Fusarium Wilt of Banana

Laboratory Diagnostics Manual

Edited by Sharon Van Brunschot

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The PCR primers and reaction conditions for molecular diagnosis of *Foc* Tropical Race 4 are pending publication and thus must be held in confidence until such time they appear in the public domain. Please also ensure these parameters do not appear in material including conference oral presentations and posters and funding body reports.

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Introduction

The history and global distribution of the disease

Diseases are a major constraint to banana production worldwide. A great number of diseases affect banana (Jones 2000), with one of the most important of these diseases being Fusarium wilt of banana.

The first record of Fusarium wilt was in Australia in 1874 (Bancroft 1876) and the disease has since been found in all banana-growing regions of the world, except for some of the countries bordering the Mediterranean Sea (see Figure 1). Fusarium wilt has had a particularly destructive history in the evolution of international banana trade. In the period 1890-1960, some 40 000 hectares of the susceptible banana cultivar Gros Michel (grown for export) were destroyed or abandoned in Central and South America and the Caribbean because of race 1 of *Foc*. In terms of crop destruction, Fusarium wilt then ranked alongside the foremost devastating plant diseases such as wheat rust and potato blight (Carefoot and Sprott 1969). Export industries were forced to replace the susceptible Gros Michel variety with Cavendish cultivars, which continue to show resistance to race 1 of *Foc* in these areas (Stover 1990).

Cavendish cultivars remain the banana varieties of international trade. However, these cultivars are not resistant to all strains of *Foc*. The 'subtropical race 4' strain of *Foc* causes losses of Cavendish cultivars in the subtropical regions of the Canary Islands, South Africa, Australia and Taiwan (Stover 1990). More importantly, in the tropical commercial and subsistence production regions of the Philippines, Indonesia, Taiwan, Malaysia, and in the southern provinces of China, a new strain of *Foc* designated 'tropical race 4' has caused widespread devastation (INIBAP 2006). Alarmingly, the disease is continuing to spread in these areas. Several incursions of this pathogen have also been recorded in Australia (Northern Territory); however these outbreaks have all been contained and have not reached the commercial growing regions situated on the east-coast of the country (Walduck 2002).

Tropical race 4 of *Foc* affects banana cultivars that comprise 80% of the world's banana production, including the important Cavendish and plantain subgroups (Ploetz 2005). The tropical race 4 strain of *Foc* could cause significant damage to the major world export production areas if introduced into Ecuador, Central America and Colombia, which are based on Cavendish cultivars. As it stands, the tropical race 4 strain poses a very real threat to the multi-billion dollar global banana trade, and the food security of millions of subsistence farmers (Ploetz 2005). Furthermore, the Cavendish variety may risk the very same fate as Gros Michel, the cultivar it replaced nearly 50 years ago because of its susceptibility to race 1 of *Foc*.

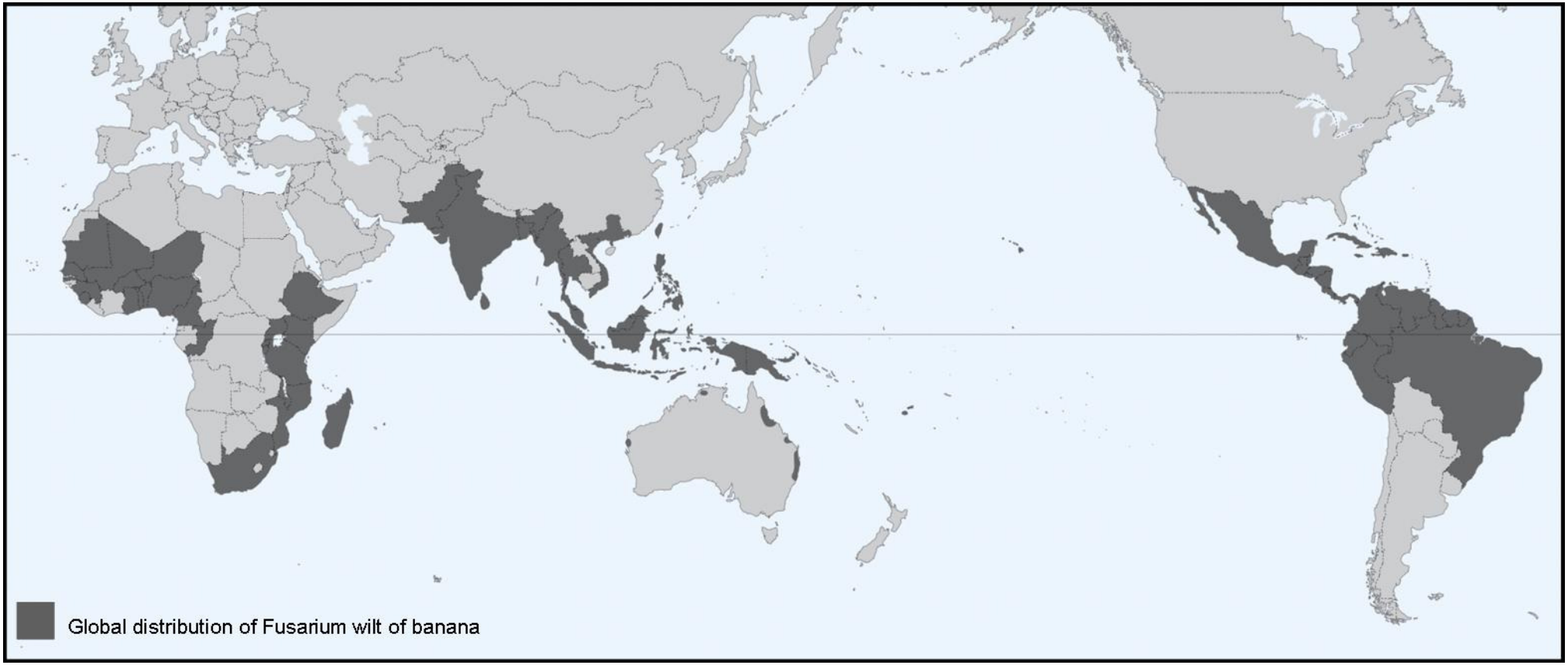


Figure 1: World map showing the global distribution of Fusarium wilt of banana.
Diagram courtesy of the CRCTPP (Sue McKell).

Fusarium wilt in Australia

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 in Australia. Races 1 & 2 of *Foc* have been found affecting banana in both northern and southern Queensland, and northern New South Wales (and race 1 has been found in Western Australia). The subtropical Australian banana industry is severely constrained by race 1 of *Foc*, 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 (Gerlach *et al.* 2000). Subtropical race 4 is found affecting Cavendish cultivars, as well as race 1 and race 2 susceptible cultivars in northern New South Wales and southern Queensland. Isolated outbreaks of tropical race 4 have occurred in the Northern Territory; the first outbreak occurred on a commercial plantation in Darwin in 1997, and the disease has since spread to other commercial plantations in the area (Walduck 2002). Strict quarantine measures are in place to limit the spread of tropical race 4 to other banana producing areas, particularly to the major Cavendish production areas of north Queensland. Tropical race 4 of *Foc* is defined as a high priority pathogen and is targeted in the surveillance programs of the Northern Australia Quarantine Strategy (NAQS) and Northwatch, and the Biosecurity Workgroup of the Department of Primary Industries and Fisheries (QDPI&F). The tropical race 4 strain of Fusarium wilt is regarded as one of the most serious threats to banana production in Australia since disease resistant replacements for Cavendish cv. Williams are not yet widely available (Gerlach *et al.* 2000).

In Australia, tropical race 4 of *Foc* poses an immediate threat to the commercial production areas in the Northern Territory, and to major production areas based along the east coast of Australia. Current disease management strategies are centred on: (a) the prevention of the movement of *Foc* into disease free areas (particularly the movement of tropical race 4), and (b) the early detection and containment of Fusarium wilt outbreaks. Rapid and accurate detection and diagnosis of the pathogen underpins the successful implementation of these management strategies. This manual provides a comprehensive guide to the suite of diagnostic techniques used in the diagnosis of isolates of *Foc*.

Disease symptoms

Fusarium wilt is a typical vascular disease causing disruption of water translocation, systemic foliar symptoms and plant collapse (Jeger *et al.* 1995). Internal symptoms are characterised by reddish-brown discolouration of the vascular tissue. 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 (see 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.

Figure 2: Disease symptoms of tropical race 4 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 forms a skirt around the based of the plant). (b) Cross section of pseudostem showing the dramatic vascular discolouration. Photographs courtesy of the CRCTPP (Dr Juliane Henderson).

The causal agent

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*).

Taxonomy

The hyphomycete genus Fusarium (Subdivision Deuteromycotina) comprises a number of species that are plant pathogens, parasites and saprophytes; more recently some species have been reported as emerging human pathogens in immunocompromised individuals (Boutati and Anaissie 1997).

Fusarium oxysporum (*Fo*), the most common species of the genus, is a soil-borne pathogen with a ubiquitous, worldwide distribution. 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 (teleomorph) 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).

Morphology

There are three types of asexual spores formed by *Fo*; macroconidia, microconidia and chlamydospores (see Figure 3) (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, and the shape of the macro- and microconidia (Leslie and Summerell 2006).

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).

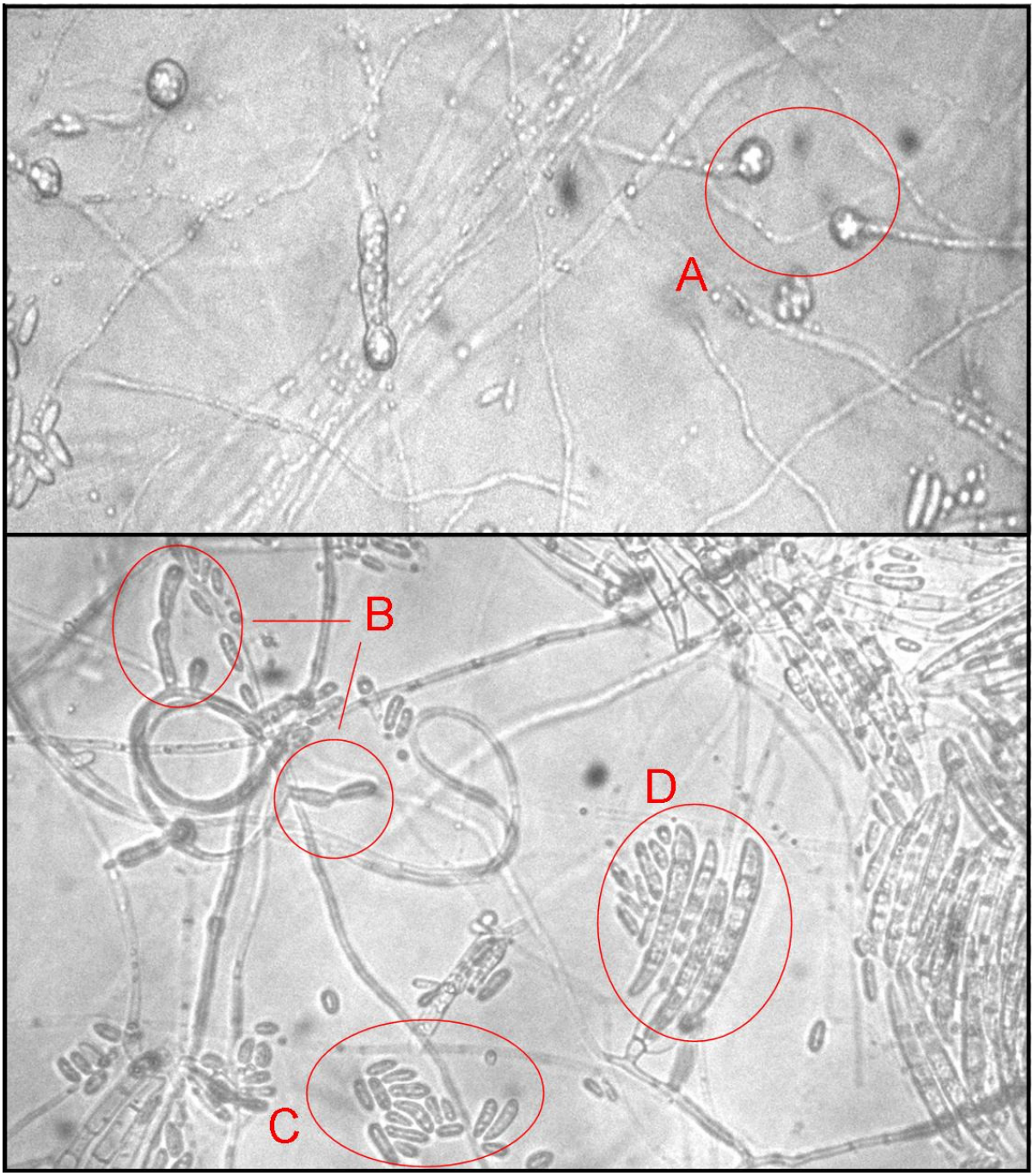


Figure 3: The three types of asexual spores produced by *Fusarium oxysporum*, as viewed under light microscopy: (a) chlamydospores, thick-walled resting spores that can persist in the soil for long periods of time (over 30 years), (b) microconidia growing from conidiophores, (c) microconidia, (d) macroconidia. Photos courtesy of QDPI&F (Wayne O'Neill).

Pathogen diversity

Foc is considered to be a genetically diverse pathogen, displaying a clonal population structure (Bentley *et al.* 1998; Koenig *et al.* 1997). Several analytical techniques have been used to study the variation in *Foc* (see Table 1). The diversity of Australian isolates of *Foc* has been well characterised, with several hundred isolates analysed by: (a) pathogenicity in the field, (b) Vegetative Compatibility Group (VCG) analysis, (c) volatile production, and (d) molecular characterisation techniques such as DNA amplification fingerprint (DAF) analysis.

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

Table 1: A summary of the techniques that have been used for the characterisation of isolates of *Foc*.

Racial classification

Isolates of *Foc* have been traditionally grouped into four physiological races, based on pathogenicity to a small number of differential banana host cultivars in the field (see Table 2). This racial classification is an artificial grouping and does not reflect a genetically defined relationship, however this terminology continues to be used (especially in field situations) as a simple and convenient way of grouping isolates of *Foc*.

Fungal vegetative compatibility

A technique that is based on the genetic relationships within fungal populations, termed vegetative compatibility group (VCG) analysis, has been 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). This modified VCG technique, based on the generation of nitrate non-utilising (*nit*) auxotrophic mutants, enables heterokaryon formation (vegetative compatibility) to be scored macroscopically and thus renders VCG analysis amenable to population studies.

Spontaneous nitrate non-utilizing (*nit*) mutants are generated on media containing potassium chlorate (colony growth on chlorate media is usually restricted, as chlorate (a nitrate analogue) is metabolised to chlorite which is toxic). *Nit* mutants appear after 7-14 days as chlorate-resistant sectors which are also unable to metabolise nitrate and typically produce restricted growth on minimal medium containing nitrate as the sole nitrogen source. When *nit* mutants from vegetatively compatible isolates are paired on minimal medium containing nitrate as the sole nitrogen source, a prototrophic heterokaryon is formed where the mycelia come in contact. The heterokaryon produces wild-type mycelium as it is able to metabolise the nitrogen in the medium.

VCGs serve as a natural means to subdivide fungal populations and therefore VCG analysis is a useful technique to measure genetic diversity within a population. In an asexual population, differences at the *vic* loci are assumed to effectively limit the exchange of genetic information to those individuals that belong to the same VCG. Since sexual recombination is not known to occur in *Foc*, members of each VCG comprise a genetically isolated subgroup and are assumed to be clonally derived populations of the pathogen (Leslie 1990).

At least 21 VCGs of *Foc* have been characterised worldwide (Moore 1994; Ploetz 1990a; Ploetz and Pegg 1999), with seven of these VCGs present in Australia, viz. VCGs 0120, 0124, 0125, 0128, 0129, 01211, and 01220 (see Table 2) (Brake *et al.* 1990; Moore *et al.* 1993; Pegg *et al.* 1995).

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

Table 2: The relationship between Australian VCGs and races of *Foc*, and the banana cultivars they infect.

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 produced easily detectable volatile odours with characteristic gas chromatogram profiles. Volatile analysis is a simple and inexpensive method of characterising isolates of *Foc* based on the biochemistry of cultures *in vivo*.

Molecular characterisation

Many molecular methods have been used to assess genetic relationships among the VCGs and races of *Foc* (see Table 1). DNA amplification fingerprinting (DAF) analysis has been used to determine the genetic relatedness between Australian isolates of *Foc*; within each VCG and among the VCGs (Bentley *et al.* 1998; Gerlach *et al.* 2000). Also, extensive sequence data of the IGS region of the rDNA of Australian isolates of *Foc* has also been collated and analysed. (S. Van Brunschot, unpublished). The genetic relationships between the different Australian VCGs of *Foc* that infect banana, inferred from DAF data and sequence data correlate well. The genetic data also supports the groupings inferred by racial classification and volatile analysis. Three major groupings have been identified: (1) VCGs 0124, 0125, 0128, and 01220 group together (isolates from races 1 &2, non-volatile), (2) VCGs 0120, 0129, and 01211 group together (subtropical race 4, volatile), and (3) VCGs 01213 and 01216 group together (tropical race 4, volatile).

Pathogen distribution

Isolates of race 1 of *Foc* belong to VCGs 0124 and 0125 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 New South Wales (NSW) and at Carnarvon in Western Australia, 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 emerging production areas of Carnarvon and Kununurra in Western Australia, or near Darwin in the Northern Territory. The strains of *Foc* known as tropical race 4 have recently (1997, 1998 and 1999) been confirmed at three sites near Darwin in the Northern Territory. Isolates of this Cavendish-competent strain of *Foc* belong to the VCGs 01213 or 01216. These are the only recorded cases of tropical race 4 of *Foc* in Australia.

Disease development and epidemiology

Spread of the pathogen

Foc is most commonly spread by the movement of infected planting material, rhizomes and suckers (and the attached soil), to new uninfected areas. This infected planting material often does not exhibit symptoms of Fusarium wilt (is asymptomatic). The pathogen can also be effectively spread by the movement of soil, running water, and farm machinery and implements. Once a site is infected, the pathogen can persist in the soil as chlamydospores for more than 30 years (Stover 1962a; Waite and Dunlap 1953). Also, it is likely that *Foc* can survive non-pathogenically on alternative hosts, such as weed species (Hennessey *et al.* 2005).

Disease cycle

The disease cycle of *Foc* begins with the entry of the pathogen into the potential host via the root tip. Substances produced by the host, in the region behind the zone of elongation of the root meristem, elicit a pathogen response which results in the germination of chlamydospores. The germinated hyphae then infect the lateral roots and progress to invade the xylem vessels. Further spread of most initial infections is usually stopped in the xylem by the vascular occluding responses of the host, which include the formation of gels, tyloses and the collapse of vessels. In susceptible cultivars, some of these infections become established in the xylem and advance ahead of these defence mechanisms. Microconidia are formed in the xylem vessels and are spread through the vascular system of the plant, streaming to new sites where they germinate. Hyphae are then produced, and this begins the invasion at this new site, thus repeating the cycle. In resistant varieties, the initial pathogen-induced occlusion reaction is further enhanced by the production of phenolic compounds, which lignifies these obstructions and limits the pathogen to the infected vessels; no further colonisation of the xylem vessels occurs. These observations were made during the inoculation of Gros Michel and Cavendish with *Foc* race 1 (Beckman 1969; Beckman 1987; Beckman 1990; Beckman and Keller 1977; Beckman and Talboys 1981; VanderMolen *et al.* 1977).

Disease control

Initially, control of Fusarium wilt in Central America was aimed at reducing the pathogen population in infested fields. However, fungicides, fumigants, flood fallowing, crop rotation and organic amendments have rarely provided long-term control in any production area. Quarantine and exclusion procedures are effective in controlling the spread of the disease by restricting the movement of corms, suckers and soil that could carry *Foc* from infested to non-infested areas. In eastern Australia, legislation provided by the Queensland Banana Industry Protection Act, which operates in association with the Plant Protection Act 1989 and the New South Wales Banana Industry Act 1987, prohibits the transfer of planting material from certain localities where the disease is prevalent or from any plantation not approved as a source of planting material. Planting material is only approved if the source plantation has no previous record of the occurrence of the disease, and is apparently free from Fusarium wilt when subjected to row-by-row inspection by trained personnel in the autumn and spring preceding the removal of the material. However, due to the presence of suppressive soils in which microbial populations suppress the pathogen population, and since infected rhizomes or suckers may not exhibit external symptoms, the pathogen may still be moved in approved planting material. In recent years, the use of certified disease-free tissue-cultured plantlets, from an approved Quality Banana Approved Nursery (QBAN) facility, has become a cost-effective way of maintaining disease-free production. Tissue-culture plants also have the added benefits of providing uniform growth and harvest times.

It is now generally accepted that the most effective means of controlling Fusarium wilt is by host resistance. Since banana is a clonally propagated, perennial crop, resistance must be enduring. Natural sources of resistance exist in wild species and cultivars of banana, and also in synthetic diploids developed by breeding programs. There are four major conventional banana breeding programs, and these are located in Honduras (FHIA), Brazil (EMBRAPA-CNPMF), Nigeria (IITA) and Guadeloupe (CIRAD-FLHOR). To date, a Cavendish replacement has not been achieved because of fertility constraints, however tetraploid hybrids can be bred to replace AAB dessert and ABB cooking banana types. The FHIA-01 (Goldfinger) variety is a good example of the hybrids produced by the FHIA breeding program in Honduras. FHIA-01, a primary tetraploid dessert banana with an acidic or 'apple' flavour, has been identified as having resistance to race 1 and race 4 of *Foc*. However, it has been hypothesised that this resistance in tetraploid hybrids may not be durable because 75% genome is derived from the disease susceptible female parent (Stover and Buddenhagen 1986). Alternative approaches are being attempted by the CIRAD-FLHOR group in Guadeloupe which involve the development of novel triploid clones from diploid stocks. Biotechnology, mutation breeding and somaclonal variations are also being used to produce resistant genotypes. These programs are likely to provide replacement clones in the future. Since Australia does not have a banana breeding program, close collaboration with

international breeding and plant improvement programs is essential for obtaining replacement clones in the future.

Disease management

Management of this disease in Australia is based around three key strategies: the early detection and containment of outbreaks of Fusarium wilt and application of protocols to prevent introduction of *Foc* into disease-free production districts; (2) adoption of disease management strategies to enable farms with Fusarium wilt to maintain production, and (3) research to evaluate and develop long-term disease management strategies for the Australian banana industry.

Local quarantine measures are in place in banana production areas of Australia to restrict the movement of banana planting material (particularly suckers and rhizome pieces). Australian banana growers require permits to move planting material to initiate new plantations and, if vegetative material is used (e.g. suckers or rhizome pieces) this must be from a certified source of "clean" planting material. All Australian banana growers and inspectors are encouraged to send specimens from any plants suspected of Fusarium wilt to the Queensland DPI laboratories at Indooroopilly for analysis. This enables new outbreaks to be mapped and the distribution of the different strains of *Foc* to be monitored, and any new or exotic strains of *Foc* to be detected (e.g. the recent outbreaks in Cavendish plantations in the Northern Territory that were caused by VCG 01213/16).

On-farm disease management

Provided the strain of *Foc* involved in an outbreak of Fusarium wilt does not pose a quarantine risk, or the property concerned is not beyond a defined local quarantine zone, banana production is allowed to continue. Australian banana growers are advised to follow simple yet practical measures to minimise the spread of the disease and to extend the productive life of the plantation.

It is essential to prevent the spread of the pathogen to other farms (e.g. no movement of planting material or infested soil and, if possible, containing or diverting associated drainage water), and to minimise the spread of *Foc* within the affected farm. The common procedure in Queensland and New South Wales for isolated cases of Fusarium wilt in an otherwise healthy block is to immediately inject the affected plants with herbicide (e.g. Roundup®/glyphosate), and let them die *in situ* without further disturbance of the plants. Often a single or double ring of healthy plants around the affected ones may also be injected or otherwise treated with herbicide and allowed to die in place. This has the two-fold effect of preventing mat-to-mat spread of the pathogen through living banana roots, and also reducing the population of the

fungus at that site by killing its preferred host plant. It is important that the affected plants and the soil around them be disturbed as little as possible in this process as any disturbance and movement of soil will increase the chances of moving the pathogen. Fencing-off the area also signals to workers or visitors to keep out, reducing the risk of movement of contaminated soil on boots, machinery or in suckers.

Banana growers must not be tempted to use planting material from known infected plantations, even if some rhizomes appear "clean". If *Foc* has been identified at a plantation, any movement of soil or plants from the affected block must be considered as an unacceptable risk.

If disease-free areas are available for banana production it is vitally important not to contaminate them through the lack of basic farm hygiene. The key to keeping disease-free areas free from *Foc* is to minimise, if not remove, all opportunities for infested soil, plant material or irrigation water (that may be carrying spores of the fungus attached to soil particles) from entering the area. Risk minimisation can be achieved in several ways:

- Using only certified disease-free vegetative planting material or tissue-cultured plants to initiate new plantations.
- Cleaning equipment, footwear and vehicles to avoid moving infested soil or plant matter from an affected block to disease-free blocks.
- Where practicable, isolating irrigation water that drains from diseased parts of a plantation. Such drainage water may be carrying spores of *Foc* and should not be used to irrigate new or disease free areas if possible. At this time no commercial treatments are known for controlling *Foc* in irrigation water or water storages.
- Educating farm workers about how the fungus is spread and providing posters or other visual material to enable symptoms of Fusarium wilt to be recognised to aid in early detection of new outbreaks.

Farmcleanse® is a detergent-based degreaser with a quaternary ammonium additive produced by the Castrol company in Australia. It has been shown to have very high efficacy against spores of *F. oxysporum* f.sp. *vasinfectum* (Atk.) Snyd. & Hans., (*Fov*), the wilt pathogen of cotton (*Gossypium hirsutum* L.) (O'Neill 1999). Farmcleanse® is applied to vehicles (particularly tyres and inside wheel arches) as a foaming spray, which is then left for about 10 minutes to allow it to soak into any soil that may still be attached before being rinsed off. It is also used in footbaths for cleaning boots when moving between infected and disease-free areas within and between properties.

Anecdotal evidence from Australian plantations suggests that fertiliser regimes that are used to "push" plants (e.g. excessive applications of nitrogen) also increase the incidence of

Fusarium wilt in affected plantations. In most plant diseases, disease expression exists in a shifting three-way balance between the host plant, the pathogen and the prevailing environmental and growing conditions. Modern agricultural practices often interfere with that balance and inadvertently hasten the spread and intensity of diseases such as Fusarium wilt. If, however, the balance favours the natural vigour of the plant, its own defence mechanisms will keep the pathogen at as low a level as possible. Where growers wish to maintain production of susceptible varieties in the presence of *Foc*, they often reduce applications of nitrogenous fertilisers, but this in turn can lead to sacrifices in bunch size, fruit quality and financial returns.

As for many diseases, resistant or tolerant banana varieties offer the only long-term and environmentally sustainable solution for control of this disease. Where such varieties have not been available, plantation owners have managed the disease in susceptible varieties for many years through a combination of hygiene and disease management practices along with early detection and containment of new outbreaks. Conversely, if incorrect practices are followed (or no measures are taken at all), the disease will continue to spread around the farm and eventually to neighbouring farms.

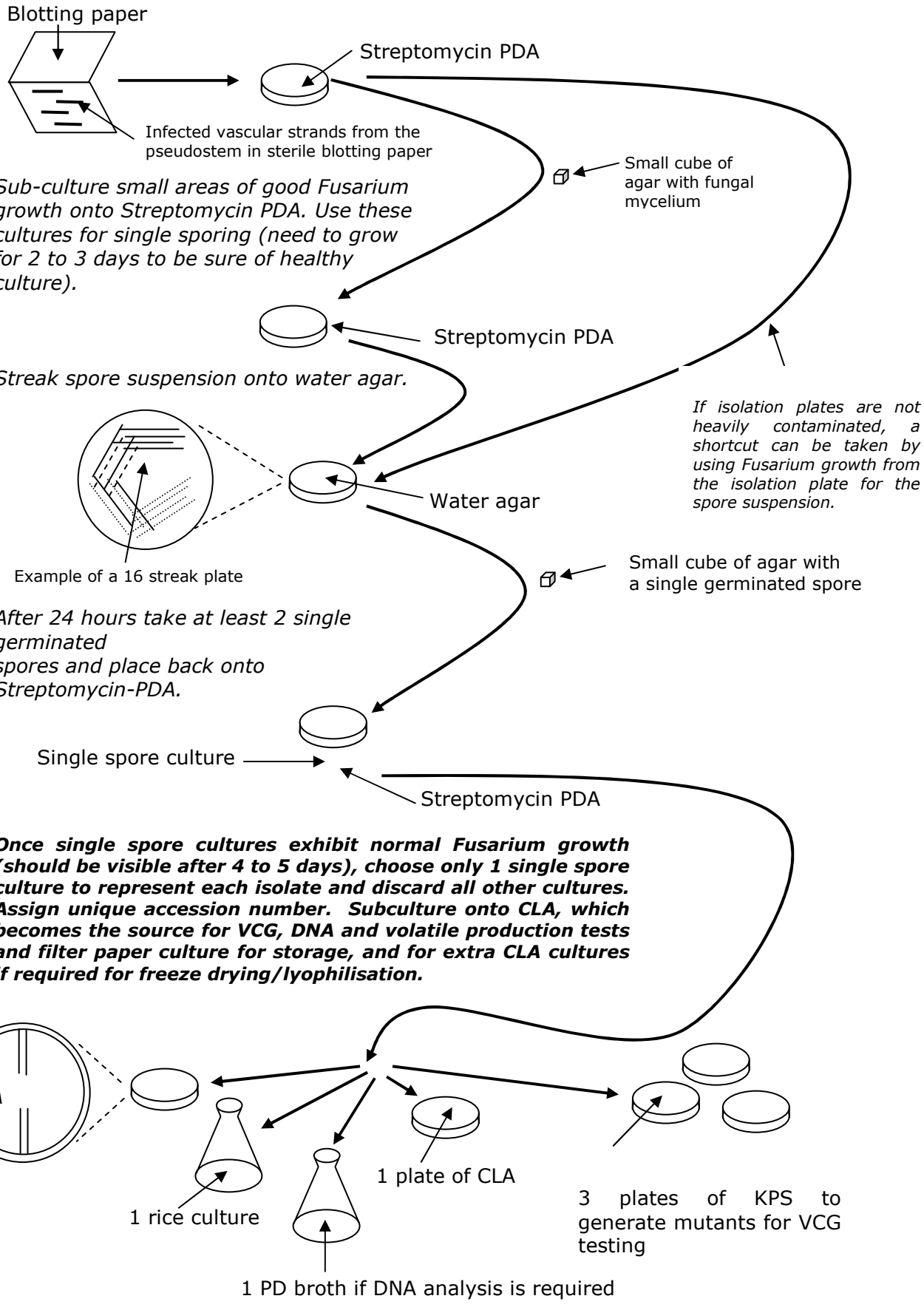
Biological diagnosis of disease

The following is a summary of the steps involved in the laboratory-based biological diagnosis of Fusarium wilt of banana:

- Receive specimen, log details and observations from grower/inspector
- Isolate from symptomatic tissue (usually 2 x Strep' PDA with 4 pieces of banana vascular tissue per plate)
- Check morphology of resultant growth (macro and microscopically)
- Subculture Fusarium growth to make spore suspension and streak onto water agar
- Select 2 x germinated single spores to initiate monoconidial cultures
- Assign unique accession number to isolate and record in specimen book and Fusarium isolate database
- The monoconidial culture can be used to:
    a) inoculate 1 x rice culture for volatile production
    b) inoculate 3 x KPS plates to generate *nit* mutants for VCG tests
    c) inoculate 1 x PD broth for DNA analysis
    d) initiate 1 x filter paper culture for medium term storage
    e) initiate 1 x CLA plate for short term storage
    f) if necessary, arrange for lyophilisation of isolate for long term storage in collection
    g) if necessary, prepare CLA cultures for lodging in a herbarium
- Conduct and record results of volatile and VCG analysis in database
- Return written reply to grower/inspector concerned using 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

The steps involved in the laboratory diagnosis have been summarised in Figure 4, and are explained in detail in the following sections.

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 to 3 days to be sure of healthy culture).*

3. *Streak spore suspension onto water agar.*

If isolation plates are not heavily contaminated, a shortcut can be taken by using Fusarium growth from the isolation plate for the spore suspension.

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

5. ***Once single spore cultures exhibit normal Fusarium growth (should be visible after 4 to 5 days), choose only 1 single spore culture to represent each isolate and discard all other cultures. Assign unique accession number. Subculture onto CLA, which becomes the source for VCG, DNA and volatile production tests and filter paper culture for storage, and for extra CLA cultures if required for freeze drying/lyophilisation.***

Figure 4: The steps involved in the isolation and biological analysis of *Foc*

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. 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 wild situation
- location (e.g. name of province or state, how far in what direction from nearest town, name of road, name of property if sample is from a commercial plantation etc.) A map with sample numbers marked on it or GPS coordinates can be very useful.
- collectors names
- other useful observations might include the source of the planting material, whether the plant is growing in water-logged soil or stressed in some other way, how many plants are affected, what other varieties are growing around the diseased plant and are these diseased or healthy?

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 dry.

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

Dissecting discoloured vascular strands from sample

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

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

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

Isolating the fungus from discoloured vascular strands

Isolation can be attempted when the strands have dried (possibly as early as the next day). Small sections (3-6mm long) of dry discoloured vascular strands are submerged into plates of quarter strength PDA medium amended with an antibacterial agent (e.g. streptomycin @ 1.2mL/240mL PDA) (see media recipes). If Fusarium is present, growth will appear from the strands in 2-4 days. However, if the sample is badly contaminated with bacteria this may mask fungal growth. Let samples dry further if this occurs and increase the strength of the antibacterial amendment in the media. A high rate of recovery of Fusarium should be expected from correctly prepared samples. Monoconidial (single-spore) cultures should be prepared from an isolate from each specimen.

Generation of monoconidial cultures

Under aseptic condition, cut two pieces of culture approximately 6mm^2 from a healthy culture isolated on quarter strength PDA (as above), and transfer these pieces to a small 9mL McCartney bottle containing sterile distilled water. After swirling the bottle gently to wash the spores into solution, flame a loop and transfer one or two loops of the spore suspension onto

one side of a water agar plate. The flamed loop is then dragged through the deposit of spore suspension several times to create 'streaks' of spore suspension across the plate either in parallel lines or in the sixteen-streak method used in microbiology. This separates the conidia from each other to enable single, separated, germinated spores to be easily identified and excise 18-24 hours later using a stereo microscope.

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

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 weak quarter-strength PDA medium to check the morphology of cultures for taxonomic purposes or for spore production. Healthy (sporodochial-type) cultures of *Foc* growing on PDA medium exhibit abundant fluffy aerial mycelium after 2 days, and produce abundant 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^{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*.

VCG analysis of isolates of *F. oxysporum* f.sp*. cubense*

Generation of nitrate non-utilising (nit) mutants

This technique was originally used by Cove (1976) for *Aspergillus nidulans*, and was 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 recipe for 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 5). 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 media recipes) (Puhalla 1985). If the sector is a true *nit* mutant it will not be able to reduce the nitrate in the medium and characteristically flat, sparse, nitrogen-deficient growth will result. If the growth that results is not sparse on MM, discard this culture, as it will be of no use in VCG tests. It is advantageous to let the sectors grow for two to three days after emerging on the KPS plates so that the fast-growing mycelia grows clear of any non-mutated mycelia which may be underneath. When each of the sectors is transferred to MM, the sector should be numbered to identify it. This becomes particularly important if the tests need to be repeated or the mutants are needed for other tests. For example, if the isolate being tested has the accession number 23532 the sectors can be sequentially numbered as 23532-1, 23532-2, 23532-3 and so forth (Figure 5).

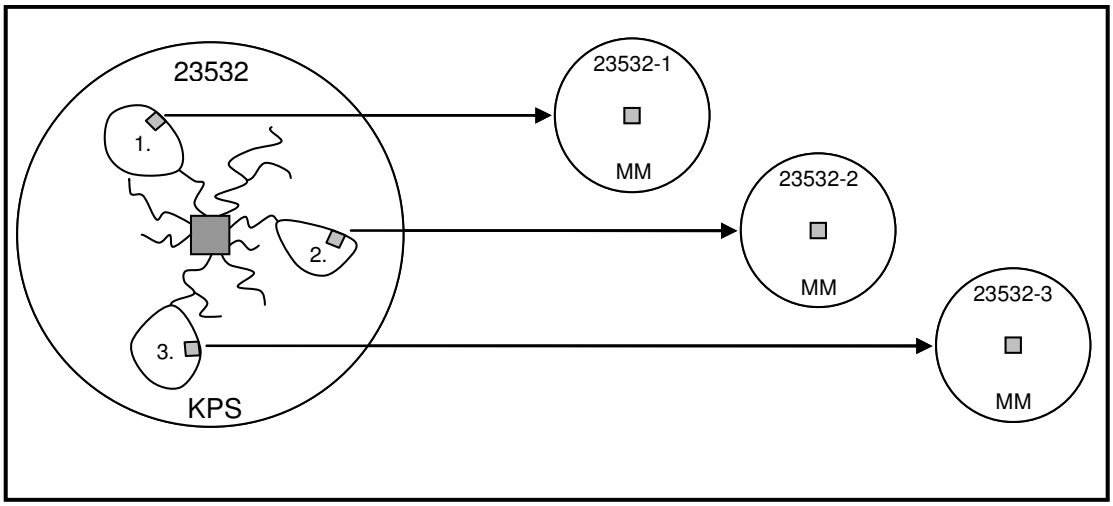


Figure 5: Fast-growing sectors emerge from a restricted colony of *Foc* on KPS medium. Mycelium from the advancing edge of each sector is transferred to Minimal Medium to test its ability to reduce nitrate.

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). 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 phenotype 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.

Pairing nit mutants in VCG tests

A small (2mm^2) piece of colonised agar from a culture of a Nit M mutant of known VCG is placed in the centre of a plate of MM. The bottom of the plate is labelled with 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 10-15mm away from the piece of Nit M culture around the edge of the plate (Figure 6). 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 mislabelled a plate, discard it and start again.

If the isolate of unknown VCG has the accession number 23532, the *nit* mutants which 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:

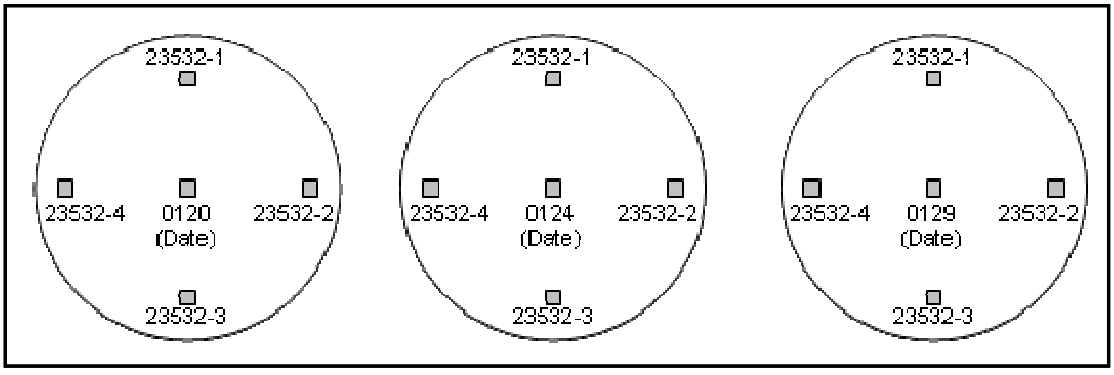


Figure 6: Pair-wise arrangement of four *nit* mutants from an isolate of unknown VCG (isolate number 23532) with Nit M testers representing VCGs 0120, 0124 and 0129. Nit M testers are placed in the centre of the plate with the *nit* mutants around the edge.

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 7-12 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 seven days the paired plates would look typically like this:

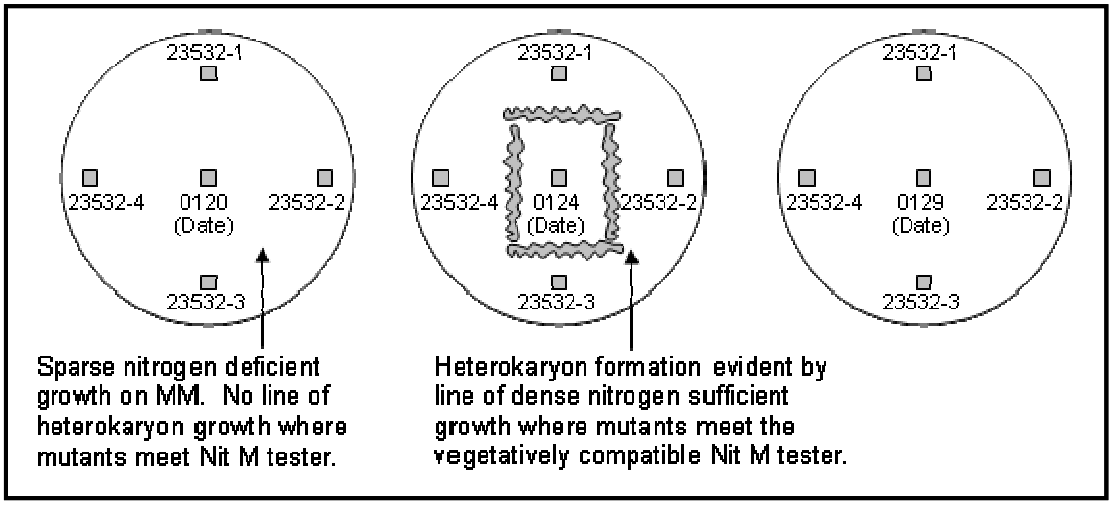


Figure 7: An example of a positive VCG test. Heterokaryon formations between the *nit* mutants of isolate 23532 and the Nit M tester representing VCG 0124 indicate that isolate 23532 belongs to VCG 0124.

An accurate, centralised record should be maintained detailing 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). Information can be added to as specimens are received and VCG tests are performed.

Volatile production

Prepare rice medium (see Media recipes). Aseptically inoculate rice with two large squares ($2cm^2$) of Fusarium culture grown on quarter strength PDA with streptomycin added at a

standard rate. Flasks do not have to be incubated and can be left on the bench top (23-28℃). Assess cultures after two weeks for production of volatiles; remove the cotton plug from the top of the flask and gently waft the air from the headspace of the culture toward your nose. A volatile odour is easily recognised as a sharp, pungent odour.

Molecular diagnosis of disease

DNA extraction

1. DNA extraction from fungal cultures

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

Consumables and equipment

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

Reagents

Reagent description
Liquid nitrogen
DNA Extraction Buffer (see below)
Phenol (stored at 4ºC)
Chloroform-isoamyl alcohol (24:1) (store at -20ºC)
3M sodium acetate (pH 5.4)
100% ethanol (ice cold, stored at -20ºC)

70% ethanol (ice cold, stored at -20ºC)
Sterile dH_2O or TE Buffer (10mM Tris, 1mM EDTA, pH 8.0)

DNA Extraction Buffer (store at 4ºC)

Reagent description
2% sodium dodecyl sulphate (SDS)
40mM EDTA
40mM sodium chloride
100mM Tris-HCl (pH 8.0)
25mM diethyldithiocarbamic acid (DIECA)

Notes

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

1. Culture single-spored isolates of *Fusarium oxysporum* f.sp. *cubense* on carnation leaf agar for approx 4-5 days.
2. Under aseptic conditions, cut out 4 agar plugs (cubes of $1cm^2$) from the growing edge of the culture and transfer to 250mL Erlenmeyer flasks containing 200mL of quarter-strength potato dextrose broth. Inoculated broths should be incubated at room temperature for 4-5 days (no longer than 7 days), shaken gently once each day.
3. Harvest mycelium by filtration through a clean glass funnel lined with Miracloth. Scrape together mycelium and store at -20°C until ready to extract DNA. Alternatively, lyophilise and store at -70°C for long-term storage.
4. Pre-warm the extraction buffer in a 37°C water bath.
5. Grind frozen mycelium (100-200mg) to a fine powder in liquid nitrogen, using a clean mortar and pestle. Transfer ground sample to a 2mL tube and add 1mL of extraction buffer. Mix by inversion and vortex lightly. Incubate in water bath at 37°C for 2 hours.
6. Add 1mL of phenol (cold), mix by inversion (20 times). Centrifuge samples in a microcentrifuge at 14000g, for 30 minutes (at 4°C).
7. Transfer upper phase to clean, labelled 2mL tube. Add 1mL of phenol (cold), mix by inversion (20 times). Centrifuge samples at 14000g, for 30 minutes (at 4°C).

8. Transfer upper phase to clean, labelled 2mL tube. Add 1mL of chloroform: IAA (cold), then mix by inversion (20 times). Centrifuge samples at 14000g, for 30 minutes (at 4℃).
9. Set up one 2mL tube for each sample that contains:
    (a) 1.2mL of ice cold 100% ethanol
    (b) 60µL of 3M sodium acetate (pH 5.4)
10. Transfer upper phase to pre-setup tubes containing ethanol/sodium acetate. Mix by inversion (10 times). Allow DNA to precipitate in ethanol solution overnight at -20℃ (or 40 minutes at -80℃).
11. Centrifuge samples at room temperature at 14000g, for 30 mins.
12. Aspirate ethanol solution by carefully pouring off. Add 500µL of 70% cold ethanol.
13. Centrifuge samples at 14000g, for 10 minutes (room temperature).
14. Carefully pour off ethanol, then dry pellet using vacuum desiccator for 15 minutes.
15. Resuspend pellet in 200µL of sterile dH_2O or TE Buffer, then store at -20℃.

2. DNA extraction from banana plant tissue

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

Consumables and equipment

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

Before starting:

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

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

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

DNA amplification fingerprinting (DAF) analysis

The DNA amplification fingerprinting system used is a modified version of the technique described in: Bentley S*, et al.* (1996) A robust DNA amplification fingerprinting system applied to analysis of genetic variation within *Fusarium oxysporum* f. sp. *cubense*. *Journal of Phytopathology* 144, 207-213.

This technique utilises arbitrary primed PCR methods to generate genome-specific DNA banding patterns. The PCR-based DNA amplification reaction is directed by a single oligonucleotide primer of arbitrary sequence. PCR products are then visualised by polyacrylamide gel electrophoresis, followed by silver staining. This method was developed to deliver robust and reproducible results that are easily transferred between different laboratories.

PCR conditions

Consumables and equipment

Item	**Description**
PCR tubes	0.2mL Eppendorf PCR tubes, strip of 8 with individual hinged lids (Quantum Scientific P/L, #. 0030 124.359)
Sterile microfuge tubes	Sterile 1.5mL microfuge tubes Quantum Scientific P/L
Pipettes: P20, P200, P1000	Pipetman P20, P100, P200 John Morris Scientific
Aerosol Resistant (plugged) tips	Axygen Maximum Recovery, racked and pre-sterilised Quantum Scientific P/L; TF-20-L-R-S (suits P20), TF-200-L-R-S (suits P200), TF-1000-L-R-S (suits P1000)
Plastic racks and boxes	PCR-dedicated racks and boxes
pens	Fine tipped permanent markers
96-well, 0.5mL plate format PCR Thermal Cycler, e.g. MJ Research PTC-100 (most brands should be suitable)	MJ Research PTC-100 (Geneworks)

Reagents

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

DAF PCR Primers

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

Precautions:

Wear clean gloves when handling all reagents and tubes to avoid contamination

Aerosol-resistant plugged tips should be used to prepare amplification reactions to avoid aerosol carryover contamination

PCR set-up areas should be physically separated from areas where DNA extraction and/or electrophoresis is performed, in order to minimise the risk of contamination

Notes:

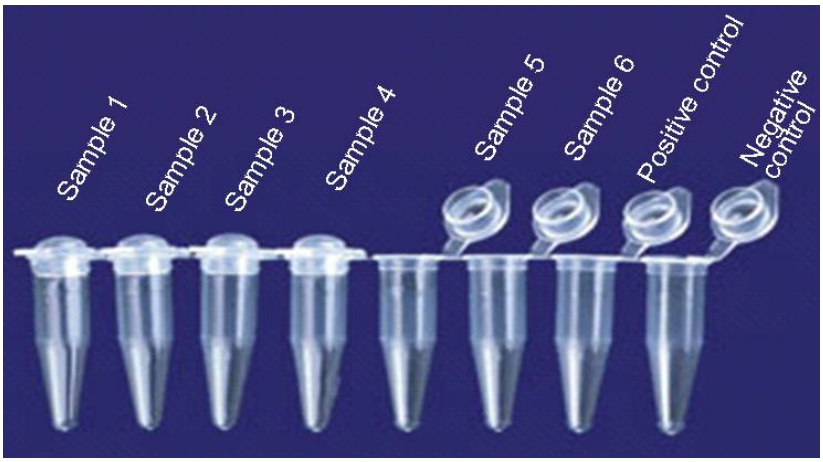
- PCR set-up should be carried out in a designated, "PCR-clean" area, using dedicated equipment. A PCR-clean laboratory coat should be worn. No movement of equipment or reagents should occur between set-up and processing of PCR products, excepting prepared reactions which may travel one-way to the template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

a) PCR set-up protocol

PCR Reaction

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

Example of PCR tube labelling and layout:



In a designated PCR set up area:

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

In a designated template addition area:

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

<u>Important:</u> take care to prevent cross-contamination between template tubes and PCR tubes. Opening template tubes with the left-hand and opening PCR tubes with the right hand is good practice in preventing cross-contamination of samples.

2. Close caps on each reaction firmly, zip spin tubes (using a capsule microcentrifuge) to draw reaction components down to the bottom of the tubes
3. Place tubes in thermal cycler and start program.

b) PCR thermal cycling procedure

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

Thermal cycling conditions:

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

Polyacrylamide Gel Electrophoresis (PAGE)

Equipment:

Item	**Description**
2 small beakers	2 X 20 ml glass beakers
Pipettes	P2 (0-2µL), P20 (0-20µL), P200 (0-200µL), P5000 (5mL)
Racked tips	white (0-2µL), yellow (0-20µL & 0-200µL) & white (5mL) tips
Mini-Protean III apparatus	BIORAD Mini-Protean III electrophoresis cell, including: lower buffer chamber, lid, inner cooling core, sandwich clamp assemblies, casting stand and plastic combs
2 pre-prepared gel sandwiches	Pre-prepared Mini-Protean III gel clamps with glass plates and GelBond® PAG polyester film in position
10mL syringes	2 X 10mL plastic syringes for gel pouring
20 ml syringes	2 X 20mL plastic syringes with needle for flushing wells
2 X loading tray	2 X 60-well H-LA tissue culture plate
GelBond	GelBond PAG Film (GE HealthCare, # 80-1129-37)
Kimwipes	Lint free tissues
pen	1 fine tipped permanent marker
discard bin	plastic pathological waste discard bin
gloves	latex gloves

Reagents:

Item	**Description**
TBE Buffer	1 X TBE (diluted from 10X liquid TBE, for consistency) (BioRad, #1610741)
Acrylamide master mix	10%T (T=total monomer), Acrylamide (Sigma, #A9099) 2%C (C=crosslinker), N,N′-Methylenebisacrylamide (Sigma, #M2022) 10% urea (Sigma, #U6504) 5% glycerol (Sigma, #G5516) in 1xTBE buffer
10% APS	Ammonium persulphate (BioRad, #1610700)
TEMED	N,N,N',N'-tetramethylethylenediamine (BioRad, #1610801)

DAF loading buffer	40% Urea (Sigma, #U6504) 3% Ficoll 400 (Sigma, #F9378) 10mM Tris-HCl (pH 8.3) 3mM EDTA 0.02% Xylene cyanol FF (Sigma, #335940) 0.02% Bromophenol blue (Sigma, #114391)
Amplisize™ Molecular Ruler	DNA size standards (50-2000bp Ladder) (BioRad, #1708200)

Acrylamide master mix:

Reagent	**Volume / Amount**
Bis (N,N′-Methylenebisacrylamide)	2g
Acrylamide	98g
Urea	100g
10 x TBE	100mL
Glycerol	50mL
MilliQ dH_20	make up to 1L

Gel casting reagents (per gel):

Reagent	**Volume**
Acrylamide master mix	4mL
TEMED	4µL
10% APS	50 µL

Precautions:

Acrylamide is a potent neurotoxin and a possible carcinogen. Always wear gloves when handling acrylamide, acrylamide solutions, or pouring acrylamide gels (Note however, that the polymerised form, polyacrylamide, is not toxic).

TEMED may be toxic if swallowed. It may also cause skin irritation. Avoid prolonged exposure to TEMED vapour.

All used tips, tubes and gloves should be discarded into the contaminated waste.

(a) Gel casting

1. As a precaution, check the gel sandwich (the two glass plates and backing film) is properly aligned at the bottom of the clamp assembly; all three surfaces should be flush.
2. Transfer each clamped assembly to the casting positions on the casting stand. Attach the clamped assembly by placing the gel sandwich against the bottom of the casting stand wall, so that the glass plates rest on the rubber mat. Gently snap the acrylic plate underneath the hinge of the casting stand by pushing on the green portions of the clamps. Do not push against the glass plates, as this could break the plates. The gel sandwiches are now ready for casting.
3. Carefully pipette 8mL of acrylamide master mix into a clean beaker.

Remember to always wear gloves when handling acrylamide solutions and label all beakers clearly with their contents.

4. Add 100µl 10% ammonium persulfate (APS) solution to the acrylamide master mix, and mix the solutions by gently swirling.
5. Add 8µl of TEMED to the acrylamide master mix, and mix the solutions by gently swirling.

Once the TEMED is added to the acrylamide mix it will start to polymerise and you must work quickly. You have about 2 minutes to mix the acrylamide, pour the gel, and position the comb.

6. Draw up the polyacrylamide solution into the 10ml plastic syringe and place the syringe tip against the top of lower glass plate of the gel sandwich. Gently expel the polyacrylamide solution into the gel space until it reaches the top of the lower glass plate. Check that there are no air bubbles in the gel. (Air bubbles can be removed by gentle flicking through the glass plate). Slide the comb into the gel so that it is horizontal and it forms wells that are 5 mm deep in the gel. It is very important that the comb is straight and that there are no air bubbles under the teeth. If there are air bubbles in your gel, quickly remove the comb and gently slide it back into place as before, being careful to avoid creating more air bubbles.
7. Once the gel has been poured and the comb is in place, leave the gels undisturbed until they polymerise. Polymerisation should be complete within 5 minutes, depending on the

temperature and humidity in the laboratory. Monitor left-over polyacrylamide left in the beaker to gauge when the gel has set.

(b) Sample preparation

1. While the gel is polymerising prepare your samples for loading. Clear labeling is essential to minimise errors when loading. Confusion can occur because the electrode assembly apparatus holds the gels backwards, which therefore requires the researcher to load the samples onto the gel from last to first. If the gels are loaded in this manner, once gels are stained and ready to analyse, the samples will then be viewed from left to right as would normally be expected.

To limit evaporation, keep the lid on the tray while not in use.

2. Add 1.4µl of loading buffer to wells of the loading tray.
3. Carefully add 1.6µl of each of the DAF PCR samples to the loading tray. If oil was used then each sample must be pipetted out from under the oil overlay. To do this, first depress the pipette plunger to the first stop, then carefully pierce the oil layer before slowly releasing the plunger to draw up the sample. Withdraw the tip from the sample tube, wipe the oil from the tip by rotating the tip inside the lip of the tube, and transfer the sample to its corresponding well in the loading tray. Mix the sample with the loading buffer by pipetting up and down twice.

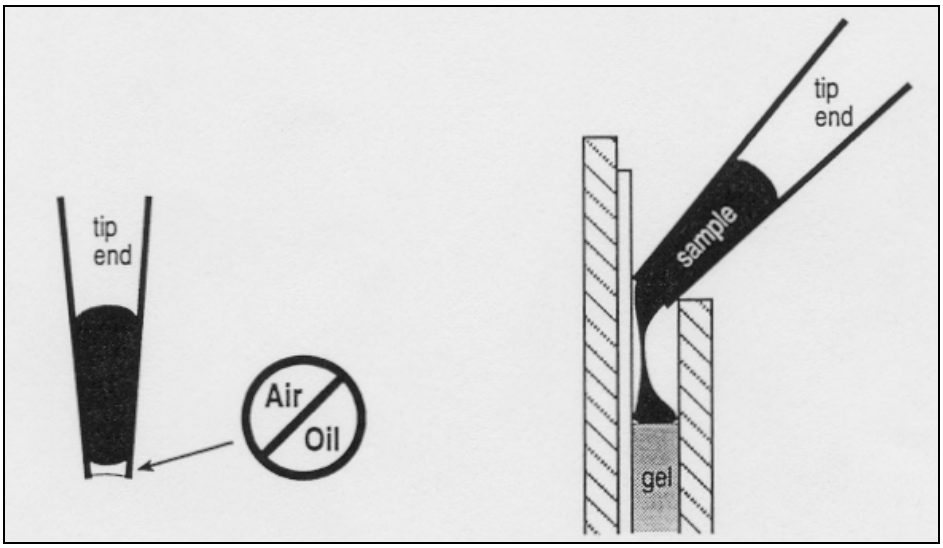
(c) Gel preparation

1. Once the gels are polymerised, remove the clamp assemblies from the casting stand. Do not remove or disturb the comb.
2. Fill the tank reservoir half full with 1 x TBE
3. Draw up 20mL of 1 x TBE with a 20mL plastic syringe, then attach the needle carefully
4. With the clamp assemblies still standing, carefully remove the comb (slide it straight out). Using the syringe, immediately rinse the wells with 1 x TBE to remove any unpolymerised acrylamide. Failure to do this may result in poorly-shaped wells and irregular DNA fingerprint bands.
5. Release the green side clamps and gently slide the gel sandwich out. Hold the gel sandwich by the edges, never in the middle. Place the gel sandwich inside the guides on the electrode assembly, making sure that the short glass plate is on the inside (gel is supposed to be back-to-front). Repeat with the other gel sandwich and gently hold the assembled electrode assembly together while placing the module into the buffer tank.

6. Fill both the inner and outer chambers of the buffer tank with 1 x TBE to a level about 5mm above the level of the inner glass plate.

(d) Gel loading and running

1. Rinse the wells again with 1 x TBE using the 20ml syringe.
2. Load 2ul of each sample from left to right (remembering to load from your last sample to your first sample due to the gel being back-to-front). Use a yellow tip attached to a P20 pipette. Be careful not to have any air in the end of the tip (see diagram below) or the sample will not expel smoothly into the well. To load, steady the barrel of the pipette with your spare hand, align the tip end over the middle of the well and, because it is too thick to fit between the glass, rest it on the edge of the small glass plate as shown below. Slowly expel the sample into the well.
3. Discard the tip and load the remaining wells similarly.
4. Attach lid to tank reservoir, and plug into power-pack (ensuring that red lead goes to red point, and black lead to black point). Start the electrophoresis. Run the gels at 300V for 33 minutes.



DNA silver staining

The silver staining method used is a modified version of that described in: Bassam BJ*, et al.* (1991) Fast and sensitive silver staining of DNA in polyacrylamide gels. *Analytical Biochemistry* **196**, 80-83.

Equipment

Item	**Description**
2 beakers	200-400ml size
Pipettes	P20 (0-20µl), P200 (0-200µl), P1000 (100-1000µl)
Tips (unfiltered)	Yellow (0-200µl), & blue (100-1000µl)
2 staining trays	Plastic sandwich boxes with lids
Silver discard trap	Bottle with salt (NaCl) added to precipitate used silver
Ethanol bottle	Squirt bottle of 95% distilled ethanol
Kimwipes	Lint-free tissues
Pen	Fine-tipped permanent marker
Discard bin	Plastic pathological waste discard bin
Gloves	Latex gloves
Plastic bags	Resealable plastic bags for gel storage

Reagents:

Item	**Description**
Fixer solution	7.5% glacial acetic acid
Silver nitrate	AR grade (Sigma, #S8157)
Sodium carbonate	AR grade (Sigma, #S7795)
Formaldehyde solution	AR grade (stock comes as 36.5-38%), then dilute to 1:2 (so actual concentration is ~17%) (Sigma, #F8775)
Stop solution	Cold 7.5% glacial acetic acid (kept at 4ºC)
Final Wash	10% acetic acid, 35% ethanol, 1% glycerol
dH_2O	MilliQ water

Precautions:

Wear gloves at all times when handling gels to help avoid staining artefacts.

Silver nitrate is toxic and should be disposed of with care. It will also stain most surfaces (including skin) so avoid spillage

Formaldehyde is toxic, carcinogenic, corrosive, and flammable. Handle with care, use only in a fume cupboard and avoid breathing formaldehyde vapour

Used tips, tubes and gloves should be discarded into the contaminated waste

(a) Gel disassembly and fixation

1. Turn off the power-pack and disconnect the apparatus by removing the lid.
2. Take the electrophoresis apparatus to a sink and remove the inner chamber (the electrode core and gel sandwich). Pour the running buffer from the inner chamber and tank into the sink. Remove the gel sandwich assemblies.
3. Disassemble the electrophoresis apparatus.
4. Pour 100mL of cold fixer solution into a staining tray.
5. Remove the polyester-backed gels from the gel sandwich assemblies. First gently prise the glass plates apart being careful not to touch the gel. Lift off the small plate. Finally, remove the backed gel from the large glass by peeling up the backing film from one corner of the plate using your (gloved) index finger.
6. Put the backed gel face-up into the shaking fixer solution. Put the tray on a rotary shaker and set it shaking (at about 60 cycles/minute) for 10 minutes.

Fixation prevents diffusion of the DNA within the gel matrix and also helps remove unwanted chemicals like urea, glycerol, and running buffer that may interfere with staining

7. Rinse the buffer tank and electrode core with dH_2O and set them aside to dry.

(b) Clean-up and reassembly of gel clamps

1. While the gel is fixing, wash and reassemble the gel clamps. Rather than store the components of the gel assemblies dry and apply the backing film immediately prior to gel casting (as is recommended by the manufacturers), it is much easier to apply the backing film wet as part of the post-electrophoresis cleanup and store the components assembled.
2. Begin by rinsing the clamp assembly and stand up vertically on its feet. Next, wash the large glass plate under running deionised water.
3. Similarly, wash a GelBond PAG film. Note that one side of the film is hydrophobic and the other is hydrophilic. Position the film hydrophilic side up on top of the large glass plate. Rub it down firmly to squeeze excess water and bubbles out from under the film.

The hydrophilic side is chemically treated to covalently bind to the acrylamide during polymerisation. If you put the GelBond the wrong way around, the gel will not stick to the film!

4. To complete the assembly, thoroughly wash the small glass plate under running deionised water. One side of this plate will make direct contact with the gel. It is very important that it is kept clean or staining artefacts will result.
5. Before being clamped in position, the components of the assembly must be aligned. Do this by standing the assembly upright and aligning the glass plates and backing film with the bench. Press down on the glass plates and backing film until their bottom edges are aligned flat with the bench.
6. Lock the clamp assembly into position using the green hinges. When secured, check the alignment of the components along the bottom edge.
7. Rinse the entire assembly down with 70% ethanol, making sure to rinse out the gel space thoroughly.
8. Shake the assembly to remove excess ethanol and stand it upright on a layer of Kimwipe tissue to dry. When dry, the assembly is ready for storage. Be sure it is stored in an upright position away from light and dust (in a closed cupboard, for example).

(c) Preparation of silver staining solutions

Reagent (per gel)	**Volume / Amount**
Silver stain	0.1g silver nitrate/100mL MilliQ water
Developer	3g sodium carbonate/100mL MilliQ water
Formaldehyde	25mL of 50% formaldehyde (fume hood)
Final Rinse	Acetic acid (10%) Ethanol (35%) Glycerol (1%) Made up as a stock.

Handle silver nitrate with care, it is toxic and will stain most surfaces

1. Prepare the developer solution. Weigh 6g of sodium carbonate into a plastic weigh boat.
2. Dissolve the sodium carbonate in 200mL of MilliQ H_2O and cool the developer solution by putting it into the fridge (cooling also helps with dissolution).
3. Prepare the silver staining solution. Weigh 0.2g of silver nitrate and dissolve it in 200mL of MilliQ H_2O.

(d) Washing and impregnation of gels

1. Wash the fixer solution from the gels. Carefully, pour the fixer into the sink, replace it with MilliQ H_2O and leave to wash for 2 minutes on shaker.
2. Repeat step 1, twice more (for a total of three x 2 minute washes in MilliQ H_2O).
3. Tip off the dH_2O and then pour on 25mL of formaldehyde solution (in fume cupboard), ensuring that you pour to the side of the gel and not directly onto gel surface (see tip below). Transfer containers (with lids attached) to shaker for 6 minutes.

Do not pour the formaldehyde directly onto the gel. This will result in dark spots on the gel. The concentration of formaldehyde after diluting with an equal volume of water is actually 17% and should always be stored at room temperature.

4. In a fume hood, tip the used formaldehyde down the sink, flushing with plenty of water.
5. Add 200mL MilliQ H_2O to each gel container and wash gels for 5 minutes on shaker.

6. Tip off MilliQ dH_2O and pour 100mL of silver solution onto each gel (again not directly onto gel surface, try to pour to one side of the gel) and leave it to shake for between 15 minutes and 1.5 hours (35 minutes is optimal).

(e) Gel image development

1. Check that the developer is cold (it should be at 4ºC).

If the developer is any warmer then image development will occur too rapidly. This will make it difficult to accurately stop the reaction and may also cause discolouration of the gel.

2. Tip off the silver solution into a silver discard (trap) bottle containing NaCl. It should turn milky in the bottle as the silver precipitates.
3. Briefly rinse residual silver solution from the surface of the gel; pour 100mL of MilliQ H_2O onto the gel and swirl for 5 -10 seconds. Tip the water off into the silver trap.

Do not rinse the gel longer than 15 seconds as this step removes silver from the gel.

4. Add about 100mL of developer to the gel and leave it to shake. Image development takes about 5 minutes.

Image development will occur slowly by reduction of silver ions with formaldehyde in the alkaline environment of the developer.

5. When the image has fully developed, stop the reaction quickly by pouring off the developer into the sink and adding 100mL of cold (4ºC) stop solution. Knowing when to stop image development takes some practice – there is always the tendency to let it go too far (resulting in a very dark gel). You need to achieve the optimum image contrast between the DNA bands and the gel itself. A slight yellow-orange background colour is acceptable (as this largely disappears when photographed in black and white). Aim to stop the reaction when the 100bp fragment is obvious (possibly sooner if the gel image is clear).

Lowering the pH is the mechanism by which the reaction is stopped. The stop solution is the same as the fixer solution (7.5% glacial acetic acid) except that it is kept cold. The stop solution is chilled so that it acts as quickly as possible (the low temperature slows image development and allows time for the acid to take effect).

6. Leave the gel in stop solution for 5 minutes
7. Tip off the stop solution and wash the gel in MilliQ H_2O for 5 minutes.
8. Tip off the MilliQ H_2O and move containers to fume cupboard. Add 50mL of Final Wash to preserve the gel, attach lids and leave to shake for 5 minutes.
9. Photograph your gel. Hold the gel by the plastic backing film (do not touch the gel directly) and take it to a light box.
10. Label your gel and hang it up to dry overnight. When dry, the gel is quite robust and it can be safely handled.

If properly stained, the gel will not fade or darken and provides a permanent record of your experiment. However, in a very dry environment, the plastic will curl up and gel will become brittle. In this state, it may crack if unduly stressed. We recommend storing the dry gel in a resealable plastic bag.

Interpretation of results

DNA Fingerprinting Analysis

This DAF analysis system is used routinely to characterise unknown isolates of *Foc* from Australia and worldwide by comparing the DNA fingerprints of these unknown isolates with those from our DNA reference collection (which spans all the currently identified VCGs of *Foc*). This DAF analysis system has been thoroughly optimised and provides an informative and reproducible platform for the molecular characterisation of unknown isolates of *Foc*.

Genome-specific DNA fingerprint patterns are analysed and scored manually (by eye); fingerprints of test samples are compared to standards (characterised isolates) by assessing the general fingerprint pattern and scoring for presence/absence of polymorphic bands (see Figure 7). Scoring is made easier by placing gels on a lightbox, which enables the discrimination of lightly stained bands. Test samples can be compared with standards from other gels by overlapping gels on the lightbox, or by digitally cutting and pasting the lanes next to one to build a new image that makes scoring easier. DAF analysis is used in conjunction with VCG testing for the characterisation of unknown isolates of *Foc*.

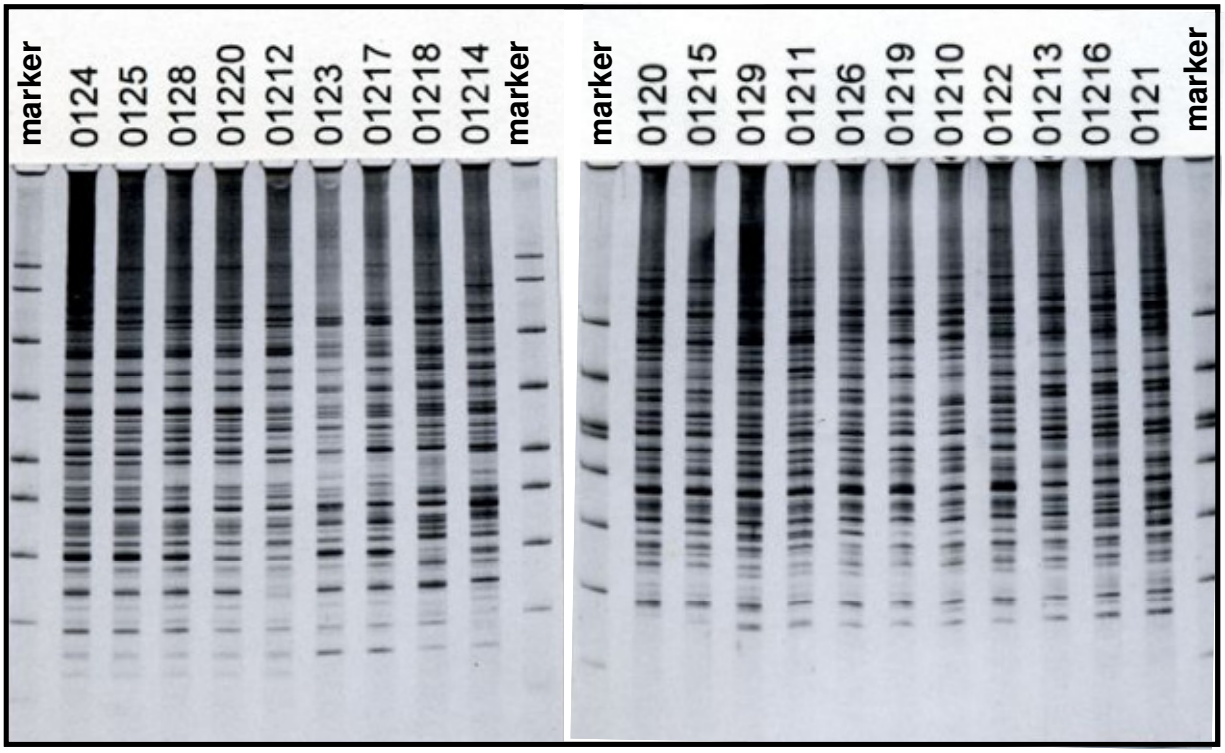


Figure 7: DNA Fingerprinting analysis of a range of Australian and overseas VCGs of *Foc*, visualised by polyacrylamide gel electrophoresis and silver staining. The DAF PCR was performed using the HIRH single oligonucleotide primer. This DAF analysis clearly shows the utility of this technique for differentiating closely related VCGs of *Foc*. Isolate information is annotated on the gel photograph. Image courtesy of the CRC for Tropical Plant Protection.

Tropical race 4 diagnostic PCR

The tropical race 4 diagnostic PCR assay specifically amplifies a fragment of the intergenic spacer (IGS) region of the ribosomal DNA of tropical race 4 isolates of *Foc*. The IGS region was chosen as the diagnostic target because: (a) it enables sensitive detection (ribosomal genes and spacers occur in tandem repeats that are thousands of copies long; thus the IGS is present in high copy number), and (b) the non-transcribed IGS region is known to be useful for inferring phylogeny between closely related taxa and for revealing intraspecific variation within species that exhibit a clonal population structure, such as *Foc*. Sequence data generated by the CRCTPP showed that the IGS region contained sequence polymorphisms that were able to be exploited for the discrimination of closely related VCGs within *Foc*. A diagnostic PCR was designed to specifically amplify a 1400bp fragment from isolates of tropical race 4 of *Foc*, using PCR primers TR4F2 and TR4R1. Both primers are required for the reaction unlike DNA fingerprinting where only one primer is required. The tropical race 4 PCR has been optimised and validated by the CRCTPP, and is suitable for diagnosing the presence of tropical race 4 of *Foc* from both fungal culture and directly from diseased plant material.

A modification of a PCR specific for isolates of *Fusarium oxysporum* (Edel *et al.* 2000) is used as an independent internal control PCR for the tropical race 4 diagnostic PCR. The *Fo* specific PCR is always performed in parallel to the tropical race 4 diagnostic test, in order to prove: (a) that the sample contains DNA from *Fo*, (b) that the test sample is able to be amplified effectively in a PCR reaction (i.e. to show that test sample does not contain PCR inhibitors). This is important for troubleshooting assays, for example, to ensure no false negative results are obtained due to PCR inhibition. This PCR specifically amplifies a 70bp fragment from isolates of *Fusarium oxysporum*, using the primers PF02 and PF03 (Edel *et al.* 2000).

PCR conditions

Consumables and equipment

Item	**Ordering Information**
Pipettes: P2, P20, P200, P1000	Pipetman P2, P20, P100, P200 (John Morris Scientific)
Aerosol resistant (plugged) tips	Axygen Maximum Recovery, racked and pre-sterilised (Quantum Scientific P/L) TF-300-L-R-S (suits P2) TF-20-L-R-S (suits P20) TF-200-L-R-S (suits P200) TF-1000-L-R-S (suits P1000)
Sterile 1.5mL microfuge tubes	Quantum 1.5 ml Microtubes (500) (Quantum Scientific P/L, #QSP505)
PCR Strip tubes with attached lids	PCR 8-strip tubes (120 strips) (Eppendorf, #0030 124.359)
PCR rack	
Microcentrifuge	
Capsulefuge (suit 8-strip PCR tubes)	
96-well, 0.5mL plate format PCR Thermal Cycler, e.g. MJ Research PTC-100.	MJ Research PTC-100 (Geneworks)

Reagents

Item	**Ordering Information**
Tth$^+$ DNA polymerase, 10 x Buffer and $MgCl_2$	*Tth*$^+$ DNA Polymerase (250Units - 5.5 Units/µL), supplied with 10 x Buffer and 25mM $MgCl_2$ (FisherBiotech, #TP1)
dNTPs (set of dATP, dCTP, dGTP, dTTP)	dNTP Set (100mM each) Roche PCR-grade dNTPs (Roche, # 1581295)
Sterile water	Millipore® or injectable grade
PCR primers	40 nmol scale, **HPLC grade** (Geneworks)

Preparation and storage of PCR stocks

Reagent	**Details**
Sterile water	• Obtain injectable water • Store in sterile vials at room temp
10X Buffer	• Supplied with enzyme • Aliquot on first thaw and be sure to mix well before using • Store –20 ºC
$MgCl_2$	• Supplied with enzyme • Aliquot on first thaw and mix well before using • Store –20 ºC
dNTP mix	• Purchased as individual stocks of dATP, dCTP, dGTP and dTTP at 100mM • Take 10µL of each and add to 460µL sterile water to give a final working solution of 2mM each dNTP • Store aliquotted at –20 ºC and avoid multiple freeze thaws where possible
Primer stocks	• Resuspend lyophilised pellet to 100µM • Dilute to 12.5µM (1:8) in sterile water and store aliquotted at –20 ºC • Do not dilute all stock as primers are more stable at higher concentrations
DNA Polymerase	• Supplied at 5.5U/µL, use 0.35µL (2U) per reaction • Store at –20 ºC in a cyclic defrost freezer

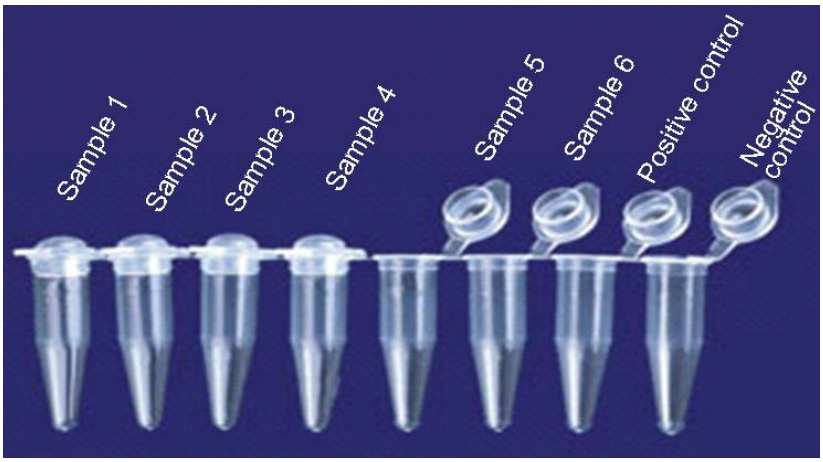
Tropical race 4 PCR primer sequences

Primer Name	**Sequence (5'→3')**
TR4F2	CGCCAGGACTGCCTCGTGA
TR4R1	CAGGCCAGAGTGAAGGGGAAT

Fo specific PCR primer sequences

Primer Name	**Sequence (5'→3')**
PFO2	CCCAGGGTATTACACGGT
PFO3	CGGGGGATAAAGGCGG

Example of PCR tube labelling and layout:



Precautions:

Wear clean gloves when handling all reagents and tubes to avoid contamination

Aerosol-resistant plugged tips should be used to prepare amplification reactions to avoid aerosol carryover contamination

PCR set-up areas should be physically separated from areas where DNA extraction and/or electrophoresis is performed, in order to minimise the risk of contamination

Notes

- PCR set-up should be carried out in a designated, “PCR-clean” area, using dedicated equipment. A PCR-clean laboratory coat should be worn. No movement of equipment or reagents should occur between set-up and processing of PCR products, excepting prepared reactions which may travel one-way to the template addition laboratory. Backwards movement of racks between the template addition area to the PCR preparation area must be avoided.
- It is recommended to only set-up one PCR experiment per day. PCR set-up should never directly follow handling of PCR products.

- The independent internal control PCR (the *Fo* specific PCR) must be set up in parallel with every diagnostic sample for quality control.
- Reagents, except for the DNA polymerase, should be thoroughly thawed, mixed and pulse-centrifuged to collect contents at the bottom of the tube.
- Preparation of mastermixes is recommended when multiple samples are to be analysed; this permits standardisation of reagents across the tubes and minimises pipetting errors. When preparing mastermixes, always include two extra tubes for positive and negative PCR controls. Also, always prepare at least 10% more reactions than are required, to account for pipetting errors caused by tip retention during aliquotting.
- PCR positive controls, i.e. DNA extracted from (a) a characterised tropical race 4 isolate of *Foc*, and (b) a *Fusarium* sp. isolate, are used to compare with unknown isolates using the *Fo* and tropical race 4 diagnostic PCRs. Also, a PCR negative control (no template) is always required for each PCR mastermix. These controls are essential for interpreting the results of the PCR, and are important for troubleshooting PCRs that fail.
- When testing DNA extracted from plant samples, always test a 1:10 and a 1:100 dilution from each extraction by PCR, as target template cannot be quantified spectrophotometrically from these mixed template samples. It is optimal to find a balance between the target DNA concentration (too much template inhibits PCR and too little will not amplify), and the concentration of PCR inhibitors (diluting extractions dilutes out the concentration of PCR inhibitors).

Tropical race 4 PCR reaction

Tropical Race 4 Specific PCR			
Reagent	**Final concentration per reaction**	**Volume per reaction (µL)**	**Master Mix for 9 reactions (µL)**
DNA template (25ng/µL)	25ng	1.0	0
Tth^+ DNA polymerase	2 units	0.35	3.15
Primer TR4F2 (12.5µM)	0.5µM	1.0	9.0
Primer TR4R1 (12.5µM)	0.5µM	1.0	9.0
dNTPs (2mM)	200µM	2.5	22.5
$MgCl_2$	3mM	3.0	27.0
10 x PCR Buffer	1x	2.5	22.5
dH_20		13.65	122.85
TOTAL		25.0	216.0

Fo specific PCR reaction

***Fo* Specific PCR**			
Reagent	**Final concentration per reaction**	**Volume per reaction (µL)**	**Master Mix for 9 reactions (µL)**
DNA template (25ng/µL)	25ng	1.0	0
Tth^+ DNA polymerase	2.75 units	0.5	4.5
Primer PF02 (12.5µM)	0.5µM	1.0	9.0
Primer PF03 (12.5µM)	0.5µM	1.0	9.0
dNTPs (2mM)	240µM	3.0	27.0
$MgCl_2$	3mM	3.0	27.0
10 x PCR Buffer	1x	2.5	22.5
dH_20		13.0	117.0
TOTAL		25.0	216.0

In a designated PCR set up area:

1. Clean PCR area with 10% bleach
2. Label 0.2mL PCR tubes
3. Assemble and thaw reagents (except for the Stoffel DNA polymerase)
4. Prepare a master mix for the tropical race 4 reactions in a 1.5mL tube; a standard mix for 8 reactions is shown in the table above (far right column). Note: always prepare at least 10% more reactions than are required, to account for pipetting error (in this case 9 reactions are prepared). **At the same time as you are setting up the tropical race 4 diagnostic assays, ensure that you set up the same panel of samples in *Fo* specific PCR (internal control PCR), for quality control.**
5. Vortex master mix thoroughly, then spin down briefly.
6. Aliquot 24µl of the master mix into each PCR tube; pipette the mix onto the bottom of the tube.
7. Add 1µL of sterile water to the negative control tube (usually the last tube as this tube stays open the longest during set-up). To ensure a reliable control against contamination during master mix preparation, the negative control tube must remain closed from this point until post-PCR analysis.

8. Transfer the tubes to the template addition section. Where possible, racks should not be removed from the PCR clean section. If this cannot be avoided, the tubes should be transferred to another rack before template addition and the "template-free" rack returned to the PCR clean laboratory immediately.

In a designated template addition area:

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

<u>Important:</u> take care to prevent cross-contamination between template tubes and PCR tubes. Opening template tubes with the left-hand and opening PCR tubes with the right hand is good practice in preventing cross-contamination of samples.

10. Close caps on each reaction firmly, zip spin tubes (using a capsule microcentrifuge) to draw reaction components down to the bottom of the tubes
11. Place tubes in thermal cycler and start program.

b) PCR thermal cycling procedure

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

Thermal cycling conditions

Tropical Race 4 Specific PCR		
Step	Temperature	Time (minutes: seconds)
1	95℃	2:00
2	95℃	0:30
3	68℃	1:30
4	Go to step 2, 29 more times	
5	72℃	3:00
6	4℃	5:00
7	End	

***Fo* Specific PCR**		
Step	Temperature	Time (minutes: seconds)
1	95℃	3:00
2	95℃	0:30
3	62℃	0:30
4	72℃	0:30
5	Go to step 2, 29 more times	
6	72℃	3:00
7	4℃	5:00
8	End	

Agarose gel electrophoresis

Consumables and equipment

Item	**Ordering Information**
Pipette: P20	Pipetman 20 (John Morris Scientific)
Pipette tips	Sterile yellow tips for P20 (Quantum Scientific P/L)
Nunc Microwell Minitrays	HLA Plate 60-well (Medos, #NUN4-52256)
Capsulefuge (suit 8-strip PCR tubes)	
Gel-electrophoresis set-up (eg. BioRad Sub-Cell GT) 20 well combs (up to 4 combs depending on throughput required)	
Gel documentation system (UV transilluminator and camera)	
Nitrile gloves	
Ethidium bromide designated staining area	

Reagents

Item	Ordering Information
Agarose	Agarose I (Biotechnology Grade; DNase, RNase, Protease Free) 500 g (Astral Scientific P/L, # AM0710)
DNA marker and 6X Gel loading buffer	Generuler™ 100bp Ladder Plus (0.05 mg) supplied with 1mL 6X Loading Dye Solution (MBI Fermentas, #SM0321)
Tris-Borate EDTA Running Buffer (10X)	TBE Buffer 10X Liquid (4 L) (Astral Scientific P/L, # AM0658)
Ethidium bromide	Ethidium Bromide (10 mg/mL) (Astral Scientific P/L, #AME406)

Notes

- A set of pipettes must be dedicated only to gel analysis. Amplified (PCR) product poses the highest risk of contamination of any template
- Gel-electrophoresis and preparation of PCR products for analysis must be kept separate at all times from areas used for DNA extraction and PCR set-up
- Visualisation of PCR products using ethidium bromide (EtBr) may be carried out by post-staining or by running EtBr in the gel. Protocols for both options are presented here
- CAUTION: EtBr is a powerful mutagen and possible carcinogen and teratogen. Refer to MSDS data sheets and safety information on the correct handling of EtBr before proceeding

Agarose gel electrophoresis procedure

1. Prepare a 1% agarose gel in 0.5X TBE. A gel with 20-wells is sufficient to run 16 samples, positive and negative PCR reactions and two molecular weight markers. If required, EtBr can be included in the gel; add 5µL of a 10mg/mL solution to 100mL (final concentration of 0.5µg/mL).
2. While the gel is setting, prepare the PCR products for analysis. To 12µL of PCR product add 2µL of 6X gel loading buffer. Prepare enough molecular weight marker for two wells by adding 2µL (500ng) of Generuler™ 100bp Ladder Plus, 8µL sterile distilled water and 2 µL 6X gel loading buffer.

3. Place the set gel into the buffer tank and cover with 0.5X TBE running buffer before removing the gel comb.
4. Load the samples (14µL) onto the gel in the following order: DNA marker, negative PCR control, test samples, positive PCR control, DNA marker.
5. Run the gel for approximately 45 minutes at 100V, until the bromophenol blue dye is three quarters along the length of the gel.
6. If EtBr has not been run in the gel, post-stain in water containing EtBr at (0.5µg/mL) for 20 min. If necessary, de-stain in water for 10-15 min.
7. Photograph gel using UV transilluminator attached to a gel documentation system.

Extreme care should be taken when handling ethidium bromide (EtBr). EtBr is carcinogenic and a powerful mutagen, and must be handled with due care. Always wear a labcoat and nitrile (blue) gloves to minimise exposure. Take care to minimise EtBr contamination by restricting the movement of contaminated items.

Ultraviolet light can damage eyes. Never look at unshielded UV light source. View only through a filter or special safety glasses that absorb harmful wavelengths. Your own glasses do not protect your eyes.

Interpretation of results

Tropical race 4 PCR

A sample is positive for tropical race 4 if a PCR product of approximately 1400bp is visible. Size can be estimated by comparison to bands of similar size in the DNA molecular marker lane. To control against cross-contamination and false positive results, the negative PCR control should not have amplified (no band present). As a control for the efficiency of the tropical race 4 PCR, a band of approximately 1400bp should be present in the positive control lane. Figure 7 illustrates typical amplification results for the tropical race 4 PCR assay.

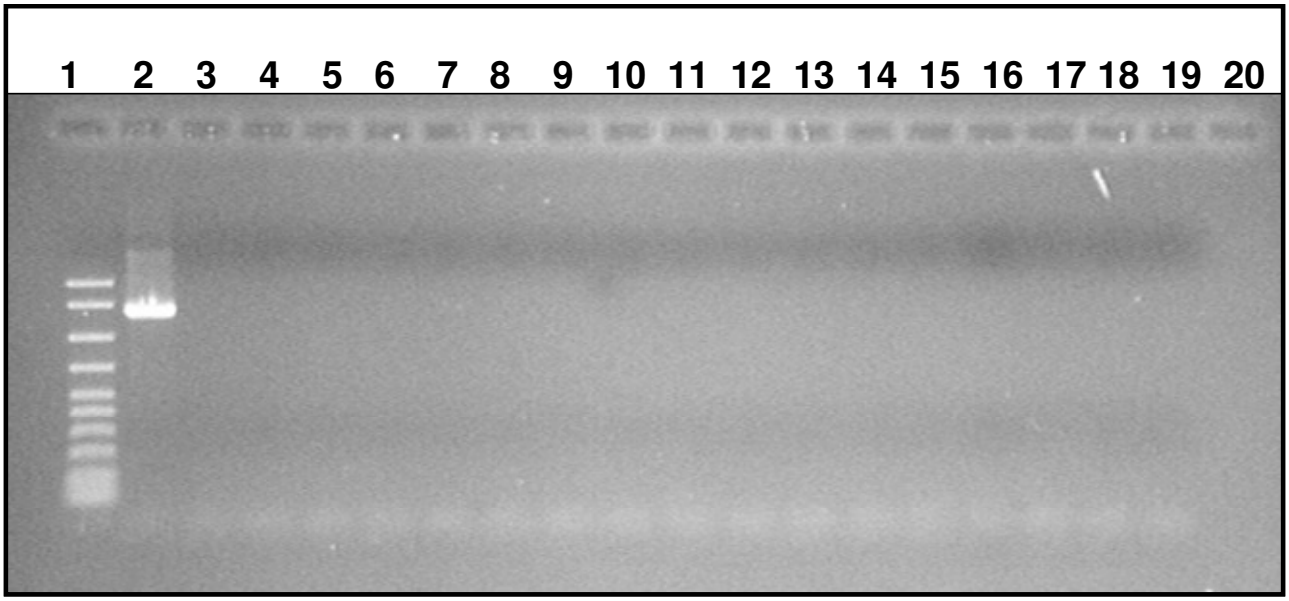


Figure 7: Agarose gel showing 1400bp specific amplicon produced from the tropical race 4 diagnostic PCR, screened against isolates of Australian VCGs of *Foc*, isolates of *Fusarium oxysporum* and *Fusarium* sp.

Lane 1: Amplisize Molecular Ruler (50-2000bp), top three bands are 1000bp, 1500bp and 2000bp, Lane 2: tropical race 4 specific band (~ 1400bp) from *Foc* VCG01213/16, Lane 3: *Foc* VCG0124, Lane 4: *Foc* VCG0125, Lane 5: *Foc* VCG0128, Lane 6: *Foc* VCG0129, Lane 7: *Foc* VCG01211, Lane 8: *Foc* VCG01212, Lane 9: *Foc* VCG0120, Lane 10: *Foc* VCG01220, Lane 11: *Foc* VCG0124, Lane 12: *F.o*. basilicum, Lane 13: *F.o.* dianthi, Lane 14: *F.o.* fragariae, Lane 15: *F.o.* vasinfectum VCG 01111, Lane 16: *F. pseudograminearum*, Lane 17: *F. solani*, Lane 18: *F. moniliforme*, Lane 19: *F. equiseti*, Lane 20: PCR negative control.

Fo specific PCR

A sample is positive for *Fusarium oxysporum* if a PCR product of approximately 70bp is visible. Size can be estimated by comparison to bands of similar size in the DNA molecular marker lane. To control against cross-contamination and false positive results, the negative PCR control should not have amplified (no band present). As a control for the efficiency of the *Fo* PCR, a band of approximately 70bp should be present in the positive control lane. Figure 8 illustrates typical amplification results for the *Fo* PCR assay.

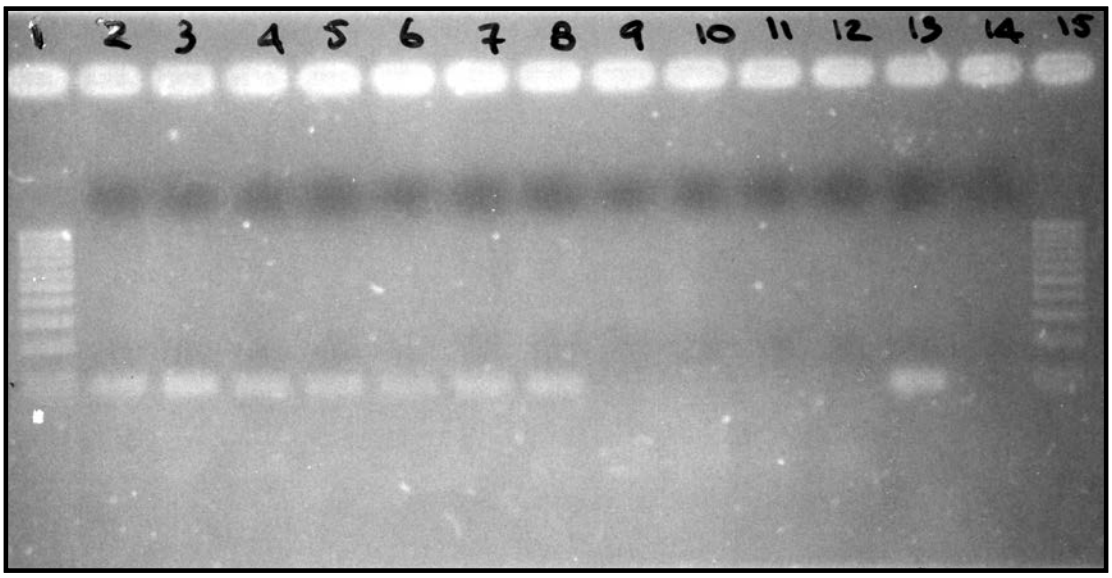


Figure 7: Agarose gel showing 70bp specific amplicon produced from the tropical race 4 diagnostic PCR, screened against isolates of Australian VCGs of *Foc*, isolates of *Fusarium oxysporum* and *Fusarium* sp.

Lane 1: Amplisize Molecular Ruler (50-2000bp), lowest band is 50bp, Lane 2: unknown sample, Lane 3: unknown sample, Lane 4: unknown sample, Lane 5: *Foc* VCG0124, Lane 6: *Foc* VCG0129, Lane 7: VCG01213/16, Lane 8: *Foc* VCG01213/16, Lane 9: *F. moniliforme*, Lane 10: *F. pseudograminearum,* Lane 11: F. solani, Lane 12: *F. equiseti,* Lane 13: Positive control (known *Fo* species, *F.o.* vasinfectum VCG 01111), Lane 14: Negative PCR control, Lane 15: Amplisize Molecular Ruler (50-2000bp).

Troubleshooting

DNA extraction (from fungal culture)

Problem	**Possible Causes**	**Suggestions**
No DNA pellet at extraction end	• Not enough sample tissue • Sample has not been macerated sufficiently • Extraction buffer has not been made up correctly • Pellet is lost after decanting off liquids after centrifugation steps	• Use 200mg of mycelial mat • Grind for longer, adding more liquid nitrogen where necessary to ensure a fine powder is formed • Check buffer preparation and/or remake • Use a pipette to remove supernatant
Gelatinous-like material present in the DNA pellet	• Excess polysaccharide in the sample	• Test 1:10 and 1:100 dilutions of the resuspended sample to dilute out possible PCR inhibitors
Difficulty resuspending DNA pellet	• Accumulation of salts, polysaccharides and other insoluble contaminants in the sample • Over drying of pellet	• Avoid using a desiccator; 15-30 min drying on the bench top is often sufficient • Complete re-suspension is not always necessary for diagnostic screening • Incubating the sample at 55℃ for 60 min may improve solubility

DNA extraction (from plant tissue)

Problem	Possible Causes	Suggestions
DNA yield is low	• Not enough sample tissue • Sample has not been macerated enough • Kit components have reached expiry date • Pellet is lost after decanting off liquids after centrifugation steps • Suboptimal elution	• Use 150mg plant material • Add a small amount of sterile sand to facilitate homogenisation • Check dates on kit and reagent bottles • Use a pipette to remove supernatant • Remember to preheat elution buffer to 70°C
DNA is degraded or of low quality	• Centrifuge speed was too high • Contaminants (e.g. phenolic compounds) were not properly removed during procedure	• Only centrifuge at a maximum speed of 11000g • Repeat washing step with buffer CW
Coloured DNA pellet	• Tannins present in sample • Commonly found when extracting from discoloured vascular strands (usually of no consequence)	• A 1:10 dilution usually amplifies well in PCR despite the extract colour
Difficulty resuspending DNA pellet	• Accumulation of salts, polysaccharides and other insoluble contaminants in the plant tissue • Over drying of pellet	• Avoid using a desiccator; 15-30 min drying on the bench top is often sufficient • Complete re-suspension is not always necessary for diagnostic screening • Incubating the sample at 55℃ for 60 min may improve solubility

PCR diagnostic tests

Problem	**Possible Causes**	**Suggestions**
Failed positive control reaction	• Degraded DNA template • Inhibitors in DNA template • Too high or too low DNA template concentration • PCR Primers degraded • dNTPs too old • PCR primer or dNTP preparation errors • *Taq* DNA polymerase inactive • Enzyme buffer or $MgCl_2$ not completely mixed • PCR thermal cycler error or power outage	• Repeat PCR with new DNA template (preferably extracted from single-spored culture to avoid PCR inhibition) • Use 1-10ng DNA as control • Repeat PCR with new aliquots of primers, dNTPs, buffers, $MgCl_2$ making sure to mix components thoroughly before addition
Failed negative control reaction	• Contamination of one or more PCR reagents	• Do not spend time trying to determine the exact source of the contamination • PCR reagents should be aliquotted such that when PCR contamination occurs, the aliquot in use at the time can be discarded • Repeat experiment with new aliquots of reagents

Electrophoresis

Agarose gel electrophoresis

Problem	Possible Causes	Suggestions
Sample escapes from bottom of the wells	• Gel comb height set too low on adjustable comb holders • Bottom of the well pierced with a tip during loading • Gel not set when comb removed	• Check comb height is 2-3mm above tray before pouring gel • Do not place tip low into well when loading • Wait until gel is set before removing comb (15-20 min)
Samples have run backwards	• Electrodes or gel tank lid has been fitted backwards.	• Check that the gel tank has been correctly attached to the voltage pack; always run gels from black (negative) to red (positive) electrodes (for DNA this is always the case).
Samples have not run out of well	• Power pack failure • Incorrect fitting of electrodes or tank lid	• Always check for voltage through the gel by looking for bubbles rising from cathode • Check current (should be ~40-50mA at 100V) • Ensure correct dilution of TBE is used in the agarose gel and tank
Gel bands wavy, fuzzy or absent	• Incorrect concentration of TBE buffer in gel or tank • Water used instead of TBE (common error) • Air bubble in agarose gel • Incomplete dissolving of agarose before pouring gel	• Take care to remove air bubbles before gel begins to set • Ensure all agarose is dissolved before cooling and pouring gel • Ensure correct dilution of TBE is used in the agarose gel and tank (0.5x).
Sample flows up and out of well	• Insufficient glycerol or sucrose in gel loading buffer (GLB)	• Check concentration of sucrose/glycerol in loading buffer or use commercially prepared

	• Too little GLB added to sample • Air bubble in gel loading tip • Sample loaded too fast • Sample well too shallow for volume added	loading buffer • Add GLB: sample in 1:6 ratio • Check pipetting technique • Check height that gel comb is set at
No bands visible on agarose gel when viewed under UV light (including molecular weight markers)	• Incorrect concentration of EtBr added to gel • Post-staining EtBr solution old	• Check concentration of EtBr added to gel • Post-stain in newly prepared EtBr solution
Uneven EtBr staining of gel (seen as patchiness or regions of intense fluorescence)	• EtBr added to agarose was not mixed properly • Post-staining with multiple gels in the tank does not allow good contact with the solution	• Swirl EtBr through cooled agarose (60℃) before pouring gel • Do not overload post-stain tank with many gels • De-stain with water and re-stain with fresh EtBr

Polyacrylamide Gel Electrophoresis (PAGE)

Problem	Possible Causes	Suggestions
PAGE wells are "hairy" with strands of polymerised acrylamide blocking the wells	• Gel was left to set for too long before rinsing out wells, and polymerised acrylamide has attached in place to the combs	• Try to clean out the wells the gels earlier (when the gel is not 100% polymerised), remove combs quickly and immediately rinse out each well thoroughly
Gels do not set	• There is a problem with your reagents, or you have added the wrong amount of reagent	• Check the concentration of solutions against manuals, make up fresh solutions and re-check calculations. All recipes listed are for 1 gel, remember to multiply this number by however many gels you are pouring
Gels leak when you pour them	• Plastic cushions have degraded and there is space allowing gels to leak, or gels were not correctly attached to casting apparatus	• Check the position of apparatus, try moulding a small amount of blue-tac in a line at the bottom of the gel rig to create a seal; or alternatively melt some agarose and apply in a line at the bottom of the gel rig to create a good seal.
Samples have run backwards	• Electrodes or gel tank lid has been fitted backwards.	• Check that the gel tank has been correctly attached to the voltage pack; black to black, red to red.
Samples have not run out of well	• Power pack failure • Incorrect fitting of electrodes or tank lid	• Always check for voltage by looking for bubbles rising from cathode • Check current • Ensure correct dilution of TBE is used (1x)
Gel has separated from GelBond Film	• GelBond was not placed in position correctly, and gel has not adhered to film	• Note that one side of the film is hydrophobic and the other is hydrophilic. Position the film hydrophilic side up on top of the large glass plate.

After staining, gels turn very dark	• Too much silver • Formaldehyde step left for too long • Development was left too long	• Double - check silver concentration of staining solution • It's important to keep very strictly to the times prescribed in this manual • Do not develop for as long, add stop solution as soon as you can make out the smallest band on your DNA ladder
No bands on gel	• If you can't see your markers, then staining did not work • If you can see the markers, but no samples, then PCR has failed	• Check all reagents and solution recipes, and go over procedure again for your own reference to make sure you haven't missed a step • re-do PCR
Bands are very orange	• Formaldehyde has gone off • Silver needs replacing	• Obtain new formaldehyde and do not reuse formaldehyde solution • Obtain new silver
Flecks on gel	• Glassware and plasticware not cleaned properly	• Always clean all equipment thoroughly with 95% ethanol and MilliQ water as residual reagents will always interfere with subsequent electrophoresis

Limitations of the technology

Due to the nature of PCR-based protocols, issues arise because of the high level of sensitivity and amplification of millions of copies of the target sequence. A few molecules of PCR-generated fragments can contaminate samples of subsequent PCR runs and result in false positives. On the other hand, a low copy number of initial target DNA sequences makes the first amplification cycles critical e.g. PCR inhibition can result in false negatives.

False positives can result from cross-amplification of non-target DNA, exogenous DNA from cells/cultures or aerosols, or from contaminating DNA originating from carry-over of previous experiments. A negative control (that contains no template DNA) should be included in all PCR diagnostic tests to identify false positive results.

False negatives can arise for many reasons, including the presence of compounds derived from extracted substrates that inhibit *Taq* polymerase, degradation of the DNA target sequence, or reagent problems. Including a positive control for DNA (a known positive DNA sample) and the presence of an amplified internal standard for each sample can protect against false negatives.

PCR reaction inhibitors

Plant-derived compounds that inhibit the PCR reaction have been well documented e.g. phenolics in banana tissue. This problem is usually easily solved by the further dilution of samples to 1:1000 in water.

Contact information

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

Quarter strength potato dextrose agar (PDA) medium

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

Ingredients per litre of distilled water:

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

Method:

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

PDA medium amended with streptomycin

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

Carnation Leaf Agar (CLA)

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

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

Preparation of Carnation leaves:

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

Potassium chlorate (KPS) medium

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

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

20g sucrose
15g $KClO_3$
20g agar

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

Rice medium

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

- Add approximately 30mL of rice and 90mL of distilled water to each 250mL Erlenmeyer flask.
- Plug with cotton wool and cover with alfoil before steaming for 1 hour on each of two consecutive days eg. in autoclave on free steaming cycle at 103^{0}C.
- Allow medium to cool before inoculation.

Minimal Medium

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

Ingredients per litre of distilled water

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

Method

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

Trace element solution

Ingredients per 95mL of sterile distilled water

5.0g	Citric acid	
5.0g	$ZnSO_4.7H_2O$	Zinc sulphate
1.0g	$Fe(NH_4)_2(SO_4)_2.6H_2O$	Ferrous ammonium sulphate
0.25g	$CuSO_4.5H_2O$	Copper sulphate
50mg	$MnSO_4.H_2O$	Manganous sulphate tetrahydrate
50mg	H_3BO_4	Boric acid
50mg	$NaMoO_4.2H_2O$	Sodium molybdate

Further reading

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