Technical Manual

Prevention and diagnostic of *Fusarium* Wilt (Panama disease) of banana caused by *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (TR4)

Luis Pérez-Vicente PhD.
Senior Plant Pathologist, INISAV, Ministry of Agriculture, Cuba.
Expert Consultant on Fusarium wilt disease of banana

Miguel A. Dita, PhD.
Research Scientist on Plant Pathology, Brazilian Research Agricultural Corporation – EMBRAPA, Brazil.
Expert Consultant on Fusarium wilt disease of banana

Einar Martínez- de la Parte, MSc
Plant Pathologist, INISAV, Ministry of Agriculture, Cuba.
Expert Consultant on Fusarium wilt disease of banana

Prepared for the Regional Workshop on the Diagnosis of Fusarium Wilt (Panama disease) caused by *Fusarium oxysporum* f. sp. *cubense Tropical* Race 4: Mitigating the Threat and Preventing its Spread in the Caribbean

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

May 2014

Technical Manual
Prevention and diagnostic of *Fusarium* Wilt (Panama disease) of banana caused by *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (TR4)

Luis Pérez-Vicente PhD.
Senior Plant Pathologist, INISAV, Ministry of Agriculture, Cuba.
Expert Consultant on Fusarium wilt disease of banana

Miguel A. Dita, PhD.
Research Scientist on Plant Pathology, Brazilian Research Agricultural Corporation – EMBRAPA, Brazil.
Expert Consultant on Fusarium wilt disease of banana

Einar Martínez- de la Parte, MSc
Plant Pathologist, INISAV, Ministry of Agriculture, Cuba.
Expert Consultant on Fusarium wilt disease of banana

Prepared for the Regional Workshop on the Diagnosis of Fusarium Wilt (Panama disease) caused by *Fusarium oxysporum* f. sp. *cubense Tropical* Race 4: Mitigating the Threat and Preventing its Spread in the Caribbean

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

May 2014

INDEX

Introduction:.....4

Fusarium wilt of banana or panama disease by *Fusarium oxysporum* f. Sp. *cubense*: a review on history, symptoms, biology, epidemiology and management.....6

Introduction.....6

Fusarium wilt symptoms.....7

Causal agent: notes on taxonomy and nomenclature 9

Morphology and anatomy 11

Pathogen variability.....12

Biology and ecology.....13

Hosts 14

Geographic distribution.....16

Fusarium wilt impact and damages..... 17

Phytosanitary risks 18

Risk management 19

Erradication of an outbreak of *Foc* TR4 20

Disease management.....22

References 23

PROTOCOLS.....32

Protocol for sampling, transport and storage of samples.....33

Protocol for the isolation of *Fusarium oxysporum f. sp. cubense*.....36

Protocol for determination of vegetative compatibility groups (VCG's).....41

Protocol for Fusarium oxysporum cultures storage.....54

Inoculation of *Fusarium oxysporum* f. sp. *cubense* in banana.....57

Protocols for DNA extraction.....61

Protocols for molecular identification of *Fusarium oxysporum* f. sp. *cubense*.....66

INTRODUCTION

Global banana production is seriously threatened by the re-emergence of a Fusarium Wilt. The disease, caused by the soil-borne fungi *Fusarium oxysporum* f. sp. *cubense* (*Foc*) and also known as "Panama disease", wiped out the Gros Michel banana industry in Central America and the Caribbean, in the mid-twentieth century. The effects of *Foc* Race 1 were overcome by a shift to resistant Cavendish cultivars, which are currently the source of 99% of banana exports.

Unfortunately, a new strain of *Foc* called Tropical race 4 (TR4) has overcome *Foc* resistance in Cavendish clones. Perhaps even more seriously, other banana cultivars such as plantains, cooking bananas and a diverse range of dessert banana varieties (not susceptible to races 1 and 2) are also susceptible to TR4. These local varieties include are mostly grown by smallholder farmers for local consumption and income generation. More than 80% of global banana and plantain production is thought to be based on TR4 susceptible germplasm. This strain of *Foc* has caused epidemics in Cavendish in the tropics different from those less-severe infections previously reported in the sub-tropics. This brings back memories of the devastating damage caused by R1 that led to losses estimated at more than a billion dollars during that era.

The devastating impact of Fusarium wilt on Cavendish plantations in Asia was first observed in Taiwan in the late 1960s, which eventually caused a significant reduction of production to just 10% of former levels, and had caused significant increases in production costs rendering its exports much less competitive. In the early 1990s, thousands of hectares of Indonesian and Malaysian Cavendish commercial plantations failed to establish due to severe epidemics of TR4, causing hundreds of millions of dollars in production losses, including from those cultivars grown by smallholder growers.

The occurrence of TR4 epidemics in Cavendish farms in China (2004) and the Philippines (2008) and more recently in Mozambique (2013) has renewed serious concerns with regard to its destructive potential in the tropics where most bananas for export and local consumption are produced. It now threatens the 400 million-dollar banana export industry of the Philippines, currently the second largest supplier of the global market after Ecuador. It is also spreading and causing damage to the predominantly Cavendish-based banana production in China, which is presently the third largest banana producer of the world after India and Brazil. Preliminary risk analysis indicated that the spread of TR4 to Africa and the Americas was only a question of time. The recent wide TR4 outbreaks reported in Oman, Jordan, Pakistan (under evaluation), and Mozambique during 2013 has proven its threat as a trans-boundary disease of special importance to other major banana producing countries in the world. This trans-boundary phenomenon threatens not only the multimillion-dollar banana export industry, but also millions of people in rural communities, who depend on bananas for their food security and livelihoods.

Planting material, water, soil particles, tools footwear and machineries can efficiently disseminate the pathogen. The fungus can survive in soil for more than 20 years, has a long latent period (it might be detected long time after the introduction) and there is no symptomatic differences among races. Besides this, cultural practices and socioeconomic factors that contribute to Race 1 epidemic still present and would contribute to a TR4 epidemic if the pathogen reaches Latin America and the Caribbean (LAC). Early detection of symptoms in the field and fast laboratory diagnostic is an essential step to either eradication or containment an eventual outbreak.

Aware on the potential threat of TR4 to LAC, MUSALAC (The Latin American Network for Research and Development of *Musa*) and Bioversity International embarked on many awareness campaigns since 2004 about TR4's threat. Raising awareness was stated as a key step to prevent the introduction of this pathogen in LAC, a highly dependent region on banana and plantains.

In 2009, an expert meeting was carried out at the headquarters of the Regional Organisation for Plant and Animal Health (OIRSA) in El Salvador. As a consequence of this meeting, specific legislations on phytosanitary and quarantine were adopted by different countries in the region. In addition, training courses and capacity building workshops on the disease have been developed. These events included relevant aspects on Fusarium wilt such as symptoms recognition, sampling procedures, pathogen diagnosis as well as available options for eradication and disease management. The first training course was carried out in Costa Rica in 2009 and subsequently Cuba (2010), México (2011), Colombia (2011, 2012), Ecuador (2011, 2013), Peru (2011), Nicaragua (2011), Puerto Rico (2013) and Dominican Republic (2013) were capacitated. In all the cases, the National Plant Protection Organizations (NPPOs) and different government agencies were involved.

As a complement to the aforementioned initiatives, a Contingency Plan for an eventual TR4 outbreak was recently developed by OIRSA, with the participation of researchers of Bioversity International, INISAV and an OIRSA expert. This document offers guidelines to NPPOs on how to deal with a putative incursion of TR4 in the region. The document has open access, can be consulted on OIRSA's web site and will be provided to the participants of this Workshop.

Fusarium wilt is currently considered (with some exceptions) a minor disease in LAC. Many of the persons that dealt with the first epidemic of race 1 in the past are no longer alive or are long retired. Therefore, there is a lack of capacity to deal with this disease at different levels and even with the efforts carried out in LAC on awareness and capacity building, the perception of many stakeholders on the potential impact of TR4 remains low.

Bananas and plantains are important to the Caribbean Islands, Guyana and Suriname. The Food and Agriculture Organization (FAO) of the United Nations has been concerned about the impact and recent dissemination of TR4 outside Asia and collaborated with relevant Agricultural Research and Development institutions to create and strengthen capacities to address Fusarium wilt in the Caribbean islands through the development of a Regional Workshop on **Prevention and diagnostic of *Fusarium* wilt of banana caused by *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (TR4)**.

We express thank to FAO, CARDI, The University of West Indies, St. Augustine in Trinidad and Tobago and all who supported the organization of this training workshop.

Workshop Facilitators

FUSARIUM WILT OF BANANA OR PANAMA DISEASE BY *Fusarium oxysporum* f. sp. *cubense*: A REVIEW ON HISTORY, SYMPTOMS, BIOLOGY, EPIDEMIOLOGY AND MANAGEMENT

Luis Pérez-Vicente [(1)] and Miguel A. Dita [(2)]

1) Instituto de Investigaciones de Sanidad Vegetal. Ministerio de Agricultura de Cuba. PO Box 634, 11300, Havana, Cuba (Email: lperezvicente@inisav.cu)
2) Brazilian Research Agricultural Corporation – EMBRAPA, Brazil. Caixa Postal 69, Jaguariúna - São Paulo - Brazil - CEP: 13820-000. (Email: miguel.dita@embrapa.br)

INTRODUCTION

Banana and plantains are essential crops in Latin America and the Caribbean (LAC), where beside economic importance, both have deep cultural roots. Although LAC is not the centre of origin of bananas, it grows 28% of the global production, estimated at 117 million tons in 2009 (Lescot, 2011). Six of the top-ten producing countries are in LAC. The sector is an important source of employment, with 1.28 million ha under cultivation (Dita *et al*., 2013). Bananas are attacked by many pests that affect productivity and sustainable production. Fusarium wilt or Panama disease, together with black Sigatoka (*Mycosphaerella fijiensis*) and bacterial wilt or Moko (caused by *R. solanacearum* race 2) is by far the most important disease of *Musa*.

The history of *Fusarium oxysporum* f. sp. *cubense* (*Foc*) has been reviewed by Stover (1962), Ploetz and Pegg, (2000), Pérez-Vicente (2004) and Ploetz (2005). Stover (1962) provided a detailed list of *Foc* reports by country.

All phylogenetic studies indicate that Fusarium wilt originated in South-East Asia (O'Donnel *et al*., 1998; Ploetz and Pegg, 2000).

The first description of Fusarium wilt in banana and plantains came from Australia; it was done by Bancroft (1876), who was unaware that he was dealing with a disease widely recognized today as one of the most destructive globally in the history of agriculture. At the beginning of the 20th century, the number of reports of the occurrence of this disease increased rapidly, mostly on commercial plantations. Its global distribution has an important anthropogenic component: the infected but symptomless rhizomes or suckers used for planting material were introduced into new areas with conventional plantation material (Ploetz and Pegg, 2000). Although the economic impact of the disease on industry is well known, its impact on subsistence agriculture is not well documented and was most likely not recorded or included in the losses reported during the 1950s. It should be noteworthy that currently approximately 20 million tons (64% of production) of *Musa* are locally consumed in LAC, (Dita *et al*., 2013).

The impact of Fusarium wilt in LAC was negligible after the introduction of Cavendish in 1950. The disease, however, still causes serious losses in subsistence agriculture in Central America, mainly in the variety Gros Michel, which is grown in agroforestry systems or in intercropping with coffee. Particular scenarios are those of Prata type (AAB) varieties and Apple (Silk, AAB), susceptible to the disease, which corresponds for more than 70% of the total (~ 500.000 ha) production in Brazil, and Bluggoe and Pisang awak (ABB) types which are cultivars that are appreciated due to their resistance to stress factors and very adapted to subsistence farming.

Fusarium wilt was reported in 1890 in Central America (Ashby, 1913). The earliest published report of the disease in the West Indies is from Cuba (Smith, 1910), although there are indications of its presence before 1900. There is evidence that the disease was present in Trinidad (Rorer, 1911), Puerto Rico (Fawcett, 1911), and Jamaica since 1903 (Ashby, 1913; Smith 1910). The chronology of spread to other Caribbean Islands is not known, but by 1925, the disease was likely present in most of them (Stover, 1962).

Stover (1962) summarized the possible origins of Fusarium wilt epidemics as follows: a) the wilt probably evolved on several susceptible varieties of edible bananas in the India-Malayan area; b) susceptible variety Silk (Apple AAB) was present in the Caribbean in 1750, while Gros Michel (AAA) was not introduced until early 1800s and was not extensively planted before 1850; the wilt was already present in some areas where Silk was planted even before Gros Michel was introduced; c) during the rapid expansion of Gros Michel in the Caribbean between 1890 and 1910, lands formerly infested with diseased Silk were planted with Gros Michel. It has been estimated that between 1890 and the mid-1950s, more than 40,000 ha of Gros Michel were destroyed (Stover, 1962). The Cavendish cultivars were only reported affected in the subtropics.

Tropical race 4 of *Foc* (VCG 01213) arose in the early 1990s in Malaysia and Indonesia (Masdek *et al*., 2003; Nasdir, 2003) and spread in South East Asia and Australia in less than a decade, causing significant losses and affecting the family income of thousands of workers and farmers in all the affected countries. Its introduction to the Cavendish plantations of America would have an even greater social and economic impact.

FUSARIUM WILT SYMPTOMS AND DAMAGES

Fusarium wilt or Panama disease of banana produces two types of external symptoms: “yellow leaf syndrome” and “green leaf syndrome” (Stover, 1962; Pérez-Vicente, 2004).

Yellow leaf syndrome: this is the most conspicuous and classic symptom of Fusarium wilt on banana. It is characterized by the yellowing border on older leaves that can at times be confused with potassium deficiency, especially in drought and cold environment. The yellowing of the leaves progresses from older to younger leaves (Figure 1A). The leaves collapse gradually, bending at the petiole, commonly close to the midrib and hang down, forming a “skirt” of death leaves around the pseudostem.

Green leaf syndrome: In contrast to the yellow leaf syndrome, the leaves of affected plants in some cultivars eventually remain predominantly green until the petioles bend and collapse (Figure 1C).

In general, younger leaves are the last to show symptoms, frequently remaining unusually erect, giving a bristle-like appearance. Growth does not stop in an infected plant and emerging leaves are of pale colour. The lamina of the emerging leaf can be markedly reduced, shrivelled and distorted. The pseudostem eventually splits longitudinally at the plant base (Figure 1B). There is no evidence of symptoms in the fruits.

A susceptible banana plant infected with Fusarium wilt will rarely recover. While it can occur, the growth is poor and the mother plant produces many infected suckers before it dies.

Internal symptoms are characterized by vascular discoloration: this begins with a yellowing of the root and rhizome vascular tissue, which progresses to develop continuous yellow, red or

brownish strands in the pseudostem. These are very characteristic of the disease (Figure 2). In susceptible cultivars, reddish coloured vessels can also be observed in petioles.

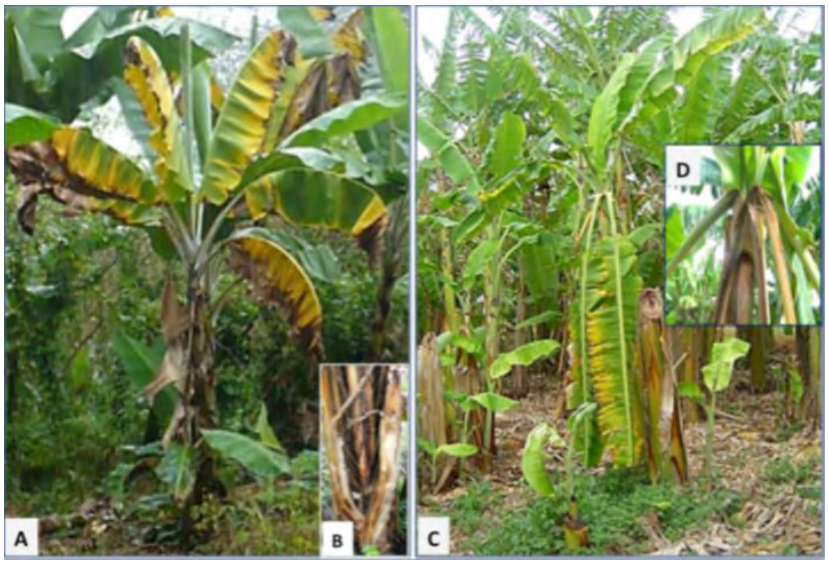

Figure 1. External symptoms of Fusarium wilt. A. Plant showing general yellowing and necrosis of leaves ('yellow leaf syndrome') in advance stage of disease. B. Pseudostem splitting. C. Plant affected by Fusarium wilt with green leaves (´green leaf syndrome´). D. Details of leaf fall down by petiole collapse (Photos: L. Pérez-Vicente and M. A. Dita; adapted from Dita *et al*., 2013).

As the plant dies, fungus grows outside the xylem in the surrounding tissues and develops abundant chlamydospores that remain in soil when plant decomposes. *Foc* colonizes and persists in secondary host roots, including those related to banana and some weed species, although these plants remain asymptomatic in the field.

There are no differences in the symptoms among different *F. oxysporum* f. sp. *cubense* races in *Musa*. Hence, the races cannot be differentiated on the basis of disease-induced symptoms (Stover, 1962; Ploetz, 1990; Ploetz and Pegg, 2000; Dita *et al.,* 2013).

In some cases, Fusarium wilt can co-exist with bacterial wilt (Moko) caused by *R. solanacearum* race 2 and symptoms of both diseases can be misinterpreted. Table 1 presents some criteria for differentiating the symptoms caused by the two diseases.

TABLE 1. Differentiation of symptoms caused by Fusarium wilt and bacterial wilt (Moko)

Fusarium wilt	**Moko**
Disease symptoms progress from older to younger leaves	Symptoms usually progress from younger to older leaves
No symptoms in young growing buds or suckers	Young emerging buds can be distorted and necrotic, eventually dying
No exudations in exposed surfaces	Bacterial ooze can be observed on exposed cut surfaces (roots, pseudostem, rachis, flowers, rhizome etc.)
No development of symptoms in fruits	Internal fruit rot and necrosis develop

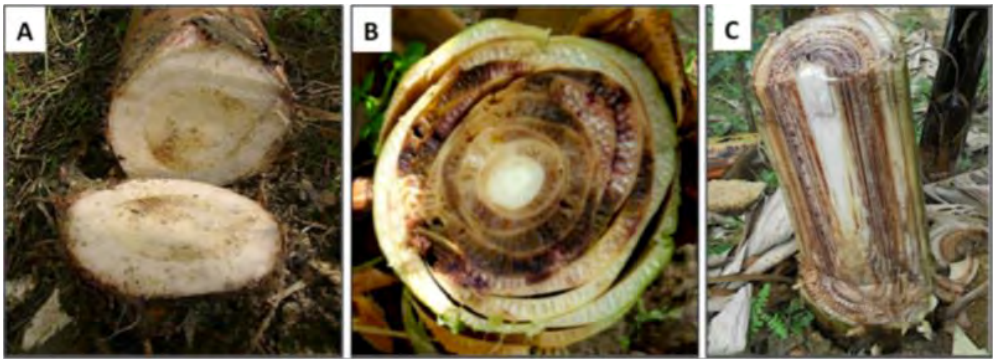

Figure 2. Internal symptoms of Fusarium wilt in banana. A. Transversal section of rhizome showing tissue necrosis. B. Transversal cut of pseudostem showing an advanced necrosis of vascular tissues. C. Longitudinal cut of pseudostem showing necrosis of the vascular strands (Photo: M. A. Dita and L. Pérez-Vicente, adapted from Dita *et al.,* 2013).

CAUSAL AGENT: NOTES ON TAXONOMY AND NOMENCLATURE

The causal agent of Fusarium wilt or Panama disease of banana is the fungus *Fusarium oxysporum* f. sp. *cubense* (E.F. Sm.) W.C. Snyder & H.N. Hansen (*Foc*). Its taxonomic position is as follows:

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

Fusarium oxysporum is a complex of anamorphic, filamentous, morphologically undifferentiated fungal species featuring saprophytes, antagonists and pathogens to plants, animals and humans (O'Donnell and Cigelnick, 1998). In the case of plant pathogens, these mostly cause wilting, damping off and root and organs necrosis and rots. From an agricultural and economical point of view, it is the most important taxon of *Fusarium* (Ploetz, 2006).

Specialization of pathogenicity to plant genera and families gave rise to the *formae speciales* (f. sp.) classification (Snyder and Hansen, 1940). Initially, it was believed that *formae speciales* were specific to one host and henc, the name was taken from the host, e.g. *betae, callistephi, apii, mori*, and about 60 others. This early concept of highly specific pathogenicity led to the establishment of several *formae speciales*, which are merely races of *formae speciales* described in other hosts. Armstrong and Armstrong (1968) demonstrated that *F. oxysporum* f. sp. *batatas* from sweet potato could also attack tobacco. Earlier studies on Fusarium wilt in Central America produced symptoms of Panama disease in Gros Michel banana with inoculation of isolates obtained from *Heliconia* sp. (R.H. Stover, personal communication, 1990). The *formae speciales* designated as *cubense* is applied, based solely on evidence from pathogenicity tests in banana.

Bancroft (1876) isolated for the first time the fungal organism from diseased banana wilt plants. Higgins (1904) noted a fungal association in banana plants suffering of a lethal wilt. Smith in 1908 (Smith, 1910) realized the first isolation of the fungus from Cuban diseased banana plants

and named the species as *Fusarium cubense*. Ashby (1913) gave the first detailed description of the causal agent in culture and Brandes (1919) confirmed Koch postulates, not only in Gros Michel (AAA) and Manzano (Apple, AAB), but also in the cultivar Bluggoe (ABB). Wollenweber and Reinking (1935) recognized that *Fusarium cubense* as a variant of the almost omnipresent *Fusarium oxysporum*. When Snyder and Hansen (1940) developed the *formae speciales* system, all species of the complex *Fusarium oxysporum* that produced wilt symptoms in *Musa* were renamed as *Fusarium oxysporum* f. sp. *cubense* (*Foc*). Phylogenetic studies reveal that *Foc* is an asexual polyphyletic fungus with various strains due to convergent evolution (Bentley et al., 1998; O'Donnell *et al*., 1998; Ploetz, 2006; Fourie *et al*., 2011).

MORPHOLOGY AND ANATOMY

Foc cannot be morphologically distinguished from other *formae speciales* that cause wilting in other hosts and other non-pathogenic *F. oxysporum* endophytes, saprophytes and antagonists (Snyder and Hansen, 1940; Messiaen and Cassini, 1968; Booth, 1972; Leslie and Summerell, 2006).

Foc is an anamorphic fungus without a known sexual stage (teleomorph). The fungus produces macroconidia, microconidia and chlamydospores for reproduction and dispersal. Macroconidia and microconidia are produced in orange structures called sporodochia. Sexual stage (teleomorph) has not been found even in isolates carrying genes Mat 1 and Mat 2 (Fourie *et al*., 2011). Macroconidia (27-55 × 3.3-5.5 μm) are abundant, falcate to erect to almost straight, of thin walls, with 3 to 5 septa (usually 3 septa). Apical cell is attenuated or hook shaped in some isolates. Basal cells are foot shaped. Macroconidia are developed in single phialids in hypha (Figure 3A). Microconidia (5-16 × 2.4-3.5 μm), usually without septa, can be oval, elliptic to kidney shaped and developed abundantly in false heads in short monophialides (Figures 3B and 3C). Chlamydospores (7-11 μm diameter), are abundantly formed in hyphae or in conidia, single or in chains, usually in pairs, but their development can be slower in some isolates (Figure 3D).

On potato-dextrose-agar (PDA) medium, colonies have a variable morphology. Mycelia can be hairy to cottony, spaced or abundant and variable from white, salmon, to pale violet. Black to violet sclerotia can be produced in some isolates.

Fusarium oxysporum usually produces pale violet to dark red color pigments in PDA (Stover, 1962; Ploetz, 1990; Pérez-Vicente *et al*., 2003). Some isolates mutate rapidly from pionnotal (with abundant greasy or brilliant conidia aggregates) to a flat humid mycelia of white-pale yellowish to peach color on a PDA culture (Stover, 1962; Ploetz, 1990).

In modified Komada media (K2), some isolates of TR4 develop laciniated radial colonies, which are not found in isolates of races 1 and 2 (Sun *et al*., 1978; Qi *et al*., 2008). However, this characteristic is not a determinant of a *Foc* TR4 diagnostic.

PATHOGEN VARIABILITY

The term and concept of race have been used to classify isolates of *Foc* since the 1950s (Stover, 1962). *Foc* races have been designated, based on pathogenicity to different reference varieties in field conditions. Four pathogenic races have been described (Stover and Waite, 1960; Stover, 1962; Moore *et al*., 1993; Su *et al*., 1998). Current classification, even when it does not represent

the full variability of the pathogen and is not based on genetic relationships of plant-pathogen interaction, has brought useful and practical information (Pérez-Vicente, 2004, Ploetz, 2006). In Table 2 the races and differential cultivars are presented.

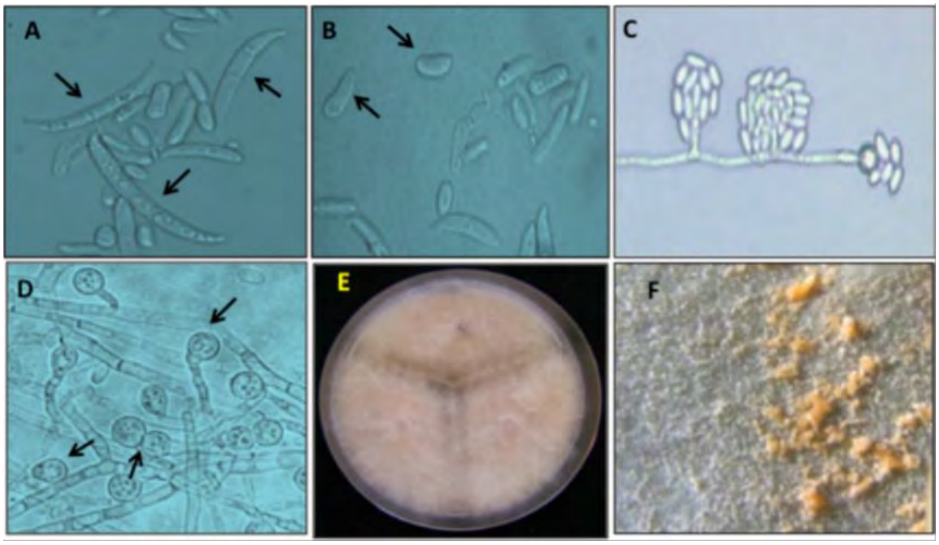

Figure 3. Reproductive structures of *Fusarium oxysporum* f. sp. *cubense.* A. Macroconidia (27 - 55 x 3.3 - 5.5 µm, 4-8 cells straight to lightly falcate and foot shaped basal cells B. Microconidia (5 - 16 x 2.4 - 3.5 µm, 1 or 2 cells from oval to kidney like shape) C. Phialides and microconidia grouped in false heads. D. Chlamydospores (7-11 µm diameter, usually globose developed isolate or in chains). E. *Fusarium oxysporum* f. sp. *cubense* tropical race 4 in PDA media. F. Orange-colored sporodochia developed in a PDA culture media (Photo: M.A. Dita and L. Pérez-Vicente).

TABLE 2. Pathogenic races of *Fusarium oxysporum* f. sp. *cubense*

Cultivars	**Race 1**	**Race 2**	**SR4**	**TR4**
Gros Michel (AAA), Manzano (AAB), Pome (AAB), Latundan Pisang awak (ABB)	+	-	+	+
Bluggoe (ABB)	-	+		+
Cavendish (AAA)			+ **(**in subtropics)	+

Race 1 attacks cultivars Gros Michel (AAA), Manzano/Apple/Latundan (Silk, AAB) and Pome (AAB); race 2 attacks Bluggoe and other cultivars (ABB genome); race 3 previously described as *Foc* (Stover, 1962; Waite and Stover, 1960) attacks Heliconia *spp*., but is no longer considered to belong to race structure of *Foc* (Ploetz, 2005 b). Race 4 is pathogenic to Cavendish and to all banana cultivars susceptible to race 1 and 2. Until the 1990s, all cases of Cavendish infection were related to stressed plants, particularly by temperature, as occurs in the subtropical banana crops in Taiwan (Su *et al*., 1986), Canary Islands, South Africa, and the South of Australia and

Brazil (Ploetz, 1990). These populations were named subtropical race 4 (SR4; Su *et al*., 1986; Grimbeek *et al*., 2001; Ploetz 2005b). With the development of banana plantations in the Equatorial regions of Indonesia and Malaysia in the late 1980s, reports began to emerge of cases of *Foc* populations pathogenic to Cavendish subgroup varieties, that were denominated as tropical race 4 (TR4). TR4 is able to infect Cavendish in both subtropical and tropical conditions plus all those that are affected by race 1 and 2 as well as other cultivars such as 'Pisang Mas' (AA) (Pegg *et al*., 1993; Ploetz, 1994; Ploetz and Pegg, 2000; Ploetz, 2006). It is a genetically distinct race, compared to the previously populations classified as subtropical race 4 (Pegg *et al*., 1994; Bentley *et al*., 1998; Koenig et al., 1997).

Due to a lack of genetic basis for the discrimination of *Foc* into races, heterocompatibility or heterokaryon development capacity between isolates has been used to characterize populations. Twenty-four vegetative compatibility groups (VCGs) have been identified, including populations from all over the world (Ploetz and Correll, 1988; Ploetz, 1990a y Ploetz, 1990b; Brake *et al*., 1990; Leslie, 1990 and 1993; Moore *et al*., 1993; Pegg *et al*. 1993; Hernández *et al*., 1993; Batlle and Pérez, 1999; Ploetz and Pegg, 2000). To date, TR4 belongs to a single group of vegetative compatibility (VCG 01213) while 9 vegetative compatibility groups have been associated with SR4. TR4 with VCG 1216 or 1213/16 complex has also been reported but current evidence indicates that it is the same group as 1213 (Dita *et al.* 2010; R.C. Ploetz & A. Viljoen personal communication, 2009).

Foc isolates are divided into different lineages (at least 8), with closely-related compatible vegetative groups (VCGs), even when are distributed over a wide geographic area. These relationships have been documented by multigenetic studies using RFLPs, AFLPs and RAPDs, electrophoretic karyotyping and phylogenies with multiple genes (Bentley *et al*., 1994; O'Donnell *et al*., 1998; Boehm *et al*., 1994; Fourie *et al*., 2009; Groenwald *et al*., 2006; Koenig *et al*., 1997; Pegg *et al*., 1995), suggesting the pathogen's clonal reproductive strategy.

BIOLOGY AND ECOLOGY

In the absence of live host tissues, the pathogen is able to survive as chlamydospores in previously colonized tissues and in soil where it can persist for long periods, latent or as a endophyte of host weeds. Stover (1972), reported that chlamydospores could survive in the soil for more than 20 years, but there is empirical evidence that this period could be even longer.

Proximity to banana roots induces chlamydospore germination. Banana infection occurs as response to primary and secondary root exudates (Li *et al*., 2009). Major roots and rhizome are not usually affected directly. After germination, hyphae adhere to and directly penetrate the epidermis; mycelia then advance intracellular through the cortex and reach the xylem vessels. Once in, the fungus remains within the xylem, producing microconidia and toxins that move upstream in the plant sap, colonizing neighbouring vessels and producing new fugal structures.

Studies of infection and pathogenesis of banana roots carried out with a green fluorescent protein (GFP)-tagged TR4 isolate (Li *et al*., 2011), showed that: a) potential invasion sites include the epidermal cells of the root caps and the elongation zone as well as natural wounds in the lateral root base; b) the fungus is capable of invading the epidermal cells of the banana roots directly; c) in banana roots, fungal hyphae were able to penetrate cell walls and grow directly inside and outside the cells; and d) fungal spores were produced in the root system and rhizome. In this

study, root exudates from highly-resistant cultivars inhibited the germination and growth of *Foc*. Moderately resistant genotypes reduced spore germination and hyphal growth, whereas the susceptible cultivars did not affect fungal germination and growth.

In artificial inoculation studies with young plants of Apple (Silk, AAB), Costa *et al.* (2013) observed infection through secondary roots, with cortex colonization 5 days after infection (dai), and xylem vessel colonization and chlamydospore development 15 dai. In more tolerant cultivars delayed colonization of tissues and lower chlamydospores formation were observed, indicating host/pathogen recognition and defence response processes and inhibition of fungal colonization and reproduction in the host.

Typical symptoms of wilting are the result of severe water stress due to occlusion of the perforated plates of the xylem vessels as well as by the combination of pathogen activities such as accumulation of mycelia, toxin production and/or host defence response including tylose production, gum and vessel shrinkage due to parenchymatic companion cell growth (Beckman, 1990). When the plant is alive, the pathogen is confined to xylem cells and some companion cells, but once the plant dies, the pathogen invades the parenchyma and sporulates profusely (Ploetz and Pegg, 2000). In conclusion, *Foc* infection is a complex phenomenon requiring a series of highly-regulated processes: 1) recognition of host roots by a signalling process not fully understood; 2) adhesion to the root surface and differentiation of the penetrating hyphae: 3) root-cortex penetration and degradation of physical barriers of the host to infection (e.g. endodermis) to reach the xylem; 4) adaptation to the host cell environment including antifungal compounds; 5) proliferation in the xylem vessels and production of reproductive structures and 6) secretion of virulence determinants, such as little polypeptides or phytotoxins (Di Pietro, *et al*., 2003).

Foc grows between 9 and 38 °C under *in vitro* conditions, with a favourable range of growth between 23 and 27 °C (Pérez *et al*., 2003).

Usually, the disease is more intense during the warmer and wet months of the year, but some factors have a preponderant influence in disease development. The most important factor is the degree of resistance/susceptibility of the *Musa* genotype, cultivar or variety present in the area. The second factor is the *Foc* pathotype present. Finally, other factors such as internal and surface drainage, environmental conditions and soil type have a decisive influence on disease development. There are some soils with physical, chemical and microbiological properties that suppress disease development. These soils were first described in the 1930's in Central America and also in Australia, Canary Islands and South Africa. Among the factors mentioned are pH (infection is lower in soils with pH of 7 or higher); use of nitrates versus ammonia (infection is lower where nitrates are used as nitrogen source instead of ammonium); high calcium content could also induce soil suppressiveness (Peng *et al*., 1999; Nel *et al*., 2006). Forsyth *et al*. (2006) reported endophytic *F. oxysporum* populations with capacity of suppression of *Foc* in greenhouse conditions in Australia. However, the strategy of biocontrol has not been successful when used alone. Pérez-Vicente *et al*. (2003) reported the reduction of *Fusarium* wilt in susceptible cultivars in Cuba by combining the use of *Trichoderma harzianum* A24 with healthy tissue-cultured plants. The use of a bio-organic compost was recently reported as highly efficient to reduce TR4 incidence in China (Shen *et al*. 2013). This compound caused a shift in the soil microbial profile, which favoured disease suppression. However, application of organic matter could not be considered alone as a factor of success for Fusarium wilt management in banana.

HOSTS

a) Primary hosts (cultivated or wild). Under field conditions, TR4 is primarily confined to the genera of *Musa* [*Musa* spp., *Musa textilis*, *Musa acuminata*, *Musa balbisiana* (Stover, 1962; CABI, 2007)] and *Heliconia* [*Heliconia* spp., *H. caribaea*, *H. psittacorum*, *H. mariae* (Stover, 1962; CABI, 2007).

b) Other hosts (cultivate or wild): have been also reported as present in different wild host some of them weeds in banana fields such as:

- *Chloris inflata* = *Chloris barbata* (purpletop chloris) (CABI, 2006; Hennessy *et al*., 2003)
- *Commelina diffusa* (spreading day flower), (Wardlaw, 1972)
- *Ensete ventricosum* (Ensete) (Wardlaw, 1972)
- *Euphorbia heterophylla*,(wild poinsettia) (CABI, 2007; Hennessy *et al.*, 2003)
- *Tridax procumbens* (coat buttons) (CABI, 2007; Hennessy *et al*., 2003).
- *Panicum purpurescens*

In all cases, infection is restricted to the vascular system in root and stem. The epidemiological importance of hosts that do not belong to *Musa* and *Heliconia* has been little documented.

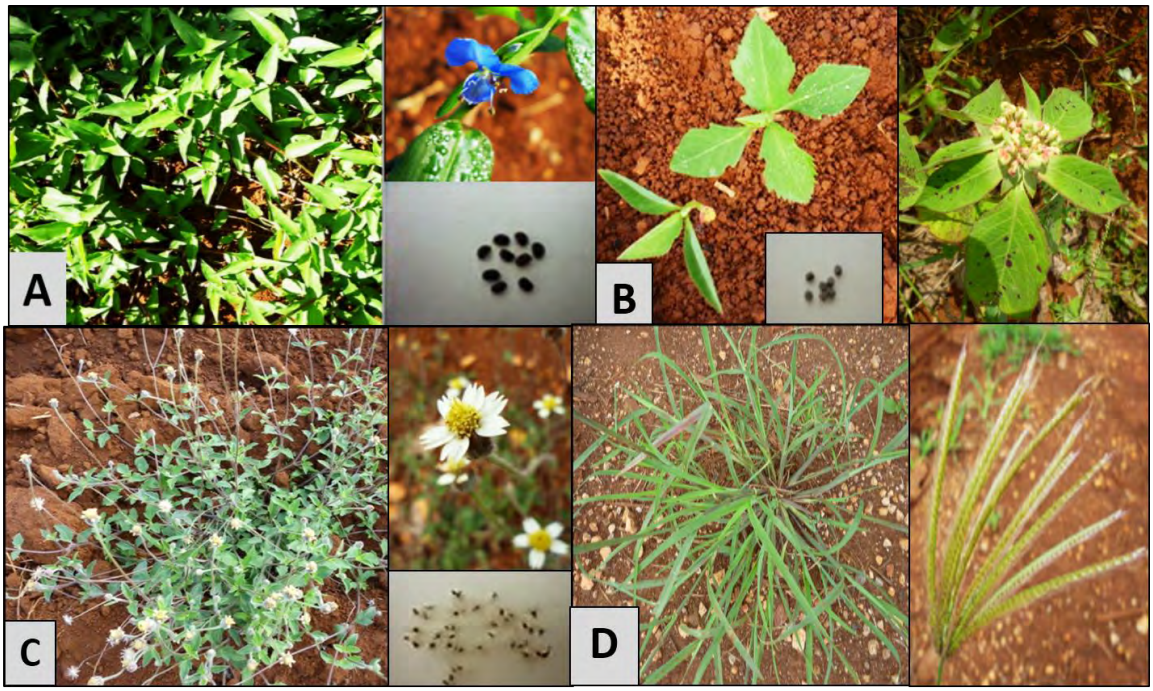

Figure 4. Weed host of *Fusarium oxysporum f sp. cubense (*adapted from Rodríguez *et al.,* 2014; INISAV)*:* A) *Commelina diffusa;* B) *Euphorbia heterophylla*; C) *Tridax procumbens;* D) *Chloris inflata*

GEOGRAPHIC DISTRIBUTION

Races 1 and 2 of *Foc* are spread worldwide (Stover, 1962; Ploetz and Pegg, 2000, Perez-Vicente, 2004). SR4 is present in Taiwan, Canary Island, South Africa and southern Brazil (Ploetz and Pegg, 2000, Perez-Vicente, 2004). Current confirmed distribution of TR4 is Taiwan (Su *et al*., 1986; Ploetz and Pegg, 2000; Hsieh and Ko, 2004), Malaysia (peninsular Malaysia and Sarawak; Ong, 1996), Indonesia (Halmahera, Irian Jaya, Java, Sulawesi, Kalimantan y Sumatra) (Nuthardi e*t al.*, 1994; Pegg *et al*., 1996; Ploetz and Pegg, 2000; Lee *et al.,* 2001; Ploetz, 2005b; O'Neill *et al.,* 2009), Philippines (Molina *et al*., 2008), People´s Republic of China (Guangdong, Guangxi, Hainan, Fujian, Yunnan) (Qi, 2001; Qi *et al*., 2008), Australia (North Territories) (Ploetz and Pegg, 2000), Jordan (García Bastidas *et al.,* 2013) and Mozambique (IITA, 2013). There are informal reports of the presence of TR4 in Oman and Pakistan. A race 1 population (VCG 0124) was reported to attack varieties of Cavendish subgroup in India (Tangavelu and Mustafa, 2010), but there is a lack of official reports on the impact. This caused certain international concern but it is important to indicate that this report cannot be considered as confirmation of the presence of TR4 in India.

DISPERSAL

Results from epidemiological studies indicate that TR4 infected plants occur with a high degree of aggregation throughout a field (Meldrum *et al*. 2013) and a high frequency of infected plant clusters are evidence of plant-to-plant dissemination of the pathogen. However, the presence of isolated plants in the field shows that other mechanisms of dispersion may also exist. *Fusarium oxysporum* f. sp. *cubense* can be dispersed through planting material and contaminated plant parts, soil and water. It is hypothesized that winds accompanied by rains could disperse *Foc*, but there are no studies that confirm this. Sporodochia formation (conidial masses) of TR4 has been confirmed in the greenhouse (Dita, unpublished), but have not yet been observed or reported under field conditions. In dry places where wind can carry contaminated dust particles, wind could also be a dissemination vehicle of *Foc*. In Caribbean countries frequently affected by hurricanes, strong winds and heavy rains causing flooding could be considered as an important vehicle for *Foc* dissemination.

There is also the possibility of dissemination by insect vectors, especially the banana weevil borer *Cosmopolites sordidus* (Coleoptera: Curculionidae). This insect is found wherever banana is grown and it moves through the soil, feeding on the roots and corm of the plants (Gold *et al.* 2001). Meldrum *et al.* (2013) confirm by PCR the presence of TR4 in exoskeleton of *C. sordidus* in banana fields in Australia.

Panama disease dispersal is rapid on susceptible banana cultivars. When Fusarium wilt (race 1) was by first observed in Jamaica in the early 1900s, 70% of 14,000 'Gros Michel' plants developed the disease within the first 2 years of planting (Cousins and Sutherland 1930).

Dispersal by plant material

Local (on-farm) or long distance (other farms, regions, or countries) *Foc* dispersal occurs mainly by the movement and planting of asymptomatic but already-infected suckers. According to

Hwang and Ko (2004), between 30 and 40% of the suckers obtained from a TR4-infected Cavendish banana rhizome are infected. However, it is possible that 100% of the suckers of a diseased plant are infected and are a potential source of pathogen dissemination. TR4 and all *Foc* races in general can also be disseminated via infected propagating material of other hosts (e.g. *Heliconia* spp. and weeds).

Pseudostem tissues and leaves of infected plants can also be ways of dispersal of *Foc*. Banana and plantain leaves and pseudostem are frequently used for wrapping or packing banana that are transported from place to place.

Dispersal through the soil

Fusarium oxysporum f. sp. *cubense* is dispersed on contaminated soil, by natural or artificial means. Natural means include soil drift due to wind or rain (erosion). Artificial means are related to soil adhering to agricultural implements, containers, tools, animals, footwear, clothes, use of soil as a substrate for nurseries.

Dispersal through water

Fusarium oxysporum f. sp. *cubense* is be efficiently dispersed through irrigation, rainfall or surface drainage waters after rainfall as well as in river streams between disease-infested and disease-free areas. If a *Foc* contaminated water reservoir is used to irrigate a disease-free area, the disease could disperse rapidly and efficiently.

As yet, there is no scientific evidence of dispersal of *Foc* in banana fruits.

FUSARIUM WILT DAMAGE AND IMPACT

The first global trade of banana relied almost exclusively on Gros Michel (Simmonds, 1960; Stover, 1962). The downfall of Gros Michel was its extreme susceptibility to race 1. Panama disease was first reported in Australia, but became most important in monocultures of Gros Michel used by exporters in the Western Hemisphere (Stover, 1962) (Panama was one of the first countries to experience major epidemics). It caused staggering losses in the banana trade before the conversion to Cavendish. Between 1940 and 1960, 30,000 ha were lost in Honduras and in a decade complete losses were recorded in operations of 4,000 ha in Suriname and 6,000 ha in Costa Rica (Ploetz, 2005). Losses caused by race 1 in Gros Michel during the first half of the 20th century were estimated at USD 2.3 billion (Ploetz, 2005) to the export companies alone and was the cause of the change in the banana export industry to Cavendish cultivars resistant to race 1 and race 2. Besides, race 1 wiped out the economic cultivation of Apple banana (Silk, Manzano) and Gros Michel in Cuba and is the main phytosanitary problem for Prata-types cultivars in Brazil. Race 1 is still present and causing damage wherever Gros Michel and Apple cultivars are cropped in America, grown alone or mixed with cacao, coffee, trees and/or other plants.

TR 4 impact in the affected countries has been as follows:

- Taiwan. The oldest international banana trade in Asia began in the early 1900s in Taiwan and the industry began to decline in the early 1970s due to high labour cost and competition from foreign producers; hence, by the 1990s only around 5,000 ha remained in production (Hwang and Ko, 2004). In the 1960s, Taiwan exported 60,000, 12-kg boxes of Giant Cavendish. The

first incidence of Fusarium wilt was reported on Cavendish cultivars in 1967 in the main banana producing area of southern Taiwan (Su *et al.* in 1977, cited by Hwang and Ko, 2004). The disease dispersed rapidly and the number of infected plants increased 5,536 in three years. In 1976, 1,200 ha were infected representing approximately 500,000 banana plants (Hwang and Ko, 2004). In 1989, it was determined that the most frequent populations in epidemics belonged to VCG 1213, confirming the presence of TR4 (Molina, 2009). Due to cold temperatures in winter and typhoons, banana plantations have to be replanted every year. Due of TR4, new lands need to be considered for planting every year.

- Peninsular Malaysia. TR4 was detected in a 392-ha Cavendish farm in 1992 and 4 years later had dispersed to 30% of plants (Meng *et al.,* 2001). Two years later, the epidemic in established plantations reached 50 plants / ha / month (Ong, 1996).
- Indonesia. In early 1990s, commercial companies such as Chiquita and Del Monte tried to establish Cavendish plantations in Indonesia and Malaysia, to take advantage of fertile soils, favourable climate and low labour costs to supply the growing markets of East Asia and Mid East. Many of these farms were previously forest areas. Within two years of establishment, these farms were severely infected with TR4. More than 8 million plants were destroyed annually and plantations had to be abandoned with annual losses over USD 75 million. This negatively affected the family income of thousands of workers and farmers (Nasdir, 2003). The pathogen dispersed from one to other islands in planting material moved by growers (Molina, 2009) causing a dramatic reduction of the production area by half in only three years (2005-2008). The government estimated that the average speed of disease dissemination in Sumatra was 100-km/ year (Plant Protection Department, 2007).
- Australia. TR4 was identified in North Australia between 1997 and 1999 and caused significant damage, limiting commercial production of the crop (Molina, 2009).
- China. The pathogen was probably introduced from Taiwan in planting material obtained from infected areas. The disease attacked >65,000 plants and is still dispersing along the Pearl River (Molina, 2009). In 2006, studies showed that TR4 had infected more than 6,700 ha. TR4 has also seriously attacked the popular local variety 'Fenjiao' (ABB, subgroup Pisang awak). It was largely concentrated in plantations in the delta of Pear River in Guangdong. (Molina, 2009). Today TR4 is distributed in Guangdong, Guangxi, Hainan, Fujian and Yunnan (Qi, 2001; Qi *et al*., 2008) affecting more than 40,000 ha (Yi *et al*., 2012).
- The Philippines. The disease was suspected to be present in the country since the 1970s, but TR4 was only confirmed in 2008. Its incidence in the surveyed farms increased from 700 cases in 2005 to 15,000 in 2007 (Molina *et al*., 2008). Large companies currently manage the disease following the protocol established for bacterial wilt (Moko, *Ralstonia solanacearum*) management, which is based on quarantine, sanitation, soil disinfection and fallows (Molina, 2009).
    - Jordan. TR4 was only confirmed in 2013 even though it could have been present since 2006 (García-Bastides *et al.* 2013). Currently, 80% of the Jordan Valley production area is affected and 20-80% of the plants are affected in the different farms.
    - Mozambique. TR4 was discovered in early 2013 and reported at the end of the year in some farms. Data of the disease impact is not yet available.

PHYTOSANITARY RISK

The main pathways for transmission of TR4 are living or dead host plants, infected plant parts and soil from infected fields, carried out of the field by persons, machinery and animals or mechanically as contaminant on articles.

Once the disease is introduced, secondary dispersal from the outbreak site could take place with soil movement with transport and irrigation water, drainage, or other water fluvial resources from regions where the disease is present to disease-free sites. It is important to emphasize that the higher risk of dispersal is via propagation material that has historically been the main dispersal mechanism.

Introduction of TR4 in any country could signify substitution of most popular banana genotypes by others of lower acceptance and introduction of new paradigms of banana production requiring different and more costly cropping practices.

RISK MANAGEMENT

Risk management is carried out via application of (1) phytosanitary measures to prevent the entry of TR4 into the country and (2) eradication-confinement or suppression-contention measures in case of an incursion.

The first step should be establishing an absolute prohibition of the entry of plant or plant parts from sites where TR4 is present. At entry points, TR4 presence can be detected by carrying out inspection of plants with wilt and vessel-necrosis symptoms or plant parts with necrotic symptoms in roots and rhizome. Such plants should be seized and sent to a diagnostic laboratory Presence of symptoms and damage (as described earlier) should be verified during inspection. Once the presence of any organism of the TR4 complex is confirmed, the material should be confiscated and immediately destroyed.

Some prevention measures are:

- TR4 should be included in the national list of quarantine pests and of obligatory declaration;
- Prohibit importation of *Musa* plants or plantlet as well as of other hosts from countries where TR4 is present. Imports of *Musa* germplasm or of plants for propagation should use the route of intermediate quarantine stations. Those materials should be adequately indexed and identified as free of TR4;
- Capacity-building and sensitization campaigns among personnel that in the line of duty, visit fields in countries where TR4 is present. This should include measures to take after field visits to prevent transfer of soil or plant parts in clothes, shoes, and/or work equipment.
- Carry out surveillance (e.g. surveys) for early detection of potential incursions of the disease.
- Determination by NPPOs reference laboratories where TR4 can be diagnosed;
- Capacity building in symptoms, sampling, treatment and manipulation of samples and diagnostics (in case laboratories capable of carrying out the diagnostic exist in the country).

- A list of national and international experts that can contribute to disease diagnosis and management of an eventual outbreak of TR4.

Once an outbreak or incursion is detected, some risk management measures are as follows:

- If TR4 is confirmed in Cavendish banana or plantain (AAB): proceed to establish quarantine in the outbreak area and delimit control area; restrict personnel, equipment and animal access; collect samples to confirm diagnosis and eliminate symptomatic plants. In the case of plants belonging to other varieties (that could be susceptible to other races also), it is advisable to wait for confirmation of TR4 diagnosis;
- Survey of personnel, equipment, animals, plant parts and soil movement from and to TR4 outbreak site.
- Record of epidemiological information to try to establish the possible initial incursion (origin) of TR4.
- After diagnostic confirmation, eradication should be carried out by destruction of affected plants and all plants in the surrounding 7.5 m radius. These should be eliminated by fire. Plants should be cut in pieces of approximately 60 -80 cm long and rhizome and roots should be extracted. All weeds in area should be cut and all material covered with a plastic shield to fumigate with methyl bromide (MB) to disinfect soil and plant materials. For the use of Methyl Bromide, national legislation needs to be taken into consideration.
- All tools used in diseased plant elimination, as well as shoes, equipment and wheels of equipment that access the infected area must be disinfected to avoid secondary dispersal. An intermediate disinfection of tools between infected and suspect plants should also be carried out during plant elimination activity. Among the active ingredients for disinfection are formaldehyde and quaternary ammonium formulations (Nel *et al,* 2006; Medrum, *et al*., 2013).
- All outbreak areas should be kept under quarantine for one-and-a-half years. Periodic surveys should be carried out to confirm if there are no new plants or re-growths to verify or declare disease eradication. Any production involving soil movement or TR4 host plants should be prohibited. The area should be prohibited for any crop that needs soil movement or establishment of nurseries.
- In case an outbreak eradication/confinement is unsuccessful, procedures of suppression / containment should be established, which in essence are the same, only changing the radius of plant elimination and use of dazomet instead Methyl Bromide.

ERADICATION OF AN OUTBREAK OF FOC TR4

To date, there are no reports on eradication of outbreaks of TR4 or other *Foc* races. The first step is to evaluate the technical feasibility of the eradication of TR4 incursion. For this, an important aspect to consider is the origin of outbreak and its history. If the outbreak is an isolated event of introduction in one site, the probability of success of eradication-confinement is higher than in case of multiple introductions or dispersion after introduction. The alternative to adoption of eradication-confinement, based on long-term suppression-containment measures can always have

a higher socio-economic impact. It is also important to take into consideration that *Foc* produces resistant chlamydospores that survive for a long time and that the alternative program of suppression-containment would have to be implemented. Another aspect to consider is information on proven efficacy of the measures to apply and the complexity of operations in terms of their capacity requirements (Dita *et al*., 2013).

Some factors to consider to deciding implementation of eradication program are:

a) How soon was the pathogen detected and diagnosed;

b) Availability of reliable information on the introduction date/period;

c) Extent and characteristic of the outbreak (banana monoculture or mixed crop with coffee or other crops that also require adoption of regulatory and control measures);

d) If it is an isolated case or secondary dispersal has probably already occurred;

e) Support expected from farmers;

f) Level of relative isolation of the outbreak area to implement restriction measures to access area;

g) Topography of the area that could enable superficial drainage and pathogen movement;

h) Possibility to implement safe movement of germplasm;

i) Possibility to use the area for other objectives;

Dita *et al*., (2013) developed a contingency plan to eradicate a potential TR4 outbreak, which establishes the organization and procedures to be followed in case of an outbreak, which are available on the website of OIRSA[1]. Important remarks on the procedures are:

1. Irrespective of the pathway of introduction, when a TR4 outbreak is found, it is very probable that a long time period has already passed between the introduction and its detection, due to the long incubation period of disease. The success of eradication-confinement strategy will be largely determined if introduction have occurred in one or several places, and if all outbreaks have been detected. It is desirable to destroy infected plants that show symptoms and have a preliminary diagnostic; this includes other host species such as weeds (see list of hosts).
2. After taking samples of symptomatic plants, it is necessary to confirm TR4 using the procedures described in the Protocols section of this manual.

- A determination of preliminary area or areas under quarantine;
- Destruction of infected plants;
- Establishment of a secured or protected area, the dimensions of which are determined after a delimitation of outbreak dimensions

To establish security area, the following aspects should be taken into account: (1) the distribution of water surface drainages and internal flux (inside the soil) with possible drift or movement of infective fungal structures; (2) soil topography and the possibility that the fungus comes in

[1] http://www.oirsa.org/aplicaciones/subidoarchivos/BibliotecaVirtual/PlandecontingenciacontraFocR4TOIRSA.pdf

contact with cropped or wild hosts. It is also necessary to consider if the outbreak has occurred upstream or downstream of a water source and the direction of currents.

The outbreak borders can be delimited until the limits of infected host (cropped or wild) are detected or until there was a high probability of pathogen dispersal.

Important facts to consider are the movement of workers, equipment and animals from or to infected fields. Quarantine restriction to access the outbreak site should be established in order to restrict secondary distribution of pathogen: a single access way and disinfection points for shoes (footbaths), tools, wheels and machineries. As personnel in charge of sampling and elimination of plants can become important vectors of the disease, strict biosecurity measures should be adopted to prevent pathogen dispersion during and after their activities.

DISEASE MANAGEMENT

In countries were the disease is present, phytosanitary management (suppression-containment activities) of *Foc* has been implemented through a similar protocol to that used to the bacterial wilt (Moko, caused by *Ralstonia solanacearum*) based on maintenance more or less permanent quarantine measures and limitation of area access, use of soil fumigants, sanitation of infected and neighbouring plants, use of soil fumigants and replanting.

Most of measures are not available to low-resource farmers. On the other hand, in the case of Taiwan, cultivars with partial resistance to TR4 can only be used in high-density plantations for a limited number of cycles that requires significant investment to replant at short periods.

The most efficient method of control is the use of resistant varieties. Due to the impact of planting material on disease dissemination, use of healthy planting material is a key component of any management system of Fusarium wilt of banana. Development of certified healthy planting material program that can be accessed by growers is important, together with adoption of other control measures. Chemical control has in general been of poor efficacy and limited success. Even though many reports on biological control alternatives have been published, most of them are related to *in vitro* and glasshouse studies and as yet, there are no biocontrol agents with proven efficacy in the field to be considered as part of management programs for TR4. Pérez-Vicente *et al*., (2003; 2009) reported the efficacy of a combination of *Trichoderma harzianum* A24 isolate applications at planting and every three months together with the use of healthy tissue culture planting material for race 2 management in conducible infected soils. These procedures allow production for more than 5 years in fields where the disease had previously destroyed Burro CEMSA plantations.

Phytosanitary management of TR4 in affected areas is directed to inoculum reduction, eliminating infected plants and delimitation of infected areas. Additionally, in Taiwan Giant Cavendish tissue culture mutants with a certain tolerance to TR4 are being cultivated in annual planting or short cycle systems (Hwang and Ko, 2004; Gus Molina, personal communication)

Crop rotation with *Foc* non-host plants has been used in the purpose to reduce *Foc* population in soil. Banana rotation with sugarcane+ fallow reduced disease incidence by 48% (Sequeira, 1962); with rice (*Oryza sativa)* disease reduction was not long-lasting (Hwang *et al*., 1985). Cassava (*Manihot esculentum*) is used by small growers in Indonesia and the Phillippines to reduce TR4 in soil (Molina, 2009; Buddenhaguen 2009). Chinese leek (*Allium tuberosum*), used in rotation

with banana, reduced TR4 incidence and severity index in Cavendish and Guangfen (AAA) cultivars by 58% and 62% respectively in China (Huang *et al*., 2012).

Stover (1962) gave a detailed summary of the results obtained from the studies of chemical and physical soil properties and its influence on incidence of Fusarium wilt of banana. There are reports on the influence of different mineral nutrition elements and pH on Fusarium wilt development in soil that can be considered as part of suppression-containment measures:

Nitrogen. Use of NO3-based fertilizers reduces disease development (Huber and Watson, 1974), whereas an increase in NH_4 favours disease development (Dominguez *et al.,* 1995). There is consensus among growers that urea use notably favours Fusarium wilt of banana.

Phosphorus. High P content in soil reduces Fusarium wilt incidence (Woltz and Jones, 1981).

Potassium. Dominguez *et al*., (2001 and 2010) reported that edaphic factors, such as structural stability of soil aggregates (200–2000 μm) and available Fe (Fe-DTPA) in soil in areas with high levels of banana disease expression, might be affected by a strong selectivity for K. Therefore, they conclude that soil-K status may exert an indirect influence on the biological mechanisms of disease expression.

Calcium. High lime (CaO) content (175-280 ppm) increments disease suppression in the soil (Volk and Gallatin in1962 cited by Stover, 1962; Höper *et al.,* 1995). High CaO content reduces chlamydospores germination (Peng *et al*., 1999). Adding calcium carbonate (CaCO3), calcium hydroxide [Ca(OH)2], calcium sulphate (CaSO4) or iron chelates such as Fe-EDDHA to the soil, reduces *Foc* germination and disease severity by one-third to one-half in soils. Smaller Ca amounts had the greatest effect and were insufficient to change soil pH (Peng *et al*., 1999)

Iron. Reduction of iron availability increase soil suppression (Scher and Baker, 1982) as well as reduce chlamydospore germination (Peng *et al*., 1999).

Manganese and Zinc. Deficiencies of manganese and zinc cause reduction of *F. oxysporum* disease in tomato (Jones and Wolf 1967; Jones *et al*., 1989).

Soil pH. pH values close to 7 are less optimal to *Fusarium* wilts (Dominguez *et al*., 2001). Suppressive soils in general have higher pH values. When pH is reduced below 6.5, there is an increase in the Fusarium wilt disease (Rishbeth, 1957; Reinking and Manns 1932b Peng *et al*., 1999). Lower pH values were significantly correlated with higher incidence of Fusarium wilt in Peru (Roman, 2012).

Soil texture. Soils with a light texture are more favourable for Fusarium wilt disease than soils with heavy clay texture (Stover, 1962).

An important measure in *Foc* management is the control of root and rhizome pests. Medrum *et al*., (2013) reported that the black weevil *Cosmopolites sordidus* can carry TR4 spores in its exoskeleton. Therefore, plans for managing *Foc* on plantations should take into consideration the presence of *C. sordidus* in the field and its possible role as a vector for *Foc.* It has been suggested that nematodes may also play an important role on Fusarium wilt of banana. While the co-infection of *Radopholus similis* with *Foc* in a susceptible cultivar Gros Michel had no influence on disease severity when compared with plants only inoculated with *Foc*, co-inoculated plants showed a significant root weight reduction (Chaves, 2014). Therefore, nematode control should be also taken into account mainly in Cavendish, which is highly susceptible to *R.similis*.

REFERENCES

Ashby, S.F. 1913. Banana disease in Jamaica. Science 31: 754-755.

Amstrong, G.M. and Amstrong, J.K., 1958. The Fusarium wilt complex as related to the sweet potato. *Plant Disease Rptr.* 42: 1319-1329.

Bancroft, J., 1876. Report of the board appointed to inquire into the cause of disease affecting livestock and plants. Queensland, 1876. *Votes and Proceedings* (3): 1011-1038.

Beckman, C.H. 1990. Host responses to infection. In: Ploetz, RC. (ed.) Fusarium wilt of banana. Minnesota, US. APS. p 93-105.

Bentley, S., Pegg, K., Dale, J.L. 1994. Optimization of RAPD-PCR fingerprinting to analyse genetic variation within populations of Fusarium oxysporum f.sp. cubense. *Phytopathology* 142: 64–78.

Bentley, S., Pegg, K.G., Moore, N.Y., Davis, R.D. and Buddenhagen, I.W. 1998. Genetic variation among vegetative compatibility groups of *Fusarium oxysporum* f. sp. *cubense* analysed by DNA fingerprinting. *Phytopathology* 88: 1283–1293.

Boehm, E.W.A., Ploetz, R.C. and Kistler, H.C. 1994. Statistical analysis of electropherickaryotype variation among vegetative compatibility groups of *Fusarium oxysporum* f.sp. *cubense Molecular Plant-Microbe Interactions* 7: 196–207.

Booth, C. 1971. The Genus *Fusarium*. Surrey, UK. CMI. 58 p.

Brake, V.M., Pegg, K.G., Irwin, J.A.G. and Langdon, P.W. 1990. Vegetative compatibility groups within Australian populations of *F. oxysporum* f. sp. *cubense* the cause of Fusarium wilt of banana. *Agricultural Research* 41: 863-870.

Brandes, E.W. 1919. Banana wilt. *Phytopathology* 9: 339-389.

Buddenhagen, I. 2009. Understanding strain diversity in *Fusarium oxysporum* f. sp. *cubense* and history of introduction of 'tropical race 4' to better manage banana production. ISHS Acta Horticulturae 828: International Symposium on Recent Advances in Banana Crop Protection for Sustainable Production and Improved Livelihoods.

CABI (CAB International). 2007. Crop Protection Compendium. Wallingford, UK.

Carlier, J., De Waele, D. and Escalant, J. 2002. Evaluación global de la resistencia de los bananos al marchitamiento por *Fusarium*, enfermedades de las manchas foliares causadas por *Mycosphaerella* y nemátodos. En Vezina, A. and Picq, C. (eds.). Guías Técnicas del INIBAP no. 6. Montpellier, FR. 67 p.

Costa, J.L., Haddad, F., Rossi, M.L., Martins, F.M., Amorim, E.P., de Figueira, A.V.O. 2013. Histopathology interaction of *Musa* spp. x *Fusarium oxysporum* f. sp. *cubense.* Proceedings, XX International Meeting ACORBAT. Sept 9 -13., Fortaleza, CE, Brazil. Pp. 267.

Cousins, H.H. and Sutherland, J.B. 1930. Plant diseases and pests. Report of the Secretary of the Advisory Committee on the Banana Industry. In: Annual Report Department of Science and Agriculture Jamaica for the year ending 31st December, 1929. Pp: 15–19

Davis, R., Moore, N,Y., Bentley, S., Gunua, T.H. and Rahamma, S. 2000. Further records of *Fusarium oxysporum* f. sp. *cubense* from New Guinea. *Australasian Plant Pathology* 29: 224.

Di Pietro, A., Madrid, M., Caracuel, Z., Delgado-Jarana, J. and Roncero, M.I., 2003. *Fusarium oxysporum*: exploring the molecular arsenal of vascular wilt fungus. *Molecular Plant Pathology* 4: 321-325.

Dita, M.A., Waalwijk, C., Buddenhaguen, I.W., Souza, M.T. and Kema, G.H.J. 2010. A molecular diagnosis for tropical race 4 of the banana Fusarium wilt pathogen. *Plant Pathology* 59: 348–357.

Dita, MA. 2011. Corrigendum. Plant Pathology 60: 384.

Dita, M.A., Garming, H., Bergh I. Van den, Staver, C. and Lescot, T. 2013. Banana in Latin America and the Caribbean: current State, challenges and perspectives. Proc. Int. ISHS-ProMusa Symp. on Bananas and Plantains: Towards Sustainable Global Production and Improved Uses Eds.: I. Van den Bergh et al. *Acta Hort. 986*, ISHS 2013: 365-380.

Dita, M.A., Echegoyen, P.E. and Pérez-Vicente, L. 2013. Plan de contingencia ante un brote de la raza 4 tropical de *Fusarium oxysporum* f. sp. *cubense* en un país de la región del OIRSA. Organismo Internacional Regional de Sanidad Agropecuaria – OIRSA. 155pp. San Salvador, El Salvador, julio de 2013.

Http://www.oirsa.org/aplicaciones/subidoarchivos/BibliotecaVirtual/PlandecontingenciacontraFocR4TOIRSA.pdf.

Domínguez-Hernández, J.D., Negrín-Medina, M.A. and Rodríguez-Hernández, C.M. 2010. Potassium selectivity in transported volcanic soils (sorribas) under banana cultivation in relation to banana-wilt expression caused by *Fusarium oxysporum* f. sp. *cubense. Soil Science and Plant Analysis*, 41:1674–1692.

Domínguez, J.; Negrín, M.A.; Rodríguez, CM. 2001. Aggregate water stability, particle size and soil solution properties in conducive and suppressive soils to Fusarium wilt of banana from Canary Islands. Soil Biology and Biochemistry 33; 349-455.

Domínguez, J.; Rodríguez, C.M, Hernández-Moreno, J.M. (1995). Iron and *Fusarium* wilts in banana crops and Andic soils. In: Abadia, J. (Ed.), Iron nutrition in soils and plants Kluwer Academic, Dordrecht, The Netherlands, pp 255-258.

Fawcett, G.L. 1911. Report of the plant pathologist. Ann. Rep. Porto Rico Exp. Stat. : 35-36

Fourie, G., Steenkamp, E.T., Gordon, T.R. and Viljoen, A. 2009. Evolutionary relationships among the vegetative compatibility groups of *Fusarium oxysporum* f. sp. *cubense*. *Applied and Environmental Microbiology* 75: 4770–4781.

Fourie, G., Steenkamp, E.T., Ploetz, R.C., Gordon, T.R. and Viljoen, A. 2011. Current status of the taxonomic position of *Fusarium oxysporum* formae speciale *cubense* within the *Fusarium oxysporum* complex. *Infection, Genetics and Evolution* 11: 533–542.

Forsyth, L., Smith, L., and Aitken, E. 2006. Identification and characterization of non-pathogenic *Fusarium oxysporum* capable of increasing and decreasing *Fusarium* wilt severity. *Mycological Research* 110: 929-935.

García-Bastidas, F. Ordóñez, N., Konkol, J., Al-Qasim, M., Naser, Z., Abdelwali, M., Waalwijk, C., Ploetz, R.C. and Kema, G.H.J. 2013. First Report of *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 associated with Panama Disease of banana outside Southeast Asia. *Plant Disease*. Published online. http://dx.doi.org/10.1094/PDIS-09-13-0954-PDN.

Grimbeek, E.J., Viljoen, A., and Bentley, S. 2001. First occurrence of Panama disease in two banana growing areas of South Africa. *Plant Disease* 85: 1211.

Groenewald, S., Van Den Berg, N., Marasas, W.F.O., and Viljoen, A. 2006. The application of high throughput AFLPs in assessing genetic diversity in *Fusarium oxysporum* f. sp. *cubense*. *Mycological Research* 110: 297–305.

Gold C.S., Pena J.E., Karamura, E.B. 2001. Biology and integrated pest management for the banana weevil *Cosmopolites sordidus* (Germar) (Coleoptera: Curculionidae). *Integrated Pest Management Review* 6 (2):79–155.

Higgins, J.E. 1904. The banana in Hawaii. Hawaii, US. *Hawaii Agricultural Experiment Station,* University of Hawaii. 51 p. (Bulletin No. 7).

Hennessy, C., Walduck, G., Daly, A. and Padovan, A. 2005. Weed hosts of *Fusarium oxysporum* f. sp. *cubense* tropical race 4 in northern Australia. *Australasian Plant Pathology* 34: 115-117.

Hernandez, J.M., Freitas. G., Ploetz, R.C. and Kendricks, C. 1993. Fusarial wilt of banana in the Canary Islands with some data regarding the Madeira Archipelago. *In*: Hwang, S.C., Ploetz, R.C., Lee, S.W., and Roa, V.N. (eds.). Proceedings: International Symposium on Recent Developments in Banana Cultivation Technology. 1992. Pingtung, TW. Taiwan Research Institute. Pp. 247-254.

Huang, Y. H., Wang, R.C., Li, C.H., Zuo, C.W., Wei, Y.R., Zhang, L. and Yi, G.J. 2012. Control of *Fusarium* wilt in banana with Chinese leek. *European Journal of Plant Pathology* 134: 87–95.

Höper, H., Steinberg, C. and Alabouvette, C. 1995. Involvement of clay type and pH in the mechanism of soil suppressivness to *Fusarium* wilt of flax. *Soil biology and Biochemistry* 27; 955-967.

Huber, D.M. and Watson, R.D. 1974. Nitrogen form and plant disease. *Annual Review of Phytopathology* 12; 139-165.

Hwang. S.C. and Ko, W.H. 2004. Cavendish banana cultivars resistant to Fusarium wilt acquired through somaclonal variation in Taiwan. *Plant Disease* 88: 580-588.

IITA, 2013. New banana disease to Africa found in Mozambique. Joint statement issued by the Mozambique Department of Agriculture, Matanuska, IITA, Stellenbosch University and Bioversity International. Press release.
http://www.iita.org/2013-press-releases/-/asset_publisher/CxA7/content/new-banana-disease-to-africa-found-in-mozambique?redirect=%2F2013-press-releases&utm_source=dlvr.it&utm_medium=twitter#.Upv_QdJQKSo.

Jones, J.P. and Woltz, S.S. 1967. Fusarium wilt (race 2) of tomato: effect of lime and micronutrient soil amendments on disease development. *Plant Dis. Reptr*. 51: 645-648.

Jones, J.P., Engelhard, A.W. and Woltz, S.S. 1989. Management of Fusarium wilt of vegetables and ornamentals by macro- and microelements. In: Soilborne Plant Pathogens: Management of Diseases with Macro and Microelements. A.W. Engelhard, (ed.) American Phytopathological Society, St. Paul, MN pp 18-32.

Katan, K. and Di Primo, P. 1999. Current Status of Vegetative Compatibility Groups in Fusarium oxysporum: Supplement (1999). *Phytoparasitica* 27(4): 273-277.

Koenig, R., Ploetz, R.C. and Kistler, H.C. 1997. *Fusarium oxysporum* f. sp. *cubense* consist of a small number of divergent and globally distributed lineages. *Phytopathology* 87: 915-923.

Lee, Y.M., Teo, L. and Ong, K.P. 2001. Fusarium wilt of Cavendish banana and its control in Malaysia. In: Molina, AB.; Nik Masdek, N.H. and Liew, K.W. (eds.) Banana Fusarium wilt management: towards sustainable cultivation. Laguna, PH. INIBAP. p. 252-259.

Lescot, T. 2011. Close-up Banana: Statistics. *FruiTrop*. 189:59-62.

Leslie, J.F. 1990. Genetic exchange within sexual and asexual populations in the genus *Fusarium*. In: Ploetz, RC. (ed.). Fusarium wilt of banana. Minnesota, US. APS. p. 37-48.

Leslie, J.F. 1993. Fungal vegetative compatibility. *Annual Review of Phytopathology* 31: 127-150.

Leslie, J.F. and Summerell, B.A. 2006. The Fusarium Laboratory Manual. Iowa, USA. Blackwell Publishing. 388 pp.

Li, C.Y., Yi, G.J., Chen, S., Sun, Q.M., Zuo, C.W., Huang, B.Z., Wei, Y.R., Huang, Y.H., Wu, Y.L., Xu, L.B. and Hu, C.H. 2011. Studies on some of the early events in the ***Fusarium oxysporum-Musa*** interaction. *Acta Horticulture* 897: 305-312.

Li, C.Y., Chen S., Zuo, C.W., Sun, Q.M., Ye, Q. & Ganjun Yi, G.J. and Huang, B.Z. 2011. *Eur. J. Plant Pathol*.131: 327–340.

Messiaen, C.M. and Cassini, R. 1968. Recherches sur les Fusarioses IV: La systématique des Fusarium. *Annals Epiphyte* 19 (8): 387-454.

Ministerio de Desarrollo Agropecuario. 2008. Resuelto No. DAL-048-ADM-08 PANAMÁ 18 DE JULIO DE 2008. Panamá, PA. Gaceta Oficial Digital No. 26130.

Meldrum, R.A., Daly, A.M., Tran-Nguyen, L.T.T., Aitken, E.A.B. 2013. The effect of surface sterilants on spore germination of *Fusarium oxysporum* f. sp. *cubense* tropical race 4. *Crop Protection* 54: 94-198.

Meldrum, R.A., Daly, A.M., Tran-Nguyen L.T.T. and Aitken, E.A.B. 2013. Are banana weevil borers a vector in spreading *Fusarium oxysporum* f. sp. *cubense* tropical race 4 in banana plantations? *Australasian Plant Pathol.* 42:543–549.

Moore, N.Y., Pegg, K., Allen, A.R. and Irvin, J.A.G. 1993. Vegetative compatibility and distribution of Fusarium oxysporum f. sp. cubense in Australia. *Australian Journal of Experimental Agriculture* 33 (6) 797 – 802.

Molina, A.B. 2009. Estado de la incidencia en Asia del marchitamiento por Raza 4 tropical de Fusarium en el cultivo del banano. In Reunión de Grupos de Interés sobre los Riesgos de la

Raza 4 tropical de *Fusarium*, BBTV y otras Plagas de Musáceas, OIRSA (San Salvador, El Salvador). Resúmenes. 71 pp.

Molina, A., Fabregar, E., Sinohin, V.G., Herradura, L., Fourie, G. and Viljoen, A. 2008. Confirmation of tropical race 4 of *Fusarium oxysporum* f. sp. *cubense*, infecting Cavendish bananas in the Philippines. In: Centennial Meeting of the American Phytopathological Society. (2008, Minnesota, US). Abstracts.

Molina, A.B., Fabregar, E.G., Ramillete, E.G., Sinohin, V.O. and Viljoen, A. 2011. Field resistance of selected banana cultivars against Tropical Race 4 of *Fusarium oxysporum* f. sp. *cubense* in the Philippines. *Phytopathology* 101: S122.

Nasdir, N. 2003. Fusarium wilt race 4 in Indonesia. Research Institute for Fruits west. Sumatra, Indonesia. Abstracts of Papers 2nd. International Symposium on Fusarium wilt on banana. PROMUSA-INIBAP/EMBRAPA. Salvador de Bahía, Brazil. 22 - 26 Sept.

Nel, B., Steinberg, C., Labuschagne, N. and Viljoen, A. 2006. Isolation and characterization of nonpathogenic *Fusarium oxysporum* isolates from the rhizosphere of healthy banana plants. *Plant Pathology* 55: 207–216.

Nel, B., Steinberg, C., Labuschagne, N. and Viljoen, A. 2006. Evaluation of fungicide and sterilizants for potential application in management of Fusarium wilt of banana.

Nurhadi, M. and Harlion. R. 1994. The disease incidence of bacterial and Fusarium wilt disease in Lampung province. *Indonesian Info. Hort.* 2(1): 35-37.

O'Donnell, K. and Cigelnik, E. 1999. A DNA sequence-based phylogenetic structure for the Fusarium oxysporum species complex. *Phytoparasitica* 27: 69.

O'Neill WT., Gulino, L.M., Pattison, A.B., Daniells, J.W., Hermanto C. and Molina, A. 2009. Vegetative compatibility group analysis of Indonesian Fusarium oxysporum f. sp. cubense isolates. In: Proceedings of the International ISHS-ProMusa Symposium on Global Perspectives on Asian Challenges. Van den Bergh, I. et al. (eds.). *Acta Horticulturae* 897. ISHS 2011.

Ong, K.P. 1996. Fusarium wilt of banana in a Cavendish banana in a commercial farm in Malaysia. In: New frontiers in resistance breeding for nematode, Fusarium and Sigatoka (1995, Kuala 70 Lumpur, MY). 1996. Proceedings. Frison, E.A., Horry, J.P. and De Waele, D. (eds.). Montpellier, FR. INIBAP. 242 pp.

Pegg, KG., Moore, N.Y. and Sorensen, S. 1993. Fusarium wilt in the Asian Pacific region. In International symposium on recent development in banana cultivation technology. (1993, Los Baños, PH). Abstracts. p. 255-314.

Pegg K.G. and Moore, N.Y. and Sorensen, S. 1994. Variability in populations of *Fusarium oxysporum* f. sp. *cubense* from the Asia/Pacific region. In: The Improvement and Testing of Musa: A Global Partnership. Jones, D.R. (ed). Proceeding of the First Global Conference of the International Musa Testing Program. HN. INIBAP. Montpellier, FR. 70–82 pp.

Pegg K.G., Moore N.Y. and Bentley, S. 1996. Fusarium wilt of banana in Australia: a review. *Australian Journal of Agricultural Research* 47: 637-650.

Peng, H.X., Sivasithamparama, K., Turner, D.W. 1999. Chlamydospore germination and Fusarium wilt of banana plantlets in suppressive and conducive soils are affected by physical and chemical factors. Soil Biology and Biochemistry 31: 1363-1374.

Pérez, L., Batlle, A. y Fonseca, J. 2003. *Fusarium oxysporum* f. sp. *cubense* en Cuba: biología de las poblaciones, reacción de los clones híbridos de la FHIA y biocontrol. En: Memorias del Taller "Manejo convencional y alternativo de la Sigatoka negra, nematodos y otras plagas asociadas al cultivo de Musáceas" Rivas, G. y Rosales, F. (Eds.). Guayaquil, Ecuador, 11- 13 de Agosto. Pp: 141-155.

Pérez-Vicente, L., 2004. Fusarium wilt (Panama disease) of bananas: an updating review of the current knowledge on the disease and its causal agent. In. Memorias de XV Reunion Internacional de ACORBAT (Oaxaca, MX). Pp: 1-14.

Pérez-Vicente, L. 2010. Estudios de poblaciones de *Fusarium oxysporum* f. sp. *cubense* en AL&C y necesidades de entrenamiento de jóvenes científicos de la región. Simposio Global sobre Avances de Investigación sobre el mal de Panamá (*Fusarium oxysporum* f. sp. *cubense*): Raza 4 Tropical y su Amenaza para la Industria Bananera de Latinoamérica y el Caribe. Guayaquil, Ecuador, Abril 18 al 22.

Pérez-Vicente, L., Batlle-Viera, A. and Chacón-Benazet, J. 2003. *Fusarium oxysporum* f. sp. *cubense* en Cuba: biología de las poblaciones, reacción de los clones híbridos de la FHIA y biocontrol. En: Memorias del Taller "Manejo convencional y alternativo de la Sigatoka negra, nemátodos y otras plagas asociadas al cultivo de Musáceas en los trópicos. (Guayaquil, EC). Rivas, G.; Rosales, F. (eds.). Pp. 141-155.

Pérez-Vicente, L.; Batlle-Viera, A.; Chacón-Benazet, J. y Montenegro-Morasén, V. 2009 Reacción de clones naturales e híbridos de la FHIA de bananos y plátanos a las poblaciones de Cuba de *Fusarium oxysporum* f. sp. *cubense* agente causal de la marchitez por Fusarium o mal de Panamá. *Fitosanidad* 13 (4): 237-242.

Pérez-Vicente, L., Batlle-Viera, A., Chacón-Benazet, J. y Montenegro-Moracén, V. 2009. Eficacia de *Trichoderma harzianum* A34 en el biocontrol de *Fusarium oxysporum f.* sp. *cubense* agente causal de la marchitez por Fusarium o Mal de Panamá de los bananos en Cuba. *Fitosanidad* 13 (4): 259-264.

Ploetz, RC. 1990. Fusarium Wilt of Banana. Minnesota, US. APS. 139 p.

Ploetz R.C. 2005a. Panama disease: an old nemesis rears its ugly head. Part 1. The beginnings of the banana export trades. Online. Minnesota, US. APS.

http://www.apsnet.orgonline/feature/panama/

Ploetz, R.C. 2005b. Panama disease: an old nemesis rears its ugly head. Part 2: The Cavendish era and beyond. Online. Minnesota, US. APS.

http://www.apsnet.orgonline/feature/panama2/default.asp

Ploetz R.C. 2006. Fusarium wilt of Banana is caused by several pathogens referred to as *Fusarium oxysporum* f. sp. *cubense*. *Phytopathology* 96: 653-656.

Ploetz, R.C.and Correll, J.C. 1988. Vegetative compatibility among races of *Fusarium oxysporum* f. sp. *cubense*. *Plant Disease* 72: 325–328.

Ploetz, R.C. and Pegg, K.G. 2000. Fusarium wilt. In: Diseases of Banana, Abaca and Enset. Jones, D.R. (Ed.). Wallingford, UK. CABI. Pp. 143-159.

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

Qi, P. 2001. Status report of banana Fusarium wilt disease in China. In Molina, A.B., Nik Masdek, N.H.; Liew, KW. (eds.) Banana Fusarium wilt management: towards sustainable cultivation. Laguna, PH. INIBAP. p. 119-120. 71

Qi, Y.X., Zhang, X., Pua, J,J., Xie, Y.X., Zhang, H.Q., Huang, S.L. 2008. Race 4 identification of *Fusarium oxysporum* f. sp. *cubense* from Cavendish cultivars in Hainan province, China. *Australasian Plant Disease Notes* 3: 46-47.

Rishbeth, J. 1957. Fusarium wilt of banana in Jamaica. I. Some observations on the epidemiology of the disease. *Ann. Bot*. 19, 293

Reinking O.A. and Manns, M.M. 1932. Significance of disease spread in the field under different conditions. Bull 44. Res. Dept. United Fruit Co.

Rorer, J.B (1911). A preliminary list of of Trinidad fungi. *Board of Agriculture, Trinidad and Tobago, Circ.* No 4: 37-44

Scher, FM.; Baker, R. 1982. Effect of *Pseudomonas putida* and synthetic iron chelator on induction of soil suppresivness to *Fusarium* wilt pathogens. *Phytopathology* 72: 1567-1773.

Sequeira, L. 1962. Influence of organic amendments on survival of *Fusarium oxysporum* f. sp. *cubense* in soil. *Phytopathology* 52: 976-982.

Simmonds, N. W. 1966. Bananas, 2nd ed. Longmans, London, UK.

Smith, E.F. 1910. A Cuban banana disease. *Science* 31: 754-755.

Snyder, W.C. and Hansen, H.N. 1940. The species concept in Fusarium. *American Journal of Botany* 27: 64-67.

Stover, R.H. 1962. Fusarium wilt (Panama disease) of bananas and other Musa species. Kew, UK. Commonwealth Mycological Institute. 177 p.

Stover, R.H., 1972. Banana, plantain and abaca diseases. Kew, UK. Commonwealth Mycological Institute. 316 p.

Stover, R.H. and Waite, B.H. 1960. Studies on Fusarium wilt of bananas V. Pathogenicity and distribution of *F. oxysporum* f. sp. *cubens*e races 1 and 2. *Canadian Journal of Botany* 38: 31-61.

Su, H.J., Hwang, S.C. and Ko, W.H. 1986. Fusarium wilt of Cavendish bananas in Taiwan. *Plant Disease* 70: 814-818.

Summerell, B., Salleh, B. and Leslie, J.F. 2003. A utilitarian Approach to Fusarium identification. *Plant Disease* 87 (2): 117-128.

Sun, E.J., Su, H.J. and Ko, W.H. 1978. Identification of *Fusarium oxysporum* f. sp. *cubense* race 4 from soil or host tissue by cultural characters. *Phytopathology* 68: 1672-1673.

Thangavelu, R., Mustaffa, M.M. 2010. First report on the occurrence of a virulent strain of Fusarium wilt pathogen (Race-1) infecting Cavendish (AAA) group of bananas in India. *Plant Disease* 94: 1379.

Waite, B.H. and Stover, R.H. 1960. Studies on Fusarium wilt of bananas. VI. Variability and cultivar concept in *Fusarium oxysporum* f. sp. *cubense*. *Canadian Journal of Botany* 38: 985–994.

Wardlaw, C.W. 1972. Banana diseases: including Plantain and Abaca. London, UK. Longman. 878 pp.

Wollenweber, H.W. and Reinking, O.W. 1935. Die Fusarien. Berlin, DE. Paul Parey. 355 pp.

Woltz, S.S. and Jones, J.P. 1981. Nutritional requirements of *Fusarium oxysporum*. Basis for a disease control system. In: Nelson, P.E., Tousson, T.A. Cook, R.J. (Ed.), Fusarium: Diseases Biology and Taxonomy. The Pennsylvania State University Press. Univ. Park. Pennsylvania, pp 340-349

PROTOCOLS

PROTOCOL FOR SAMPLING FUSARIUM WILT OR PANAMA DISEASE BY *Fusarium oxysporum f. sp. cubense* INFECTED PLANTS AND TISSUES.

(Procedure adapted from Natalie Moore, QDPI).

Luis Pérez Vicente and Alicia Batlle

Procedures for sampling from of Fusarium wilt or Panama disease infected plants.

1. **Sampling preparation.**

- Samples should consist of a section of pseudostem of wilted banana plants with evident continuous coloured vascular strands.
- Take the sample as low and close to the centre of the pseudostem as possible, but not from sector with advanced rotting, in opposite sense to the base of most external leaf sheaths (Figure 1). As banana tissue is very humid, risk of bacterial contamination is high, particularly in hot humid weather and samples can be rapidly deteriorated. The more the sample deteriorates the possibility of recovering pure *Fusarium oxysporum f. sp. cubense* (*Foc*) cultures is simultaneously reduced.

Figure 5. Procedure for sample collection from suspected infected banana plants by *Fusarium oxysporum* f. sp. *cubense* in disease free areas. A and B Cut of a pseudostem fragment. C. View if the pseudostem fragment severed showing necrotic vascular strands. D. Fragments of affected tissue inside a flask or envelop hermetically closed. E. Dissected vascular strands of pseudostem showing necrosis caused by pathogen. F and G. Sampled plant with reposition of the fragment cut in the original place and covered by adhesive tape to avoid exposition of tissues and exudates at environment. (Photos from P. E. Echegoyen taken from Dita *et al.,* 2013).

- Keep the sample in strong paper bags, paper envelop or glass vials until coloured vessel strands can be extracted. Avoid use of plastic bags because they make the samples sweat and promote bacterial growth.

Take precise notes of each simple such as:

a) Sample number (one sample per plant)

b) Date

c) Cultivar name of host plant including local names (and uses if known)

d) Genomic constitution of the host (i.e. AAA, AAB, etc.). This is not as important as the precise identification of cultivar.

e) If the sample plant is taken from a garden, commercial plantation, city, town or community or if it is from the wild

f) Locality (i.e. province name, state, distance from the closest city, name of the road, name of the property and if it is a commercial plantation, etc.

g) Name of the collector.

h) Other useful observations to be included such as source of planting material, if soils are waterlogged, how many diseased plants are affected, which other varieties are growing close by, and if they are healthy or diseased.

i) A small piece of rhizome (5 cm × 5cm) that shows coloured streams of vessels can be used as sample, but this is only recommended where rhizome rot are not advanced. This piece of rhizome should also be covered in paper or placed on a paper sheet to dry.

NOTE: When affected wilted plants are observed, it is better take the samples from established plants (mature plants or plantations) than one with recently planted plants.

2. Dissection of coloured vascular strands of the sample.

- Dissect the coloured vascular strands of the sample in the same day as collected or as soon as possible after collection. Sterile paper filter use is recommended and an aseptic technique for disease vessel dissection from the sample.
- Firstly, sterilize sample surface by submerging in 70% alcohol. When processing different samples, use a different filter paper for each sample and the scalpel blades and forceps should be flamed or at least submerged in alcohol between the different samples.
- Place the extracted coloured vessels on sterile filter paper in a paper envelop in order to dry it under natural conditions. Usually a few days of drying is enough
- **NOTE:** Do not expose the strands to heat or high temperature (i.e. direct sunlight or the back of the car), because this can kill the fungus. **Do not dry the sample in an oven!**

Fusarium specimens do not need to be kept in a refrigerator; laboratory room temperature is enough. Leaf samples do not need to be enveloped in a moist paper. Dry paper is better.

To send samples by mail.

- If it is necessary to mail samples for analysis and isolation, send the sample in a paper envelope as soon as it is dry enough, with sample number and details of each sample. Include a copy of the official import permit in case there are international samples inside the parcel.

NOTE: If there is any chance that the samples becoming mixed or if details of some samples are missing or confusing or suspected to be incorrect, the sample should be discarded and a new sample should taken.

PROTOCOL FOR THE ISOLATION OF *Fusarium oxysporum f. sp. cubense* FROM TISSUE SAMPLES OF FUSARIUM WILT OR PANAMA DISEASE AFFECTED BANANA PLANTS AND SOIL

Luis Pérez Vicente, Einar Martínez and Miguel A. Dita.

ISOLATION OF THE FUNGUS FROM PLANT DISEASED COLLECTED MATERIAL.

Fungal isolation from affected colored strands.

- The isolation can be attempted as soon as the strands with vessels are dry (possibly the day after the collection)
- Plate small sections (3-6 mm long) of the tissues with vascular vessels in Petri plates with ¼ strength potato dextrose agar (PDA) or water agar (WA) with an antibacterial agent (i.e. streptomycin sulfate1.2 mL / 240 mL of PDA).
- If *Fusarium* is present, it will grow out from the vessels in 2-4 days (see Figure 6).
- If the sample is contaminated with bacteria, the fungal growth could be masked. If this occurs, allow the sample dry more and increase the streptomycin sulfate in the media.
- From samples that have been prepared correctly, a high rate of *Fusarium* recovery is possible.
- Prepare single conidia cultures of each specimen.

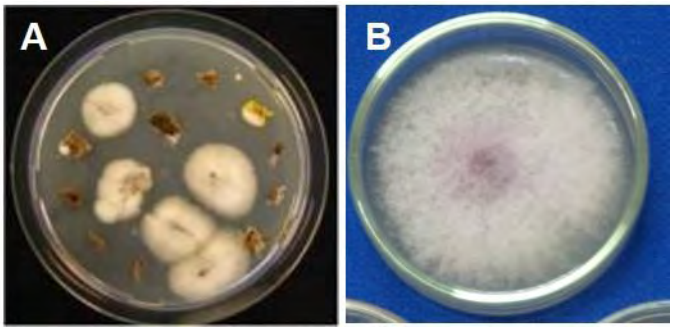

Figure 6. *Foc* growth in agar plates. A. *Foc* colonies from pseudostem discoloured strands in Water Agar. B. *Foc* single conidia culture in PDA plate.

1. Isolation from soil

- Collect a soil sample from the first 25 cm depth and store in a paper bag.
- Let samples air dry in the more aseptic conditions for 24-48 hours
- Grind the larger particles in a mortar

- Prepare a soil suspension in sterile water in a proportion of 1:50 soil weight / water volume (If the suspension is too concentrated because of high *Fusarium* population in the sample, a 1:100 proportion can be prepared). Shake the suspension for better release and distribution of soil particles and fungal structures.
- Dilute 1 mL suspension in 10 cm Petri plates with modified K2 media at close to melting temperature to achieve a good dispersion of the soil in the culture media.
- 1 mL of suspension can also be distributed on the surface of the plate with solidified K2 media. Agar should be allowed to dry for 3-4 days in the plates before plating the spore suspension so that it can absorb a higher amount of the spore suspension.
- Distribute the suspension as uniformly possible and allow it to stand for two minutes. Remove excess soil suspension from the plate and incubate it at 27°C upside down.
- Recovered colonies are transferred to other appropriate media to obtain single conidial isolates.

2. Single spore isolations (single conidia).

Fusarium oxysporum single spore isolations are obtained by the plate dilution method and streaking plates (showed ahead). For both methods:

- Collect a scrape of sporulating hyphae from cultures growing on PDA (¼ strength) and dissolve in 10 mL sterile distilled water in test tubes.
- From an initial suspension, a dilution serial can be prepared. Pipette or streak 1 mL of each of the dilutions on water agar.
- Incubate plates overnight at 25°C with caps in upside position
- Check the plates under a dissecting microscope the following day to localize germinated conidia and transfer with a sterile needle or scalpel single conidia isolated from the water agar to new 90 mm plates with ¼ strength PDA.
- Additionally, single-spore cultures can also be obtained by dissecting the tip of a single growing hypha of an old culture grown in carnation leaf agar (CLA).

CULTURE MEDIA FOR THE ISOLATION AND CULTURE OF *F. oxysporum*

1. Potato dextrose agar ¼ strength (PDA ¼).

(Ainsworth, G.C., 1971. Ainsworth and Bisby's Dictionary of the Fungi. 6th. Ed. Commonwealth Mycological Institute, Kew Surrey, England, 663 pp).

Ingredients for a litre of distilled water**.**

Peeled pieces of potatoes	100 g
Dextrose	10 g
Agar.	20 g

Method. Boil the potatoes in the distilled water for an hour and filter through eight cheese cloth layers. Discard the solid portion; then add dextrose and agar to the liquid portion, dissolve well and return to heat until the agar is fully dissolved (around 40-50 min). Withdraw the media from heat, dispense in flasks or bottles and autoclave immediately (humid cycle, 100 kPa at 121°C for 20 min.) When fresh, tighten the caps and mark the flasks or bottles with **PDA** and date.

2. PDA supplemented with streptomycin.

Proceed to melt the required number of 240 mL PDA bottles in a water bath. When media has melted, place the bottles in a water bath at 50°C for 20 min or until the media reaches 50°C. For each 240 mL of media, add 1.2 mL of streptomycin solution (1g of streptomycin sulfate powder to 100 mL distilled water) just before dispensing the media in the Petri plates.

3. Carnation leaves agar (CLA).

(Burguess, L.W., Liddell, C.M. and Summerell, B.A. 1988. Laboratory Manual for Fusarium Research, 2nd Edition, University of Sydney, Australia, 156 pp.)

Method. Four to ten sterilized pieces of carnation leaves are placed on water agar surface before media hardens (solidifies). After the media has solidified, the plates with CLA are stored in a refrigerator at 4°C.

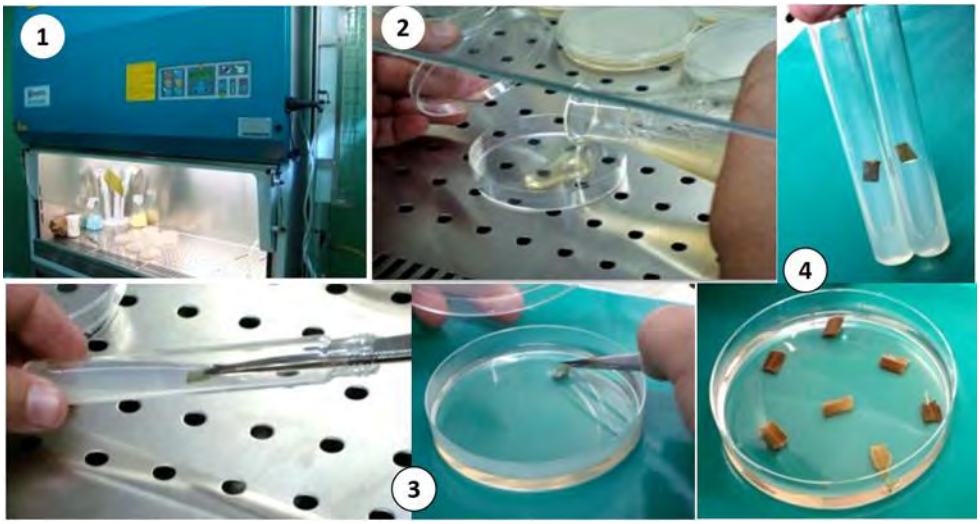

Figure 7. Preparation of Carnation Leaf Agar. 1. Biological safety cabinet. 2. Water Agar 2% poured in plates. 3. Sterilized Carnation leaf fragments placed in plates and tubes. 4. Plates and tubes with CLA ready for inoculation

Preparation of carnation leaves (Figure 7)*:*

- Fresh carnation leaves not treated with agrochemicals, are cut in 8 x 3 mm pieces before being placed in an oven at 70°C to dry.
- When dry, place it in containers that are suitable to receive Gamma radiations (i.e. glass, polystyrene containers or Petri plates sealed with Parafilm). Note that after repeated exposure to Gamma radiations, plastic will degrade.

- Radiate containers in a Gamma cell for a total rate of 2.5 Mega Rad.
- Store sterile pieces in a refrigerator at 4 °C until use.

4. Komada modified media (K2)

(Sun, E.J., Su, H.J and Ko, W.H., 1978. Identification of *Fusarium oxysporum f. sp. cubense* race 4 from soil or host tissue by cultural characters. *Phytopathology* 68: 1672-1673).

Ingredients for 900 mL of distilled water:

D-galactose	10.0 g
L-Asparagine	2.0 g
KH_2PO_4	1.0 g
KCl	0.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
FeNa EDTA	10.0 mg
Agar	20.0 g
Distilled H_2O	900 mL

- Sterilize at 120°C for 20 min.
- Adjust pH to 3.8 with 10% phosphoric acid.
- Add at a temperature close to 50°C, 100 mL of a solution is sterilized by filtration with:

Streptomycin sulfate	0.3 g
Oxgall	0.5 g
$N_{a2}B_4O_{7.}$	0.5 g
PCNB (75% PH)	0.9 g

Inoculate plates with a 0.5 ml diluted suspension of soil in sterile water.

5. Spezieller Nährstoffarmer agar (SNA).

KH_2PO4	1 g
KNO_3	1 g
$MgSO_4 \cdot 7H2O$	0.5 g
KCl	0.5 g
Glucose	0.2 g
Sucrose	0.2 g
Distilled H_2O	1L

Sporulation is stimulated if sterile filter paper Whatman # 1 pieces are included.

This media is appropriate for producing microconidia in a stable way. Suitable for chlamydospores detection.

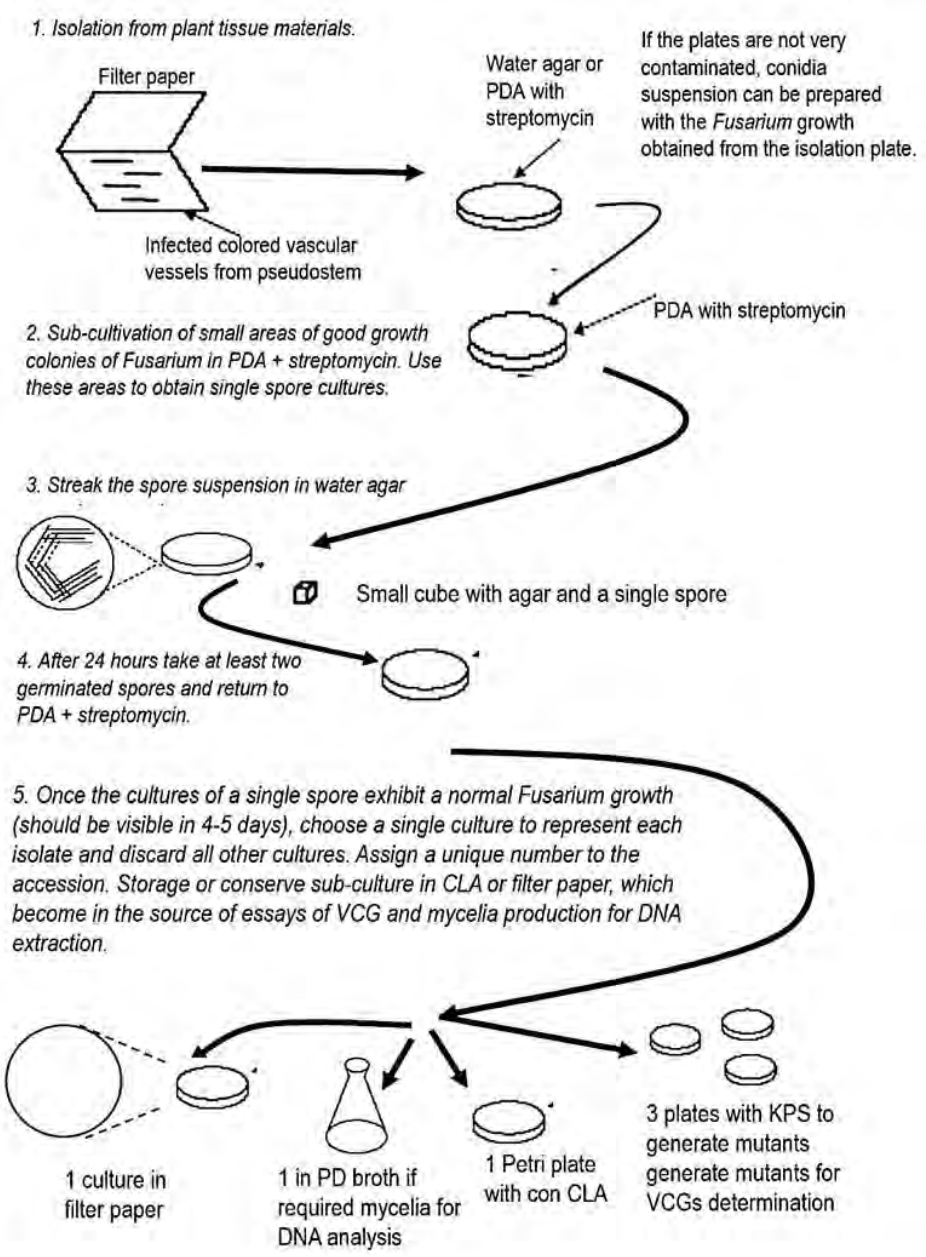

Figure 8. Scheme of the process of isolation, obtain single spore isolates and store for different purposes.

PROTOCOL FOR DETERMINATION OF VEGETATIVE COMPATIBILITY GROUPS (VCGs): TECHNIQUE OF PUHALLA (1985) AND CORRELL *ET AL*. (1987) BASED ON THE GENERATION OF AUXOTROPHIC MUTANTS THAT DO NOT USE NO_3 (*NIT* MUTANTS*)*.

Luis Pérez Vicente, Alicia Batlle and Miguel A. Dita.

Introduction.

Heterocompatibility and heterokarion development.

Heterokarion formation is a process that normally haploid fungi can use to get the benefit of a functional diploidy (as are complementation and heterosis) and constitute the first step in a parasexual cycle for character transmission (Leslie, 1990; 1993). Fungi capable of developing such heterokaryons are known as vegetative compatible. The vegetative compatibility has been used to identify genetically isolated sub populations of fungal pathogens (Puhalla, 1985).

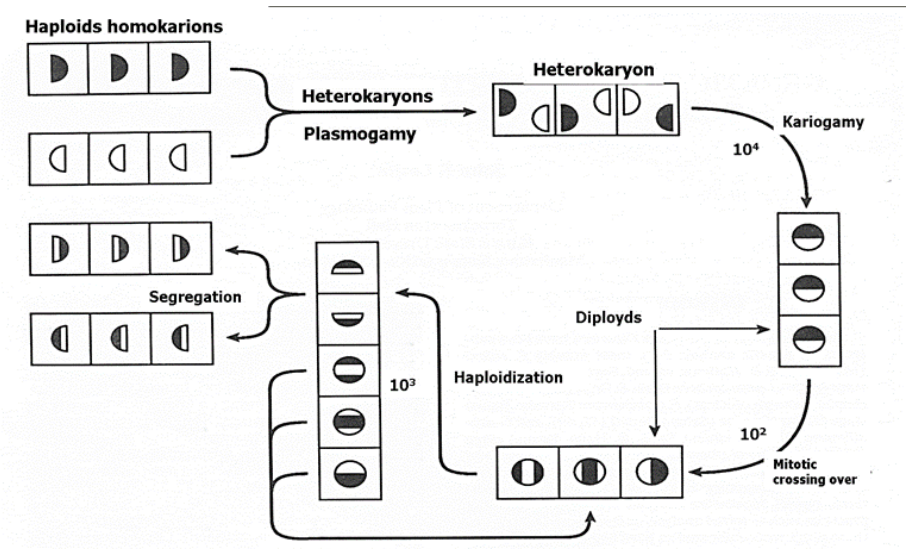

Figure 9. Scheme of heterokarions (according Leslie 1990 (Ploetz, 1990)

Vegetative compatibility results are a useful technique to study relationships among fungi with asexual reproduction as *Foc.* The technique was developed by Cove (1976) and refined by Puhalla (1985) and Correll *et al.* (1987). As vegetative compatibility in many fungi (including *Fusarium* species) requires that alleles of at least 10 different loci (*vic* loci) be identical (Puhalla and Spieth, 1985), the members of a vegetative compatibility group (VCG), constitute clonally derived sub-populations and hence closely related (vegetative compatibility among lines of different *formae speciales* has never been observed). It is improbable that individuals having the same VIC alleles complement are not clonally related. This then indicates that two vegetative compatible individuals should be identical for many genes including those responsible for

pathogenicity, ecological fitness and other characteristics that affect their roles as banana pathogens. Due to this, association between vegetative compatibility and other characters genetically controlled, VCGs are strong indicators of pathogenic behaviour and a powerful tool in the study of biology and genetics of populations (particularly in the case of *Foc-Musa* where genetic analysis of pathogenicity is practically impossible).

Generation of mutants that do not use nitrate (nit).

This technique was originally used with *Aspergillus nidulans* by Cove (1976) and modified by Puhalla (1985) and Correll *et al*. (1987), for use with *Fusarium oxysporum*.

- Cultures grown in CLA media and/or PDA are used to inoculate Petri plates in a media that contains potassium chlorate. Potassium chlorate is an analog of potassium nitrate and is taken and processed through the metabolic route of reductase nitrate (Correll *et al.,* 1987). This process results in the production of chlorite that is toxic to the fungus (in substitution of nitrite that is beneficial to the fungus) giving place to characteristic colonies of slow growth with unrestricted nodose mycelia.
- After 5-12 days, fast-growing sectors begin to grow from the slow growing sectors of these restricted colonies (Figure 10). The mycelia of these fast-growing sectors have a mutation that allows the fungus to resist the chlorate (and hence also the toxic chlorite). However the mutation also determines that the fungus is unable to reduce nitrate. So, these sectors are known as mutants that do not use nitrate and are *nit* mutants in abbreviate form.
- To test if fast growing sectors are able to use nitrate, a small mycelia piece (2 × 2 mm) is taken from the border of the sector. This is transferred to a medium that contains nitrogen only in nitrate form, such as **Minimal Media** (MM) (Puhalla, 1985). If the sector is a true *nit* mutant, it will not be capable of reducing the nitrate in the media and will grow as a characteristic N-deficient, poor sparse mycelia. If the resulting growth in MM is not sparse, the culture is discarded because it will not be useful in VCG tests (complementation assays).
- It is advantageous to allow the sectors to grow for two or three days after emergence of the mutated sector in plates with the chlorate media (**KPS**), so that the growth in the fast-growing sectors is clean with no mutated mycelia that could grow underneath. Each unrestricted colony that is grown on the KPS media can give up to five separated sectors. When each sector is transferred to a MM media, the sector should be labelled for identification. This is particularly important if the tests have to be repeated or the mutants are required for other tests. For instance, if the isolate that is being tested has the access number 2354, the sectors should be sequentially number as 2354-1, 2354-2, 2354-3, etc. (Figure, 10).

Figure 10. Foc colonies of restrict growth in media with potassium chlorate, with a mutated fast growth

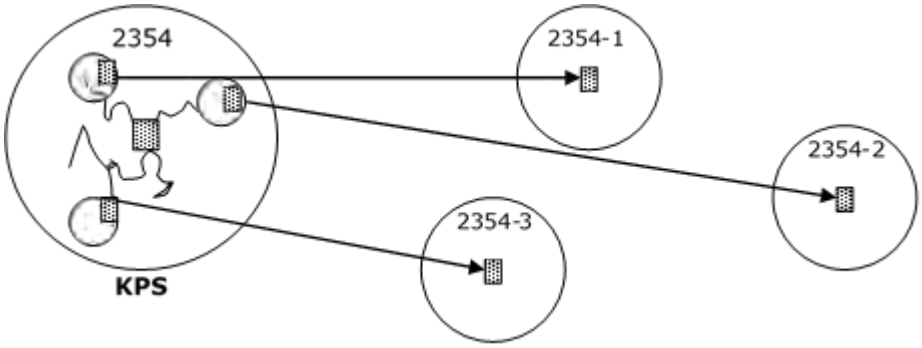

Figure 11. Fast growing sectors that emerge of a *Foc* restrict colony in KPS media. Mycelia of the advance axis are transferred to a MM media to test the capacity to reduce nitrate.

1. Determination of the mutant phenotype.

Some *nit* mutants are better than others to be used in VCG tests. The *nit* mutant phenotype is determined by the type of growth (sparse- / nitrogen deficient or dense nitrogen-sufficient) that is produced when the nit mutant is transferred to a single N source (Correll *et al.*, 1987). For a more comprehensive explanation about phenotypes and which combinations are better to be used in complementation tests, consult Correl *et al.,* (1987). It is advantageous to generate 3-5 *nit* 1 or *nit* 3 mutants of each isolate to mate in combination with Nit M testers (mutants of known VCG). The mutants of phenotypes *nit* 1 and *nit* 3 are the more frequently-generated mutants. Nit M mutants are the less commonly generated and better used as reference for VCGs testers.

Table 3.Tests to determine mutant phenotypes (Correl *et al.* 1987)

Phenotype	ClO_3	NO_3	NO_2	Hypoxanthine	NH4
Wild type	-	+	+	+	+
nit 1	+	-	+	+	+
nit 3	+	-	-	+	+
Nit M	+	-	+	-	+
crn	+	+	+	+	+

Nitrate Minimal Media (MM)

Nitrite Basal media + 0.5 g $NaNO_2$/L

Hypoxanthine Basal media + 0.2 g hypoxanthine.

Ammonia Basal media + 1.0 g ammonium tartrate/L.

2. **Nit *mutant mating in VCGs tests.***

- A little colonized fragment of agar (2 × 2 mm) of a Nit M mutant culture of a known VCG, is placed in the centre of a plate with MM.
- The bottom of the plate is marked with the VCG number that represents the Nit M.
- Small similar pieces of mutant *nit* cultures that have been generated with the unknown VCG isolates are then placed in at least 10-15 mm of distance from Nit M fragment around the border of the plate (Figure 12). Before proceeding to transfer the *nit* mutants the bottom of the plate should be marked (using permanent markers that do not dissolve) with the number of the sample, to avoid confusions.

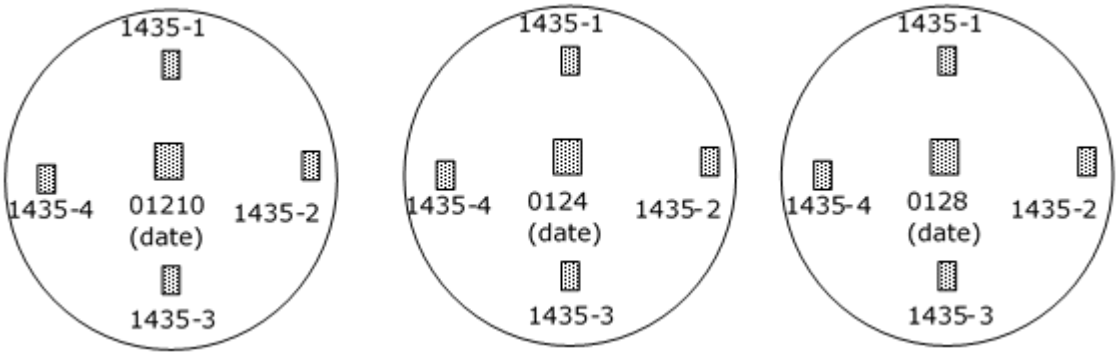

Figure 12. Disposition of the mating test of four *nit* mutants belonging to an unknown VCG (isolate No. 1435) with Nit M testers of VCGs 1210, 0124 and 0128 located in the centre of the plates with MM media and the *nit* mutants around the border.

- If it is considered that a *nit* mutant fragment has been placed in an incorrect position, the plate should be discarded and operation repeated.
- If the unknown isolate has the access 1435, the *nit* mutants that are generated from it should be numbered 1435-1, 1435-2, 1435-3 and 1435-4. If these are mated with Nit M that belong to VCGs 01210, 0124 or 0128 in Petri plates with MM, the complementation test to determine the VCG should looks like as the following form:

- The plates with the mated isolates are kept in an incubator at 25°C and are checked every two days to detect heterokarion growth.
- If heterokarion develops (if the isolate is vegetative compatible with one of the Nit M testers), a dense line of mycelial growth will form after 7-12 days where the hyphae of *nit* have contacted hyphae of Nit M representative of the VCG to which the isolate under study belong. If a line of heterokaryotic growth is not evident, it is considered that the isolate does not belong to the tested VCG. For instance, if the isolate 1437 belongs to VCG 1210, after 12 days the plate of the test will appear in the following manner (Figure 13):

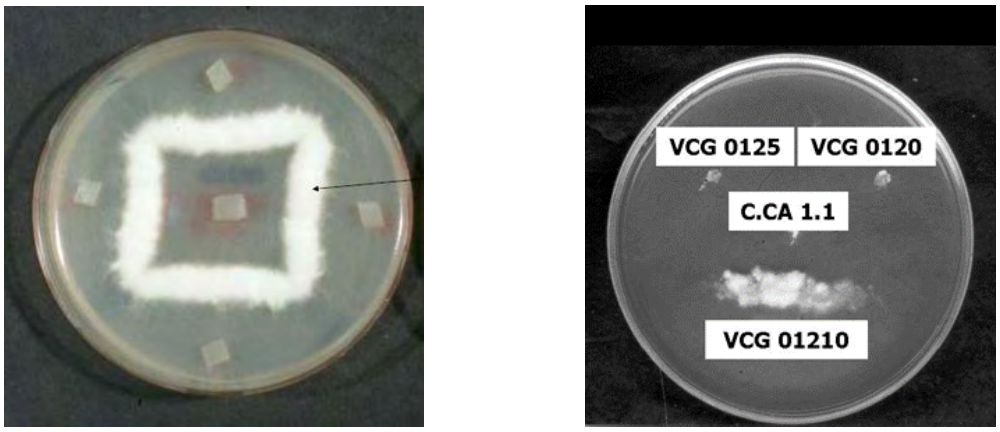

Figure 13. Example of a positive complementation test for VCG determination. There is formation of heterokaryons between the *nit* mutant and VCG 1210 Nit M tester. Isolate C.Ca 1.1 belongs to VCG 1210 because develop a heterokaryon (shown by the dense mycelia en MM media

CULTURE MEDIA TO DETERMINE VCGs

1. **KPS Media.** (Puhalla, J.E., 1985. Classification of Fu*sarium oxysporum* on the basis of vegetative compatibility. *Canadian Journal of Botany* 63, 179-183.)

Boil 200 g peeled potatoes and cut in pieces in 1L of distilled water for 50 min. Filter liquid using 8 layers of cheese cloth and discard solid portion. Complete the liquid to a 1 L volume and add:

Sucrose	20 g
$KClO_3$	15.0 g
Agar.	20.0 g

Mix agar under heat until it has dissolved. Remove the media from the heat immediately and dispense 240 mL in bottles of 250 mL. Plug with autoclavable caps (left the caps loose to release vapor). Sterilize in an autoclave (humid cycle of 100 kPa at 121 °C for 20 min). When fresh, tighten the caps and label the bottles with KPS and a date.

2. **Minimal Media -MM** (Puhalla J.E. and Speigh, P.T., 1983. Heterokaryosis in *Fusarium moniliforme*. Experimental Mycology 7, 328-335.)

Ingredients for 1 L of distilled water:

Sucrose	30 g
BBL agar or of similar analytic grade	20 g
KCl (potassium chlorate)	0.5 g
NaNO3 (sodium nitrate)	2 g
KH2PO4 (di acid potassium phosphate)	1 g
MgSO4.7H20 (magnesium sulfate heptad hydrated)	0.5 g
FeSO4.7H20 (ferrous sulfate)	10 mg
Sterile solution of trace elements	0.2 mL

(Add this solution after media is melted and before autoclaving).

Method. Introduce the media in a water bath shaking from time to time until agar dissolve (approximately 1 hour). Add trace elements (previously prepared and stored in 1L containers in a freezer). Dispense approximately 240 mL of media. Plug with autoclavable caps (left the caps loose to release the vapour) and autoclave to sterilize (humid cycle 100 kPa a 121 °C by 20 min.). When cool, tighten the caps and label with MM, month and year.

Trace elements solution

Ingredients for 95 mL of distilled water:

Citric acid	5.0 g
$ZnSO_4.7H_20$ (zinc sulfate)	5.0 g
$Fe\ (NH_4)_2(SO_4)_2.6H_20$ (ferrous ammonia sulfate)	1.0 g
$CuSO_4.\ H_20$ (copper sulfate)	0.25 g
$MnSO_4.\ 4H_2O$ (Manganese sulfate tetra hydrated)	50 mg
H_3BO_4 (boric acid)	50 mg
$NaMoO_4.\ 2H20$ (sodium molybdate)	50 mg

3. **Nitrite media** (Correll, J.C., Klittich, C. J. R. and Leslie, J.F., 1987. Nitrate non-utilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. *Phytopathology* 77, 1640-1646).

Sucrose	30 g
BBL Agar or similar analytic grade	20 g
KCl (potassium chloride)	0.5 g
$NaNO_2$ (sodium nitrite)	0.5 g

$KH_2\ PO_4$ (de acid potassium phosphate)	1 g
MgSO4.7H20 (heptad hydrated magnesium sulfate)	0.5 g
FeSO4.7H20 (hepta hydrated ferrous sulfate)	10 mg
Sterile solution of trace elements	0.2 mL

(Add this solution after media is melted and before autoclaving).

Method.

Introduce the media in a water bath, shaking from time to time until the agar has dissolved (approximately 1 hour). Add trace elements (previously prepared and stored in 1L containers in a freezer). Dispense approximately 240 mL of media. Plug (with caps not tight) and autoclave to sterilize (humid cycle 100 kPa a 121 °C for 20 min.). When cool, tighten the caps and label with **NM**, month and year.

Trace elements solution

Ingredients for 95 mL of distilled water:

Citric acid	5.0 g
$ZnSO_4.7H_20$ (zinc sulfate)	5.0 g
Fe $(NH_4)_2(SO_4)_2.6H_20$ (ferrous ammonia sulfate)	1.0 g
$CuSO_4$. H_20 (copper sulfate)	0.25 g
$MnSO_4$. $4H_2O$ (tetra hydrated manganese sulfate)	50 mg
H_3BO_4 (boric acid)	50 mg
$NaMoO_4$. 2H20 (dehydrated sodium molibdate)	50 mg

4. **Hypoxanthine media** (Correll, J.C., Klittich, C. J. R. and Leslie, J.F., 1987. Nitrate non-utilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. *Phytopathology* 77, 1640- 1646).

– Sucrose	30 g
– BBL agar or of analytic similar grade	20 g
– KCl (potassium chloride)	0.5 g
– Hypoxanthine	0.2 g
– $KH_2\ PO_4$ (Di acid potassium phosphate)	1 g
– Mg SO4.7H20 (heptad hydrated magnesium sulfate)	0.5 g
– Fe SO4.7H20 (heptad hydrated ferrous sulfate)	10 mg
– Sterile traces of microelement solution	0.2 mL

(Add this solution after media is melted and before autoclaving).

Method

Introduce the media in a water bath shaking from time to time until the agar has dissolved (approximately 1 hour). Add trace elements (previously prepared and stored in 1L containers in a

freezer). Dispense approximately 240 mL of media. Plug (with caps not tight) and autoclave to sterilize (humid cycle 100 kPa a 121 °C by 20 min.). When cool, tighten the caps and label with **HX**, month and year.

Trace elements solution

Ingredients for 95 mL of distilled water:

Citric acid	5.0 g
$ZnSO_4.7H_20$ (zinc sulfate)	5.0 g
Fe $(NH_4)_2(SO_4)_2.6H_20$ (ferrous ammonia sulfate)	1.0 g
$CuSO_4$. H_20 (copper sulfate)	0.25 g
$MnSO_4$. $4H_2O$ (tetra hydrated manganese sulfate)	50 mg
H_3BO_4 (boric acid)	50 mg
$NaMoO_4$. 2H20 (di hydrated sodium molibdate)	50 mg

5. **Ammonia media** (Correll, J.C., Klittich, C. J. R. and Leslie, J.F., 1987. Nitrate non-utilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. *Phytopathology* 77, 1640- 1646).

– Sucrose	30 g
– BBL agar or of analytic similar grade	20 g
– KCl (potassium chloride)	0.5 g
– Ammonium tartrate	1 g.
– KH_2 PO_4 (Di acid potassium phosphate)	1 g
– Mg SO4.7H20 (heptad hydrated magnesium sulfate)	0.5 g
– Fe SO4.7H20 (heptad hydrated ferrous sulfate)	10 mg
– Sterile traces of microelement solution	0.2 mL

(Add this solution after media is melted and before autoclaving).

Method

Introduce the media in a water bath shaking from time to time until the agar has dissolved (approximately 1 hour). Add trace elements (previously prepared and kept in 1L containers in a freezer). Dispense approximately 240 mL of media. Plug (with caps not tight) and autoclave to sterilize (humid cycle 100 kPa a 121 °C by 20 min.). When cool, tighten the caps and label with **HX**, month and year.

Trace elements solution

Ingredients for 95 mL of distilled water:

Citric acid	5.0 g
$ZnSO_4.7H_20$ (zinc sulfate)	5.0 g
Fe $(NH_4)_2(SO_4)_2.6H_20$ (ferrous ammonia sulfate)	1.0 g

$CuSO_4$. H_20 (copper sulfate)	0.25 g
$MnSO_4$. $4H_2O$ (tetra hydrated manganese sulfate)	50 mg
H_3BO_4 (boric acid)	50 mg
$NaMoO_4$. 2H20 (di hydrated sodium molibdate)	50 mg

6. **Uric acid media.** (Correll, J.C., Klittich, C. J. R. and Leslie, J.F., 1987. Nitrate non-utilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. *Phytopathology* 77, 1640- 1646).

- KH_2 PO_4 (Di acid potassium phosphate) 1 g
- Mg SO4.7H20 (heptad hydrated magnesium sulfate) 0.5 g
- Fe SO4.7H20 (heptad hydrated ferrous sulfate) 10 mg
- Sterile traces of microelement solution 0.2 mL

(Add this solution after media is melted and before autoclaving).

Method.

Introduce the media in a water bath shaking from time to time until the agar has dissolved (approximately 1 hour). Add trace elements (previously prepared and stored in 1L containers in a freezer). Dispense approximately 240 mL of media. Plug (with caps not tight) and autoclave to sterilize (humid cycle 100 kPa a 121 °C by 20 min.). When cool, tighten the caps and label with **HX**, month and year.

VCG compatibility codes of *F. oxysporum* listed by *formae speciales* (Katan, 1999).

No.	HOST	*F. SPECIALIS*	VCGs	NUMBER
001	*Apium*	*apii*	010-012	(3)
002	*Dianthus*	*dianthi*	020-022, 025,027, 028	(6)
003	*Lycopersicon*	*lycopersici*	030-033	(4+)
004	*Medicago*	*medicaginis*	040-041	(2)
005	*Chrysanthemum*	*chrysanthemi*	050-051	(2)
006	*Vigna*	*tracheiphilum*	060	(1)
007	*Pisum*	*pisi*	070	(5? +)
008	*Citrullus*	*niveum*	080-082	(3)
009	*Lycopersicon*	*radicis-lycopersici*	090-094, 096-099	(9+)
010	*Brassica*	*conglutinans*	0100, 0101, 0104	(3?)
011	*Gossypium*	*vasinfectum*	0110-0119, 0111-0112	(12+)
012	*Musa*	*cubense*	0120-0126, 128-01224	(24+)
013	*Cucumis melo*	*melonis*	0130-0136, 0138	(8+)
014	*Elaeis*	*elaeidis*	0140, 0141	(5+)
015	*Cyclamen*	*cyclaminis*	0151-0153	(3)
016	*Phaseolus*	*phaseoli*	0161-0165	(5+)
017	*Phoenix dactylifera*	*albedinis*	0170	(1)
018	*Cucumis sativus*	*cucumerinum*	0180-0183	(6+)
019	*Lilium*	*lilii*	0190	(1)
020	*Ocimum*	*basilici*[z]	0200	(1)
021	*Matthiola*	*matthioli*[x]	0210	(1)
022	*Raphanus*	*raphani*	0220	(1)
023	*Tulipa*	*tulipae*	0230	(1)
024	*Phoenix canariense*	*canariensis*	0240	(1+)
025	*Papaver*	*papaveris*		
026	*Cucumis sativus*	*radicis- cucumerinum*	0260, 0261	(2)
027	*Beta*	*betae*		(7+)
028	*Cicer*	*ciceris*	0280	(1)
029	*Erythroxylum*	*erythroxyli*		(2)
030	*Lactuca*	*lactucum*		(1)
031	*Lupino*	*lupini*	031-	(2 ?)
032	*Solanum melongena*	*melongenae*[w]	032	(1)
033	*Spinacia*	*spinaceae*		(3)
034	*Gladiolus*	*gladioli*	0340-0343	(4)
035	*Solanum tuberosum*	*tuberoci*		(6)
036	*Ipomea*	*batatas*		(2)
037	*Nicotiana tabacum*	*nicotianae*		(2)

z: Previously VCG 016-
y: Previously VCG 001-
x: Previously included in the VCG 010
w: Previously included en in the VCG 017-
+: The new VCGs are not compatible with the established VCGs.
?: Two or more VCGs can overlap.

WORLD DISTRIBUTION OF FOC VCGS (Pérez, 2004).

VCG .	Complexes of VCGs	Distribution by countries[2]
0120	0120 - 01215	South Africa, Australia, Brazil, Costa Rica, Canary Islands, Guadeloupe, Honduras, Indonesia, Jamaica, Malaysia, Taiwan (there are isolates belonging to subtropical race 4).
0121	No	Indonesia, Malaysia, Taiwan.
0122	No	Philippines.
0123	0123 a – 0123 b	Philippines, Indonesia, Malaysia, Thailand, Taiwan, Vietnam.
0124 India,	0124-0125-0128-01220	Australia, Burundi, Brazil, Cuba, EUA, Honduras, Jamaica, Kenya, Malaysia, Malawi, Nicaragua, Philippines, Thailand, Uganda, Tanzania, Vietnam.
0125	0124-0125-0128-01220	Australia, Brazil, Jamaica, Honduras, India, Kenya, Malaysia, Philippines, Thailand, Uganda, Zaire.
0124/ 0125	0124-0125- 0128-01220	Australia, Brazil, Cuba, EUA, Honduras, India, Indonesia Jamaica, Kenya, Malaysia, Malawi, Nicaragua, Philippines, Thailand, Uganda, Vietnam.
0126	No	Honduras, Indonesia (Papua New Guinea?), Philippines
0128	0124-0125-0128-1220	Australia, Comoro's Islands, Cuba, Kenya, India, Thailand.
0129	No	Australia.
01210	No	Cuba, USA (Florida).
01211	No	Australia.
01212	No	Kenya, Tanzania, Uganda.
01213	01213-01216	Australia, Indonesia, Malaysia (tropical race 4)
01214	No	Malawi.
01215	0120-01215	Costa Rica, Indonesia, Malaysia.
01216	01213-01216	Australia, Malaysia, Indonesia (tropical race 4).
01217	No	Malaysia.
01218	No	Indonesia, Malaysia, Las Filipinas, Thailand.
01219	No	Indonesia,
01220	0124-0125-0128-1220	Australia, India, Kenya, Thailand.
01221		Thailand
01222	No	India, Kenya, Uganda, Australia (*Heliconia chartacea)*

[2] Data of Ploetz (1990 a y c), Bentley et al. (1998); Battle and Pérez, (1999; 2003); Magnaye, (1999); Singburaudom, (1999); Rutherford, (1999); Kangire and Tushemereirwe (2003), Viljoen et al. (2003), Thangavelu et al. (2003), Masdek et al., (2003).

Additional works on VCG analyses (Bentley *et al.*, 1998) have classified a set of *Foc* isolates that do not develop heterokaryons with the above listed VCGs. As a comprehensive VGG analysis comprising a global population of *Foc* is missing, new groups might be identified considering the genetic diversity of the pathogen.

REFERENCES AND BIBLIOGRAPHY USEFUL TO CONSULT

Ainsworth, G.C., 1971. Ainsworth and Bisby's Dictionary of the Fungi. 6th. Ed. Commonwealth Mycological Institute, Kew, Surrey, England, 663 pp.

Batlle A. y Pérez L., 1999. Grupos de compatibilidad vegetativa de las poblaciones de *Fusarium oxysporum* Schlecht. f. sp. *cubense* (E. F. Smith) Snyd. and Hans. presentes en Cuba. INFO*MUSA* 8 (1): 22 – 23.

Batlle, A. and Pérez, L., 2003. Population Biology *of Fusarium oxysporum f. sp. cubense* in Cuba. In: 2nd. International Symposium on Fusarium wilt on banana. PROMUSA-INIBAP/EMBRAPA. Salvador de Bahía, Brazil. 22-26 Sept.

Bentley, S., Pegg, K.G., Moore, N.Y., Davis, D.R. and Buddenhagen, I.W. 1998. Genetic variation among vegetative compatibility groups of *Fusarium oxysporum f. sp. cubense* analyzed by DNA fingerprinting. *Phytopathology* 88, 1283- 1293.

Burguess, L.W., Liddell, C.M. and Summerell, B.A. 1988. Laboratory manual for Fusarium research, 2nd Edition, University of Sydney, Australia, 156 pp.

Cove, D.J., 1976. Chlorate toxicity in *Aspergillus nidulans*: the selection and characterization of chlorate resistant mutants. *Heredity* 36, 191-203.

Correll, J.C., Klittich, C.J.R. and Leslie, J.F. 1987. Nitrate non-utilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. *Phytopath*. 77, 1640-1646.

Kangire, A. and Tushemereirwe, W. 2003. Status of *Fusarium* wilt of banana in Uganda. 2nd. International Symposium on Fusarium wilt on banana. PRO*MUSA*-INIBAP/EMBRAPA. Salvador de Bahía, Brazil, 22-26 Sept.

Katan, T., 1999. Current status of vegetative compatibility groups in *Fusarium oxysporum. Phytoparasitica* 27(1).

Leslie, J.F., 1993. Fungal vegetative compatibility. *Ann. Rev. Phytopath.* 31, 127-150.

Magnaye, L., 1999. Status of Panama disease in Philippines. In: Fusarium wilt management: Towards sustainable cultivation. Proceedings of the International Workshop on the banana Fusarium wilt disease. (Ed). Molina, A.B., Masdek, N., and Liew, K.W. INIBAP-ASNET/MARDI. Pp: 50- 57.

Masdek, N., Mahmood, M., Molina, A., Hwang, S.C., Dimyati, A., Tangaveli, R. and Omar, I., 2003. Global significance of Fusarium wilt: Asia. Abstracts of Papers 2nd. International Symposium on Fusarium wilt on banana. PROMUSA-INIBAP/EMBRAPA. Salvador de Bahía, Brazil. 22 - 26 Sept.

Pérez, L. 2004. Fusarium wilt (Panama disease) of bananas; an updating rewiew of the current knowledge of the disease and its causal fungus. Proceedings of the XV ACORBAT, Oaxaca, México. Oct 2004. Pp 1-14.

Ploetz, R.C., 1990 a. Variability in *Fusarium oxysporum f. sp. cubense*. *Can. J. Botany* 68, 1357 – 1363.

METHODS FOR STORAGE OF CULTURES of *Fusarium oxysporum*

Luis Pérez Vicente and Einar Martínez de la Parte.

1. Storage in filter paper.

Method.

- Whatman #1 (5 cm of diameter) filter papers discs are sterilized in autoclave in glass Petri plates.
- Discs are then aseptically placed on surface PDA ¼ strength media in Petri plates.
- Fusarium isolates to be stored are grown in CLA for 7-10 days.
- *F. oxysporum* mycelial plugs (3 mm diameter) are then placed on the sterile filter paper and allowed to grow for 7-10 days in the plates until the mycelial growth completely covers the filter paper dishes.
- The filter paper with the fungal growth is raised from the agar, and placed on another sterile piece of paper filter and allowed to dry for one day.
- After dry, it is cut into small pieces (5 mm diameter) and placed in cryovials.
- Cryovials are marked with the isolate number and stored at 5°C until used.

***Storage period recommended*: 3 months-1 year**

2. Storage in slants with Carnation leaves agar (CLA).

Method.

- Fresh carnation leaves not treated with agrochemicals are cut in small pieces of 8 × 3 mm before placed to dry in an oven at 70° C until dry.
- When dry, leaf fragments are placed in containers appropriate for Gamma radiation (i.e.; glass or polystyrene containers with caps or Petri dishes sealed with Parafilm). Note that Gamma radiation will degrade plastics after repeated exposures.
- Containers are placed in a Gamma cell and irradiated at a total rate of 2.5 Mega Rad.
- The sterile pieces are stored in a refrigerator at 4°C until ready to be used.
- Prepare water agar (AA), dissolving 20 g agar in a 1L of distilled water and sterilize in autoclave at 20°C for 20 min.
- After autoclaving, aliquots of 10 ml of water agar are taken and filled in 25-50 ml sterile bottles or tubes in a lamina flux bench.
- The bottles are placed lying-down with support in a tray at a 45° angle until culture media slants solidify.

- A piece of carnation leaf is placed on agar surface. The isolate is then placed close to the edge of the carnation leaf piece in the water agar and allowed to grow at 25°C for a week.
- All cultures are clearly marked with the number of isolate and stored at 5°C until ready to use.

***Storage period recommended*: 3 months -2 years.**

3. Deep freezing.

Method.

- First, a glycerol stock solution is prepared and sterilized in autoclave
- *F. oxysporum* is cultured for 7-10 days in ¼ strength PDA at 25°C
- 10 mL of 15% glycerol is pipetted over the fungal growth in a Petri plate in a sterile air flow bench cabin. The spores and some hyphae are released gently with a sterile and cool scalpel
- Aliquots of 1mL are pipette into 2 mL cryovials tubes.
- Each one of the cryovials is carefully marked and stored in cryoconservation boxes at -80°C.
- When it is necessary to recover the isolate, small amounts of the freeze solution in the cryovial are scratched with a scalpel and placed in a culture media.

***Storage period recommended*: Until 5 years.**

4. Storage in soil.

Method:

- The soil is primarily sterilized in small bottles or tubes.
- Cultures are then grown in ¼ strength PDA plates for 7-10 days.
- Distilled water (20 mL) is poured on each culture under an air lamina flow bench cabinet; spores are gently released with a sterile scalpel or spatula.
- 10 mL of spore suspension is transferred aseptically from Petri plate cultures to the soil in the glass bottles and tubes.
- All glass tubes and bottles are clearly labelled with isolate number and stored at room temperature.
- The isolate is recovered placing a small amount of soil in culture media.

***Storage period recommended*: Up to 5 years.**

5. Lyophilization of *Fusarium* cultures.

Method (Fisher *et al.* 1982. Carnation leaves as a substrate and for preserving cultures of *Fusarium* species. *Phytopathology* Volume 72 (1): 151 – 153).

- Isolates for lyophilisation are grown in CLA or PDA in Petri plates for 7-10 days. In CLA the fungus colonize leaves and sporulate.
- Some fractions of leaves or small pieces of mycelia and spores are transferred aseptically to each one of five 5 mL sterile glass vials that are tagged with the isolate number.
- An aliquot of 0.5 mL of sterile skim milk is added to each vial.
- Vials are plugged with gum plug caps with small channels that allows air evacuation.
- Vials are placed in a tray and frozen rapidly by pouring liquid N in the tray.
- A Lucita plaque is placed slightly higher than the used tray, on the upper part of the partially plugged tubes.
- A lyophilizer chamber is use for drying.
- The tray is placed in the cold plate in the dry chamber and submitted to vacuum.
- When refrigeration is complete, the heater is turned on while the samples are gradually drying
- Vials at then sealed in vacuum, inflating the gum diaphragm of chambers on the tray which press down the Lucita plate and force the gum tubes to seal the vials.
- After lyophilisation, vials are tapped, labelled and marked at -20°C.

Storage period recommended: **up to 20 years.**

INOCULATION OF *Fusarium oxysporum* f. sp. *cubense* CAUSAL AGENT OF FUSARIUM WILT IN BANANA

Miguel Angel Dita Rodriguez [(1)], Luis Pérez Vicente[(2)], Einar Martínez[(2)]

[1]Brazilian Research Agricultural Corporation – Embrapa, Brazil
[2]Instituto de Investigaciones de Sanidad Vegetal, INISAV, Cuba

Introduction

Use of resistant genotypes is the best control practice for Fusarium wilt (FW) of banana also called Panama disease. Genetic improvement plays an important role in transferring disease resistance alleles to the genetic background of elite genotypes. However, banana breeding is a complex and time-consuming process. One of the major and critical steps of the breeding programmes is the selection process. For instance, screening for FW resistance carried out at the Embrapa Cassava and Fruits is carried out in a highly-infested field area (Cordeiro et al., 1993). While 'Silk' (AAB) plants, used as susceptible control, mostly show high and consistent levels of FW (more than 90%), in some cases some plants remain symptomless, suggesting the possibility of escape. In addition, field tests are time consuming (3 years) and demanding in terms of manpower and space. Hence, a rapid and reliable method for early detection of resistant genotypes is essential. One additional bottleneck of banana breeding for FW resistance is the lack of knowledge about the genetic and molecular basis of resistance. Detailed studies to obtain such information are necessarily dependent on reliable greenhouse bioassay for Foc-banana interaction. Finally, core studies on pathogenicity, biological control and epidemiology are also dependent on reliable and standardized bioassays. Several protocols to infect banana with Foc under greenhouse conditions have been reported (Sun and Su, 1984; Mohamed et al., 2000; Smith et al., 2008, Ribeiro et al 2011, Dita et al. 2011). The following protocol describes a rapid and reliable bioassay for Foc-banana interaction, which comprises validated experiences on what have been developed so far. The objective of this protocol is produce symptoms of FW in banana and was specifically described for this training course. For protocols to be used on detailed plant-pathogen interactions for resistance screening purposes, see referred literature.

Planting Material

1. Use acclimatized 45-days-old tissue-culture plantlets of a susceptible variety ['Silk' (AAB), Gros Michel AAA, and Pisang awak (ABB) are susceptible to race 1. Bluggoe type varieties (ABB) are susceptible to race 2 and Cavendish type varieties are susceptible to tropical race 4]. Check the reaction to *Foc* of a set of banana genotypes on Table 1.

Observations: Plants should be grown in a disease-free environment with no contact with *Foc*, which may eventually provoke accidental or cross-contamination. Plants should be at least 15-25 cm high and shows no nutrient deficiency.

Inoculum production

1. Inoculate the *Foc* single-spore isolates to be studied on Petri plates with Potato-

Dextrose-Agar (PDA) half-strength for one week [For other inoculum sources see Smith *et al*. 2008; Ribeiro *et al.* 2011, Dita *et al.* 2011]

2. Collect conidia (macro and micronidias) and adjust inoculum concentration to 10^6 conidia/ml.

Inoculation procedures

Before the inoculation, a double-pot system should be prepared (Figure 13).

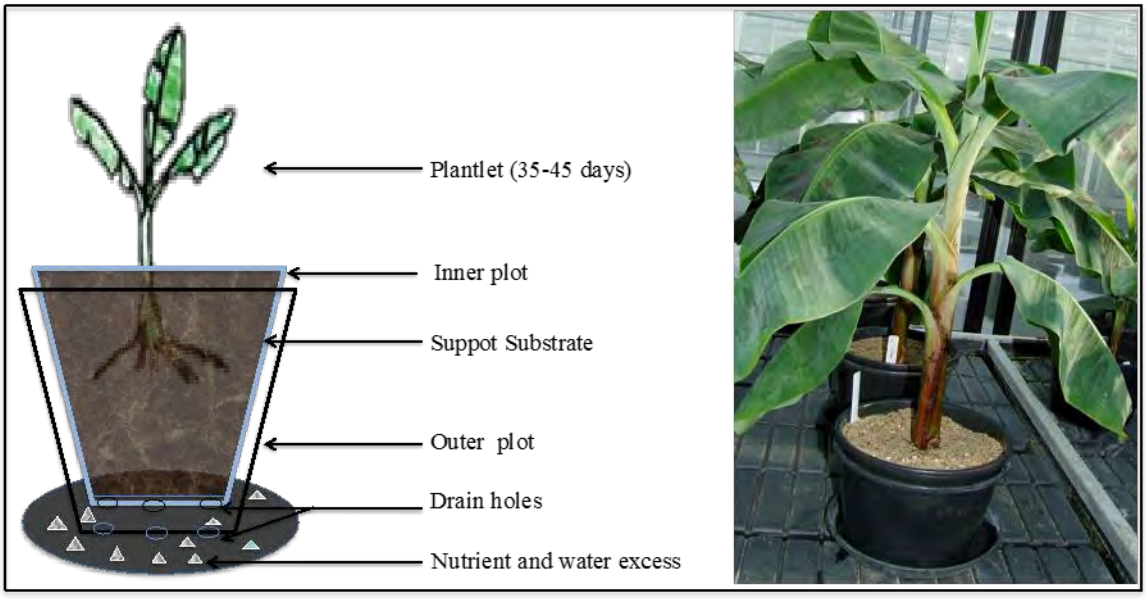

Figure 13. Double-pot system for inoculation of *Fusarium oxysporum* f. sp. *cubense* in banana.

Inoculation by root dipping

1. Remove plantlets (10 plantlets) from pots and make sure most of substrate is removed form the roots. Wash with tap water when necessary. Cut excessive root growth as necessary. Avoid plant stress during this process and keep plants with adequate water levels.
2. Prepare a recipient with an appropriate volume to cover the whole plant root systems and fill with inoculum suspension adjusted to 10^6 conidia/ml.
3. Leave the plants in contact with the inoculum suspension for 30 minutes. Then remove the plants from the inoculation recipient and allow the roots system dry out.
4. Place plants in the double-pot systems. The substrate on the inner pot should preferably be autoclaved. If autoclaved, substrate should be air-dried for at least 72 hour to allow the elimination of gases formed during sterilization process. Phytotoxic effects have been observed when using autoclaved soil on banana experiments without appropriate post-autoclave aeration.
5. Apply regular watering to maintain the substrate at field capacity level.

Evaluation consists on visual observations of typical symptom of Fusarium wilt. The incubation period (the period from inoculation to the first symptoms observation) might be variable depending on inoculation procedures, banana genotypes used, aggressiveness of the *Foc* isolate or environmental condition. Scales for both external and internal (rhizome discoloration) symptoms may be used to assess disease severity when needed (Figure 14).

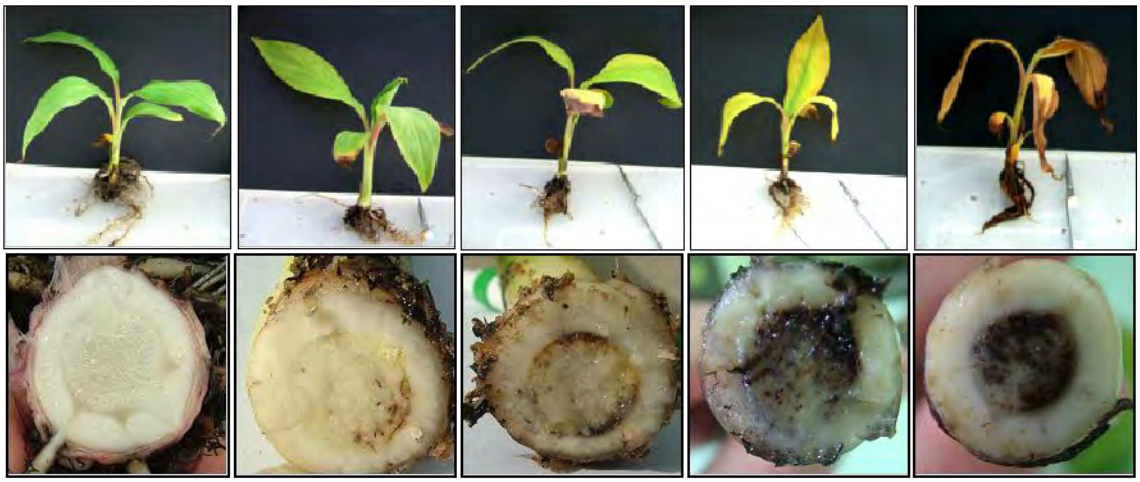

Figure 14. Scale for evaluation of Fusarium wilt of banana in greenhouse conditions based on external (upper panel) and internal symptoms (lower panel). Classes for external symptoms are: 1: No symptoms; 2: Initial yellowing mainly in the lower leaves; 3: Yellowing of all the lower leaves with some discoloration of younger leaves; 4: All leaves with intense yellowing; 5: Plant dead. Class for internal symptoms are: 1: No symptoms; 2: Initial rhizome discoloration; 3: Slight rhizome discoloration along the whole vascular system; 4: Rhizome with most of the internal tissues showing necrosis; 5: Rhizome totally necrotic.

Final recommendations:

- Avoid visiting disease-free areas such as acclimatization rooms, or other banana experiments after working in *Foc* inoculation area.
- Keep plants watered at field-capacity and the irrigation water confined to the double –pot system to avoid contamination on the greenhouse surfaces
- Once the experiment is finished, carry out extensive sterilization of pots, substrate and other tools used in the experiment. Contaminated soil/substrate should be autoclaved twice with an interval of 24 h.

REFERENCES

Cordeiro, Z., Shepherd, K., Soares Filho, W. and Dantas, J.L.L. 1993. Avaliação de resistência ao mal-do-Panamá em híbridos tetraplóides de bananeira. *Fitopatol. Bras*. 18:478-483.

Dita, M.A., Waalwijk, C., Paiva, L.V., Souza, Jr. M.T. and Kema, G.H.J. 2011. Agreenhouse bioassay for the *Fusarium oxysporum* f. sp. *cubense* x 'Grand Naine'

(Musa, AAA, Cavendish subgroup) interaction. *Acta Hort.* 897:377-380.

Mohamed, A.A., Mak, C., Liew, K.W. and Ho, Y.W. 2000. Early evaluation of banana plants at nursery stage for Fusarium wilt tolerance. p.174-185. In: A.B. Molina, N.K. Masdek and K.W. Liew (eds.), Banana Fusarium Wilt Management: towards Sustainable Cultivation, Proc. Int. Workshop on the Banana Fusarium Wilt Disease, 384 Genting Highlands Resort, Malaysia, 18-20 October 1999.

Ribeiro, L.R., Amorim, E.P., Cordeiro, Z.M., Silva, S. de Oliveira e and Dita, M.A. 2011. Discrimination of banana genotypes for Fusarium wilt resistance in greenhouse. *Acta Hort*. 897:381-385

Smith, L.J., Smith, M.K., Tree, D., O'Keefe, D. and Galea, V.J. 2008. Development of a small-plant bioassay to assess banana grown from tissue culture for consistent infection by *Fusarium oxysporum* f. sp. *cubense*. *Australas. Plant Path*. 37: 171-179.

PROTOCOLS FOR DNA EXTRACTION of *Fusarium oxysporum.*

Einar Martínez de la Parte[1], Miguel A. Dita[2] and Luis Pérez Vicente [1]

[1]Instituto de Investigaciones de Sanidad Vegetal (INISAV). Ministry of Agriculture.Cuba

[2]Brazilian Research Corporation - EMBRAPA

INTRODUCTION

A high quality DNA is essential to carry out molecular procedures. DNA extraction protocols included in this manual have been reported in studies with *Fusarium oxysporum* and/or other pathogens.

Mycelia or spores from single-spore isolates cultures are obtained from solid (media with agar) or liquid (culture in broth) media. (Check "Protocol to obtain and store single spore *Fusarium* cultures")

DNA extraction can be carried out by different protocols or by using specific DNA extraction kits following indications of the kit employed.

EXTRACTION PROTOCOL OF Lin *et al. (2008)*

This protocol is a modification of the protocol described by Dellaporta *et al*. (1983) and used by Lin *et al*. (2008) to developed molecular markers for *Foc* race 4 identification.

Reactive and solutions

Chloroform /isoamylicalcohol

Potassium acetate 5M

Sodium acetate 3M (pH 5.4)

Isopropanol (ice cold)

Ethanol absolute (ice cold)

Ethanol 75%

TE Buffer (10mM Tris, 1mM EDTA, pH 8.0)

TNE Extraction buffer

Sodium dodecyl sulphate (SDS) 1%

Na_2EDTA 50mM

NaCl 50mM

Tris-HCl (pH 8.0) 100mM

β-mercaptoethanol 8 µM

1 RNAase 10 µg/mL

Method

1. Grind the frozen mycelium (1g) of each sample in liquid nitrogen into a fine powder. Transfer to a 1.5 mL tube.
2. Add 5mL of modified TNE buffer.
3. Incubate at65 ºC for 30 minutes.
4. Add 0.33 volumes (~1.65 mL) of5 M potassium acetate. Mix gently
5. Centrifuge at 20000x g for 5 minutes at4°C.
6. Transfer the supernatant to a clean tube
7. Add equal volume of isopropanol to precipitate crude DNA.
8. Incubate at−20°C for 20 minutes.
9. Centrifuge at 20000x g for 20 minutes at4°C.
10. Eliminate supernatant carefully.
11. Re-suspend the pellet in 200 µL of H_2O and add 200 µL of chloroform/isoamylic alcohol (24:1). Mix thoroughly.
12. Centrifuge at20000x g for 5 minutes at4°C.
13. Transfer upper aqueous phase to a fresh tube (1. 5mL).
14. Add 0.1 volume (~20 µL) of3M sodium acetate (pH 6.5) and 2.5 volumes(~500 µL) of absolute ethanol. Mix gently.
15. Centrifuge at 20000x g for 5 minutes at4°C.
16. Carefully discharge supernatant to avoid loses the pellet.
17. Wash with 300 µL of ethanol 75%.
18. Centrifuge at 20000x g for 5 minutes at4°C.
19. Discharge supernatant and dry the pellet.
20. Re-suspend in 1× TE buffer.

EXTRACTION PROTOCOL OF Lin* et al*., (2001).

This protocol is fast, simple and yields high molecular weight DNA. It has been used with good results to extract DNA of sugarcane orange rust (*Puccinia kuehnii*), soybean Asian rust (*Phakokpsora pachyrhizi*) (Pérez-Vicente *et al*., 2009 a; b) and *Fusarium oxysporum* f. sp. *cubense* (Pérez Vicente *et al*., 2014 in edit). It is recommended for DNA extraction from plant tissue.

Reactive and solutions

Phenol/chloroform/isoamylic alcohol (25:24:1)

Chloroform/isoamylic alcohol (24:1; v: v)

Ammonium acetate 1M

Absolute ethanol

Ethanol 70%

Polyvinyl pyrrolidone PVP 10%

SDS 20%
B-mercaptoethanol
RNAase A

Extraction Buffer

EDTA pH-8 50mM

Tris-HCl (pH 8.0) 100mM

MNaCl 500ml

Method

1. Grind 100 mg of sample. Transfer to a new tube of 1.5 mL.
2. Add 470µL of extraction buffer, 60 µL of SDS 20%, 60 µL of PVP 10%, 12 µL of ß-mercaptoethanol. Mix gently and incubate at 65°C for 15 min.
3. Centrifuge at 12000 g for 10 min at room temperature.
4. Transfer supernatant to a new tube.
5. Add equal volume of phenol-chloroform-isoamylic alcohol (25:24:1), mix by inverting the tube.
6. Centrifuge at 12000xg for 4 min at room temperature.
7. Transfer upper aqueous phase to a fresh tube.
8. Add equal volume of chloroform-isoamylic alcohol (24:1).
9. Centrifuge at 12000 x g for 4 min at room temperature. Transfer upper aqueous phase to a fresh tube.
10. Add 2 µL of RNAase A, incubate at 37°C for 10 min.
11. For DNA precipitation, add 300 µL of isopropanol (cold). Incubate at –20°C for 10 min.
12. Centrifuge at12000 g for 10 min and pour out the supernatant.
13. Wash the pellet (× 2) with 600µL of ethanol 70% (cold). Centrifuge at 12000x g for 5 min.
14. Dry the pellet and re-suspend in 50 µL of 1X TE buffer. Keep at –20°C.

PROTOCOL FOR PLANT DNA EXTRACTION

This protocol constitutes a modification of protocols described by Sreelakshmi *et al.* (2010) and Zhou *et al*. (2010). It is fast, simple and yields high molecular weight DNA. It has been used with good results to extract DNA from citrus and other crops (Canales *et al*., 2014 in edit).

a) **Extraction Buffer:** 100 mM Tris HCl pH 7.5; 500 mM de NaCl; 50 mM EDTA pH 8.0; 1.25% SDS (p/v); 60 µL PVP; 0.2M ß mercaptoetanol.

1. Grind 100 mg of sample (≈10 pseudostem vascular strand of 1cm length from symptomatic plants) in liquid nitrogen. Transfer to a tube of 2 mL.
2. Add 20 mg PVPP and 750 µL of buffer extraction [(a)].
3. Incubate at 65°C for 30 min and for 5 min at room temperature
4. Add 400 mL of NH_4Ac 6M cold. Incubate on ice for 15 min.
5. Centrifuge 10 min at 12000 x g
6. Transfer upper aqueous phase (≈650 µL) to a fresh tube of 1.5 mL.
7. Add 4 µL of RNAse; incubate at 37°C for 30 min.
8. Add equal volume (650 µL) of isopropanol cold.
9. Incubate at -20 °C (for at least 2 hours) or at -30°C for half hour. Centrifuge at 12,000 x g for 10 min and discharge supernatant.
10. Wash with Ethanol 70% (1mL). Centrifuge at 12000 x g for 5 min.
11. Repeat the wash with Ethanol 70% (0.5 mL). Centrifuge 1 min and discharge supernatant.
12. Dry the pellet and re-suspend in 100-200 µL de TE; incubate at 4 °C overnight.

Several extraction kits in the market are used to yield high quality DNA. Some of them as DNeasy Mini Plant kit (QIAGEN), Gentra PureGene cell kit (QIAGEN), ISOPLANT DNA extraction kit (Nippon Gene, Tokyo, Japan) and Wizard Magnetic DNA Purification System for Food kit (PROMEGA) have been successfully used in DNA extractions in *Fusarium oxysporum* studies (Hirano and Arie, 2009; Leong *et al*., 2009; 2010; Dita *et al*., 2010; Elliot *et al*., 2011).

REFERENCES

Dellaporta, S. L., Wood, J. & Hicks, J. B. (1983). A plant DNA mini-preparation: version II. *Plant Molecular Biology Reporter*. 1:19–21. doi:10.1007/BF02712670.

Dita, M.A., Waalwijk, C; Buddenhaguen, I.W., Souza, M.T., Kema, G.H.J. (2010). A molecular diagnosis for tropical race 4 of the banana Fusarium wilt pathogen. Plant Pathology. Doi: 1111/j.1365-3059.2009.02221.x

Elliott, M. L., Des Jardin, E. A., O'Donnell, K., Geiser, D. M., Harrison, N. A., and Broschat, T. K. (2010). *Fusarium oxysporum* f. sp. *palmarum*, a novel forma specialis causing a lethal disease of *Syagrus romanzoffiana* and *Washingtonia robusta* in Florida. Plant Dis. 94:31-38.

Hirano, Y. and Arie, T. (2009). Variation and phylogeny of *Fusarium oxysporum* isolates based on Nucleotide Sequences of Polygalacturonase Genes. *Microbes Environ.*, 24(2): 113-120.

Leong, S.K.; Latiffah, Z. and Baharuddin. S. (2009). Molecular Characterization of *Fusarium oxysporum* f. sp.*cubense* of Banana. *American Journal of Applied Sciences* 6 (7): 1301-1307.

Leong, S.K.; Latiffah, Z. and Baharuddin. S. (2010).Genetic Diversity of Fusarium oxysporum f sp. Cubense isolates from Malaysia. *African Journal of Microbiology Research*, 4 (11):1026-1037.

Li RC, Ding ZS, Li LBandKuang TY.2001. A rapid and efficient DNA minipreparation suitable for screening transgenic plants. *Plant Molecular Biology Reporter***19**, 379a-379c.

Pérez-Vicente L., Martínez-de la Parte E., Pérez-Miranda M., Martín-Triana E.L., Borrás-Hidalgo O. and Hernández-Estévez I. 2009a. First report of Asian rust of soybean caused by *Phakopsora pachyrhizi*in Cuba. BSPP *New Diseases Report*, en prensa.

Pérez-Vicente L., Martín-Triana E.L., Barroso F., Martínez-de la Parte E., Borrás-Hidalgo O. and Hernández Estévez I. 2009b. Definitive identification of orange rust of sugarcane caused by *Puccinia kuehnii* (W. Krüger) e. j. Butler in Cuba.BSPP New Diseases Report, vol. 20 ().

Sreelashmi, Y., Gupta, S., Bodanapu, R., Singh Chauhan, V., Hanjabam, M., Thomas, S.,Mohan, V., Sharma, S., Srinivasan, R., and Sharma R. 2010. NEATTILL: A simplified procedure for nucleic acid extraction from arrayed tissue for tilling and other high-throughput reverse genetic applications. *Plant Methods*, 6:3. doi:10.1186/1746-4811-6-3

Zhou, L.,Powell, C.A.,Hoffmann, M.T., Li., W., Fan, G., Liu, B., Lin, H. Duan, Y. 2011. Diversity and plasticity of the intracellular plant pathogen and insect symbiont Candidatus *Liberobacter asiaticus*" as revealed by hyper variable prophague genes with intragenic tandem repeats. *Applied and Environmental Microbiology*, 77: 6663-6675.

PROTOCOLS FOR MOLECULAR IDENTIFICATION OF *Fusarium oxysporum f. sp. cubense*

Einar Martínez-de la Parte[1], Luis Pérez-Vicente[1] and Miguel A. Dita Rodríguez[2].

1) Instituto de Investigaciones de Sanidad Vegetal (INISAV) Ministry of Agriculture. Cuba

2) Brazilian Research Agricultural Corporation - EMBRAPA

INTRODUCTION.

Fusarium oxysporum is a complex of species that comprises morphologically indistinguishable *formae speciales,* which may be pathogenic or non-pathogenic strains.

Panama disease or Fusarium wilt of banana, caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*), is one of the most important fungal disease of banana and a serious constraint to both commercial production of banana and cultivation for subsistence agriculture (O'Donnell *et al*., 1998). The fungus has a long latent period and symptoms can appear long after infection. Inoculation procedures for determining pathogenicity and "races" are time-consuming. Infected planting material, contaminated soil attached to farming tools, machinery and footwear are the main ways of disease dissemination. Early and fast identification to delimiting disease spreading and establish appropriate quarantine and disease management procedures is a top priority. To achieve these actions, an accurate and quick diagnostic protocol of *Foc* is essential. Rapid and reliable diagnostic can avoid the propagation of infected, but symptomless planting materials and support the development of epidemiology-based management procedures (Lin *et al*., 2008, Dita et al 2013).

Disease diagnostic and pathogen identification by traditional methods including pathogen isolation and characterization through inoculation tests are laborious and time-consuming (Alves-Santos *et al*., 2002). Identification of *Foc* was usually based on morphological characteristics, which require an accurate knowledge of *Fusarium* taxonomy (Jurado *et al*., 2006). Though morphology-based methods still play an important role in phytopathological diagnostic of these species, advanced molecular methods are able to categorize at race level.

Polymerase Chain Reaction (PCR) technique, described in the 1980s, has revolutionized molecular biology (Saiki *et al*., 1985; Mullis y Faloona, 1987) and phytopathological diagnostic procedures.

PCR-based diagnostics have a high analytic sensitivity in differentiating *Fusarium oxysporum* strains (Grajal-Martín *et al*., 1993; Assigbetse *et al*., 1994; Kelly *et al*., 1994; Bentley *et al*., 1995; Migheli *et al*., 1995; 1998; Alves-Santos *et al*., 2002; Lin *et al*., 2008). These methods allow processing a great number of samples in short time periods and the pathogen detection in complex mixes even when mycelia is not detected under microscope (Jurado *et al*., 2006).

I. **Genomic regions used in phylogenetic analysis and characterization of fungi**

DNA ribosomal nuclear genes: The ribosomal DNA (DNAr) has an important function in protein synthesis and its variability can reflect the genomic evolution (Otero *et al*., 2004) and also provide a useful intra and inter polymorphism in eukaryotic organisms (Kim *et al*., 2001). Within nuclear DNA, ribosomal genes are in multiple copies separated by non-codifier spacer. Each repetitive unit of rDNA consists in 18S, 5.8S and 28S genes of rRNA and two Internal Transcribed Spacer (ITS1 and ITS2) that are located between these genes (Figure 1). Major intergenic spacers (IGS, intergenic spacer) or non-transcribed spacers (NTS) are found between the regions codifying for larger and minor subunits of consecutive cistrons (Reed *et al*., 2000). The IGS that separates repetitive units of DNAr are the spacer regions that evolve faster and that´s why narrowly related spaces can show a considerable diversity, showing frequent variations with regard to length and sequences (Hills *et al*., 1991) (Figure 15).

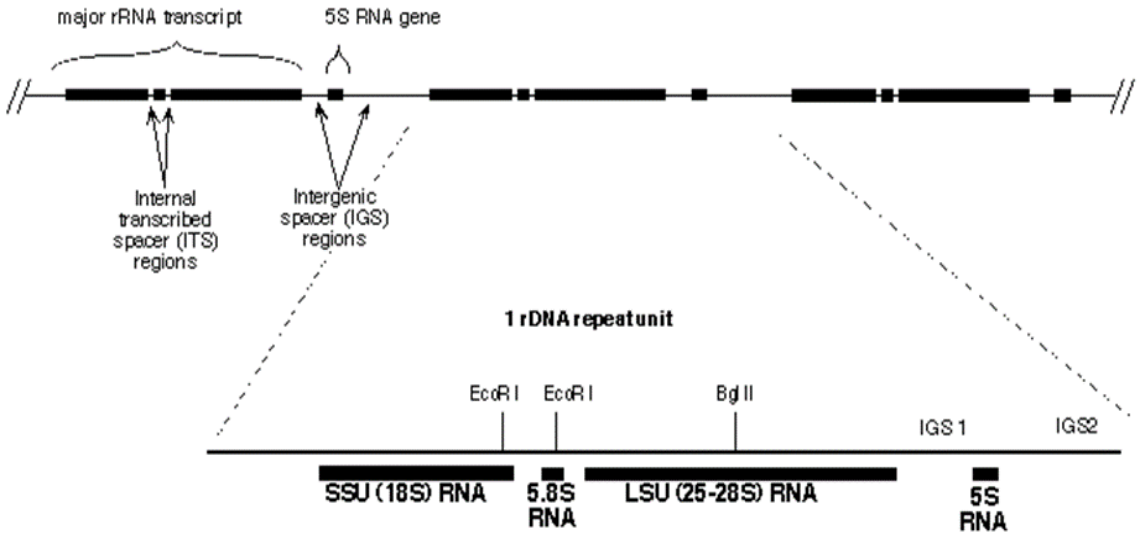

Figure 15. Structure of ribosomal DNA (rDNA) with some sites of cut of restriction enzymes (reproduced from Vilgalys *et al*., 1994).

Every repetitive unit of rDNA contains regions that codify for 18S, 5.8S y 28S genes of RNAr as well as for the two transcribed internal spacers (**I**nternal **T**ranscribed **S**pacer; ITS1 and ITS2) that are between these genes (Figure1). Even when not translated inside of the protein, ITS regions have a critical role in functional rRNA (Sugimoto *et al*., 2001). ITS constitute an ideal target for specific primers development due to are highly variable in length and nucleotide development, presenting differences between species and in occasions in the same specie as shown in *Metharrizium* (Entz *et al*., 2005). At intraspecific level however the variability of sequences is low or not detected (Lee and Taylor, 1992).

The ITS sequences are the ideal targets for specific primers development due to their high number of copies, their relative fast evolution and being highly variable in length and sequences among closely- related species, allowing differentiation of species or of isolates of the same species. (Esteve-Zarzoso *et al.*, 1999; Entz *et al*., 2005). These regions are now the ones more widely sequenced of fungal DNA.

The rDNA genes and spacer regions have been extensively employed in genetic studies with fungi to examine the relationship between genera and species closely related or of the same species (Moore and Frazer, 2002). ITS have been used frequently in phylogenetic studies wherever used rDNA (Lee and Taylor, 1992; Bidochka *et al*., 1999; Collins *et a*l., 2003; Fahleson *et al*., 2003; Sugimoto *et al*., 2003). Recent studies indicate however, that intraspecific heterogeneity of rDNA is higher in IGS region and hence, this region has been used for characterization and discrimination of fungal species. such as *Verticillum* spp. (Collins *et al*., 2003; Sugimoto *et al*., 2003, Qin *et al*., 2006), *Fusarium* spp. (Edel *et al.,* 1995; Kim *et al*., 2001; 2005; Cai *et al*., 2003; Lori *et al*., 2004) and *Sclerotinia* spp. (Freeman *et al*., 2002) among others.

Besides DNAr, other genes have been frequently used in phylogenetic studies. The **ß-tubulin gene** has been successfully used in phylogenetic studies among fungi (Moore and Frazer, 2002), including phytopathogenic species such as Fusarium spp. (Appel and Gordon, 1996; O´Donnell *et al.,* 2000; Skovgarard *et al*., 2001; Kim *et al*., 2005), *Phytophthora* spp. (Kroon et al., 2005) and Verticillium spp. (Qin *et al*., 2006; Otero *et al*., 2004).

Extrachromosomic information can be useful in the clarification of taxonomic positions (Collins *et al*., 2003; Otero *et al*., 2004; Skovgarard *et al.,* 2003):

LSU and SSU rRNA. The mitochondrial genome has genes that codify for mitochondrial proteins, as well as for all tRNA and rRNA subunits (LSU y SSU rRNA). Mitochondrial RNA genes have evolved in fungi sixteen times faster than nuclear rDNA, so inter and intra specific variations can be potentially detected and is the reason why they are used together with ITS and IGS rDNA regions (Skovgarard et al., 2001; Kroon et al., 2005).

Enterobacterial Repetitive Intergenic Consensus (ERIC). Primers ERIC 1R and 2F are based in sequences of enterobacterial consensus repetitive regions (ERIC, Versalovic et al. 1991) and have been used to group isolates of *Fusarium oxysporum* (Edel et al. 1995; Smith-White et al., 2001) and *F. oxysporum* f. sp. *cubense* characterization (Leong et al., 2009).

Elongation factor -1α gen (EF-1α). Other markers used in genetic variability analysis and phylogenetic analysis are the elongation factor-1α gen (EF-1α, O´Donnell et al., 1998) and elements of transposition or transposons that seems to be highly distributed in *Fusarium oxysporum* and that show a high level of genetic variability (Hua-Van et al., 2001).

II. Genes commonly used in phylogenetic analysis and characterization of *Fusarium oxysporum* f. sp. *cubense.*

Different studies have been carried out to determine variability of *Fusarium oxysporum* species complex (O'Donnell *et al*., 1998). The genome regions more useful to develop specific markers have been the sequences of the Inter-Genic Spacer (IGS) and Internal Transcribed Spacer (ITS) of ribosomal operon, the elongation translation factor-1α (TEF-1α), histone (H3) genes and mitochondrial β-tubulin genes, (*tub-2)*

Lin *et al*. (2008) developed a molecular marker for the identification of *Foc* race 4. These authors achieved the specific detection of *Foc* race 4 by PCR amplification with primers Foc-1(5′-CAGGGGATGTATGAGGAGGCT-3′) and Foc-2 (5′-GTGACAGCGTCGTCTAGTTCC-3′). *Foc* race 4 was detected on DNA samples from

both pure isolates and infected plant material. This method yields an amplification product of 242 bp that is specific to *Foc* race 4 (both subtropical and tropical). Dita *et al*. (2010) demonstrate that this primer set reacted with isolates of 10 different VCGs (0120, 0121, 0122, 0126, 0129, 01210, 01211 and 01215) including those belonging to the 01213 group, which is indicative of tropical race 4 is (TR4). Although that method can detects Foc TR4, it is not specific for TR4 and can react with isolates present in Latin American and the Caribbean, which are not pathogenic to Cavendish under tropical conditions.

Based on two single nucleotide polymorphisms present in the IGS region of *Foc*, Dita *et al*. (2010), developed a PCR-based diagnostic tool to specifically detect isolates from TR4 (VCG 01213). Primers FocTR4-F (5´-CACGTTTAAGGTGCCATGAGAG-3´) and FocTR4-R (5′-GCCAGGACTGCCTCGTGA-3′) yield an amplification product of 462 bp specific to VCG 01213 (Foc TR4). Validation of the specificity of these primers involved TR4 isolates from different geographic regions, as well as *Foc* isolates from 19 other VCGs, other fungal plant pathogens and DNA samples from infected tissues of the Cavendish banana cultivar Grand Nain (AAA).

Pérez *et al*. (2012), using primers developed from IGS sequences, developed a real time PCR diagnostic method able to differentiate isolates of Foc TR4, Foc R1 and Foc R2 races present in Cuba.

In the present manual PCR and qPCR methods for diagnostic of *Foc* TR4 and R1 and R2 are described.

PCR IDENTIFICATION OF *Foc* TR4 (Dita *et al*., 2010).

For PCR identification of *Foc* TR4 by PCR with primers FocTR4-F/FocTR4-R we should follow the work scheme showed in Figure 16.

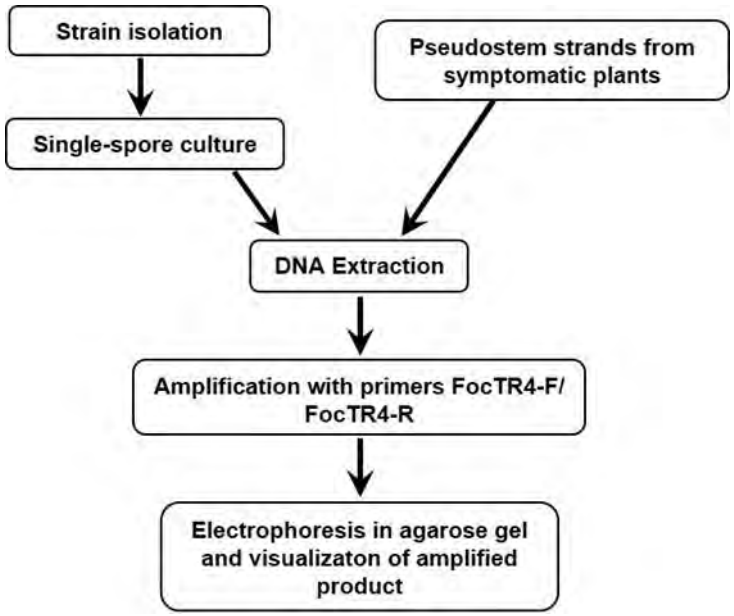

Figure 16**.** Work scheme for PCR identification of *Foc* TR4 with primers FocTR4-F/FocTR4-R.

DNA Extraction

For DNA extraction from *Foc* strains, Dita *et al*. (2010; 2011) used the Wizard Magnetic DNA Purification System for Food kit (Promega). Nevertheless, other methods can be used for DNA extraction (see "DNA extraction protocols for *Fusarium oxysporum* strains"). The selection of DNA extraction protocol depends on the equipment and reactive available in the laboratory.

Amplification reaction

Reaction conditions

For a final volume of 25 µL, add:

DNA	2 µL.
primer FocTR4-F	1.0 µL (10 µM).
primer FocTR4-R	1.0 µL (10 µM).
Buffer Pfu	2.5 µL.
DNTPs	0.5 µL (4 µM).
Taq DNA Pol Pfu	0.25 µL.
Water	17.75 µL.

DNA is amplified with primers FocTR4-F/FocTR4-R and the following program:

1. Initial denaturation 95°C for 5 min.
2. Denaturation 95°C for 1min.
3. Annealing 60°C for 1min. } 30 cycles
4. Extension 72°C for 3min.
5. Final extension 72ºC for 10 min.

Electrophoresis

PCR products are subjected to an agarose gel (1.5%) electrophoresis. With primers FocTR4-F/FocTR4-R a 463 pb amplification product will be obtained specifically for *Foc* TR4 (see Dita *et al*., 2010.).

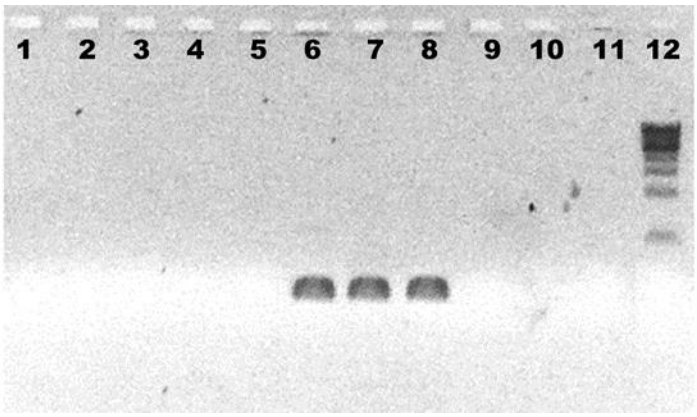

Figure 15. DNA amplification of six Foc VCGs with FocTR4-F / FocTR4-R primers (Dita *et al*., 2010; 2011). Line 1- Nit M-24322 (0120), Line 2- Nit M- PHL-18 (0123), Line 3- MoGU3 (0124), Line 4- SC-3 (0128), Line 5-CAM-3 (01210), **Line 6- Nit M- CV-3-5 (01213), Line 7- Nit M-CV-1-5 (01213), Line 8- Nit M-Indo 19-1 (01213)**, Line 9- Nit M-Mal 25A (0124/0125), Line 10- *F. oxysporum* from necrotic banana roots, Line 11- *F. pallidoroseum*, Line 12- 1 DNA ladder plus. Number between parentheses indicate VCG.

IDENTIFICATION OF *FOC* RACES USING REAL TIME PCR.

For DNA extraction from fungal strains, several methods can be used (see "DNA extraction protocols for *Fusarium oxysporum* strains").

Amplification reaction

Pérez-Vicente *et al*. (2013) developed two primer combinations for *Foc* races TR4 (qFocR4T-f and qFocR4T-r1) and R1/R2 (qFocR1R2-f and qFocR1R2-r) identification by qPCR.

Reaction conditions

Components	Final volume	
	15 µL	**20 µL**
DNA	3 µL.	3 µL.
primer combination*	1.0 µL (10 µM).	0.75 µL (10 µM).
SYBR	10 µL	7.5 µL
Water	6.0 µL.	3.75 µL.

*primer combination for TR4= qFocR4T-f + qFocR4T-r1

primer combination for R1/R2= qFocR1R2-f + qFocR1R2-r

DNA is amplified with primers qFocR4T-f and qFocR4T-r1 and the following program:

1) 95°C for 15 min.
2) 94°C for 15 sec.
3) 69°C for 30 sec. } 30 cycles
4) 72°C for 30 sec.

In case of R1/R2 detection with qFocR1R2-f and qFocR1R2-r primers the program will be:

1) 95°C for 15 min.
2) 94°C for 15 sec.
3) 57°C for 30 sec. } 22 cycles
4) 72°C for 30 sec.

REFERENCES

Alves-Santos F.M., Ramos, B., García-Sánchez, M.A., Eslava, A.P. y Díaz-Mínguez, J.M. 2002. A DNA-based procedure for in plant detection of *Fusarium oxysporum* f. sp. *phaseoli*. *Phytopathology* 92: 237-244.

Appel, D.J. and Gordon. T.R. 1996. Relationship among pathogenic and nonpathogenic isolates of *Fusarium oxysporum* based on the partial sequence of the Intergenic Spacer Region of the ribosomal DNA. *MPMI*. 9(2):125-136.

Assigbetse, K.B., Fernández, D., Dubois, M. P., and Geiger, J.P. 1994. Differentiation of *Fusarium oxysporum* f. sp. *vasinfectum* races on cotton by random amplified polymorphic DNA (RAPD) analysis. *Phytopathology* 84:622-626.

Bentley, S., Pegg, K. G., and Dale, J.L. 1995. Genetic variation among a worldwide collection of isolates of *Fusarium oxysporu*m f. sp. *cubense* analyzed by RAPD-PCR fingerprinting. *Mycol. Res*. 99:1378-1384.

Bidochka, M.J., St. Leger, R.J., Stuart, A. and Gowanlock, K. 1999. Nuclear rDNA phylogeny in the fungal genus *Verticillium* and its relationship to insect and plant virulence, extracellular proteases and carbohydrases. *Microbiology*. 145, 955–963.

Cai, G., Gale, L.R., Schneider, R.W., Kistler, H.C., Davis, R.M., Elias, K.S., and Miyao, E.M. 2003. Origin of race 3 of *Fusarium oxysporum* f. sp. *lycopersici* at a single site in California. *Phytopathology* 93:1014-1022.

Collins, A., Okoli, C.A.N., Morton, A., Parry, D., Edwards, S.G., and Barbara, D.J. 2003. Isolates of *Verticillium dahliae* pathogenic to crucifers are of at least three distinct molecular types. *Phytopathology* 93:364-376.

Dellaporta, S.L., Wood, J. & Hicks, J.B. 1983. A plant DNA mini-preparation: version II. *Plant Molecular Biology Reporter*. 1:19–21. doi:10.1007/BF02712670.

Dita, M.A., Waalwijk, C., Buddenhagen, I.W., Souza Jr., M.T. and Kema, G.H.J., 2011. Corrigendum. *Plant Pathology* 60: 384.

Dita, M.A., Waalwijk, C., Buddenhaguen, I.W., Souza, M.T., Kema, G.H.J. 2010. A molecular diagnosis for tropical race 4 of the banana *Fusarium* wilt pathogen. *Plant Pathology*. Doi:1111/j.1365-3059.2009.02221.x

Edel, V., Steinberg, C., Avelange, I., Laguerre, G. and Alabouvette, C. 1995. Comparison of three molecular methods for the characterization of Fusarium oxysporum strains. *Phytopathology*. 85:579-585.

Entz S.C., Johnson, D.L. and Kawchuk L.M. 2005. Development of a PCR-based diagnostic assay for the specific detection of the entomopathogenic fungus *Metarhizium anisopliae* var. *acridum*. *Mycol. Res*. 109 (11):1302–1312.

Esteve-Zarsoso, B., Belloch, C., Uruburu, F. and Querol, A. 1999. Identification of yeast by RFLP analysis of 5.8S rRNA gene and the two ribosomal internal transcribed spacers. *International Journal of Systematic Bacteriology.* 49: 329-337.

Fahleson, J., Lagercrantz, U., Hu, Q., Steventon, L. A., and Dixelius, C. 2003. Estimation of genetic variation among *Verticillium* isolates using AFLP analysis. *Eur. J. Plant Pathol*. 109: 361-371.

Freeman, J. Ward, E., Calderon, C. and McCartney, A. 2002. A Polymerase Chain Reaction (PCR) Assay for the Detection of Inoculum of *Sclerotinia sclerotiorum*. *European Journal of Plant Pathology*, 108 (9): 877-886.

Grajal-Martín, M.J., Simon, C.J., and Muehlbauer, F.J. 1993. Use of random amplified polymorphic DNA (RAPD) to characterize race 2 of *Fusarium oxysporum* f. sp. *pisi*. *Phytopathology* 83:612-615.

Hills, D.M. and Dixon, M.T. 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. *Q. Rev. Biol.* 66, 411-453.

Hua-Van, A., Langin, T. and Daboussi, M.J. 2001. Evolutionary history of the *Impala* Transposon in *Fusarium oxysporum Mol. Biol. Evol.* 18(10):1959–1969.

Jurado, M., Vázquez, C., Marín, S., Sanchis, V., & González-Jaéna, M. T. 2006. PCR-based strategy to detect contamination with mycotoxigenic *Fusarium* species in maize. Systematic and *Applied Microbiology*, 29, 681–689.

Kelly, A., Alcalá-Jiménez, A.R., Bainbridge, B.W., Heale, J.B., Pérez-Artés, E., and Jiménez-Díaz, R. M. 1994. Use of genetic fingerprinting and random amplified polymorphic DNA to characterize pathotypes of *Fusarium oxysporum* f. sp. *ciceris* infecting chickpea. *Phytopathology* 84:1293-1298.

Kim H.J. Choi Y.K. and Min, B.R. 2001. Variation of the Intergenic Spacer (IGS) Region of Ribosomal DNA among *Fusarium oxysporum* formae speciales. *Journal of Microbiology*. 39(4): 265-272.

Kim, Y., Hutmacher, R.B., and Davis, R.M. 2005. Characterization of California isolates of *Fusarium oxysporum* f. sp. *vasinfectum. Plant Disease*. 89: 366-372.

Kroon, L.P.N.M., Bakker, F.T., van den Bosch, G.B.M., Bonants, P.J.M., and Flier, W.G. 2005. Phylogenetic analysis of *Phytophthora* species based on mitochondrial and nuclear DNA sequences. *Fungal Genet. Biol*. 41:766-782.

Lee, S.B. and Taylor, J.W. 1992. Phylogeny of five fungus-like proctistan *Phytophthora* species, inferred from internal transcribed spacer of ribosomal DNA. *Mol. Biol. Evol.* 9:636-653.

Leong S.K., Latiffah, Z. and Baharuddin, S. 2009. Molecular Characterization of *Fusarium oxysporum f. sp. cubense* of Banana. *American Journal of Applied Sciences* 6 (7): 1301-1307

Lin, Y.H., Chang J.Y., Liu E.T., Huang J.W and Chang P.F.L. 2008. Development of a molecular marker for specific detection of Fusarium oxysporum f. sp. cubense race 4. *Eur J Plant Pathol*. DOI 10.1007/s10658-008-9372-4.

Lori, G., Edel-Hermann, V., Gautheron, N., and Alabouvette, C. 2004. Genetic diversity of pathogenic and nonpathogenic populations of *Fusarium oxysporum* isolated from carnation fields in Argentina. *Phytopathology* 94:661-668.

Migheli, Q., Berio, T., Gullino, M.L., and Garibaldi, A. 1995. Electrophoretic karyotype variation among pathotypes of *Fusarium oxysporum* f. sp. *dianthi*. *Plant Pathol.* 44:308-315.

Migheli, Q., Briatore, E., and Garibaldi, A. 1998. Use of random amplified polymorphic DNA (RAPD) to identify races 1, 2, 4 and 8 of *Fusarium oxysporum* f. sp. *dianthi* in Italy. *Eur. J. Plant Pathol.* 104:49-57.

Moore, D., and Frazer, L.N. 2002. Essential Fungal Genetics. Springer Verlag, UK.

Mullis, K.B. and Faloona, F. 1987. Specific synthesis of DNA *in vitro* via polymerase catalyzed chain reaction. *Methods Enzymol.* 55:335-350.

O'Donnell, K., Kistler, H., Cigelnik, E. and Ploetz, R.C. 1998. Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. *Applied Biological Sciences*. *Proc. Natl. Acad. Sci. USA*. 95: 2044–2049.

O'Donnell, K., Kistler, H.C., Tacke, B.K., and Casper, H.H. 2000. Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium*

graminearum, the fungus causing wheat scab. *Proc. Natl. Acad. Sci.* USA 97:7905-7910.

Otero, L., Ducasse, D. & Miller, R.N.G. 2004. Variability in ribosomal DNA genic and spacer regions in *Verticillium dahliae* isolates from different hosts. *Fitopatologia Brasileira* 29:441-446.

Qin, Q.M., Vallad, G.E., Wu, B.M., and Subbarao, K.V. 2006. Phylogenetic analyses of phytopathogenic isolates of *Verticillium* spp. *Phytopathology* 96:582-592.

Qi, Y.X, Zhang, X., Pu, J. J., Xie, Y. X., Zhang, H.Q. and Huang, S. L. 2008. *Australasian Plant Disease Notes* 3, 46-47

Reed, K.M., Hackett, J.D. and Phillips. R.B. 2000. Comparative analysis of intra-individual and inter-species DNA sequence variation in *Salmonid* ribosomal DNA cistron. *Gene* 249, 115-125.

Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A., Arnheim, N. 1985. Primer-directed enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230, 1230-1350.

Shen, Z., Zhong, S., Wang, Y., Wang, B., Mei, X., Li, R., Ruan, Y. and Shen Q. *European Journal of Soil Biology* 57 (2013) 1- 8

Skovgaard, K., Nirenberg, H. I., O'Donnell, K., and Rosendahl, S. 2001. Evolution of *Fusarium oxysporum* f. sp. *vasinfectum* races inferred from multigene genealogies. *Phytopathology* 91:1231-1237.

Skovgaard, K., Rosendahl, S, O'Donnell, K. and Nirenberg, H.I. 2003. *Fusarium commune* is a new species identified by morphological and molecular phylogenetic data. *Mycologia,* 95(4): 630–636.

Smith-White J. L., Gunn L. V. and Summerell B. A. 2001. Analysis of diversity within *Fusarium oxysporum* populations using molecular and vegetative compatibility grouping. *Australasian Plant Pathology.* 30:153–157

Sreelakshmi, Y., Gupta, S., Bodanapu, R., Chauhan, V., Hanjabam, M., Thomas, S., Mohan, V. Sharma, S., Srinivasan, R. and Sharma, R. 2010. NEATTILL: A simplified procedure for nucleic acid extraction from arrayed tissue for TILLING and other high-throughput reverse genetic applications**.** *Plant Methods*, 6:3.

Sugimoto M, Koike M and Naga H. 2001. Ribotyping of Entomopathogenic *Verticillium lecanii* in Japan. *Phytoparasitica* 29 (5):1-8

Sugimoto, M., Koike, M., Hiyama, N. and Nagaob, H. 2003. Genetic, morphological, and virulence characterization of the entomopathogenic fungus *Verticillium lecanii*. *Journal of Invertebrate Pathology* 82:176–187

Versalovic, J., Koeuth, T., Lupski, J.R. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acid Research* **19**, 6823–6831.

Vilgalys, R. Hopple, J.S. and Hibbett D.S. 1994. Phylogenetic implications of generic concepts in fungal taxonomy: The impact of molecular systematic studies. *Mycologica Helvetica* 6: 73-91.

Zhou, L.,Powell, C.A.,Hoffmann, M.T., Li., W., Fan, G., Liu, B., Lin, H. Duan, Y. 2011. Diversity and plasticity of the intracellular plant pathogen and insect symbiont Candidatus *Liberobacter asiaticus*" as revealed by hyper variable prophague genes with intragenic tandem repeats. *Applied and Environmental Microbiology*, 77: 6663-6675.