BANANA FUSARIUM WILT

Field and laboratory diagnostics training manual

Authors: Altus Viljoen and Diane Mostert

Food and Agriculture Organization of the United Nations

UNIVERSITEIT iYUNIVESITHI STELLENBOSCH UNIVERSITY 1918-2018

Faculty of AgriSciences

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Field and greenhouse diagnostics training manual

Prof. Altus Viljoen
Department of Plant Pathology, Stellenbosch University, Stellenbosch 7600, South Africa

E-mail: altus@sun.ac.za

Dr. Diane Mostert
Department of Plant Pathology, Stellenbosch University, Stellenbosch 7600, South Africa

E-mail: diane@sun.ac.za

Stellenbosch University (SU) is amongst South Africa's leading tertiary institutions based on research output, student pass rates and rated scientists, and is recognised internationally as an academic institution of excellence. The university is home to an academic community of 29 000 students (including 4 000 foreign students from 100 countries) as well as 3 000 permanent staff members (including 1 000 academics) on five campuses. As research partner, SU participates in various international academic networks. The scenic beauty of the Stellenbosch area; state-of-the-art, environmentally friendly facilities and technology; and the visionary thinking about the creation of a sustainable 21st-century institution, makes for the unique character of Stellenbosch University.

This volume contains all the information required for this workshop. This includes means to identify and manage banana Fusarium wilt, and protocols for all the laboratory sessions. Additional material and reprints of scientific publications would be made available for the purpose of the workshop, if required.

Contents

Introduction

Fusarium wilt, caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* (Foc), is considered the most destructive disease of bananas. The disease was discovered in Australia in 1876, but became prominent when it destroyed the international banana export industry, based on Gros Michel bananas, in the 1900s. The Fusarium wilt epidemic was brought under control when Gros Michel bananas were replaced with Cavendish cultivars; a banana variety that is not affected by the Foc strain that caused the Gros Michel demise (Foc race 1). Foc race 1 also does not affect cooking bananas and plantains grown on the Africa continent. Since 1990, however, Cavendish bananas became severely affected by a different strain of Foc in tropical Asia, called Foc TR4. Two decades after its discovery, Foc TR4 has been detected in several countries outside Asia, including northern Mozambique. The ability of African cooking bananas and plantains to resist Foc TR4 is largely unknown.

Foc comprises three races and 24 vegetative compatibility groups (VCGs). Races refer to Foc strains that affect a specific group of banana cultivars, and VCGs to strains that can anastomose (fusion of cells from different strains, but not their nuclei). Nine VCGs of Foc are present in East and Central Africa (ECA), of which VCGs 0124 and 0125 are widespread (Karangwa, 2015). Fusarium wilt is moved with infected planting materials (Stover, 1962), and their occurrence in Africa is strongly related to the distribution of susceptible banana cultivars (Blomme *et al*., 2013). The disease is therefore mostly observed in areas where susceptible sweet bananas are grown. Foc TR4 is found only in northern Mozambique where it was introduced in 2013. This strain is, however, expected to spread. It is also expected to affect both dessert and cooking bananas on the continent.

Two major strategies are available to mitigate Fusarium wilt of banana: prevention and the use of resistant varieties. Fusarium wilt can be prevented by the planting of tissue culture bananas, and by implementing phytosanitary practices supported by empowered regulatory authorities. The planting of tissue culture bananas is possible for large-scale commercial growers, but seldom for small-scale and subsistence growers who cannot afford them and are forced to establish new banana fields by using suckers. For these farmers, resistant varieties, if available, are the only option to continue farming bananas. The occurrence of Foc TR4 in northern Mozambique, and its subsequent detection in a small grower field and in Mayotte Island of the African east coast, suggest that Foc TR4 has already spread beyond the borders of the contaminated farms in northern Mozambique. This information gap requires regional surveillance, increased awareness, and the capacity to identify and manage the fungus on a national and regional basis to enable farmers, NARS and government officials to rapidly respond to the spread of the fungus.

A brief history

Banana Fusarium wilt was first discovered in Australia by Bancroft in 1874, with reports of disease outbreaks in tropical America following soon thereafter (Costa Rica and Panama in the 1890s). Because of its early discovery in Panama, the damage it caused to plantations in the country and the unknown nature of its cause, the disease became popularly known as Panama disease. The disease-causing agent was isolated by Dr Erwin Smith after inspecting a Cuban sample in 1910, and the responsible fungus named *Fusarium cubense*. Brandes (1919) who first demonstrated its pathogenicity to banana when he inoculated bananas in steam-sterilised soil infested with the fungus. Panama disease did not attract much further research attention until a severe epidemic broke out in tropical Latin America subsequent to the establishment of vast monoculture plantations of the highly prized 'Gros Michel' banana for export. Three decades after its isolation the pathogen was recognised to be a variant of the ubiquitous *F. oxysporum* species, and was renamed *F. oxysporum* f. sp. *cubense*.

It is believed that the introduction of Foc into Africa and the Americas is linked to the dissemination of planting material, since the pathogen is often introduced into new areas with infected rhizomes free of visual symptoms. The fungus might have been introduced into the West Indies with the Silk variety that came from South India, and from there spread to Central and South America at the time when Gros Michel was widely planted for export in the area. Between 1926 and 1959 the export trade in Central and South America was well established and successful, but suffered huge losses due to the rapid spread of Foc and the susceptibility of Gros Michel (Fig. 1). Gros Michel was eventually replaced with resistant Cavendish cultivars during the 1960s. Cavendish cultivars, though, were soon found to be susceptible to Foc in the subtropics. They succumbed to the disease in the Canary Islands in the 1920s, followed by reports of Fusarium wilt in South Africa (1940s), Australia (1950s) and Taiwan (1970s). A devastating new strain of Foc was discovered in Cavendish plantations in Indonesia and Malaysia in 1990. This strain, called Foc TR4, spread to northern Australia in 1997, and was thereafter reported from Taiwan, the Philippines and China. After being confined to Asia for almost two decades, Foc TR4 has spread to the Middle East and Mozambique.

Based on pathogenicity to different banana cultivars, three races of *Foc* have been recognised. Race 1 causes disease of Gros Michel (AAA) and Silk (AAB) cultivars. Race 2 attacks Bluggoe (ABB), and race 4 infects Cavendish (AAA) cultivars and most cultivars susceptible to Foc races 1 and 2. Foc race 4 is further subdivided into tropical and subtropical strains, the former attacking Cavendish banana in the tropics while the latter attacks Cavendish bananas grown in the subtropics, where cold winters are thought to predispose these cultivars to infection. Race 3 has been omitted as a pathogen of banana, as it only attacks *Heliconia* spp. All evidence on the genetic diversity and ancestry of Foc points towards

an Asian origin for the banana pathogen, as most isolates of the pathogen from outside Asia are related to the Asian population. However, independent evolution could also have occurred outside this centre of origin, with a variant of the fungus isolated from a specific geographical area in Malawi being genetically distinct from other isolates of Foc.

Fusarium wilt can be found in all banana-producing countries of the world, except the South-Pacific islands, parts of Melanesia, and countries around the Mediterranean Sea and Somalia. Most recently the disease was reported from New Guinea and Yap in the Federated States of Micronesia. Once the fungus is introduced into a disease-free plantation, it can spread with contaminated irrigation water and soil attached to implements, shoes and vehicles. Heavy rainfall can lead to increased spread of the pathogen from plant to plant and from the surface down to the roots. The run-off water may contaminate the irrigation reservoirs and increase the spread of the fungus through the plantation.

Since the discovery of Fusarium wilt of banana, various control methods have been attempted to curb the damage caused by the disease. Yet, no long-term control measures are available other than the planting of resistant cultivars. Soil fumigation, fungicides, crop rotation, flood-fallowing and organic amendments are some of the control strategies that were investigated in the past (Fig. 1). Studies on biological control and soils that are naturally suppressive to Fusarium wilt of banana due to beneficial micro-organisms have yielded unconvincing results. Many effective biological control agents can be found for Fusarium wilt diseases of other crops, which make biological control a promising option for the integrated management of Fusarium wilt of banana. Current practices for the prevention of Fusarium wilt of banana include the use of disease-free tissue culture plantlets and proper sanitation methods. The treatment of vehicles, machinery, tools and footwear with effective surface disinfectants is particularly important. In fields where Foc is already present, the planting of resistant cultivars is essential, if such cultivars are acceptable to the local markets.

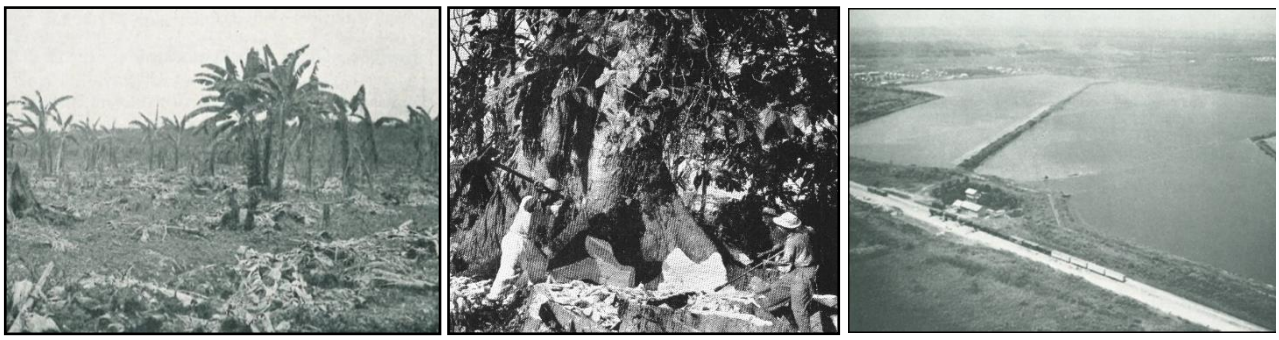


Figure 1. Fusarium wilt destroyed thousands of hectares of Gros Michel bananas in Central America during the 1900s. This led to the destruction of virgin forests to free up new land for planting, and the flooding of infested fields to eradicate the fungus.

Symptoms

Banana plants with Fusarium wilt can be identified by the conspicuous yellowing and wilting of older leaves (Fig. 2A), which progress to the youngest leaves until the affected plants are killed (Fig. 2B). The disease should, however, not be identified only on external symptoms only, as other biotic (bacterial wilt, Armillaria, banana weevil) and abiotic (nutrient deficiency, water-logging) stresses of banana can result in leaf yellowing and wilting of banana plants. For this reason, Fusarium wilt in suspect plants needs to be identified by also inspecting internal symptoms in the pseudostem and rhizome, and by isolating Foc from affected tissue.

External symptoms

The most characteristic symptom of banana Fusarium wilt is chlorosis (yellowing) of older banana leaves that progresses upward. The yellowing most often starts on the leaf margins, from where it progresses to the leaf midriff (Fig. 2A). In some banana varieties, such as Cavendish bananas affected by Foc race 4, the chlorosis is followed by necrosis (browning caused by death of leaf tissue). In others, such as Pisang Awak, the browning of affected leaves are seldom observed. In Gros Michel affected by Foc race 1, yellowing might not be noticed on affected leaves (Fig. 3). Despite the variety involved, two more indicators are always associated with leaves affected by Fusarium wilt: the symptoms progress upwards to the younger leaves, and the petioles buckle and hang down the pseudostem.

A second external symptom often linked to banana Fusarium wilt is the splitting of the pseudostem (Fig. 2C). The splitting is caused by the inability of dead leaf bases to expand as the plant grows, thus splitting open as the inner pseudostem swells. Pseudostem splitting is not always associated with Fusarium wilt, and there might be other causes for the splitting of pseudostems too. This symptom, therefore, has to be considered along with leaf yellowing and wilting in suspect plants.

Internal symptoms

Fusarium wilt results in very characteristic internal symptoms in the rhizome and pseudostem, irrespective of the cultivar affected. When pseudostems are cut through horizontally, reddish- to dark-brown lesions can be spotted inside the leaf bases (Fig. 2D). The bunch stalk, however, will always be clean of such symptoms. It is always advisable to split the pseudostems longitudinally through the discoloured lesions, as their progression through the vascular tissue then becomes clear. The youngest infection in banana pseudostems is often yellow, and later becomes dark red; both limited to the xylem vessels only. This is the best material to collect for the isolation of Foc. The older the infection becomes, the more expanded and darker the lesions will become, and such lesions might be co-colonised by secondary

contaminants. Despite the age of lesions, it is important that they are continuous, which separates internal symptoms caused by Foc from those caused by other biotic stresses.

When external symptoms are visible but internal symptoms are absent from the pseudostem, it becomes important to inspect the rhizome. The plant then has to be cut open at soil level to expose the pseudostem base. Diseased plants will have a very characteristic yellow to dark-red discolouration of the inner rhizome that starts at the edges and progresses inwards (Fig. 2E). Very often only part of the inner rhizome is affected, but as the disease progresses, it might affect the entire inner rhizome. The outer rhizome is never affected. If the pseudostem is pushed over once the rhizome had been split open (not cut), the inner rhizome will display yellow strands that are attached to both the top and bottom halves of the rhizome. When no discolouration is observed, the external symptoms were caused by something different than Foc. In such cases, the inner rhizome might display black spots instead of the continuous yellow to reddish-brown discolouration associated with Fusarium wilt.

Figure 2. Disease symptoms of banana Fusarium wilt. Banana leaves become yellow (A) and plants eventually die (B). In some cases, the base of pseudostems splits open (C). Internally, the vascular bundles in the pseudostem will turn yellow to reddish-brown (D), while a deep golden discolouration of the inner rhizome develops (E).

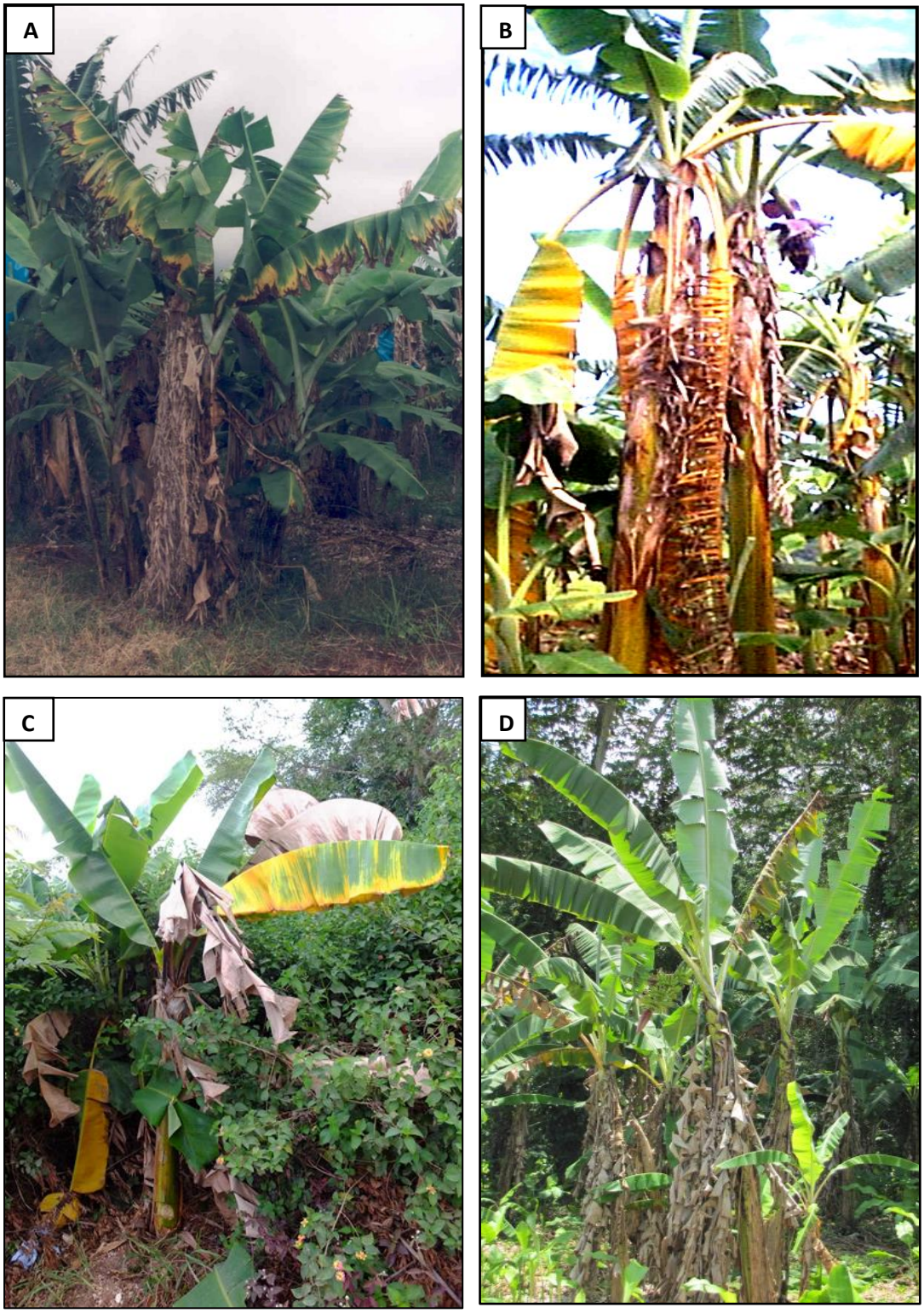


Figure 3. Disease symptoms of Fusarium wilt on different banana varieties: (A) Cavendish bananas in South Africa, (B) Pisang Awak in Uganda, (C) Pisang Awak in Mozambique, and (D) Gros Michel in Costa Rica.

Sample collection

Taking a sample from the diseased host plant

Samples for the identification of the cause of Fusarium wilt should be taken from the inside of the pseudostem of wilted banana plants where continuous discoloured vascular strands are evident (Fig. 4). The sample should be taken from as low in the pseudostem as possible, but not from areas where decay is advanced. Also, the sample should be taken from as close to the centre of the pseudostem as possible, as opposed to the outermost leaf bases. As banana tissue is very wet, the risk of bacterial contamination of samples is high, particularly in warm weather, and samples can 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 until the strands can be excised. Avoid plastic bags as this causes the samples to sweat and promotes growth of bacteria. Accurate notes must be taken for each sample where applicable, 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.).
- Age of plant/plantation.
- Whether plants sampled are grown in a garden, commercial plantation, village or the wild.
- Size of the diseased area where the plant was collected, with photos.
- Location (e.g. name of province/state, how far and in what direction from nearest town, name of road, name of property if sample is from a commercial plantation). A map with sample numbers marked on it and GPS coordinates are essential.
- Collector's names, and the required phytosanitary certificate/importation permit.
- Other useful observations might include the source of the planting material, whether the plant is growing in water-logged soil, how many plants are affected, what other varieties are growing in the vicinity and are these diseased or healthy?

A small (5x5 cm) piece of rhizome tissue showing typical discoloured vascular strands may be used as a sample, but this is not recommended if decay in the rhizome is advanced. This piece of rhizome tissue should also be wrapped in paper or placed in a paper envelope to dry.

Note: When looking for wilt-affected plants, it is better to take samples from established plantings of bananas rather than recently planted young plants.

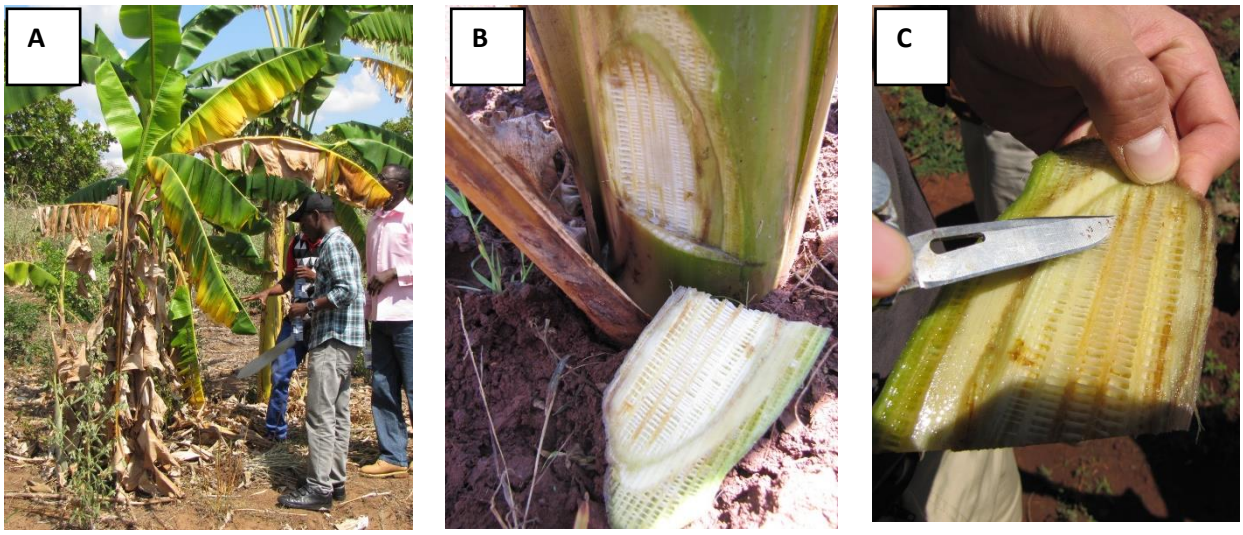


Figure 4. Vascular strands are collected from banana pseudostems with typical Fusarium wilt symptoms (A). This can be achieved in a non-destructive way by (B) slicing open part of the pseudostem on the side of yellow leaves, and by (C) dissecting out discoloured xylem tissue.

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 techniques should be applied to the dissection of strands. Samples should first be surface-sterilised by wiping with 70% alcohol or surgical spirits. Where several samples are being prepared, a fresh piece of blotting paper should be used for each sample, and scalpel blades should be flamed or at least wiped with 70% alcohol between samples. The excised strands 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 OK. They do not need to be wrapped in moist paper like leaf specimens – dry paper is best.

Posting of samples

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 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.

Pathogen isolation

The banana Fusarium wilt fungus

Foc, the banana Fusarium wilt fungus, is a soil-inhabiting filamentous fungus that belongs to the section Elegans in the genus *Fusarium*. In culture it can produce a surprising range of colours, textures and aromas, but nothing that distinguishes the banana pathogen from *F. oxysporum* strains causing disease to other crops. On potato dextrose agar, colony morphology can range from sparse to cottony growth and the colour from deep purple to cream to peach. Certain members of Foc are able to produce volatiles and a star-like growth. The fungus produces three spore types, which include: macroconidia, microconidia and chlamydospores.

The macroconidia are nearly straight, slender and thin-walled with three to four septa, a foot-shaped basal cell and a curved tapered apical cell. They are produced from short phialides on conidiophores. Microconidia are one- or two celled, oval- to kidney-shaped and are produced in false heads. Chlamydospores are usually globose and are formed singly or in pairs in hyphae or conidia. They are resistant to desiccation and unfavourable environmental conditions, and enable the fungus to survive for more than 30 years in the soil after their host has been removed. In the presence of roots, chlamydospores or conidia germinate and penetrate susceptible plants.

Foc is believed to have co-evolved with its *Musa* hosts in Southeast Asia and from there it was introduced into new banana-producing countries by the movement of infected planting material. In studies of global collections of Foc, two major groups of the Fusarium wilt fungus were identified: one group generally associated with banana cultivars with partial B genomes (i.e. at least one chromosome derived from *M. balbisiana*; e.g. Lady finger AAB and Bluggoe ABB), and the other associated with banana cultivars with pure A genomes (i.e. all chromosomes derived from *M. acuminata*; e.g. Cavendish AAA and Gros Michel AAA). These groups are evidence for independent evolutionary origins of Foc in Southeast Asia. One VCG of Foc, VCG 01214, may have originated outside the Indo-Malayan region. Isolates of this VCG affect ABB cooking bananas in northern Malawi, and are genetically distinct from other Foc lineages/VCGs. Foc isolates are further divided into distinct lineages with clusters of closely related VCGs, even when they are distributed over a broad geographic area. These relationships have been documented with RAPDs, RFLPs, AFLPs, electrophoretic karyotypes and multi-gene phylogenies, and suggest a clonal reproductive strategy for this pathogen.

Isolating the fungus from discoloured vascular strands

Isolations can be attempted when the strands have dried. Small sections (3-6 mm long) of dry discoloured vascular strands are placed on ½ strength potato dextrose agar (PDA) medium

amended with an antibacterial agent (e.g. streptomycin @ 1.2 mL/240 mL PDA). If present, Fusarium growth will appear from the strands in 2-4 days (Fig. 5). 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. Single-spore (monoconidial) cultures should be prepared from an isolate from each specimen.

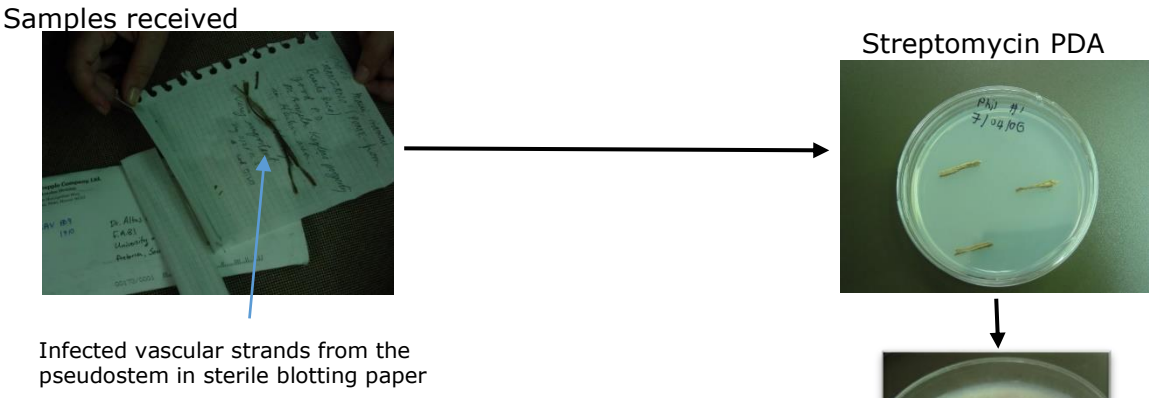
Single-sporing of isolates

Single-spore isolates of *F. oxysporum* are obtained from spore suspensions (Fig. 5). Firstly, a small scrape of sporulating hyphae are collected from cultures grown on ½-strength PDA plates, and dissolved in 10-mL sterile distilled water in test tubes. From the initial spore suspension, a series of dilutions can be prepared. One ml of each of the dilution series is then pipetted onto water agar, and the water agar plates incubated upside-down overnight at 25^{o}C. The plates are viewed for germination of conidia under a dissecting microscope the following morning, and single-conidia cut from the water agar with a surface-sterilised scalpel and transferred to new 90-mm ½-strength PDA plates. Additionally, single-spore cultures can also be obtained by dissecting the very tip of single growing hyphae from an older culture grown on carnation leaf agar (CLA).

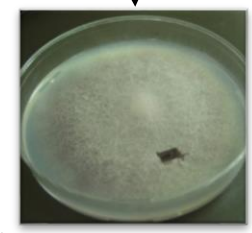
Maintenance of healthy cultures

Healthy cultures of Foc from single spores are maintained on carnation leaf agar (CLA). Cultures can be initiated on weak-strength PDA medium (e.g. ½ strength) (Ainsworth, 1971) to check the morphology of cultures for taxonomic purposes or for spore production (Fig. 5). Healthy (sporodochial-type) cultures of Foc growing on PDA medium exhibit abundant fluffy aerial mycelium after 2 days and produce abundant microconidia. Some macroconidia 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 (Nelson *et al*., 1983, Windells, 1992). 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.

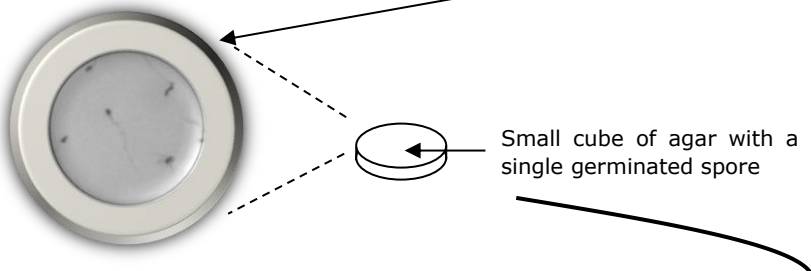
1. *Isolation from plant material.*



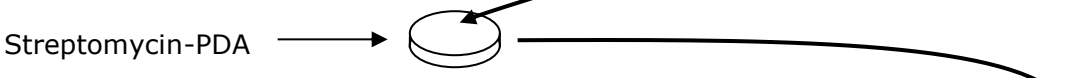
2. *Sub-culture small areas of good Fusarium growth onto Streptomycin PDA. Use these cultures for single-sporing (need to grow for 2-3 days to be sure of healthy culture).*



3. *Place a spore suspension onto water agar.*



4. *After 24 hours take at least 2 single germinated spores and place back onto Streptomycin-PDA.*



5. *Once single spore cultures show normal Fusarium growth (should be visible after 4-5 days), choose only 1 single spore culture to represent each isolate and discard all other cultures. Assign this culture with a unique accession number. Subculture this isolate onto CLA, which will become the source for morphological, molecular and VCG identification, pathogenicity testing and storage.*

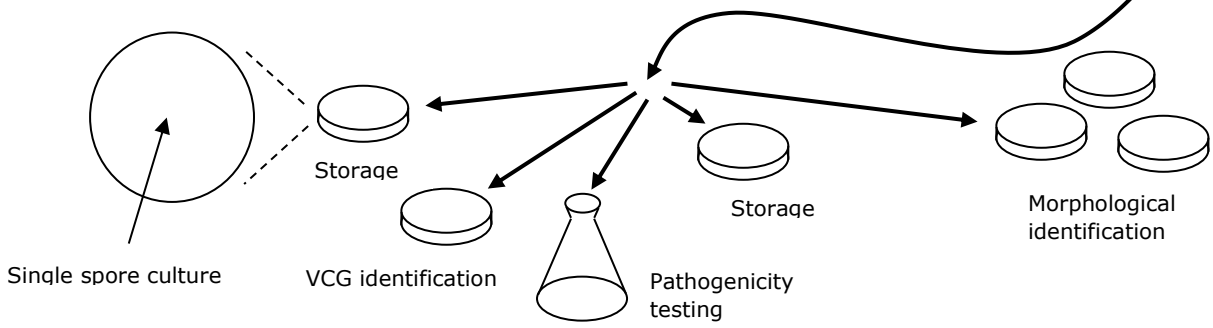


Figure 5. Isolation of *Fusarium oxysporum* f. sp. *cubense* from diseased banana tissue.

Pathogen identification

The banana Fusarium wilt fungus is a member of the *Fusarium oxysporum* species complex (FOSC) in the genus *Fusarium*. In culture the fungus produces a surprising range of colours and forms, but nothing that distinguishes it from *F. oxysporum* causing disease to other crops. On potato dextrose agar, colony morphology can range from sparse to cottony growth and the colour from deep purple to cream to peach. The fungus produces three spore types, which include macroconidia, microconidia and chlamydospores. The macroconidia are nearly straight, slender and thin-walled, with three to four septa, a foot-shaped basal cell and a curved tapered apical cell. They are produced from short phialides on conidiophores. Microconidia are one- or two celled, oval- to kidney-shaped and are produced in false heads. Chlamydospores are usually globose and are formed singly or in pairs in hyphae or conidia.

Steps involved in laboratory diagnosis of Fusarium wilt of banana

- Receive specimen, log details and observations from grower/inspector.
- Isolate from symptomatic tissue (usually 2x Streptomycin-amended PDA plates with four pieces per plate).
- Check morphology of resultant growth (macro- and microscopically).
- Subculture *Fusarium* colony to make spore suspension and plate onto water agar (WA).
- Select 2x germinated single spores to initiate monoconidial cultures.
- Assign unique accession number to isolate and record in specimen book and *Fusarium* isolate database.
- The mono-conidial culture is used to:
  a) Inoculate 2x PDA plates for cultural identification
  b) Initiate 2x carnation leaf agar (CLA) plates for morphological identification
  c) Inoculate 3x chlorate medium (CM) plates to generate *nit* mutants for VCG tests
  d) Inoculate 1x PDA plate for DNA analysis
  e) Initiate 1x PDA plate for long-term storage
  f) If necessary, prepare CLA slants for medium-term storage
  g) If necessary, arrange for lyophilisation of isolate for long-term storage in collection
- Conduct and record results of volatile and VCG analysis in database.
- Return written reply to grower/inspector concerned using a Plant Disease Report form, recording the date and details of reply in specimen book (usually phone results also).
- Maintain isolate collections and records in database.

Cultural and morphological characteristics of *F. oxysporum* f. sp. *cubense* (Fig. 6)

- Produce abundant microconidia that are single-celled, oval to kidney-shaped.
- Microconidia are produced in false heads on branched and unbranched monophialides.
- Macroconidia are sickle-shaped with an attenuated apical cell and foot-shaped basal cell.
- Chlamydospores are present and formed singly or in pairs.
- No perfect (sexual) stage of *F. oxysporum* is known.
- On PDA, fungal colonies produce white aerial mycelia that may turn purple in the centre. Isolates may differ in their cultural morphology.
- Cream to orange sporodochia are formed on carnation leaves on CLA.

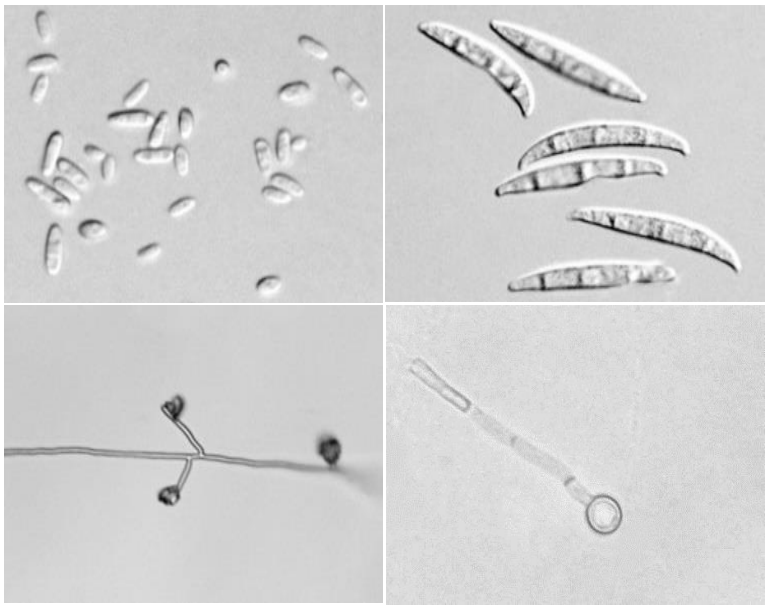


Figure 6. Morphology of *Fusarium oxysporum* f. sp. *cubense*: A. Microconidia, B. Macrocondidia, C. Production of microconidia in false heads on short monophialides, and D. Chlamydospores formed at the end of a hypha.

Vegetative compatibility testing

Vegetative compatibility is a trait of filamentous ascomycetes governing non-self recognition during vegetative growth. This is achieved through a process called anastomosis, in which two individuals' hyphae are fused to form a heterokaryon (Leslie and Summerell, 2006). These individuals must have identical alleles at all their *vic* loci to be compatible. To date, 24 VCGs or VCG complexes have been described in Foc, which is an unusually large number compared to other *formae speciales* of *F. oxysporum* (Katan, 1999; Katan and Di Primo, 1999). Of these, VCG 01214 is found exclusively found in Malawi, and VCG 01212 exclusively in east Africa.

1. Generation of nitrate non-utilising (*nit*) mutants

Cultures growing on CLA or PDA medium are used to inoculate plates with a medium containing potassium chlorate. Potassium chlorate is an analogue of nitrate, and through the nitrate reductase pathway 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 result. After 5 to 12 days, fast-growing sectors begin to emerge from the restricted colonies (Fig. 7). 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 (2x2 mm) of mycelium is taken from the advancing edge of the sector. This is then transferred to a medium that contains nitrogen only in the form of nitrate, such as Minimal Medium (Puhalla, 1985). If the sector is a true *nit* mutant it will not be able to reduce the nitrate in the medium and characteristically 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.

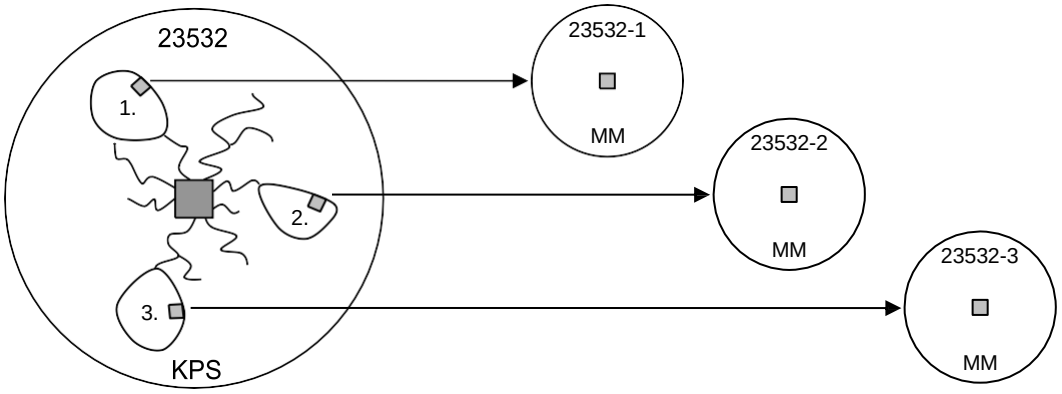


Figure 7. Fast-growing sectors emerge from a restricted colony of *Fusarium oxysporum* f. sp. *cubense* on KPS medium. Mycelium from the advancing edge of each sector is transferred to Minimal Medium to test its ability to reduce nitrate.

It is advantageous to let the sectors grow for 2-3 days after emerging on KPS plates so that the fast-growing mycelia grows clear of any non-mutated mycelia which may be underneath. Each restricted colony growing on KPS medium may yield up to five separate sectors. 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 (Fig. 7).

2. Phenotyping of *nit*-mutants

Some *nit* mutants are more reliable than others for use in VCG tests. The phenotype of the *nit* mutant can be determined by the type of growth (sparse, nitrogen-deficient or dense, nitrogen-sufficient) when the *nit* mutant culture is transferred to media that has nitrogen present in one of four forms (Correll *et al*. 1987). It is advantageous to generate several (at least four or five) *nit* 1 or *nit* 3 mutants from each isolate to pair in combinations with the Nit M testers (mutants of known VCG). Mutants of the *nit* 1 or *nit* 3 phenotypes are the most commonly generated type of mutant. Mutants of the Nit M phenotype are less commonly generated and are best used as the 'testers' of known VCG.

3. Pairing of *nit*-mutants

A small (2x2 mm) piece of colonised agar from a culture of a Nit M mutant of a known VCG is placed in the centre of a plate of MM. The bottom of the plate is labelled with the number of the VCG that this Nit M represents. Similar small pieces of culture of the *nit* mutants that have been generated from the isolate of unknown VCG are then placed at least 10-15 mm away from the piece of Nit M culture around the edge of the plate (Fig. 8). These must also be labelled on the bottom of the plate (using permanent ink that will not dissolve!). Labelling the base of the plate before transferring the *nit* mutants saves time and avoids confusion. If you think you have placed a mutant in the wrong position or mis-labelled a plate, discard it and start again.

If the isolate of an unknown VCG has the accession number 23532, the *nit* mutants that are generated from this isolate would be numbered 23532-1, 23532-2, 23532-3 and 23532-4. If these mutants are paired with Nit M testers representing VCGs 0120, 0124 and 0129 on MM plates, the finished VCG test plates would look like this:

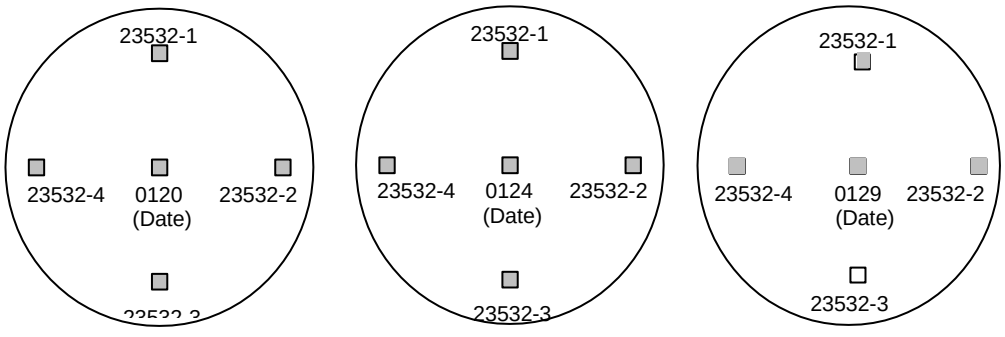


Figure 8. Pair-wise arrangement of four *nit* mutants from an isolate of unknown VCG (isolate number 23532) with Nit M testers representing VCGs 0120, 0124 and 0129. Nit M testers are placed in the centre of the plate with the *nit* mutants around the edge.

The paired plates are kept in an incubator at 25°C and checked every 2 days for the formation of heterokaryon growth. If a heterokaryon is going to develop (i.e. if the isolate is vegetatively compatible with one of the Nit M testers) a line of dense nitrogen-sufficient growth will start to form in 7 to 12 days where the hyphae of the *nit* mutants meet the hyphae of the Nit M mutant, representing the VCG to which that isolate belongs. If no line of heterokaryon growth is evident by 12-14 days, the isolate is said not to belong to that VCG. For example, isolate 23532 belongs to VCG 0124, and in 7 days the paired plates would look typically like this (Fig. 9):

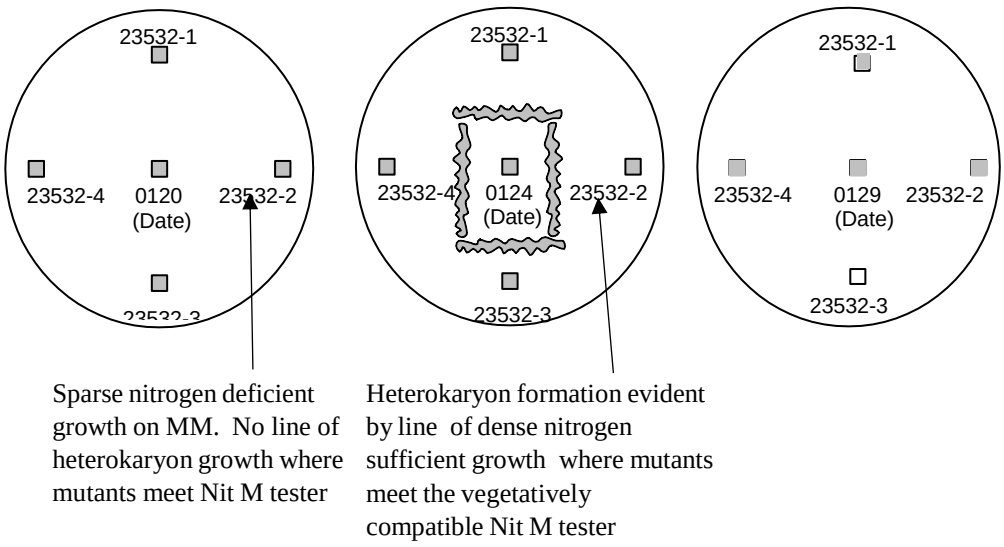


Figure 9. Example of a positive VCG test. Heterokaryon formations between the *nit* mutants of isolate 23532 and the Nit M tester representing VCG 0124 indicate that isolate 23532 belongs to VCG 0124.

Please keep an accurate, centralised record of all VCG tests performed, including the dates and which of the *nit* mutants tested produced heterokaryons with which Nit M testers. Grids for recording the results of VCG tests can easily be drawn up and copied for use. VCG test results, along with accurate information on the host variety, location, the grower's and specimen collector's names and other information can be easily maintained in an electronic database (e.g. Microsoft Access), which is easy to set up and add information to as specimens are received and VCG tests are performed.

Table 1. Vegetative compatibility groups, races and origins of strains of *Fusarium oxysporum* f. sp*. cubense*.

VCG	Race	Origins
0120/01215	1, 4	Australia, Brazil, Costa Rica, France (Guadeloupe, Guiana), Honduras, Indonesia (Java), Jamaica, China, Malaysia (Sarawak), Nigeria, Portugal (Madeira), South Africa, Spain (Canary Islands), Taiwan, USA (Florida)
0121	4	Indonesia (Sumatra, Kota), Taiwan
0122	4?	Philippines
0123	1	Malaysia (peninsular and Sarawak), Philippines, Taiwan, Thailand, China
0124/ 0125/ 0128/ 01220/ 01222	1, 2	Australia, Brazil, Burundi, China, Cuba, Democratic Republic of Congo, Haiti, Honduras, India, Jamaica, Malawi, Malaysia, Mexico, Nicaragua, Rwanda, Tanzania, Thailand, Uganda, USA (Florida), Zanzibar, Kenia
0126	1?	Honduras, Indonesia (Irian Jaya, Sulawesi), Papua New Guinea, Philippines, China
0129/ 01211	1, 2	Australia
01210	1	Cayman Islands, Cuba, USA (Florida)
01212	?	Tanzania
01213/ 01216	4	Australia, Indonesia (Agam, Dharmasraya, Halmahera, Irian Jaya, Java, Solok Sulawesi, Sumatra, Pariaman, Tanag Datar), Malaysia (peninsular),Taiwan, Jordan, Oman, Mozambique, China, Philippines
01214	None	Malawi
01217	None	Malaysia
01218	None	China, Indonesia (Java, Sumatra), Malaysia (peninsular), Thailand
01219	None	Indonesia (Java, Sumatra)
01221	None	Thailand, China
01223	None	Malaysia
01224	None	Malaysia

Sources: Ploetz and Pegg (2000); Ploetz (2005); Lodwig *et al*. (1999)

[1]Vegetative compatibility groups (VCG), a phenotypic marker used to characterise fungal isolates based on heterokaryon formation (Puhalla 1985)

[2]Isolates in a VCG are compatible with isolates in different VCG, forming a VCG complex

[3]Possible race 4

[4]Race undetermined

[5]Foc TR4

Molecular identification of Foc

Deoxyribonucleic acid (DNA) consist of four nucleotide bases namely adenine (A), thymine (T), guanine (G) and cytosine (C). DNA forms a double-stranded helix with two individual strands running in opposite directions bound together at the core with hydrogen bonds between the nucleotide bases and an outer negatively charged phosphate backbone. Adenine and thymine are joined together with two hydrogen bonds and guanine and cytosine with three. This double helix structure was first observed by Watson and Crick in the 1950s.

DNA isolation can be subdivided into four steps: (1) isolation of cells; (2) cell lysis; (3) removal of protein and RNA and (4) isolation of pure DNA.

Step 1 and 2: Cells are isolated and lysis takes place by both physical and chemical actions. Physically the cells are disrupted with grinding of mycelia in liquid nitrogen as well as heat shocked. Chemically the cells are disrupted by the addition of SDS that lyses the nuclear membrane. SDS is also added to avoid cleavage of DNA by magnesium-independent nucleases that might be present. EDTA is added as a chelating agent to exclude magnesium ions that serve as a cofactor for all common deoxyribonucleases.

Step 3: Phenol and chloroform are used to denature and remove proteins form nucleic acid solution. RNase is added to digest RNA.

Step 4: DNA is concentrated by precipitation with absolute ethanol and NaAc. The re-suspended DNA is lastly washed with 70% ethanol to remove excess salts. This is a very important step in the DNA isolation method because excess salts might interfere with further utilisation of DNA for example with PCR and restriction enzyme digestions. This is because many of these enzymes used in these techniques are salt sensitive.

DNA isolation method

Rapid extraction from plants and fungal cultures

- DNA can be extracted either directly from planting material or from pure culture isolates.
- If extracted from planting material, the plant tissue is crushed to a fine powder in liquid nitrogen using a mortar and pestle. A quantity of 0.1 ml powder is then transferred into a 2-ml micro-centrifuge tube.
- If extracted from pure culture isolates, the fungus is grown for 7 days on ½ strength potato dextrose agar at 25°C. The mycelia is then scraped from the plate with a spatula and placed into a 2-ml micro-centrifuge tube.

- A 500-µl volume of 0.5 M NaOH and glass beads are then added to either the mycelia or ground powder. The volume of NaOH can be increased if the volume seems too small.
- To lyse the cells, the suspension is then shaken in Tissuelyser for 5 min.
- The suspension is then centrifuged for 1 min at 14 000 rpm to pellet the debris.
- Five µl of the supernatant is thereafter diluted in 495 µl of 100 mM Tris-HCL.
- One µl of the diluted DNA will then be used for the PCR reaction.

Conventional DNA isolation

- Cultures are grown at 25°C in potato dextrose broth or ½ PDA for approximately 1 week.
- Mycelia are harvested and ground with a mortar and pestle by using liquid nitrogen.
- Dried mycelia are immediately placed into 1.5-ml Eppendorf tubes and 500 µl of DNA extraction (Reador and Broda, 1985) buffer is added to each tube.
- Samples are heat shocked by either placing isolates in a 60°C water bath for 5 min or incubation for 30 min at 37°C.
- 700 µl of 1:1 phenol-chloroform (see addendum) is added to the Eppendorf tubes and samples are centrifuged at 14 000 rpm at 4°C for 7 min.
- The supernatant is transferred to a new tube and the washing step is repeated until the interphase disappears (at least three times).
- The DNA is precipitated by adding 0.1 volume of 3 M NaAc and three volumes of cold absolute ethanol and centrifugation at 14 000 rpm for 10 min.
- The supernatant is discarded and the pellet is washed by adding 1 ml of cold 70% ethanol. The tube is inverted five times until the white ball appears.
- The samples is left for 5 min and then centrifuged at 10 000 rpm for 5 min. After washing, the 70% ethanol is removed and the samples are vacuum dried.
- The DNA pellet is re-suspended in sterile nuclease-free H_2O.
- An RNase treatment is performed by adding 5 µl RNase (10 µg/µl) to the re-suspended DNA and placing the samples into a water bath at 37ºC for 3 hrs.
- DNA is visualised by agarose gel electrophoresis and ethidium bromide staining.

Agarose gel electrophoresis

Preparation of gels:

- Weigh off 0.6 g of agarose for a 60-ml gel.
- Add 60 ml 1 x TAE and swirl to mix.
- Bring to boil; microwave for approximately 2-3 min or use a hot plate.
- Make sure that the agarose is properly dissolved.

- Let the gel stand for about 5 min or until the solution is cooled to 60°C.
- Add 5 µl ethidium bromide (see addendum) or 0.5 µg/ml, swirl to mix.
- Slowly add to gel tank and leave until it is set.
- Remove the gel comb.
- Pour 1x TAE into the gel tank to submerge the gel.
- Prepare the DNA sample; mix with the appropriate loading dye and load the mixture into the wells. Always use one part of dye to five parts of DNA.
- Load the appropriate DNA ladder.
- Close the gel tank and switch on the power-source. Run for approximately 20 min. DNA is negatively charged and will run towards the anode. Always make sure the gel tank is set up in the correct orientation.
- View gel under UV light or you could say: visualise the DNA under UV light.

Additional information

- Agarose is a polysaccharide isolated from seaweed and is used as a matrix in gel electrophoresis. Movement through the matrix is dependent on both the charge and size of the fragment.
- The percentage agarose gel that needs to be prepared depends on the approximate size of the fragment that needs to be viewed or separated. The lower the gel percentage the bigger the pore sizes of the gel and the larger the fragments that can be separated. The amount of gel that will be prepared will depend on the size of the gel tank.
- Loading dye is mixed with DNA samples before the samples are loaded into the wells. The loading dye has three distinct purposes: it increase the density of the sample, it ensure that the DNA sink to the bottom and it add colour to the sample so that it can be monitored. Bromophenol blue is used for dye.
- Ethidium bromide is a chemical that fluoresces under UV light and intercalates between the bases of DNA. It is ideal to use for staining DNA to fluoresce under UV light.
- DNA ladders are loaded to determine DNA size and approximate concentration.

Essential components of a PCR

A thermostable DNA polymerase. The polymerase is responsible for the template dependent synthesis of DNA.

1. A pair of synthetic oligonucleotides to prime DNA synthesis. The correct design of primers is very important for amplification of the target DNA and to suppress the amplification of unwanted sequences. The primers will bind to the target fragment at opposite ends and amplification will follow in both directions.

2. Deoxynucleoside triphosphates (dNTPs). The dNTPs mixture consists of equal amounts of dATP, dTTP, dCTP and dGTP and it serves as the building blocks for DNA synthesis.
3. Divalent cations (Mg^{2+}). The *Taq* polymerase requires free divalent cations in order to function adding from 1–5 mM. In many instances the Mg^{2+} is already included in the PCR buffer, if not we need to add it separately.
4. PCR buffer, which is added to maintain the pH
5. DNA template

Programming the polymerase chain reactions

A PCR programme consists of three elements: denaturation of the usually double stranded DNA template by heat; annealing of the oligonucleotide primers to the single stranded target sequence followed by primer extension and therefore template synthesis

1. Denaturation of the template is dependent on the G+C content of the target sequence. The higher the G+C content the higher the denaturation temperature that is required and the longer the sequence, the more time for denaturation is required. If denaturation is not completed, the sequence will reanneal when the temperature is lowered. Denaturation of linear DNA is usually carried out at 92-94^{o}C for 45 seconds. This is the highest temperature the *Taq* polymerase enzyme can endure and also efficient temperatures because the average G+C content of linear DNA is 55%. An initial denaturation step is carried out and the time of this initial step is dependent on the length of the target sequence.
2. The annealing temperature that is used to anneal the primers to the target sequence is critical. If the temperature is too high, the primers will not anneal, however if the temperature is to low, non-specific primer annealing will take place. Annealing is usually 3-5^{o}C lower than the melting temperature of the primers ($T_m = 2(A+T) + 4(G+C)$).
3. Extension is usually carried out at temperatures ranging from 72-78^{o}C; this is the optimal temperature for DNA synthesis, catalysed by *Taq* polymerase.
4. Number of cycles required for amplification (25-45 cycles) is dependent on the amount of template DNA that is added to the reaction mixture. Optimisation is required and is dependent on the target sequence and ultimate DNA concentration.

- Proteinase K, phenol and EDTA are common culprits that can inhibit PCR reactions. This problem can be easily overcome by cleaning of the DNA template.
- When one designs PCR primers, there are a few universal rules that needs to be followed, however, even if all the rules are adhered to it does not necessarily ensure the design of successful, usable primers.

Property	Optimal design
Base composition	G+C content should be between 40–60%
Length	18–25 base pairs
Repeated and self-complementary sequences	No inverted repeats or self-complementary sequences of more that 3 base pairs should be used. These sequences could form hairpin structures that will prevent the primer from annealing.
Complementarity between primers	The 3' terminal should not be able to bind to any site on the other primer.
Melting temperature	The melting temperatures of the two primers should not differ more than 5^{o}C. This is important in order to use an annealing temperature that will be effective for both primers.

PCR amplification with Foc race 1 and 4 molecular markers

To identify Foc Lineage VI (race 1):

- Each PCR reaction contains:
  - 12 μL of Kapa ready mix
  - 0.5 μL of 10 mM solution of each primer
  - 2 μL of 20 ng/μl concentration DNA
- This multiplex assay use two sets of primers as forward and reverse primers for the amplification of Foc Lineage VI, respectively:
  - FocLin6bF (5'-CGACAATGAGCTTATCTGCCATT-3') and FocLin6bR (5'-CATCGAGGTTGTGAGAATGGA-3') (Karangwa, 2015)
  - FocLin VI-F (5'-AGGGACTGGATTTCTACCCT-3') and FocLinVI-R (5'-GTGTCACTTGGTCCTCGTAT-3') (Ndayihanzamaso, 2020).
- All reactions are made up to a final volume of 25 μl with sterile nuclease-free H_2O.
- A negative control, containing water instead of DNA template as well as a positive control (CAV 968), a DNA template the will amplify needs to be included in the experiment.
- Programme: Initial denaturation step of 2 min at 95^{o}C, followed 35 cycles of denaturation for 30 seconds at 95^{o}C, annealing at 62^{o}C for 30 seconds and elongation at 72^{o}C for 30 seconds. The PCR is ended off with a final extension step at 72^{o}C for 5 min.
- Successful amplification is confirmed with 1% agarose gel electrophoresis in 1x Tris Acetic acid EDTA (TAE, pH 8.0) and ethidium bromide staining (Fig. 10).

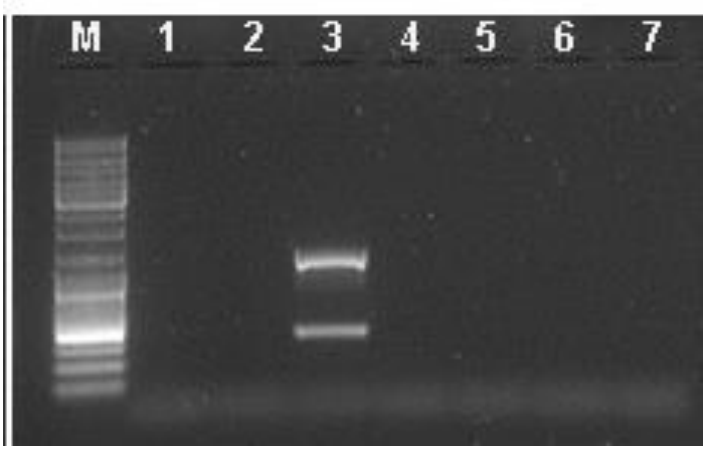


Figure 10. Both 300-bp and 1002-bp fragments of Foc amplified in a multiplex PCR assay. Lanes 1-7: isolates representative of Foc Lineages.

To identify Foc Race 4 (Lin *et al*., 2009):

- Each PCR reaction contains:

  2 μL of 1x Buffer

  1.5 μL of 50 mM $MgCl_2$

  0.6 μL of 10 mM dNTPs

  0.5 μL of 10 mM solution of each primer

  0.3 μL of 5 U/□l Taq

  2 μL of 20 ng/μL concentration DNA
- Foc-1 (CAGGGGATGTATGAGGAGGCT) and Foc-2(GTGACAGCGTCGTCTAGTTCC) developed by Lin *et al*. (2009) are used as forward and reverse primers for the amplification of the Foc Race 4.
- All reactions are made up to a final volume of 20 μl with sterile nuclease-free H_2O.
- A negative control, containing water instead of DNA template as well as a positive control (CAV 789), a DNA template the will amplify needs to be included in the experiment.
- Programme: Initial denaturation step of 5 minutes at 94°C, followed 25 cycles of denaturation for 30 seconds at 94°C, annealing at 68°C for 30 seconds and elongation at 72°C for 1 min. The PCR is ended off with a final extension step at 72°C for 4 minutes.
- Successful amplification is confirmed with 1% agarose gel electrophoresis in 1x Tris Acetic acid EDTA (TAE, pH 8.0) and ethidium bromide staining.

To identify Foc TR4:

- Each PCR reaction contains:
  - 4 μL of 1x Buffer
  - 3.5 μL of 50 mM $MgCl_2$
  - 1.3 μL of 10 mM dNTPs,
  - 2 μL of 20 mg/ml BSA
  - 1 μL of 10 mM solution of each primer
  - 0 . 6 μL of 5 U/μL Taq
  - 2 μL of 20 ng/μL concentration DNA
- FocTR4-F(CACGTTTAAGGTGCCATGAGAG) and FocTR4-R(GCCAGGACTGCCTCGTGA) are used as forward and reverse primer, respectively, for the amplification of the Foc TR4 (Dita *et al*., 2010).
- All reactions are made up to a final volume of 50 μl with sterile nuclease-free H_2O.
- A negative control, containing water instead of DNA template as well as a positive control (CAV 789), a DNA template the will amplify needs to be included in the experiment.
- Programme: Initial denaturation step of 5 min at 94°C, followed 25 cycles of denaturation for 45 seconds at 94°C, annealing at 62°C for 45 seconds and elongation at 72°C for 1 min. The PCR is ended off with a final extension step at 72°C for 5 min.
- Successful amplification is confirmed with 1% agarose gel electrophoresis in 1x Tris Acetic acid EDTA (TAE, pH 8.0) and methyl bromide staining (Fig. 11).

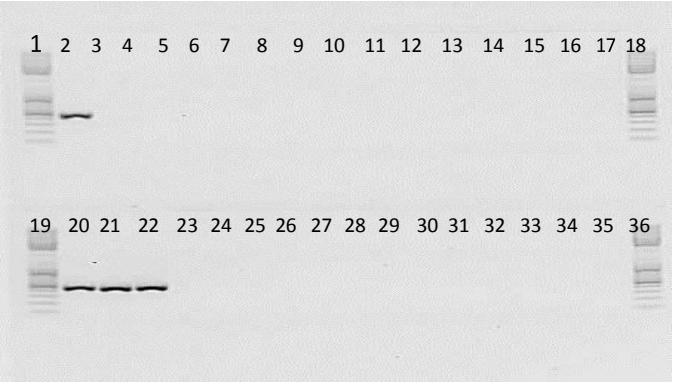


Figure 11. PCR amplification of *Fusarium oxysporum* f. sp. *cubense* vegetative compatibility groups with the primer set VCG 01213/16 F1 and VCG 01213/16 R2. Lines 1. Ladder; 2. VCG 01213/16; 3. VCG 0120; 4. VCG 01215; 5. VCG 0120/15; 6. VCG 0121; 7. VCG 0122; 8. VCG 0123; 9. VCG 0124; 10. VCG 0125; 11. VCG 0124/5; 12. VCG 0126; 13. VCG 0128; 14. VCG 0129; 15. VCG 01210; 16. VCG 01211; 17. VCG 01212; 18. Ladder; 19. Ladder; 20. VCG 01213/16; 21. VCG 01213; 22. VCG 01216; 23. VCG 01217, 24. VCG 01218; 25. VCG 01219; 26. VCG 01220; 27. VCG 01221; 28. VCG 01222; 29-34. Non-pathogenic *F. oxysporum*; 30. Non-template control; 36. Ladder.

Detection of Foc TR4 by real-time PCR

The real-time PCR protocol described below is based on the publication Aguayo *et al*. (2017). It targets two Foc race 4 VCGs*:* the highly aggressive VCG 01213/16 that is responsible for the epidemics of Cavendish bananas in the tropics, and VCG 0121, a less virulent strain that may also affect this variety in the tropics. The technique targets a gene region associated with fungal virulence.

The goal of the real-time PCR protocol is to detect Foc TR4 in symptomatic tissue collected from Cavendish banana pseudostems. The method is qualitative, and has not yet been validated for quantification of the inoculum. Samples that give negative results are either not contaminated with Foc TR4, or contain very low levels of the pathogen. Further testing of these samples is required to ensure that the symptoms are not caused by Foc TR4.

To identify Foc TR4 using real-time PCR

- Each qPCR reaction contains:
  - 10 µl of SYBR no-Rox mix (Bioline, Fremont, USA)
  - 0.6 µl of 10 mM stock solution of each primer
  - 0.2 µl of 10 mM stock probe solution
  - 2 µL of DNA
- The qPCR assay uses a set of primers as forward and reverse primers, as well as a fluorescently-labelled probe for amplification:
  - FWB-TR4 F (5'-CGGTCTCGGCCAAATCTGATT-3')
  - FWB-TR4 R (5'-ACGACTTATCTAGCGGTTGATGTG-3')
  - FWB-TR4 P (5' FAM- ACCCTTCAACTCCACTCGATCGCA –BHQ1 '3)
- All reactions are made up to a final volume of 20 µl with sterile nuclease-free H_2O.
- A non-template control (NTC) that contains water instead of DNA, as well as a positive control containing DNA of a known Foc TR4 culture, need to be included in the experiment.
- Programme: Initial denaturation step of 10 min at 95°C and activation of DNA polymerase, followed by 40 cycles of denaturation for 10 seconds at 95°C, hybridization polymerization at 60°C for 45 seconds and FAM fluorescence reading.

Important considerations for real-time identification of Foc TR4

- The method has been developed to identify Foc TR4 in symptomatic stem samples from Cavendish bananas. It may also be used to identify Foc TR4 in other banana varieties, but this has not been tested.
- Total DNA (plant DNA, fungal DNA, or eventually DNA from other organisms) was extracted and purified by Aguayo *et al*. (2017) using a DNeasy plant mini kit™ from Qiagen in Cuortaboeuf, France. This kit is not exclusively recommended for use, as other products may be give the same result.
- The detection method needs to include positive controls to ensure that:
  - The laboratory operator has followed the protocol correctly
  - The quality of consumables and reagents is adequate
  - The quantity of DNA extract is enough to be amplified by the qPCR
  - Detection of accidental contaminated samples do not occur
- The recommended controls that should be used are:
  - A quality control for DNA extraction to detect potential PCR inhibitors in each tube. Ioos *et al*. (2009) report on the use of 18S qPCR protocol to this end. The 18S gene region is a common housekeeping gene that is present in all fungi and plants. If the DNA extraction was not efficient, amplification with this qPCR assay will not be recorded.
  - A negative DNA extraction control that consists of an empty sample tube, which will follow the same steps (including sample preparation, lysing, DNA extraction and PCR) as the tube with the banana sample. The negative control will be used to check the absence of contamination. The negative control should be tested in duplicate during the qPCR reaction. It will be used to check the absence of cross contamination between samples or any external contamination during the DNA extraction step.
  - A positive control at the estimated limit of detection that needs to be tested in duplicate during the qPCR reaction to check that the PCR reaction was performed correctly (thermodynamic, volumetric and chemical conditions) to detect the lowest quantity of the target pathogen. The control consists of a calibrated genomic DNA (gDNA) solution extracted from a known Foc TR4 strain or a calibrated bacterial plasmid solution cloned with the genomic target of the FWB-TR4 test. This control must be characterized by the laboratory under its own conditions. In practice, this control is defined as lowest quantity of target resulting in positive result. The positive control should be added at the end of the qPCR setup to prevent possible contamination.
  - A no-template control that is systematically introduced in duplicate in each qPCR reaction. This negative control consists of adding water in one of the reactional tubes

instead of the banana sample DNA. It is used to check the absence of contamination during the steps of PCR mix preparation and template load.

- Quality control is performed by analysing the fluorescent curves measured by the qPCR machine and generated with the different controls. This is achieved when:
  - None of the negative controls has generated a FAM fluorescence higher than the fixed threshold line determined by the operator. This means that no accidental cross contamination occurred during the grinding and DNA extraction steps of the samples or during the preparation of the reactional mix, and when sample DNA were added.
  - None of the NTC generated a FAM fluorescence higher than the fixed threshold line determined by the operator => this means that no accidental contamination occurred during the preparation of the reactional mix, and when sample DNA were added.
  - The positive control duplicates at limit of detection generated a FAM fluorescence higher than the fixed **threshold line determined by the operator** => the PCR conditions and the reactional mix enabled a specific amplification of the target, with a correct PCR run execution.
- Sample test results can be interpreted for the various samples in the same PCR run. For each PCR run, the positive control at the limit of detection must be checked. In general, all the tested samples with values superior to the limit of detection control will be considered as positive. The results of the tested samples are analysed individually, and can be interpreted as follows:
  - A sample is considered positive if one of the tested samples is positive. The result may be for example expressed as "Suspected presence or detection of Foc TR4 in the sample".
  - A sample is considered negative if all the tested samples are negative. The result may be for example expressed as "Foc TR4 not detected in the sample".
  - A sample is considered undetermined if one of more DNA extracts are suspect of having inhibitors or low DNA levels, and when no other sample from the same plant is positive. The result may then be expressed as "Undetermined", and the reasons for the results explained.

Storage of *F. oxysporum* cultures

Storage on sterile filter paper

Filter papers disks (5 cm in diameter) are autoclaved in glass Petri dishes. The disks are then aseptically placed on ¼-strenth PDA in Petri dishes. *Fusarium* isolates are cultured on CLA for 7-10 days. Rectangular agar pieces colonised by *F. oxysporum* (3 mm in diameter) are then placed on the sterile filter papers, and grown for 7-10 days until the entire filter paper is covered by mycelia. The filter paper with fungal growth is lifted off the PDA, placed in sterile Petri dishes, and left for 1 day to dry. It is then cut into smaller pieces (5 mm in diameter) and placed in cryovials. The cryovials are all clearly labelled with the isolate number, and stored at 5^{o}C until use.

Recommended period of storage: 3-12 months

Storage on CLA slants

Water agar (WA) is prepared by dissolving 20 g agar in 1 L distilled water. The WA is then autoclaved at 121^{o}C for 20 minutes. After autoclaving, aliquots of 10 ml of the WA are poured into sterile 20-ml bottles under sterile airflow. The bottles are placed in a tray, and the tray kept at an angle of 45^{o}C until the WA is solidified. A single sterile carnation leaf is placed on top of the agar. The isolate is then placed next to the carnation leaf on the water agar, and grown at 25^{o}C for 1 week. All cultures are clearly marked with the isolate number, and stored at 5^{o}C until further use.

Recommended period of storage: 3-24 months

Deep-freezing

A 15% glycerol stock solution is first prepared and autoclaved. The *F. oxysporum* isolates are then grown on ¼-strength PDA plates at 25^{o}C for 7-10 days. Ten ml of the 15% glycerol are thereafter pipetted onto the fungal growth in the Petri dishes in a sterile flow cabinet. The spores and some hyphae are carefully dislodged with a surface-sterilised scalpel. One-ml-aliquots of the spore suspension are then pipetted from the Petri dishes into 2-ml cryovials. Each of the cryovials are carefully labelled, placed into cryovial boxes, and stored at –80^{o}C. When the isolate needs to be recovered, small quantities of the frozen suspension is scraped from the cryovial with a sterile scalpel, and placed onto the culture medium.

Recommended period of storage: Up to 5 years

Storage in soil

Soil is first sterilised in small glass bottles or tubes. The cultures are then grown on ¼-strength PDA plates for 7-10 days. Sterile distilled water (20 ml) is poured onto each culture in a flow cabinet, and the spores discretely dislodged with a surface-sterilised scalpel. Ten ml of the fungal spore suspension is then pipetted from the Petri dishes, and aseptically transferred onto the soil in the glass bottles and the tubes. All the glass tubes and bottles are clearly marked with the isolate number, and stored at room temperature. The isolate is recovered by placing a small amount of soil onto culture medium.

Recommended period of storage: Up to 5 years

Lyophilisation of *Fusarium* cultures

Isolates to be lyophilised are grown on carnation-leaf agar in Petri dishes for 7-10 days. Several colonised carnation-leaf pieces are then transferred to each of five replicated sterile 5-ml glass vials labelled with the isolate number. A 0.5-ml aliquot of sterile skim milk is added to each vial. The vials are then stoppered with split rubber stoppers, which allow for evacuation of air. The stoppered vials are placed in a tray and quickly frozen by pouring liquid nitrogen into the tray. A Lucite plate slightly larger than the tray is placed on top of the partially stoppered vials.

A drying chamber on a refrigerated freeze-dryer is used for lyophilisation. The tray is placed on the pre-cooled shelf in the drying chamber, and a vacuum is pulled. When refrigeration is completed, the heat is turned on, while the samples dry gradually. After lyophilisation, the vials are sealed under vacuum by inflation of the rubber diaphragm in the chambers over the tray, which presses down the Lucite plate and forces the rubber stoppers to seal the vials. After lyophilisation, vials are capped and labelled, and the vials stored at -20^{o}C.

Recommended period of storage: Up to 20 years

Culture mites and mycology laboratory hygiene

The following notes have been adapted from “Fungi and Food Spoilage” by John Pitt and Ailsa Hocking, CSIRO Division of Food Research, Sydney. 1985. Academic Press.

Culture mites

A major hazard in growing and maintaining fungal cultures is the culture mite. Mites are small (0.05-0.15 mm long) and just visible by the naked eye. They are arachnoids, related to spiders. Many mite species live on fungal hyphae as their main or sole diet in nature, and find culture collections an idyllic environment. They crawl from culture to culture, contaminating them with fungi and bacteria as they go or eat them entirely. Each mite leaves a trail of eggs about half-adult size as it moves. Eggs hatch with 24 hours, and reach adulthood within 2-3 days. The damage an unchecked mite plague can do to a bacterial or fungal culture collection is enormous and they represent a real threat to culture collections.

The most common sources of mites are plant and soil specimens, contaminated fungal cultures and mouldy foodstuffs left in laboratories and near incubators. Mites can also be carried on large dust particles. Building work near a laboratory almost always induces a mite infestation – be on the lookout!

The avoidance of losses due to mites requires constant vigilance. Infestation by mites can be minimised by hygienic laboratory practices (e.g. avoiding accumulation of dust or old cultures in the laboratory). It is also good practice to handle and store food, plant and soil samples ***in a separate area*** to where fungi are inoculated and incubated. Subculture all plates at least every 2 weeks (e.g. your *nit*-M tester and CLA culture collections). Always watch for tell-tale signs, such as contaminants growing around tile edges of a Petri dish, a ‘moth-eaten’ appearance to colonies or ‘tracks’ of bacterial colonies across agar. Examination of suspect material or cultures under the stereomicroscope will readily reveal the presence of mites and mite eggs.

To control a mite plague, remove all contaminated material, including cultures. Freeze Petri dishes and culture tubes, as freezing rapidly kills adult mites, and mite eggs will only survive 48-72 hours at -20°C. Cultures can then be sub-cultured from uninfected portions of the culture with the aid of the stereomicroscope. Plates with mites can also be autoclaved, steamed or flooded with alcohol. Clean benches thoroughly with sodium hypochlorite (approximately 3%) or 70% ethanol. Incubators can be disinfested with aerosol insecticides or a solution of thymol in alcohol (alcohol alone does not kill mites).

Hygiene in the mycology laboratory

Like any other microbiological laboratory, a mycological laboratory should be kept in a clean condition. Discard unwanted cultures frequently, and dispose of them by steaming or autoclaving. Practical plant pathologists are in the business of controlling diseases, not spreading them around! Wipe bench tops regularly with ethanol (70-95%). Laboratory floors and associated walkways should be frequently wet-mopped, or polished only with machines equipped with efficient vacuum cleaners and dust filters. Where possible, store food, plant and soil materials away from the laboratory. Open Petri dishes carefully. Transport Petri dishes to the stereomicroscope stage before removing lids. Transport culture plates in study containers with lids and wipe with ethanol (70-95%) before and after each use.

Contrary to popular belief, a well-run mycological laboratory is not a source of contamination to bacteriological laboratories. The air in a mycological laboratory should not carry a significant population of fungal spores if correct procedures are followed. The reverse problem can occur, however, because bacteria multiply much more rapidly than do fungi. Bacterial spores are often present in food laboratories, readily infect fungal plates, and can rapidly outgrow and inhibit the formation of fungal colonies *in vitro*.

If for any reason fungal spore concentrations do build up in a laboratory and cause an unacceptable level of contamination, the air should be purified. The simplest technique is to spray with an aerosol before the laboratory is closed in the evening. Any aerosol spray, such as a room deodoriser or air freshener, is effective. Aerosol droplets entrain fungal spores very efficiently and carry them to the floor. A more drastic and effective treatment in cases of severe contamination is to spray a solution of thymol in ethanol around the room, and close it for a weekend. The spray is rather pungent, and while not harmful to humans, it effectively kills fungal spores (and mites).

Prevention and management

Fusarium wilt is moved to new growing areas with infected planting materials, in soil attached to shoes and vehicles, and in water (Stover, 1962). The disease is mostly observed in areas where susceptible bananas are grown, but not in fields planted with disease resistant varieties. Once established the fungus cannot be eradicated and can survive in soil for decades. This makes banana Fusarium wilt difficult to control with conventional plant protection techniques such as crop rotation and the use of fungicides. The most efficient way to deal with the disease, therefore, is by preventing the fungus from being introduced into banana fields, or by replacing susceptible varieties with resistant ones. The early and accurate detection of Foc TR4, therefore, is of great importance to protect bananas against this devastating pathogen. Some progress had been made in breeding disease-resistant plants using conventional (Rowe and Rosales, 2000) and unconventional methods (Hwang and Ko, 2004), but these plants often do not meet the end-user preference for flavour, taste and texture, production qualities, or are not immune.

Banana Fusarium wilt can be prevented and managed by means of enhanced awareness, legislation, regulation, quarantine, avoidance and treatment of infested fields. These actions can take place before Foc enters countries and farms, or once it has been introduced into countries and onto farms. The activity chosen to deal with Fusarium wilt depends on the availability, affordability, geographical area and production systems in countries/farms affected and under threat of being affected, as well as general knowledge of the disease and its management. Protecting bananas against Foc TR4 (or other foreign strains) is the responsibility of a number of stakeholders, including national and regional authorities, research scientists, extension officers and producers. Activities include:

Prevention of banana Fusarium wilt

Pre-border proactive activities:

- Assessment of national biosecurity legislation and regulations.
- Assessment of a country's ability to prevent and respond to incursions of Foc.
- Obtaining sufficient knowledge on Foc and means to deal with it outside country borders.
- Raising awareness about Foc TR4 among policy makers, government and quarantine officials, the public, researchers, banana producers and other stakeholders.
- Developing standardised training manuals, identification and surveillance protocols, as well as methods to deal with Foc incursions nationally and regionally.
- Developing national capacity and infrastructure to deal with incursions.

- Training plant health officials, scientists, extension officers, border control and quarantine people, and producers on Foc identification and management.
- Distribution of posters, brochures and information materials on Foc TR4 and other races.
- Preparing technical materials on the prevention, detection, contention and eradication of Foc-affected plants.
- Introducing an emergency fund to rapidly respond to incursions.
- Developing an entrance risk analysis and identify high-risk entry points.

On-border activities:

- Evaluating quarantine measures and strengthen border control.
- Including Foc TR4 as quarantine pest on national lists.
- Developing legislation and phytosanitary regulation for bananas and/or parts of bananas introduced from Foc TR4-affected countries or countries at risk.
- Strictly controlling the importation of banana and plantain plants and plant parts from countries affected or at risk of Foc TR4 through national quarantine stations.
- Requesting *in vitro* plants to be accompanied by certificates for disease indexing.
- Identifying and strengthening high risk entry points for banana plants infected with Foc.
- Training scientists in the use of reliable diagnostics for Foc TR4 identification.

Post-border off-farm activities:

- Mapping of the distribution of Foc TR4 and other Foc races in banana-growing countries.
- Gathering of epidemiological data to establish means of introduction and spread.
- Assessing, training and introducing surveillance systems and teams in-country.
- Introducing legislation to regulate the movement of banana planting materials and other risky materials within country borders.
- Setting up quarantine zones to prevent the movement of infected planting materials and other possible risky materials in-country.
- Collaborating nationally and regionally to prevent and manage Foc TR4 in the region.
- Organising training workshops and expert consultations with international *Fusarium* specialists.

Post-border farm-border activities:

- Obtaining clean planting and propagation material from reputable sources, preferentially tissue culture bananas.
- Putting up highly visible and clearly understandable signs at farm entrances to notify visitors about farm biosecurity.

- Cleaning all vehicles visiting farms by hosing-off clay and plant parts and disinfection at wash-down areas before entering or leaving farm gates.
- Managing the movement of farm visitors and vehicles entering farm borders by:
  - Allowing visitors onto the farm only by appointment and upon signing in
  - Disinfecting the shoes and vehicles of visitors
  - Using only on-farm vehicles and boots provided to visitors in plantations
- Enquiring about the employment history, nationality and movement of all farm workers.
- Avoiding sharing farm machinery, equipment and field tools with other growers.
- Strictly controlling access of contractors and service providers to farm.

Early detection and eradication of banana Fusarium wilt

- Training and employing scouts to identify and immediately report any suspect plants.
- Properly isolating and eradicating any outbreaks of banana Fusarium wilt by fencing-in of diseased and neighbouring plants, killing of such plants and burning, and preventing the movement of soil and water outside affected areas.
- Restricting and controlling movement of farm workers and farm equipment into and out of Foc-infested fields, and use disinfectants to clean shoes and plantation tools. These shoes and plantation tools should preferentially not used in other areas on-farm.
- Limiting vehicle movement to designated roads and regulate routes according to areas affected and non-affected by Foc.
- Abandoning further banana production in newly affected sites.
- Growing ground covers to prevent erosion and movement of soil.
- Introducing protocols to identify, isolate and monitor Fusarium wilt outbreaks. This should include means to distinguish between Fusarium wilt and closely related diseases.
- Preventing flow of irrigation or rainwater out of affected areas by redirecting drainage canals.
- Regularly testing irrigation water and planting materials to prevent further spread of banana Fusarium wilt.

Management of banana Fusarium wilt

The management of banana Fusarium wilt in fields where Foc is present requires expert advice from experienced field plant pathologists.

Biological control:

- Using of mycorrhizae, *Trichoderma*, non-pathogenic *Fusarium oxysporum*, bacteria and actinomycetes.

Chemical control:

- Fungicide dipping for root protection during planting, stem injections.
- Surface sterilants for disinfection purposes.
- Soil fumigation.
- Plant activators.

Cultural control:

- Clean planting material: tissue culture and clean suckers/bits.
- Proper sanitary and phytosanitary actions.
- Crop rotation and fallow periods.
- Soil amendments and fertilizers: pH, nitrogen, calcium, silicon, iron, organic matter.
- Flood fallowing and bio-fumigants: leek, brassicas, anaerobic fumigation.
- Establishment of persistent ground covers that stimulate microbial activity and prevent movement of soil and drainage water.
- Suppressive soil properties: consider soil biology, chemistry and physical properties.
- Proper irrigation schedules and drainage.
- Fencing-in of infested fields to prevent unnecessary pedestrian and animal movement.

Host resistance:

- Planting popular local varieties with natural resistance to Foc TR4.
- Planting hybrids developed by conventional plant breeding: ex. FHIA varieties.
- Planting varieties developed by mutation breeding: Somaclonal variants, chemical mutations and irradiation.
- If acceptable to markets, plant genetically modified bananas with proven resistance to Foc TR4 (with anti-fungal genes, resistance genes, anti-stress genes, HIGS).

Integrated disease management:

- Employ mixed cropping systems.
- Employ plant resistance and soil amendments.
- Fumigation and the use of biological control products.

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Appendix A: Methodology and Media

Method for the isolation and culturing of *F. oxysporum*

Melt the PDA in 1-L bottles in an autoclave. When melted, place bottles in a water bath at 50°C for 20 minutes or until media reaches 50°C. To each 1-L bottle of medium add 0.04g streptomycin solution just before dispensing into Petri dishes.

Half strength potato dextrose agar (PDA) medium amended with antibiotics

- 19 g potato dextrose agar (PDA)
- 15 g agar
- 1 litre of distilled water

Carnation leaf agar (CLA) (Burgess *et al*., 1994)

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°C.

Preparation of Carnation leaves

Fresh, healthy carnation leaves, which have not been treated with fungicides or other chemicals, are cut into pieces approximately 10x3 mm before placing in paper bags to dry. When dry, place leaf pieces in containers suitable for Gamma-irradiation (e.g. 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°C until required.

Method for VCG analysis

Place media in an oven until agar has dissolved (approximately 1 hr), shaking occasionally. Add the trace element solution (this is pre-made and kept in 1-mL containers in the freezer). Autoclave to sterilise (e.g. wet cycle: 100 kPa at 121°C for 20 min), and dispense into Petri dishes clearly marked with the medium name and date.

Trace element solution

5.0 g	Citric acid	
5.0 g	$ZnSO_4.7H_2O$	Zinc sulphate
1.0 g	$Fe(NH_2)_2(SO_4)_2.6H_2O$	Ferrous ammonium sulphate
0.25 g	$CuSO_2.5H_2O$	Copper sulphate
50 mg	$MnSO_2.H_2O$	Manganous sulphate tetrahydrate
50 mg	$H1BO_2$	Boric acid
50 mg	$NaMoO_4.2H_2O$	Sodium molybdate
95 mL		Sterile distilled water

Minimal medium (MM medium)

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

Nitrate medium (NO_3 medium)

30 g	Sucrose	
20 g	BBL agar (or similar analytical grade agar)	
0.5 g	KCl	Potassium chloride
0.5 g	NaNO3	Sodium nitrate
1 g	KH2PO4	Potassium dihydrogen orthophosphate
0.5 g	$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
10 mg	$FeSO_4.7H_2O$	Ferrous sulphate
0.2 mL	Sterile trace elements solution (add this after medium has melted and before autoclaving)	
1 L	Distilled water	

Hypoxanthine medium (HX medium)

30 g	Sucrose	
20 g	BBL agar (or similar analytical grade agar)	
0.5 g	KCl	Potassium chloride
0.2 g	Hypoxanthine	
1 g	KH2PO4	Potassium dihydrogen orthophosphate
0.5 g	$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
10 mg	$FeSO_4.7H_2O$	Ferrous sulphate
0.2 mL	Sterile trace elements solution (add this after medium has melted and before autoclaving)	
1 L	Distilled water	

Ammonium medium (NH_4 medium)

30 g	Sucrose	
20 g	BBL agar (or similar analytical grade agar)	
0.5 g	KCl	Potassium chloride
1 g	Ammonium tartrate	
1 g	KH2PO4	Potassium dihydrogen orthophosphate
0.5 g	$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
10 mg	$FeSO_4.7H_2O$	Ferrous sulphate
0.2 mL	Sterile trace elements solution (add this after medium has melted and before autoclaving)	
1 L	Distilled water	

Nitrite medium (Ni medium)

30 g	Sucrose	
20 g	BBL agar (or similar analytical grade agar)	
0.5 g	KCl	Potassium chloride
0.2 g	$NaNO_2$	
1 g	KH_2PO_4	Potassium dihydrogen orthophosphate
0.5 g	$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
10 mg	$FeSO_4.7H_2O$	Ferrous sulphate
0.2 mL	Sterile trace elements solution (add this after medium has melted and before autoclaving)	
1 L	Distilled water	

Media and solutions for molecular studies

0.5 M NaOH

- Add 1 g of NaOH pellets to 30 mL of distilled water
- Dissolve pellets and make up to 50 mL
- Autoclave

1 M Tris-HCL, pH8

- 121.1 g Tris in 800 ml H_2O
- Adjust the pH 8
- Fill up to 1 litre
- Autoclave

100 mM Tris

- Add 10 mL of 1 M Tris to 90 mL of distilled water
- Autoclave

DNA extraction buffer

- 250 mM Tris-HCL (pH 8)
- 250 mM NaCL
- 25 mM EDTA
- 0.5% SDS
- Autoclave

0.5 M EDTA, pH8

- 186.1 g disodium ethylenediaminetetra-acetate H_2O
- Adjust the pH 8
- Fill up to 1 litre

10% SDS

- 100 g sodium dodecyl sulphate
- Fill up to 1 litre

3 M NaAc (Sodium acetate)

- 246.09 g NaAc
- 800 ml water

50x TAE

- 242 g of Tris base
- 57.1 ml acetic acid
- 100 ml of 0.5 M EDTA (pH 8)
- Make up to 1 litre

1x TAE

- 20 ml of 50x TAE
- Make up to 1 L

Ethidium bromide

Prepared as 10 $mg.ml^{-1}$ in H_2O

Bromophenol blue

- 0.25% bromophenol blue
- 15% ficol
- 1x TAE

DNA ladder

- As provided by company

Calculation example:

Preparation of 500 ml DNA extraction buffer:

200 mM Tris.HCl

$C_1V_1 = C_2V_2$

1 x X = 0.2 x 500 ml

X = 100 ml

Note: 200 mM = 0.2 M

150 mM NaCl

$C_1V_1 = C_2V_2$

1 x X = 0.15 x 500 ml

X = 75 ml

Note: 150 mM = 0.15 M

25 mM EDTA

$C_1V_1 = C_2V_2$

0.5 x X = 0.025 x 500 ml

X = 25 ml

0.5% SDS

$C_1V_1 = C_2V_2$

10% x X = 0.5% x 500 ml

X = 25 ml

Total: 500 ml

Tris HCL: 100 ml

NaCl: 75 ml

EDTA: 25 ml

SDS: 25 ml

Water: 275 ml