Plant Pathology (2010) **59**, 348–357 Doi: 10.1111/j.1365-3059.2009.02221.x

A molecular diagnostic for tropical race 4 of the banana fusarium wilt pathogen

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This study analysed genomic variation of the translation elongation factor 1α (*TEF-1α*) and the intergenic spacer region (IGS) of the nuclear ribosomal operon of *Fusarium oxysporum* f. sp. *cubense* (Foc) isolates, from different banana production areas, representing strains within the known races, comprising 20 vegetative compatibility groups (VCG). Based on two single nucleotide polymorphisms present in the IGS region, a PCR-based diagnostic tool was developed to specifically detect isolates from VCG 01213, also called tropical race 4 (TR4), which is currently a major concern in global banana production. Validation involved TR4 isolates, 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 Naine (AAA). Subsequently, a multiplex PCR was developed for fungal or plant samples that also discriminated *Musa acuminata* and *M. balbisiana* genotypes. It was concluded that this diagnostic procedure is currently the best option for the rapid and reliable detection and monitoring of TR4 to support eradication and quarantine strategies.

Keywords: *Fusarium oxysporum* f. sp. *cubense in planta* detection, *Musa* spp., Panama disease, PCR-based diagnostic, vegetative compatibility groups

Introduction

Banana and plantain (*Musa* spp.) are among the most important crops in the world, serving as a staple food and source of income in many developing countries. Banana is also the world's leading fruit crop and consequently an important export commodity for several agricultural-based economies in Latin America, Africa and Asia, and represents the fifth most important agricultural crop in world trade (Aurore *et al.*, 2009). Among the major global constraints on production are several diseases such as black Sigatoka or black leaf streak disease caused by *Mycosphaerella fijiensis* and Panama disease or fusarium wilt caused by *Fusarium oxysporum* f. sp. *cubense* (Foc) (Stover, 1962; Ploetz, 2006). Symptoms of fusarium wilt start with yellowing and wilting of the older leaves, which progresses to the younger leaves until the death of the entire plant. Internally, plants with advanced infection show discoloration of the rhizome and necrosis of xylem vessels in the pseudostem. Foc is a soilborne pathogen that produces chlamydospores, enabling the fungus to persist in soil in the absence of the host. Hence, once soil is infested with Foc, susceptible varieties cannot be successfully replanted for up to 30 years (Stover, 1962, 1990). As a result, fusarium wilt wiped out the banana industry based on cv. Gros Michel in Central America in the middle of the last century. This forced the trade to shift to resistant cultivars of the Cavendish subgroup (AAA) (Stover, 1962, 1990; Ploetz, 2006). Cavendish cultivars solved the problems for the banana export trade from Latin America, where tropical race 4 (TR4, see below) is absent, but not in Asian countries, where TR4 is present. Hence, fusarium wilt continues to be a constraint to susceptible varieties and is still considered a major threat to banana production because, unlike black leaf streak disease, it cannot be controlled with fungicides.

Early attempts to rationalize pathogen diversity resulted in the designation of race 1 and race 2, differentially pathogenic on cvs Gros Michel (AAA) and Bluggoe (ABB) from observations in Honduras (Waite & Stover, 1960). Later, in Taiwan, Cavendish bananas were affected and a race 4 was designated. However, this pathotype could also cause disease in banana cultivars susceptible to races 1 and 2 (Su *et al.*, 1986). Before 1990, isolates that were classified as race 4 only caused serious losses in Cavendish genotypes in subtropical regions of Australia, the Canary Islands and Taiwan (Su *et al.*, 1986; Pegg *et al.*, 1996). Since then,

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Published online 12 January 2010

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a new variant that severely affects Cavendish cultivars in the tropics was identified. Thus, two types of Foc race 4, viz. subtropical race 4 (ST4) and tropical race 4 (TR4) were designated. However, while ST4 isolates cause disease in Cavendish in the subtropics, mainly when plants are exposed to abiotic stress, TR4 isolates are pathogenic under both tropical and subtropical conditions (Buddenhagen, 2009).

Since its appearance, TR4 has caused severe damage to Cavendish cultivars in Malaysia, Indonesia, South China, the Philippines and the Northern Territory of Australia (Ploetz, 2006; Molina *et al.*, 2008; Buddenhagen, 2009). Control strategies of TR4 are based on visual monitoring for early symptom appearance, eradication of infected plants and isolation of infested areas to reduce pathogen dissemination. However, these strategies are often impractical and therefore not carried out. Additionally, identification is further complicated by the above mentioned race concept, which does not adequately capture genetic variation. Therefore, alternative characterization strategies have been implemented. Vegetative compatibility group (VCG) analyses (Correll *et al.*, 1987; Ploetz & Correll, 1988; Moore *et al.*, 1993) and phylogenetic studies based on molecular data (Koenig *et al.*, 1997; Bentley *et al.*, 1998; O'Donnell *et al.*, 1998; Groenewald *et al.*, 2006; Fourie *et al.*, 2009) revealed more genetic variation in Foc. At least 21 different VCGs of Foc have been characterized, with the majority of groups present in Asia, where the pathogen is thought to have evolved (Ploetz & Pegg, 1997; Fourie *et al.*, 2009). While TR4 isolates are designated as VCG 01213 (or VCG 01216, which is a different designation for the same VCG) isolates classified as ST4 belong to VCGs 0120, 0121, 0122, 0129 and 01211 (Buddenhagen, 2009). Therefore, VCG tests are useful for TR4 diagnosis, but require time-consuming generation and characterization of *nit* mutants and the availability of testers.

This paper describes the development of a rapid and reliable PCR diagnostic for Foc TR4/VCG 01213 that can also be used for *in planta* detection. It is anticipated that it will be used to support national and international quarantine measures in order to avoid further dissemination of TR4.

Materials and methods

Fusarium oxysporum isolates and cultural conditions

In total, 82 Foc isolates originating from different banana production areas and comprising 20 VCGs were analysed (Table 1). Samples from geographic regions known to be infested by TR4 were received as dry pseudostem strands and were sectioned into pieces (2 cm long and 0·5 cm wide), transferred to Komada's medium (Komada, 1975) and incubated at 25°C. After 3–5 days, when fungal growth appeared as white and pink aerial mycelia, isolated colonies were examined by light microscopy for the presence of macroconidia and microconidia diagnostic of *F. oxysporum*. Positive samples were transferred to plates with potato-dextrose agar (PDA) and stored for further analyses.

Vegetative compatibility group analyses

Nitrate-nonutilizing (*nit*) mutants of the wild-type Foc strains were generated in minimal medium (MM) (Puhalla, 1985) amended with 1·5–4·5% $KCl0_3$ and incubating for 7–14 days at 25°C. Spontaneous $KClO_3$-resistant sectors were transferred to MM. Those that grew as thin colonies with no aerial mycelium were classified as *nit* mutants and were further characterized on media containing one of four different sources of nitrogen (Correll *et al.*, 1987). Finally, VCGs of all mutants were determined by pairing on MM with tester *nit* mutants from strains with known VCGs (Correll *et al.*, 1987). Complementation between different *nit* mutants resulted in dense aerial growth at the contact zone between the two colonies. None of the isolates tested was self-incompatible.

DNA isolation, PCR amplification and sequencing

For DNA isolation, a single-spore culture of each isolate (Table 1) was grown in Petri plates (6 cm diameter) containing PDA and incubated at 25°C for 5 days. To facilitate the harvest of mycelia, a cellophane disc (5·5 cm diameter) was placed on the medium surface prior to inoculation. Mycelium was harvested by scraping the cellophane disc and was subsequently stored in 2-mL tubes at −80°C. After addition of a tungsten bead, the mycelium was lyophilized and ground by vigorous shaking of the tubes in a MM300 mixer mill (Retch). Total genomic DNA was extracted using the Wizard Magnetic DNA Purification System for Food kit (Promega) according to the manufacturer's instructions. DNA samples were diluted to 10 ng μL^{-1} and stored at −20°C until use. DNA samples from isolates of *Fusarium oxysporum* f. sp. *passiflorae*, *F. guttiforme*, *F. graminearum* and *F. verticillioides* (Table 1) were used for specificity tests. The translation elongation factor 1α gene, *TEF-1α*, was amplified with primers EF-1 and EF-2 (O'Donnell *et al.*, 1998) using the following programme: 95°C for 2 min and 35 cycles of 95°C for 30 s, 57°C for 30 s and 72°C for 1 min, followed by an additional extension time for 10 min at 72°C. The intergenic spacer (IGS) region of the nuclear ribosomal operon was amplified using primers iNL11 (5′-AGGCTTCGGCTTAGCGTCTTAG-3′) and iCNS1 (5′-TTTCGCAGTGAGGTCGGCAG-3′) and the following programme: 95°C for 5 min and 30 cycles of 95°C for 1 min, 62°C for 1 min and 72°C for 3 min, followed by an additional extension time for 10 min at 72°C. PCR products were directly sequenced using Big Dye Terminator (v3.1; Applied Biosystems). The *TEF-1α* gene was sequenced using the aforementioned primers. The IGS regions of the nuclear ribosomal operons were sequenced with primers iNL11, iCNS1, NLa (5′-TCTA GGGTAGGCKRGTTTGTC-3′) and CNSa (5′-TCTCA TRTACCCTCCGAGACC-3′).

Table 1 Origin of isolates of *Fusarium oxysporum* f.sp. *cubense* and other species, their known or determined vegetative compatibility groups (VCG), race classification and response to known and newly developed PCR diagnostics

							PCR diagnostic	
Code	Received as	VCG[b]	Race[c]	Host[d]	Location	Source[e]	Foc-1/ Foc-2	FocTR4-F/ FocTR4-R
Focu1	Foc	0120		Mons Mari	Queensland	Australia, 2	+	–
Focu2	Foc	0121		Gros Michel		Costa Rica, 2	+	–
NRRL36102	Foc	0121		Cavendish		Taiwan, 3	+	–
NRRL25603	Foc	0122		Cavendish		Australia, 3	+	–
NRRL36103	Foc	0122		Cavendish		Philippines, 3	+	–
NRRL26022	Foc	0123		Pisang Awak		Thailand, 3	–	–
NRRL36101	Foc	0123	R1	Mons Mari		Australia, 3	+	–
NRRL36104	Foc	0123		Kluai Namwa Sai Deng		Thailand, 3	–	–
Focu3	Foc	0124		Bluggoe		Honduras, 2	–	–
Focu4	Foc	0124		Bluggoe		Jamaica, 2	–	–
NRRL25607	Foc	0124	R2	Bluggoe		USA, 3	–	–
NRRL36105	Foc	0124		Bluggoe		Honduras, 3	–	–
Focu5	Foc	0125		Lady Finger	Currumbin, Queensland	Australia, 2	–	–
NRRL36106	Foc	0125		Pome		Australia, 3	–	–
Focu6	Foc	0126		Maqueño		Honduras, 2	+	–
NRRL36107	Foc	0126		Maqueño		Honduras, 3	+	–
NRRL36111	Foc	0128		Bluggoe		Australia, 3	–	–
NRRL36110	Foc	0129		Cavendish		Australia, 3	+	–
Focu7	Foc	01210		Apple	Florida	USA, 2	+	–
NRRL26029	Foc	01210	R1	Silk	Florida	USA, 3	+	–
NRRL36109	Foc	01211		SH3142		Australia, 3	+	–
NRRL36108	Foc	01212		Ney Poovan		Tanzania, 3	–	–
NRRL36114[a]	Foc	01213	TR4	Pisang Manurung		Indonesia, 5	+	+
Focu8	Foc	01214		Harare	Misuki Hills, Karonga,	Malawi, 2	–	–
NRRL25609	Foc	01214		Harare		Malawi, 3	–	–
NRRL36113	Foc	01214		Bluggoe		Malawi, 3	–	–
NRRL36112	Foc	01215		Cavendish		South Africa, 3	+	–
NRRL36120	Foc	01218		Pisang Awak		Thailand, 3	–	–
NRRL36118	Foc	01221		Pisang Awak		Thailand, 3	–	–
NRRL36117	Foc	01222		Pisang Awak Legor		Malaysia, 3	–	–
NRRL36116	Foc	01223		Pisang Keling		Malaysia, 3	–	–
NRRL36115	Foc	01224		Pisang Ambon		Malaysia, 3	–	–
BPI-0901	Field samples (petiole)	0120*		Cavendish	Java	Indonesia, 6	+	–
Foc19508	Foc	0120*	R1	Gros Michel	Guapiles	Costa Rica, 4	+	–
FocST498	Foc	0120*	ST4	Dwarf Cavendish	Canary Islands	Spain, 1	+	–
BPS1.1	Field samples (pseudostem)	01213*		Cavendish	Kuta-village Bali	Indonesia, 6	+	+
BPS3.1[a]	Field samples (pseudostem)	01213*		Cavendish	Darwin	Australia, 6	+	+
BPS3.2[a]	Field samples (pseudostem)	01213*		Cavendish	Darwin	Australia, 6	+	+
BPS3.3[a]	Field samples (pseudostem)	01213*		Cavendish	Darwin	Australia, 6	+	+
BPS3.4[a]	Field samples (pseudostem)	01213*		Cavendish	Darwin	Australia, 6	+	+
Foc-T105	Foc	01213*	R4	Cavendish	Nantow	Taiwan, 7	+	+
Foc-T14	Foc	01213*	R4	Cavendish	Taitung	Taiwan, 7	+	+
Foc-T202	Foc	01213*	R4	Cavendish	Nantow	Taiwan, 7	+	+
II-5[a]	Foc	01213*	TR4	Pisang Manurung		Indonesia, 5	+	+
BPI-0902	Field samples (pseudostem)	†		Silk	Mariana Islands (Saipan), Farm: Lucy Norita	Indonesia, 6	–	–
BPI-0903	Field samples (pseudostem)	†		Silk	Mariana Islands (Rota CNMI) Farm: Frank Calvo	Indonesia, 6	–	–
BPI-0904	Field samples (pseudostem)	†		Silk	Mariana Islands (Rota CNMI)	Indonesia, 6	–	–

Table 1 *Continued*

Code	Received as	VCG[b]	Race[c]	Host[d]	Location	Source[e]	PCR diagnostic Foc-1/ Foc-2	PCR diagnostic FocTR4-F/ FocTR4-R
BPI-0905	Field samples (pseudostem)	†		Silk	Mariana Islands (Tinian Island),	Indonesia, 6	–	–
Foc_R1	Foc	†	R1	Silk	Cruz das Almas, Bahia	Brazil, 9	–	–
Foc_R2	Foc	†	R2	Monthan	Cruz das Almas, Bahia	Brazil, 9	–	–
BPS4.1	Field samples (pseudostem)			Awak	Namulon	Uganda, 4	–	–
BPS5.1	Field samples (pseudostem)			Sukara	NE Kampala	Uganda, 6	–	–
BPS5.2	Field samples (pseudostem)			Sukara	NE Kampala	Uganda, 6	–	–
BPS5.3	Field samples (pseudostem)			Sukara	NE Kampala	Uganda, 6	–	–
BPS5.4	Field samples (pseudostem)			Sukara	NE Kampala	Uganda, 6	–	–
BPS5.5	Field samples (pseudostem)			Sukara	NE Kampala	Uganda, 6	–	–
Foc05	Foc		R1	Prata	Janaúba Minas Gerais	Brazil, 8	–	–
Foc49	Foc		R1	Prata Anã	Cruz das Almas, Bahia	Brazil, 9	–	–
Foc97	Foc		R1	Silk	Botucatu, SP	Brazil, 9	–	–
FocYB	Foc		R1	Yamgambi	Botucatu, SP	Brazil, 9	+	–
FT1	Foc			Pisang Awak		Uganda, 8	–	–
FT12	Foc			Pelipita		Uganda, 8	–	–
FT13	Foc			Pelipita		Uganda, 8	–	–
FT14	Foc			Gros Michel		Uganda, 8	–	–
FT23	Foc			Pisang Ceylan		Uganda, 8	–	–
FT24	Foc			Pisang Ceylan		Uganda, 8	–	–
FT3	Foc			Pisang Awak		Uganda, 8	–	–
IMI 141103	Foc		R2			10	–	–
IMI 141109	Foc		R1			10	–	–
T91-1A	Foc					Taiwan, 2	+	–
T91-1B	Foc					Taiwan, 2	+	–
T91-1C	Foc					Taiwan, 2	+	–
T91-2	Foc					Taiwan, 2	+	–
T91-4A	Foc					Taiwan, 2	+	–
T91-4B	Foc					Taiwan, 2	+	–
T91-4C	Foc					Taiwan, 2	+	–
T91-5A	Foc					Taiwan, 2	+	–
T91-5C	Foc					Taiwan, 2	+	–
T91-6A	Foc					Taiwan, 2	+	–
T91-6B	Foc					Taiwan, 2	+	–
T91-6C	Foc					Taiwan, 2	+	–
T91-7	Foc					Taiwan, 2	+	–
Fop-08-1	*F. o.* f. sp. *passiflorae*			Passion fruit		Brazil, 9	–	–
Fgt-08-1	*F. guttiforme*			Pineapple		Brazil, 9	–	–
Fg820	*F. graminearum*			Wheat		Netherlands, 11	–	–
M2	*F. verticillioides*			Maize		Netherlands, 11	–	–

[a]Isolates BPS3.1, BPS3.2, BPS3.3, BPS3.4 came from different pseudostem strands of the same plant; isolates II-5 and NRRl36114 were obtained from different sources, but were thought to be clones.

[b]Vegetative compatibility groups (VCGs) were assigned using *nit* mutants according to Correll *et al.* (1987). *Isolates with VCG determined in this study; †Isolates not complemented with VCG 01213 testers.

[c]Race designation as provided by supplier. R1, race 1; R2, race 2; ST4, subtropical race 4; TR4, tropical race 4.

[d]Banana cultivars are inter- and intraspecific diploid or triploid hybrids of *M. acuminata* (AA) and *M. balbisiana* (BB). Ploidy levels and constitutions of cultivars as follows: AA, SH3132; AAA, Cavendish, Dwarf Cavendish, Gros Michel, Lady Finger, Mons Mari, Pisang Ambon, Yamgambi; AAB, Apple, Maqueño, Pisang Ceylan, Pisang Keling, Pisang Manurung, Pome, Prata, Prata Anã, Silk, Sukara; AB, Ney Poovan; ABB, Awak, Bluggoe, Harare, Kluai Namwa Sai Deng, Monthan, Pelipita, Pisang Awak, Pisang Awak Legor.

[e]Source: 1, Julio Hernandez, Instituto de Investigaciones Canarias, Spain; 2, Marie-Jo-Daboussi, Université Paris Sud, Paris, France; 3, Kerry O'Donnell, National Center for Agricultural Utilization Research, USDA, Peoria, IL, USA; 4, Mauricio Guzmán, Corbana, Guapiles, Costa Rica; 5, Corby Kistler, ARS-USDA, Cereal Disease Laboratory, St Paul, MN, USA; 6, Ivan Buddenhagen; 7, Pi-Fang Linda Chang, Department of Plant Pathology, National Chung Hsing University, Taiwan; 8, Jim Lorenzen, International Institute of Tropical Agriculture, Uganda; 9, Embrapa Cassava & Tropical Fruits, Brazil; 10, Mycotheque de l'Universite Catholique de Louvain, Belgium; 11, Plant Research International, Wageningen University, the Netherlands.

Sequence analyses and TR4 primer design

Sequences were manually edited using the SEQMAN module of DNASTAR 6.0 to generate a consensus sequence. Alignment was performed using the CLUSTALW tool in the MEGALIGN module of DNASTAR 6.0. DNA sequences of the IGS region and the *TEF-1α* gene were used, both as individuals and as a combined dataset for the 82 Foc isolates. In addition, a dataset containing *TEF-1α* and IGS sequences from 848 *F. oxysporum* isolates (O'Donnell *et al.*, 2009) was used for comparative analyses. Single nucleotide polymorphisms (SNPs) were identified and used for primer design. The primer set FocTR4-F/FocTR4-R for specific detection of TR4 (VCG 01213) was designed to generate a unique amplicon of 463 base pairs (bp). Amplification conditions were as described above for IGS amplification, except the annealing temperature, which was fixed to 60°C. In addition, the Foc-1/Foc-2 primer set (5′-CAGGGGATGTATGAGGAGGCT-3′/5′-GTGACAGCGTCGTCTAGTTCC-3′) reported for specific detection of Foc race 4 was tested (Lin *et al.*, 2008).

Plant inoculation and *in planta* detection

Hardened 3-month-old tissue-cultured banana plants of cv. Grand Naine were inoculated with three TR4 isolates (NRRL36114, BPS3.4 and II-5) and with one race-1 isolate (Foc_R1) that is pathogenic on cv. Silk (AAB) (Table 1). Plants were inoculated by root dipping (30 min, 10^6 conidia per mL) and then transferred to pots (8 L) partially filled with sand supplemented with 20 maize kernels colonized (after sterilization) with each isolate for 10 days. During acclimatization and after inoculation plants were maintained in a greenhouse at 28°C, 80% relativity humidity and 16 h light. Rhizome and pseudostem samples collected 40 days after inoculation (d.a.i.), were cut in half, with one half plated on Komada's medium for selective isolation of Foc and the other half used for DNA extraction. Total genomic DNA from plant tissues was extracted using the aforementioned kit. *In planta* detection for TR4 was performed using the FocTR4-F/FocTR4-R primer set as described above for fungal DNA on cv. Grand Naine and additionally on six AA diploid, five BB diploid and two AAB triploid banana genotypes (Table 2).

Multiplex PCRs

Using the amplification conditions fixed for the FocTR4-F/FocTR4-R primers, multiplex PCRs were developed to detect in one single reaction false negatives in either fungal or plant samples. For fungal DNA, the multiplex PCR incorporated the *TEF-1α* primer set (EF-1 and EF-2) as internal positive control. For plant samples, the banana actin gene AF285176.1 (http://www.ncbi.nlm.nih.gov) was used to design the BanActin2-F (5′-ACAGTGTCTGGATTGGAGGC-3′) and BanActin2-R (5′-GCACTTCATGTGGACAATGG-3′) primers that amplified a 217-bp product as internal positive control.

Table 2 Banana genotypes used for PCR amplifications

Cultivar	Genome composition	Species
Borneo	AA	*Musa acuminata*
Mandang	AA	*Musa acuminata*
Born Pisan Mas	AA	*Musa acuminata*
Calcutta 4	AA	*Musa acuminata*
Selangor	AA	*Musa acuminata*
Z6Fb	AA	*Musa acuminata*
Etikehel	BB	*Musa balbisiana*
Singapuri	BB	*Musa balbisiana*
Tani	BB	*Musa balbisiana*
Buthonan	BB	*Musa balbisiana*
MPL	BB	*Musa balbisiana*
Grand Naine	AAA	*Musa acuminata*
Silk	AAB	*Musa* spp.
Prata Anã	AAB	*Musa* spp.

Results

Genetic diversity of *Fusarium oxysporum* f. sp. *cubense*

Foc was not recovered from some samples received as dry pseudostem from the field, but most samples produced typical *Fusarium* colonies on Komada's medium. This resulted in 16 field isolates being selected for further analyses in this study (Table 1). VCG tests were performed for most of the isolates from areas where TR4 is reported, which were suspected to belong to VCG 01213 (Table 1). Several isolates (NRRL36110, NRRL36111, NRRL 36112, Foc_R1 and Foc_R2) clearly showed increased levels of resistance to $KCLO_3$ even at 4·5%, but eventually *nit* mutants could be generated. Foc isolates Foc_R2 and Foc_R1 did not complement any VCG tester of the collection.

High-quality genomic DNA was obtained for all isolates and the primers and amplification conditions resulted in high-quality DNA sequences of the *TEF-1α* gene and IGS region. Phylogenetic analyses of IGS and *TEF-1a* revealed polymorphisms between the Foc isolates, but for the *TEF-1α* gene these were insufficient to allow a reliable discrimination of VCG 01213 from other VCGs. For instance, isolates of VCGs 0120, 0121, 0129 and 01211 showed 100% similarity with VCG 01213 isolates representative of TR4 (data not shown). Comparative analysis of the IGS region showed a higher SNP frequency (Fig. 1) and was therefore, along with data from 848 isolates of *Fusarium* spp. (O'Donnell *et al.*, 2009), used for primer design. These analyses revealed that VCG 01213 isolates are closely related to VCG 1210 (NRRL26029), VCG 0129 (NRRL36110), VCG 0120 (NRRL25603) and VCG0126 (NRRL36107) isolates, but differences were sufficient for specific primer design (Fig. 1).

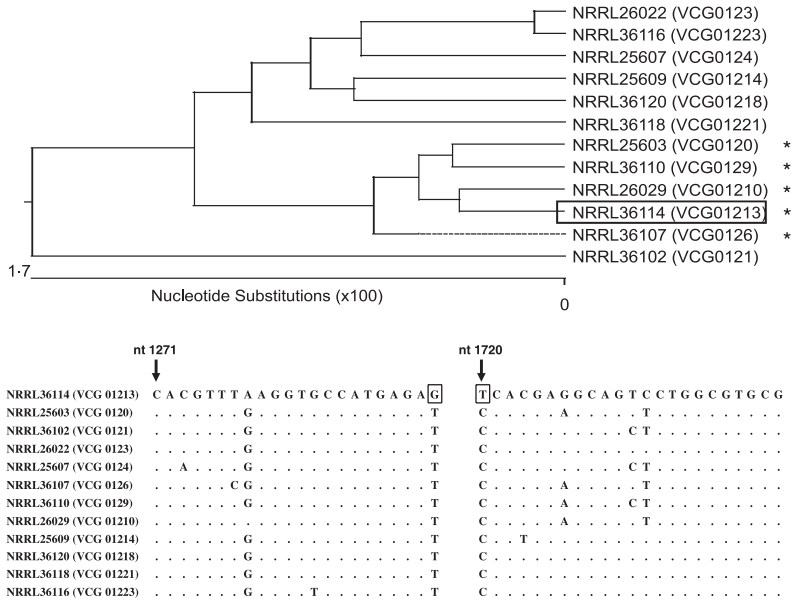

Figure 1 Genetic relationship of representative isolates of *Fusarium oxysporum* f. sp. *cubense* (Foc) related to NRRL36114 (VCG 01213; TR4) based on DNA sequences of the intergenic spacer region of the ribosomal operon (upper panel). Isolates positive for the Foc-1/Foc-2 primer set (Lin *et al.*, 2008) are indicated by asterisks. The alignment of the representative IGS sequences of Foc isolates related to NRRL36114 shows the two single nucleotide polymorphisms that were used for primer design (lower panel).

Specificity of the FocTR4-F/FocTR4-R primer set

The designed primer set for TR4, FocTR4-F (5′-CAC GTTTAAGGTGCCATGAGAG-3′) and FocTR4-R (5′-CGCACGCCAGGACTGCCTCGTGA-3′), produced the predicted 463-bp amplicon that was confirmed by gel electrophoresis (Fig. 2). PCR amplification only generated this diagnostic 463-bp amplicon in VCG 01213 isolates (Table 1, Fig. 2). The Foc-1/Foc-2 primer set of Lin *et al.* (2008) amplified bands in Foc isolates belonging to at least nine VCGs. These comprised VCG 01213, as well as VCGs 0120, 0121, 0122, 0126, 0129, 01210, 01211 and 01215, plus isolates of unknown VCGs from Brazil (FocYB) and Taiwan (Table 1).

Disease development and *in planta* detection

In TR4-inoculated plants, typical external yellowing appeared 7 d.a.i. and internal rhizome discoloration occurred 14 d.a.i. At 40 d.a.i. TR4-inoculated plants showed severe wilting and internal necrosis, even in the pseudostem (Fig. 3). No symptoms were observed in plants inoculated with Foc_R1 or in those used as non-inoculated controls. The three TR4 isolates caused similar symptoms, with no differences regarding incubation period or severity. All three TR4 isolates were successfully recovered from rhizomes with symptoms on Komada's medium. DNA (20 ng) from infected plants was successfully used for PCR amplification of the diagnostic

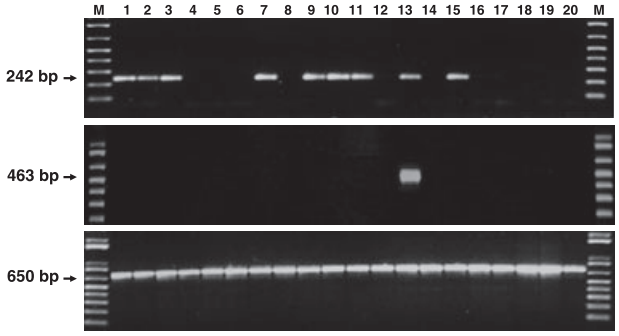

Figure 2 Amplification of PCR products of 20 representative vegetative compatibility groups (VCGs) of *Fusarium oxysporum* f. sp. *cubense* (Foc) using primer set Foc-1/Foc-2 (upper panel), FocTR4-F/FocTR4-R (middle panel) and EF-1/EF-2 (lower panel). Lane 1, NRRL36101 (0120); 2, NRRL36102 (0121); 3, NRRL36103 (0122); 4, NRRL36104 (0123); 5, NRRL36105 (0124); 6, NRRL36106 (0125); 7, NRRL36107 (0126); 8, NRRL36111(0128); 9, NRRL36110 (0129); 10, NRRL26029 (01210); 11, NRRL36109 (01211); 12, NRRL36108 (01212); 13, NRRL36114 (01213); 14, NRRL36113(01214); 15, NRRL36112 (01215); 16, NRRL36120 (01218); 17, NRRL36118 (01221); 18, NRRL36117 (01222); 19, NRRL36116 (01223); 20, NRRL36115 (01224). Numbers in parentheses are VCGs. Specific DNA bands for Foc race 4 (242 bp), Foc TR4 (463 bp) and elongation factor 1α (648 bp) are indicated on the left. M, molecular marker 1-kb DNA ladder plus.

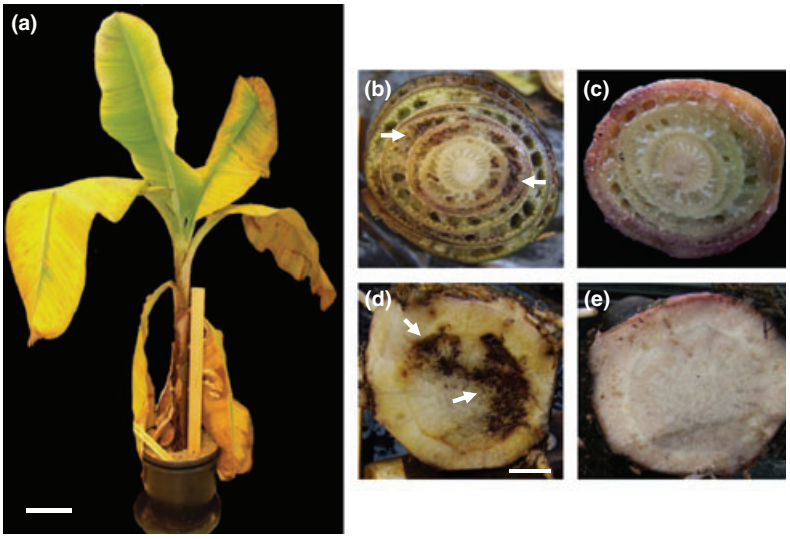

Figure 3 Banana cv. Grand Naine 40 days after inoculation with TR4 isolate NRRL36114 of *Fusarium oxysporum* f. sp. *cubense* (Foc). (a) Plant showing fusarium wilt symptoms; bar = 10 cm. (b–d) Cross sections of pseudostem (b, c) and rhizome (d, e) of inoculated (b, d) and non-inoculated (c, e) plants; arrows show necrosis caused by Foc TR4; bar = 1 cm.

463-bp amplicon using the FocTR4-F/FocTR4-R primer set. No amplicons were observed from samples of non-inoculated cv. Grand Naine plants and the 13 additional banana genotypes that were tested (data not shown).

Duplex PCR using fungal DNA generated two fragments in TR4 isolates, one belonging to the *TEF-1α* gene (648 bp) and the VCG 01213 diagnostic 463-bp amplicon. Samples from isolates of other VCGs only generated the *TEF-1α* amplicon (Fig. 2). For *in planta* detection, the duplex PCR generated the VCG 01213 diagnostic 463-bp amplicon only in TR4-infected samples (Fig. 4). The amplicon derived from the banana actin gene was successfully amplified in all the banana DNA samples. Interestingly, the banana actin amplicons were specific for either *Musa acuminata* (A genome, 217 bp) or *M. balbisiana* (B genome, ~280 bp) banana genotypes, whereas AAB triploids showed both fragments (Fig. 4).

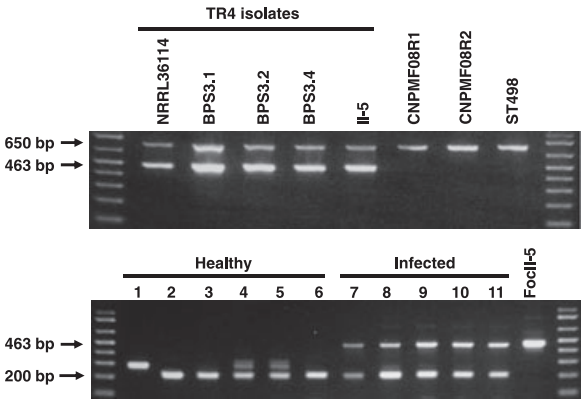

Figure 4 Amplification products of duplex PCRs using DNA from pure-culture *Fusarium oxysporum* f. sp. *cubense* (Foc; upper panel) or banana plants (lower panel) as templates. Duplex PCRs for Foc cultures were performed using the elongation factor-1α (EF-1/EF-2) primer set as internal control in combination with the TR4-specific primers FocTR4-F/FocTR4-R (upper panel). Duplex PCRs of banana samples used the banana actin (BanAct2-F/BanAct2-r) and TR4-specific (FocTR4-F/FocTR4-R) primer sets (lower panel). Lane 1, *Musa balbisiana* cv. Buthohan (BB); 2, *Musa acuminata* cv. Pisang Mas (AA); 3, Grand Naine (from leaf of tissue-cultured plants); 4, Silk (AAB); 5, Prata Anã (AAB); 6, rhizomes from non-inoculated Grand Naine plants; 7–9, infected rhizomes from Grand Naine plants inoculated with Foc TR4 isolates NRRL36114, BPS3.4 and II-5; 10-11, Infected pseudostems from cv. Grand Naine plants inoculated with Foc TR4 isolates NRRL36114 and BPS3.4; 12, positive control using DNA from a pure culture of isolate NRRL36114. Specific DNA bands for Foc TR4 (463 bp), elongation factor 1α (648 bp) and the banana actin gene (217 bp) are indicated on the left. M, molecular marker 1-kb DNA ladder plus.

Discussion

Considering the history of Panama disease (Stover, 1962, 1990; Ploetz, 1994) and the Cavendish-dependence of export trades, TR4 is currently a major threat to the global banana industry. If TR4 enters the major banana plantations in Latin America, the Caribbean and West Africa, a multibillion dollar production and export industry will be facing devastation. Moreover, the food security of millions of people depending on smallholder production will be in danger. In the absence of resistant cultivars, delimiting the dissemination of the disease is a top priority that relies on accurate diagnosis. *Fusarium oxysporum* comprises morphologically indistinguishable pathogenic as well as non-pathogenic strains. Therefore, identification to the species, *forma specialis* and strain levels is highly desired (Lievens *et al.*, 2008), particularly for quarantine pathogens that are of high economic importance, such as Foc TR4. This study reports a new PCR diagnostic that uniquely amplifies a 463-bp amplicon in

isolates belonging to Foc VCG 01213, which encompasses TR4 (Ploetz, 2006; Buddenhagen, 2009).

Until now, Foc race diagnosis relied exclusively on pathogenicity trials and VCG testing. It has been repeatedly stated that the lack of a universally acceptable greenhouse inoculation technique is an important bottleneck for the characterization of Foc isolates (Bentley *et al.*, 1998; Groenewald *et al.*, 2006; Smith *et al.*, 2008). The inoculation procedure used in this study was efficient and reliable, not only with TR4 isolates on cv. Grand Naine, but also for race 1. When more differentials become available, the Foc complex of banana might be better resolved by testing a range of Foc isolates from different VCGs on a panel of diverse banana genotypes with different ploidy levels, as was shown for *F. oxysporum* f. sp. *dianthi* in carnation (Aloi & Baayen, 1993).

Field data for TR4 or ST4 occurrence should be interpreted with caution. TR4 is more aggressive than ST4 (Ploetz, 2006; Buddenhagen, 2009), but the latter can also cause severe damage in Cavendish cultivars, particularly under abiotic stress, such as low temperatures and waterlogging (Su *et al.*, 1986; Pegg *et al.*, 1996; Buddenhagen, 2009). This is not always known by growers and extension officers, who may consider such infections as TR4, resulting in false alarms and needless eradication measures. An example is isolate BPI-0901, a suspect TR4 isolate obtained from Indonesian Cavendish samples. It was negative in the present PCR-based diagnosis and only after time-consuming (6 months) successive attempts were *nit* mutants obtained. Subsequent VCG characterization resulted in VCG 0120, further validating the molecular TR4 diagnostic.

VCG analyses have contributed to an improved understanding of genetic variation in Foc, but the lack of an accessible international VCG tester collection complicates its use for diagnosis. Molecular studies have shown the existence of different genotypic groups and clonal lineages of Foc that were largely VCG-specific, but no correlation between these data and race designations was observed (Koenig *et al.*, 1997; Bentley *et al.*, 1998; O'Donnell *et al.*, 1998; Gerlach *et al.*, 2000; Groenewald *et al.*, 2006; Fourie *et al.*, 2009). Mutations in the *vic* locus, could, however, render isolates within the same VCG incompatible (Bentley *et al.*, 1995, 1998). In addition, some VCGs of Foc can produce heterokaryons between separate groups, such as VCGs 0120 and 01215 (Bentley *et al.*, 1998; Gerlach *et al.*, 2000; Groenewald *et al.*, 2006). Therefore, TR4 diagnostics should focus on genetic specificity based on molecular data.

Sequences of IGS and *TEF-1α* were used to study genetic diversity of Foc, with the aim of identifying SNPs for specific primer design. The *TEF-1α* gene has been widely used in *Fusarium* spp. for both phylogenic (O'Donnell *et al.*, 1998; Fourie *et al.*, 2009) and identification purposes (Bogale *et al.*, 2007; Mehl & Epstein, 2007). In the present study, however, *TEF-1α* revealed insufficient polymorphisms for reliable discrimination of VCG 01213 from other VCGs. Instead, the results showed that the higher SNP frequency of the IGS region provides a rich source of genetic diversity in this *forma specialis*, which was successfully exploited to develop a Foc TR4 diagnostic PCR. Moreover, it can also be used to further elucidate phylogenetic relationships among Foc populations. This confirms the results of Fourie *et al.* (2009), who also showed that restriction fragment length polymorphisms of the IGS region (IGS-RFLP) were more discriminative than three other genome regions, including *TEF-1α*, for Foc lineages. As the higher copy number of IGS increases the sensitivity of PCR-based diagnostics, this region also has been used to develop diagnostics for other plant pathogenic *Fusarium* spp., such as *F. circinatum* (Schweigkofler *et al.*, 2004) and *F. oxysporum* f. sp. *vasinfectum* (Zambounis *et al.*, 2007).

Specificity of diagnostics is required for the unequivocal detection of quarantine organisms. The diagnostic developed here was specific for TR4 on pure-culture DNAs of VCG 01213 isolates that were either characterized prior to or after the PCR test, the latter including isolates from infected banana tissues (BPS1.1, BPS3.1) from Indonesia and pure cultures from Taiwan (T-14, T105 and T-202). The Foc-1/Foc-2 primer set recently published by Lin *et al.* (2008), considered to be specific for Foc race 4 (both ST4 and TR4), was also tested. However, this primer set reacted with isolates of 10 different VCGs, including those belonging to the 01213 group (TR4). These results are in agreement with those of Buddenhagen (2009), who reported that ST4 isolates belong to VCGs 0120, 0121, 0122, 0129 and 01211. In addition, the present results suggest that isolates of VCG 01215 also affect Cavendish in subtropical areas. Interestingly, isolates from Brazil, Costa Rica, Honduras and the USA were also positive with the Foc-1/Foc-2 primer set. Whilst positive results for isolates from Taiwan or other countries where ST4 is present (Su *et al.*, 1986; Ploetz, 2006; Lin *et al.*, 2008) were expected, it was intriguing to also find positives in areas where ST4 is not officially reported. This suggests that ST4 is present in Central and Latin America, as well as the USA. This ambiguity illustrates the drawback of the current race designation system for Foc in banana. An isolate may be classified as ST4 in subtropical areas (where it affects Cavendish), but as race 1 in tropical areas, such as Brazil, Costa Rica and Honduras (where it is unable to affect Cavendish).

Although this system initially helped to discriminate Foc populations, it is currently outdated and leads to erroneous conclusions, hampering decision making. From a diagnostic and regulatory perspective, methods that are repeatable, highly specific, sensitive for the target pathogen and also can be used on infected plant tissue, without the need for pathogen isolation and culture, would be strongly preferred (Martin *et al.*, 2009). The TR4 diagnostic developed here unambiguously detected TR4 in infected tissues of banana cv. Grand Naine. In comparison with traditional agar plating and pathogen purification from infected samples, VCG analysis and pathogenicity tests, which may take weeks or months, the *in planta* detection method described here provides a receipt-to-result efficiency of

about 6 h. This is comparable to *in planta* detection methods previously reported for other plant pathogenic fungi and oomycetes (Alves-Santos *et al.*, 2002; Wang *et al.*, 2007; Vincelli & Tisserat, 2008). It is concluded that this PCR diagnostic is currently the only option for rapid, reliable and specific detection of TR4. Application enables the monitoring of the disease and supports management and eradication strategies.

Acknowledgements

We thank Drs Jim Lorenzen (IITA, Uganda), Julio Hernández (Instituto de Investigaciones Canárias, Spain), Kerry O'Donnell (National Center for Agricultural Utilization Research, USA), Marie-Jo Daboussi (Université Paris Sud, France), Mauricio Guzmán (Corbana, Costa Rica), Pi-Fang Linda Chang (National Chung Hsing University, Taiwan) and Corby Kistler (ARS-USDA, USA) for providing isolates or samples used in this study. We also thank Dr Irie Vroh (IITA, Nigeria) for supplying part of the banana DNA used for PCRs, Caucasella Diaz (Plant Research International, the Netherlands) for tissue-culture acclimatized cv. Grand Naine plants and Ineke de Vries for technical support. MAD is grateful to CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for the post-doctoral fellowship. This research was partially funded by the Dutch Dioraphte Foundation and the EU Endure programme.

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