THE INTERNATIONAL PLANT DIAGNOSTIC NETWORK

International Plant Diagnostic Network

Standard Operating Procedure for Plant Diagnostic Laboratories

Banana Fusarium wilt/Panama disease

Fusarium oxysporum f. sp. *cubense*

Table of Contents

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1.0 **Background: Disease and Pathogen**

Fusarium wilt, a lethal fungal disease of banana (Musa spp.) is caused by *Fusarium oxysporum* f. sp. *cubense* (E.F. Smith) Snyder & Hansen (1945). It was first reported from Panama as early as 1890 and thus has been commonly known as Panama disease (Stover, 1962). The disease probably originated in Southeast Asia, but was first reported in Australia in 1876 (Ploetz and Pegg, 2000). It is found in all banana-producing regions except the Mediterranean, Melanesia, Philippines, Somalia, and some islands in the South Pacific (Ploetz, 2005) ((Figure 1). In Africa Foc has been confirmed to occur in Burundi, Cameroon, DR.Congo, Ghana, Guinea, Kenya, Madagascar, Malawi, Nigeria, Rwanda, Sierra Leone, South Africa, Tanzania and Uganda Fusarium wilt is a highly destructive disease that has, and continues to, cause major losses in many parts of Africa (see below and Figure 1). Recent surveys undertaken in Kenya have shown that incidence of Fusarium wilt in some areas is as high as 80%, due to extensive cultivation of the highly susceptible clone Gros Michel. It is also a major concern to bananas and plantains, which are essential to the nutritional and economic well being of millions of people throughout the developing world.

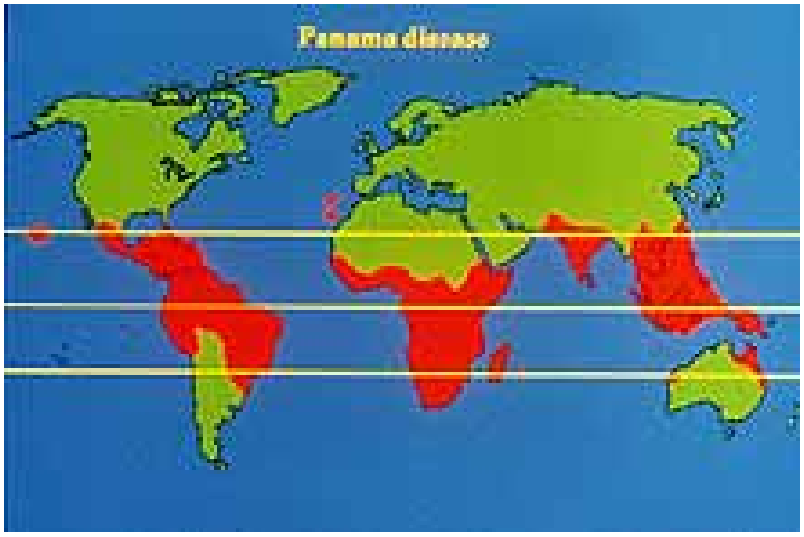

Figure 1.Geographical distribution of Panama disease (Courtesy of Randy Ploetz)

The causal pathogen attacks the banana's vascular system. As diseased plants die, the fungus grows out of the xylem into surrounding tissues. Many chlamydospores are formed which are returned to the soil as the plant decays. Chlamydospores are stimulated to germinate and infect nearby banana roots. Following germination, mycelium is produced from which conidia form in 6-8 hours and chlamydospores in 2-3 days. Small secondary or tertiary roots are invaded. No banana cultivars are immune and the fungus is able to establish itself in the vascular system of the root. In susceptible plants, the fungus is not blocked by the host defence mechanisms and the infection becomes systemic through the vascular system of the corm, pseudostem and fruit stalk. In resistant cultivars, the fungus becomes blocked by vascular occluding responses of the host and cannot advance into the corm.

1.1 *Fusarium. oxysporum* f.sp. *cubense Races*

Race has been used to classify strains of F. *oxysporum* f.sp. *cubense* since the mid-1900s (Stover, 1962). The 'race' system of distinguishing the different pathotypes of *F. oxysporum* f. sp. *cubense* has proved limiting as exceptions are known, which indicate that more races may exist. Extensive inoculation studies utilizing many isolates of the pathogen and very many different banana clones would be necessary to obtain the data necessary to clearly define the various pathotypes that exist (Ploetz and Pegg, 2000). Although it is an imperfect measure of pathogenic diversity in this complex, it does, nonetheless, provide useful information. Races *F. oxysporum* f. sp. *cubense* are not defined genetically, but are groups of isolates that attack differential cultivars in the field. *F. oxysporum* f. sp. *cubense* strains have been classified into four physiological races based on pathogenicity to host cultivars in the field, that is, race 1, race 2, race 3, and race 4 (Persley and De Lanhe, 1987).

- Race 1 attacks Gros Michel and 'Lady Finger' (AAB). Also affected by race 1 are abacá, 'Maqueño', 'Silk' (AAB), 'Pome', and 'Pisang Awak'.
- Race 2 affects the hybrid triploid Bluggoe (ABB), some bred tetraploids and enset;
- Race 3 attacks *Heliconia* spp. and is non-pathogenic or weakly pathogenic on Musa spp.
- Race 4 is capable of attacking 'Cavendish' (AAA) as well as other varieties of banana affected by races 1 and 2. Race 4 is further divided into 'sub-tropical' and 'tropical' strains. 'Tropical' race 4 is a more virulent form of the pathogen and is capable of causing disease in 'Cavendish' growing under any conditions, whereas 'subtropical' race 4 generally only causes disease in plants growing sub-optimally (cool temperatures, water stress, poor soil). Race 4 is still restricted to Asia and northern Australia, but has caused grave concern in the western Cavendish-dependent export trades and wherever plantains are important local foods.

2.0 Identification of *Foc*

2.1.1 General Identification Overview

The hyphomycete genus Fusarium (Subdivision Deuteromycotina) comprises a number of species that are plant pathogens, parasites and saprophytes; more recently some species have been reported as emerging human pathogens in immunocompromised individuals (Boutati and Anaissie, 1997). *Fusarium oxysporum* (*Fo*), the most common species of the genus, is a soil-borne pathogen with a ubiquitous, worldwide distribution.

Classification

Domain: Eukaryota; Kingdom: Fungi; Phylum: Ascomycota; Class: Ascomycetes; Subclass: Sordariomycetidae; Order: Hypocreales.

2.1.2 Key Contacts for *Foc* identification

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NOTE: Samples should first be sent to your local county extension service or NPDN laboratory. Contact the individual receiving the sample prior to shipment of out-of-country material.

3.0 Protocols in the Field

3.1 Field Sampling and Shipping:

3.1.1 Field Sampling for *Foc* Analysis

a) Dissect discoloured vascular strands from sample

b) Ideally, the discoloured vascular strands should be dissected from the sample on the same day that it is collected, or as soon as possible after collection. Samples should be taken from 3-4 randomly selected plants in an infected field site.

c) Cut out the section of tissue from low part of pseudostem where many discolored strands are present.

d) Dissect out 5-8 individual discolored vascular strands and place between sterile blotting paper to air dry. The use of sterile blotting papers is recommended and aseptic technique should be applied to the dissection of strands. Samples should first be surface-sterilised by wiping with 70% alcohol or surgical spirit.

e) When dry, place the blotting paper and strands in a paper envelope (never a plastic bag), seal and label with the sample number, cultivar name, locality, GPS co-ordinates, collector's name and date.

N.B Where several samples are being prepared, a fresh piece of blotting paper should be used for each sample, and scalpel blades should be flamed if possible or at least wiped with 70% alcohol between samples. The excised strands, with as little as possible of the adjacent tissue, should then be placed between sterile blotting papers in a paper envelope to dry naturally. A few days are usually sufficient.

Remember.

- Do not let the strands get too hot (e.g. in direct sunlight or in the boot of a car) as this may kill the fungus.
- Do not dry them in an oven! Fusarium wilt specimens do not need to be kept in the fridge - room temperature is acceptable.
- They do not need to be wrapped in moist paper like leaf specimens - dry paper is best.
- If posting the strands for isolation and analysis, please post in a paper envelope as soon as the strands are dry enough with sample numbers and details clearly written on or with each sample envelope. Please include a copy of the relevant quarantine import permit /phyto sanitary Certificate (SPS) inside the package if this is required.

Note: If there is any possibility that samples have been mixed up and the details for some samples may be incorrect, discard the samples concerned.

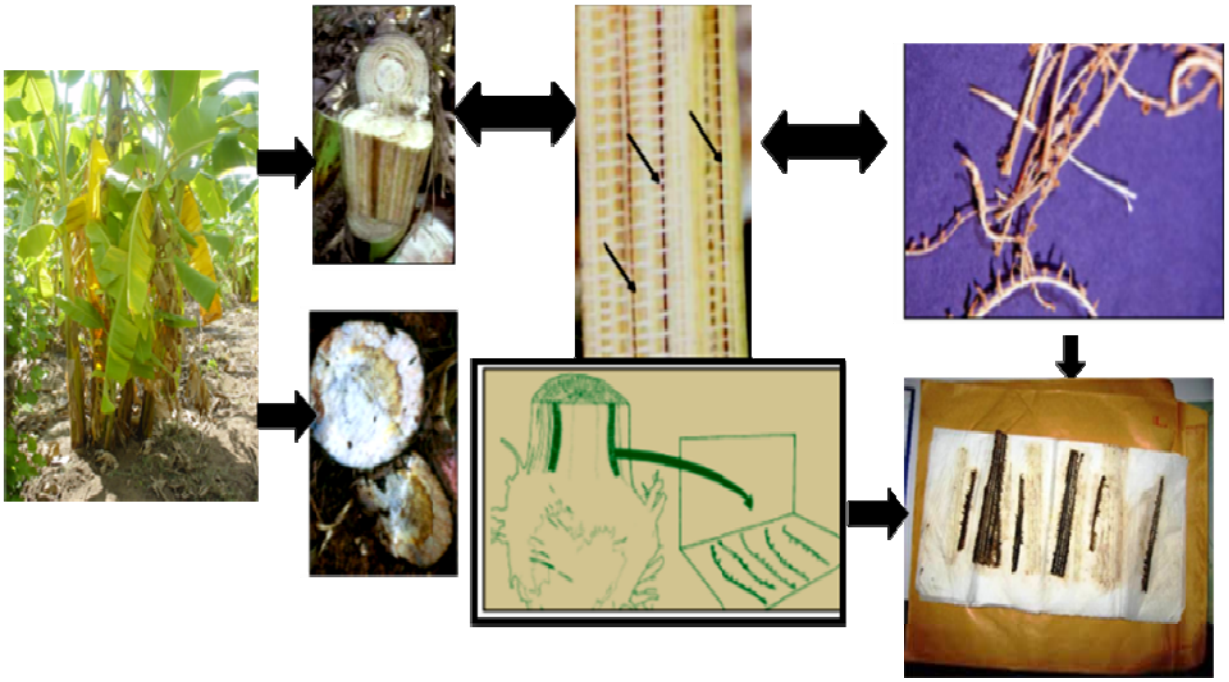


Figure 2.Sampling procedure for Fusarium Wilt (Source: IITA)

3.1.2 Symptoms typical and not typical of Panama disease

The picture below shows symptoms very typical of Panama disease. This is clearly demonstrated in the red and yellow xylem vessels with CONTINUOUS discoloration. In some instances you might find brown-red blotches inside banana pseudostems (as I indicated on the picture) that is NOT CONTINUOUS. These are caused by other factors, often insects, but also might relate to toxicities in a phenomena sometimes referred to as 'false Panama disease'. When you collect tissue to send for isolation, always try to dissect the discoloured yellow and red vessels that contain the spores.

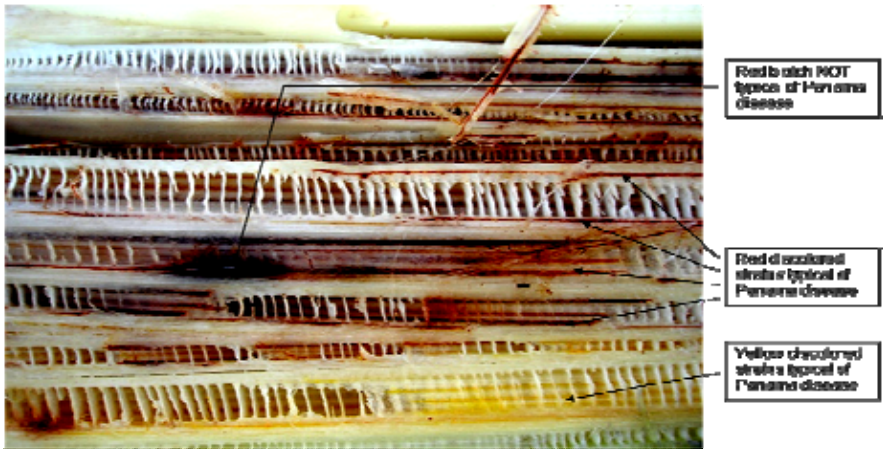


Figure 3. ABB genotypes in the Rusizi valley in Burundi showing symptoms typical and not typical to Panama disease (Source: Guy Blomme, Biodiversity)

3.2 Shipping Samples for *Foc* Analysis

Sample submission may be directly from a grower questioning the cause of symptomatic plants or from regulatory personnel that have reasons for suspecting a possible infection.

1. Suspect plant material must be placed in double ziplock bags and stored in at damp free cabin at room temperature awaiting shipment to a diagnostic facility. The preferred method for shipment is triple packaging, two ziplock bags and an outer container. Tubes and plates should be sealed with tape. Also, shatter proof containers should be used for the cultures. The outer shipping container should be an approved cardboard shipping box. The seams of the box should be closed with approved shipping tape.
2. If submitted by regulatory personnel, the inspector will label and complete the appropriate forms. The inspector should record the Country, identifier, the grower's license number (if applicable), the host(s), the inspector's initials as well as the location and date of inspection. If submitted by the Country's Department of Agriculture, please include the Department of Agriculture designation. Upon

receipt of the sample, this number will be placed in the notes section of the laboratory's database program so that it can be cross referenced with the original source

3. If there is no visible surface growth of the suspected fungus, specimen should be surface sterilized with 70% ethanol solution, rinsed in sterile water and teased out fragments laid on agar slants or plates or added to liquid medium and poured on to plates. Suppress bacterial growth by adding penicillin or streptomycin to the media. When shipping slants, ensure that they are 1) packaged well in a standard mailing tube or strong crush-proof container 2) the seal on the vial is secure and is not likely to be disturbed during shipping and 3) host plant information is also included with this sample submission.

1. Collect multiple, representative samples.
2. Digital diagnosis may also be useful for screening suspect vector samples.

4.0 Receipt and Examination

Upon arrival, contact submitting entity and acknowledge receipt of sample. The suspect plant material should be examined within a certified biological safety cabinet. Any tools, supplies, and miscellaneous materials used during the examination must be separated and placed in sealed plastic bags awaiting sterilization by a certified autoclave. The surface of all materials must be disinfected prior to the removal from the biological safety cabinet.

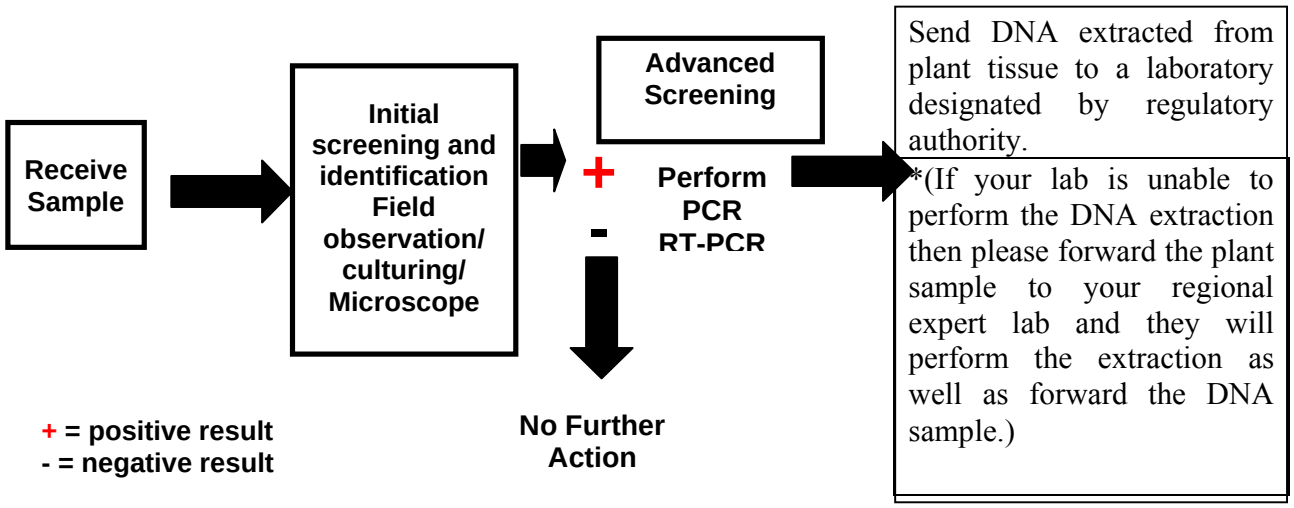
5.0 Storage

While examination and testing are being conducted, suspect plant material must be stored in access controlled cabinets and/or refrigerators. Keeping the suspect plant material for extended periods of time is not recommended. Plant material should be destroyed using the methods described in section no.7, Sample Destruction. Sample destruction is recommended within 2 weeks of submission to your facility if no confirmation has been reported.

6.0 Screening:

Both initial and advanced screening steps are listed below. Initial screening includes both serological testing methods and isolation of pathogens. Initial screening will be carried out by triage laboratories. If these facilities cannot perform the initial screening steps, samples can be referred to the appropriate regional center. Upon completion of the initial screening, advanced screening may be required depending upon initial screening results.

Disease Sample Protocol Flowchart



7.0 Protocols in the laboratory

7.1.1 Initial Screening by Triage Laboratories

7.1.2 Microscopy : Key Identification Characteristics

Fusarium wilt of bananas is caused by *Fusarium oxysporum* f.sp. *cubense*, a common soil inhabitant. The formae speciales of *F. oxysporum* each produce three types of asexual spores. The macroconidia (22-36 x 4-5 µm) are produced most frequently on branched conidiophores in sporodochia on the surface of infected plant parts or in artificial culture. Macroconidia may also be produced singly in the aerial mycelium, especially in culture. The macroconidia are thin-walled with a definite foot cell. *Fusarium oxysporum* f.sp. *cubense* cannot be distinguished reliably in culture from other formae speciales. Oval or kidney-shaped microconidia (5-7 x 2.5-3 µm) occur on short microconidiophores in the aerial mycelium and are produced in false heads. Both macro and microconidia may also be formed in the xylem vessel elements of infected host plants, but the microconidia are usually more common (Figure 3). The fungus may be spread by macroconidia, microconidia and mycelium within the plant as well as outside the plant. Illustrations of the conidia have been published (Nelson *et al.*, 1983). Chlamydospores (9 x 7 µm) are thick-walled asexual spores that are usually produced singly in macroconidia or are intercalary or terminal in the hyphae. The contents are highly refractive. Chlamydospores form in dead host-plant tissue in the final stages of wilt development and also in culture. These spores can survive for an extended time in plant debris in soil (Figure 4).

Mutation in culture is a major problem for those working with vascular wilt isolates of *Fusarium oxysporum*. The sporodochial type often mutates to a 'mycelial' type or to a 'pionnotal' type. The former has abundant aerial mycelium, but few macroconidia, whereas the latter produces little or no aerial mycelium, but abundant macroconidia. These cultures may lose virulence and the ability to produce toxins. Mutants occur more frequently if the fungus is grown on a medium that is rich in carbohydrates.

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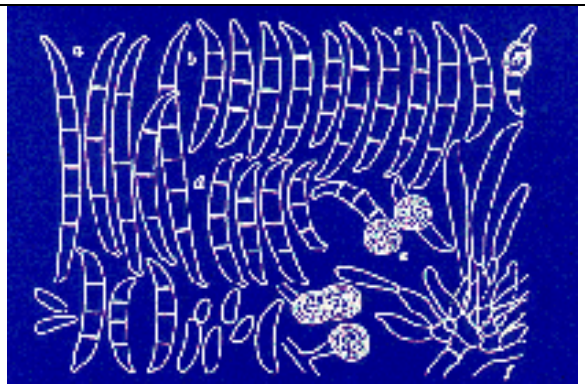
<tr><td>  </td><td>  </td></tr>
<tr><td>Figure 4 Chlamydospores, conidiophores and micro- & macroconidia of FOC</td><td>FOC in-vitro</td></tr>
<tr><td>  </td><td>  </td></tr>
<tr><td>Vascular discoloration on pseudostem</td><td>Vascular discoloration on pseudostem</td></tr>
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Figure 4.Typical symptoms for Fusarium Wilt (Photographs from Google images)

7.1.3 Symptomology of Panama disease

The first internal symptoms are a reddish brown discoloration of the xylem that develops in feeder roots and the initial sites of infection (Ploetz and Pegg, 2000). This varies from one or two strands in the oldest and outermost pseudostem leaf sheaths in the early stages of disease to heavy discoloration throughout the pseudostem and fruit stalk in the later disease stages. Vascular discoloration progresses to the rhizome and is most prominent where the stele joins the cortex, and ultimately proceeds up to and includes large portions of the pseudostem. Discoloration varies from pale yellow in the early stages to dark red or almost black in later stages. The infection may or may not pass into young budding

suckers or mature 'daughter' suckers. Where wilt is epidemic and spreading rapidly, suckers are usually infected and seldom grow to produce fruit. On plants that are more than 4 months old, the oldest leaves yellow or split longitudinally at the base. Eventually younger leaves wilt and collapse until the entire canopy consists of dead or dying leaves (Ploetz, 2005). Above- and below-ground parts of affected plants eventually rot and die. However, only above-ground parts fall when dead.

Figure 5: Banana cultivar Bluggoe with yellowing symptoms on lower leaves

Figure 6: Splitting of the base of the pseudostem of the Cavendish cultivar

Figure 7. Vascular discoloration inside the pseudostem of a Cavendish cultivar

Figure 8: Discoloration of vascular tissues in the rhizome

Photographs from Uganda (IITA and NARL)

Splitting of the base of the pseudostem is another common symptom as is necrosis of the emerging heart leaf. Other symptoms include irregular, pale margins on new leaves and the wrinkling and distortion of the lamina. Internodes may also shorten (Moore *et al*., 1995). Since infected rhizomes are often symptom less, they effectively spread the pathogen when used as seed pieces (Stover, 1962). The pathogen also moves in soil and running water, and on farm implements and machinery. It survives up to 30 years in the absence of banana, and non-host weed species that are infected by the pathogen are inoculum reservoirs (Stover, 1962; Waite and Dunlap, 1953).

7.1.4 Isolating the fungus from discoloured vascular strands/Culture Techniques

(See Annex 1 for preparing a sample from the diseased host plant). Isolation can be attempted when the strands have dried (possibly as early as the next day). Small sections (3-6mm long) of dry discoloured vascular strands are submerged into plates of selected media

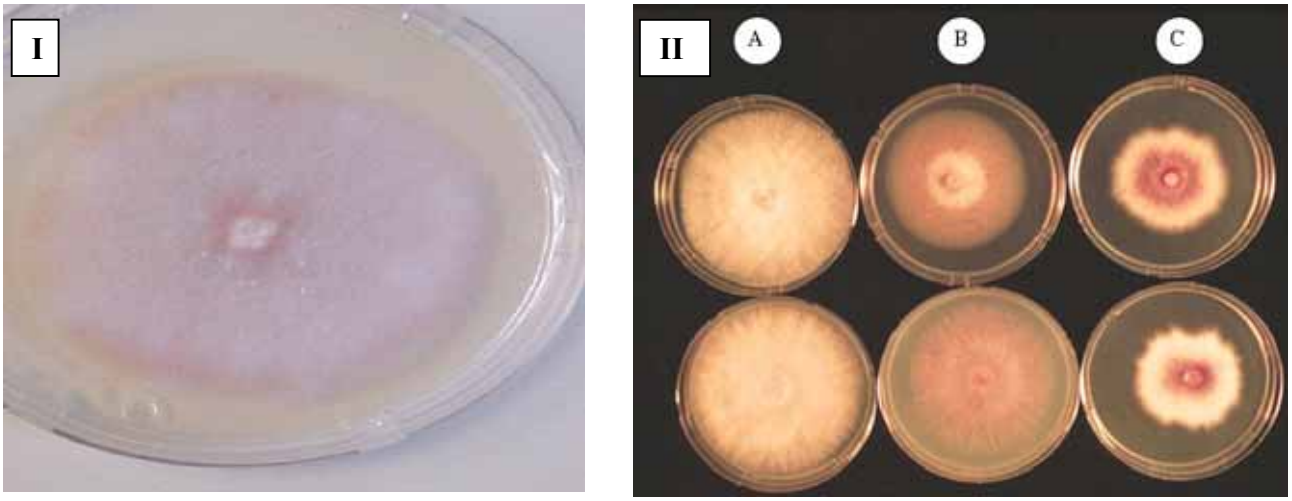
Media for the isolation and culturing of *F. oxysporum* (See Annex 2 for Details on preparation of different Media

I. Quarter strength PDA medium amended with an antibacterial agent (e.g. streptomycin @ 1.2mL/240mL PDA) (see media recipes).

Results. If Fusarium is present, growth will appear from the strands in 2-4 days. However, if the sample is badly contaminated with bacteria this may mask fungal growth. Let samples dry further if this occurs and increase the strength of the antibacterial amendment in the media. A high rate of recovery of Fusarium should be expected from correctly prepared samples. Mono-conidial (single-spore) cultures should be prepared from an isolate from each specimen.

Media recipes

II. Quarter strength potato dextrose agar (PDA) medium
III. PDA medium amended with streptomycin
IV. Carnation Leaf Agar (CLA).
V. Potassium chlorate (KPS) medium.
VI. Rice medium
VII. Minimal Medium



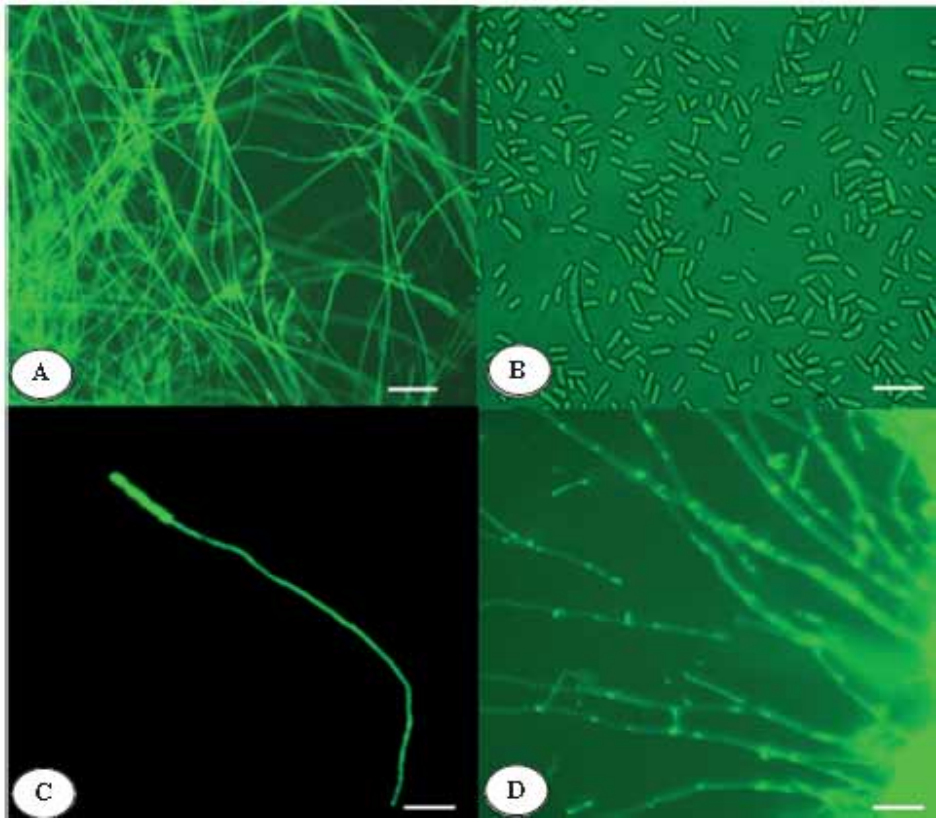
Cultural appearance of (I) Appearance of any form of *Fusarium oxysporum* f.sp. *cubense* raised on PDA media and (II) three isolate of Foc Sub-tropical race 4 on PDA plates. A illustrate cultures that produced pink colonies with abundant aerial mycelia, plate B dark pink colonies with aerial mycelia, and plate C cultures with a near purple colony colour

Source: University of Pretoria. Susan Groenewald

7.1.5 Morphological Methods. Generation of monoconidial cultures

1. Under aseptic condition, cut two pieces of culture approximately 6 mm^2 from a healthy culture isolated on quarter strength PDA (as above), and transfer these pieces to a small 9 mL McCartney bottle containing sterile distilled water.
2. After swirling the bottle gently to wash the spores into solution, flame a loop and transfer one or two loops of the spore suspension onto one side of a water agar plate.
3. The flamed loop is then dragged through the deposit of spore suspension several times to create 'streaks' of spore suspension across the plate either in parallel lines or in the sixteen-streak method used in microbiology. This separates the conidia from each other to enable single, separated, germinated spores to be easily identified and excise 18-24 hours later using a stereo microscope.

Germinated spores are observed by looking for a germ tube growing from them under stereo microscopy. Once a single germinated spore has been located, use a flamed scalpel to transfer it to an individual plate of quarter strength PDA. Repeat this procedure 4 times for each isolated culture to ensure a pure monoconidial culture is obtained. After three days, mycelial growth should be observed. Choose a culture that is healthy and showing typical growth as the representative isolate of that culture and discard the others. This culture should immediately be transferred to CLA for short-term storage, or filter paper for long-term storage.



Structure of fusarium oxysporum f.sp cubense (Foc) fluorescing bright green. (A) Fluorescing hyphal mass (B) Typical size and shape of macroconidia and Macroconidia of Foc. (C) A germinating macroconidium on a glass slide confirming viability of the fungus. Cross section of banana pseudostem showing net works of hyphae of Foc through the plant size (Scale bar: 10 mm= 24 µm)
Source: Marinda Visser , University of Pretoria

7.1.6 Racial classification

Isolates of *Foc* have been traditionally grouped into four physiological races, based on pathogenicity to a small number of differential banana host cultivars in the field (see Table 1). This racial classification is an artificial grouping and does not reflect a genetically defined relationship, however this terminology continues to be used (especially in field situations) as a simple and convenient way of grouping isolates of *Foc*.

Fungal vegetative compatibility

A technique that is based on the genetic relationships within fungal populations, termed vegetative compatibility group (VCG) analysis, has been utilized to group isolates of *Foc* (Moore 1994). Vegetative compatibility differentiates isolates that have identical alleles at the loci that govern heterokaryon formation, commonly referred to as het or vic, and thus vegetative compatibility (Leslie, 1993). VCG analysis was first described for the analysis of Aspergillus nidulans (Cove 1976), and was later adapted for use in *Fo* (Correll *et al*. 1987). This modified VCG technique, based on the generation of nitrate non-utilizing (nit) auxotrophic mutants, enables heterokaryon formation (vegetative compatibility) to be scored macroscopically and thus renders VCG analysis amenable to population studies.

Steps to follow

- Cultures growing on CLA or PDA medium are used to inoculate plates of a medium containing potassium chlorate (KPS) (see **Media recipes**). Potassium chlorate is an analogue of nitrate and is taken up and processed through the nitrate reductase pathway (Correll *et al*. 1987). This process results in the production of chlorite which is toxic to the fungus (instead of nitrite which is useful to the fungus), and characteristically slow-growing colonies with restricted, 'knotted' mycelial growth are observed.
- After 5 to 12 days, fast-growing sectors begin to emerge from the restricted colonies. The mycelium in these fast-growing sectors has sustained a mutation which enables the fungus to resist chlorate (and therefore also the toxic chlorite). However, the mutation also renders the fungus unable to reduce nitrate. Thus, these sectors are known as nitrate non-utilising mutants or nit mutants for short.

- To test if the fast-growing sectors are unable to use nitrate, a small piece ($2mm^2$) of mycelium is taken from the advancing edge of the sector. This is then transferred to a medium which contains nitrogen only in the form of nitrate, such as Minimal Medium (see Media recipes) (Puhalla, 1985).
- If the sector is a true nit mutant it will not be able to reduce the nitrate in the medium and characteristically flat, sparse, nitrogen-deficient growth will result. If the growth that results is not sparse on MM, discard this culture, as it will be of no use in VCG tests. It is advantageous to let the sectors grow for two to three days after emerging on the KPS plates so that the fast-growing mycelia grows clear of any non-mutated mycelia which may be underneath. When each of the sectors is transferred to MM, the sector should be numbered to identify it. This becomes particularly important if the tests need to be repeated or the mutants are needed for other tests. For example, if the isolate being tested has the accession number 23532 the sectors can be sequentially numbered as 23532-1, 23532-2, 23532-3 and so forth

VCGs serve as a natural means to subdivide fungal populations and therefore VCG analysis is a useful technique to measure genetic diversity within a population. In an asexual population, differences at the vic loci are assumed to effectively limit the exchange of genetic information to those individuals that belong to the same VCG. Since sexual recombination is not known to occur in Foc, members of each VCG comprise a genetically isolated subgroup and are assumed to be clonally derived populations of the pathogen (Leslie 1990). At least 21 VCGs of Foc have been characterized worldwide (Moore 1994; Ploetz 1990a; Ploetz and Pegg 1999), with seven of these VCGs present in Australia, viz. VCGs 0120, 0124, 0125, 0128, 0129, 01211, and 01220 (see Table 1) (Pegg *et al*. 1995).

Table 1. The relationship between Australian VCGs and races of *Foc*, and the banana cultivars they infect.

Race	1	2	3	Subtropical race 4	Tropical race 4
Host	Lady Finger (AAB) Gros Michel (AAA)	Bluggoe (ABB)	*Heliconia* spp.	Cavendish (AAA) & all other cultivars susceptible to races 1 & 2	
VCG	0124+ 0125 01220*	0124+ 0128	-	0120 0129 01211	01213/16
Volatility	Non-volatile	Non-volatile	-	Volatile	Volatile

* Isolates of VCG01220 are unique because they were initially classified as belonging to subtropical race 4, as they were isolated from diseased Cavendish plants. However, subsequent biological and genetic characterisation has revealed that VCG01220 is more closely related to races 1 & 2 of *Foc* (Pegg *et al*. 1995).

\+ Isolates of VCG0124 have been isolated from both Race 1 and Race 2 susceptible cultivars (Gerlach *et al*. 2000).

7.1.7 Volatile production

Isolates of *Foc*, when cultured on a starch substrate such as steamed rice or millet, either produce or do not produce a characteristic volatile odour. This trait of volatile production has been used to group isolates of *Foc* (Stover 1962). Isolates are classified as either 'odoratum' or 'inodoratum', depending on the presence or absence of volatile substances in the headspace above the culture. These volatiles can be analysed by gas chromatography, or more conveniently detected by nose. Volatile analysis has been used to characterise Australian and Asian isolates of *Foc* (Pegg *et al*. 1993). There studies showed there was an absolute correlation between the productions of volatile substances, VCG and pathogenicity in the Australian isolates; race 1 and race 2 isolates did not produce a detectable volatile odour and gave gas chromatogram profiles with no peaks, while race 4 isolates produced easily detectable volatile odours with characteristic gas chromatogram profiles. Volatile analysis is a simple and inexpensive method of characterising isolates of *Foc* based on the biochemistry of cultures in vivo.

7.2 Advanced Screening by Expert Laboratory

N.B. At the moment there are no specific primers that can be used to detect for *Fusarium oxysporum* f. sp. *cubense*. Race 1, 2 and 3 that are known to occur in the region. However primers for Tropical Race 4 have been developed and are being used in other region such as Asia and Australia apart from Africa. Therefore there is a lack for molecular diagnosis for Race 1, 2 and 3.
Information presented below applies to a Quarantine Race 4 that does not exist in Africa.

DNA Testing

See Annex 4. For different *Foc* DNA Extraction Protocols
See Appendix 2. Consumable and Reagent for *Foc* DNA extraction

7.2.1 Molecular diagnosis for tropical race 4 (TR 4)

Preparation

1. Samples from geographic regions known to be infested by TR4 are received as dry pseudostem strands and should be sectioned into pieces (2 cm long and 0.5 cm wide).
2. These are then transferred to Komada's medium (See Annex 2 for Recipe) and incubated at 25 ^{0}C. After 3–5 days, when fungal growth appeared as white and pink aerial mycelia, isolated colonies are examined by light microscopy for the presence of macro conidia and micro conidia diagnostic of *F. oxysporum*.
3. Positive samples are then transferred to plates with potato-dextrose agar (PDA) and stored for further analyses.

DNA isolation and PCR amplification for Race 4 Foc

1. For DNA isolation, a single-spore culture of each isolate is grown in Petri plates (6 cm diameter) containing PDA and incubated at 25^{0}C for 5 days. To facilitate the harvest of mycelia, a cellophane disc (5.5 cm diameter) is placed on the medium surface prior to inoculation.
2. Mycelium is harvested by scraping the cellophane disc and is subsequently stored in 2-mL tubes at) 80 ^{0}C. After addition of a tungsten bead, the mycelium is lyophilized and ground by vigorous shaking of the tubes in a MM300 mixer mill (Retch). Total genomic DNA is extracted using the Wizard Magnetic DNA Purification System for Food kit (Promega) according to the manufacturer's instructions. DNA samples are then diluted to 10 ng lL) 1 and stored at) 20 ^{0}C until use.
3. The primer set FocTR4-F / FocTR4- R for specific detection of TR4 (VCG 01213) designed to generate a unique amplicon of 463 base pairs (bp). FocTR4-F (5'-CACGTTTAAGGTGCCATGAGAG-3') & FocTR4-R (5'CGCACGCCAGGACTGCCTCGTGA-3'). Amplification conditions follows a program: 95 ^{0}C for 5 min and 30 cycles of 95^{0}C for 1 min, 68 ^{0}C for 1 min and 72 ^{0}C for 3 min, followed by an additional extension time for 10 min at 72 ^{0}C. Alternatively using a shorter version of FocTR4-R (5¢-GCCAGGACTGCCTCGTGA-3¢) together with FocTR4-F works with the annealing temperature set at 60 ^{0}C).

7.2.2 DNA amplification fingerprinting (DAF) analysis

The DNA amplification fingerprinting system is a modified version of the technique described in: Bentley *et al*. (1996). A robust DNA amplification fingerprinting system applied to analysis of genetic variation within *Fusarium oxysporum* f. sp. *cubense*. *Journal of Phytopathology* **144**, 207-213.This technique utilises arbitrary primed PCR methods to generate genome-specific DNA banding patterns. The PCR-based DNA amplification reaction is directed by a single oligonucleotide primer of arbitrary sequence. PCR products are then visualised by polyacrylamide gel electrophoresis, followed by silver staining. This method was developed to deliver robust and reproducible results that are easily transferred between different laboratories

<u>See Annex 5 for PCR conditions: Precautions to undertaken during preparation for PCR and Master Mix Composition</u>

4. **Thermal cycling conditions:**

DNA Fingerprinting PCR

Step	Temperature	Time (minutes: seconds)
1	94°C	5:00
2	94°C	0:30
3	52°C	1:00
4	51°C	1:00
5	50°C	1:00
6	49°C	1:00
7	48°C	1:00
8	Go to step 2, 34 more times	
9	72°C	10:00
10	End	

See Appendix 2 for Reagents and DAF PCR Primers for DNA amplification fingerprinting (DAF) analysis

DNA Fingerprinting Analysis

Genome-specific DNA fingerprint patterns are analysed and scored manually (by eye); fingerprints of test samples are compared to standards (characterised isolates) by assessing the general fingerprint pattern and scoring for presence/absence of polymorphic bands (see Figure 8). Scoring is made easier by placing gels on a light box, which enables the discrimination of lightly stained bands. Test samples can be compared with standards from other gels by overlapping gels on the light box, or by digitally cutting and pasting the lanes next to one to build a new image that makes scoring easier. DAF analysis is used in conjunction with VCG testing for the characterisation of unknown isolates of *Foc*.

7.3 A summary of the techniques that have been used for the characterisation of isolates of Foc.

Technique used for	Description of technique	Reference
Vegetative compatibility grouping analysis	Differentiates *Foc* isolates that share identical alleles at the loci that govern heterokaryon formation	(Brake *et al.* 1990; Moore 1994; Moore *et al.* 1993; Ploetz 1990b; Ploetz and Correll 1988)
Production of volatile organic compounds	Differentiates isolates of *Foc* grown on a starch substrate by the presence/absence of volatiles in the headspace above the culture	Brandes 1919; Moore *et* 1991; Stover 1962b)
Electrophoretic karyotype	Used to compare chromosome number and genome size	(Boehm *et al.* 1994; Miao 1990)
Restriction fragment length polymorphism (RFLP) analysis	Analysis of genetic similarity by measuring polymorphisms in the genome	(Koenig *et al.* 1997)
Random amplified polymorphic DNA (RAPD) analysis	Estimates genetic relatedness by comparing polymorphisms in the genome	(Bentley *et al.* 1995)
DNA amplification fingerprinting (DAF) analysis	Generates genome-specific banding patterns for estimating genetic relatedness	(Bentley and Bassam 1996; Bentley *et al.* 1998)
DNA sequencing of nuclear and mitochondrial genes	Comparison of sequence information to estimate phylogenetic relationships between isolates	(O'Donnell *et al.* 1998)

7.4 Key Contact Person For diagnosis of Foc samples:

Lisa Gulino Plant Pathologist Qld Department of Primary Industries & Fisheries Plant Pathology Building 80 Meiers Road Indooroopilly, Qld, 4068 Ph: +61 7 38969337 Fax: +61 7 38969533 Email: lisa-maree.gulino@dpi.qld.gov.au	**Dr Juliane Henderson** Research Fellow (Banana disease diagnostics) Centre for Tree Pathology The University of Queensland Qld Department of Primary Industries & Fisheries Plant Pathology Building 80 Meiers Road Indooroopilly, Qld, 4068 Ph: +61 7 38969348 Fax: +61 7 38969533 Email: juliane.henderson@dpi.qld.gov.au

8.0 Communication:

If the PCR results in a suspect positive ID, follow this communications protocol. If a negative result is produced, no further communications are necessary.

9.0 Confirmation:

- **a.** Diagnosticians will be notified of the results by the diagnostic laboratory or any idenfited laboratory that carries out *Foc* diagnosis
- **b.** Notify the Regional /National Disease inspector of confirmed results.
- **c.** Country officials will be notified of the results by the institutional office. Once confirmation is made, country regulatory officials will handle any actions dealing with containment and eradication.
- **d.** Notify your Institution's Environmental and Health Safety Official.

10. Sample Destruction:

Plant material and/or supplies used in the examination and isolation of the suspect sample must be destroyed using a biologically monitored autoclave. The autoclave must be set at a minimum of 15 psi, 121 °C for 30 minutes.

All tools and other equipment must be sanitized and/or sterilized before re-use.

Appendix 1: Sampling Methods

- **Complete a sample form**.

Note total number of symptomatic plants in your report by stock. Take a picture of the samples before removing it from the plant if possible. Photograph the entire plant, as well as close-ups of the symptomatic plant part. Step back and photograph the nursery block etc. to gain a perspective of where the symptomatic plant is located with respect to other plants in the nursery. A minimum of 3 photographs per symptomatic plant will be typical, unless several plants in a block are symptomatic, in which case discretion is advised.

- **Sanitation**

Decontaminate all equipment you use to take samples before leaving a nursery. Use a spray bottle containing a dilute (5%) Clorox® solution or 70% or stronger of ethanol over all tools before leaving the site. Spray boots or shoes with solution in spray bottle before leaving each site.

Follow decontamination procedures before and after taking each sample.

- **Notification**

Ensure that transportation and laboratory facilities have been arranged such that samples will be processed and plated within 24 -48 hours of collection. See Supervisor on where to submit samples.

- **Preparing Samples**

✓ Samples should be bagged in a moisture-retaining container, such as a polyethylene bag to prevent drying.

✓ Decontaminate hands and place sample bag in a second protective bag.

✓ Always write out the identifying label remarks on the outside of the bag.

✓ Keep the sample cool and out of the sun (have a foam Cooler available).

✓ After you have double bagged the sample, fill out a pest and disease sample form and attach it to the bag.

- **Labeling and Documenting Samples**

After care has been exercised to secure a good sample, it is vital to protect and label the sample properly. The label should contain the complete information pertaining to the sample submitted. Use one of the black felt tipped water proof pens provided. The label must include:

1. Name and Number of the Nursery or address of property
2. Time and Date of sampling
3. Surveyor's ID (alpha-numeric characters) or submitter's name and contact information.
4. Sample ID - This should be the inspection record ID plus a two character alpha-numeric sample code. Example: [GRN][FL][123][35]

3 program code, 2-letter state code, 3-numeric site code (Nursery) and 2 alpha-numeric sample code.

Appendix 2: Specific Equipment and Reagent Information for DNA Extractions

DNA Extraction Kits:

- DNeasy Plant Mini Kit (Qiagen Cat # 69104)

Reagents and Equipment to Be Supplied by User

- Equipment for disrupting plant tissue. Such as a Bead Beater (see below)
- Ethanol (96–100%)
- Liquid nitrogen

For the DNeasy Plant Mini Kit:

- Microcentrifuge tubes (1.5 ml)
- Microcentrifuge with rotor for 2 ml tubes
- Screw-cap plastic tubes – 2 ml volume (general lab vendors)
- Glass beads 5mm diameter (Fisher Scientific cat. # 11-312C).
- Mini Bead-beater (Cole Parmer, Cat# A-36270-02)

Vendors:

Cole Parmer (800-323-4340) www.coleparmer.com (search for Mini Bead Beater, then select the Mini Bead Beater $847.00)

Qiagen (800-426-8157) www1.qiagen.com/products (select Genomic DNA Stabilization and Purification, then select DNA purification from Animal and Plant Samples, be sure to select the MINI kit)

Fisher Scientific (800-766-7000) www.fishersci.com

Consumables and equipment needed for *Foc* DNA extraction

Item	**Description / Ordering Information**
Pipettes: P20, P200, P1000	Pipetman P20, P100, P200 (John Morris Scientific)
Sterile 2.0mL microfuge tubes	Quantum 2.0mL Micro-tubes (Quantum Scientific P/L)
Aerosol resistant (plugged) tips	Axygen Maximum Recovery, racked and pre-sterilised (Quantum Scientific P/L); TF-20-L-R-S (suits P20),TF-200-L-R-S (suits P200), TF-1000-L-R-S (suits P1000)
Miracloth	Calbiochem® Miracloth (Merck, #475855)
Heating water bath (set to 37 ºC)/heater blocks	
Small liquid nitrogen Dewar/flask	
Vacuum desiccator	
Micro centrifuge	

Reagents

- Liquid nitrogen
- DNA Extraction Buffer (see below)
- Phenol (stored at 4ºC)
- Chloroform-isoamyl alcohol (24:1) (store at -20ºC)
- 3M sodium acetate (pH 5.4)
- 100% ethanol (ice cold, stored at -20ºC)
- 70% ethanol (ice cold, stored at -20ºC)
- Sterile dH_2O or TE Buffer (10mM Tris, 1mM EDTA, pH 8.0)

DNA Extraction Buffer (store at 4ºC)

Reagent description

2% sodium dodecyl sulphate (SDS)

40mM EDTA

40mM sodium chloride

100mM Tris-HCl (pH 8.0)

25mM diethyldithiocarbamic acid (DIECA)

Consumables and equipment for DNA Extraction using NucleoSpin DNA Extraction Kit

Item	Description / Ordering Information
NucleoSpin® Plant DNA extraction kit	NucleoSpin® Plant DNA extraction kit (Scientifix, #740570.50)
Pipettes: P20, P200, P1000	Pipetman P20, P100, P200 (John Morris Scientific)
Sterile 1.5mL and 2mL microfuge tubes	Quantum 1.5mL and 2mL Micro-tubes (Quantum Scientific P/L)
Aerosol Resistant (plugged) tips	Axygen Maximum Recovery, racked and pre-sterilised (Quantum Scientific P/L); TF-20-L-R-S (suits P20),TF-200-L-R-S (suits P200), TF-1000-L-R-S (suits P1000)
Micro-centrifuge	
Heating water bath/heater blocks	
Small liquid nitrogen Dewar/flask	
Vacuum desiccator	
Mortars and pestles	
Liquid nitrogen	

Appendix 2 for Reagents for DNA amplification fingerprinting (DAF) analysis

Item	Description
DNA polymerase (Stoffel enzyme)	AmpliTaq® DNA Polymerase, Stoffel fragment (10 units/µl) (Applied Biosystems, #N8080038)
10 x DAF Buffer	Made in-house: 50mM $MgCl_2$ 100mM KCl 100mM Tris-HCl pH 8.3
dNTPs (set of dATP, dCTP, dGTP, dTTP)	• dNTP Set (100mM each) • Roche PCR-grade dNTPs (Roche, # 1581295)
Primer (300µM)	• Sequencing/PCR grade, synthesised by Gene Works, Australia. • Re-suspend lyophilised pellet to 300µM, and aliquot stocks into small amounts and freeze.
dH_20	• Sterile deionised water • injectable water is optimal

DAF PCR Primers

Primer Name	**Sequence (5'-3')**
ILOE	GATGAGCC
HIRH	ACGTCCAC
DINQ	CTGGCCCA
NRKI	CCTCGTGG
NROI	CCTGGTGG
IMBR	GTAACGCC

Appendix 3: Useful References

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Ploetz, R. C. 2005. *Panama disease: an old nemesis rears its ugly head. Part 2.* The Cavendish era and beyond. *APSnet.* http://www.apsnet.org/online/feature/panama/.

Puhalla, J.E., 1985. Classification of strains of Fusarium oxysporum on the basis of vegetative compatibility. Canadian Journal of Botany 63: 179-183.

Snyder WC and Hansen HN, 1940. The species concept in Fusarium. American J. Bot. 27: 64-67.

Stover R.H., 1962. Fusarial Wilt (Panama Disease) of Bananas and other Musa species, 117pp. Kew, Surrey, UK: Commonwealth Mycological Institute.

Annex 1: Preparing a sample from the diseased host plant

The sample should consist of a 10 cm cross section of the pseudostem of the wilted banana plant where typical continuous darkened vascular strands are evident. The sample should be taken from as high in the pseudostem as is possible and not from areas where decay is advanced. The sample should also be taken from as close to the centre of the pseudostem as is possible, as opposed to the outermost leaf bases. For plants in early stages of infection the discoloured vascular strands may only be apparent in the corm or rhizomes. In this case a small piece of rhizome or corm (10 cm cube) with typical discoloured vascular strands should be collected as a sample. Again it is not recommended to obtain samples from tissue where decay is advanced.

As banana tissue is very wet, the risk of bacterial contamination of samples is high, particularly in warm weather as samples will deteriorate rapidly. The chance of recovering healthy cultures of Foc decreases as the sample deteriorates. Samples should be kept in heavy paper bags or wrapped in paper (e.g. newspaper) until the strands can be excised. Avoid using plastic bags as this causes the samples to sweat and promotes growth of bacteria. Accurate notes must be taken for each sample including:

- sample number (one sample number per plant)
- date
- the variety of the host plant, including local names (and uses if known)
- Genomic constitution of host if known (e.g. AA, AAB, ABB etc.). This is not as important as an accurate identification of the variety.
- whether the plants sampled are growing in a garden, commercial plantation, village or wild situation
- location (e.g. name of province or state, how far in what direction from nearest town, name of road, name of property if sample is from a commercial plantation etc.) A map with sample numbers marked on it or GPS coordinates can be very useful.
- collectors names
- other useful observations might include the source of the planting material, whether the plant is growing in water-logged soil or stressed in some other way, how many plants are affected what other varieties are growing around the diseased plant and are these diseased or healthy?

NOTE: When looking for wilt-affected plants, it is better to take samples from established plantings of bananas (plantations or mature mats) rather than recently planted young plants.

Dissecting discoloured vascular strands from sample

Ideally, the discoloured vascular strands should be dissected from the sample on the same day that it is collected, or as soon as possible after collection. The use of sterile blotting papers is recommended and aseptic technique should be applied to the dissection of strands. Samples should first be surface-sterilised by wiping with 70% alcohol or surgical spirit. Where several samples are being prepared, a fresh piece of blotting paper should be used for each sample, and scalpel blades should be flamed if possible or at least wiped with 70% alcohol between samples. The excised strands, with as little as possible of the adjacent tissue, should then be placed between sterile blotting papers in a paper envelope to dry naturally. A few days are usually sufficient. Do not let the strands get too hot (e.g. in direct sunlight or in the boot of a car) as this may kill the fungus. Do not dry them in an oven! Fusarium wilt specimens do not need to be kept in the fridge - room temperature is acceptable. They do not need to be wrapped in moist paper like leaf specimens - dry paper is best.

If posting the strands for isolation and analysis, please post in a paper envelope as soon as the strands are dry enough with sample numbers and details clearly written on or with each sample envelope. Please include a copy of the relevant AQIS quarantine import permit inside the package if this is required.

Note: If there is any possibility that samples have been mixed up and the details for some samples may be incorrect, discard the samples concerned.

Annex 2. Media for the isolation and culturing of *F. oxysporum*

Quarter strength potato dextrose agar (PDA) medium

Ainsworth GC (1971) 'Ainsworth and Bisby's Dictionary of the Fungi.' (Commonwealth Mycological Institute: Kew, Surrey, England)

Ingredients per litre of distilled water:

- 100g peeled and diced potatoes
- 10g dextrose
- 20g agar

Method:

Steam potatoes in the distilled water for one hour then strain through eight layers of cheesecloth. Discard the solid portion. Add dextrose and agar to the liquid portion, stir well and return to the steamer until agar dissolves (approximately 40-50 min). Remove media from steamer and immediately dispense 240mL into 250 mL glass bottles; cap with autoclavable caps (vented). Sterilise in an autoclave (wet cycle: 100 kPa at 121^{o}C for 20 min). When cool, tighten caps and label bottles with PDA and the date.

PDA medium amended with streptomycin

Melt the required number of bottles of 240mL PDA medium in a steamer. When melted, place bottles in a water bath at 50^{o}C for 20 minutes, or until media reaches 50^{o}C. To each 240mL media add 1.2mL streptomycin solution (1g streptomycin sulfate powder per 100mL sterile distilled water) just before dispensing into Petri dishes.

Carnation Leaf Agar (CLA)

Burgess LW, *et al*. (1988) 'Laboratory Manual for Fusarium Research.' (University of Sydney: Sydney, Australia)

Four to ten pieces of sterilised carnation leaf are placed onto the surface of freshly poured water agar plates just before the agar sets. When set, the CLA plates are stored upside down in a refrigerator or cold room at 4^{o}C.

Preparation of Carnation leaves:

Fresh, healthy carnation leaves, which have not been treated with fungicides or other chemicals, are cut into pieces approximately 10 mm x 3 mm before placing in paper bags to dry. When dry, place leaf pieces in containers suitable for Gamma-irradiation (eg. glass or hard polystyrene containers with lids or polyethylene Petri dishes sealed with Parafilm). Note that Gamma radiation will degrade plastics after repeated exposure. The containers are placed in a Gamma cell for a total dose of 2.5 Mega Rad. Store containers of Gamma-sterile leaf pieces in refrigerator or cold room at 4^{o}C until required.

Potassium chlorate (KPS) medium

Puhalla JE (1985) Classification of strains of *Fusarium oxysporum* on the basis of vegetative compatibility. *Canadian Journal of Botany* **63**, 179-183.

Cook 200g peeled and diced potatoes in 1 L of distilled water in a steamer for 50 minutes. Pass the liquid through 8 layers of cheesecloth and discard the solid portion. Make the liquid volume up to 1 L with distilled water and add:

- 20g sucrose
- 15g $KClO_3$
- 20g agar

Return the media to the steamer until agar has dissolved. Remove media from steamer and immediately dispense 240mL into 250mL glass bottles; cap with autoclavable caps (vented). The capped bottles of media are then sterilised in an autoclave (wet cycle: 100 kPa at 121^{o}C for 20 min). When cool, tighten caps and label bottles with KPS and the date.

Rice medium

Moore, N.Y., Hargreaves, P.A., Pegg, K.G. and Irwin, J.A.G. (1991). Characterisation of strains of Fusarium oxysporum f. sp. cubense by production of volatiles. *Australian Journal of Botany* **39**: 161-166.

- Add approximately 30mL of rice and 90mL of distilled water to each 250mL Erlenmeyer flask.
- Plug with cotton wool and cover with alfoil before steaming for 1 hour on each of two consecutive days eg. in autoclave on free steaming cycle at 103^{o}C.
- Allow medium to cool before inoculation.

Minimal Medium

Puhalla JE, *et al*. (1983) Heterokaryosis in *Fusarium moniliforme*. *Mycology* **7**, 328-335.

Ingredients per litre of distilled water:

- 30g Sucrose
- 20g BBL agar (or similar analytical grade agar)
- 0.5g KCl Potassium chloride
- 2g $NaNO_3$ Sodium nitrate
- 1g KH_2PO_4 Potassium dihydrogen orthophosphate
- 0.5g $MgSO_4.7H_2O$ Magnesium sulphate heptahydrate
- 10mg $FeSO_4.7H_2O$ Ferrous sulphate
- 0.2mL sterile trace elements solution (add this after medium has melted and before autoclaving)

Method

Place in steamer until agar has dissolved (approximately 1 hour), shaking occasionally. Add trace element solution (this is pre-made and dispensed into 1mL aliquots, stored in the freezer). Dispense approximately 240mL of medium into media bottles. Cap (with caps vented) and autoclave to sterilise (e.g. wet cycle: 100kPa at 121^{o}C for 20 min). When cool, tighten caps and label bottles with MM and the month and year.

Trace element solution

Ingredients per 95mL of sterile distilled water. 5.0g Citric acid

- 5.0g $ZnSO_4.7H_2O$ Zinc sulphate
- 1.0g $Fe(NH_4)_2(SO_4)_2.6H_2O$ Ferrous ammonium sulphate

- 0.25g $CuSO_4.5H_2O$ Copper sulphate
- 50mg $MnSO_4.H_2O$ Manganous sulphate tetrahydrate
- 50mg H_3BO_4 Boric acid
- 50mg NaMoO4.2H2O Sodium molybdate

Komada's Medium preparation

Komada's medium is recommended for the selective isolation of *F. oxysporum* from soil (Komada, 1975). *F. oxysporum* colonies are pigmented. Other Fusarium species are suppressed. The basal medium contains the following constituents in 1L distilled water and is autoclaved (121°C for 15m) and cooled to 55°C before antimicrobial agents are added.

- K2HPO4 1.0g
- KCl 0.5g
- MgSO4.7H2O 0.5g
- F3Na EDTA 0.01g
- L Asparagine 2.0g
- D Galactose 20.0g
- Agar 15.0g

To the basal medium is added in 10ml sterile distilled water:

- PCNB as Terrachlor® 1.0g
- Oxgall 0.5g
- Na2B4O7.10H2O 1.0g
- Streptomycin sulfate 0.3g

The pH is adjusted to 3.8±0.2 with 10% phosphoric acid.

Annex 3. Procedure for maintaining of healthy cultures

Healthy (sporodochial-type) monoconidial cultures of *Fusarium oxysporum* f. sp. *Cubense* (*Foc*) should be maintained on carnation leaf agar (CLA) to prevent mutation. Cultures can be initiated on weak quarter-strength PDA medium to check the morphology of cultures for taxonomic purposes or for spore production. Healthy (sporodochial-type) cultures of *Foc* growing on PDA medium exhibit abundant fluffy aerial mycelium after 2 days, and produce abundant micro-conidia. Some macro-conidia may also be produced on PDA, although this type of spore is more commonly produced on CLA medium.

Cultures of Foc should NOT be kept on PDA medium for longer than 4 or 5 days as mutations can rapidly occur and these cannot be reversed. Mutated cultures (e.g. slimy pionnotal mutants) should be discarded. Cultures are normally maintained in an incubator at 25^{o}C. Black light is generally not required for cultures of *Foc* to sporulate. Various methods are used for long-term (e.g. lyophilisation), medium-term (e.g. colonised filter paper in cold storage) and short-term (e.g. CLA) storage of cultures of *Foc*.

Annex 4. Sample preparation for DNA extraction and Different DNA Extraction Protocols/Methods

DNA Extraction Protocols: Plant DNA Extraction Protocol

a. Sample Preparation for DNA Extraction

NB: *Contamination of PCR samples can start prior to the PCR test during both sample selection and preparation, and DNA extraction.*

To minimize the potential for contamination during the sample selection make sure that the area used in the lab to select leaves is separated from the DNA extraction area. Do not extract DNA on the same bench where PCR products are amplified or analyzed.

Make sure that disposable lab mats are used on the benches where samples are processed, and samples are cut in weigh boats changed between each sample. Do not cut on cutting boards or the bench directly, as this will result in contamination of the sample. Gloves should be worn to select leaves and cut samples and changed between each sample to avoid contaminating the following sample. A good rule of thumb is to treat each sample as if it is infected and as if the next sample to be processed is healthy and take measures to minimize contamination of that next sample. Cutting implements should either be disposed off between samples, or dipped in ethanol and flamed between samples to avoid direct contamination.

Leaf tissue for DNA extraction should be collected from the midrib and petiole of symptomatic leaves.

- A disposable blade can be used. Dispose of blades, or flame sterilizes the cutting implements between each sample. The midrib of each leaf should be cut out. The maximum amount of tissue used in the DNeasy Plant Mini kit is 100mg (the midribs of 5-6 leaves, each midrib cut into 6-12 pieces).
- The best results have been obtained by cutting out half to two-thirds of the midrib, closest to the petiole, and using just that portion for PCR.
- Make sure to keep leaves and their respective midrib pieces separate, unless pooling the leaves.

b. DNA Extraction

DNA must be extracted using one of the validated protocols: the Qiagen DNeasy Plant Mini kit, or the FL DNA extraction protocol that finishes with the Qiagen DNeasy Plant DNA kit.

IMPORTANT NOTES:

- In order to avoid cross contamination designate a separate room or lab area for the DNA extraction.
- Also, use separate designated sets of pipettes (especially if your lab conducts PCR). Use aerosol-resistant barrier pipette tips.
- Centrifuge any DNA-containing tubes before opening so that any liquid near the rim of the tube is removed; centrifuge rotors designed for aerosol containment are recommended.
- Use microfuge tube openers to avoid contact with the top rim of the microfuge tubes. Your fingers should never touch the top of the microfuge tube.
- It is a good practice to store plant samples or extracts in a separate freezer or freezer compartment from PCR reaction components. If samples are contaminated with soil rinse them in sterile water and pat-dry them with a hand towel.
- Wear gloves and change them often, particularly between different segments of the DNA extraction procedures.
- Use disposable lab mats to cover bench areas and change them between each set of extractions.
- All racks, tube openers, and other plastic materials used in the procedure should be decontaminated between each set of extractions by soaking in a 10% bleach solution (a 1:10 dilution of commercial bleach in water) for 30 minutes followed by 2 rinses with water to remove the bleach solution. Bench areas, pipettes, centrifuge rotors, lab chairs, drawer handles, and other knobs, etc. in the environment of the bench used to do DNA extractions should be wiped down every couple of days with a DNA elimination solution such as DNA Away (bioexpress.com).
- Samples should remain in a refrigerator or freezer prior to packaging and immediate shipping for PCR testing. Do not have boxes sitting at room temperature for several hours before shipment.

1) Plant DNA Extraction using Qiagen DNEasy Plant Mini Kit (Cat# 69104)

This protocol is based on the Qiagen's protocol for total DNA extraction from plant tissue using the mini columns. A booklet is included with each kit and the protocol is on page 16. Please, read carefully all information provided in the booklet before starting work. A DNeasy Plant Extraction Worksheet that provides the protocol and places to record sample information is available in Appendix 4. A list of supplies for this procedure is in Appendix 3.

There have been a few changes made in the protocol noted in the section addressing the extraction protocol. Here are some additional helpful tips:

- Consider extracting as many samples at once as your centrifuge rotor can hold. For example, one person can process 12 samples at one time. Too many samples processed simultaneously can result in some columns beginning to dry before the next step in the procedure.
- Prepare and label all necessary tubes and columns in advance (columns should be labeled on the top of the lid, not on the side of the collection tubes). Place them on a rack in rows:
    - First row – the QIAshredder columns (lilac colored, columns supplied)
    - Second row – 1.5 ml tubes
    - Third row – DNeasy columns (white columns supplied, make certain the column is labeled since you will move this, not the tube during its use in the procedure)
    - Fourth row – 1.5ml tubes with lids cut off (if necessary).
    - Fifth row – 1.5 ml tubes for the DNA extracts clearly labeled with the sample ID and date of extraction.
- Set up a water bath or heating block at 65ºC.
- The first time you use the kit add the appropriate volume of 95-100% ethanol to both the AW and AP3/E buffers. Record on the bottle that EtOH was added and the date added. (If a date does not exist on an opened bottle and you doubt that EtOH has been added, smell if the bottle contains ethanol.)
- Always check buffers AP1 and AP3/E for precipitates that can form upon storage. To dissolve the precipitate, warm the buffers up to 65ºC prior to use.
- Wear gloves during extraction. Change them often and if you have any reason to believe they have been contaminated with sample extracts.
- Open tubes and columns carefully to avoid aerosol formation and spills. Use a tube opener. When you transfer samples do not touch the edge of the tubes with the pipettle tip.
- When handling columns, please hold them by the top of the collection tube, not by the column. The column sits inside the collection tube. If you hold it by the column only, the collection tube may disconnect and drop (potential contamination or loss of sample). When transferring columns from one collection tube to another, please do not touch the bottom of the column (this is where the sample will elute and is a potential area to introduce contamination to the sample).
    - Once you start the extraction, please do not stop or leave your samples sitting longer than prescribed by the protocol.
- Pre-warm a portion of the AE buffer to 65ºC in water-bath or heating block.
- Centrifugation steps are carried out at room temperature.

- Most centrifugation steps are to be performed at 20,000 x g or 14,000 rpm. If you do not have a centrifuge capable of that speed then double the time.

2) DNeasy Extraction Protocol

- First thing, set up a water bath or heating block to 65ºC.
- Pre-warm a portion of the AE buffer to 65ºC in the water bath or heating block.

(*Note: The protocol has been modified slightly from the manufacturers' recommendations.*)

1. Select 2-3 symptomatic leaves and excise 100mg of midribs and petioles in a sterile petri dish using a sterile scalpel blade. Cut sample into small pieces and place in a 2 ml screw-cap tube. Flame sterilize blade with ethanol between each sample.
2. Place 3 glass beads (2.5mm diameter) into each tube. Tightly close the cap and flash freeze in liquid nitrogen. Homogenize for 60 seconds using the Mini Bead-Beater. Put tubes on ice while homogenizing the next sample. Repeat homogenization 2 more times. Alternatively, one can use a mortar and pestle to grind samples with liquid nitrogen and then place the ground sample in tubes. Make sure to remove the toweling under the mortar between samples and decontaminate the mortar and pestle, as well as the device used to move the sample from the mortar to the tube, between each grind. Decontaminate by washing, followed by soaking in a 10% bleach solution for 30 minutes, and finally rinsing well to remove the bleach solution).
3. Add 400µ1of buffer AP1 and 4µ1 of RNase A to each tube, mix by vortexing and incubate at 65°C for 10 min. Mix tubes 2-3times by inverting during incubation.
4. Add 130 µ1 of buffer AP2 to each sample, mix well by vortexing and incubate on ice for 5 min.
5. Centrifuge samples for 5 min at full speed (14,000 rpm).
6. Transfer supernatant to QIA shredder spin columns (lilac colored tubes) placed in 2ml collection tubes and centrifuge for 2 min at max. speed.
7. Transfer flow-through fraction into a new 1.5 ml tube (try not to disturb pellets formed at the bottom of the tubes). Usually about 450 µl is recovered. Note volume transferred for next step.
8. Calculate and add 1.5 volumes of AP3/E buffer to each sample (for example, for 450 µl of flow-through add 675 µl of AP3/E). Immediately mix well by carefully pipetting up and down.
9. Pipette 650ul of sample onto a DNeasy spin column (white columns) and centrifuge for 1 min at 8000 rpm. Discard flow–through and repeat with any remaining sample. (Hazardous waste note: The flow-through contains AP3/E buffer with guanidine hydrochloride that is hazardous. Please, discard this fraction according to your lab hazardous waste regulations).

10. Place DNeasy columns into new 2ml collection tubes.
11. Add 500 µ1of Buffer AW and centrifuge for 1 min at 8000 rpm. Discard flow through.
12. Add 500 µ1of Buffer AW and centrifuge for 2 min at 14000 rpm. Make sure membrane is dry, if not return to centrifuge for an addition 30-60 sec. Discard flow- through and keep the column.
13. Place the column in a new 1.5ml tube (you may have to cut off the tube cap to close your centrifuge). Pipette 50µl of pre-heated (65ºC) sterile distilled DNase and RNase free water directly to the membrane at the bottom of the column and take care not to punch through the membrane with the pipettle tip. Incubate for 5 min at room temperature. Spin for 1 min at 6,000 x g (8,000 rpm). Discard the column and keep the flow-through (this is your DNA). You may want to do a second elution with 200µl of water.
14. Pipette the flow-through (total plant DNA extract) to a new, properly labeled 1.5ml tube and store it at -20ºC.

3) <u>DNA extraction from fungal cultures</u>

The DNA extraction protocol used below is a modified version of the protocol described by: Bentley *et al*. (1994) for Optimization of RAPD-PCR fingerprinting to analyse genetic variation within populations if *Fusarium oxysporum* f. sp. *cubense*. *Journal of Phytopathology* **142**, 64-78.

Notes

- DNA extraction should be carried out in an area which is physically separated from areas used for PCR preparation and PCR analysis, using equipment dedicated to extraction only
- Pre-warm extraction buffer at 37ºC
- Pipetting for steps 6-10 should be carried out in a fume cupboard

1. Culture single-spored isolates of *Fusarium oxysporum* f.sp. *cubense* on carnation leaf agar for approx 4-5 days.
2. Under aseptic conditions, cut out 4 agar plugs (cubes of 1cm2) from the growing edge of the culture and transfer to 250 mL Erlenmeyer flasks containing 200 mL of quarter-strength potato dextrose broth. Inoculated broths should be incubated at room temperature for 4-5 days (no longer than 7 days), shaken gently once each day.

3. Harvest mycelium by filtration through a clean glass funnel lined with Miracloth. Scrape together mycelium and store at -20°C until ready to extract DNA. Alternatively, lyophilise and store at -70°C for long-term storage.
4. Pre-warm the extraction buffer in a 37°C water bath.
5. Grind frozen mycelium (100-200mg) to a fine powder in liquid nitrogen, using a clean mortar and pestle. Transfer ground sample to a 2mL tube and add 1mL of extraction buffer. Mix by inversion and vortex lightly. Incubate in water bath at 37°C for 2 hours.
6. Add 1mL of phenol (cold), mix by inversion (20 times). Centrifuge samples in a micro centrifuge at 14000g, for 30 minutes (at 4°C).
7. Transfer upper phase to clean, labelled 2mL tube. Add 1mL of phenol (cold), mix by inversion (20 times). Centrifuge samples at 14000g, for 30 minutes (at 4°C).
8. Transfer upper phase to clean, labelled 2mL tube. Add 1mL of chloroform: IAA (cold), then mix by inversion (20 times). Centrifuge samples at 14000g, for 30 minutes (at 4°C).
9. Set up one 2 mL tube for each sample that contains:
    a) 1.2 mL of ice cold 100% ethanol.
    b) 60 µL of 3M sodium acetate (pH 5.4)
10. Transfer upper phase to pre-setup tubes containing ethanol/sodium acetate. Mix by inversion (10 times). Allow DNA to precipitate in ethanol solution overnight at -20°C (or 40 minutes at -80°C).
11. Centrifuge samples at room temperature at 14000g, for 30 mins.
12. Aspirate ethanol solution by carefully pouring off. Add 500µL of 70% cold ethanol.
13. Centrifuge samples at 14000g, for 10 minutes (room temperature).
14. Carefully pour off ethanol, then dry pellet using vacuum desiccator for 15 minutes.
15. Re-suspend pellet in 200µL of sterile dH2O or TE Buffer, then store at -20°C.

4) DNA extraction from banana plant tissue

DNA can be extracted efficiently and reliably from banana plant tissue (usually discoloured vascular strands) using the NucleoSpin® Plant DNA Extraction kit. The NucleoSpin® Plant kit offers an optimised system for the isolation of genomic DNA from plant tissue samples that contain phenolic compounds and polysaccharides (which are known inhibitors of PCR). The NucleoSpin® Plant DNA Extraction kit is reliable, fast and easy to use.

Before starting:

1. Prepare buffer C4: Transfer the total contents of buffer C2 to buffer C3 and mix well. The resulting buffer (C4) is stable for 4 months at room temperature.
2. Prepare buffer C0: Add the total contents of solution C to powder concentrate C0 and mix well. The resulting buffer (C0) is stable for one year at room temperature. Preheat buffer C0 for 10 minutes at 45ºC, and mix well before use.
3. Prepare buffer C5: Add the given volume of ethanol (indicated on the bottle) to buffer C5 before use.
4. Prepare RNase A: Add the given volume of water (indicated on the tube) to RNase A. Store at 4ºC for up to 3 months.

5) Genomic DNA isolation and purification with NucleoSpin® Plant (lysis buffer C0)

1. Homogenise 100-150mg plant material (vascular strands/corm material) in liquid nitrogen using a clean mortar and pestle.
2. Transfer the ground powder to a clean 1.5mL tube. Add 400µL of preheated (45ºC) buffer C0. Vortex the mixture thoroughly.
3. Add 10µL RNase A and incubate the suspension for 30 minutes at 60ºC.
4. Centrifuge the mixture for 5 minutes at 10000g. Transfer the clear lysate to a clean 1.5mL tube.
5. Add 400µl buffer C4 and 300µl ethanol. Mix by inversion (4 times).
6. Place a NucleoSpin® Plant column into a clean 2mL centrifuge tube and load 700µL of sample.
7. Centrifuge for 1 minute at 10000g. Discard flow through and re-attach column to the same tube. Repeat steps 6 and 7 until all the lysate has passed through the column.
8. Pipette 400µl buffer CW onto the column. Centrifuge for 1 minute at 10000g. Discard flow through and re-attach column to the same tube.
9. Pipette 700µl buffer C5 onto the column. Centrifuge for 1 minute at 10000g. Discard flow through and re-attach column to the same tube.
10. Pipette another 200µl buffer C5 onto the column. Centrifuge for 2 minutes at 10000g. This step removes buffer C5 completely.
11. Place the column in a new 1.5mL centrifuge tube. Pipette 100µl elution buffer CE (preheated to 70ºC) evenly onto the column. Incubate for 5 minutes at room temperature. Centrifuge for 1 minute at 10000g to collect the DNA.
12. Store DNA at -20ºC.

Annex 5. PCR Conditions, Master Mix Composition and preparation

PCR Conditions

Precautions:

- Wear clean gloves when handling all reagents and tubes to avoid contamination.
- Aerosol-resistant plugged tips should be used to prepare amplification reactions to avoid aerosol carryover contamination.
- PCR set-up areas should be physically separated from areas where DNA extraction and/or electrophoresis is performed, in order to minimise the risk of contamination

Notes:

- PCR set-up should be carried out in a designated, "PCR-clean" area, using dedicated equipment. A PCR-clean laboratory coat should be worn. No movement of equipment or reagents should occur between set-up and processing of PCR products, excepting prepared reactions which may travel one way to the template addition laboratory. Backwards movement of racks between the template addition areas to the PCR preparation area must be avoided.
- It is recommended to only set-up one PCR experiment per day. PCR set-up should never directly follow handling of PCR products.
- DAF PCRs are directed by a single oligonucleotide primer; so when setting up your PCR it is necessary to choose one primer (per reaction) from the table above. Each primer yields a unique banding pattern; each of the primers listed in the table above yield fingerprints that enable the discrimination between *Foc* VCGs. For definitive characterisation of an unknown sample it is essential to screen samples and controls with a minimum of 3 primers.
- Reagents, except for the DNA polymerase, should be thoroughly thawed, mixed and pulse-centrifuged to collect contents at the bottom of the tube.
- Preparation of master mixes is recommended when multiple samples are to be analysed; this permits standardisation of reagents across the tubes and minimises pipetting errors. When preparing master mixes, always include two extra tubes for positive and negative PCR controls. Also, always prepare at least 10% more reactions than are required, to account for pipetting errors caused by tip retention during aliquotting.
- A PCR positive control (DNA extracted from a characterised isolate of *Foc*), and PCR negative control (no template) is always required for interpreting the results of the PCR, and are important for troubleshooting PCRs that fail.

a) PCR set-up protocol

PCR Reaction

DNA Fingerprinting PCR			
Reagent	**Final concentration per reaction**	**Volume per reaction (µL)**	**Master Mix for 9 reactions (µL)**
DNA template (25ng/µL)	25ng	1.0	0
Stoffel enzyme (10 units/µL)	3 units	0.3	2.4
Primer (300µM)	15µM	1.0	8.0
dNTPs	200µM	2.0	16.0
10 x PCR Buffer	1x	2.0	16.0
dH_20		13.7	109.6
TOTAL		**20.0**	**152.0**

In a designated PCR set up area:

1. Clean PCR area with 10% bleach
2. Label 0.2mL PCR tubes
3. Assemble and thaw reagents (except for the Stoffel DNA polymerase)
4. Prepare a master mix for your reactions in a 1.5mL tube; a standard mix for 8 reactions is shown in the table above (far right column). Note: always prepare at least 10% more reactions than are required, to account for pipetting error (in this case 9 reactions are prepared).
5. Vortex master mix thoroughly, then spin down briefly.
6. Aliquot 19µl of the master mix into each PCR tube; pipette the mix onto the bottom of the tube.
7. Add 1µL of sterile water to the negative control tube (usually the last tube as this tube stays open the longest during set-up). To ensure a reliable control against contamination during master mix preparation, the negative control tube must remain closed from this point until post-PCR analysis.
8. Transfer the tubes to the template addition section. Where possible, racks should not be removed from the PCR clean section. If this cannot be avoided, the tubes should be transferred to another rack before template addition and the "template-free" rack returned to the PCR clean laboratory immediately.

In a designated template addition area:

1. Using filter tips, add 1µL template DNA to individual PCR tubes, according to your reaction layout. The use of plugged tips at template addition ensures the source of the DNA being tested and prevents cross-contamination from pipette barrels. Open only one PCR tube at a time and close the

lid immediately following template addition. Add 1µL of positive control DNA to appropriate tubes, according to your reaction layout.

Important:

1) Take care to prevent cross-contamination between template tubes and PCR tubes. Opening template tubes with the left-hand and opening PCR tubes with the right hand is good practice in preventing cross-contamination of samples.
2) Close caps on each reaction firmly, zip spin tubes (using a capsule micro centrifuge) to draw reaction components down to the bottom of the tubes.
3) Place tubes in thermal cycler and start program.

b) PCR thermal cycling procedure

1. Thermal cycling should be carried out in a 96-well plate thermal cycler with a hot-lid attachment (ensure the hot-lid function is "enabled" when starting each PCR).
2. Completed reactions may be stored at 4ºC until ready to analyse by gel electrophoresis