
- Borne on branched conidiophores or on the surface of sporodochia (small compact mass of hyphae)
- Thin walled, 3-5 septate (dividing cell walls)
- 3 septate, 27-46 x 3-4.5 µm; 5 septate, 35-60 x 3-5 µm

- Fusoid-subulate and pointed at both ends. Hooked apex and a pedicellate (foot shaped) base

Microconidia (small)

- Abundant and variable in shape and size
- Borne on simple phialides arising laterally on the hypae or from short sparsely branched conidiophores
- Oval or ellipsoid, straight to kidney shaped, with no septa (dividing cell walls)
- 5-12 x 2.2-3.5 µm
- Microconidia are ovoid (oval shaped) and can be slightly curved. They are produced in slimy droplets on short conidiogenous cells (monophialides)

Chlamydospores

- Generally abundant
- Smooth and rough walled
- Forming both terminally (at the end of hyphae) and intercalary (along the length of the hyphae)
- Usually solitary but occasionally formed in pairs or in chains

A key for identifying the most common Fusarium species of most economic importance has been developed by CABI can be found in Annex III.

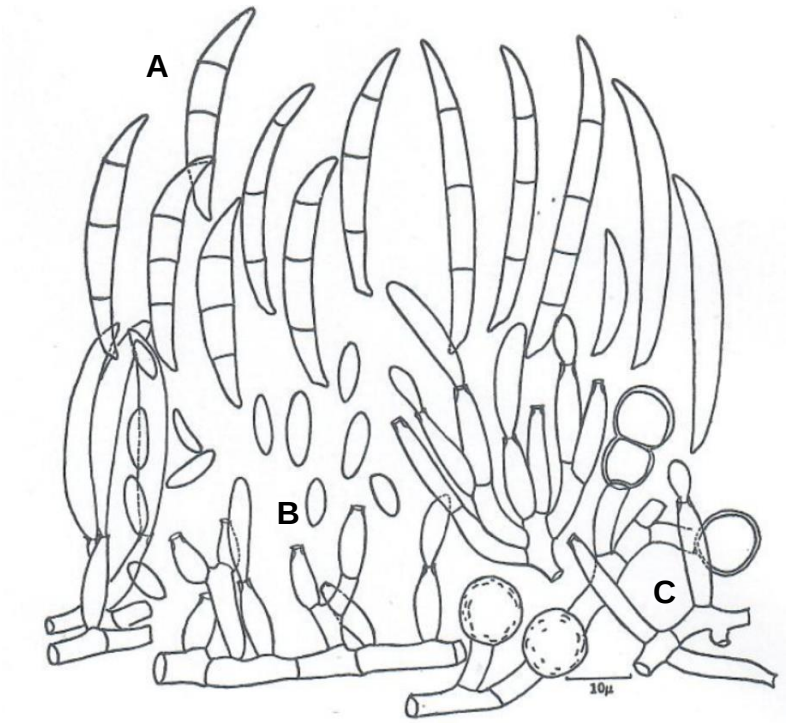


Figure 7. Different types of spores produced asexually by *F. oxysporum*. A: macroconidia; B: microconidia; C: chlamydospores; (Illustration from Booth, 1971[20])

4.6 Storage and preservation of Foc cultures

When kept on nutrient rich culture media such as PDA and PSA cultures of Foc can degenerate quickly (a few days in some cases). When this occurs Foc cultures become flat and slimy (pionnotal) and should be discarded as the condition cannot be reversed. Healthy cultures of Foc should have fluffy aerial mycelium. To avoid degeneration, cultures of Foc should be ideally be maintained on carnation leaf agar (CLA). However, not every laboratory has the facilities to produce CLA and in that case SNA + filter paper is a good, inexpensive

alternative. PDA and PSA can be used to observe certain cultural characteristics of Foc but must not be used to maintain or store Foc even in the short-term.

Cultures can be kept on slopes of SNA or CLA in universal bottles for short-term storage. For long-term storage other preservation techniques need to be considered such as freeze-drying (lyophilisation) or storage in liquid nitrogen but these require specialist equipment. More cost-effective ways to store Fusarium cultures have been tried with some success such as on pieces of sterile filter paper, refrigerated in vials of sterile silica gel or inoculated into sterile soil. The use of mineral oil or serial transfer on culture medium will cause degeneration and is not advised.

4.7 Molecular diagnosis

To confirm an identification of Foc TR4 a number of steps have to be carried out the first of which is a DNA extraction. This can be performed directly on the sampled plant material or the isolated, purified fungal culture. DNA extraction from isolated, purified fungal culture is preferred as the resulting DNA is of better quality. When a rapid diagnosis is required DNA extraction from plant material can be carried out much more quickly. To determine whether or not DNA from Foc TR4 is present in the sample primers for several molecular markers have been developed using techniques such as PCR, qPCR and LAMP. Its recommended using two sets of independent markers when identifying Foc TR4 to avoid false positives.

DNA extraction

As noted above DNA can be extracted directly from the plant material sample or the fungal isolate itself. Many commercial kits are available to perform DNA extractions e.g., Quiagen DNeasy, Wizard Magnetic DNA Purification System (Promega) and ClearDetections. A DNA extraction method published by FA García-Bastidas et al.[6] can be found in Annex VI.

Markers for molecular identification

PCR

- FocR4T developed by Dita et al. (2010)[21]
- W2987 developed by Li et al. (2013)[22]
- SIX genes developed by Carvalhais et al. (2019)[23]

qPCR

- FWB-TR4 Aguayo et al. (2017)[24]
- ClearDetections comercial kit

LAMP assay

- Developed by Ordóñez et al. (2019)[25]

4.8 Preparing samples to send to a diagnostic laboratory

Plant samples, fungal cultures or DNA can be sent to a diagnostic laboratory for confirmation of FocTR4 depending on the facilities locally available to prepare the samples and those in the receiving laboratory. It is important to contact the laboratory identified to receive the samples before the samples are collected as the laboratory will need time to prepare for their arrival. The laboratory may have specific requests on the number of samples to be sent and how the samples are collected. If the laboratory is an international laboratory, it is likely that they will have to provide documents to be completed by the exporting country that need to be included in the shipment i.e. submission forms, import permits and CBD declaration for access and benefit-sharing obligations of the Convention on Biological Diversity (CBD).

Sending plant samples

- Samples should be shipped as soon as possible after collection to maintain the integrity of the sample.
- Ensure that you collect and send sufficient material that clearly exhibits the symptoms that you are concerned about so there is sufficient material to work with
- Try to collect plant material that shows early to middle stages of symptom development and not material that has severe symptoms or is badly damaged or dead.
- Samples should show the area between healthy and unhealthy plant tissues, as this is usually where plant pathogens are present and most active.
- Fresh samples (collected the same day) offer the best opportunity to isolate and identify pests and diseases. Most fungi can tolerate the drying process well. Samples should be air dried for up to several days depending upon their size and water content. **Do not** dry samples in an oven.
- Wrap the samples in paper and cardboard, never place them in plastic as this will promote bacterial growth and speed up the decay process. Exceptions include when nematodes are suspected to be present in the material.
- Individual samples should be wrapped separately and, where appropriate, each sample labelled with the sender's own reference number for the sample.
- Pack all samples carefully in an outer crush-proof container such as a strong cardboard box. Place additional paper, cardboard or polystyrene granules around the samples to cushion them against damage during shipping.
- Avoid thin absorbent paper like tissues as these disintegrate, become damp and become difficult to remove.
- Clearly mark the outside of the package(s) with ‘Perishable Biological Material. Keep in Shade. Keep material cool but do not refrigerate’.

Sending fungal cultures

- Cultures should be pure, containing one organism only.
- Fungal cultures can be sent in Petri dishes or on agar slopes in bottles or test tubes.
- In the case of fungi, send established cultures.
- Ensure petri dishes are sealed with tape.
- Fit test tubes with a sterile cotton-wool bung or use tubes with screw-tops.
- Label each isolate with a reference number or code.
- Place each sample in a separate paper enveloped labelled with your own reference number.
- Do not use plastic bags for sending samples as these can cause condensation and result in spoilage.
- Send samples in a crush-proof box to avoid damage in transit.
- Retain a representative collection of each sample as material is not normally returned.

It is also useful to send photographs with any request as these are useful to when making a diagnosis.

5. Other useful resources

CABI TR4 Portal
www.cabi.org/isc/tr4

FAO Technical Manual Prevention and diagnostic of Fusarium Wilt (Panama disease) of banana caused by Fusarium oxysporum f. sp. cubense Tropical Race 4 (TR4)
https://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/caribbeantr4/13ManualFusarium.pdf

FAO's TR4 Global Network (TR4GN)
https://www.fao.org/tr4gn/en

Guía Andina Para el Diagnóstico de Fusarium Raza 4 Tropical (TR4) Fusarium oxysporum f. sp. Cúbense (syn. Fusarium odoratissimum) Agente Causalde la Marchitez por Fusarium en Musáceas (Plátanos y Bananos)
https://bit.ly/3mpTYYl

IPPC, Prevention, preparedness and response guidelines for Fusarium Tropical Race 4 (TR4) of banana
https://www.ippc.int/en/publications/92050/

IPPC, Workshop Series: Fusarium TR4 Diagnostic, Surveillance, Inspection and Simulation Exercises
https://www.ippc.int/en/news/workshops-events/webinars/workshop-series-fusarium-tr4-diagnostic-surveillance-inspection-and-simulation-exercise/

Isolation of Fusarium Tropical Race 4 from plant tissue
www.youtube.com/watch?v=XnK03qXvJfs&t=149s&ab_channel=ferchuckygarcia

Masterclass on Fusarium wilt of banana (Promusa)
www.youtube.com/channel/UCsxm3SYT-7ZGOJYx5N9Ahww

Panama disease (Wageningen UR)
www.youtube.com/playlist?list=PLXv9pwN0ObKUwfKGATz6MGQT6ivNCbhK3

Panama tropical race 4 (Biosecurity Queensland Government)
www.youtube.com/playlist?list=PLpiCDHV-IjhHhQEteJfg5xpOJDkvOZEpu

6. Annexes

Annex I. Media for isolation of Foc

Low nutrient medium is preferred for isolating fungal pathogens from plant material. The fungi emerging from the pieces of plant tissue grow more sparsely than on nutrient rich media. Low nutrient media also restricts the growth of other saprophytic fungi allowing the pathogen to be isolated more easily.

Tap Water Agar (TWA)

Low nutrient medium good for making isolations from plant material and culture purification through single sporing.

20g agar
1 litre tap water
Dissolve agar in water. Autoclave at 121^{o}C for 20 mins. Mix well before pouring plates.

¼ Strength Potato Dextrose Agar (¼ PDA)

¼ PDA is a low nutrient medium which can be homemade from common available ingredients or bought as a pre-prepared commercial product and amended with additional agar for lower strength. Many recipes for PDA are available.

125ml potato extract*
5g glucose
20g agar
875ml distilled water

Mix the water and potato extract* together then add glucose and agar. The mixture is heated slowly until the agar is dissolved and the pH adjusted if necessary to 6.5 with calcium carbonate. Dispense into bottles and autoclaved at 121^{o}C for 20 mins.

* Potato extract is prepared from 600g of mature main crop potatoes (not new potatoes) peeled, diced and placed in muslin in 1.5 litres of water and boiled for 10 min. The potatoes are then discarded, and the liquor placed in large glass containers and autoclaved at 121^{o}C for 20 min. It can be stored in a refrigerator for use as required.

Antibiotics

TWA and ¼ PDA can be supplemented with antibiotics to reduce bacterial contamination during isolation. The broad-spectrum antibiotic streptomycin sulphate is commonly used at a final concentration of 100 mg/L or 100 ppm. To make a stock add 1g streptomycin sulphate to 100 ml of sterile distilled water. The antibiotic solution can be made in smaller volumes and sterilised using a syringe filter disc. When the media is below 50^{o}C or conformable to hold in your hand all 1ml of stock solution for each 100 ml of media. Mix well and pour the plates.

Annex II. Growth media for identification of Foc

It is rarely possible to reliably identify Fusaria to species level directly from isolation plates as colony and spore morphologies are not typical. Also, several Fusarium species may be present in the same host tissue. Cultures should therefore be purified by sub-culturing onto fresh agar plates.

Several different types of media can be used for growing Fusaria cultures but their culture characteristics and morphology can vary greatly depending on the media used. When using an identification manual or key it is important to use the same media that is specified in the manual being used[19]. Standard recipes may also vary so it is important to use the recipe referred to in the specific manual or key. Below are several media used for maintaining and identifying Fusaria species.

Spezieller Nahrstoffarmer Agar (SNA)

A nutrient weak medium for general growth and maintenance of isolates and to aid identification. A transparent medium induces good sporulation and regular spore shape

1.0g Potassium dihydrogen phosphate (KH_2PO_4)
1.0g Potassium nitrate (KNO_3)
0.5g Magnesium sulphate ($MgSO_4.7H_2O$)
0.5g Potassium chloride (KCl)
0.2g Glucose
0.2g Sucrose
20g Agar
1 litre distilled water

Autoclave at 121^{o}C for 20 mins
One or two pieces of sterile filter paper (approximately 1 cm^2) can be placed onto the agar surface in order to enhance sporulation.

Potato Dextrose Agar (PDA)

A nutrient rich medium primarily used for isolate identification, excellent for inducing pigment production by isolates. Commercially prepared PDA media can be purchased and offer more consistent results.

500ml potato extract*
20g glucose
20g agar
500ml distilled water

Mix the water and potato extract* together then add glucose and agar. The mixture is heated slowly until the agar is dissolved and the pH adjusted if necessary to 6.5 with calcium carbonate. Dispense into bottles and autoclaved at 121^{o}C for 20 mins.

* Potato extract is prepared from 1.8kg of mature main crop potatoes (not new potatoes) peeled, diced and placed in muslin in 4.5 litres of water and boiled for 10 min. The potatoes are then discarded, and the liquor placed in large glass containers and autoclaved at 121^{o}C for 20 min. It can be stored in a refrigerator for use as required.

Potato Sucrose Agar (PSA)

500ml potato extract*
20g sucrose (for PSA) or glucose (for PDA)

20g agar
500ml distilled water

The water and potato extract* are mixed together and the sucrose/glucose and agar added. The mixture is heated slowly until the agar is dissolved and the pH adjusted if necessary to 6.5 with calcium carbonate. It is then dispensed into suitable bottles and autoclaved at 121^{o}C for 20 mins.

* Potato extract is prepared from 1.8 kg of mature main crop potatoes peeled, diced and suspended in muslin in 4.5 litres of water and boiled for 10 min. The potatoes are then discarded and the liquor placed in large glass containers and autoclaved at 121^{o}C for 20 min. It can be stored in a refrigerator until needed.

Carnation Leaf Agar (CLA)

Healthy carnation (*Dianthus caryophyllus* L.) leaves, free from fungicide or insecticide residues, are cut into 5 mm pieces immediately after collection and then dried at ca. 70^{o}C for 2hr.

Leaves are then sterilized using gamma irradiation (2.5 Mega Rads). Sterile leaf pieces are then placed aseptically into Petri dishes and molten 2% TWA added. About one leaf piece per 2ml of agar is adequate.

Annex III. Teaching key to common species of *Fusarium*

(Cultures grown on SNA and PSA for 10d at 25C in a 12h light/dark cycle)

		Go to:
1	Microconidia abundant.	2
1	Microconidia sparse or absent.	12
2	Microconidia formed from elongated (>50µm) conidiogenous cells (visible at low power on SNA as Mucor-like structures); collecting in slimy droplets.	3
2	Microconidia formed on shorter conidiogenous cells; in slimy drops, chains or dry heads.	4
3	Colonies on PSA with white/grey aerial mycelium, colony reverse white or beige, sometimes with steel blue rings, sometimes with brick-red diffusing pigment. Macroconidia in buff sporodochia, having Fusarium foot cell. Hyaline chlamydospores formed in mature cultures.	***F. solani***
3	Colonies on PSA with white or brown aerial mycelium, colony reverse becoming brown. Macroconidia with rounded apex and lacking oblique foot cell. Chlamydospores (if present) brown.	cf. ***Cylindrocarpon spp***
4	Microconidia formed in chains (visible by viewing SNA plate directly at low power).	5
4	Microconidia not in chains.	7
5	Colonies on PSA strongly carmine red; macroconidia thick walled, 5-10 septate, 60-100 x 43-8µm, forming in creamy yellow sporodochia. Usually on woody hosts in the tropics.	***F. decemcellulare***
5	Colonies on PSA white or pink with reverse the same colour or violet (not carmine red); macroconidia (if present) thin walled, 3-7 septate, 30-60 x 3-5µm.	6
6	Microconidia forming long, fragile chains, arising from cells with a single conidiogenous locus (mono-phialides).	***F. moniliforme***
6	Microconidia forming short chains, arising from cells with one or several conidiogenous loci (polyphialides).	***F. proliferatum***
7	Microconidia forming in dry heads in the aerial mycelium.	8
7	Microconidia forming in slimy droplets, not dry heads.	9
8	Microconidia uniform, clavate, 0-1 septate, 2.5-4µm wide, with rounded apex and pointed base. Usually from warm climates.	***F. chlamydosporum (= F. fusarioides)***
8	Microconidia of 2 shapes: pyriform, 5-6.5µm wide and fusoid, 3-4µm wide. Usually from cool climates.	***F. sporotrichioides***
9	Colonies on PSA vinaceous and with pyriform/globose microconidia present.	10
9	Colonies on PSA with violet pigment or non-pigmented; microconidia ovoid or ellipsoid.	11
10	Microconidia globose/subglobose, mostly 0-septate, arising from short, fat phialides (8-18 x 3-6µm). Cultures on PSA with strong, fruity odour.	***F. poae***
10	Microconidia pyriform and fusoid, 0-1 septate, arising from phialides 10-30 x 1.5-3µm. No odour on PSA.	***F. tricinctum***
11	Microconidia arising from simple, short, lateral mono-phialides. Chlamydospores present in mature cultures.	***F. oxysporum***
11	Microconidia arising from both mono- and polyphialides. Chlamydospores not present.	***F. sacchari***
12	Dry macroconidia present in aerial mycelium on SNA (seen by viewing plate directly at low	13
12	Macroconidia borne in slimy aggregates or sporodochia, not dry	14
13	Cultures on PSA with vinaceous red pigment, aerial mycelium also becoming red with age. Orange, slimy sporodochia forming on agar surface. Spores from sporodochia slender, filiform, thin-walled. Dry spores from polyblastic cells fusiform, lacking oblique foot cell.	***F. avenaceum***
13	Cultures on PSA with pink, floccose aerial mycelium, reverse similarly coloured. Many strains darkening to brown at maturity. Sporodochia sparse or absent. Macroconidia of 2 types: dry spores (from polyblastic conidiogenous cells) with conical basal cell; slimy spores with oblique foot cell, arising from phialides.	***F. pallidoroseum (= F. semitectum)***
14	Colonies on PSA white, pink beige, brown, or orange, (not vinaceous or carmine red)	15

14	Colonies on PSA with vinaceous or carmine red pigment (degenerated strains may lose their pigmentation)	17
15	Colonies on PSA slow growing (2-4 cm after 10d) with lobed margin. Colony reverse pink, beige or brown, sometimes with orange or yellow diffusing pigment. Orange sporodochia abundant. Macroconidia mainly 3-5 septate, straight or slightly curved, fusiform to cylindrical, not markedly swollen in their middle, apical cells often hooked or beaked, not elongated. Chlamydospores sparse or absent. Usually on woody hosts.	***F. lateritium***
15	Colonies on PSA not as above, fast growing (over 6 cm in 10d).	16
16	Colonies on PSA with abundant, floccose aerial mycelium, pink often becoming beige/brown, reverse the same colour. Macroconidia in scattered bright orange sporodochia on SNA. Spores distinctly curved with elongated apical cell, swollen middle and distinctly pedicellate foot cell. Chlamydospores common in most strains.	***F. equiseti***
16	Colonies on PSA with abundant, floccose aerial mycelium, pink, reverse the same colour, sometimes producing yellow pigment. Macroconidia in pale orange sporodochia on SNA; spores thick-walled, fusiform, not markedly swollen in their middle; apical cell papillate, not elongated; foot cell not having exaggerated "toe".	***F. sambucinum*** (non-pigmented strains)
17	Macroconidia from sporodochia on SNA distinctly curved/falcate, central cells swollen, apical cell elongated and foot cell markedly pedicellate ("Gibbosum" shape).	18
17	Macroconidia from sporodochia on SNA with conical, hooked or papillate apical cell, not extended into an elongated point. Spores fusiform, straight or slightly curved, foot cell pedicellate or wedge shaped.	20
18	Macroconidia formed in large (5mm) bright orange sporodochia on SNA. Spores with elongated, whip-like apical and foot cells; apical cell as long as the main part of the spore.	***F. longipes***
18	Apical cell shorter than main part of spore.	19
19	Sporodochia on SNA pale orange, macroconidia falcate, widest near the middle and tapering evenly towards each end. Spores 3.5-4µm wide.	***F. acuminatum***
19	Sporodochia on SNA bright orange, macroconidia swollen, often widest in upper third of spore, giving hump-backed appearance. Spores 4-5µm wide	***F. compactum***
20	Colonies on PSA with felted, white or pink aerial mycelium; slow growing (3-5 cm in 10d) with carmine red pigment below. Sporulation initially in aerial mycelium, giving colonies a powdery appearance; small, scattered sporodochia later formed on agar surface. Macroconidia mostly 5-7 septate, thin-walled, fusiform, straight or slightly curved, 65-90 x 4-4.5µm, with conical pointed or beaked apical cell. Causes bark disease of *Coffea* in Africa.	***F. stilboides***
20	Colonies on PSA fast growing (>6cm in 10d), with vinaceous pigment on PSA.	21
21	Brown sporodochia formed on SNA. Macroconidia thick-walled, curved, with distinctly pedicellate foot cell and conical, pointed, apical cell.	***F. crookwellense***
21	Orange sporodochia formed on SNA.	22
22	Macroconidia from sporodochia on SNA with very distinctly pedicellate foot cell. Macroconidia with almost straight ventral surface and slightly curved dorsal surface, 5-septate, 3.5-5µm wide. Apical cell pointed, gradually tapering.	***F. graminearum***
22	Macroconidia arising from sporodochia on SNA having indistinctly pedicellate foot cell, spores curved.	23
23	Macroconidia from sporodochia on SNA 3-5 septate, with strongly curved dorsal surface, stout (5-7µm wide). Apical cell short, abruptly pointed. Basal cell ~shaped, lacking a distinct "toe".	***F. culmorum***
23	Macroconidia from sporodochia on SNA thick-walled, fusiform, 3-5 septate, 4-6µm wide; apical cell papillate. Typically isolated from woody plants	***F. sambucinum*** (pigmented strains)

Annex IV. DNA extraction from banana plant material and *Fusarium*.

From Guía Andina Para El Diagnóstico De Fusarium Raza 4 Tropical (R4T)[6] (Standardised by F.A. Garcia-Bastidas 2013 - Adapted from Bernatzky and Tanksley, 1990)

Materials:

- Mortar and pestle, liquid nitrogen (equipment for lyophilized, zirconium beads)
- Microcentrifuge tubes
- Water bath or hot plate
- 1000, 200 and 10 µl pipettes and tips
- Racks, markers, gloves

Plant material or fungal mycelium/spores:

1. Place at least 100 mg of material into a 2 ml tube
2. Add liquid nitrogen to the tube and macerate with a pestle (freeze-drying or any other lysis procedure is acceptable
3. Immediately add 320 µl of sorbitol extraction buffer[1] + 100 µl of sarcosine buffer[2] + 320µl of CTAB nuclear lysis buffer[3] shake for a few minutes and spin in the centrifuge
4. Preheat the water bath to 65°C and immerse the tubes in racks for one hour (30 minutes minimum) inverting every 15 minutes. Cool before continuing with the next step.
5. In a gas extraction chamber add chloroform:isoamyl alcohol (24:1) in proportion 1:1 to the homogenate, approximately 620µl (from now on it is very important that the tubes are properly labelled, the reagents used from now on can easily erase the ink.
6. Centrifuge for 10 to 15 minutes at ~13000 rpm.
7. Take the aqueous phase (620 µl approx.), transfer it to a new 1.5 mL tube and wash again with 1 volume of chloroform:isoamyl alcohol. Invert to mix.
8. Centrifuge at maximum speed for 20 minutes. Take the aqueous phase and transfer it to a new tube.
9. Add 1 volume of isopropanol in relation 1:1 and 50µl of 5M potassium acetate, pH 5.5 (optional). Invert once gently.
10. Incubate for 5-30 minutes at -20°C to -30°C. Observe whether strands of DNA are formed, 1 hr 20 mins should be sufficient time (Do not exceed the time limit to avoid DNA degradation).
11. Mix by inversion and centrifuge at full speed for 15 minutes.
12. Discard the isopropanol, then perform a wash with absolute ethanol and a wash with 500 µl 70% ethanol with. Centrifuge at maximum speed for 6-10 minutes between each washing, detaching the pellet from the tube before spinning.
13. Let the pellet dry overnight or as long as necessary to evaporate the ethanol residues. Alternatively, use of a SpeedVac is recommended.
14. Resuspend DNA in 100-200 µl of PCR type sterile water or TE[4] Buffer according to the pellet size (the larger the size, the greater the amount of water) and 2-3 µl of RNase (4 mg/ml). Incubate for 30 minutes at 37°C +/- 0.5 °C.
15. Store the extracted DNA in a freezer -20°C to -30°C.
16. To guarantee the quality of the DNA obtained, it should be quantified. Alternatively DNA integrity can be checked on a 0.8% agarose gel 55 V constant for 90-120 minutes.

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[1] 350 mM of Sorbitol (63.77g/1L); 100 mM de Trizma base (12.10g/1L); 5 mM of EDTA (1.86 g/1L); 0.2% of 2-mercaptoethanol (2mL/1L). Prepare 1L of ddH2O and adjust the pH to 8.2. Autoclaving is not required, keep at room temperature and refrigerate at 4°C prior to using.

[2] N-Lauroyl Sarcosine 5%.

[3] 55 mM CTAB (20 g/1L), 200 mM Trizma base (24.22 g/1L), 50 mM EDTA (18.61 g/1L), 2 M NaCl (116.88 g/1L). prepare 1L with ddH2O and adjust to pH 7.5. Not required to be autoclaved, keep at room temperature.

[4] 10 mM Trizma base (0.1211 g/100 mL); 1 mM EDTA (0.0372 g/100mL); pH 8.0. Autoclave and store at room temperature.

7. References

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