National Diagnostic Protocol

Fusarium wilt of Banana caused by *Fusarium oxysporum* f. sp. *cubense TR4*

NDP 49 V1

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Purpose

National Diagnostic Protocols (NDPs) are diagnostic protocols for the unambiguous taxonomic identification of plant pests. NDPs:

- are a verified information resource for plant health diagnosticians
- are consistent with ISPM No. 27 – Diagnostic Protocols for Regulated Pests
- provide a nationally consistent approach to the identification of plant pests enabling transparency when comparing diagnostic results between laboratories; and,
- are endorsed by regulatory jurisdictions for use (either within their own facilities or when commissioning from others) in a pest incursion.

Where an International Plant Protection Convention (IPPC) diagnostic protocol exists it should be used in preference to NDPs, unless it is shown that the NDP has improved procedures for Australian conditions. NDPs may contain additional information to aid diagnosis. IPPC protocols are available on the IPPC website:

https://www.ippc.int/core-activities/standards-setting/ispms

Process

NDPs are facilitated and endorsed by the Subcommittee on Plant Health Diagnostics (SPHD). SPHD reports to Plant Health Committee and is Australia's peak technical and policy forum for plant health diagnostics.

NDPs are developed and endorsed according to Reference Standards developed and maintained by SPHD. Current Reference Standards are available at https://www.plantbiosecuritydiagnostics.net.au/work/subcommittee-on-plant-health-diagnostics/

NDPs are living documents. They are updated every 5 years or before this time if required (i.e. when new techniques become available).

Document status

This version of the National Diagnostic Protocol (NDP) for *Fusarium oxysporum* f. sp. *cubense* TR4 is current as at the date contained in the version control box below.

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Further information

Inquiries regarding technical matters relating to this protocol should be sent to: sphd@agriculture.gov.au

Contents

1 INTRODUCTION

The causal agent of Fusarium wilt disease of banana is the soil-borne ascomycete fungus *Fusarium oxysporum* f. sp. *cubense* (E.F. Smith) Snyder and Hansen (*Foc*). 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 (Nucci and Anaissie 2007). *Fusarium oxysporum* (*Fo*), the most common species of the genus, is a soil-borne pathogen with a ubiquitous, worldwide distribution. Strains within *Fo* cause wilt diseases on a broad range of agricultural and ornamental host plant species (Armstrong and Armstrong 1981; Beckman 1987), and cannot be distinguished by morphological features. The sexual state of *Fo* has never been observed. Strains within *Fo* possessing the same limited host range are grouped together and assigned to a forma specialis; currently, over 120 formae speciales have been described for *Fo* (Armstrong and Armstrong 1981). Only *Foc* is able to cause Fusarium wilt on banana plants (Armstrong and Armstrong 1981; Snyder and Hansen 1940); where banana plant hosts are defined as species from the genus *Musa* (Family *Musaceae*, Order *Zingiberales*) and from the genus *Heliconia* (Family *Heliconiaceae*, Order *Zingiberales*) (Jones 2000).

The history and distribution of Fusarium wilt in Australia has been well documented (Gerlach *et al.* 2000; Moore *et al.* 2001; Pegg *et al.* 1996). All four races of *Foc* are present. Races 1 and 2 of *Foc* have been found affecting banana in both northern and southern Queensland, and northern New South Wales (NSW), and race 1 has been found in Western Australia (WA). The subtropical Australian banana industry is severely constrained by race 1, which is predominantly based on the production of the highly susceptible Lady finger variety. Race 3, a pathogen of *Heliconia* spp., has been found in the Northern Territory (NT) (Gerlach *et al.* 2000). Subtropical race 4 is found affecting Cavendish cultivars, as well as race 1 and race 2 susceptible cultivars in northern NSW and southern Queensland. Tropical race 4 (TR4) is the most recent and most devastating race of the pathogen as it affects Cavendish cultivars. It is regarded as one of the most serious threats to banana production in Australia and worldwide. Banana plants bred for TR4 resistance, developed in Taiwan, have been trialled by Australian growers, however they are not yet widely available (ABGC 2015).

Several detections of TR4 have occurred in the NT. The first was on a commercial plantation in Darwin in 1997 (Walduck 2002) with detections reported from additional locations since. TR4 was detected in the Tully Valley, north Queensland in March 2015. Although various attempts have been made to confine the disease to a single farm even including closing down the infected property, the fungus has been detected in other four properties in Tully Valley to date.

This protocol provides a comprehensive guide to the suite of diagnostic techniques used in the diagnosis of isolates of *Foc TR4.*

2 TAXONOMIC INFORMATION

Kingdom:	Fungi
Phylum:	Ascomycota
Class:	Sordariomycetes
Order:	Hypocreales
Family:	Nectriaceae
Genus:	*Fusarium*
Species:	*Fusarium oxysporum*
Forma specialis:	*Fusarium oxysporum* f. sp. *cubense* (E.F. Sm.), W.C. Snyder & H.N. Hansen
Race:	*Fusarium oxysporum* f. sp. *cubense* tropical race 4

Synonyms:

- *Fusarium cubense* E.F. Sm.
- *Fusarium cubense* var. *inodoratum* E.W. Brandes
- *Fusarium* var. *cubense* (E.F. Sm.) Wollenw.
- *Fusarium odoratissimum* (Maryani *et al.*, 2019)

Common names:

- Panama disease of banana
- Panama TR4
- Fusarium wilt of banana
- Vascular wilt of banana and abaca
- **Spanish:** mal de Panamá
- **French:** fusariose du bananier; maladie de Panama

2.1.1 Racial classification

Isolates of *Fusarium oxysporum* f. sp. *cubense (Foc)* have been traditionally grouped into four physiological races, based on pathogenicity to a small number of differential banana host cultivars in the field (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*.

2.1.2 Fungal vegetative compatibility

A technique that is based on the genetic relationships within fungal populations, termed vegetative compatibility group (VCG) analysis, has been utilised to group isolates of *Foc* (Brake *et al.* 1990; Moore 1994; Moore *et al.* 1993; Ploetz 1990b; Ploetz and Correll 1988). 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; Puhalla 1985).

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 24 VCGs of *Foc* have been characterised worldwide (Moore 1994; Ploetz 1990a; Ploetz and Pegg 1999, Fourie *et al.* 2011), with eight of these VCGs present in Australia, viz. VCGs 0120, 0124, 0125, 0128, 0129, 01211, 01213/16 and 01220 (Table 1) (Brake *et al.* 1990; Moore *et al.* 1993; Pegg *et al.* 1995). Isolates of *Foc* race 1 belong to VCGs 0124 and 0125 and are widespread throughout eastern Australia. Strains in the very closely related VCGs 0128 and 01220 are narrowly distributed at a small number of sites in northern Queensland and NSW and at Carnarvon in WA, respectively. The Cavendish-competent subtropical race 4 isolates belong to VCGs 0120, 0129 and 01211, and are at present restricted to the banana growing regions of southern Queensland and northern NSW. They have not yet been recorded in the major Cavendish production areas of northern Queensland, or the production areas of Carnarvon and Kununurra in WA, or near Darwin in the NT.

Strains of *Foc* TR4 have been confirmed at various sites since the first detection in Darwin in 1997 (Walduck 2002). These include detections Humpty Doo (2006), Elizabeth Valley (2007), Rapid Creek (2009), Yirrikala (2009), Lake Bennett (2010) and Nightcliff (2011) (Tran-Nguyen pers comm). TR4 has since spread to north Queensland with four detections from the same property in the Tully Valley in 2015 (Queensland DAF 2016a) and the fungus was later also detected in other four properties also in the Tully Valley to date. Strains of TR4 belong to VCGs 01213/16.

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

Race	1	2	3	Subtropical race 4	Tropical race 4
Host name (genomic constitution)	Lady finger (AAB) Gros Michel (AAA)	Bluggoe (ABB)	*Heliconia* spp.	Cavendish (AAA) and all other cultivars susceptible to races 1 and 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 and 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).

3 DETECTION

Currently, a definitive positive detection of *Foc* TR4 must include a combination of multiple molecular tests (Section 4.7) concurrent with VCG testing (Section 4.6). This is due to cross-specific reactions of molecular protocols found with some Australian isolates of *Fusarium oxysporum.* The TR4 specific diagnostic developed by Dita *et al.* (2010), based on two single nucleotide polymorphisms in the intergenic spacer region of the nuclear ribosomal operon (IGS) was shown to be conspecific with non-TR4 isolates in the Australian samples. This produced false positives for TR4 in north Queensland, causing associated legal and economic impacts (McKillop 2016, Deloitte 2015). As a result, the suite of molecular and non-molecular assays were adopted for TR4 diagnostics to ensure the reliability of results. The Deloitte (2015) report also recommended developing a more reliable molecular test for TR4 in Australia, and that a record be kept of all decisions made involving statutory requirements under the *Biosecurity Act 2014* in Queensland.

3.1 Symptoms

Fusarium wilt is a typical vascular disease causing disruption of water translocation, systemic foliar symptoms and plant collapse (Jeger *et al.* 1995). External symptoms are characterised by a yellowing of the leaf margins of older leaves, the collapse of leaves at the petiole and the splitting of the pseudostem base (Figure 1). Internal symptoms are characterised by reddish-brown or yellow discolouration of the vascular tissue with continuous vascular streaking (Figure 2). Disease progression results in the collapse of the crown and pseudostem, and ultimately plant death (Stover 1962a).

Banana suckers that are less than about four months old do not develop visible symptoms of Fusarium wilt. The lack of visible symptoms on suckers has assisted in the movement of the pathogen to new regions through the movement of these asymptomatic suckers to new areas as planting material. The fruit of the banana plant does not show any specific disease symptoms.

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Figure 1. Disease symptoms of TR4 affecting Cavendish clones at the Coastal Plains Banana Quarantine Station, Northern Territory: (a) Banana plant showing typical symptoms of Fusarium wilt, yellowing, necrosis and collapse of leaves (notice that leaves form a skirt around the base of the plant). (b) Cross section of pseudostem showing the dramatic vascular discolouration (photograph provided by Juliane Henderson).

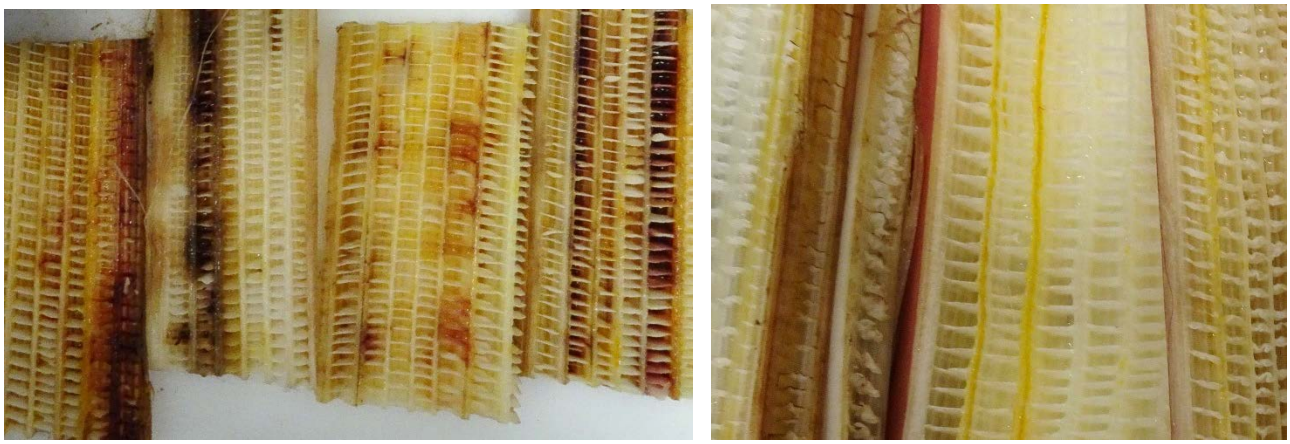

Figure 2. Internal symptoms of TR4 infected Cavendish plants characterised by reddish-brown (left) or yellow (right) discolouration of the vascular tissue of pseudostems with continuous vascular streaking (photograph taken by the Panama TR4 diagnostic team at the Plant Biosecurity Laboratory, Department of Agriculture and Fisheries).

3.2 Sampling and culturing from plant material

3.2.1 Preparing a sample from the diseased host plant

The sample should consist of a section from the pseudostem of the wilted banana plant where typical continuous discoloured vascular strands are evident (Figure 2). The sample should be taken from as low in the pseudostem as is 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 is 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 (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 in the wild
- 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.) and GPS coordinates if available
- 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 whether these are diseased or healthy.

A small (5cm x 5cm) piece of rhizome tissue showing typical discoloured vascular strands may also 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 absorb excessive sap from the tissue.

Fresh pseudostem or rhizome specimens should be double-bagged with zip lock plastic bags, placed in Styrofoam eskies with some ice blocks to keep the samples in cold conditions. The Styrofoam eskies are then placed inside a carton box and sent to the laboratory for diagnostics. Samples should arrive within five days from collection in the field. If transportation may take longer than five days, discoloured vascular strands can be dissected from the sample and dried before sending to the laboratory for diagnostics.

3.2.2 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. This should be preferably performed in a biosecurity containment laboratory to prevent *Foc* TR4 being released into the environment in the event the samples are infected with Panama TR4 disease. 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 is 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, post in a paper envelope inside the triple packaging as soon as the strands are dry enough with sample numbers and details clearly written on or with each sample envelope. Include a copy of the relevant quarantine import permit inside the package if this is required. If there is any possibility that samples have been mixed up and the details for some samples may be incorrect, discard the samples concerned.

4 IDENTIFICATION

The steps involved in the laboratory diagnosis of Panama TR4 have been summarised below (Figure 3) and are explained in detail in the following sections. For accurate identification, VCG testing is necessary in combination with the molecular testing due to issues with specificity with Australian isolates of *Foc*.

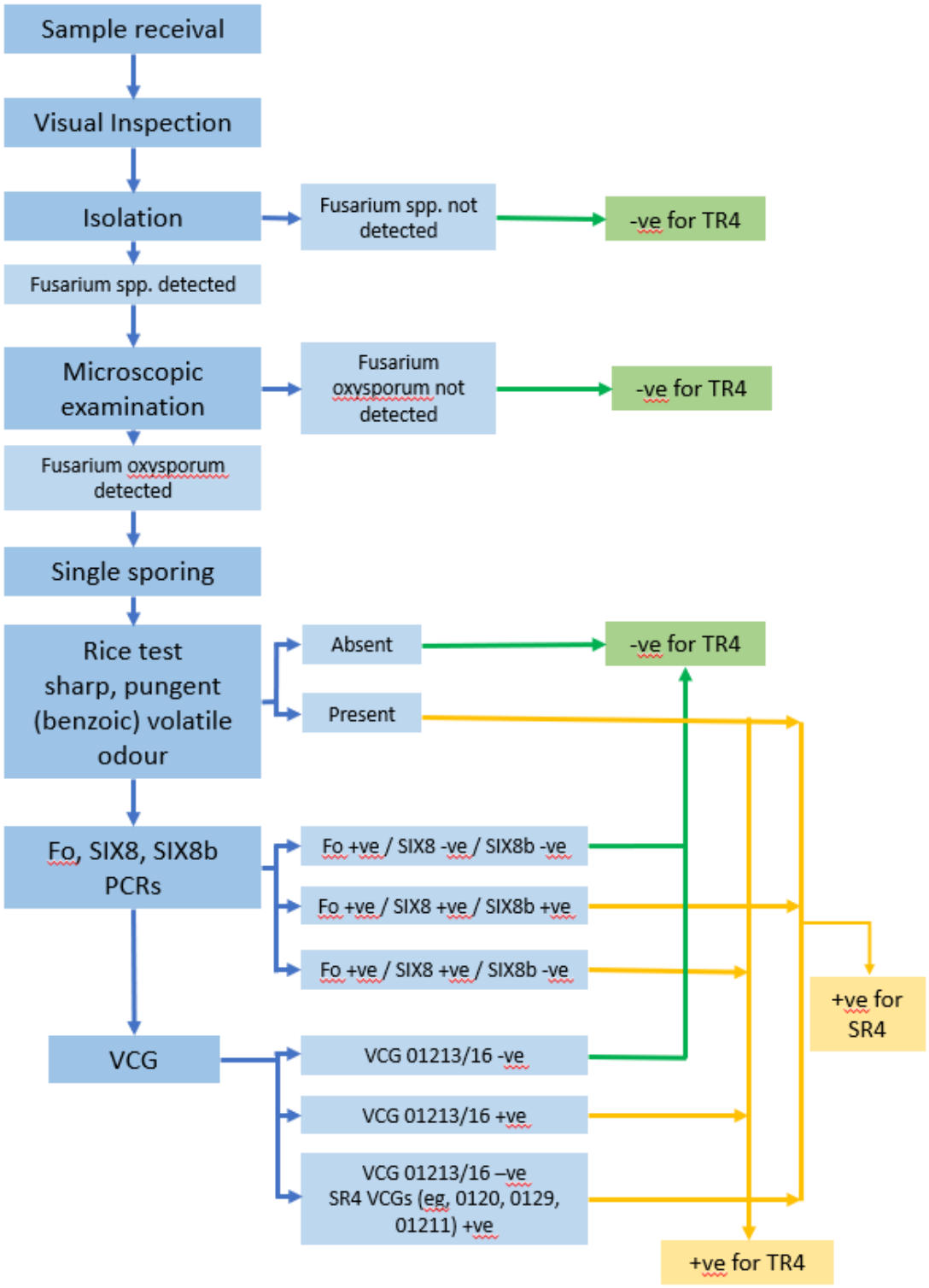

Figure 3. Panama TR4 diagnosis workflow.

4.1 Isolation of the fungus from discoloured vascular strands

Isolation of *Foc* TR4 from discoloured vascular strands should be performed by trained technicians in biosecurity containment laboratories. For dry discoloured vascular strands, the tissues are cut into small segments approximately 0.5 – 1 cm x 0.3 - 0.5 cm. Half of each piece are plated onto paired half strength Potato Dextrose Agar amended with Streptomycin (SPDA) and Nash-Snyder (NS) Medium (see Appendices 8.1.1 and 8.1.2 for SPDA and NS media preparation). The number of isolation pieces should be limited to a maximum of five per plate, equally spaced apart. For fresh banana pseudostem or corm, discoloured vascular strands dissected from these samples can be plated directly on SPDA and NS plates without letting them dry. If NS media is not used, tissue should be surface sterilized with 80% (v/v) ethanol, rinsed with sterile distilled water and dried using sterile plotting paper before plating. 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 dissected strands dry in sterile blotting paper if this occurs and increase the strength of the antibacterial concentration in the media. A high rate of recovery of *Fusarium* should be expected from correctly prepared samples. Isolation of *Fusarium oxysporum* is generally consistent from symptomatic tissue. Colony morphology should be checked by microscopy (Section 4) before isolates are progressed for further analysis. Monoconidial (single-spore) cultures should be prepared from an isolate from each specimen.

4.2 Morphological identification by microscopic examination

There are three types of asexual spores formed by *Fo*; macroconidia, microconidia and chlamydospores (Figure 4) (Nelson 1991). Macroconidia are formed from monophialides on branched conidiophores in sporodochia, and to a lesser extent from monophialides on hyphae (Leslie and Summerell 2006). Macroconidia are four to eight celled, sickle-shaped, thin-walled and delicate, with foot-shaped basal attenuated apical cells (Jones 2000). Microconidia are abundantly borne on false heads on short monophialides, are one or two celled, oval- to kidney shaped. The dimensions of the macro- and microconidia are (typically) in the range of 27-55µm x 3.3-5.5µm and 5-16µm x 2.4-3.5µm, respectively. Chlamydospores are thick-walled, asexual, globose spores 7-11µm, usually formed singularly or in pairs, but may also be found in clusters or short chains (Jones 2000; Leslie and Summerell 2006). The critical morphological features of *Fo* include the production of microconidia on false heads on short phialides formed on the hyphae, the production of chlamydospores, no polyphialides, micro-conidia have oval shape and no septate (Leslie and Summerell 2006) (Figure 5).

Fusarium species are well-known for their tendency to mutate in culture thereby making it difficult to maintain the morphology and virulence of isolates (Nelson 1991). On initial isolation, most wild-type cultures produce macroconidia in sporodochia. The sporodochial type fungus often mutates in culture and to a lesser extent in nature. Cultures may become more mycelial and produce fewer sporodochia, or may mutate to the pionnotal forms which produce less aerial mycelium and macroconidia from conidiophores that form a slimy sheet, giving the culture a wet appearance. Mutation can often result in the production of different shaped macroconidia and loss of virulence. Cultural mutation can be minimised by culturing using the single-spore or hyphal-tip methods, by avoiding carbohydrate-rich media (e.g. PDA) and minimising prolonged subculturing. Short term maintenance of cultures should be on Carnation Leaf Agar (CLA) (Burgess *et al.* 1994). Long term maintenance of cultures should be by lyophilisation or in liquid nitrogen (Nelson 1991).

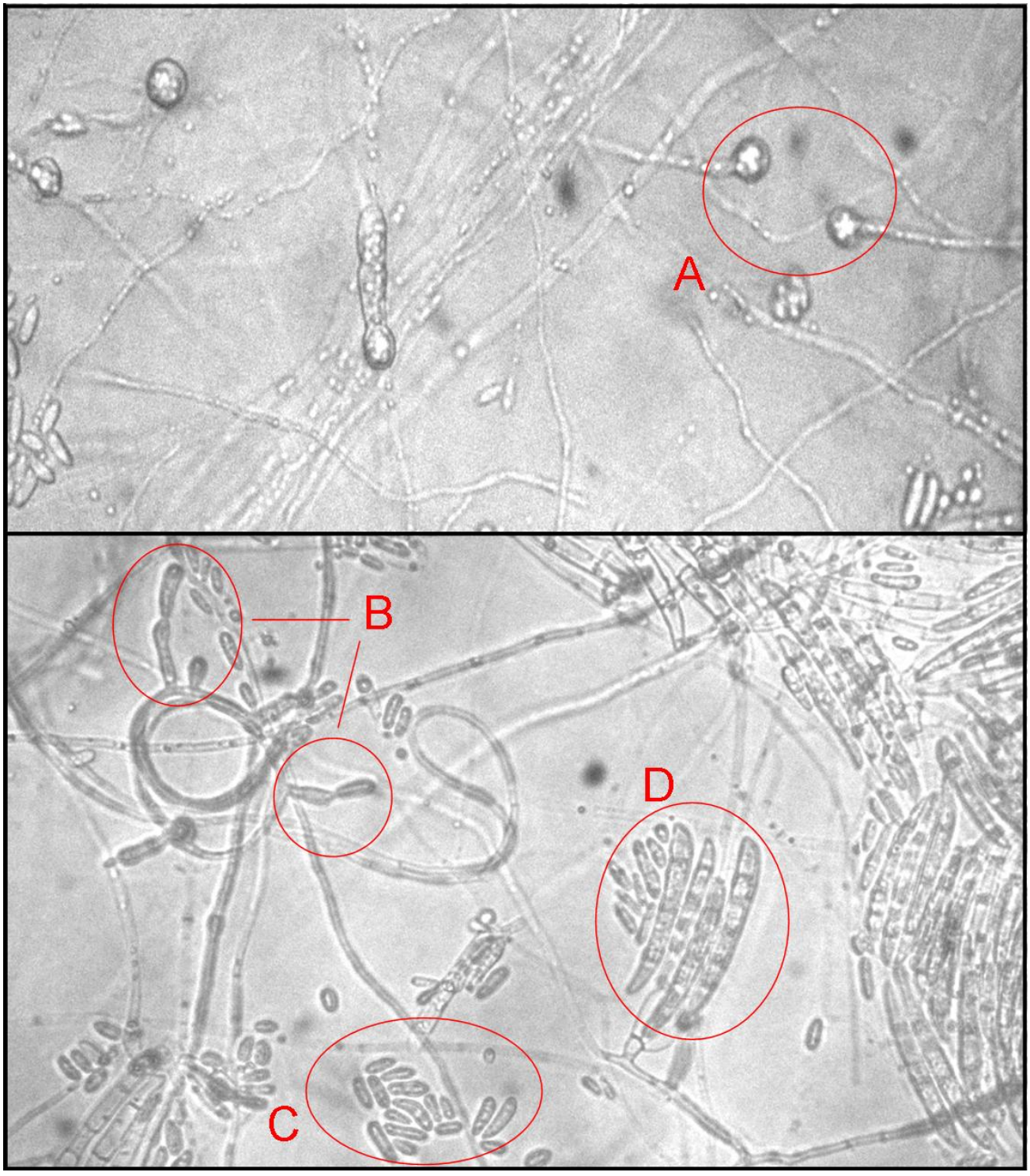

Figure 4. The three types of asexual spores produced by *Fusarium oxysporum*. (A) chlamydospores, thick-walled resting spores that can persist in the soil for long periods of time (over 30 years), (B) microconidia growing from conidiophores, (C) microconidia, (D) macroconidia. Photograph courtesy Wayne O'Neill, Queensland Department of Agriculture and Fisheries.

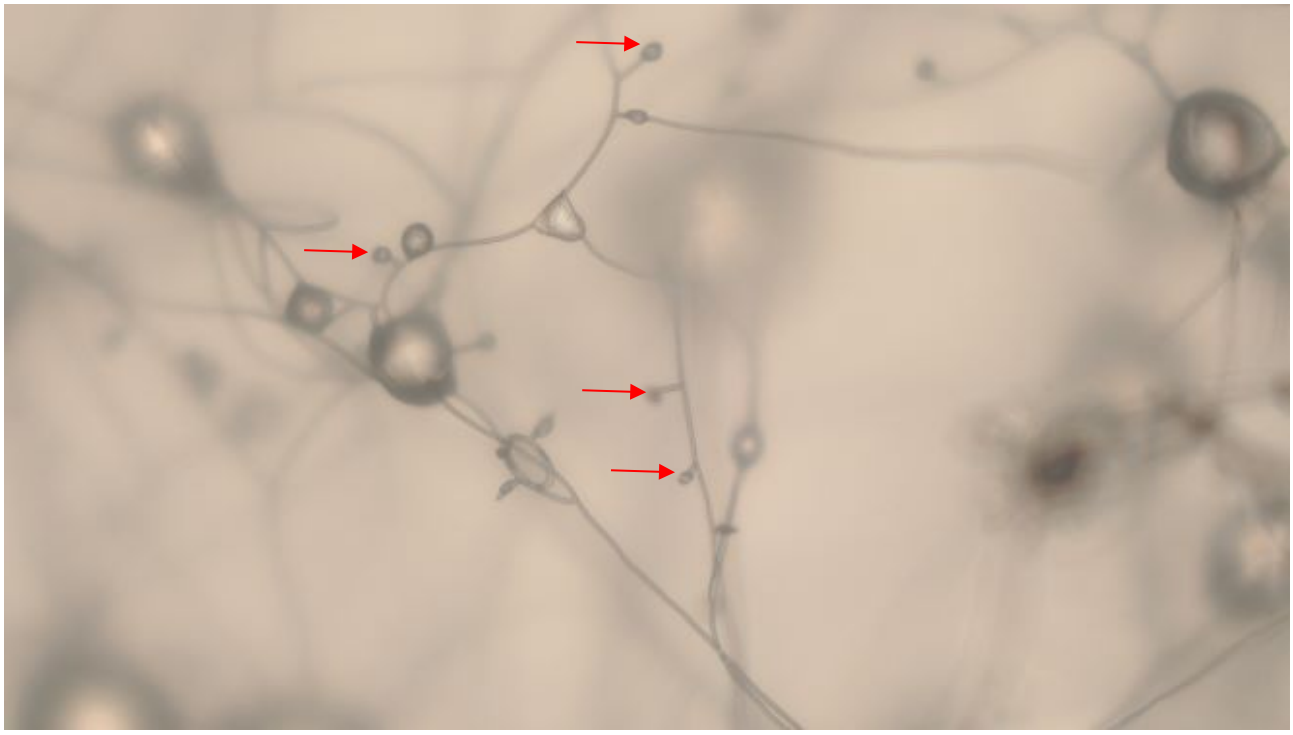

Figure 5. *Fusarium oxysporum* microconidia formed as false heads (as pointed by red arrows) on short phialides on the hyphae (photograph taken by the Panama TR4 diagnostic team at the Plant Biosecurity Laboratory, Department of Agriculture and Fisheries).

4.3 Generation of monoconidial cultures

Generation of monoconidial cultures (single spored cultures) creates a pure fungal culture and ensures rice testing, molecular and VCG are all performed on the same isolate. To generate monoconidial cultures, the following process is performed using sterile instruments and aseptic technique under a naked flame:

4.3.1 Streaking spore suspensions on water agar

i. Cut out two squares, approximately 6mm^2 each in size, of the SPDA subculture plate or isolation plate using a surface sterilised scalpel.
ii. Place the two squares into approximately 9mL of sterile distilled water and shake approximately 10 times to create a spore suspension.
iii. Streak the spore suspension onto a water agar plate using a sterile loop (see Figure 6). A basic two step streaking pattern (a straight line close to one edge which is distributed across the plate in a zig-zag) is sufficient to distribute the spores. Ensure that the suspension has been transferred to the plate and is not retained in the loop by surface tension.
iv. Seal the plate with Parafilm and store for approximately 8 - 30 hours at 25°C.

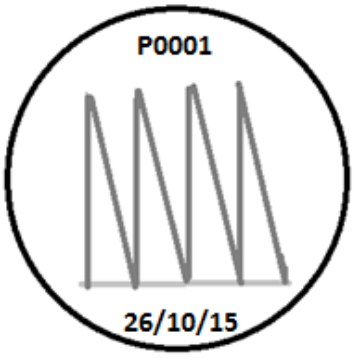

Figure 6: Pattern for streaking spore suspensions

4.3.2 Selection and excision of a single germinating spore

Following 8 - 30 hours of incubation at 25°C, the following process is performed under a microscope. Care should be taken to minimise the amount of time between sterilising the scalpel blade and cutting out/transferring each single germinating spore. The target spore should be selected under the microscope before sterilising the blade.

i. Set up the dissecting microscope using the bottom light source. The angle of the mirror may need to be adjusted while viewing the plate in order to get enough contrast to make the spores visible.
ii. A dense line of germinating spores should be visible at the edge where the loop was first applied to the plate in a straight line. Once the spores are in focus, the plate can be moved to an area where the spores are less dense (in the zig-zag streak) until single germinated conidia are visible.
iii. Dissect and pick out a single germinated conidia using a sterilised scalpel size 11 (Figure 7).
iv. Transfer two single spores to SPDA plates (one spore per plate) labelled with the sample number and date on the base.
v. Seal the SPDA plates with Parafilm and place them into the incubator at 25˚C. Allow the single-spore cultures to grow in the incubator for five days.

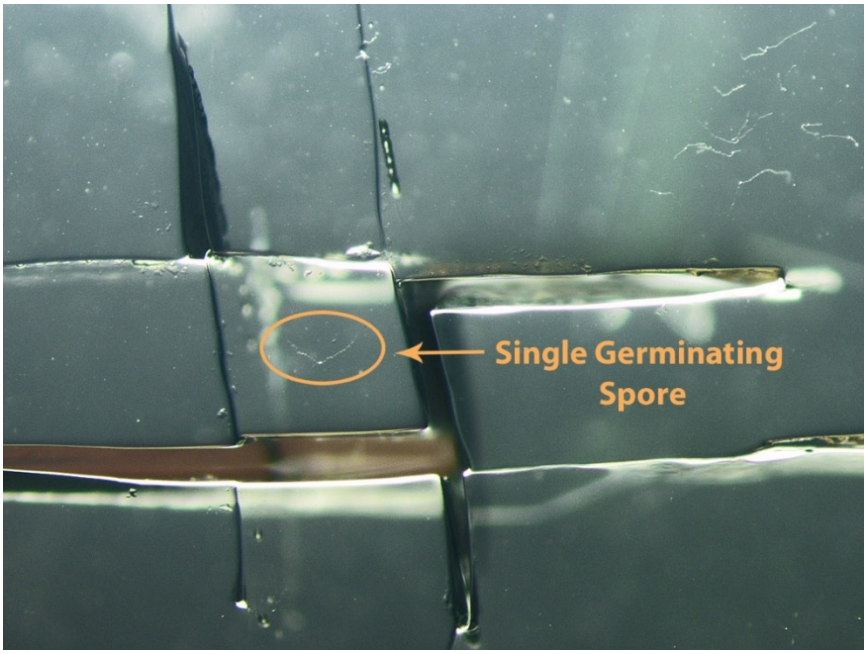

Figure 7. Selection and excision of a single germinating spore (photograph provided by the Panama TR4 diagnostic team at the Plant Biosecurity Laboratory, Department of Agriculture and Fisheries)

4.4 Maintenance 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 ½ SPDA 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 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. Mutated cultures (e.g. slimy pionnotal mutants) should be discarded. Cultures are normally maintained in an incubator at 25°C. Black light is generally not required for cultures of *Foc* to sporulate. Various methods are used for long-term (e.g. lyophilisation, in glycerol at -80°C), medium-term (e.g. colonised filter paper in cold storage) and short-term (e.g. CLA) storage of cultures of *Foc*.

4.5 Rice test for volatile analysis

4.5.1 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* (Brandes 1919; Stover 1962b). 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* (Moore *et al.* 1991; Pegg *et al.* 1993). These studies showed there was an absolute correlation between the production 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 including both TR4 and SR4 produced easily detectable volatile odours with characteristic gas chromatogram profiles (Table 1). Volatile analysis is a simple and inexpensive method of characterising isolates of *Foc* based on the biochemistry of cultures *in vivo*.

4.5.2 Rice test procedure for volatile analysis

i. Prepare rice medium (see Appendix 8.1.7).
ii. Aseptically inoculate rice with two squares (approx. 1cm^2) of *Fusarium* culture grown on SPDA.
iii. Cut out two squares approximately 6mm^2 of the sample and place into the flask of rice medium.
iv. Re-seal the flask with its cotton wool plug and aluminium foil cover.
v. Place the flask in the incubator at 25˚C for 7-14 days.
vi. After 7-14 days, assess the odour of volatiles produced by the culture. Remove the cotton plug and waft the air from the top of the flask towards your nose. A volatile odour is easily recognised as a sharp, pungent (benzoic) volatile odour.

4.6 Vegetative compatibility group (VCG) analysis of *Foc*

As discussed in Section 2.1.2, vegetative compatibility group (VCG) analysis has been utilised to group isolates of *Foc* (Brake *et al.* 1990; Moore 1994; Moore *et al.* 1993; Ploetz 1990b; Ploetz and Correll 1988). A modified VCG technique is used, based on the generation of nitrate non-utilising (*nit*) auxotrophic mutants. This technique was originally developed by Cove (1976) for *Aspergillus nidulans*, and was later modified for use with *Fusarium oxysporum* (Correll *et al.* 1987; Puhalla 1985). Cultures growing on CLA or PDA medium are used to inoculate plates of a medium containing potassium chlorate (KPS, see Appendix 8.1.5 for preparation of KPS media). 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 (Figure 8). 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 (MM, see Appendix 8.1.8 for media preparation) (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 M1011, the sectors can be sequentially numbered as M1011-1, M1011-2, M1011-3, and so forth.

4.6.1 *Generation of nitrate non-utilising (*nit*) mutants*

i. Label three KPS medium plates (containing potassium chlorate) with sample number and date on the base.
ii. Retrieve the single-spore cultures from the incubator.
iii. Select the healthiest single-spore culture. If there is a difference in size, choose the largest colony, or if one plate has a contaminant choose the other. Cut out three (one for each KPS plate) approximately 1 cm^2 squares of SPDA with mycelium using a surface sterilised scalpel.
iv. Position each piece near the edge of the plate, mycelium side down and slide the square across the centre of the plate, spreading the spores across the agar. This will provide a large surface area for the mutant sectors to develop.
v. Seal the KPS plates with Parafilm and store in the incubator at 25°C.
vi. If an isolate does not produce mutant sectors within seven days, it is transferred to plates of KPS with a higher proportion of $KClO_3$ (e.g. 'KPS 45', 45 g/L $KClO_3$) by subculturing approximately 1 cm^2 square plug of mycelia from a KPS plate and sliding it across the surface of the KPS 45 plate, mycelium side down.

4.6.2 *Transferring the* nit *mutants to minimal medium*

i. Observe KPS plates daily to assess the growth type on the media. Initially, the plates will only have slow-growing colonies with restricted, 'knotted' mycelial growth (due to chlorite toxicity), but after approximately five days, fast-growing sectors (*nit* mutants) will be generated.
ii. Once at least four sectors are generated across the three KPS plates, mark the outer edge of each *nit* mutant sector (as far away from the unmutated, restricted growth as possible) with a dot and number them sequentially on the underside of the KPS plate.
iii. Label four MM plates e.g. M1011-1, M1011-2, M1011-3 & M1011-4, etc.
iv. Using the dot as a guide, remove a small (approximately 2 mm^2) square of the *nit* mutant and transfer this to the corresponding MM plate (Figure 8).

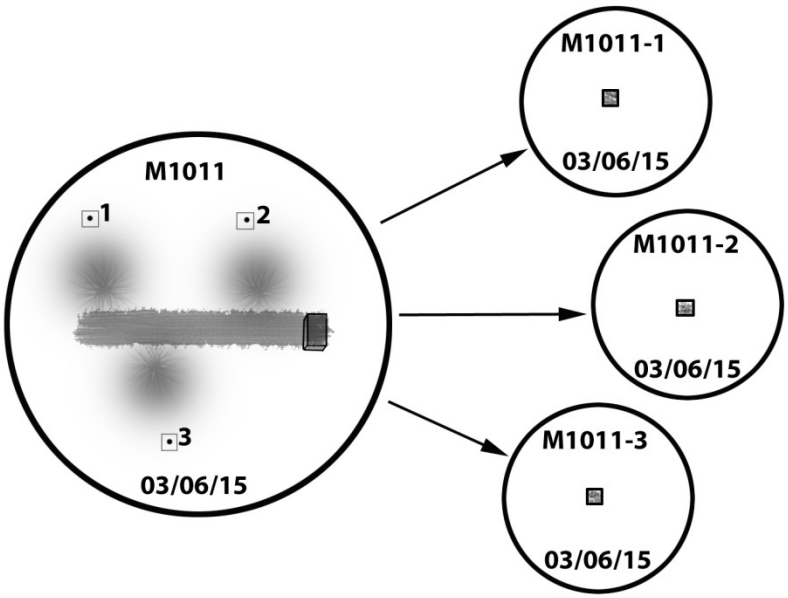

Figure 8. Generating and transferring *nit* mutants

4.6.3 *Identification of true* nit *mutants using minimal media*

i. Observe the growth on the MM plates daily. If the sector is a true *nit* mutant, it will not be able to utilise the nitrogen in the MM plates and the growth on the plates will remain flat and sparse.
ii. Discard any cultures that produce fluffy mycelial growth. A minimum of three *nit* mutants are required to set up a reliable VCG test so subculture more mutant sectors from the KPS plates if required until a sufficient number of true *nit* mutants have been obtained.
iii. After approximately three days of growth on MM the *nit* mutant colonies will be large enough to identify as true *nit* mutants and provide sufficient material for VCG testing.

4.6.4 *Determining the phenotype 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) which is produced when the *nit* mutant culture is transferred to media that has nitrogen present in only one of four forms (Correll *et al.* 1987). For further explanation of the phenotypes and which combinations are best to use in VCG tests refer to Correll *et al.* (1987) and Leslie and Summerell (2006). It is advantageous to generate several (at least four or five) *nit1* or *nit3* mutants from each isolate to pair in combinations with the NitM testers (mutants of known VCG). Mutants of the *nit1* or *nit* 3 phenotype are the most commonly generated type of mutant. Mutants of the NitM phenotype are less commonly generated and are best used as the 'testers' of known VCG.

The phenotype of *nit* mutants can be determined based on their different abilities to use various nitrogen sources (Table 2). Four phenotyping media that differ in their nitrogen source are used for determining the *nit* mutant phenotype, including MM + $NaNO_3$, MM + $NaNO_2$, MM + hypoxanthine, and MM + ammonium tartrate (see Appendix 8.2 for media preparation). Wild-type strains can utilize all of the nitrogen sources but are sensitive to ClO_3. *nit1* strains can grow on all of the media except for those with NO_3 as the sole nitrogen source. *nit3* mutants can grow on all media except for those with NO_3 or NO_2 as the nitrogen source. NitM mutants can grow only on media with NO_2 or NH_4 as the nitrogen source. *crn* (ClO_3-resistant NO_3-utilizing) mutants can grow on all of the media and are both ClO_3-resistant and NO_3-utilizing. In essence the medium containing NH_4 serves as a positive control, and the medium with NO_3 as a negative control (Leslie and Summerell, 2006).

Table 2. Determining the phenotype of nit mutants based on their ability to utilise different nitrogen sources (Leslie and Summerell, 2006).

	Medium supplement				
Strain type	**NH_4**	**NO_3**	**NO_2**	**Hypoxanthine**	**ClO_3**
Wild type	+	+	+	+	-
nit1	+	-	+	+	+
nit3	+	-	-	+	+
NitM	+	-	+	-	+
crn	+	+	+	+	+

4.6.5 Pairing nit mutants in VCG tests

i. A positive control plate is set up for each batch of VCG testing. The positive control consists of TR4 positive control mutants (*nit1*) mated with one of the TR4 NitM testers used for the diagnostic sample(s).

ii. Line up the VCG testers (the *Foc* NitM mutants of known VCG) to be used and place new MM plates, face down, adjacent to each tester on the bench.

iii. Label each of the plates with:
- NitM tester VCG code, accession number and date (in the middle of the plate)
- numbers for the unknown *nit* mutants around the outside of the plates (up to four unknown *nit* mutants per plate).
- Media batch number.

iv. Turn the plates over and, using the labels on the underside of the plate as a guide, place small approximately 4 mm^2 piece of colonised agar from the MM cultures into the new VCG plate, on top of their corresponding label. Sterilise the scalpel before subculturing each piece. The finished VCG plates should look like Figure 9.

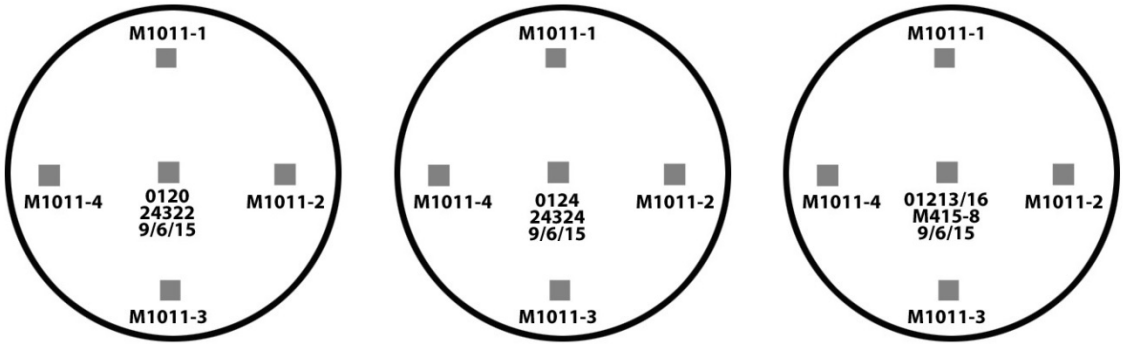

Figure 9. Example of pair-wise arrangement of four nit mutants. NitM testers (VCGs 0120, 0124 and 01213/16) are placed in the centre of the plate with the nit mutants around the edge (isolate number M1011).

v. Keep the paired VCG plates in the incubator at 25°C, checking every day for the formation of heterokaryon growth. The heterokayron is a line of dense, fluffy nitrogen-sufficient growth that forms within approximately seven days if the isolate is vegetatively compatible with one of the NitM testers (Figure 10).

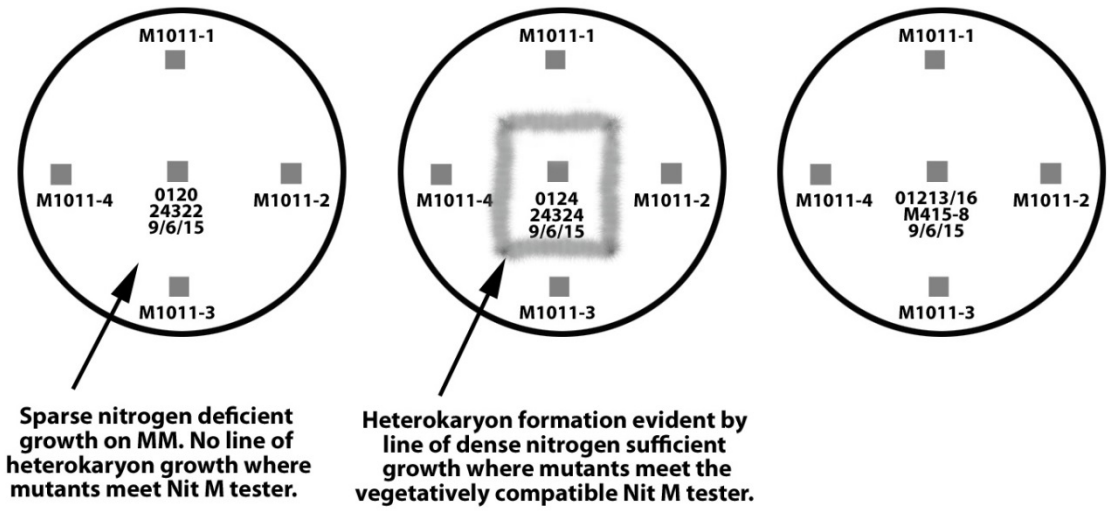

Figure 10. An example of a positive VCG test.

4.7 Molecular identification

Many molecular methods have been used to assess genetic relationships among the VCGs and races of *Foc*. Next generation sequencing (NGS) particularly whole genome shotgun sequencing, DArTseq, RNA-Seq, digital gene expression profiling and Miseq (Ordonez *et al*. 2015, Guo *et al*. 2014, Fraser-Smith et al. 2014, Wang *et al*. 2012). Other molecular methods used to determine the genetic relatedness between isolates of *Foc,* within each VCG and among the VCGs include analysis of the intergenic spacer region of rDNA (IGS; Dita *et al.* 2010), and DNA amplification fingerprinting (DAF) (Bentley *et al.* 1998; Gerlach *et al.* 2000). However, there have been issues with specificity and resolution of the Dita IGS protocol when applied to Australian isolates of *Foc* (McKillop 2016). Therefore the protocol cannot be used in isolation for *Foc* diagnostics. VCG testing is required with the use of additional molecular diagnostics.

Vegetative compatibility grouping (VCG) is considered the gold standard for a definitive diagnosis of the strain of *Foc* causing Panama disease. However, a matrix of molecular assays targeting two loci, 28S rDNA (Fo PCR, Edel *et al*. 2000) and *SIX8* gene (SIX8a/SIX8b PCR, Fraser-Smith *et al*. 2014) in the pathogen are also used to support VCG analysis and provide an interim diagnosis of TR4 (Table 3).

Table 3. Molecular diagnostic matrix for detection of TR4

	Fo PCR	SIX8 PCR	SIX8b PCR	TR4 VCG	TR4 Diagnosis
TR4 *Foc*	+ve	+ve	-ve	+ve	Positive
SR4 *Foc*	+ve	+ve	+ve	-ve	Negative
R1 *Foc*	+ve	-ve	-ve	-ve	Negative
Other *Fo*	+ve	-ve	-ve	-ve	Negative
Other *Fusarium*	-ve	-ve	-ve	-ve	Negative

The following protocols for molecular diagnostics have been adapted from the SOPs developed by the Plant Biosecurity Laboratory (PBL) for diagnosis of TR4. All molecular tests are performed on DNA extracted from monoconidial cultures. Extraction of DNA from monoconidial cultures is performed using commercial plant DNA extraction kit, e.g. DNeasy Plant Mini Kit (Qiagen) as per manufacturer's instructions.

4.7.1 Fusarium oxysporum PCR

The *Fusarium oxsporum* PCR (Edel *et al.* 2000) has been designed for the detection of *Fusarium oxysporum.* Based on sequence data from the large subunit (28S) of ribosomal DNA (rDNA), primers PFO2 and PFO3 were designed by Edel *et al.* (2000) and tested on 16 strains of *F. oxysporum* and 80 strains of 23 other species of *Fusarium*. The PCR specifically amplified a 70bp DNA amplicon from the *F. oxysporum* strains tested and not from the other species.

Reagents

- 95% and/or absolute ethanol
- Primers

Primer	Name	Sequence 5'-3'
Forward Primer	PFO2	CCC AGG GTA TTA CAC GGT
Reverse Primer	PFO3	CGG GGG ATA AAG GCG G

- Sterile Reagent Grade Water (Nuclease-free)
- 5x MyTaq™ Reaction Buffer Red
- MyTaq™ HS DNA Polymerase
- Bioline agarose tablets
- GelRed Nucleic Acid Gel Stain, 20,000X in water
- 0.5xTBE buffer
- Bioline Easy Ladder I

Preparation of Reaction Mixture

- Remember to include controls: Positive TR4 Control, Positive SR4 Extraction Control, Negative extraction Control, Negative Template Control (NTC). *Foc* SR4 BRIP 42107a (VCG 0120), BRIP 44622a (VCG 0129), and BRIP 44073a (VCG 01211), which can be sourced from Queensland Plant Pathology Herbarium (BRIP), can be used as positive SR4 extraction controls.
- Label thin-walled snap-strip PCR tubes
- Allow all reagents to thaw properly, briefly vortex and microfuge (5-10 sec pulse) all reagents and mixtures before opening tubes.
- Return reagents to the -20°C freezer as quickly as possible when finished.
- Prepare mastermix in sterile microfuge tubes in descending order as follows:

Reagent	Final Conc.	X1 (µl)	(n) +2 §
Water	-	16.6	16.6 * (n + 2)
5X MyTaq Reaction Buffer Red	1x	5	5 * (n + 2)
Primer PF02 (12.5µM)	0.5µM	1	1 * (n + 2)
Primer PF03 (12.5µM)	0.5µM	1	1 * (n + 2)
MyTaq HS DNA Polymerase (5U/µl)	2U	0.4	0.4 * (n + 2)
Total		24	24 * (n + 2)

§ n = the number of PCR reactions; samples are run in duplicate and therefore n = number of samples and controls * 2

- Dispense 24µL of final mix into each PCR reaction tube and cap.
- Place these in the fridge until sample addition.

Addition of samples

- All samples are tested in duplicate.
- Remove the caps from the NTC open tubes. Add 1µL sterile RNase/DNase-free water to these tubes and leave uncapped until all samples and controls have been added.
- Add 1µL of test samples (including negative extraction control) to their respective PCR tubes containing 24µL of reaction mixture.
- Positive control should be added last.
- Recap the NTC open tubes.
- Transfer the reaction tubes to the designated area for amplification.

Amplification

- PCR should be performed on a calibrated thermocycler with heated lid
- The following parameters are used for amplification:

Stage 1	1x	95°C/3min
Stage 2	30X	95°C/30sec
		62°C/30sec
		72°C/30sec
Stage 3	1x	72°C/3min
	1x	16°C/∞

Preparation of 1.5% agarose gels

To prepare a 1.5% agarose gel, add 1.5g agarose (3 x 0.5g tablets) to 100mL of 0.5x TBE buffer in a 250mL Schott bottle and leave to dissolve. These can be prepared in advance and left on the shelf for later use.

Electrophoresis

- Add 3µL of Easy Ladder I molecular weight marker to the first and last wells.
- Starting at well 2, pipette 5µL of sample into the wells.
- Add 5µL of positive control to the final sample well.
- Run the gel at 100V until adequate separation of the indicator dyes has occurred i.e. until the quickest dye has run to within 2cm of the bottom of the gel.
- Photograph gel.

Expression of results

- *Fo* positive reactions contain a 70bp product.
- The test has passed if there is no product in the negative extraction control and no template control and a 70bp product in the positive extraction control and positive template control.
- The presence of a PCR product in the negative controls and/or the absence of a 70bp PCR product in positive controls indicate the test has failed and must be repeated to obtain a valid result.

4.7.2 SIX8/SIX8b PCRs

The SIX8/SIX8b Polymerase Chain Reaction (PCR) (Fraser-Smith *et al.* 2014) has been designed for the detection of subtropical race 4 (SR4) and tropical race 4 (TR4) strains of *Fusarium oxysporum* f. sp. *cubense*. The presence of *SIX8* genes in race 4 *Fusarium oxysporum* f. sp. *cubense* isolates differentiates them from races 1 and 2. Furthermore, isolates of TR4 and SR4 can be distinguished from each other based on the presence of *SIX8* homologs. The SIX8 PCR amplifies a 770bp DNA amplicon from both SR4 and TR4, while the SIX8b PCR assay amplifies a 595bp DNA amplicon from SR4 only.

The presence of an amplicon in SIX8 and absence in SIX8b indicates the sample to be TR4.

Reagents

- 95% and/or Absolute ethanol
- Primers:

Primer	Name	Sequence 5'-3'
SIX8 Forward	Foc-SIX8-F	CGA AGT GCG CCA TAT AAG ACT
SIX8 Reverse	Foc-SIX8-R	CAC CTG CTT GCT CCT TAT CC
SIX8b Forward	Foc-SIX8b-F	CGT CCT TAC TTA TAT ACC CTC TCA A
SIX8b Reverse	Foc-SIX8b-R	GGC CTA ATC CAC ACA ACA

- Sterile Reagent Grade Water (Nuclease-free)
- 5x MyTaq™ Reaction Buffer Red (Bioline)
- MyTaq™ HS DNA Polymerase (Bioline)
- Bioline agarose tablets
- GelRed Nucleic Acid Gel Stain, 20,000X in water
- 0.5xTBE buffer
- Bioline Easy Ladder I

Preparation of Reaction Mixture

- Remember to include controls: Positive TR4 Template Control, Positive SR4 Template Control, Negative *Foc* R1 or R2 Template Controls, Negative Extraction Control, and Negative Template Control (NTC) open. *Foc* SR4 BRIP 42107a (VCG 0120), BRIP 44622a (VCG 0129), and BRIP 44073a (VCG 01211), which can be sourced from Queensland Plant Pathology Herbarium (BRIP), can be used as positive SR4 template controls.
- Ensure all reagents are thawed properly, briefly vortex and microfuge (5-10 sec pulse) all reagents and mixtures before opening tubes.
- Return reagents to the -20°C freezer as quickly as possible when finished.
- Prepare two mastermixes in sterile microfuge tubes in descending order as follows:

SR4/TR4 Diagnostic (Foc-SIX8-F/Foc-SIX8-R) 770bp			
Reagent	Final Conc.	x1 (µl)	(n) +2 §
Water	-	16.6	16.6 * (n + 2)
5X MyTaq Reaction Buffer Red	1x	5	5 * (n + 2)
Primer Foc-SIX8-F (10µM)	0.4µM	1	1 * (n + 2)
Primer Foc-SIX8-R (10µM)	0.4µM	1	1 * (n + 2)
MyTaq HS DNA Polymerase (5U/µl)	2U	0.4	0.4 * (n + 2)
Total		24	24 * (n + 2)

SR4 Diagnostic (Foc-SIX8b-F/Foc-SIX8b-R) 595bp			
Reagent	Final Conc.	x1 (µl)	(n) +2 §
Water	-	16.6	16.6 * (n + 2)
5X MyTaq Reaction Buffer Red	1x	5	5 * (n + 2)
Primer Foc-SIX8b-F (10µM)	0.4µM	1	1 * (n + 2)
Primer Foc-SIX8b-R (10µM)	0.4µM	1	1 * (n + 2)
MyTaq HS DNA Polymerase (5U/µl)	2U	0.4	0.4 * (n + 2)
Total		24	24 * (n + 2)

§ n = the number of PCR reactions; samples are run in duplicate and therefore n = number of samples and controls * 2

- Dispense 24µL of final mix into each PCR reaction tube and cap.
- Place these in the fridge until sample addition.

Addition of samples

- All samples are tested in duplicate.
- Sample addition is performed as instructed as in the section “Addition of samples” for *Fo* PCR (Section 4.7.1) as above.

Amplification

- PCR should be performed on a calibrated thermocycler with heated lid.
- The following parameters are used for both PCRs:

Race 4 Diagnostic (770bp and 595bp)		
Stage 1	1x	95°C/3 Min
Stage 2	35x	95°C/15sec
		60°C/15sec
		72°C/10sec
Stage 3	1x	72°C/2min
	1x	16°C/∞

Preparation of 1.5% agarose gels and electrophoresis

- Preparation of 1.5% agarose gels and electrophoresis are performed as instructed as in the sections "Preparation of 1.5% agarose gels" and "electrophoresis" for *Fo* PCR (Section 4.7.1) as above.

Expression of results

- Positive SIX8 reactions contain a 770bp product.
- Positive SIX8b reactions contain a 595bp product.
- SIX8/SIX8b test samples with a 770bp product and 595bp product respectively, are positive for SR4.
- SIX8/SIX8b test samples with a 770bp product only are positive for TR4.
- For SIX8 the test has passed if there is no product in the *Foc* R1 *Foc* R2 template control, negative extraction control and no template control, and a 770bp product in the TR4 and SR4 positive controls.
- For SIX8b the test has passed if there is no product in the *Foc* R1 or R2 template control, TR4 template control, negative extraction control, and no template control and a 595bp products is observed in the SR4 template controls.
- The presence of a PCR product in any of the negative controls for either the SIX8 or SIX8b PCR indicates the test has failed and must be repeated to obtain a valid result.
- The absence of a PCR product in any of the positive controls for either the SIX8 or SIX8b PCR indicates the test has failed and must be repeated to obtain a valid result.

4.7.3 Elongation factor (EF) internal control PCR

This EF PCR assay has been designed for the detection of the translation elongation factor-1α (EF) from fungal species. The TEF PCR amplifies a 660bp DNA amplicon from *Fusarium* species and can be used as a positive internal control PCR for diagnostic specimens to confirm 1) the integrity of the template is suitable for PCR amplification and, 2) negative PCR results in subsequent *Fo* and *SIX8* PCRs

are not due to the presence of inhibitory factors. Positive amplicons may then be sequenced for identification of most, but not all species within the *Fusarium* genus.

Reagents

- 95% and/or absolute ethanol
- Primers

Primer	Name	Sequence 5'-3'
Forward Primer	EF1	ATG GGT AAG GAR GAC AAG AC
Reverse Primer	PFO3	GGA RGT ACC AGT SAT CAT GTT

- Sterile Reagent Grade Water (Nuclease-free)
- 2x Phusion High-Fidelity PCR Master Mix with HF Buffer (New England Biosciences; NEB, Catalog # M0531S) or Phusion High-Fidelity PCR Kit (Catalog # E0553L).
- 0.5 g agarose tablets (Bioline) or equivalent agarose powder (Certified Molecular Biology Agarose of Biorad, Cat # 1613102)
- GelRed Nucleic Acid Gel Stain, 10,000X in water
- 0.5xTBE buffer
- Easy Ladder I or equivalent ladder (eg. hyper ladder)(Bioline)
- GelPilot 6x loading dye (Bioline)

Preparation of Reaction Mixture

- Remember to include the following controls: Positive TR4 Template Control, Negative extraction Control, Negative Template Control (NTC).
- Label thin-walled snap-strip PCR tubes
- Allow all reagents to thaw properly, briefly vortex and microfuge (5-10 sec pulse) all reagents and mixtures before opening tubes.
- Return reagents to the -20°C freezer as quickly as possible when finished.
- Prepare mastermix in sterile microfuge tubes in descending order as follows:

Reagent	**Final Conc.**	**X1 (µl)**	**(n) +2 §**
Water	-	19	19 * (n + 2)
2x Phusion High-Fidelity PCR Master Mix with HF Buffer (NEB) (Cat # M0531S)	1x	25	25 * (n + 2)
Primer EF1 (10µM)	0.5µM	2	2 * (n + 2)
Primer EF2 (10µM)	0.5µM	2	2 * (n + 2)
Total		48	48 * (n + 2)

§ n = the number of PCR reactions; samples are run in duplicate and therefore n = number of samples and controls * 2

- Dispense 48µL of final mix into each PCR reaction tube and cap.
- Place these in the fridge until sample addition.

Addition of samples

- Remove the caps from the NTC open tubes. Add 2µL sterile RNase/DNase-free water to these tubes and leave uncapped until all samples and controls have been added.
- Add 2µL of test samples (including negative extraction control) to their respective PCR tubes containing 48µL of reaction mixture.
- Positive control should be added last.
- Recap the NTC open tubes.
- Transfer the reaction tubes to the designated area for amplification.

Amplification

- PCR should be performed on a calibrated thermocycler with heated lid
- The following parameters are used for amplification:

Stage 1	1x	98°C/30sec
Stage 2	30X	98°C/10sec
		55°C/30sec
		72°C/60sec
Stage 3	1x	72°C/5min
	1x	16°C/∞

Preparation of 1.5% agarose gels

To prepare a 1.5% agarose gel, add 1.5g agarose (3 x 0.5g tablets) to 100mL of 0.5x TBE buffer in a 250mL Schott bottle and leave to dissolve. These can be prepared in advance and left on the shelf for later use.

Electrophoresis

- Add 3µL of Easy Ladder I molecular weight marker to the first and last wells.
- Starting at well 2, pipette 5µL of sample into the wells. The remaining PCR products can be used for DNA sequencing if needed.
- Add 5µL of positive control to the final sample well.
- Run the gel at 100V until adequate separation of the indicator dyes has occurred i.e. until the quickest dye has run to within 2cm of the bottom of the gel.
- Photograph gel.

Expression of results

- Amplification is confirmed through visualisation of a 660 bp product.

- The test has passed if there is no product in the negative extraction control and no template control and a 660bp product in the positive template control.
- The presence of a PCR product in the negative controls and/or the absence of a 660bp PCR product in the positive template control indicate the test has failed and must be repeated to obtain a valid result.

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The protocol was reviewed by S Mintoff, NT Department of Industry, Tourism and Trade.

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8 APPENDICES

8.1 Media for the isolation and culturing of *Fo*

8.1.1 Half strength Potato Dextrose Agar amended with streptomycin (½ SPDA)

½ SPDA is media used for the isolation and culturing of fungi. Potato Dextrose Agar is a general-purpose medium that is supplemented with antibiotics (200 ppm streptomycin) to inhibit bacterial growth. Half strength potato dextrose is used to slow the growth of fungal colonies by restricting nutrients, and in order to reduce the occurrence of mutations.

Personal protective requirements

- Lab coat
- Disposable latex or nitrile gloves
- Safety glasses (for handling hot media)
- Insulated or heat resistant gloves (for handling hot media)
- Plastic apron (if handling waste)
- Enclosed shoes

Ingredient	Structure	Hazardous	1 L	500 mL
Distilled water	H_2O	None	1000 mL	500 mL
Potato Dextrose Agar (commercial mix)		None	19.5 g	9.75 g
Agar/Solidifier *Difco*		Low	7.5 g	3.75 g
Streptomycin sulfate (4 mg/mL)	$C_{42}H_{84}N_{14}O_{36}S_3$	Moderate	5 mL	2.5 mL

Method

1. Prepare 4mg/mL antibiotic streptomycin sulfate stock solution.
2. Filter sterilise streptomycin sulfate stock solution using a 0.22 µm filter membrane. The streptomycin sulfate stock solution can be stored in the 4°C fridge and used for a month.
3. Weigh dry ingredients on balance in weigh boat separately, transferring into bottle after each ingredient.
4. Add distilled water and mix well.
5. Autoclave media with lids loosened at 121°C for 20 min.
6. Remove bottles containing media from autoclave, tighten lids and allow media to cool to approximately 55°C in water bath.
7. Add 2.5 mL of 4 mg/mL antibiotic stock solution to 500mL of media in laminar flow and pour plates inside the laminar flow cabinet.

8.1.2 Nash-Snyder (NS) medium

Nash-Snyder (NS) medium was specifically developed to select for *Fusarium* species. NS is used for the isolation of *Fusarium* from plant and soil. It contains the fungicide, pentachloronitrobenzene (PCNB) and antibiotics streptomycin and neomycin that supress the growth of non-target fungi and bacteria but allows the growth of *Fusarium*.

Safety Precautions

PCNB and neomycin are highly hazardous therefore dust masks should be worn and handling these chemicals should take place inside a fume cupboard.

Personal protective requirements

- Lab coat
- Disposable latex or nitrile gloves
- Safety glasses (for handling hot media)
- Insulated or heat resistant gloves (for handling hot media)
- Plastic apron (if handling waste)
- Enclosed shoes

Ingredient	Structure	Hazardous	1 L	400 mL	500 mL
Distilled water	H_2O	None	1000 mL	400 mL	500 mL
Magnesium sulphate heptahydrate	$MnSO_4.7H_2O$	Low	0.5 g	0.2 g	0.25 g
Potassium dihydrogen phosphate	KH_2PO_4	Low	1 g	0.4 g	0.5 g
PCNB (Terraclor 75% wettable powder)		Moderate	1 g	0.4 g	0.5 g
Peptone		Low	15 g	6.0 g	7.5 g
Agar		Low	15 g	6.0 g	7.5 g
Streptomycin sulphate		Moderate	1 g	0.4 g	0.5 g
Neomycin sulphate		High	0.12 g	0.048 g	0.06 g

Method

1. Add distilled water to desired volume.
2. Weigh dry ingredients on balance in weigh boat separately, transferring into bottle after each ingredient. Magnesium sulphate should be the first dry ingredient added and dissolved then potassium dihydrogen orthophosphate and the rest of the dry ingredients, minus agar and antibiotics can be added.
3. Adjust pH to 6.0 ± 0.5 using either HCl (5M or 1M) or NaOH (5M or 1M) unless specified otherwise.
4. Add agar.
5. Autoclave bottles/tubes with lids loosened at 121°C for 20 min.
6. Prepare streptomycin and neomycin in 10 mL sterile water and the solutions are filter sterilized using a 0.22um point of use filter.
7. Once cooled to approximately 55°C add the streptomycin and neomycin solution and pour plates inside the laminar flow cabinet.

Notes

- Selective for *Fusarium*.
- NS is light sensitive, wrap in foil for storage.
- Prepared in 90 mm Petri dish unless otherwise specified.

8.1.3 Water agar

Water agar is used to single-spore isolates. It slows down the growth of the fungi, and allows single germinated spores or single hyphae to be dissected from the medium with greater ease.

Personal protective requirements

- Lab coat
- Disposable latex or nitrile gloves
- Safety glasses (for handling hot media)
- Insulated or heat resistant gloves (for handling hot media)
- Plastic apron (if handling waste)
- Enclosed shoes

Ingredient	Structure	Hazardous	1 L	400 mL
Distilled water	H_2O	None	1000 mL	400 mL
Agar		None	15 g	6 g

Method

1. Weigh agar and transfer into bottle.
2. Add distilled water heat and mix until the agar has dissolved.
3. Autoclave media with lids loosened at 121°C for 20 min.
4. Remove bottles containing media from autoclave, tighten lids and allow media to cool to approximately 53°C in water bath.
5. Pour in 90 mm Petri dishes inside the laminar flow cabinet.

8.1.4 Carnation Leaf Agar (CLA; Burgess et al. 1988)

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 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°C until required.

8.1.5 Potassium chlorate Potato Sucrose (KPS) medium (Puhalla 1985)

KPS medium is potato dextrose medium amended with potassium chlorate. It is used in the VCG procedure to produce chlorate resistant mutant sectors.

Personal protective requirements

- Lab coat
- Disposable latex or nitrile gloves
- Safety glasses (for handling hot media)
- Insulated or heat resistant gloves (for handling hot media)
- Plastic apron (if handling waste)

- Enclosed shoes

Ingredient	Structure	Hazardous	1 L	500 mL	400 mL
Distilled water	H_2O	None	1000 mL	500 mL	400 mL
Potato Dextrose Agar		None	39 g	19.5 g	15.6 g
Potassium chlorate	$KClO_3$	Moderate	15 g	7.5 g	6 g

Method

1. Weigh dry ingredients on balance in weigh boat separately, transferring into bottle after each ingredient.
2. Add distilled water and mix well.
3. Autoclave media with lids loosened at 121°C for 20 min.
4. Remove bottles containing media from autoclave, tighten lids and allow media to cool to approximately 53°C in water bath.
5. Pour plates inside the laminar flow cabinet.

8.1.6 Potassium chlorate Potato Sucrose 45 (KPS 45) medium

KPS medium is potato dextrose medium amended with potassium chlorate. It is used in the VCG procedure to produce chlorate resistant mutant sectors. KPS 45 has an increased concentration of potassium chlorate and is used when a *Fusarium* isolate is very fast growing on KPS and will not produce chlorate resistant sectors.

Personal protective requirements

- Lab coat
- Disposable latex or nitrile gloves
- Safety glasses (for handling hot media)
- Insulated or heat resistant gloves (for handling hot media)
- Plastic apron (if handling waste)
- Enclosed shoes

Ingredient	Structure	Hazardous	1 L	400 mL	300 mL
Distilled water	H_2O	None	1000 mL	400 mL	300 mL
Potato Dextrose Agar (commercial mix)		None	39 g	15.6 g	11.7 g
Potassium chlorate	$KClO_3$	Moderate	45 g	18 g	13.5 g

Method

1. Weigh dry ingredients on balance in weigh boat separately, transferring into bottle after each ingredient.
2. Add distilled water and mix well.
3. Autoclave media with lids loosened at 121°C for 20 min.
4. Remove bottles containing media from autoclave, tighten lids and allow media to cool to approximately 53°C in water bath.
5. Pour plates inside the laminar flow cabinet.

8.1.7 Rice medium (Moore et al. 1991)

Rice medium is used for the rice test which assesses the production of volatiles. When *Fusarium oxysporum* f. sp*. cubense* TR4 (and subtropical race 4) grows on rice, the culture produces a distinctive benzoic odour.

Personal protective requirements

- Lab coat
- Disposable latex or nitrile gloves
- Safety glasses (for handling hot media)
- Insulated or heat resistant gloves (for handling hot media)
- Plastic apron (if handling waste)
- Enclosed shoes

Ingredient	Structure	Hazardous	250 mL
Distilled water	H_2O	None	90 mL
Rice		None	30 g

Method

1. Weigh rice into a 250 mL Erlenmeyer flask.
2. Add distilled water and mix well.
3. Plug with cotton wool wrapped in gauze and cover with aluminium foil.
4. Autoclave for 1 h twice, on two consecutive days, in autoclave on free steaming cycle at 103°C.
5. Allow medium to cool before inoculation.

8.1.8 Minimal medium (Puhalla et al. 1983)

Minimal medium is used to identify *nit* mutants and to determine VCGs through complementation tests. When a true *nit* mutant grows on MM, the growth of the culture will remain sparse until it anastomoses with a complementary *nit* of the same VCG at which time the culture will revert to the fluffy, nitrogen-sufficient wild type growth (i.e. a heterokaryon is formed).

Personal protective requirements

- Lab coat
- Disposable latex or nitrile gloves
- Safety glasses (for handling hot media)
- Insulated or heat resistant gloves (for handling hot media)
- Plastic apron (if handling waste)
- Enclosed shoes

Ingredient	Structure	Hazardous	1 L	400 mL
Distilled water	H_2O	None	1000 mL	400 mL
Magnesium sulphate heptahydrate	$MgSO_4.7H_2O$	Low	0.5 g	0.2 g
Potassium dihydrogen phosphate	KH_2PO_4	Low	1 g	0.4 g
Potassium chloride	KCl	Low	0.5 g	0.2 g
Ferrous sulphate heptahydrate	$FeSO_4.7H_2O$	Moderate	10 mg	4 mg
Sodium nitrate	$NaNO_3$	Moderate	2 g	0.8 g
Sucrose	$C_{12}H_{22}O_{11}$	Low	30 g	12 g
Sterile trace elements (see 8.1.9 for solution preparation)		Moderate	200 µL	80 µL

Agar/Solidifier Difco		Low	15 g	6 g

Method

1. Add distilled water to desired volume.
2. Weigh dry ingredients on balance in weigh boat separately, transferring into bottle after each ingredient. Magnesium sulphate heptahydrate should be the first dry ingredient added and dissolved, and then potassium dihydrogen phosphate and the rest of the dry ingredients except the agar/solidifier.
3. Adjust to pH 6.7 ± 0.5 using either HCL (5M or 1M) or NaOH (5M or 1M) unless specified otherwise.
4. Add the agar/solidifier and mix and top up to final volume.
5. Loosen the lid and place in the autoclave for a 30-minute free steaming cycle. At the end of the cycle, ensure that all ingredients are dissolved and molten.
6. Add trace elements using an autopipette.
7. Autoclave media with lids loosened at 121°C for 20 min.
8. Remove bottles containing media from autoclave, tighten lids and allow media to cool to approximately 53°C in water bath.
9. Pour plates inside the laminar flow cabinet.

8.1.9 Trace element solution

This solution is used as an ingredient in minimal media.

Personal protective requirements

- Lab coat
- Disposable latex or nitrile gloves
- Safety glasses (for handling hot media)
- Insulated or heat resistant gloves (for handling hot media)
- Plastic apron (if handling waste)
- Enclosed shoes

Ingredient	**Structure**	**Hazardous**	**100 mL**
Sterile distilled water	H_2O	Nil	95 mL
Citric acid	$C_6H_8O_7$	Medium	5 g
Zinc sulphate heptahydrate	$ZnSO_4.7H_2O$	Medium	5 g
Ferrous ammonium sulphate hexahydrate	$(NH_4)_2Fe(SO_4)_2.6H_2O$	Moderate	1 g
Copper sulphate	Cu_2SO_4	Moderate	0.25 g
Manganous sulphate tetrahydrate	$MnSO_4.4H_2O$	Moderate	50 mg
Boric acid	H_3BO_4	Medium	50 mg
Sodium molybdate	Na_2MoO_4	Medium	50 mg

Method

1. Add distilled water to desired volume.
2. Weigh dry ingredients on balance in weigh boat separately, transferring into bottle after each ingredient.
3. Dissolve.
4. Store in the freezer at -18°C.

Notes
This solution is made up in bulk and used as required as an ingredient in other media.

References
Correll, J.C., Klittich, C.J.R. and Leslie, J.F. (1987) Nitrate nonutilising mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. *Phytopthology* **77**, 1640-1646.

8.2 Media for nit mutants phenotypic determination

8.2.1 Nitrite medium (Ni)

Nitrite medium is used to phenotype *nit* mutants. It is based on minimal medium with 0.5 g of sodium nitrite substituted for the sodium nitrate.

Safety Precautions
Sodium nitrite is highly hazardous therefore dust masks should be worn and handling these chemicals should take place inside a fume cupboard.

Personal protective requirements
- Lab coat
- Disposable latex or nitrile gloves
- Safety glasses (for handling hot media if not in laminar flow)
- Insulated or heat resistant gloves (for handling hot media)
- Plastic apron (if handling waste)
- Enclosed shoes

Ingredient	Structure	Hazardous	1 L	400 mL
Distilled water	H_2O	None	1000 mL	400 mL
Magnesium sulphate heptahydrate	$MgSO_4.7H_2O$	Low	0.5 g	0.2 g
Potassium dihydrogen phosphate	KH_2PO_4	Low	1 g	0.4 g
Potassium chloride	KCl	Low	0.5 g	0.2 g
Ferrous sulphate heptahydrate	$FeSO_4.7H_2O$	Moderate	10 mg	4 mg
Sodium nitrite	$NaNO_2$	High	0.5 g	0.2 g
Sucrose	$C_{12}H_{22}O_{11}$	Low	30 g	12 g
Sterile trace elements (see 8.1.9 for solution preparation)		Moderate	200 µL	80 µL
Agar/Solidifier Difco		Low	20 g	8 g

Method
1. Add distilled water to desired volume.
2. Weigh dry ingredients on balance in weigh boat separately, transferring into bottle after each ingredient. Magnesium sulphate heptahydrate should be the first dry ingredient added and dissolved then potassium dihydrogen phosphate and the rest of the dry ingredients except the agar/solidifier can be added.
3. Adjust pH to 6.7 ± 0.5 using either HCl (5M or 1M) or NaOH (5M or 1M) unless specified otherwise.
4. Add the agar/solidifier and mix and top up to final volume.
5. Loosen the lid and place in the autoclave for a 30-minute free steaming cycle. At the end of the cycle, ensure that all ingredients are dissolved and molten.
6. Add trace elements using a pipette (Gilson style).

7. Autoclave media with lids loosened at 121°C for 20 min.
8. Remove bottles containing media from autoclave, tighten lids and allow media to cool to approximately 55°C in water bath.
9. Pour plates inside the laminar flow cabinet.

Notes
Prepare in 60 mm petri dishes unless otherwise specified.

8.2.2 Hypoxanthine medium (Hx)

Hypoxanthine medium is used to phenotype *nit* mutants. It is based on minimal medium with 0.2 g of hypoxanthine substituted for the sodium nitrate.

Personal protective requirements
- Lab coat
- Disposable latex or nitrile gloves
- Safety glasses (for handling hot media)
- Insulated or heat resistant gloves (for handling hot media)
- Plastic apron (if handling waste)
- Enclosed shoes

Ingredient	Structure	Hazardous	1 L	400 mL
Distilled water	H_2O	None	100 0mL	400 mL
Magnesium sulphate heptahydrate	$MgSO_4.7H_2O$	Low	0.5 g	0.2 g
Potassium dihydrogen phosphate	KH_2PO_4	Low	1 g	0.4 g
Potassium chloride	KCl	Low	0.5 g	0.2 g
Ferrous sulphate heptahydrate	$FeSO_4.7H_2O$	Moderate	10 mg	4 mg
Hypoxanthine	$C_5H_4N_4O$	Moderate	0.2 g	0.08 g
Sucrose	$C_{12}H_{22}O_{11}$	Low	30 g	12 g
Sterile trace elements (see 8.1.9 for solution preparation)		Moderate	200 µL	80 µL
Agar/Solidifier Difco		Low	20 g	8 g

Method
1. Add distilled water to desired volume.
2. Weigh dry ingredients on balance in weigh boat separately, transferring into bottle after each ingredient. Magnesium sulphate heptahydrate should be the first dry ingredient added and dissolved then potassium dihydrogen orthophosphate and the rest of the dry ingredients except the agar/solidifier can be added.
3. Adjust to pH 6.7 ± 0.5 using either HCl (5M or 1M) or NaOH (5M or 1M) unless specified otherwise.
4. Add the agar/solidifier and mix and top up to final volume.
5. Loosen the lid and place in the autoclave for a 30-minute free steaming cycle. At the end of the cycle ensure that all ingredients are dissolved and molten.
6. Add trace elements using an autopipette.
7. Autoclave media with lids loosened at 121°C for 20 min.
8. Remove bottles containing media from autoclave, tighten lids and allow media to cool to approximately 53°C in water bath.
9. Pour plates inside the laminar flow cabinet.

Notes
Prepared in 60 mm plates unless otherwise specified.

8.2.3 Ammonium medium (Am)

Ammonium medium is used to phenotype *nit* mutants. It is based on Minimal Medium with 1.6 g of ammonium tartrate substituted for the sodium nitrate.

Personal protective requirements

- Lab coat
- Disposable latex or nitrile gloves
- Safety glasses (for handling hot media)
- Insulated or heat resistant gloves (for handling hot media)
- Plastic apron (if handling waste)
- Enclosed shoes

Ingredient	Structure	Hazardous	1 L	400 mL
Distilled water	H_2O	None	1000 mL	400 mL
Magnesium sulphate heptahydrate	$MgSO_4.7H_2O$	Low	0.5 g	0.2 g
Potassium dihydrogen phosphate	KH_2PO_4	Low	1 g	0.4 g
Potassium chloride	KCl	Low	0.5 g	0.2 g
Ferrous sulphate heptahydrate	$FeSO_4.7H_2O$	Moderate	10 mg	4 mg
Ammonium tartrate	$C_4H_{12}N_2O_6$	Low	0.2 g	0.08 g
Sucrose	$C_{12}H_{22}O_{11}$	Low	30 g	12 g
Sterile trace elements (see 8.1.9 for solution preparation)		Moderate	200 µL	80 µL
Agar/Solidifier Difco		Low	20 g	8 g

Method

1. Add distilled water to desired volume.
2. Weigh dry ingredients on balance in weigh boat separately, transferring into bottle after each ingredient. Magnesium sulphate heptahydrate should be the first dry ingredient added and dissolved then potassium dihydrogen phosphate the rest of the dry ingredients except the agar/solidifier can be added.
3. Adjust pH to 6.7 ± 0.5 using either HCl (5M or 1M) or NaOH (5M or 1M) unless specified otherwise.
4. Add the agar/solidifier, mix, and top up to final volume.
5. Loosen the lid and place in the autoclave for a 30-minute free steaming cycle. At the end of the cycle, ensure that all ingredients are dissolved and molten.
6. Add trace elements using an autopipette.
7. Autoclave media with lids loosened at 121°C for 20 min.
8. Remove bottles containing media from autoclave, tighten lids and allow media to cool to approximately 53°C in water bath.
9. Pour plates inside the laminar flow cabinet.

Packaging
Prepared in 60 mm Petri dish unless otherwise specified.

8.2.4 Minimal media with chlorate (MMC)

MMC medium is a minimal medium amended with potassium chlorate. It is used in the VCG procedure to produce chlorate resistant mutant sectors. This particular medium is used in preference over KPS when attempting to generate a higher proportion of 'NitM' mutants.

Personal protective requirements

- Lab coat
- Disposable latex or nitrile gloves
- Safety glasses (for handling hot media)
- Insulated or heat resistant gloves (for handling hot media)
- Plastic apron (if handling waste)
- Enclosed shoes

Ingredient	**Structure**	**Hazardous**	**1 L**	**400 mL**
Distilled water	H_2O	None	1000 mL	400 mL
Magnesium sulphate heptahydrate	$MgSO_4.7H_2O$	Low	0.5 g	0.2 g
Potassium dihydrogen phosphate	KH_2PO_4	Low	1 g	0.4 g
Potassium chloride	KCl	Low	0.5 g	0.2 g
Ferrous sulphate heptahydrate	$FeSO_4.7H_2O$	Moderate	10 mg	4 mg
Sodium nitrate	$NaNO_3$	Moderate	2 g	0.8 g
Sucrose	$C_{12}H_{22}O_{11}$	Low	30 g	12 g
L-asparagine	$C_4H_8N_2O_3$	Low	1.6 g	0.64 g
Potassium chlorate	$KClO_3$	Moderate	15 g	6 g
Sterile trace elements	-	Moderate	200 µL	80 µL
Agar/Solidifier Difco	-	Low	15 g	6 g

Method

1. Add distilled water to desired volume.
2. Weigh dry ingredients on balance in weigh boat separately, transferring into bottle after each ingredient. Magnesium sulphate heptahydrate should be the first dry ingredient added and dissolved, and then potassium dihydrogen phosphate and the rest of the dry ingredients except the agar/solidifier.
3. Adjust to pH 6.7 ± 0.5 using either HCl (5M or 1M) or NaOH (5M or 1M) unless specified otherwise.
4. Add the agar/solidifier and mix and top up to final volume.
5. Loosen the lid and place in the autoclave for a 30-minute free steaming cycle. At the end of the cycle, ensure that all ingredients are dissolved and molten.
6. Add trace elements using an auto- pipette.
7. Autoclave media with lids loosened at 121°C for 20 min.
8. Remove bottles containing media from autoclave, tighten lids and allow media to cool to approximately 53°C in water bath.
9. Pour plates inside the laminar flow cabinet.