Eur J Plant Pathol (2013) 135:395–405
DOI 10.1007/s10658-012-0096-0

A molecular diagnosis method using real-time PCR for quantification and detection of *Fusarium oxysporum* f. sp. *cubense* race 4

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Accepted: 10 September 2012 / Published online: 26 September 2012

Abstract The Fusarium genus causes devastating plant diseases worldwide, in which *Fusarium oxysporum* is the most serious crop pathogen. Disease monitoring is the basis of integrated pest management of any disease. The lack of rapid, accurate, and reliable device to detect and identify plant pathogens is one of the main limitations in integrated disease management. This study describes an efficient and quantifiable diagnosis method for the specific detection of *F. oxysporum* f. sp. *cubense* (Foc) race 4 in field-infected banana. With the optimized PCR parameters using the SCAR (sequence characterized amplified region) primers FocSc-1/FocSc-2 and a real-time PCR strategy, the developed method showed high reproducibility and was very sensitive to detect extremely low quantities of Foc genomic DNA (gDNA). We also found that Foc gDNA in severely symptomatic banana pseudostems and leaves were 6946-fold and 26.69-fold more than in those of mild-symptomatic banana, respectively.

Keywords *Fusarium oxysporum* f. sp. *cubense* · Fusarium wilt · Molecular detection · Reliable diagnosis · Real-time PCR · SCAR

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Introduction

Fusarium wilt disease caused by *Fusarium oxysporum* (Fo) has been considered as one of the most important fungal vascular diseases of crops (Forsyth et al. 2006; Snyder and Hanson 1940). The pathogen has several specialized forms (known as *forma specialis*, f. sp.) according to the host it specifically infects, such as *F. oxysporum* f. sp. *cubense vs*. banana (*Musa* spp.), *F. oxysporum* f. sp. *niveum vs*. watermelon (*Citrullus lanatus* (Thunb.) Matsum & Nakai), and *F. oxysporum* f. sp. *lactucae vs*. lettuce (*Lactuca sativa* L.) (Smith 2007).

Differentiation of Fo from other *Fusarium* sp. is typically based on morphological characteristics, which requires a great knowledge of Fusarium taxonomy

(Jurado et al. 2006). The conventional manner for identifying various *formae speciales* of Fo undoubtedly depends on the presence of evident symptoms, the isolation and culturing of the pathogen, and further pathogenicity testing against the corresponding host. Taking *F. oxysporum* f. sp. *cubense* (Foc) on banana, the targeted pathogen in this study, as an example, diagnosis of Fusarium wilt diseases on banana is relatively easy when typical symptoms including foliage chlorosis, necrosis and ultimately drooping of the leaves (Beckman 1990) are distinct and recognizable. However, the reliability and accuracy of these methods rely on experience and subjective judgement of the diagnostician. Furthermore, Fusarial symptoms are not always consistent and unique and sometimes could even be confused with other diseases of banana. In addition, diagnosis of this pathogen in asymptomatic infected banana tissues is very difficult. Traditional isolation and pathogenicity tests require weeks or longer (Daniells et al. 1995), which often gives the pathogen enough time to spread dramatically and cause severe epidemics (Schaad et al. 2003). Therefore, in order to improve the management of Fusarium wilt of banana, development of an efficient, sensitive, and specific diagnostic method for rapid identification of Foc is essential (Yergeau et al. 2005).

Based on the specificity of pathogenicity against various banana cultivars, Foc can be grouped into three races (Groenewald et al. 2006). Race 1 is capable of damaging "Gros Michel (genome type = AAA)" and "Silk (AAB)" (Stover and Malo 1972; Su et al. 1986), while race 2 infects "Bluggoe" (AAB) and other closely related cooking bananas (Moore et al. 1995; Waite and Stover 1960). Race 4, which is divided into tropical (TR4) and subtropical (ST4) groups according to where it was collected (Dita et al. 2010; Groenewald et al. 2006), mainly causes disease in Cavendish cultivars as well as those susceptible to race 1 and race 2 (Hwang and Ko 2004; Su et al. 1977). Based on the statistics of the FAO (Food and Agriculture Organization of the United Nations), banana was the fourth largest fruit crops in the world, with a cultivation area of 4,923,584 ha and a yield of 97,378,272 tons in 2009 (FAO 2011). Unfortunately, almost all presently popular cultivars are attackable by Foc race 4. Hence, the race 4 of Foc, a devastating wilt disease pathogen on banana historically, is considered one of the major threats to banana production (Dita et al. 2010; Lin et al. 2009).

Random amplification of polymorphic DNA (RAPD) technology is one of the powerful DNA fingerprinting tools for fungal pathogen identifications (Fungaro et al. 2004; Mes et al. 1999). Polymerase chain reaction (PCR) with specific primers has been routinely applied to diagnose pathogens in complex mixtures even when fungal mycelia are not visible under the microscope (Jurado et al. 2006). A 242-bp DNA fragment (named as Foc_{242}) specific to Foc race 4 was earlier published (Lin et al. 2009). The Foc_{242} DNA marker amplified by primer set Foc-1/Foc-2 was originally collected based on RAPD analysis and has been confirmed to be Foc race 4 specific.

Molecular methods such as PCR assay have been frequently applied for identification and detection of Fusarium plant pathogens (Klemsdal and Elen 2006; Möller et al. 1999; Parry and Nicholson 1996; Schilling et al. 1996; Wilson et al. 2004; Yoder and Christianson 1998). A more reliable PCR-based tagging technique known as sequence characterized amplified region (SCAR) was described by Paran and Michelmore (1993). SCAR primers are longer primers extending from the 3′ ends of the original primer sites based on the sequence of the polymorphic DNA marker. The SCAR primers will bind to the original DNA marker and specifically amplify the SCAR marker, which can increase the reproducibility (Clercq et al. 2007), sensitivity (Dias et al. 2007), and specificity (Lin et al. 2010) of PCR assays. In addition, real-time PCR is a time-saving protocol that is sensitive and specific and can increase the likelihood of quantitative detection of plant pathogens *in planta*. Therefore, we developed the SCAR primer set FocSc-1/FocSc-2 to tag the Foc_{242}. In this study, this SCAR primer set was confirmed to not only have higher specificity but also be more sensitive than the primer set Foc-1/Foc-2. In addition, we have developed a more reliable molecular diagnosis based on real-time PCR by using the primer set FocSc-1/FocSc-2 to tag and quantify Foc DNA in field-infected banana tissues.

Materials and methods

Fusarium isolates and growth condition

Fusarium wilt pathogens including: 12 Foc race 4 and 5 Foc race 1 isolates in Taiwan, confirmed by pathogenicity tests; nine reference Foc isolates (ATCC76247 and ATCC96285, race 1; ATCC76243, ATCC76257 and ATCC96288, race 2; ATCC38741, ATCC76262, ATCC96289, and ATCC96290, subtropical race 4);

and 11 other *F. oxysporum formae speciales* were used to reconfirm the specificity of the Foc_{242} marker amplified by the primer sets Foc-1/Foc-2 and FocSc-1/FocSc-2 (Table 1). A single spore culture of each tested isolate was grown on a Nash-PCNB plate (1.5 % peptone, 2 % agar, 0.1 % KH_2PO_4, 0.05 % $MgSO_4 \cdot 7H_2O$, 0.1 % pentachloronitrobenzene, 0.03 % streptomycin, and 0.1 % neomycin) (Nash and Snyder 1962).

Conidia collection and DNA isolation

Dried fungal mycelium (1 g) and field-infected banana tissues (0.3 g) were snap frozen in liquid nitrogen and ground to fine powder using a mortar and pestle. Genomic DNA (gDNA) was extracted according to Lin et al. (2009), and the DNA samples were dissolved in 0.1× TE buffer (1 mM Tris-HCl, pH 8; 0.01 mM EDTA) for further analysis.

In order to accurately quantify conidia of Foc, 0.25-μl drops of the spore suspension were placed on a glass haemacytometer and the spores were counted under a microscope. Those quantified drops were individually transferred with a micropipette to microtubes for further real-time PCR analysis.

Primer design and PCR amplification

The SCAR primer set FocSc-1/FocSc-2 (5′-CAGGGGATGTATGAGGAGGCTAGGCTA /5′-GTGACAGCGTCGTCTAGTTCCTTGGAG) was designed according to the sequence of a 242 bp DNA fragment (named as Foc_{242}). The Foc_{242} had been confirmed with high specificity to Foc race 4 (Lin et al. 2009). The other primer set which could amplify the same DNA marker of Foc_{242}, Foc-1/Foc-2 (5′-CAGGGGATGTATGAGGAGGCT/5′-GTGACAGCGTCGTCTAGTTCC), was also used for comparison with FocSc-1/FocSc-2 for its specificity and amplification efficiency of Foc_{242}. The PCR conditions used were as described by Lin et al. (2009). In addition, the FnSc-1/FnSc-2 primer set (5′-TACCACTTGTTGCCTCGGCGGATCAG/5′-TTGAGGAACGCGAATTAACGCGAGTC), which generates the 327 bp DNA fragments (named as $FnSc_{327}$) from all Fo isolates following the PCR program described by Lin et al. (2010), was also used. The $FnSc_{327}$ was used as an internal control in the specificity assay of PCR.

PCR products were subjected to electrophoresis in 2.0 % agarose gels, and the DNA bands were observed on UV box (312 nm, Vilber Lourmat, Eberhardzell, Germany) after ethidium bromide staining.

Real-time PCR amplification and quantification

The Foc_{242} fragment was gel-purified, cloned into pGEM®-T Easy vector (Promega Co, Madison, WI, USA), and sequenced. The sequenced DNA was re-amplified by Foc-1/Foc-2 from the plasmid and purified using Micro-Elute DNA Clean/Extraction Kit (GeneMark Tech., Co., Ltd., Tainan, Taiwan). The concentration of the target DNA (Foc_{242}) was determined by spectrophotometer (GeneQuant proRNA/DNA Calculator, Amersham Biosciences, Piscataway, NJ, USA). Ten-fold serial dilutions of the target DNA, ranging from 1 ng to 10^{-6}ng per reaction, were performed for standard curve plotting and melting-curve analysis of real-time PCR amplification and yielded linear and reliable results (correlation coefficient, $R^2 > 0.95$; Bookout and Mangelsdorf 2003).

The primer sets Foc-1/Foc-2 and FocSc-1/FocSc-2 were evaluated via standard curve plotting and melting-curve analysis of real-time PCR amplification in four replicates with a serial dilution of above-mentioned target DNA as template. Real-time PCR was monitored on Rotor-Gene® Q-Pure Detection System (Software Ver. 2, Qiagen Inc., Valencia, CA, USA). The standard curve was created by plotting the target DNA amount against the Threshold cycle (Ct) value exported from the Rotor-Gene® Q. For real-time PCR assay, a 10 μl reaction mixture contained template DNA dilution, each of 100 nM amplification primers, and 1X QuantiFast SYBR® Green PCR Master Mix (Qiagen). The parameters for real-time PCR were as follows: 95 °C for 5 min (denaturation and hot start activation), 40 cycles of 95 °C for 10 s and 60 °C for 30 s. After the real-time PCR, melting curves (65 °C to 99 °C) of the PCR products was analyzed to verify their specificity.

For reproducibility and sensitivity assay of real-time PCR, the novel and more reliable primer set FocSc-1/FocSc-2 was assessed in six replicates by using serial dilutions of gDNA (ranging from 10^{-1}ng to 10^{-4}ng per reaction) and conidia (ranging from 2000 to 20 per reaction) of Foc-24 isolate as template. DNA amount of tested samples was quantified by comparing the obtained Ct value with the standard curve.

Table 1 *Fusarium* isolates used for PCR amplification

Species	Races[a]	Original hosts	Geographic locations	Providers[b]
F. oxysporum f. sp. *cubense*		Banana (*Musa* sp.)		
ATCC76247	1	Banana	Honduras	ATCC
ATCC96285	1	Banana	SE. Queensland, Australia	ATCC
ATCC76243	2	Banana	SJ. Queensland, Australia	ATCC
ATCC76257	2	Banana	Honduras	ATCC
ATCC96288	2	Banana	N. Queensland, Australia	ATCC
ATCC38741	ST4	Banana	Taiwan	ATCC
ATCC76262	ST4	Banana	Taiwan	ATCC
ATCC96289	ST4	Banana	SE. Queensland, Australia	ATCC
ATCC96290	ST4	Banana	SE. Queensland, Australia	ATCC
Foc-132	1	Banana	Chiyi, Taiwan	TBRI
Foc-135	1	Banana	Nantow, Taiwan	TBRI
Foc-136	1	Banana	Nantow, Taiwan	TBRI
Foc-137	1	Banana	Pingtung, Taiwan	TBRI
Foc-138	1	Banana	Pingtung, Taiwan	TBRI
Foc-24	TR4[c]	Banana	Taiwan	TBRI
Foc-3-1	TR4[c]	Banana	Pingtung, Taiwan	TBRI
Foc-3-3	TR4[c]	Banana	Pingtung, Taiwan	TBRI
Foc-4-2	TR4[cd]	Banana	Kaohsiung, Taiwan	TBRI
Foc-6-2	TR4[c]	Banana	Hualien, Taiwan	TBRI
Foc-7-7	TR4[c]	Banana	Taitung, Taiwan	TBRI
Foc-7-9	TR4[c]	Banana	Chiyi, Taiwan	TBRI
Foc-7-13	TR4[c]	Banana	Nantow, Taiwan	TBRI
Foc-T105	TR4[c]	Banana	Nantow, Taiwan	J.-W. Huang
Foc-T14	TR4[c]	Banana	Taitung, Taiwan	TDARES
Foc-T44	TR4[c]	Banana	Taitung, Taiwan	TDARES
Foc-TN3	TR4[c]	Banana	Kaohsiung, Taiwan	ARI
F. oxysporum f. sp. *cepae*				
Foce		Onion (*Allium cepa* L.)	Taiwan	H.-L. Wang
F. oxysporum f. sp. *chrysanthemi*				
Foch-11-28		Garland Chrysanthemum (*Chrysanthemum coronarium* L.)	Changhua, Taiwan	J.-W. Huang
F. oxysporum f. sp. *cucumerium*				
Focu-100		Cucumber (*Cucumis sativus* L.)	Nantow, Taiwan	Y.-S. Lin
F. oxysporum f. sp. *gladioli*				
Fog-50		Gladiolus (*Gladiolus* sp.)	Taiwan	W.-H. Hsieh
F. oxysporum f. sp. *lactucae*				
ATCC76616		Lettuce (*Lactuca sativa* L.)	California, USA	ATCC
Fola-104-1		Lettuce	Yunlin, Taiwan	J.-W. Huang
Fola-106-1		Lettuce	Yunlin, Taiwan	J.-W. Huang
F. oxysporum f. sp. *lilii*				
Foli-001		Lily (*Lilium* Oriental hybrid 'Casa Blanca')	Taiwan	W.-H. Hsieh
Foli-F16		Lily	Changhua, Taiwan	J.-W. Huang
Foli-G16		Lily	Changhua, Taiwan	J.-W. Huang
F. oxysporum f. sp. *luffae*				
Folu-S167		Loofah (*Luffa cylindrica* (L.) Roem.)	Nantow, Taiwan	Y.-S. Lin

Table 1 (continued)

Species	Races[a]	Original hosts	Geographic locations	Providers[b]
Folu-114		Loofah	Nantow, Taiwan	Y.-S. Lin
F. oxysporum f. sp. *lycopersici*				
Foly-4		Tomato (*Solanum lycopersicum* L.)	Taiwan	AVRDC-WVC
Foly-8		Tomato	Taiwan	AVRDC-WVC
Foly-11A		Tomato	Taiwan	AVRDC-WVC
Foly-19		Tomato	Taiwan	AVRDC-WVC
Foly-26		Tomato	Taiwan	AVRDC-WVC
F. oxysporum f. sp. *melonis*				
Fome-111		Muskmelon (*Cucumis melo* L.)	Taiwan	W.-H. Hsieh
F. oxysporum f. sp. *niveum*				
ATCC18467		Watermelon (*Citrullus lanatus* (Thunb.) Matsum & Nakai)	South Carolina, USA	ATCC
ATCC44293		Watermelon	California, USA	ATCC
ATCC42006		Watermelon	Taiwan	ATCC
ATCC64104		Watermelon	Taiwan	ATCC
Fon-H0103		Watermelon (*Citrullus lanatus* (Thunb.) Matsum & Nakai)	Miaoli, Taiwan	J.-W. Huang
F. oxysporum f. sp. *tracheiphilum*				
Fot-1211		Asparagus bean (*Vigna unguiculata* subsp. *unguiculata*)	Pingtung, Taiwan	C.-H. Kuo

[a] *ST4* subtropical race 4; *TR4* tropical race 4

[b] *ATCC* American type culture collection (Manassas, VA, USA); *TBRI* Taiwan banana research institute (Pingtung, Taiwan); *TDARES* Taitung district agricultural research and extension station (Taitung, Taiwan); *ARI* Agricultural research institute (Taichung, Taiwan); *AVRDC-WVC*=AVRDC-The world vegetable center (Tainan, Taiwan)

[c] Tropical race 4 of Foc was identified according to the method by Dita et al. (2010)

[d] Confirmed with VCG test as VCG 01213/16 (tropical race 4) according to Miss Linda J. Smith from the Plant Protection Unit, Queensland Department of Primary Industries, QDPI, Australia {Lin et al. (2009)}

Detection of Foc in field-banana samples

The real-time PCR assay was used to detect Foc in field-infected banana. The field-infected banana samples were surface-sterilized with 0.1 % Clorox bleach for 1 min, rinsed with sterile water three times, and air-dried. The surface-sterilized dried samples were then cut into 1 cm^2 section (0.5×2 cm^2) each and put onto Nash-PCNB agar media for a plate-out assay. Simultaneously, the nearby area of each section was picked for PCR amplification. The tested DNA samples were extracted from symptomatic and symptomless pseudostems and leaves of bananas (0.3 g each) collected from the fields for diagnostic PCR assay.

Results

Specificity of the PCR amplification

In order to develop a realizable molecular detection system for Foc race 4 isolates, the novel SCAR PCR primer set FocSc-1/FocSc-2 was developed according to the sequence of Foc race 4 specific marker Foc_{242} by expanding original amplification primers from 21 nt-long (Foc-1/Foc-2) to 27 nt-long (FocSc-1/FocSc-2). Both primer sets, FocSc-1/FocSc-2 (Fig. 1a) and Foc-1/Foc-2 (Fig. 1b), were able to amplify the target DNA (Foc_{242}) only from DNA of Foc race 4 isolates {including 12 isolates in Taiwan and 4 reference isolates from

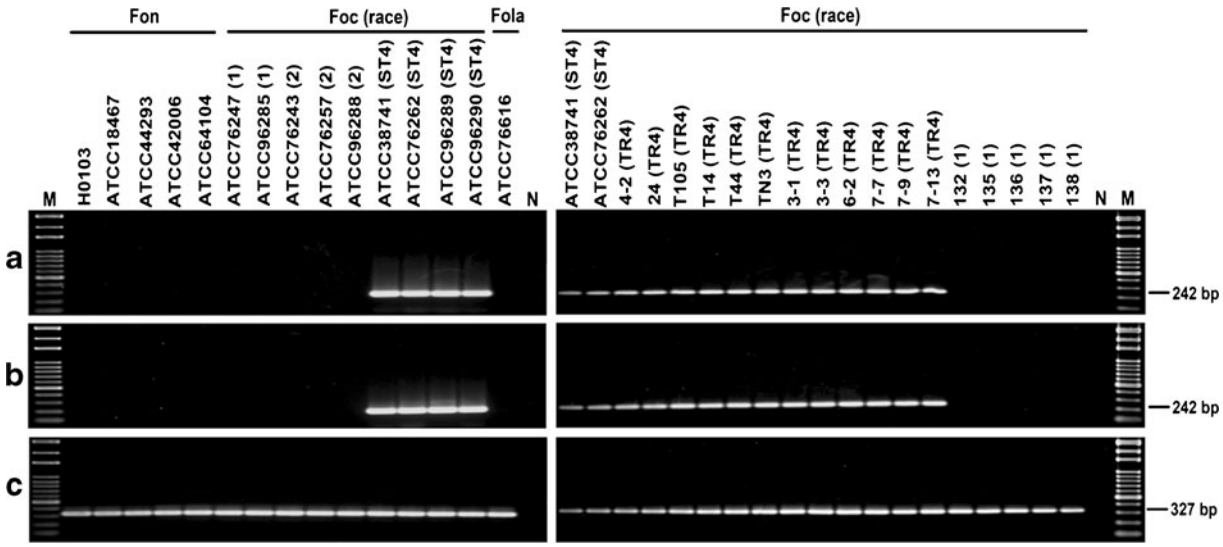


Fig. 1 PCR amplification of nine American Type Culture Collection (ATCC) and 17 Taiwanese isolates of *Fusarium oxysporum* f. sp. *cubense* (Foc) with three different primer sets FocSc-1/FocSc-2 (**a**), Foc-1/Foc-2 (**b**), and FnSc-1/FnSc-2 (**c**). PCR amplifications were performed using 10 ng genomic DNA extracted from each isolate (see Table 1). *F. oxysporum* f. sp. *niveum* (Fon) and *F. oxysporum* f. sp. *lactucae* (Fola) isolates were used for comparison. The locations of corresponding 242 bp, 242 bp, and 327 bp DNA bands were indicated on the right. *N* = negative control using sterile dH_2O as the template; *M* = molecular markers of Gen-100 DNA ladder (GMbiolab Co. Ltd., Taichung, Taiwan)

the American Type Culture Collection (ATCC)} but not from DNA of any other tested isolates {including 5 Foc race 1 isolates in Taiwan, 1*F. oxysporum* f. sp. *niveum* isolate Fon-H0103, and 10 reference isolates from ATCC such as *F. oxysporum* f. sp. *cubense* race 1 (ATCC76247 and ATCC96285) and race 2 (ATCC76243, ATCC76257, and ATCC96288), *F. oxysporum* f. sp. *niveum* (ATCC18467, ATCC44293, ATCC42006, and ATCC64104), and *F. oxysporum* f. sp. *lactucae* (ATCC76616)}. In addition, FocSc-1/FocSc-2 (Fig. 2a) and Foc-1/Foc-2 (Fig. 2b) did not amplify the Foc_{242} marker in the DNA samples of all tested *Fusarium oxysporum* isolates infecting other crops such as loofah (Folu), garland chrysanthemum (Foch), onion (Foce), cucumber (Focu), tomato (Foly), muskmelon (Fome), lily (Foli), gladiolus (Fog), asparagus bean (Fot), and lettuce (Fola). Therefore, the Foc_{242} marker could be amplified by both primer sets FocSc-1/FocSc-2 and Foc-1/Foc-2 from the tested samples of Foc race 4 isolates but not from that of any other isolates (Figs. 1 and 2). In conclusion, these data indicated that both primer sets FocSc-1/FocSc-2 and Foc-1/Foc-2 were applicable to develop a molecular diagnostic method for rapid identification of Foc race 4 by using PCR assay.

Primer selection for real-time PCR amplification

In order to appraise the sensitivity of the above-mentioned primer sets, serial dilutions of Foc_{242}, ranging from 1 to 10^{-6} ng, were performed in real-time PCR assays (Fig. 3). According to the plotted standard curves (Fig. 3a) and the Ct values (Fig. 3b) of primer sets FocSc-1/FocSc-2 and Foc-1/Foc-2, the data presented an average difference of 1.564 in Ct values between the primer sets. These data showed that the real-time PCR efficiency of the primer set FocSc-1/FocSc-2 was 2.96-fold higher than that of the primer set Foc-1/Foc-2. In addition, the presence of a single peak (at 85 °C) of amplicon in dissociation curves (data not shown) and a high correlation coefficient (R^2=0.99125) (Fig. 3a) indicated that the real-time PCR assay using primer set FocSc-1/FocSc-2 offers a novel method for high specificity and reproducibility to detect Foc race 4. Therefore, the SCAR primer set was the first priority choice for further detection purposes.

Reproducibility and sensitivity of real-time PCR amplification

Serial dilutions of gDNA (ranging from 10^{-1} to 10^{-4} ng) and conidia (amount ranging from 2000

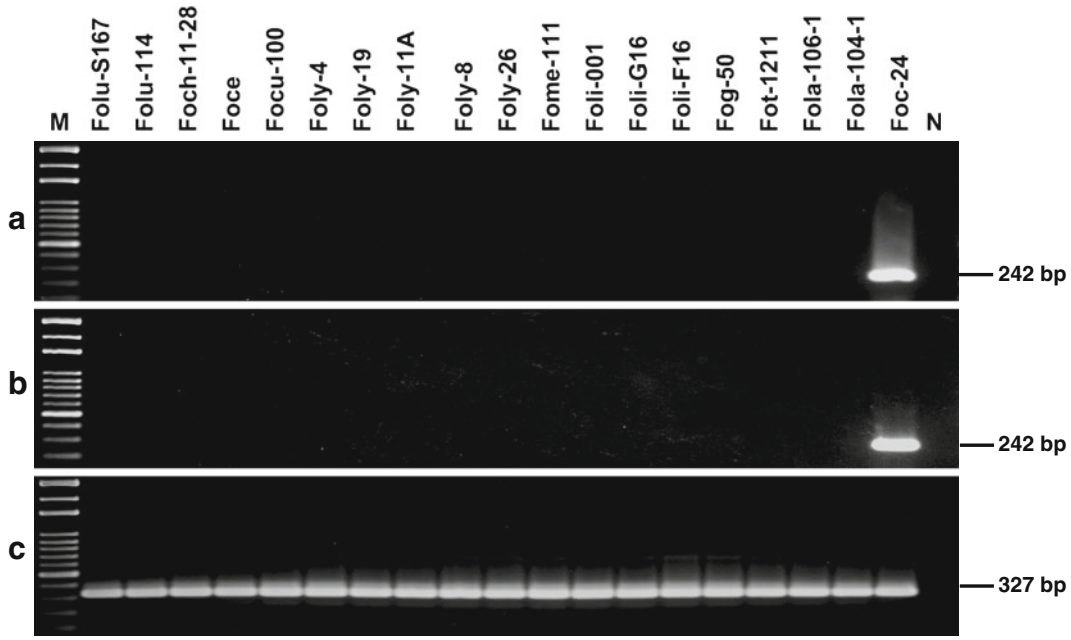


Fig. 2 PCR amplification of eleven *formae speciales* of *Fusarium oxysporum* (Fo) with three different primer sets FocSc-1/FocSc-2 (**a**), Foc-1/Foc-2 (**b**), and FnSc-1/FnSc-2. PCR amplifications were performed using 10 ng genomic DNA extracted from each isolate in Taiwan (see Table 1). *F. oxysporum* f. sp. *cubense* race 4 isolate (Foc-24) was used for comparison. The locations of corresponding 242 bp, 242 bp, and 327 bp DNA bands were indicated on the right. *N* = negative control using sterile dH_2O as the template; *M* = molecular markers of Gen-100 DNA ladder (GMbiolab Co. Ltd., Taichung, Taiwan)

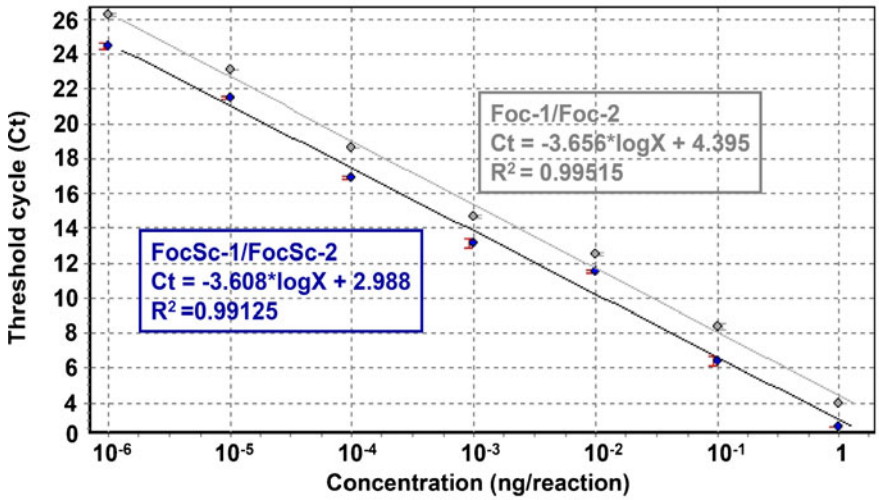
to 20) of Foc-24 isolate were prepared to test the reproducibility and sensitivity of real-time PCR assay using the FocSc-1/FocSc-2 primer set (Fig. 4). We were able to obtain the standard curve with high reproducibility by plotting Ct values *versus* corresponding gDNA amounts even when the tested gDNA template was as low as 10^{-4}ng (Fig. 4a). In addition, when using Foc-24 conidia as templates, the real-time PCR data demonstrated that a conidia amount of 2000 (group A), 200 (group B), and 20 (group C) corresponded to average Ct values of 24.29±0.24, 27.91±0.34, and 32.47±0.32, respectively; and the Ct values could be estimated from DNA amounts of $(2.18\pm0.30)\times10^{-2}$, $(2.43\pm0.52)\times10^{-3}$, and $(1.55\pm0.29)\times10^{-4}$ng (Fig. 4b).

Real-time PCR detection in naturally infected banana tissues

Real-time PCR amplification with the FocSc-1/FocSc-2 primer set, the quantifiable detection method for Foc race 4, was used to test the gDNA samples collected from each of the two natural field-infected banana plants (Table 2). It appeared that the target DNA fragment could be detected in gDNA of symptomatic banana pseudostems and leaves. Furthermore, the data indicated that in tested banana tissue samples, the Foc gDNA contained per μg of total DNA of infected-banana pseudostems and leaves ranged from 14.24±6.49 ng to $(2.05\pm1.06)\times10^{-3}$ng and $(4.75\pm3.37)\times10^{-2}$ng to $(1.78\pm0.68)\times10^{-3}$ng, respectively. In addition, according to the diagnostic results obtained by the real-time PCR assay, we found that Foc gDNA in severely symptomatic pseudostems (14.24±6.49 ng per μg of banana gDNA) and leaves $\{(4.75\pm3.37)\times10^{-2}$ng per μg of banana gDNA$\}$ were 6946-fold and 26.69-fold more than in those of mild-symptomatic pseudostems $\{(2.05\pm1.06)\times10^{-3}$ng per ng of banana gDNA$\}$ and leaves $\{(1.78\pm0.68)\times10^{-3}$ng per ng of banana gDNA$\}$, respectively (Table 2). However, we recovered Foc only from the samples of symptomatic banana tissues but not from those of symptomless banana tissues using the plate-out assay, even though positive real-time PCR results were obtained from those symptomless banana tissues.

These data indicated that the real-time PCR assay with the SCAR primer set has high sensitivity, and the diagnostic PCR results was supported by the symptomatic

a



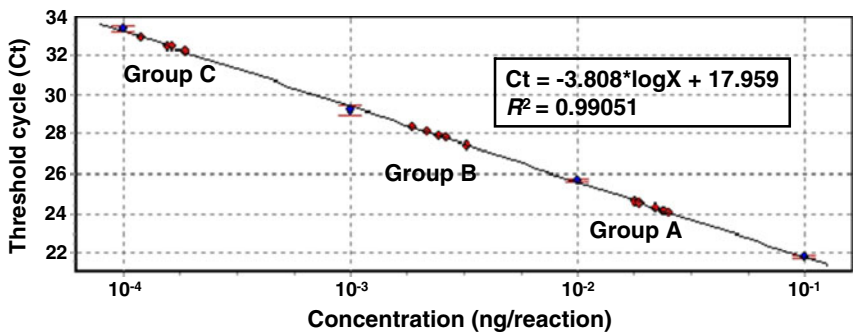
b

	Values of Ct						
Foc-1/Foc-2	26.27±0.18	23.11±0.06	18.59±0.08	14.69±0.09	12.5±0.16	8.39±0.31	3.92±0.05
FocSc-1/FocSc-2	24.43±0.28	21.49±0.11	16.92±0.16	13.15±0.54	11.5±0.20	6.40±0.61	2.63±0.01

Fig. 3 Standard curves (**a**) and threshold cycle (Ct) values (**b**) from real-time PCR of the amplification products using primer sets FocSc-1/FocSc-2 and Foc-1/Foc-2. The standard curves were created by plotting the target DNA (Foc_{242}) amount (10^{-6} to 1 ng) against the corresponding Ct value. Error bars represent standard deviations from tetraplicate reactions

characteristics of the infected banana. Therefore, the real-time PCR assay is suitable to detect Foc race 4 in banana tissues even if the infected bananas exhibit mild symptoms.

a



b

	Group C	Group B	Group A
Ct values	32.47±0.32	27.91±0.34	24.29±0.24
Calc. Conc. (ng/reaction)	(1.55±0.29) × 10^{-4}	(2.43±0.52) × 10^{-3}	(2.18±0.30) × 10^{-2}

Fig. 4 Detection sensitivity of real-time PCR assay using the primer set FocSc-1/FocSc-2 in genomic DNA (blue spots) and conidia (red spots) of *Fusarium oxysporum* f. sp. *cubense* (Foc) race 4. Serial dilutions of Foc-24 genomic DNA ranging from 10^{-1} to 10^{-4}ng and conidia numbers ranging from 2,000 to 20 (grouped A to C, respectively), were used as templates. The standard curves were created by plotting the genomic DNA amount against the threshold cycle (Ct) value. Error bars represent standard deviations from six replicate reactions

Table 2 Detection and quantification of *Fusarium oxypsorum* f. sp. *cubense* race 4 in symptomatic and symptomless leaves and pseudostems of naturally field-infected banana using real-time PCR method

Samples	Symptoms[a]	Calculated *Fusarium oxypsorum* f. sp. *cubense* DNA amounts (ng per μg banana total DNA)[b]
Symptomatic banana		
Pseudostems	+++	14.24±6.49
Pseudostems	++	$(4.11\pm2.23)\times10^{-1}$
Pseudostems	+	$(2.05\pm1.06)\times10^{-3}$
Leaves	+++	$(4.75\pm3.37)\times10^{-2}$
Leaves	++	$(1.13\pm1.08)\times10^{-2}$
Leaves	+	$(1.78\pm0.68)\times10^{-3}$
Symptomless banana		
Pseudostems	−	$(1.84\pm0.72)\times10^{-4}$
Leaves	−	$(1.55\pm0.29)\times10^{-4}$

[a] −= no symptoms on pseudostems or leaves; + = mild symptoms (less than 1/3 area of pseudostem necrosis or leaf yellowing); ++ = moderate symptoms (less than 2/3 but equal or more than 1/3 area of pseudostem necrosis or leaf yellowing); +++ = severe symptoms (equal or more than 2/3 area of pseudostem necrosis or leaf yellowing)

[b] mean ± standard deviation is presented from tetraplicate samples collected from two separate leaves or pseudostems from each of the two banana plants

These results indicate that this detection protocol, based on real-time PCR amplification using the primer set FocSc-1/FocSc-2, may lead to efficient disease management practices for Fusairum wilt disease of banana caused by Foc race 4.

Discussion

Thus far commercial banana cultivars resistant to Foc race 4 have not been extensively planted. Therefore, prevention of Foc race 4 from contaminating uninfected plantations, avoidance of banana planting in infected fields, and prevention of dissemination of the pathogen from diseased to healthy banana, are the top priorities in disease management for a multibillion dollars' worth of banana industry (Wilson and Otsuki 2004). This study described a real-time PCR assay with high sensitivity and reproducibility for detection of Foc race 4. Comparison with conventional field identification methods using time-consuming and laborious pathogenicity test, the PCR assay provide a rapid, efficient, and reliable diagnosis of this devastating pathogen.

In this study, we reported a new developed SCAR primer set FocSc-1/FocSc-2 which presents higher PCR amplification efficiency than Foc-1/Foc-2 (average 2.96-fold). Both primer sets, FocSc-1/FocSc-2 and Foc-1/Foc-2, could amplify the Foc_{242} marker, and the specificity of the SCAR primer set FocSc-1/FocSc-2 was comparable to the reported primer set Foc-1/Foc-2. In addition, the Foc_{242} had been proved to be a molecular marker specific to Foc race 4 after being tested with over a hundred isolates of *Fusarium oxysporum* (Lin et al. 2009).

The standard curve of FocSc-1/FocSc-2 was plotted against Foc-24 gDNA concentrations ranging from 10^{-1} to 10^{-4}ng in real-time PCR (Fig. 4). Under the tested gDNA concentrations, the real-time PCR assay with primer set FocSc-1/FocSc-2 has high reproducibility with low average standard deviation (0.34) and a correlation coefficient approaching 1 (0.99051). In addition, by using the PCR assay, the number of conidia could be used to calculate corresponding amounts of gDNA of Foc-24.

The real-time PCR was able to identify, detect, and quantify Foc race 4, simultaneously. Even when the conidia number of Foc-24 was as low as 20 per reaction (group C of Fig. 4), the PCR detection results of the group C were very reproducible (average standard deviation of 0.32 within average Ct values of 32.47); while the other two assays of groups A and B also obtained similar results with low average standard deviations of 0.24 and 0.34, respectively. Therefore, these data suggested that the real-time PCR amplification with primer set FocSc-1/FocSc-2 was applicable and quantifiable to detect low amounts of Foc race 4 such as gDNA and conidia for as low as 10^{-4}ng gDNA and 20 conidia, respectively.

Conventional methods applied to diagnose Foc *in planta* usually involve traditional agar plating and pathogenicity test which may take days, weeks, and even months. Pathogen isolation combined with microscopic techniques can be an accurate method to identify *Fusarium* species, however, it requires sufficient knowledge of the morphological characteristics of the pathogens. Furthermore, differentiating *formae speciales* of *F. oxysporum* by using pathogenicity tests are time-consuming and labor-intensive, and requires a trained plant pathologist or professional technician

who can perform artificial inoculations of the pathogen and recognition of the symptom development. Moreover, identification of races of Foc completely depends on the pathogenicity test against distinctive cultivars of bananas. Therefore, conventional methods provide no rapid identification of Foc race 4, and no possibility of performing diagnosis and quantification of the pathogen *in planta* simultaneously.

As well as being considered a powerful tool for diagnosis of plant pathogens (Lopez et al. 2003; Ward et al. 2004), real-time PCR can reduce the use of ethidium bromide which is an environmental hazard (Demontis et al. 2008). Therefore, in this study, we implemented a real-time PCR assay to develop the molecular diagnostic method using FocSc-1/FocSc-2 primer set for detection and quantification of Foc race 4 *in planta*.

Compared with the diagnostic method of Foc race 4 previously described by Lin et al. (2009), the novel assay in this study can increase at least a hundred-fold detection limit with highly reproducible results. The molecular detection assay could quantify Foc gDNA in pseudostems and leaves of banana with the detection data conforming to the corresponding symptomatic characteristics of infected-banana. Therefore, these results indicated that the diagnosis protocol undoubtedly was superior to screening Foc-infected banana samples.

A rapid, specific, sensitive, and quantifiable detection method can help plant pathologists to study pathogen distribution *in planta.* Moreover, the assay will provide a significant and objective evidence for confirming successful pathogen inoculation. If the artificial inoculation results show confusing symptom development, researchers are unable to readily study the interactions between pathogens and their hosts. Thus, evidence for tagging presence of pathogens especially in mild-symptomatic hosts is vitally important for research with time-course experiments. For these reasons, an effective and accurate pathogen-tagging method certainly will help researchers to speed up their studies. The developed diagnostic assay is simple with high specificity, and is capable of rapid tagging and accurate quantification of Foc race 4 in infected banana tissues, even with the mild symptoms. Therefore, it may be a powerful tool that can be beneficial for quarantine purposes, breeding programs, as well as in other research in epidemiology, stress biology, and phytopathology.

Acknowledgements We are grateful to Drs H.-L. Wang (National Kaohsiung Normal University), Y.-S. Lin (National Chung Hsing University, NCHU), W.-H. Hsieh (NCHU), C.-H. Kuo (National Chiayi University), Taitung District Agricultural Research and Extension Station, Agricultural Research Institute and AVRDC-The World Vegetable Center for providing the tested microorganisms. We also thank Miss Y.-L. Wan and Miss. Y.-R. Chen for technical assistance. This research was supported in part by Council of Agriculture, Taiwan, R.O.C. under grant numbers 93AS-1.9.2-BQ-B1(1), 94AS-13.3.2-BQ-B1(6), 96AS-4.1.2-IC-I1(2), 97AS-4.1.2-ICI1(6), 98AS-4.1.1-IC-I1(1), and 99-AS-9.3.1-BQ-B2(6); by National Science Council, Taiwan, R.O.C. under grant numbers 98-2313-B-005-025-MY3, 99-2622-B-005-006-CC2, and 101-2313-B-005-028-MY3; by the Ministry of Education, Taiwan, R.O.C. under the ATU plan; and also by National Chung Hsing University, Taiwan, R.O.C. Dr. Ying-Hong Lin was supported with the postdoctoral fellowships provided by National Science Council, Taiwan, R.O.C. under grant numbers 098-2811-B-005-024, 099-2811-B-005-025, 100-2811-B-005-017, and 101-2811-B-005-011.

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