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NOVEL SPECIFIC PRIMERS FOR THE SPECIFIC DETECTION OF *FUSARIUM OXYSPORUM* F. SP. *CUBENSE* BASED ON SYBR GREEN REAL-TIME PCR

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ABSTRACT

Fusarium oxysporum f. sp. *cubense* (*Foc*) is the causal agent of Fusarium wilt of banana worldwide. *Foc* is transmitted to another banana plantation via an infected banana sucker. A specific and sensitive detection assay could be used for disease-free propagule screening to prevent disease dispersal effectively. In this study, SYBR green-based real-time PCR assay was developed based on novel specific primers targeting the large subunit of RNA polymerase II (*RPB1*) gene. The partial *RPB1* gene was amplified and sequenced from *Foc* isolate FOC1708 and SK3-2. Regions specific for *Foc* were identified and used for designing real-time PCR primers. The specificity of the designed primers was evaluated on genomic DNA from *Foc* isolates and non-target microorganisms. The results showed that the designed primers were specific to only *Foc* isolates from race 1 and tropical race 4 (TR4). The detection efficiency of the designed primers was compared to other published primers through optimized SYBR green-based real-time PCR assay and nested PCR. The new primers could detect the *Foc* genomic DNA at a minimum of 5 pg and target pathogen in a symptomless banana sucker. The specificity and sensitivity of the new primers were comparable to the published real-time PCR primers and the nested PCR assay. This developed assay with these novel primers can aid the disease quarantine for effective prevention and control of the Fusarium wilt of banana.

Keywords: Banana; Detection; Fusarium; Panama disease; Real-time PCR

INTRODUCTION

Fusarium wilt of banana (Panama disease) caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*) is one of the most significant threats to banana production (*Musa* spp.) worldwide (**Bentley *et al.*, 1998**). Based on the pathogenicity on different banana cultivars, *Foc* can be divided into races (**Stover, 1990**), including *Foc* race 1, race 2, race 3, and race 4. *Foc* race 1 occurs worldwide and causes disease on Gros Michel (genome type=AAA) and a range of other cultivars carrying the AAB genome. *Foc* race 2 causes disease on those race 1-susceptible cultivars, as well as the hybrid triploid 'Bluggoe' (AAB). *Foc* race 4 is separated into tropical race 4 (TR4) and subtropical race 4 (STR4). STR4 causes disease in Cavendish in the subtropics, whereas TR4 targets Cavendish under tropical and subtropical conditions (**Buddenhagen, 2009**).

The disease pathogen infects healthy bananas through their roots and spreads to the upper plant parts and vascular bundles. The pathogen causes leaf yellowing and wilt symptoms by blocking the banana vascular bundle (**Ploetz, 2006**). As the pathogen is transmitted through vascular tissues, the pathogen can be transmitted to a banana sucker (**Stover, 1962; Li *et al.*, 2011**). The symptomless infected suckers can be a source of inoculum and cause disease dispersal from farm to farm or between countries (**Dita *et al.*, 2018**). Therefore, an effective and essential strategy to prevent the disease is to use disease-free propagating materials.

PCR based detection assays including PCR (**Li *et al.*, 2012**), nested PCR (**Prachaiboon *et al.*, 2020**), real-time or quantitative PCR (**Yang *et al.*, 2015; Lin *et al.*, 2013**) were used to detect the *Foc* from banana propagules before planting or in disease quarantine. Conventional PCR is widely used to detect *Foc*; however, its sensitivity is lower than nested PCR (**Prachaiboon *et al.*, 2020**) and real-time PCR (**Lin *et al.*, 2013**). Additionally, the PCR assay, including nested PCR, requires time and labor to perform the PCR (**Schaad and Frederick, 2002**). Real-time PCR has been developed to address the limitations of PCR and can be used to quantify pathogen DNA in a plant, water, and soil samples (**Matthews *et al.*, 2020**)

Most real-time PCR assays developed for *Foc* detections are mainly based on TaqMan-based systems (**Yang *et al.*, 2015; Lin *et al.*, 2016; Aguayo *et al.*, 2017; Matthews *et al.*, 2020)** that provide higher sensitivity than other assays and detect different races in one reaction. However, TaqMan-based detection requires a fluorogenic probe which increases running costs and assay set up. SYBR green-based detection is an optional system comparable to TaqMan-based detection in performance and quality (**Tajadini *et al.*, 2014**). In addition, SYBR green-based real-time PCR is relatively cost-benefit and easy to use and technically based on binding the fluorescent dye to double-stranded deoxyribonucleic acid (dsDNA) (**Tajadini *et al.*, 2014**). Therefore, this research aimed to develop a SYBR green-based real-time PCR assay for the detection of *Foc*. This study designed new primers targeting the largest subunit of RNA polymerase II (*RPB1*) gene. The sequence of the *RPB1* gene has been used for phylogenetic analysis of *Fusarium* species (**O'Donnell *et al.*, 2013; Czislowski *et al.*, 2018**). *The RPB1* gene reflects the horizontal transmission of one family of effectors, *the Secreted In Xylem* (*SIX*) genes and could be a potential source to improve the *Foc* diagnostic (**Czislowski *et al.*, 2018**).

MATERIAL AND METHODS

Fungal isolates and mycelial DNA extraction

Foc race 1 isolate FOC1708, used as a reference strain, was provided by the Department of Agriculture, Thailand. Besides, *Foc* race 1 isolate SK3-2 and NP1-4 were collected from infected bananas from Sakon Nakhon and Nakhon Phanom province, Thailand, and were identified as *Foc* based on microconidia, macroconidia, and chlamydospore characteristics (**Leslie & Summerell, 2008**), pathogenicity on banana seedling, and PCR assay with *Foc* race 1 specific primers (**Yang *et al.*, 2015**). *Foc* isolates were maintained on potato dextrose agar at 4 °C. Genomic DNA was extracted from mycelia using the Qiagen kit or CTAB method. The DNA concentration was determined using a Thermo Scientific™ NanoDrop™ lite spectrophotometer.

Real-time PCR primer design

The gene encoding the largest subunit of RNA polymerase II (*RPB1*) was selected as a target sequence for real-time PCR primer design. Partial *RPB1* gene was amplified from *Foc* isolates FOC1708 and SK3-2 using primers Fa (**Hofstetter *et al.*, 2007**)/G2R (**Benjamin Hall, unpublished data**) and Fa/R8 reported by **O'Donnell *et al.* (2010)**. The PCR reaction contained 0.2 µM each primer, 0.2 µM dNTPs, 1X buffer S, 1 U of the *Taq* polymerase (Vivantis, Malaysia), and 50 ng *Foc* DNA. The PCR was performed using the following thermocycling conditions: 95 °C for 5 min; 35 cycles of 95 °C for 45 s, 55 °C for 45 s, and 72 °C for 60 s; and a final extension at 72 °C for 5 min. The PCR products were purified using BioFact™ PCR Purification Kit, then were sequenced by Macrogen. Multiple alignments of partial *RPB1* sequences from *Foc* isolate FOC1708, SK3-2, and other *Fusarium* species derived from the NCBI database were constructed using BioEdit Sequence Alignment Editor (**Hall, 1999**). Regions specific for *Foc* were identified. Primers were designed and analyzed using the Primer3 software (**Koressaar and Remm, 2007**).

SYBR green-based real-time PCR assay

The real-time PCR assays were performed to amplify the partial *RPB1* gene of *Foc* using SensiFAST™ SYBR No-ROX Kit (Bioline, UK). The assay was carried in a 10 µl volume solution containing 1X SensiFAST SYBR No-Rox Mix, 400 nM forward and reverse primers, and 1 µl of purified DNA. The optimized thermocycling conditions were 1 cycle of 95 °C for 2 min and 30 cycles of 95 °C for 30 s and 70 °C for 30 s, followed by a melting curve analysis program according to the real-time PCR machine instruction. All the reactions were performed in triplicate on the PCRmax Eco 48 real-time PCR system (PCR max, UK).

The specificity of the assay

The specificity of the designed primers to detect *Foc* was evaluated on genomic DNA from *Foc* race 1 isolates, including FOC1708, SK3-2, NP1-4, and *Foc* tropical race 4 (TR4) isolate PM-HTS-Fo56, PM-HTS-Fo57, and PM-HTS-Fo58 provided by Associate Professor Dr. Ying-Hong Lin, Department of Plant Medicine, National Pingtung University of Science and Technology. *Fusarium oxysprum* f. sp. *lycopersici* (*Fol*), *Trichoderma asperellum*, *Pestalotiopsis* sp., *Colletotrichum capsici*, *C. gloeosporioides*, *Ralstonia solanacearum*, and *R. syzygii* subsp. *celebesensis* were used in the specificity test. The real-time PCR reaction and thermal cycle condition were followed the above condition.

Sensitivity, standard curve, and amplification efficiency of the assay

The newly developed real-time PCR primer set's sensitivity was examined by testing a series of 1:10 dilutions of the pGEM®-T Easy vector (Promega Corporation, Madison, WI, USA) containing the partial *RPB1* gene (pGEM®-T-RPB1) ranging from 10^7 to 10 copies/µl. The obtained quantitation cycle (Cq) values were plotted against the target gene copy number to create a standard curve. Amplification efficiency was calculated using QPCR Standard Curve Slope to Efficiency Calculator available at https://www.chem.agilent.com/store/biocalculators/calcSlopeEfficiency.jsp.

Detection efficiency of the assay

Detection efficiency of the newly developed real-time PCR primers, RPB11 primer set, targeting the *RPB1* gene in genomic DNA of *Foc*, varied from 50 ng to 0.5 pg, was compared with published primers for *Foc* detection, including real-time PCR primers, Foc1-0422F1/Foc1-0422R1 (Table 1) designed by **Yang *et al.* (2015),** and nested PCR primers developed by **Prachaiboon *et al.* (2020)**. The real-time PCR reaction for primer Foc1-0422F1 and Foc1-0422R1 was the same as the RPB11 primer set, although the thermal cycling differed. The optimized thermocycling conditions for the Foc1-0422 primer set were 1 cycle of 95 °C for 2 min and 30 cycles of 95 °C for 30 s and 66 °C for 30 s, followed by a melting curve analysis program. The nested PCR was performed by using primer W1805F and W1805R (Table 1) **(Li *et al.*, 2012)** for the first round PCR and primer W18051F3 and W18051B3 (Table 1) in the second-round PCR. The PCR reaction contained 0.2 µM each primer, 0.2 µM dNTPs, 2 mM MgCl2, 1X buffer A, 1 U of the Taq polymerase (Vivantis, Malaysia), and 1 µl DNA sample. The first-round PCR was performed using the following thermocycling conditions: 95 °C for 2 min; 30 cycles of 95 °C for 30 s, 58.4 °C for 30 s, and 72 °C for 2 min; and a final extension at 72 °C for 5 min. The first-round PCR product was diluted at 1:20 then used as a second-round PCR template. The second-round PCR was performed using the following thermocycling conditions: 95 °C for 2 min; 30 cycles of 95 °C for 30 s, 63 °C for 30 s, and 72 °C for 2 min; and a final extension at 72 °C for 5 min. The nested PCR products were checked by 1 % agarose gel electrophoresis.

To examine the detection efficiency, novel RPB11F/RPB11R and published Foc1-0422F1/Foc1-0422R1 primers with SYBR green-based real-time PCR assay as well as nested PCR were performed to detect *Foc* in banana suckers. Ten symptomless banana suckers were collected from *Foc* infected parent plants. Three roots, 1 cm in length next to the base of a sucker, were sampled from each sucker then the sampling roots were used for DNA extraction by the CTAB method. The detection efficiency using novel RPB11F/RPB11R real-time PCR primers compared with the real-time PCR with primer Foc1-0422F1/Foc1-0422R1 and the nested PCR. The real-time PCR and nested PCR reaction and thermal cycling conditions were mentioned above.

RESULTS

The specificity of the real-time PCR primer

Multiple alignments of partial *RPB1* sequences revealed specific regions among *Foc* isolates but not other *Fusarium* species (Figure 1). The regions were used to design new real-time PCR primers (RPB11) based on the *RPB1* gene, including primer RPB11F and primer RPB11R (Figure 1). Primer sequences were listed in Table 1. For determining the specificity of the designed primers, DNA from *Foc*, *Fusarium* species, plant pathogenic fungi, microbes that were probably found in the banana rhizosphere were used for real-time PCR with RPB11 primer set following the real-time PCR reaction described in MATERIAL AND METHODS. The results showed that the PPB11 primer set specifically detected all isolates of *Foc* race 1 and TR4. However, there was a difference in the Quantification cycle (Cq) and melting temperature (Tm) value between isolates of *Foc* race 1 and TR4. The RPB11 primer set generated a positive amplification signal for *Foc* race 1 isolates at Cq 13 to 15, but the primer showed a positive signal for *Foc* TR4 isolates at Cq 26 to 28 (Figure 2A). The Tm values of the amplified products of *Foc* race 1 isolates were 84 °C, but the *Foc* TR4 isolates were around 84.7 - 85 °C (Figure 2B). The RPB11 primer set did not generate a positive signal for non-target microbes (Table 2).

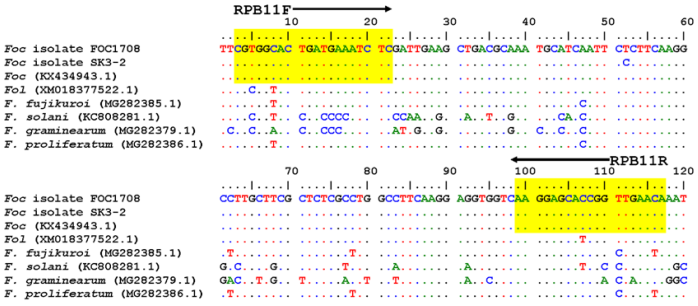


Figure 1 Multiple nucleotide sequence alignment of *RPB1* gene from *Fusarium oxysporum* f. sp. *cubense* and *Fusarium* spp.. The sequence of primer RPB11F and RPB11R are highlighted with yellow colour.

Table 1 Sequences of primers used in this study

Assay	Primer name	Sequence (5'- 3')	Reference
SYBR green real-time PCR	RPB11F	CGTGGCACTGATGAAATCTC	This study
	RPB11R	TGTTCAACCGGTGCTCCTT	This study
	Foc1-0422F1	AGGTGAGAAATCTGTTGAGTCTCGAT	**Yang *et al.* (2015)**
	Foc1-0422R1	AACTCCTTCACCAGCCTTTCG	**Yang *et al.* (2015)**
Nested PCR	W1805F	GTTGAGTCTCGATAAACAGCAAT	**Li *et al.* (2012)**
	W1805R	GACGAGGGGAGATATGGTC	**Li *et al.* (2012)**
	W*18051F3*	CTGGTGAAGGAGTTGTCCG	**Prachaiboon *et al.* (2020)**
	W*18051B3*	TTGTGATGTCGGCATGAGAT	**Prachaiboon *et al.* (2020)**

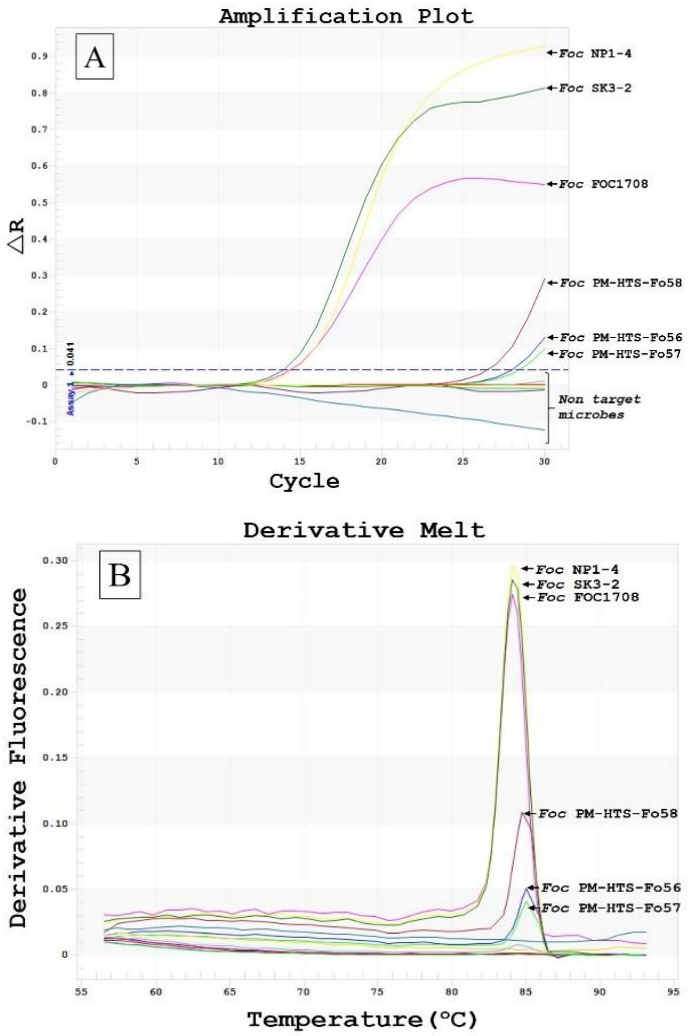


Figure 2 RPB11 specificity test by real-time PCR using genomic DNA of *Fusarium oxysporum* f. sp. *cubense* race 1, TR4, and non-target microorganisms (Sample's names are listed in Table 2). A: amplification signal and B: melting temperature.

Table 2 Specificity of the developed real-time PCR assay targeting *RPB1* gene

Sample name	Cq	Tm(ºC)
Foc race 1 isolate FOC1708	14.94±0.50	84±0.17
Foc race 1 isolate SK3-2	13.87±0.08	84±0.17
Foc race 1 isolate NP1-4	14.53±0.07	84±0.17
Foc TR4 isolate PM-HTS-Fo56	27.93±0.17	85±0.30
Foc TR4 isolate PM-HTS-Fo57	28.43±0.11	85±0.30
Foc TR4 isolate PM-HTS-Fo58	26.53±0.10	84.7±0.3
Fol isolate Fol101	-	-
Fol isolate Fol401	-	-
Colletotrichum capsici isolate CCC5	-	-
C. gloeosporioides isolate CGC7	-	-
Pestalotiopsis sp. isolate III	-	-
Trichoderma asperellum isolate TPK101	-	-
Ralstonia solanacearum isolate 832	-	-
Ralstonia syzygii isolate RM1201	-	-
Negative control	-	-

The sensitivity, standard curve, and amplification efficiency of the developed real-time PCR assay

For the sensitivity test, serial dilutions of pGEM®-T Easy vector containing the partial *RPB1* gene (pGEM®-T-RPB1) were used to examine the sensitivity of the real-time PCR assay with RPB11 primer set. The results showed that the assay with 10^7 to 10^3 pGEM®-T-RPB1 copies as the templates generated a positive amplification signal, but not with 10^2 pGEM®-T-RPB1 copies (Figure 3A). The Cq values corresponding to 10^3 pGEM®-T-RPB1 copies were 33.69 ± 1.2. The Tm values of the amplified products from the assay with 10^7 to 10^3 pGEM®-T-RPB1 copies were 84.24 ± 0.1 °C (Figure 3B). Therefore, the detection limit of the real-time PCR assay with RPB11 primer set for the sensitivity test of pGEM®-T-RPB1 was 10^3 copies. The standard curves showed a dynamic linear range across at least 5 log units of pGEM®-T-RPB1 copy number. Linear regression analysis revealed that the correlation coefficients (R^2) were 0.976 with a slope value of 3.29, and the amplification efficiency = 101.55 % (Figure 3C). These results showed an excellent linear correlation in the regression line and can be reliably used to calculate the target gene or *Foc* concentration.

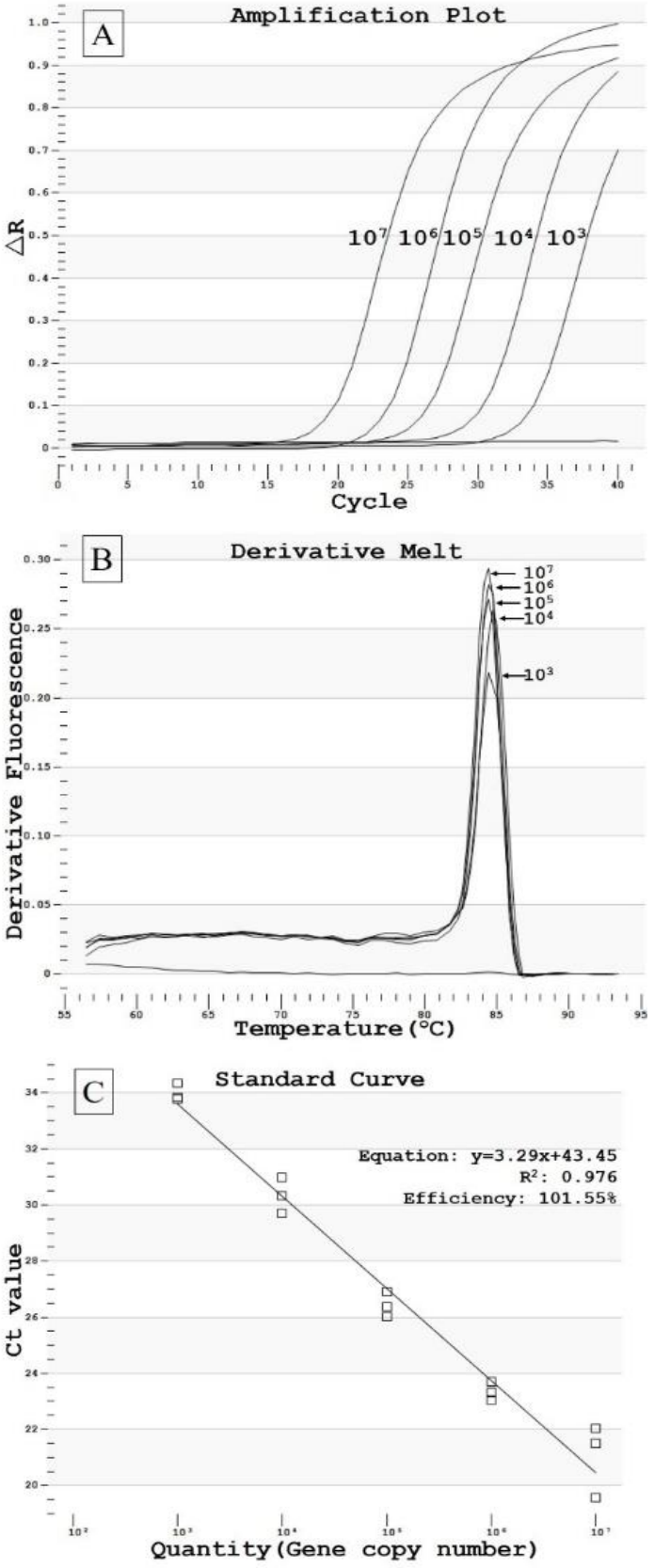


Figure 3 Sensitivity of the developed real-time PCR assay targeting *RPB1* gene for *Fusarium oxysporum* f. sp. *cubense* detection. A: amplification signal, B: melting temperature, and C: standard curve.

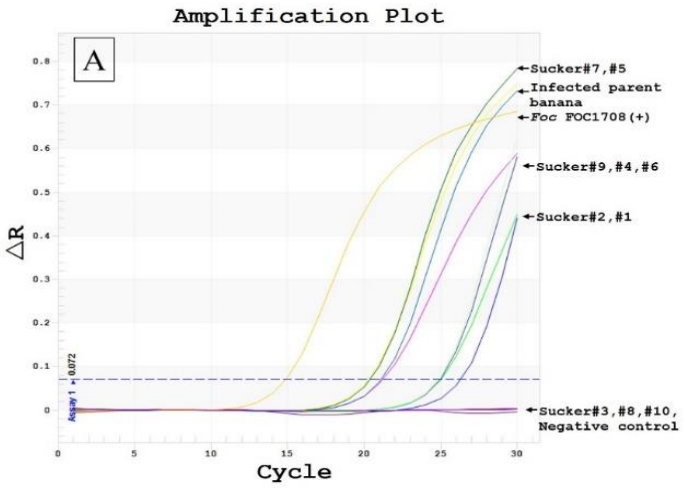
Detection efficiency of the newly developed real-time PCR primers

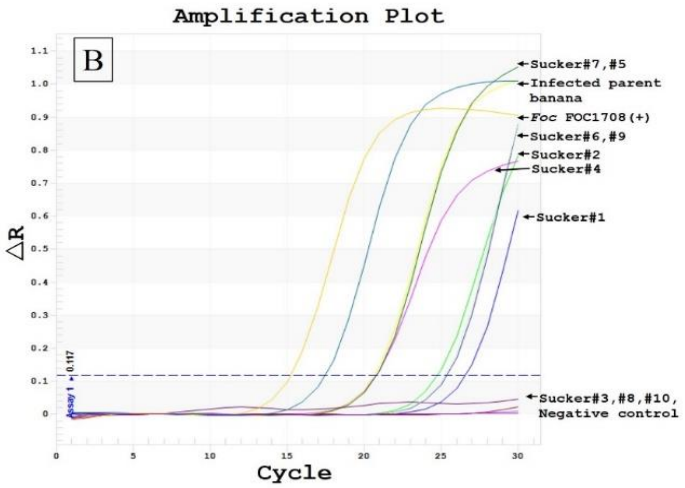
The detection efficiency of the developed real-time PCR assay using novel and published primers, and nested PCR assay were compared. *Foc* DNA at different amounts was used as a template for all assays. The results showed that the newly developed real-time PCR primers could detect the *Foc* DNA at a minimum of 5 pg. The Cq values were related to the amount of DNA template. The Tm values of all detected samples were 84.22 ± 0.1 °C. The detection efficiency was comparable to nested PCR developed by **Prachaiboon *et al*. (2020)** and real-time PCR developed by **Yang *et al.* (2015)** (Table 3).

Table 3 Detection efficiency of the developed real-time PCR assay targeting *RPB1* gene and other published assays for *Fusarium oxysporum* f. sp. *cubense* detection.

***Foc* DNA (pg)**	**The developed real-time PCR primers** (This study)		**Published assays**		
			Nested PCR (**Prachaiboon *et al*., 2020**)	Real-time PCR primers (**Yang *et al*., 2015**)	
	Cq	Tm(ºC)		Cq	Tm(ºC)
50000	14.73±0.34	84.3	+	14.95±0.06	82.5
5000	19.79±0.18	84.2	+	19.05±0.03	82.4
500	23.24±0.02	84.3	+	26.59±0.44	82.4
50	29.67±0.12	84.1	+	30.64±0.2	82.5
5	33.69±1.22	84.2	+	34.19±0.8	82.3
0.5	-	-	-	-	-

To evaluate the efficiency of the newly developed real-time PCR assay for detecting *Foc* in the symptomless banana suckers, a total of 10 sucker samples were tested using novel RPB11F/RPB11R primers and the other assays mentioned above. Seven out of ten samples showed a positive amplification signal based on novel RPB11F/RPB11R primers (Figure 4A), published Foc1-0422F1/Foc1-0422R1 primers (**Yang *et al*., 2015**) (Figure 4B), and nested PCR assay (**Prachaiboon *et al*., 2020**) (Figure 4C). The positive control samples, *Foc* isolate FOC1708, and infected parent bananas showed positive signals in all assays. The negative control samples did not generate a signal in any assays. *Foc* positive samples were detected by our assay and also the others (Figure 4). All assays presented the results in the same way.





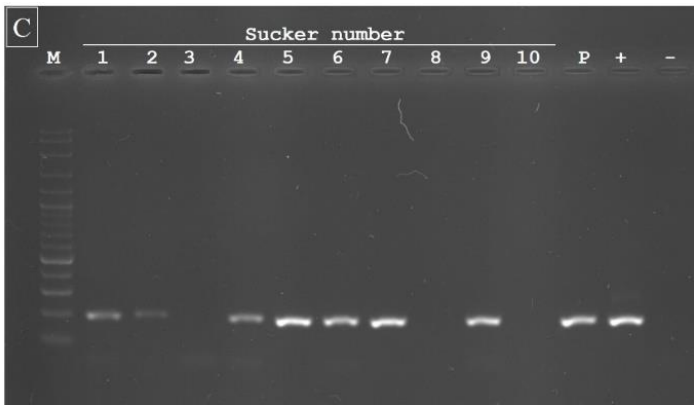


Figure 4 Detection of *Fusarium oxysporum* f. sp. *cubense* in banana suckers derived from infected parent plants. A: The developed real-time PCR assay targeting the *RPB1* gene. B: Real-time PCR assay with primers developed by **Yang *et al.* (2015)** and C: Nested PCR assay (**Prachaiboon *et al*., 2020**), P: infected parent banana, positive control (+): *Foc* isolate FOC1708, and negative control (-): non-infected banana.

DISCUSSION

Fusarium wilt or Panama disease of banana caused by *Foc* is a significant disease for banana production worldwide. The SYBR green-based real-time PCR assay in this finding provides an accurate and efficient assay for the *Foc* detection in the infected and symptomless banana suckers.

The SYBR green-based real-time PCR assay we developed for the *Foc* detection uses the specific primers designed based on the partial sequence of the new target gene *RPB1*, the large subunit of RNA polymerase II. This gene plays a vital role in the initiation and elongation of mRNA (**Roeder, 1996**) and is commonly used to be a marker gene for *Fusarium* phylogenetic analysis in addition to other conserved regions (**O'Donnell *et al*., 2010; O'Donnell *et al.*, 2013; Maryani *et al*., 2019**). In addition, RPB1 is highly conserved within fungal species and successfully used to design specific primers in several fungi such as *Colletotrichum truncatum* (**Tian *et al*., 2017**), *Penicillium italicum* (**Chen *et al.*, 2019**), etc. The partial *RPB1* gene sequences of our *Foc* samples and *Foc* sequences from NCBI provided a potential sequence for designing a specific primer for *Foc*.

The newly developed RPB11 primer set generated positive amplification signals from isolates belonging to *Foc* race 1 and TR4. The Cq values of *Foc* race 1 isolates were less than Cq values of *Foc* TR4 because of the low concentration of DNA template. We found the difference between Tm of amplification products of *Foc* race 1 and TR4. This was because the G-C content of the target *RPB1* region between *Foc* race 1 and TR4 may be different same as the finding of **Matthews *et al.* (2020)** reported that the melting point of target amplicons, the DNA-directed RNA polymerase III subunit beta (*RPC2*), are related to their race. From our result, we can use this finding to improve our assay further to clearly distinguish *Foc* race through High-Resolution Melting (HRM) analysis (**Ratti *et al*., 2019; Schiwek *et al*., 2020**).

The comparison of detection efficiency between the newly developed primers, the published primers, and the nested PCR assay confirmed that the SYBR based real-time PCR with the new primers was comparable to SYBR real-time PCR with primers published by **Yang *et al.* (2015)** and the nested PCR developed by **Prachaiboon *et al.* (2020)**. The newly developed SYBR green real-time PCR assay could detect *Foc* in material propagation, especially banana suckers, one of the most critical factors for disease dissemination (**Ploetz, 2005; Dita *et al*., 2010; Pérez-Vicente *et al*., 2014**). *Foc* is transmitted from infected mother plants to suckers which are usually symptomless (**Stover, 1962**). Three symptomless banana suckers did not generate any positive signal tested by all assays from our experiment, although the samples were collected from the symptom mother plant. We assume that *Foc* inoculum had not translocated to the new suckers yet, or the inoculum concentration was lower than the detection limit of our assay.

CONCLUSION

The novel real-time PCR primers, RPB11F and RPB11R, were designed from *RPB1* gene. Optimized SYBR green-based real-time PCR with the new primers is *Foc* specific detection assay. The sensitivity and efficiency of detecting target pathogens in a symptomless banana sucker were comparable to the published real-time PCR primers and the nested PCR assay. This developed assay will be a compelling choice for disease-free propagating materials screening and disease quarantine procedures.

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